Candidate Genes for Late Diabetic Complications

Lindholm, Eero

2007

Link to publication

Citation for published version (APA):
Lindholm, E. (2007). Candidate Genes for Late Diabetic Complications Endocrinology
Candidate Genes for Late Diabetic Complications

Academic dissertation

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With the permission of the Medical Faculty of Lund, to be presented for public examination at the Clinical Research Centre, Malmö University Hospital, December 4, 2007 at 9.15 a.m.

Faculty Opponent

Professor Knut Borch-Johnsen
Department of Epidemiology
Steno Diabetes Centre
Gentofte, Denmark
Wer sie nicht kennte
Die Elemente,
Ihre Kraft
Und Eigenschaft,
Wäre kein Meister
Über die Geister.

Johann Wolfgang von Goethe
Faust

(Den som ej känt
vart element,
ej vet att märka,
huru de verka,
kan ej befalla
andanne alla)

(översättning Viktor Rydberg)
Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The new WHO criteria for classification of diabetes takes into account also clinical stages dividing the diabetic patients into not insulin requiring (NIR), insulin requiring for control (IRC) and insulin requiring for survival (IRS) subgroups. Diabetic complications are the result of chronically elevated blood glucose. Genetic factors are believed to play role in pathogenesis of diabetic complications.

The aim of this study was
1) To test the usefulness of the new WHO criteria for clinical staging of diabetes in the characterization of diabetic patients.
2) To test a putative association between late diabetic complications and candidate gene polymorphisms.

In study I we could show that the WHO clinical staging of diabetes could discriminate between clinically meaningful subgroups. The IRC patients represented a group with more severe diabetes than acknowledged in the etiological classification with high frequency of diabetic complications. In study II we demonstrated that polymorphisms in the UCP1-3 genes did not play a major role in the development of micro- or macroalbuminuria in Scandinavian diabetic patients. In study III we showed that a polymorphism in the MHC class II transactivator gene (MHC2TA) was associated with cardiovascular mortality and predictors of cardiovascular mortality, microalbuminuria and metabolic syndrome. In study IV and V we showed that polymorphisms in the LTA, TNF and AGER genes were associated with diabetic complications. The association was complex and dependent on the HLA-DQB1 genotypes, with partly different alleles conferring susceptibility in type 1 and type 2 diabetic patients. We cannot exclude that these genes are a part of a large haplotype block that also includes HLA-DQB1 risk genotypes.

Although this study revealed several associations with putative candidate gene polymorphisms and diabetic complications, the studied polymorphisms can only explain part of the genetic risk factors for diabetic complications. More studies are needed to enable mapping of the susceptibility genes for diabetic complications. Revealing the genetic risk factors could help us to identify the patients at risk and understand the pathogenesis of diabetic complications and making it possible to find novel treatments for diabetic complications.

Key words: diabetes mellitus, nephropathy, retinopathy, neuropathy, genetic, UCP, HLA-DQB1, MHC2TA, AGER, TNF, LTA

Classification system and/or index terms (if any):

Supplementary bibliographical information:

<table>
<thead>
<tr>
<th>Language</th>
<th>ISSN and key title</th>
</tr>
</thead>
<tbody>
<tr>
<td>English</td>
<td>ISSN 1652-8220</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Recipient’s notes</th>
<th>Number of pages</th>
<th>Price</th>
<th>Security classification</th>
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<td></td>
<td>117</td>
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V. **Lindholm E**, Bakhatadze E, Cilio CM, Agardh E, Groop L, Agardh C-D. Linkage disequilibrium between *LTA, TNF* and *AGER* polymorphisms and their association to late diabetic complications. (*Submitted*), 2007.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACE</td>
<td>angiotensin-I converting enzyme</td>
</tr>
<tr>
<td>AER</td>
<td>Urinary albumin excretion rate</td>
</tr>
<tr>
<td>AGER</td>
<td>Gene encoding the receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>DAG</td>
<td>1,2-diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DME</td>
<td>Diabetic macular edema</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNs</td>
<td>Diabetic neuropathies</td>
</tr>
<tr>
<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>GADA</td>
<td>Glutamic acid decarboxylase antibody</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRC</td>
<td>Insulin requiring for control</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin requiring for survival</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of the odds</td>
</tr>
<tr>
<td>LTα</td>
<td>Lymphotoxin alpha</td>
</tr>
<tr>
<td>MDC</td>
<td>Malmö Diet and Cancer Study</td>
</tr>
<tr>
<td>MHC2TA</td>
<td>Major histocompatibility complex II transactivator gene</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NIR</td>
<td>Not insulin requiring</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin angiotensin aldosterone system</td>
</tr>
<tr>
<td>RBX</td>
<td>Ruboxistaurin</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TNF</td>
<td>Gene encoding for tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
</tbody>
</table>
1. Introduction

The earliest known record of diabetes mentioned comes from the 3rd dynasty Egyptian papyrus by the physician Hesy-Ra [1]. The word diabetes means “going through” and was used by Aretaeus of Cappadocia in the 2nd century AD. The word mellitus is latin and refers to the sweetness of the urine from a diabetic subject [1]. The British physician George Harley commented in 1866 that “there are at least two distinct forms of the disease” and the French physician Etienne Lancereaux made a distinction between fat and thin diabetes: “Le diabete gras et le diabete maigre” [2]. This was indeed an important distinction as most of the children and young adults with diabetes died within a few months whereas the older persons who were only treated with diet could survive for years.

Until the early twentieth century the chances of a young diabetic surviving long enough to develop diabetic complications were poor, and the chances of an older patient to survive the attendant vascular complications long enough to develop diabetic nephropathy or retinopathy was equally poor.

Despite the obvious difficulties, the discovery of diabetic complications stretches back to the eighteenth and nineteenth centuries. Domenico Cotugno (1736-1822) wrote in 1770 probably the first proper description of proteinuria, noticing that he had seen diabetics with coagulable urine. The first one to suggest that the albuminuria seen in patients was caused by diabetes (and not the opposite) was the German physician Wilhelm Griesinger (1817-1868) [3].

Diabetic retinopathy was first documented by Eduard Jaeger in 1855 and proliferative retinopathy was described in 1876 by Wilhelm Manz [4]. The first clinical description of diabetic neuropathy was done by John Rollo of London in 1798, who described the pain and paresthesia in the legs of diabetic patients [5].

The discovery of insulin by Banting and Best in 1921 did indeed revolutionize the treatment of diabetes, but it also transformed the diabetes from an acute fatal illness to a chronic disease with serious long-term complications. Dr. Elliot P. Joslin wrote in 1931, only ten years after the discovery of insulin: “With the advent of insulin, we moved from the era of diabetic coma to the era of diabetic complications” [6].

Paul Kimmelstiel and Clifford Wilson published in 1935 a paper describing details of nodular renal lesions in eight maturity-onset (48-68 year old) diabetic patients. It was however Arthur Allen that in 1941 established these lesions to be specific for diabetes and by no means rare [3]. Similarly, although diabetic retinopathy was described earlier, it was first in 1943 that Arthur James Ballantyne suggested that diabetic retinopathy is a unique vasculopathy and not only a product of hypertension or atherosclerosis [4]. The final acceptance for the concept that diabetic microangiopathy was specific for diabetes came after work of Knud Lundbæk [7].

The prevalence of proliferative diabetic retinopathy (PDR) in the Wisconsin epidemiologic study of diabetic retinopathy (1980-1982) was 60% after 35 years of diabetes. The risk of severe visual loss from PDR is approximately 40% six years after onset of PDR if not treated with laser photocoagulation. The prevalence of legal blindness in the Wisconsin study was 3% in patients with a diabetes duration over 15-19 years and increased to 12% in those with a duration ≥ 30 years [9].

Several factors such as self monitoring of blood glucose, screening program and laser treatment of proliferative diabetic retinopathy and macular edema, more effective management of blood pressure treatment with ACE inhibitors have all contributed to better management of diabetes and decline in both nephropathy [10-14] and retinopathy [11, 14] in type 1 diabetic (T1D) patients as well as in type 2 diabetic (T2D) patients. Discovery of
glycated hemoglobin (HbA1c) finally made it possible to show in large studies such as the Diabetes Control and Complications Trial (DCCT) and the UK Prospective Diabetic Study (UKPDS) that better metabolic control could prevent diabetic complications in both T1D and T2D patients, respectively [15, 16].

The last decades have also meant new challenges as the prevalence of T2D is increasing (www.who.int) and better management of cardiovascular complications in T2D patients means longer survival and sufficient time to develop severe microvascular complications such as diabetic nephropathy and proliferative retinopathy. Diabetic nephropathy is today the most common cause of new cases of end stage renal disease (ERDS) in need for dialysis or renal replacement therapy in Sweden and the number of T2D patients with ERDS is increasing [17]. Diabetic retinopathy remains a major cause of severe visual impairment in the Western world [18].
2. Diabetes mellitus- diagnosis and classification

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Table 1 summarizes changes in the WHO diagnostic criteria for diabetes mellitus and intermediate hyperglycemia over time. The first WHO guidelines for the diagnosis and classification of diabetes was published in 1965 [19]. The first widely accepted classification system was published by WHO in 1980 [20] and modified in 1985 [21]. This classification included two major types of diabetes; insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetes mellitus. It was a compromise between clinical and etiological classifications and allowed classification of patients even when the specific cause or etiology was unknown. As more data on etiology and the importance of intermediate non-diagnostic glucose values emerged, a new diagnostic criteria and classification system was introduced by WHO in 1999 [22].

Table 1. Changes in WHO diagnostic criteria for diabetes and intermediate hyperglycemia over time.

<table>
<thead>
<tr>
<th>Year</th>
<th>1965</th>
<th>1980</th>
<th>1985</th>
<th>1999</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting P-Glucose</td>
<td>Not specified</td>
<td>Not defined</td>
<td>Not defined</td>
<td>&lt;6.1 mmol/l</td>
</tr>
<tr>
<td>2-h P-glucose</td>
<td>&lt;6.1 mmol/l</td>
<td></td>
<td></td>
<td>Not specified but &lt;7.8 mmol/l implied</td>
</tr>
<tr>
<td>IFG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting P-Glucose</td>
<td>Not defined</td>
<td>Not defined</td>
<td>Not defined</td>
<td>≥6.1 and &lt;7.0 mmol/l and &lt;7.8 mmol/l</td>
</tr>
<tr>
<td>2-h P-glucose</td>
<td></td>
<td></td>
<td></td>
<td>&lt;7.8 mmol/l (if measured)</td>
</tr>
<tr>
<td>IGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting P-Glucose</td>
<td></td>
<td>&lt;8.0 mmol/l and</td>
<td>&lt;7.8 mmol/l and</td>
<td>&lt;7.0 mmol/l and</td>
</tr>
<tr>
<td>2-h P-glucose</td>
<td>6.1–7.1 mmol/l</td>
<td>≥8.0 and &lt;11.0 mmol/l</td>
<td>≥7.8 and &lt;11.1 mmol/l</td>
<td>≥7.8 and &lt;11.1 mmol/l</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting P-Glucose</td>
<td>Not specified</td>
<td>≥8.0 mmol/l and / or</td>
<td>≥7.8 mmol/l or</td>
<td>≥7.0 mmol/l or</td>
</tr>
<tr>
<td>2-h P-glucose</td>
<td>≥7.2 mmol/l</td>
<td>≥11.0 mmol/l</td>
<td>≥11.1 mmol/l</td>
<td>≥11.1 mmol/l</td>
</tr>
</tbody>
</table>


Diagnostic criteria were based on either a fasting plasma glucose concentration above 7.0 mmol/l on two different days or a 2-hour 75 g oral glucose tolerance test (OGTT) value above
Diabetes mellitus

11.0 mmol/l [22]. A new category of impaired fasting glucose (IFG) was introduced and defined as fasting plasma glucose values below the diagnostic cut-off for diabetes (<7.0 but ≥6.1 mmol/l). Impaired glucose tolerance (IGT) was now defined as a 2-h glucose ≥7.8 but <11.1 mmol/l and fasting plasma glucose <7.0 mmol/l.

The diagnostic fasting plasma glucose concentration was lowered because several studies showed increased risk of microvascular [24] and macrovascular complications [25] in patients with normal 2-h OGTT values but fasting plasma glucose values ≥7.0 mmol/l.

Fasting plasma glucose of 7.0 mmol/l will also in most subjects represent a diagnostic 2-h post-load concentration [24, 25] and seems as an optimal cut off level to separate the bimodal frequency distribution of fasting plasma glucose levels [24].

In 2003 the American Diabetes Association (ADA) reviewed its diagnostic criteria and recommended a plasma glucose level of 5.6 mmol/l as a new threshold for IFG. The latest WHO report recommends that the current diagnostic criteria for diabetes should be maintained [23]. Because lack of evidence for benefits of lowering the cut off the study group recommended that the current cut off for IFG should be left unchanged. They also recommended the use of OGTT as a diagnostic test because it is the only way to identify IGT and because fasting plasma glucose alone fails to diagnose 30% of the patients with diabetes [23].

![Figure 1. Disorders of glycemia, etiological types and clinical stages. From [22].](image)

The new classification system of diabetes encompasses both etiological types and clinical stages of diabetes and different categories of hyperglycemia [22] (Figure 1). The etiological types are: 1) T1D, usually autoimmune, sometimes idiopathic, 2) T2D, which may range from predominantly insulin resistant with a relative insulin deficiency to a predominantly secretory defect with or without insulin resistance, 3) other specific types of diabetes, including monogenic forms, diseases in endocrine pancreas and 4) gestational diabetes.

Besides etiological processes, the new WHO criteria for classification of diabetes takes into account also clinical stages based upon the degree of glycemia and mode of treatment. The pre-diabetic stages are IFG and/or IGT. Diabetes mellitus is subdivided into three clinical
Diabetes mellitus

stages: not insulin requiring (NIR), insulin requiring for control (IRC) and insulin requiring for survival (IRS). All patients with diabetes mellitus can be categorized according to clinical stages and the stage of glycemia may change over time so that individual patients can move from one stage to another in either direction. The clinical classification is therefore a complement to the etiological classification, even though the WHO classification does not clearly define the difference between IRC and IRS stages [22].
3. Diabetic complications

3.1 Epidemiology

3.1.1 Natural history and epidemiology of diabetic nephropathy

The renal involvement in diabetes mellitus is a gradual process and rather well defined in T1D. Mogensen [26] suggested that the development of renal changes may be divided into five different stages (Table 2). Stage 1 is present at diagnosis of diabetes and includes hyperfunction/hypertrophy of the kidneys. Urinary albumin excretion rate (UAE) may be increased, however not permanently. Most of the abnormalities seen at this stage may be reversed with improvement of metabolic control by initiation of insulin treatment. Stage 2 usually lasts at least five years from diagnosis. The glomerular filtration rate (GFR) is increased and exercise-induced microalbuminuria may be present. Blood pressure is however normal. Stage 3 is present typically after 6-15 years of diabetes and UAE is 20-200 μg/min. Overt nephropathy (stage 4) occurs usually 15-25 years from the onset of T1D. GFR declines 10 ml/min per year and if not treated, the blood pressure is high. Stage 5 represents the final outcome of diabetic kidney disease with ESRD and usually occurs 25-30 years or more after diagnosis of diabetes. GFR is now <10 ml/min and blood pressure is always high if untreated.

Table 2. Stages of diabetic nephropathy.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Chronology</th>
<th>GFR</th>
<th>Baseline UAE</th>
<th>Exercise induced UAE</th>
<th>Main structural changes</th>
<th>BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Hyperfunction / hypertrophy</td>
<td>At diagnosis</td>
<td>↑↑</td>
<td>Normal or ↑</td>
<td>↑</td>
<td>Kidney hypertrophy, increased glomerular size</td>
<td>Normal</td>
</tr>
<tr>
<td>2 Normo-albuminuria</td>
<td>First five years</td>
<td>↑↑</td>
<td>Normal</td>
<td>↑</td>
<td>Increasing basement membrane thickness and mesangium expansion</td>
<td>Normal, Increase by 1 mmHg/ year</td>
</tr>
<tr>
<td>3 Incipient diabetic nephropathy</td>
<td>After 6-15 years from diagnosis (in ≈ 35% of patients)</td>
<td>↑</td>
<td>20-200 μg/min</td>
<td>↑</td>
<td>Increasing glomerular occlusion and severe mesangial expansion</td>
<td>High</td>
</tr>
<tr>
<td>4 Proteinuria</td>
<td>After 15-25 years from diagnosis.</td>
<td>↓↓</td>
<td>&gt;200 μg/min, increasing</td>
<td>↑↑↑</td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>5 End-stage renal failure</td>
<td>After 25-30 years from diagnosis.</td>
<td>↓↓↓</td>
<td>Decline</td>
<td>Not studied</td>
<td></td>
<td>High</td>
</tr>
</tbody>
</table>

Adapted from [26]. BM=basement membrane, UAE=urinary albumin excretion rate, BP= blood pressure

The course of kidney disease seems to be similar in T2D [26] with some important exceptions. High blood pressure is normally present in T2D patients even before the onset of
Diabetic complications

diabetic nephropathy. Because T2D can remain undiagnosed several years, microvascular complications like nephropathy may be present already at the time of diagnosis. The peak incidence of nephropathy in T1D is 15 years from the onset of diabetes. Patients who do not develop nephropathy during the first 20-25 years of diabetes have a very low risk to develop nephropathy later on (about 1 % per year) [27]. It has been estimated that diabetic nephropathy will ultimately develop in 35 % of the patients with T1D [8]. The incidence of diabetic nephropathy in T1D patients is however declining as shown in several populations [10-14]. The prevalence of diabetic nephropathy has previously been reported to be lower in T2D than in T1D patients. The prevalence of diabetic nephropathy seems to differ between different ethnic groups. Because proteinuria is a risk factor for cardiovascular disease it is possible that previous studies underestimate the prevalence of diabetic nephropathy. It seems that the risk for proteinuria at any given duration of diabetes is similar in both T1D and T2D. As 90% of the diabetic patients have T2D and the diabetes prevalence is increasing, there has also been a rise in the prevalence of diabetic nephropathy and diabetes is now the most common single cause of ESRD in Europe [28].

3.1.2 Natural history and epidemiology of diabetic retinopathy

The early stage of diabetic retinopathy (DR) is characterized by loss of retinal pericytes. This is followed by development of weakness in the capillary wall that leads to formation of microaneurysm and leakage from capillaries as their walls become more permeable. Impaired vascular function gradually develops leading to areas of ischemia and infarction. In response to these changes local growth factors are secreted that contribute to new vessel formation.[29]. Macular edema (ME), and proliferative retinopathy are the two major sight threatening manifestations of diabetic retinopathy and they represent the end manifestations of increased vascular permeability and vascular occlusion. Several clinical classifications have been proposed and in 2002 the Global Diabetic Retinopathy Project Group agreed upon a retinopathy scale, which consists of five different stages (Table 3).

Table 3. Diabetic Retinopathy Disease Severity Scale*

<table>
<thead>
<tr>
<th>Proposed disease severity level</th>
<th>Findings observable on dilated ophtalmoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No apparent</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>Mild nonproliferative diabetic retinopathy</td>
<td>Microaneurysms only</td>
</tr>
<tr>
<td>Moderate nonproliferative diabetic retinopathy</td>
<td>More than just microaneurysms but less than severe nonproliferative diabetic retinopathy</td>
</tr>
<tr>
<td>Severe nonproliferative diabetic retinopathy</td>
<td>Any of the following: more than 20 intraretinal hemorrhages in each of 4 quadrants; definite venous beading in 2+ quadrants; Prominent intraretinal microvascular abnormalities in 1+ quadrant and no signs of proliferative retinopathy</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>One or more of the following: neovascularization, vitreous/preretinal hemorrhage</td>
</tr>
</tbody>
</table>

*From [30].

Macular edema (if present) is divided in three categories: 1) Mild with some retinal thickening or hard exudates in the posterior pole but distant from the center of the macula, 2) Moderate with retinal thickening or hard exudates approaching the center of the macula but
not involving the center, and 3) Severe with retinal thickening or hard exudates involving the center of the macula [30].

DR is still the leading cause of blindness in older adults (45-74 years) accounting for more than one third of the cases and the fourth common cause of blindness in younger adults (15-44 years) in the Western world [18]. The prevalence of diabetic retinopathy is correlated to diabetes duration but unlike nephropathy, retinopathy shows no decline in incidence after 15-20 years of diabetes duration. The Wisconsin study reported a 70% overall prevalence of diabetic retinopathy in T1D patients (onset of diabetes before 30 years) and 39% in insulin treated T2D patients (age at onset ≥30 years). Prevalence of proliferative diabetic retinopathy (PDR) was 23% and 14%, respectively, and clinically significant ME 14% and 11% in T1D and insulin treated T2D patients, respectively [31]. With increased duration retinopathy prevalence reaches almost 100% in T1D patients and 85% in patients with insulin treated T2D [32]. The incidence of severe retinopathy seems to be declining, perhaps due to better metabolic control [11, 14]. In a 14-year follow-up to the Wisconsin Epidemiologic Study of Diabetic Retinopathy, the cumulative incidence of PDR over a period of 15 years in persons with T1D was still 37% but there appeared to be a decline in the estimated annual rates of progression to proliferative retinopathy and the incidence of ME in the last 4-year period of the study compared to earlier periods of the study [33]. A Swedish study from Linköping showed also that the cumulative proportion of severe retinopathy in T1D patients diagnosed in childhood is declining [11].

3.1.3 Natural history and epidemiology of diabetic neuropathy

Diabetic neuropathy encompasses a wide range of nerve abnormalities and is common, with prevalence rates reported between 5–100% depending on the diagnostic criteria [34-36]. Due to the variety of clinical manifestation there is no universally accepted classification of diabetic neuropathy. Neuropathy is often divided into sensorimotor and autonomic neuropathy (Table 4).

### Table 4. Classification of diabetic neuropathy

<table>
<thead>
<tr>
<th>Sensorimotor neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal symmetric polyneuropathy</td>
</tr>
<tr>
<td>Focal neuropathy</td>
</tr>
<tr>
<td>Diabetic mononeuropathy (cranial, truncal, peripheral nerves)</td>
</tr>
<tr>
<td>Mononeuropathy multiplex</td>
</tr>
<tr>
<td>Diabetic amyotrophy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autonomic neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoglycemic unawareness</td>
</tr>
<tr>
<td>Abnormal pupillary function</td>
</tr>
<tr>
<td>Cardiovascular autonomic neuropathy</td>
</tr>
<tr>
<td>Vasomotor neuropathy</td>
</tr>
<tr>
<td>Sudomotor neuropathy (sweat glands)</td>
</tr>
<tr>
<td>Gastrointestinal autonomic neuropathy</td>
</tr>
<tr>
<td>Gastric atony</td>
</tr>
<tr>
<td>Diabetic diarrhea or constipation</td>
</tr>
<tr>
<td>Fecal incontinence</td>
</tr>
<tr>
<td>Genitourinary autonomic neuropathy</td>
</tr>
<tr>
<td>Bladder dysfunction</td>
</tr>
<tr>
<td>Sexual dysfunction</td>
</tr>
</tbody>
</table>

From [37].
In this study we have only considered distal symmetric polyneuropathy also known as diabetic peripheral neuropathy (DPN). DPN is a causal factor in most foot ulcers and the incidence of foot ulcers increases three-fold in patients with DPN [38]. Several studies have suggested that improved glycemic control will be beneficial in preventing diabetic nephropathy [15, 39, 40]. There are however no population based studies on trends in the incidence of neuropathy. Treatment strategies of diabetic neuropathy based on pathogenetic mechanisms like aldose reductase inhibitors or protein kinase C inhibitor (ruboxistaurin) have been disappointing so far [41, 42].

3.1.4 Natural history and epidemiology of macrovascular complications

Macrovascular disease is the leading cause of mortality in subjects with diabetes and also a major cause of morbidity [43]. The major manifestations of macrovascular disease include heart disease, cerebrovascular disease and peripheral vascular disease. The prevalence and incidence of macrovascular disease in diabetes mellitus is still not very well studied [43] and there are many sources for potential biases. Both diabetes mellitus and macrovascular disease are common conditions and can therefore occur together by chance. Both diseases can also be subclinical for years and silent coronary heart disease is more common in diabetic than non-diabetic subjects [44] which might underestimate the prevalence. T2D patients tend to have a more severe diabetes with longer duration and studies based on subjects with already known diabetes are likely to give falsely high prevalence of macrovascular disease in T2D patients [43]. The risk for coronary heart disease is almost 10-fold in T1D men and even greater in women compared to non-diabetic subjects [43]. T2D male patients have a 2-3 fold risk of coronary heart disease, and the risk is even greater in women. The risk for stroke is approximately 2-fold increased in persons with known or newly diagnosed diabetes [45]. Diabetes is also a well known risk factor for peripheral vascular disease. The prevalence of intermittent claudication was 3 times higher in diabetic men and almost 6 times higher in diabetic women than in non-diabetic subjects of the same sex [46]. The prevalence of peripheral vascular disease in the UKPDS was 1.2% at diabetes diagnosis and increased to 12.5% by 18 years [47] with hyperglycemia, dyslipidemia, blood pressure and smoking as associated risk markers.

3.2 Pathogenesis of diabetic complications

3.2.1 Environmental factors

The occurrence of hyperglycemia is an absolute condition for development of diabetic nephropathy, retinopathy and neuropathy and several large studies have shown the importance of glycemic control in preventing microvascular complications [15, 16]. In contrast, the role of glycemic control in macrovascular complications is not as obvious. It seems though as diabetes mellitus itself is a risk factor for macrovascular disease and some studies have also shown association between glycemic control and the risk for coronary heart disease [48-50], whereas more aggressive treatment of glycemia in the UKPDS study did not show any benefits in terms of lower prevalence of MI [39]. However, the DCCT study group could recently show that T1D patients in the intensive treatment group had significantly less atherosclerosis than the conventionally treated group suggesting that better glycemic control might prevent atherosclerosis in T1D [51].

Diabetes duration [52] and smoking [53] are both risk factors for micro- and macrovascular complications. Male gender has been a risk factor for diabetic nephropathy [54] but not for diabetic retinopathy in most of studies [55].

Elevated blood pressure is a risk factor for diabetic nephropathy, retinopathy and macrovascular disease [52]. Elevated blood pressure is usually present at the time of
Diabetic complications

diagnosis of T2D, whereas in T1D blood pressure usually rises at the onset of microalbuminuria [56]. In the UKPDS tight blood pressure control was shown to cause a 34% reduction in progression of retinopathy and a 47% reduced risk of deterioration in visual acuity of three lines (ETRDS scale) in association with a 10/5 mm Hg reduction in blood pressure [57]. The same study showed after six years a 29% reduction in risk of having urinary albumin concentration >50 mg/l.

There are also complex interrelationships between different diabetic complications so that presence of one diabetic microvascular complication influences the risk of developing a second complication. Proliferative retinopathy is associated with microalbuminuria [58] although 35% of T1D patients with proliferative retinopathy did not show any signs of nephropathy [59] which suggests that at least partially different patophysiological mechanisms might be operative behind retinopathy and nephropathy.

Severity of diabetic nephropathy is associated with the severity of retinopathy and prevalence of diabetic neuropathy. Microalbuminuria is also known to be a risk marker for macrovascular disease and presence of retinopathy in T2D is associated with increased risk for coronary heart disease, independent of other known risk factors [60]. Presence of autonomic neuropathy has been associated with both diabetic nephropathy and retinopathy [61].

Ethnic background has been suggested to play role for development of diabetic nephropathy. The incidence and severity of diabetic nephropathy are increased in blacks, Mexican-Americans, and Pima Indians with T2D compared to the white population. Although some of the variation is due to differences in prevalence of hypertension and socio-economic background it seems that even after correction for hypertension and socio-economic status there is still an increased risk of ESRD caused by diabetic nephropathy in blacks [62].

Pregnancy is known to be a risk factor both for development and progression of diabetic retinopathy and sudden improvement in poorly controlled diabetes may cause a rapid worsening of retinopathy [63].

Lipid abnormalities are already present in the microalbuminuric stage and increased serum triglyceride levels are an independent risk factor for diabetic nephropathy in T1D [64] and T2D [65].

3.2.2 Major pathogenic pathways

Protein kinase C

Protein kinase C (PKC) constitutes a superfamily of serine-threonine kinase isoenzymes, many of which are activated by cofactors such as diacylglycerol (DAG) and phosphatidylserine [66]. The DAG-PKC pathway is activated in diabetes mellitus because of an increased de novo synthesis of DAG. High glucose can through PKC dependent mechanisms induce formation of reactive oxygen species (ROS) in the cell [66]. Activation of PKC leads to phosphorylation of a variety of target proteins and PKC appears to be important in the pathogenesis of diabetic complications. The first clinical studies on the selective PKC-β inhibitor ruboxistaurin mesylate (RBX) showed some beneficial effects on diabetic complications; urinary albumin excretion was reduced and GFR maintained during one year treatment with RBX [67]. RBX had no effect on progression of diabetic retinopathy even if it seemed to reduce the risk of visual loss [68]. RBX did not have any effect on vibration threshold or neurological symptoms, although patients with less severe neuropathy showed some improvement in symptoms and nerve function [42].
**Diabetic complications**

**Polyol Pathway**
Reduction of glucose by aldose reductase (AR) leads to the formation of sorbitol, which, in some tissues is further oxidized to fructose upon sorbitol dehydrogenase-catalyzed oxidation. Conversion of glucose to fructose results in utilization of NADPH and NAD⁺ which might lead to a state of pseudohypoxia with depletion of NADPH and accumulation of reduced NAD. Increase in NADH due to increased polyol pathway activity could lead to synthesis of DAG and activation of the protein kinase C pathway. Accumulation of sorbitol in the cell also leads to osmotic stress and membrane damage [69]. Inhibition of the polyol pathway with specific aldose reductase inhibitors seemed a promising pharmacological approach to prevent microvascular diabetic complications, but the outcomes of clinical trials have been disappointing so far. Most studies showed only modest improvement with multiple side effects [69].

**Glycoxidation**
Advanced glycation end products (AGEs) are formed as a result of a complicated series of reactions between glucose, fructose, or glycolytic intermediates and amino groups of proteins, lipids, or nucleic acids [70]. The reaction begins with glucose attachment to amino groups, thus forming a reversible Schiff base adduct. This reaction occurs over a period of hours, and the Schiff base in turn undergoes a slow intramolecular rearrangement to form Amadori products. Amadori products were previously thought to be practically irreversible [70], but a new mechanism involving enzymatic deglycation has recently been discovered [71]. Glycated proteins can undergo further reactions to form AGEs. Formation of AGEs is non-enzymatic and therefore dependent only on concentration and temperature. Macrophages and other cellular systems can endocytose and degrade AGEs via receptor or non-receptor pathways, resulting in low molecular weight AGE peptides which can be catabolised and excreted through the kidneys [72] and AGEs will therefore accumulate in kidney failure.
AGEs constitute a heterogenous group of molecules and they can cause tissue damage by cross-links that disrupt the structure and function of proteins and lipids [72]. AGE cross-links kidney matrix proteins lead to changes in their structure and function. These changes can be inhibited in diabetic animals by administration of the cross-link breaker ALT-711 [73]. AGEs accumulate in the retina of patients with diabetic retinopathy and AGE accumulation is also found in different nervous tissues and vascular wall where AGEs form cross-links [72].
AGEs can also cause tissue damage by interacting with cell surface receptors which leads to altered intracellular events that induce oxidative stress and inflammation [72]. AGE receptors have been found in renal mesangial cells and binding of AGE to its receptors leads to increased extracellular matrix production, induction of oxidative stress and activation of PKC-β [72]. RAGE overexpression in diabetic mice resulted in increased albuminuria, renal hypertrophy, elevated serum creatinine, mesangial expansion and glomerulosclerosis [74]. The galectin-3 (AGE-R3) knockout mice showed accelerated AGE-induced glomerular injury [75] suggesting protective effect of the galectin-3 receptor against AGE induced renal injury. Retinal endothelial cells exposed to AGE overproduce vascular endothelial growth factor (VEGF) through oxidative stress induction, PKC activation and abnormal endothelial nitric oxide synthase (eNOS) expression. In a recent study systemic administration of sRAGE significantly inhibited blood-retinal barrier breakdown, leukostasis, and expression of ICAM-1 in the retina in the diabetic C57/BJ6 and RAGE-transgenic mice [76].
Several ways of reducing AGE-induced damage in diabetes have been proposed. Better glycemic control will reduce formation of AGEs and this can be further reduced by smoking cessation and dietary measures [72]. Antioxidants can potentially protect against glycation...
derived free radicals and some of them like pyridoxamine and benfotiamine can inhibit AGE formation; the latter is now in phase II trials [77]. Some of the drugs already used in treatment of diabetes mellitus including metformin, pioglitazone, ACE inhibitors and angiotensin II receptor inhibitors, have shown to also have AGE inhibiting activity [77]. Acetylsalicylic acid has shown to reduce glycation in vitro and in animal experiments and salicylate based anti-inflammatory drugs can inhibit the early lesions of diabetic retinopathy in rats [77]. The best studied specific AGE blocker is aminoguanidine. It reacts with with carbonyl groups from reducing sugars and 3-deoxyglucosones and in diabetic animals aminoguanidine can prevent/reduce formation of diabetic nephropathy [78], retinopathy [79] and neuropathy [80].

In a clinical trial aminoguanidine has been shown to have protective effects against diabetic retinopathy and nephropathy [83]. A similar trial on T2D patients was, however, prematurely stopped due to serious side effects [70].
Oxidative stress - a common mediator?

Nishikawa et al. have shown that inhibition of mitochondrial superoxide production could block all three major pathways of hyperglycemic damage [84]. They blocked the formation of reactive oxygen species (ROS) by an inhibitor of electron transport chain complex II, by an uncoupler of oxidative phosphorylation and by uncoupling protein-1 and by manganese superoxide dismutase (MnSOD). In each case they could prevent glucose induced activation of PKC, formation of AGEs and sorbitol accumulation in the cell. They suggest that formation of superoxide in the mitochondria is a causal link between elevated glucose and each of the three main pathways responsible for hyperglycemic damage. Recently Vecchione et al. showed that some of the beneficial effects of statins are mediated through reduction of oxidative stress in diabetic vasculature [85].

Inflammation

Inflammation may play a role in the pathogenesis of obesity, insulin resistance and T2D [86]. In diabetic nephropathy, several pro-inflammatory cytokines are secreted by blood-borne cells mainly as monocytes and macrophages, as well as intrinsic renal cells [87]. Upregulation of monocyte chemotactic protein-1 is a feature of diabetic renal injury and is associated with macrophage recruitment and progression of diabetic nephropathy [88, 89]. Intracellular adhesion molecule-1 (ICAM-1) is involved in the activation of leukocytes and macrophages to sites of inflammation and patients with nephropathy have elevated concentrations of ICAM-1 [90, 91]. The proinflammatory cytokines IL-1β, IL-6, IL-18 and TNF-α have also been associated with diabetic nephropathy both in experimental models of diabetes and in clinical studies [87]. IL-1β stimulates mesangial cell proliferation and extracellular matrix synthesis, which would lead to expansion of the mesangium and thickening of the glomerular basement membranes. IL-1β has also been involved in the development of intraglomerular microcirculatory abnormalities related to the stimulation of prostaglandin synthesis by mesangial cells and it increases endothelial procoagulant activity and endothelial permeability [87]. IL-6 has been related to increased glomerular basement membrane thickening and it enhanced fibronectin expression. It also affects extracellular matrix dynamics at both mesangial and podocyte level, stimulates mesangial cell proliferation, and increases endothelial permeability [87]. IL-18 has been independently associated with urinary albumin excretion rate in T2D subjects [92]. TNF-α on the other hand, plays a critical role in the development of microvascular diabetic complications, including nephropathy [93]. It does not only mediate the inflammatory response but also reduces glomerular blood flow and filtration rate, induces damage to the glomerular permeability barrier which, in turn, will lead to albuminuria, apoptosis, recruitment of inflammatory cells to the kidney and increased coagulant activity [93].

Diabetic retinopathy shows many features of chronic inflammation, i.e., increased nitric oxide production, ICAM-1 upregulation, leukostasis and increased vascular permeability. Chronic hyperglycemia can cause a low-grade chronic inflammation in the retina which in turn leads to increased production of cytokines, tissue damage and cell death [94]. The role of inflammation in diabetic neuropathy is not as well studied as in retinopathy and nephropathy but a recent study by González-Clemente et al. showed that the activity of the TNF-α system is increased in subjects with T1D and diabetic neuropathy suggesting that inflammation may play a pathogenic role also in the development of diabetic neuropathy [95]. Inflammation plays also a crucial role in diabetic macrovascular disease by promoting and destabilizing atherosclerotic plaques [96]. Several inflammatory markers have been linked to cardiovascular disease progression, but their clinical usefulness is still unclear [96].
3.2.3 Genetic factors

Familiar clustering and heritability

The challenge of family studies on diabetic complications is that there must be an aggregation of both diabetes and the specific complication in the family why studies on familial aggregation of diabetic complications are rare. Evidence that genetic susceptibility plays an important role in diabetic nephropathy in T1D was first presented by Seaquist et al. [97] who could show that 83% of siblings of probands with diabetic nephropathy also suffered from renal disease, compared with 17% of those without nephropathy. Borch-Johnsen et al. [98] also showed that there was a familiar clustering of diabetic nephropathy and concluded that this could be either due to shared genetic or environmental factors as the HbA1c correlated within sib-pairs. The heritability of urinary albumin excretion rate in Finnish nuclear families was 30% being strongest from mothers to sons [99]. Both albumin-creatinine ratio and GFR showed strong familiality, having a heritability of 75% and 46% in Caucasians with T2D, respectively [100]. The cumulative risk of diabetic nephropathy in T1D patients after 30 years duration was 71.5% in the siblings of index cases with nephropathy and 25.4% in siblings of index cases without nephropathy implicating familial aggregation of diabetic nephropathy in T1D [101]. Retinopathy, on the other hand seems to affect all patients to some extent. The DCCT found familiar clustering of severe diabetic retinopathy and nephropathy in T1D patients [102]. Familial aggregation of severe retinopathy was also demonstrated in Mexican American T2D patients [103]. A recent study of 322 families with at least two T2D siblings from Southern India reported a 3-fold increased risk for retinopathy in siblings of probands with retinopathy compared to siblings of those without [104].

Previous studies suggest an ethnic predisposition to diabetic neuropathy. T1D Algerians develop diabetic neuropathy earlier and more often than French T1D patients [105]. However, no studies on heritability of diabetic neuropathy has been performed so far and our knowledge of genetic risk factors for diabetic neuropathy is mainly derived from different association studies on candidate genes.

In contrast to neuropathy, the heritability of cardiovascular disease is well studied. The Framingham Heart Study demonstrated that a positive family history of a parent [106] or a sibling [107] is a risk factor for coronary artery disease (CAD) and the familial risk is greater the lower the age at first manifestation of the disease in affected family members [108]. A family history of stroke and MI was associated with stroke at a younger age [109]. A recent study suggested that heritability of ischemic stroke was greater in women than in men, with an excess of affected mothers and sisters in female probands independently of traditional vascular risk factors [110]. A family history of peripheral vascular disease [111] and premature cardiovascular disease [112] are risk factors for peripheral vascular disease in young adults.

Genome-wide scans

A genome-wide linkage analysis requires no previous knowledge of the putative gene. In a genome-wide scan, markers randomly spread over the entire genome cover all chromosomes. If one marker is inherited from parents more often to affected than to unaffected individuals than predicted, the region is considered linked to the disease and may harbor a susceptibility gene. Linkage is reported as a logarithm of the odds (LOD) score and a genome wide linkage requires a LOD score >3.6 and suggestive evidence for linkage a LOD score of 2.2 [113]. The first genome scan search for nephropathy and retinopathy loci was performed in 98 Pima Indian sibling pairs concordant for diabetic nephropathy. The strongest evidence for linkage
to nephropathy was observed on chromosome 7q (LOD score 2.7) [114]. Vardarli et al. reported a strong linkage (LOD score 6.6) to nephropathy on chromosome 18q22.3-23 in Turkish T2D patients [115]. A genome scan for diabetic nephropathy in 206 African American T2D sibling pairs concordant for severe diabetic kidney failure (ESRD or advanced diabetic nephropathy) from 166 families showed no LOD scores above 2.0. In an ordered subset analysis there was nominal support for susceptibility locus on chromosome 18q (LOD score 3.72) [116].

A linkage analysis in 63 families with multiple members with T2D found support for linkage to albumin-creatinine ratio on chromosome 22q (LOD score 3.7) and chromosome 7q (LOD score 3.1). When the analysis was restricted to 59 Caucasian families, support for linkage in all relatives increased and became significant for 5q (LOD score 3.4) [117].

Genome-wide scan for T1D nephropathy in Finns did not show any significant linkage. However, one locus on 3q reached suggestive linkage (LOD score 2.7) [118]. In a genome-wide search for linkage to renal function in West Africans with T2D there was observed to creatinine clearance on chromosome 16 (LOD score 3.6) [119]. Recently, the first results from a large genome-wide scan from the Family Investigation of Nephropathy and Diabetes (FINDD) study were published [120]. This study found nominal evidence for linkage to diabetic nephropathy on chromosomes 7q21.3, 10p15.3, 14q23.1, and 18q22.3, and to albuminuria on 2q14.1, 7q21.1 and 15q26.3. Results regarding 7q, 10p and 18q were replications from linkage to diabetic nephropathy in other populations [120].

The first genom-wide scan on diabetic retinopathy in Pima Indians suggested nominal linkage between regions on chromosomes 3 and 9 and the occurrence of retinopathy in 136 affected siblings (103 affected pairs) with T2D, with a maximum multipoint LOD score of 1.46 for the region on chromosome 9 [114]. Recently, a larger scan for diabetic retinopathy susceptibility genes in 393 Mexican American families showed suggestive linkage for any retinopathy on chromosomes 3 (LOD score 2.4) and 12 (LOD score 2.5). In an ordered subset analysis ranking families by average age of diabetes diagnosis LOD scores > 2.0 for any retinopathy occurred on chromosomes 12 (LOD score 4.47), 15 (LOD score 3.65) and 20 (LOD score 2.67) while LOD scores >2.0 was seen for either moderate-to-severe nonproliferative or proliferative retinopathy on chromosomes 5 (LOD score 2.53), 6 (LOD score 2.2), and 19 (LOD score 2.21) [121]. A genome-wide linkage analysis that was conducted in 211 Pima Indian sibships showed suggestive linkage for diabetic retinopathy on chromosome 1p (LOD score 3.1) [122].

Several genome-wide scans have tried to find linkage to cardiovascular disease but none of them has been specifically conducted in diabetic patients. Genome scans suggested loci linked to coronary artery disease on chromosome 2q21-22 (LOD score 3.2), Xq23-26 (LOD score 3.5) in a Finnish population [123] and on 16p13 (LOD score 3.0) in North-Eastern Indian populations [124]. Other scans performed in German [125] and US populations [126] suggested linkage between MI and regions on chromosome 14q (LOD score 3.9) and 1p34-36 (LOD score 11.68), respectively, whereas stroke was linked to to a region on chromosome 5q (LOD score 4.4) in an Icelandic population [127].

**Candidate genes for diabetic nephropathy**

A large number of genes have been associated with diabetic nephropathy, but most of the studied gene variants has not been convincingly replicated in different populations. The candidate genes in the majority of the studies have been chosen on the basis of complex pathways that are believed to be involved in the pathogenesis of diabetic nephropathy including increased activity of a variety of growth factors and cytokines (i.e., transforming growth factor beta (TGF-β), growth hormone (GH), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF)); activation
of PKC isoforms; increased release of renin, angiotensin, endothelin, and bradykinin; formation of ROS; increased formation of AGEs; increased activity of the aldose reductase pathway; and abnormalities in glucose transport mechanisms [128]. Best studied are genes that are involved in the renin-angiotensin-aldosterone system (RAAS), especially angiotensin-I converting enzyme insertion/deletion (ACE I/D) polymorphism. Despite a plethora of studies, the role of ACE I/D polymorphism in the pathogenesis of diabetic complications is still unclear. A recent meta-analysis of 47 studies published between 1994-2004 including 14,727 subjects, suggested that the ACE II polymorphism is protective against diabetic nephropathy in Asian T2D patients but not in Caucasian T1D or T2D patients [129]. The ACE I/D polymorphism seems, however, to affect the response to treatment with ACE-inhibitors, and the renoprotective effect is mainly seen in patients with the II genotype [130, 131]. Table 5 summarizes some of the candidate genes for diabetic nephropathy. All of the genes presented have been shown to be associated with diabetic nephropathy in at least two different populations.

Candidate genes for diabetic retinopathy
Candidate genes for diabetic retinopathy have also been selected from the proposed pathogenic pathways that include the polyol pathway, AGEs, the renin-angiotensin system, growth factors (VEGF, GH and IGF-1), oxidative damage and ROS, PKC, GLUT1, PPARγ, extracellular matrix homeostasis and tissue matrix metalloproteinases, inflammation, trombogenesis, apolipoprotein (a) and vitamin D [132]. VEGF is a central regulator of both physiological and pathological angiogenesis and plays an important role in neovascularisation of proliferative retinopathy and increased vascular permeability of diabetic macular edema [133]. Several studies have shown associations between polymorphisms in the VEGF gene and diabetic retinopathy [134-137] and treatment with anti-VEGF aptamer (Pegaptanib) has in a phase II study shown to have beneficial effects in treatment of diabetic macular edema [138].

A (A-C)n repeat polymorphism in the aldose reductase gene (AKR1B1) has been associated with diabetic retinopathy in different ethnic populations [132]. Clinical trials with aldose reductase inhibitors have, however, been a disappointment [139]. The ACE I/D polymorphism has been intensively studied also in diabetic retinopathy, mostly with negative results [132]. Table 6 lists candidate genes and studies, that have shown association with diabetic retinopathy in more than one population.

Candidate genes for diabetic neuropathy
There are few studies on candidate genes for diabetic neuropathy and most of the positive associations lack confirmation in other populations. Perhaps the best studied gene is the aldose reductase gene [145-149], but low sample size and different ways of characterizing diabetic neuropathy have given conflicting results. The D allele of the ACE I/D polymorphism has been associated with increased risk for diabetic neuropathy in Turkish T2D patients [140] and in female but not male British T2D patients [141]. Also the genes for apolipoprotein E (APOE) [142], uncoupling protein 2 (UCP2) [143], genes encoding the enzymes Mn-SOD, the extracellular superoxide dismutase (EC-SOD) [144], catalase [145], Na/K ATPase (ATP1-A1) [146], tumor necrosis factor receptor 2 (TNFRSF1B) [147] and human leukocyte antigen (HLA) [148] have been associated with diabetic neuropathy.

Table 7 summarizes some of the candidate genes for diabetic nephropathy all of which have been associated with diabetic neuropathy in at least one population.
Diabetic complications

Candidate genes for diabetic macrovascular disease

Atherosclerosis is a very complex disease or condition with probably hundreds of susceptibility genes [149]. In a recent review on susceptibility genes for MI the authors could find almost 5000 studies on candidate genes for CAD and MI [150]. Positive and reproducible findings were shown for 192 polymorphisms from 102 genes in at least two independent populations. Most studies have investigated the renin-angiotensin system, lipid metabolism, inflammation and the clotting cascade [150]. The situation is even more complicated by the fact that many of the cardiovascular risk factors like blood pressure, diabetes, and lipid levels have themselves a significant genetic component [149] and candidate genes for these traits might therefore affect susceptibility to cardiovascular disease.

Several genome-wide scans for CAD and stroke have identified loci of interest [151]. However, only the locus on 2p11 has been replicated in two independent studies, one American [126] and one British [152]. Three genes responsible for MI and/ or stroke have been identified in genome-wide scans: a four marker SNP haplotype of ALOX5AP-5 lipooxygenase activating protein (FLAP) [153], a five to seven SNP marker haplotype of leukotrien A4 hydroxylase (LTA4H) [154] and a gene encoding phosphodiesterase 4D (PDE4D) [127]. Two genome-wide association studies have been conducted. The first one by Ozaki et al. assessed 92,788 SNPs in 13,738 genes. They mapped a susceptibility locus to the lymphotixin-a gene with an odds ratio of 1.8 for MI. In a follow up study they showed that variation in the gene encoding for galecin-2 (LGALS2) was associated with MI. Shiffman et al. [155] used 11,053 SNPs in 6891 genes in a genome-wide association study to identify four gene variants associated with MI: Palladin (a cytoskeletal protein), ROSI (a tyrosine kinase) and two G-protein coupled receptors TAS2R50 and OR13G1. In a follow up study they tested 11,647 SNPs in three case and control cohorts [156]: two variants in a gene modulating platelet degranulation (VAMP8) and a gene encoding for ribonuclear protein (HNRPUL-1) were associated with MI. The palladin and ROSI SNPs were tested also in the follow up study but were not associated with MI. A recent genome-wide association study could identify three susceptibility locus that were found associated with coronary artery disease in the Wellcome trust case control consortium and replicated in a German MI family study [157]. The strongest association was found on chromosome 9p21.3 (SNP, rs1333049) (p=1.80x10-14 and p=6.12x10-5, respectively) and two other loci were on chromosome 6q25.1 (rs6922269, p=6.33x10-6 and p=0.009, respectively ) and on chromosome 2q36.3 (rs2943634, p=1.19x10-5 and p=0.03, respectively).
### Table 5. Candidate genes for diabetic nephropathy, *

<table>
<thead>
<tr>
<th>Gene</th>
<th>OMIM name</th>
<th>Role</th>
<th>Reference</th>
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<tbody>
<tr>
<td>AGE receptor</td>
<td>AGER</td>
<td>Glycation</td>
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<td>AKR1B1</td>
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<td>[164, 165]</td>
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<td>[166, 167]</td>
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<td>[175, 176]</td>
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<td>Free radical scavenger ?</td>
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<td>[179-181]</td>
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<td>SLC2A1</td>
<td>Glucose transport in glomeruli</td>
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<td>HFE</td>
<td>Diabetes, iron overload in kidney</td>
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<tr>
<td>Lipoprotein Lipase</td>
<td>LPL</td>
<td>Lipid metabolism</td>
<td>[171, 197-199]</td>
</tr>
<tr>
<td>Manganese superoxide dismutase (Mn-SOD) (Mitochondrial superoxide dismutase)</td>
<td>SOD2</td>
<td>free radical scavenging enzyme</td>
<td>[200-202]</td>
</tr>
<tr>
<td>Matrix metalloproteinase-9</td>
<td>MMP9</td>
<td>Inflammation</td>
<td>[183, 203, 204]</td>
</tr>
<tr>
<td>Methylene tetrahydrofolate reductase</td>
<td>MTHFR</td>
<td>Hyperhomocysteinemia, endothelial dysfunction</td>
<td>[205-207]</td>
</tr>
<tr>
<td>Nitric oxide synthase 3 (eNOS)</td>
<td>NOS3</td>
<td>Blood pressure</td>
<td>[208-210]</td>
</tr>
<tr>
<td>Paraoxonase 1</td>
<td>PON1</td>
<td>Lipid oxidation</td>
<td>[211, 212]</td>
</tr>
<tr>
<td>Paraoxonase 2</td>
<td>PON2</td>
<td>LDL oxidation, associated with CAD.</td>
<td>[213, 214]</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor-gamma</td>
<td>PPARG</td>
<td>Inflammation, insulin resistance, type 2 diabetes matrix turnover</td>
<td>[215, 216]</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1</td>
<td>PAI1</td>
<td>Fibrinolysis, extracellular matrix turnover</td>
<td>[217, 218]</td>
</tr>
<tr>
<td>Protein kinase C beta</td>
<td>PRKCB1</td>
<td>PKC pathway</td>
<td>[219, 220]</td>
</tr>
<tr>
<td>Solute carrier family 12 (sodium/chloride transporters), member 3</td>
<td>SLC12A3</td>
<td>Blood pressure</td>
<td>[221-223]</td>
</tr>
<tr>
<td>Transforming growth factor beta 1</td>
<td>TGFB1</td>
<td>extracellular matrix turnover</td>
<td>[224, 225]</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha</td>
<td>TNF</td>
<td>Inflammation</td>
<td>[226, 227]</td>
</tr>
</tbody>
</table>

*The candidate genes are derived from a medline search using key words [diabetes or diabetic], [gene or genetic] and [nephropathy].
Diabetic complications

Table 6. Candidate genes for diabetic retinopathy.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>OMIM name</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE receptor</td>
<td>AGER</td>
<td>Glycation</td>
<td>[158, 228, 229]</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>AKR1B1</td>
<td>Polyl pathway</td>
<td>[230-233]</td>
</tr>
<tr>
<td>Angiotensin I converting enzyme</td>
<td>ACE</td>
<td>RAAS</td>
<td>[234, 235]</td>
</tr>
<tr>
<td>Nitric oxide synthase 2 (iNOS)</td>
<td>NOS2A</td>
<td>Blood pressure</td>
<td>[236, 237]</td>
</tr>
<tr>
<td>Nitric oxide synthase 3 (eNOS)</td>
<td>NOS3</td>
<td>Blood pressure</td>
<td>[238-240]</td>
</tr>
<tr>
<td>Integrin alpha-2/beta-1 (or platelet glycoprotein Ia/Iib)</td>
<td>ITGAV2</td>
<td>Cell surface glycoprotein</td>
<td>[241, 242]</td>
</tr>
<tr>
<td>Intercellular adhesion molecule-1</td>
<td>ICAM1</td>
<td>Inflammation</td>
<td>[243, 244]</td>
</tr>
<tr>
<td>Methylenetetrahydrofolate reductase</td>
<td>MTHFR</td>
<td>Hyperhomocysteinemia, endothelial dysfunction</td>
<td>[245-247]</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>NPY</td>
<td>Angiogenesis</td>
<td>[248, 249]</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor 1</td>
<td>PAI1</td>
<td>Fibrinolysis, extracellular matrix turnover</td>
<td>[250, 251]</td>
</tr>
<tr>
<td>Paraoxonase 1</td>
<td>PON1</td>
<td>Lipid oxidation</td>
<td>[211, 213]</td>
</tr>
<tr>
<td>Tumor necrosis factor alpha</td>
<td>TNF</td>
<td>Inflammation</td>
<td>[252, 253]</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>VEGF</td>
<td>Neovascularization</td>
<td>[134-137]</td>
</tr>
</tbody>
</table>

*The candidate genes are derived from a medline search using key words [diabetes or diabetic], [gene or genetic] and [retinopathy] and from reference [132].

Table 7. Candidate genes for diabetic Neuropathy.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>OMIM name</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldose reductase</td>
<td>AKR1B1</td>
<td>Polyl pathway</td>
<td>[254-256]</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase 2</td>
<td>ALDH2</td>
<td>Vulnerability to oxidative stress</td>
<td>[257]</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>AGT</td>
<td>RAAS</td>
<td>[140, 141]</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>APOE</td>
<td>Lipid metabolism</td>
<td>[142]</td>
</tr>
<tr>
<td>Catalase gene</td>
<td>CAT</td>
<td>Antioxidant enzyme</td>
<td>[145]</td>
</tr>
<tr>
<td>Extracellular superoxide dismutase (EC-SOD)</td>
<td>SOD3</td>
<td>Free radical scavenging enzyme</td>
<td>[144]</td>
</tr>
<tr>
<td>Human leukocyte antigen</td>
<td>HLA</td>
<td>Inflammation</td>
<td>[148]</td>
</tr>
<tr>
<td>Manganese superoxide dismutase (Mn-SOD) (Mitochondrial superoxide dismutase)</td>
<td>SOD2</td>
<td>Free radical scavenging enzyme</td>
<td>[144]</td>
</tr>
<tr>
<td>Na/K ATPase gene (ATP1-A1)</td>
<td>ATP1A1</td>
<td>Reactive oxygen species</td>
<td>[146]</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor 2</td>
<td>TNFRSF1B</td>
<td>Inflammation</td>
<td>[147]</td>
</tr>
<tr>
<td>Uncoupling protein 2</td>
<td>UCP2</td>
<td>Reactive oxygen species</td>
<td>[143, 258]</td>
</tr>
</tbody>
</table>

*The candidate genes are derived from a medline search using key words [diabetes or diabetic], [gene or genetic] and [neuropathy].
4. Aims of the present study

The aims of this study were:

1. To test the usefulness of the new WHO criteria for clinical staging of diabetes in the characterization of diabetic patients.

2. To study whether there is an association between polymorphisms in the UCP1-3 genes and diabetic nephropathy.

3. To study whether there is an association between MHC2TA -168 A→G polymorphism and cardiovascular morbidity and mortality, microalbuminuria and the metabolic syndrome.

4. To study whether AGER -374 T/A polymorphism is associated with diabetic nephropathy, retinopathy, neuropathy or macrovascular disease.

5. To study whether polymorphisms in LTA, TNF and AGER genes alone or together (as haplotypes) increase the risk for diabetic nephropathy, sight-threatening retinopathy and macrovascular disease.
5. Subjects and methods

5.1 Diabetes registry
A diabetes registry in Southern Sweden (Diabetes 2000) was initiated in 1996 and hitherto 7,461 patients have been registered. The majority of the patients (4,981) have been registered at the Department of Endocrinology, University hospital MAS, Malmö, the remaining were registered at the Trelleborg hospital or health care centers in the Malmö and Trelleborg regions. The registry includes information on onset of diabetes, and mode of treatment. At registry inclusion and at least once a year thereafter the following measurements are performed: body weight, height, blood pressure, fasting concentrations of plasma glucose, HbA1c, serum total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in addition to the urinary albumin excretion rate (AER) and P-creatinine. Plasma glucose, C-peptide and GAD antibodies (GADA) are measured at the registry inclusion. At annual follow-ups, signs of retinopathy, nephropathy, neuropathy and macrovascular disease are recorded. All patients gave their informed consent and the registry was approved by the Swedish Data Inspection Board and the Ethics Committee of Lund University.

5.2 The Botnia population and the myocardial infarction case-control population from the Malmö diet and cancer study
The Botnia Study was initiated in 1990 and represents a large population-based T2D family study in Finland and Sweden, aiming at identification of genes increasing susceptibility to T2D, metabolic syndrome and associated complications [259, 260].

The Malmö Diet and Cancer study population (MDC) [261] includes 28,098 randomly selected men (born 1923–1945) and women (born 1923–1950) living in the city of Malmö (population 250,000) in Sweden. A baseline examination was carried out between 1991 and 1996 encompassing a comprehensive assessment of lifestyle factors, heredity, medication as well as previous and current diseases.

5.3 Assessment of complications

5.3.1 Classification of nephropathy
The urinary albumin concentration was determined by immunonephelometry (Beckman Instruments, Fullerton, CA, USA) up to 1998 and thereafter by an immunoturbimetric method (Beckman Coulter, Beckman Instruments). Albuminuria was reported either as μg/min (AER), mg/24 h or as a urinary albumin:creatinine ratio (g/mol). Microalbuminuria was defined as 20–200 μg/min, 30–300 mg/24 h or 2.0–25 g/mol in men and 2.8–25 g/mol in women. For the definition of macroalbuminuria we also considered older values given as the urinary albumin concentration measured in a first morning specimen. Values of 30–300 mg/l were considered as microalbuminuria. Values above the upper limit were indicative of macroalbuminuria.

Diabetic nephropathy was defined as the presence of macroalbuminuria. Macroalbuminuria was considered present when at least two values above the cut-off limit for macroalbuminuria were recorded. One positive measurement only was considered as macroalbuminuria if the patient thereafter was treated with ACE inhibitors or angiotensin II receptor blockers or if the patient previously had had persistent microalbuminuria. Patients with known other kidney diseases were excluded from the analysis. Normoalbuminuria required that all urinary albumin measurements were in the normal range, otherwise the albuminuria status was
Subjects and methods

5.3.2 Classification of retinopathy
Fundus photography (2-4 fields per eye) or fundus examination by biomicroscopy revealed the degree of diabetic retinopathy. In study I, retinopathy was defined as any type of retinopathy affecting at least one eye. In study IV and V patients were divided into two groups; subjects with no or non-proliferative retinopathy without macular edema requiring photocoagulation and subjects with sight-threatening retinopathy, which included patients with proliferative retinopathy and/or photocoagulation treatment (panretinal and/or focal/grid for macular edema). The duration of sight-threatening retinopathy was defined from the first information of diagnosis or laser treatment. When calculating the genotype frequencies in patients without sight-threatening retinopathy only those with a diabetes duration ≥10 years were included.

5.3.3 Classification of neuropathy
Peripheral sensory neuropathy was assessed by measuring vibration sensation thresholds by a biothesiometer on the medial malleoli (Bio-Thesiometer; Bio-Medical Instruments, Newbury, OH, USA) and defined as a threshold ≥25 V. The duration was calculated from the first value ≥25 V. When calculating the genotype frequencies for patients with vibration threshold <25 V, only patients with a diabetes duration ≥10 years were included.

5.3.4 Classification of macrovascular disease
Diabetes registry. Information on macrovascular disease was obtained from medical records. In study I, III and IV macrovascular disease was defined as previous MI and/or stroke. In paper V macrovascular disease was defined as previous MI, angina pectoris, transitory ischemic attack (TIA), stroke and/or peripheral vascular disease.
Botnia study. In the Botnia study (study III) a structured questionnaire was completed by specially trained nurses, covering information about diseases other than T2D (particularly hypertension, coronary heart disease, MI and stroke) and data on smoking habits at the baseline examination. Diagnosis of MI was always established in the hospital. Total and cardiovascular mortality were assessed with a median follow up time of 7.9 years and the mortality data was obtained from the central death-certificate registry in Finland. Cardiovascular mortality was classified using the 9th revision of the International Classification of Diseases (cardiovascular diagnosis codes 390–459) before 1997 and the 10th revision (codes 100–199) thereafter. Causes of death were classified as 1) cardiovascular death (coronary heart disease), cerebrovascular disease (including both thrombotic and hemorrhagic stroke) or other cardiovascular (including pulmonary embolism, abdominal aortic aneurysm, hypertensive complications, general atherosclerosis and peripheral artery disease with gangrene), or 2) other causes of death (neoplasm, violent or other).

The MI case-control population from the Malmö Diet and Cancer Study (MDC). On December 31st, 2000 the study population was matched against the Swedish National Board of Health and Welfare's National Patient Registry and Cause of Death Registry. MI cases (first MI) were identified in the Swedish Patient Registry or in the Swedish Cause of Death Registry; using ICD 9–10 codes 410 and I21 in the Swedish Patient Registry and 410–414 and I21–I25 in the Swedish Cause of Death Registry.
Subjects and methods

Two age- (±1 year) and gender-matched controls without MI from MDC were assigned to each MI patient, resulting in a case-control material consisting of 1,244 MI patients and 2,488 control subjects.

5.4 Genotyping

Study II

Genotyping of the UCP1 -3286 A→G and UCP3 -55 C→T polymorphisms was performed using PCR and thereafter cleavage with appropriate restriction enzymes and separation of the alleles on agarose gel. Genotyping of the UCP2 I/D polymorphism was performed with PCR and following separation of the alleles on agarose gel. A total of 434 diabetic patients and 106 non diabetic control subjects were genotyped for the polymorphisms in the UCP1-3 genes. The genotyping success rate was 99.0% for UCP1 and UCP2 and 98.9% for UCP3.

Study III

The -168 A/G polymorphism (rs3087456) was genotyped using an allelic discrimination method on the ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA). Totally 11,064 individuals were successfully genotyped. The genotyping success rate was 97.9, 98.0 and 99.0% in Botnia, MDC and Diabetes registry, respectively.

Study IV

AGER –374 T/A polymorphism (rs1800624) was genotyped using an allelic discrimination method on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A total of 867 T1D, 2 453 T2D patients and 205 non-diabetic control subjects were genotyped for the AGER –374 T/A polymorphism. The genotypic success rate was 98.4% and regenotyping was performed in 115 samples with 100% genotyping concordance rate.

The AGER 63 bp insertion/deletion polymorphism was genotyped in a randomly selected subset of T1D (n=390) and T2D (n=410) diabetic patients with AGER –374 T/T or A/A genotypes with PCR amplification resolving the PCR products on a 2% agarose gel. The HLA-DQB1 genotyping was performed using a primer-pair with a biotinylated 3’ primer, the 158 bp second exon of the HLA-DQB1 gene was amplified by PCR. The amplification product was bound to streptavidin-coated microtitration plates and denatured with NaOH. After washing, bound DNA was assessed using two different hybridisation mixtures (A and B) with lanthanide (III) chelate-labelled DNA probes specific for the HLA-DQB1 alleles. Mixture A contained a europium (Eu)-labelled internal reporter probe for DQB1 *0602 and *0603 alleles (*0602–*0603), samarium (Sm)-labelled probe for *0603 and *0604 (*0603–*0604) alleles, and terbium (Tb)-labelled consensus sequence specific probe (Tb-DQB1 control) as a control for PCR amplification. Mixture B contained Tb-, Sm- and Eu-labelled probes specific for DQB1*02, *0301, *0302 alleles. When the sample is positive for both the Eu-labelled probe (*0602–*0603) and the Sm-labelled probe (*0603–*0604) and the second HLA-DQB1 allele is any DQB1 allele except *02, *0302 and *0301 (we used the symbol X to mark an unknown DQB1 allele), then the whole genotype is denoted as 0602-03-04/X. Thus, the HLA-DQB1 genotype is HLA-DQB1*0603/X, if it is homozygous for the *0603 allele, or HLA-DQB1*0602*0603 or HLA-DQB1*0602*0604 or HLA-DQB1*0603*0604. The HLA-DQB1 genotyping was done in 825 T1D, 1 179 T2D diabetic patients and 205 non diabetic control subjects.
Subjects and methods

Study V
The AGER -374 T/A polymorphism and HLA-DQB1 polymorphism were genotyped as previously described [158]. In addition LTA T60N C→A (rs1041981) and TNF -308 G→A (rs1800629) polymorphisms were genotyped using the allelic discrimination method on the ABI 7900 instrument (Applied Biosystems, Foster City, CA, USA). A total of 726 T1D, 2,920 T2D patients and 200 non-diabetic control subjects were successfully genotyped for LTA polymorphism and 729 T1D, 2,927 T2D patients and 205 non-diabetic control subjects were genotyped for TNF polymorphism. The genotyping success rates were 99.0% for the LTA and 98.7% for the TNF polymorphisms. Regenotyping was done in 133 (LTA) and 136 (TNF) samples with a 100% genotyping concordance rate.

5.5 Statistical methods
Descriptive data, unless otherwise stated, are expressed as mean ± standard deviation, or median value [25th-75th percentile]. Categorical variables were compared by chi-square test. Normally distributed continuous variables were subjected to Student’s t-test, while non-normally distributed were analyzed by the Mann-Whitney U-test. In order to assess factors associated with diabetic complications, a multiple logistic regression analysis with forward selection was performed. All data were analyzed with a NCSS (NCSS statistical software, Kaysville, UT, USA). A p-value <0.05 was considered statistically significant. To evaluate putative haplotype blocks in study V, linkage disequilibrium (LD) between the SNPs was analyzed using Haploview 3.32 and D’ values were calculated with 95% confidence intervals (CI) when the genotype frequencies were in Hardy-Weinberg equilibrium [262]. A corrected p-value was obtained after 100,000 permutations of individual SNPs and haplotype blocks including the TNF, LTA and AGER polymorphisms. Power analysis was made using Genetic Power calculator [263]. HW-QuickCheck software [264] was used for testing of putative excess of heterozygous/homozygous patients.

Genetic Power
Study II: A post-hoc power calculation shows that the genetic power to detect differences in UCP1 allele frequencies between cases and controls assuming dominant model and genotype relative risk of 1.2 was 42.4% for UCP1, 40.6% for UCP2 and 40.9% for UCP3.
Study III: The statistical power to detect differences in risk of MI according to genotype assuming dominant model and a genotype relative risk of 1.2 was 32.0% in Botnia, 95.1% in the MDC cohort and 36.5% for Swedish T2D patients (from Diabetes 2000).
Study IV&V: Power assuming α=0.05 and a relative risk of 1.3 was 11%, 31% and 32% for T1D patients and 62%, 81% and 80% for T2D patients with or without diabetic nephropathy for the LTA, TNF and AGER polymorphisms. The power for retinopathy was 68%, 86% and 85% in T1D and 58%, 73% and 73% in T2D and for macrovascular disease 20%, 28% and 28% in T1D and 97%, 99% and 99% in T2D.
6. Results

6.1. WHO clinical stages (I)

This study evaluated whether the clinical stages proposed by WHO in 1999 [22], especially if they can discriminate between clinically meaningful diabetic subgroups. We defined the clinical stages as following: patients still on diet and/or oral treatment were considered as not insulin requiring (NIR), patients who required insulin therapy after one year from diagnosis were considered to be insulin requiring for control (IRC) and patients who because of deteriorating hyperglycemia within one year required insulin were considered as insulin requiring for survival (IRS).

Table 8. Clinical characteristics in different clinical stages.

<table>
<thead>
<tr>
<th></th>
<th>NIR</th>
<th>IRC</th>
<th>IRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>711</td>
<td>543</td>
<td>743</td>
</tr>
<tr>
<td>Males/Females (n)</td>
<td>427/284</td>
<td>295/248</td>
<td>404/339</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.1±12.0*</td>
<td>62.4±11.6</td>
<td>43.6±14.7***</td>
</tr>
<tr>
<td>Age at diabetes diagnosis (years)</td>
<td>52.1±12.0***</td>
<td>48.7±12.9</td>
<td>22.7±15.3***</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>7.0 (4.0-13.0)***</td>
<td>13.0 (8.0-18.8)</td>
<td>19.0 (10.0-29.0)***</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>29.3±5.2**</td>
<td>28.0±4.8</td>
<td>24.2±3.4***</td>
</tr>
<tr>
<td>f-P-Glucose (mmol/L)</td>
<td>12.3±4.0***</td>
<td>12.9±4.6</td>
<td>13.4±5.9</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.21±1.65***</td>
<td>7.62±1.61</td>
<td>7.64±1.50</td>
</tr>
<tr>
<td>P-C-Peptide (nmol/L)</td>
<td>0.98 (0.72-1.31)***</td>
<td>0.56 (0.28-0.94)</td>
<td>0.05 (0.05-0.05)*</td>
</tr>
<tr>
<td>GADA positivity (n ;%)</td>
<td>38 (5.5)***</td>
<td>87(16.8)</td>
<td>318(44.5)*</td>
</tr>
<tr>
<td>S-Cholesterol (mmol/L)</td>
<td>5.39±1.08</td>
<td>5.44±1.14</td>
<td>5.09±1.03</td>
</tr>
<tr>
<td>S-HDL Cholesterol (mmol/L)</td>
<td>1.18±0.34*</td>
<td>1.27±0.40</td>
<td>1.58±0.46***</td>
</tr>
<tr>
<td>S-Triglycerides (mmol/L)</td>
<td>1.96 (1.36-2.92)</td>
<td>1.69 (1.12-2.53)</td>
<td>0.98 (0.74-1.37)***</td>
</tr>
<tr>
<td>S-LDL Cholesterol (mmol/L)</td>
<td>3.25±0.93</td>
<td>3.27±0.95</td>
<td>2.97±0.86</td>
</tr>
<tr>
<td>U-Albumin (μg/min)</td>
<td>14 (8-47)*</td>
<td>18 (9-74)</td>
<td>10 (6-23)*</td>
</tr>
<tr>
<td>P-Creatinine (μmol/L)</td>
<td>83.0 (74.0-94.0)***</td>
<td>90.0 (77.0-109.0)</td>
<td>86.0 (77.0-96.0)</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>146±21.2</td>
<td>147±20.5</td>
<td>134±19.9</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>80.8±11.8</td>
<td>78.9±9.6</td>
<td>75±6.8</td>
</tr>
</tbody>
</table>

Figures are given as mean±SD or median (interquartile range). *p<0.05, **p<0.01, ***p<0.001 Compared with IRC. P-values adjusted for age, duration sex and BMI. NIR = Not insulin requiring, IRC = Insulin requiring for control, IRS = Insulin requiring for survival.

The three clinical stages showed clearly different features (Table 8). NIR patients were older at the time of diabetes diagnosis (p<0.05), had a higher BMI (p<0.01, adjusted for age, sex and duration), higher C-peptide concentrations (p<0.001) and lower frequency of GADA (p=0.001) than the IRC patients. They also had lower HbA1c concentrations (p<0.001), lower HDL-cholesterol concentrations (p<0.05), lower AER (p<0.05) and lower P-creatinine than IRC patients. Data were adjusted for age, sex, BMI and duration, where appropriate.
Table 9. Presence of complications in patients belonging to the clinical stages.

<table>
<thead>
<tr>
<th></th>
<th>NIR</th>
<th>IRC</th>
<th>IRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microalbuminuria</td>
<td>211(36.6)*</td>
<td>179 (43.4)</td>
<td>158 (25.0)**</td>
</tr>
<tr>
<td>Macroalbuminuria</td>
<td>78 (17.6)***</td>
<td>107 (31.5)</td>
<td>90 (16.0)***</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>60 (14.5)***</td>
<td>179 (43.9)</td>
<td>417 (62.1)***</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>186 (59.0)*</td>
<td>211 (68.1)</td>
<td>238 (42.7)***</td>
</tr>
<tr>
<td>Macrovascular disease</td>
<td>77 (20.5)*</td>
<td>116 (28.2)</td>
<td>66 (10.4)***</td>
</tr>
</tbody>
</table>

Data are given as number of patients (n) and frequency (%). *p<0.05, **p<0.01, ***p<0.001, compared with IRC. NIR = Not insulin requiring, IRC = Insulin requiring for control, IRS = Insulin requiring for survival.

The IRC patients had a higher prevalence of micro- or macroalbuminuria (p<0.05 and p<0.001), retinopathy (p<0.001), neuropathy (p<0.05), and macrovascular disease (p<0.05) than the NIR (Table 9). In a subset of patients (n=426) matched for diabetes duration, age and sex, the IRC patients had still higher frequency of macroalbuminuria (29.7 vs. 17.2%, p<0.001) and retinopathy (40.0 vs. 17.2%, p<0.001) than IRS patients.

The IRC patients were older at the time of diabetes diagnosis (p<0.001), had higher BMI (p<0.001, adjusted for age, sex, BMI and duration) and higher C-peptide concentrations (p<0.001) and lower frequency of GADA (16.8 vs. 44.5%; p<0.001) than the IRS patients. The IRC patients had a higher frequency of micro- and macroalbuminuria (p<0.001), neuropathy (p<0.001) and macrovascular disease (p<0.001) but lower prevalence of retinopathy (62.1 vs. 43.9%) than the IRS patients (p<0.001).

In conclusions this study suggest that the WHO clinical staging of diabetes can discriminate clinically meaningful subgroups and the IRC patients represent more severe from of diabetes than acknowledged in the etiological classification.

6.2. The role of UCP1-3 genes in diabetic nephropathy (II)

Increased production of reactive oxygen species (ROS) has been suggested as a cause of diabetic complications and blocking the mitochondrial superoxide production in vivo can block all three main pathways behind diabetic complications [86]. The uncoupling proteins (UCPs) represent a family of proteins that are able to dissipate the proton gradient in the inner mitochondrial membrane thereby uncoupling the oxidative phosphorylation and reducing formation of ROS. The aim of this study was therefore to study whether variation in the UCP1, UCP2 and UCP3 genes was associated with increased risk of diabetic nephropathy.

No differences in allele and genotype frequencies of the UCP1–3 polymorphisms were seen between healthy control subjects, diabetic subjects with normal AER, and diabetic subjects with micro- or macroalbuminuria (Table 10). The UCP3 C/T polymorphism was, however, associated with BMI.

We concluded that the the studied polymorphisms in UCP1–3 genes do not play a major role in the development of diabetic nephropathy in Scandinavian patients.
Table 10. UCP1-3 genotype and frequencies.

<table>
<thead>
<tr>
<th></th>
<th>UCP1</th>
<th>UCP2</th>
<th>UCP3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/G or A/G</td>
<td>A/A</td>
<td>I/I or I/D</td>
</tr>
<tr>
<td>Control subjects</td>
<td>38</td>
<td>68</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>(35.8%)</td>
<td>(64.2%)</td>
<td>(55.7%)</td>
</tr>
<tr>
<td>Diabetic subjects</td>
<td>97</td>
<td>121</td>
<td>115</td>
</tr>
<tr>
<td>Normo-albuminuria</td>
<td>(44.5%)</td>
<td>(55.5%)</td>
<td>(52.8%)</td>
</tr>
<tr>
<td>Micro- or macro-albuminuria</td>
<td>84</td>
<td>132</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>(38.9%)</td>
<td>(61.1%)</td>
<td>(48.6%)</td>
</tr>
</tbody>
</table>

Data are given as number of patients (n) and frequency (%). The UCP2 I/D and UCP3 C → T (-55) polymorphisms were in linkage disequilibrium (93 % of the subjects homozygous for the D/D genotype were homozygous for the UCP3 C allele p< 0.0005).

6.3. MHC2TA gene polymorphism, the metabolic syndrome and cardiovascular mortality (III)

Variation in the MHC class II transactivator gene (MHC2TA) has recently been shown to be associated with increased susceptibility to MI [265]. The aim of this study was therefore to try to confirm this association in three different populations and also to study the role of the MHC2TA polymorphism in microalbuminuria, the metabolic syndrome and cardiovascular mortality. Patients were selected from three large populations in Finland and Sweden; the Botnia study, the Malmö Diet and Cancer Study (MDC) and the Diabetes Registry in Southern Sweden (Diabetes 2000) (DR).

The genotype and allele frequencies of the MHC2TA -168 A/G polymorphism were similar in patients with or without MI in all three study populations. The MHC2TA polymorphism was not associated with cardiovascular mortality in the whole population, but in a subgroup of patients with previous history of MI the MHC2TA AG/GG genotypes were associated with cardiovascular death (Figure 3).

The MHC2TA AG/GG genotypes were more frequently found among patients with the metabolic syndrome (40.1 vs. 36.9%, p=0.030) as well as among non-diabetic individuals with microalbuminuria in the Botnia cohort (50.0% vs. 36.0%, p=0.003, Table 11). In contrast, the AG/GG genotypes were not associated with microalbuminuria among T2D patients, neither in the Botnia, nor in the DR cohort (Table 11).
Table 11. The genotype frequencies of the MHC2TA – 168 A/G polymorphism in different study populations according to history of previous MI and microalbuminuria status.

<table>
<thead>
<tr>
<th></th>
<th>MI+</th>
<th>MI-</th>
<th>p</th>
<th>Microalbuminuria</th>
<th>Normoalbuminuria</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-diabetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Botnia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2D subjects</td>
<td>112 (63.4/33.0/3.6)</td>
<td>2686 (62.8/33.3/3.9)</td>
<td>0.90</td>
<td>99</td>
<td>1940 (64.0/32.4/3.6)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>184 (59.2/38.0/2.7)</td>
<td>1326 (59.7/36.0/4.2)</td>
<td>0.90</td>
<td>756 (58.9/38.8/2.3)</td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>T2D subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>1071 (58.0/35.7/6.3)</td>
<td>2312 (55.7/37.4/6.9)</td>
<td>0.21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T2D subjects</td>
<td>151 (45.7/43.7/10.6)</td>
<td>123 (52.8/42.3/4.9)</td>
<td>0.24</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes registry</td>
<td>T2D subjects</td>
<td>316 (57.0/37.3/5.7)</td>
<td>1974 (54.4/39.1/6.4)</td>
<td>0.44</td>
<td>827 (53.8/39.4/6.8)</td>
<td>1311 (56.4/37.1/6.4)</td>
</tr>
</tbody>
</table>

Data are given as number of patients and allele frequencies. P-values are for the frequency of risk alleles (AG or GG) with or without MI or albuminuria respectively.

Taken together, we could not confirm an association between MHC2TA and MI, however we showed that the AG/GG genotypes of the MHC2TA -168 A→G polymorphism were associated with microalbuminuria and features of the metabolic syndrome and increased risk of cardiovascular mortality in patients with previous MI.

Figure 3. Kaplan-Meir curve of cardiovascular mortality in the Botnia cohort in patients with previous MI. The data has been treated as left truncated and right censored.
6.4. Genetic risk factors for diabetic complications- the role of the HLA region on chromosome 6p (IV and V)

The HLA locus located on the short arm of chromosome 6 is among the most polymorphic regions in the human genome. Many of the genes are involved in inflammatory responses and may therefore be considered as candidate genes for late diabetic complications. Previous association studies on this chromosomal region have given conflicting results concerning risk for late diabetic complications [159, 228, 229, 253, 266, 267]. The aim of the first study (IV) was to investigate a putative association between AGER -374 T/A polymorphism and diabetic nephropathy, retinopathy, neuropathy and macrovascular complications. We could show that T1D patients had higher frequency of the AGER -374 A/A or T/A genotypes than T2D patients (51.1% vs. 44.9%, p=0.002) and control subjects (51.1% vs. 47.6%, p=0.0006). The RAGE -374 T → A polymorphism was associated with HLA-DQB1 genotypes; patients with HLA-risk genotypes had higher frequency of the A/A or T/A genotypes than patients with other HLA-DQB1 genotypes (60.3% vs. 40.3%, p=0.000001). In T1D patients, the frequency of the A/A or T/A genotypes was higher in patients with than without diabetic nephropathy (61.1% vs. 46.8%, p=0.006) and with than without sight-threatening retinopathy (56.1% vs. 47.6%, p=0.03). In T2D patients with Hba1c below the median, the T/T genotype was more frequent in patients with than without diabetic nephropathy (54.3% vs. 38.2%, p=0.02). We could not demonstrate any association between AGER polymorphism and diabetic neuropathy or macrovascular complications.

We concluded that the AGER -374 T → A was associated with diabetic nephropathy and possibly retinopathy in T1D patients but not in T2D patients. We could however not exclude that other genes in the region like TNF or LTA could influence susceptibility to diabetic nephropathy or retinopathy.

In the follow up study we therefore included LTA T60N C → A and TNF -308 G → A polymorphisms. The study population was partly the same as in the previous study, however, because of the previously shown association of AGER polymorphism with HLA-DQB1 high risk genotypes, a special attention was made to exclude possible LADA patients. We therefore excluded all GADA positive T2D patients as well as T1D patients with an age at diagnosis over 35 years. Our results showed that the AGER -374 A allele was more common in T1D patients with than without diabetic nephropathy (31.2 vs. 28.4%, p=0.007) independently of LTA or TNF genotypes (Table 12). In a logistic regression analysis however, the LTA polymorphism (and not the AGER) was associated with increased risk of diabetic nephropathy in T1D patients. The AGER -374 A allele was associated with increased risk for macrovascular disease in T1D patients (OR 2.05 [1.19-3.54], p=0.01), but with decreased risk in T2D patients (OR 0.66 [0.49-0.90], p=0.009). The AGER A allele was independently associated with sight-threatening retinopathy in T2D (OR 1.65 [1.11-2.45], p=0.01). The TNF A allele was associated with increased risk for macrovascular complications in T2D (OR 1.53 [1.04-2.25], p=0.03), but not in T1D patients (Figure 4).

In addition the AA haplotype of TNF and LTA was more common in T2D patients with than without macrovascular disease (21.5% vs. 18.1%, p=0.003).

Taken together this study showed that the LTA, TNF, AGER genes increased the risk of diabetic micro- and macroangiopathy either alone or in concert. The association was very complex and the genes might be part of a large haplotype block that also includes HLA-DQB1 risk genotypes.
Table 12. Allelic association of LTA T60N (C→A), TNF -308G→A and AGER -374 T→A polymorphism with diabetic nephropathy, retinopathy and macrovascular complications.

<table>
<thead>
<tr>
<th></th>
<th>T1D</th>
<th>T2D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephropathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTA T60N C→A</td>
<td>Controls: N=340</td>
<td>Controls: N=442</td>
</tr>
<tr>
<td></td>
<td>p=0.317; p*=0.918</td>
<td>p=0.205; p*=0.592</td>
</tr>
<tr>
<td></td>
<td>Cases: N=113</td>
<td>Cases: N=314</td>
</tr>
<tr>
<td></td>
<td>27.9/53.2/18.9</td>
<td>33.3/47.3/19.5</td>
</tr>
<tr>
<td></td>
<td>18.6/63.7/17.7</td>
<td>37.2/45.9/16.9</td>
</tr>
<tr>
<td>TNF -308 G→A</td>
<td>Controls: N=342</td>
<td>Controls: N=438</td>
</tr>
<tr>
<td></td>
<td>p=0.503; p*=0.983</td>
<td>p=0.559; p*=0.970</td>
</tr>
<tr>
<td></td>
<td>Cases: N=113</td>
<td>Cases: N=314</td>
</tr>
<tr>
<td></td>
<td>47.7/46.5/5.8</td>
<td>63.9/30.4/5.7</td>
</tr>
<tr>
<td></td>
<td>44.2/48.7/7.1</td>
<td>65.0/30.9/4.1</td>
</tr>
<tr>
<td>AGER -374 T→A</td>
<td>Controls: N=345</td>
<td>Controls: N=584</td>
</tr>
<tr>
<td></td>
<td>p=0.007; p*=0.023</td>
<td>p=0.119; p*=0.509</td>
</tr>
<tr>
<td></td>
<td>Cases: N=114</td>
<td>Cases: N=315</td>
</tr>
<tr>
<td></td>
<td>52.2/41.2/6.7</td>
<td>54.2/37.6/8.2</td>
</tr>
<tr>
<td></td>
<td>34.2/57.9/7.9</td>
<td>58.1/37.1/4.8</td>
</tr>
<tr>
<td>Retinopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTA T60N C→A</td>
<td>Controls: N=310</td>
<td>Controls: N=609</td>
</tr>
<tr>
<td></td>
<td>p=0.154; p*=0.461</td>
<td>p=0.34; p*=0.122</td>
</tr>
<tr>
<td></td>
<td>Cases: N=310</td>
<td>Cases: N=885</td>
</tr>
<tr>
<td></td>
<td>29.4/53.5/17.1</td>
<td>34.1/49.8/16.1</td>
</tr>
<tr>
<td></td>
<td>21.6/60.6/17.7</td>
<td>37.5/45.6/16.9</td>
</tr>
<tr>
<td>TNF -308 G→A</td>
<td>Controls: N=307</td>
<td>Controls: N=611</td>
</tr>
<tr>
<td></td>
<td>p=0.573; p*=0.982</td>
<td>p=0.368; p*=0.878</td>
</tr>
<tr>
<td></td>
<td>Cases: N=315</td>
<td>Cases: N=886</td>
</tr>
<tr>
<td></td>
<td>50.2/43.6/6.2</td>
<td>65.0/30.7/4.3</td>
</tr>
<tr>
<td></td>
<td>46.0/48.9/5.1</td>
<td>67.8/28.8/3.4</td>
</tr>
<tr>
<td>AGER -374 T→A</td>
<td>Controls: N=313</td>
<td>Controls: N=549</td>
</tr>
<tr>
<td></td>
<td>p=0.295; p*=0.774</td>
<td>p=0.734; p*=0.998</td>
</tr>
<tr>
<td></td>
<td>Cases: N=315</td>
<td>Cases: N=503</td>
</tr>
<tr>
<td></td>
<td>48.9/45.3/5.8</td>
<td>54.9/36.5/8.6</td>
</tr>
<tr>
<td></td>
<td>44.8/47.9/7.3</td>
<td>50.3/44.0/5.7</td>
</tr>
<tr>
<td>Macrovascular complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTA T60N C→A</td>
<td>Controls: N=609</td>
<td>Controls: N=1802</td>
</tr>
<tr>
<td></td>
<td>p=0.034; p*=0.122</td>
<td>p=0.102; p*=0.456</td>
</tr>
<tr>
<td></td>
<td>Cases: N=112</td>
<td>Cases: N=381</td>
</tr>
<tr>
<td></td>
<td>27.6/55.2/17.2</td>
<td>38.1/47.5/14.4</td>
</tr>
<tr>
<td></td>
<td>15.2/64.3/20.5</td>
<td>35.0/48.6/16.4</td>
</tr>
<tr>
<td>TNF -308 G→A</td>
<td>Controls: N=611</td>
<td>Controls: N=1804</td>
</tr>
<tr>
<td></td>
<td>p=0.426; p*=0.916</td>
<td>p=0.003; p*=0.017</td>
</tr>
<tr>
<td></td>
<td>Cases: N=113</td>
<td>Cases: N=614</td>
</tr>
<tr>
<td></td>
<td>49.3/44.0/6.7</td>
<td>67.3/29.2/3.5</td>
</tr>
<tr>
<td></td>
<td>42.5/52.2/5.3</td>
<td>61.4/34.1/4.5</td>
</tr>
<tr>
<td>AGER -374 T→A</td>
<td>Controls: N=617</td>
<td>Controls: N=1809</td>
</tr>
<tr>
<td></td>
<td>p=0.299; p*=0.797</td>
<td>p=0.010; p*=0.052</td>
</tr>
<tr>
<td></td>
<td>Cases: N=111</td>
<td>Cases: N=544</td>
</tr>
<tr>
<td></td>
<td>48.8/44.6/6.6</td>
<td>54.4/38.1/7.5</td>
</tr>
<tr>
<td></td>
<td>41.4/52.3/6.3</td>
<td>58.8/35.9/5.3</td>
</tr>
</tbody>
</table>

p*= Corrected p-values after 100,000 permutations (Haploview).
**Results**

Figure 4. Logistic regression analysis in T1D (4a) and T2D (4b) patients with LTA T60N (C→A), TNF -308 G→A, AGER -374 T→A polymorphisms and HLA-DQB1 risk genotypes as independent and diabetic complications as dependent variable. Age, systolic and diastolic blood pressure, sex, previous/current smoking were included in all models. BMI was included in the models for nephropathy and macrovascular disease, duration in the models for nephropathy and retinopathy and age at diagnosis in the model for macrovascular disease.
7. Discussion

7.1 Study Population

The patients in our studies were mainly recruited from the local diabetes registry (Diabetes 2000). In the first study the population included 1,997 patients and in the last study a total of 5,474 patients. The majority of the patients were recruited in Malmö and 22% of the patients were other than Scandinavian origin and therefore not included in the genetic studies. The majority of the patients were recruited at the Department of Endocrinology in Malmö and at the Hospital in Trelleborg. As the majority of the T1D patients are treated at the hospital outpatient clinics, the diabetes registry covered most of the T1D patients in the region. On the other hand the T2D patients are usually referred to the hospital clinic due to problems with metabolic control or diabetic complications and consequently, one could expect an enrichment of complications in this material.

According to the Swedish national guidelines for diabetes (1999) all patients with hypertension or microalbuminuria should be treated with ACE inhibitors or angiotensin receptor II (AT2) blockers. Because a majority of the T2D patients have hypertension a vast majority was treated with ACE inhibitors/AT2 blockers. We can therefore not exclude that some of the patients in the control group with normal AER might have developed micro- or macroalbuminuria if not treated. Treatment with RAAS-blockade could also have beneficial effects in preventing diabetic retinopathy [268] and therefore influence studies on diabetic retinopathy.

Similarly, wide spread use of statins in treatment of hyperlipidemia in diabetic patients is a confounding factor in studies regarding macrovascular complications. Previous data would also suggest that approximately 30% of the T2D patients without diabetic retinopathy and macroalbuminuria have other kidney diseases than diabetic nephropathy [269]. Since all patients with nephropathy due to diabetes also have retinopathy it would be possible to separate between diabetic nephropathy from other causes excluding T2D patients without diabetic retinopathy. This procedure, however did not change the results, possible because we had already excluded all patients with previously known other kidney diseases.

In studies II-V we applied the candidate gene approach. The *UCP1-3* genes have been previously studied for association with obesity but not with diabetic complications. All other genes i.e. *AGER*, *TNF*, *LTA*, *HLA* and *MHC2TA* had previously been associated with diabetic complications in other populations. Previous experience has demonstrated the difficulty in reproducing results in other populations. Several reasons have been suggested, e.g small sample size and ethnic differences etc. One additional problem is publication biases, i.e. positive association are more likely to be published than negative ones. A good example of this so called “winners curse” is the ACE I/D polymorphism, which has been extensively studied for a role in diabetic complications. A recent meta-analysis of 47 studies suggested that the II genotype is protective for diabetic nephropathy especially in Asian T2D patients [129]. The majority of the studies are were, however, small and how this polymorphism modifies the effect of ACE inhibitors is not clear and the effect may be different between Asian and Caucasian populations [270].

A special concern in studies of diabetic complications is a possible survival bias. Genes that may influence susceptibility to e.g diabetic nephropathy may be the same that influence the risk for macrovascular complications as seen in studies III and V. This could, in turn lead to survival biases.
7.2 Study I
This is the first and to our knowledge the only study on the clinical staging proposed by WHO 1999. The advantages of the clinical staging are obvious: it offers a way to classify diabetes without any knowledge of the etiological type of diabetes and does not require any measurement of beta-cell function, auto-antibodies or DNA testing and can therefore be applied worldwide. In this study the clinical stages were defined as not insulin requiring (NIR) if the patient was currently treated with diet/oral agents only. The definition between insulin requiring for control (IRC) and insulin requiring for survival (IRS) was based on the timepoint for insulin treatment. If the insulin treatment was started within one year of diabetes, the patient was considered to require insulin for survival, otherwise only for control.

This kind of definition of the clinical stages creates an obvious problem because the patients that are considered as IRC cannot later be defined as IRS. This is especially true for the GADA positive patients in the IRC group, who in most cases would represent latent autoimmune diabetes in adults (LADA) and who after several years will develop $\beta$-cell failure [271]. Still, the classification seemed to discriminate between three clinically meaningful subgroups. The NIR group represented a classical T2D population with high BMI, high c-peptide levels and high lipid levels. IRS patients were mainly T1D patients with low c-peptide levels and high frequency of GADA. The IRC group turned out to be a high risk group for diabetic complications, with a high frequency of both micro– and macrovascular complications. We conclude that the IRC patients clearly represent a more severe form of diabetes than acknowledged in the etiological classification.

7.3 Study II
Studies on UCP2 and UCP3 knock out mice suggest that UCP2 and 3 are more important for the regulation of ROS than for energy metabolism as previously thought [272, 273]. This would make them obvious candidate genes for diabetic complications. In this study we tested the hypothesis that polymorphisms in the UCP1-3 genes could be associated with diabetic nephropathy. We concluded that it was not the case at least not in this population. The study population was well matched for metabolic control, sex and duration of diabetes. The power was not enough to detect small differences in allele frequencies and we can therefore not exclude that UCP2 or UCP3 genes could have some effect on the risk for diabetic nephropathy. Rudofsky et al. found an association between diabetic neuropathy and polymorphisms in the UCP2 (-866 G→A) and UCP3 (-55 C→T) genes [154]. No association was however found with diabetic nephropathy or retinopathy. The study population was even smaller, 227 patients, than in our study and larger studies would be needed to be able to definitely solve this issue. Recently Rudofsky et al. could show that UCP2 and UCP3 polymorphisms were associated with diabetic neuropathy in T1D [143] but not in T2D [274]. In contrast, they could not see any association with between diabetic nephropathy or retinopathy and UCP2-3 polymorphisms [274].

7.4 Study III
One previous study suggested that a polymorphism in the gene (MHC2TA) is associated with MI [265]. The investigators also suggested that the same polymorphism was associated with susceptibility of rheumatoid arthritis and multiple sclerosis.

Our study included 11,064 individuals from three different populations with a large number of MI cases (651 with T2D and 1,183 without diabetes mellitus) and was thus well powered. Nevertheless, we could not confirm any association between this gene and MI. The MHC2TA polymorphism was however associated with mortality in individuals with a previous MI and
also with features of the metabolic syndrome. The lack of association with MI in our study could be due to a differences in the ascertainment of MI as the information on MI was collected retrospectively in our study. The MHC2TA polymorphism was associated with microalbuminuria only in non-diabetic subjects. This could reflect the fact that in diabetic subjects other factors including hyperglycemia may influence the day-to-day variation in albumin excretion. Our results also suggest that the G-allele of the MHC2TA polymorphism (and in particular the AG genotype) could be a risk factor for cardiovascular mortality after MI, although the mechanism remains unclear.

7.5 Study IV
The gene for the receptor for advanced glycation end-product (AGER) has previously been associated with diabetic nephropathy, retinopathy and also with MI. We could show that the genotype frequencies of the AGER -374 T→A polymorphism differed between different ethnic groups and between T1D and T2D patients. A natural explanation for this is that the AGER is located in the HLA region and also associated with HLA-DQB1 risk genotypes, the frequencies of which are known to differ between populations. Our finding that the A allele is a risk factor for diabetic nephropathy is in conflict with previous studies where the A allele has been protective in T1D patients with high HbA1c. This could be explained by other genes in the HLA-region that are in linkage disequilibrium with AGER -374 T→A polymorphism.

7.6 Study V
Variants in the lymphotoxin alpha (LTA) and TNF-alpha (TNF) genes have previously been associated with diabetic complications. As they are located in the region that harbors genes of the MHC class III and also the AGER gene, association between variants in any of these genes might depend on variation in other genes. In this study we confirmed that all three genes (LTA, TNF and AGER) are associated with T1D HLA-DQB1 risk genotypes. Because of excess of heterozygosity in T1D patients, we could not calculate LD between the three loci. In T2D patients the LTA and TNF were in tight LD, whereas the AGER was not. We conclude that the gene polymorphisms studied were associated with diabetic complications in a very complex way. They are probably part of a larger haplotype block that includes the HLA-DQB1 risk genotypes. Although the short arm of chromosome 6 still remains as a very interesting region in the search for candidate genes for diabetic complications, there are many issues to be solved and future studies must therefore be larger and include more extensive genotyping than hitherto.
Conclusions

The WHO clinical staging of diabetes can discriminate between clinically meaningful subgroups. The insulin requiring for control patients represent a group with more severe diabetes than acknowledged in the etiological classification with high frequency of diabetic complications.

The $UCP1$ -3862 A→G, $UCP2$ insertion/deletion (I/D) polymorphism in exon 8 and the $UCP3$ -55 C→T polymorphisms do not play a major role in the development of micro- or macroalbuminuria in Scandinavian diabetic patients.

The -168A→G polymorphism in the MHC class II transactivator gene ($MHC2TA$) is associated with cardiovascular mortality, microalbuminuria and the metabolic syndrome.

Polymorphisms in the $LTA$, $TNF$ and $AGER$ genes are associated with diabetic complications. The association is complex and dependent upon the $HLA-DQB1$ genotypes, with partly different alleles conferring susceptibility in type 1 and type 2 diabetic patients. We cannot exclude the possibility that the genes are part of a large haplotype block that also includes $HLA-DQB1$ risk genotypes.
Populärvetenskaplig sammanfattning

Diabetes karakteriseras av kroniskt förhöjt blodsocker som beror på bristande insulininsöndring och/eller bristfällig känslighet för insulin i kroppens vävnader. Diabetes kan indelas i olika typer beroende på orsak (etiologiskt typ). Förutom tidigare kända typ 1 och typ 2 diabetes finns nu även väl kända mindre vanliga former av diabetes där den bakomliggande genförändringen som orsaker sjukdomen är känd (t.ex. MODY).


Kroniskt förhöjt blodsocker har skadliga effekter i olika organ som t.ex. i njurar (nefropati), ögon (retinopati), nerver (neuropati) och i blodkärlen (makrovasculära komplikationer). Orsakerna till diabeteskomplikationer är delvis okända, man vet dock att nivån av blodsocker och blodtryck spelar en viktig roll i uppkomsten av olika komplikationer. Det finns dock patienter som har haft diabetes flera decennier utan att drabbas av allvarliga diabeteskomplikationer. Tidigare studier har också kunnat fastställa att det finns en anhopning av nefropati och retinopati i vissa familjer vilket tyder på att det kan finnas genetiska orsaker till diabeteskomplikationer. Det samma gäller risken för hjärtsjukdom och även den verkar ha ärftliga orsaker.

Målsättning med denna studie var att undersöka:

1) Hur användbar är den av WHO föreslagna kliniska stadieindelningen för att identifiera kliniskt meningsfulla diabetiska undergrupper?
2) Hur påverkar variationen i olika kända gener risken att drabbas av diabeteskomplikationer?

Studie I visade den föreslagna kliniska stadieindelningen på ett meningsfullt sätt karakterisera patienter med kliniska undergrupper oberoende av etiologisk typ. IRC visade sig ha den högsta risken att utveckla diabeteskomplikationer och hade dålig sockerkontroll, högt blodtryck och höga blodfetter.

Studie II visade att variationen i UCP1, UCP2 och UCP3 generna inte är associerade med diabetesnefropati hos svenska diabetespatienter.

Studie III visade att variation i MHC2TA genen är associerad med utsöndring av äggvita hos finska kontrollpersoner utan diabetes. Samma genvariation var också associerad med metabolt syndrom och med ökad mortalitet hos patienter som tidigare haft en hjärtinfarkt.

Studie IV och V visade att variationer i LTA, TNF och AGER generna var associerade med diabetes nefropati, retinopati och makrovasculära komplikationer på ett komplicerat sätt och att samband i vissa fall var olika vid typ 1 och typ 2 diabetes och dessutom beroende på vilken s.k. vävnadstyp (HLA-typ) patienten hade.

Trots att dessa studier visar en del positiva samband mellan diabetes komplikationer och variation i de studerade generna, kan de förklara endast en liten del av den ärftliga risken. Det krävs flera studier för att kunna kartlägga de ärftliga faktorerna bakom diabeteskomplikationer och för att kunna dra några definitiva slutsatser angående orsak och verkan.
Yleistieteellinen yhteenveto

Acknowledgements

I would like to thank my supervisor Professor Carl-David Agardh and my co-supervisor Leif Groop for their support, enthusiasm, constructive criticism and source of inspiration.

I am also grateful to Professor Elisabet Agardh for her advice, ideas and comments as well as to associate professor Marju Orho-Melander for her support, critical comments and advice with paper III.

I would like to thank all my other co-workers Ekaterine Bakhtadze, Göran Berglund, Corrado Cilio, Mia Klannemark, Olle Melander, Marketa Sjögren and Tiina-Maija Tuomi for their ideas and critical comments.

Peter Almgren (also a co-author) and Timo Kanninen for their help in statistical, database and programming issues. Aki Suomalainen for help with programming and Johan Hultman for his help with computers.

I am also grateful to people in the lab especially Margareta Svensson and Esa Laurila for their skillful assistance and for Britt Bruveris-Svenburg and Anita Svensson for GADA analysis and Lena Rosberg who did the genotyping in paper III.

Bodil Israelsson and Lovisa Johansson for all help with practical issues with my experiments.

Henrik Jansson, Bengt Hallengren, Camilla Cervin, Barbro Holm, Targ Elgzyri, Devjith Tripathy, Åsa-Linda Lethagen, Harvest Feng Gu and Hayan Li for co-operation in studies that are not included in this work.

Maj-Lis Smith for interesting scientific discussions.

Professor Åke Lernmark for his efforts to explain to me the secrets of the HLA.

Leif Persson at the department of clinical chemistry for his help with Diabetes 2000 registry.

All people from the old floor 3 at Wallenberg laboratory and department of endocrinology during these years: Avinash Abhyankar, Gertrud Ahlqvist, Kristina Bengtsson, Anna Berglund, Kerstin Berntorp, Philippe Burri, Maria Carlsson, Emma Carlsson, Anders Dahlén, Mozghan Dorkhan, Malin Eliasson, Karl-Fredrik Eriksson, Jenny Fredriksson, Charlotte Granhall, Barbro Gustavsson, Per Hagert, Forouzan Haghanitar, Barbro Holm, Johan Holmkvist, Xudong Huang, Lennart Hulthén, Martins Kalis, Markku Lehto, Charlotte Ling, June Ljungberg, Holger Luthman, Valeriya Lyssenko, Marie Nilsson, Hemang Parikh, Martin Ridderstråle, Nael Shaat, Malin Svensson, Mona Svardh, Jianping (Jesse) Weng, Ylva Wessman, Fredrik von Wowern.

I also want to specially mention the late Göran Sundkvist, Professor, who always took time to answer questions whether they yielded practical clinical management or scientific issues.

Last but not least thanks to my wife Beata and my son André – Your are always in my heart!
References


