Innate immune system and adiponectin in diabetic nephropathy in type 1 diabetes

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“One never knows, what awaits one “

Laurie Lee 1936
List Of Original Publications

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


### Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACEinhibitor</td>
<td>Angiotensin converting-enzyme inhibitor</td>
</tr>
<tr>
<td>AER</td>
<td>Albumin excretion rate</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II receptor blocker</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complication Study</td>
</tr>
<tr>
<td>eGDR</td>
<td>Estimated glomerular disposal rate</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FinnDiane</td>
<td>Finnish Diabetic Nephropathy Study</td>
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<tr>
<td>GC-SF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>hsCRP</td>
<td>Highly sensitive C-reactive protein</td>
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<tr>
<td>HNP</td>
<td>Human neutrophil peptides</td>
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<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
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<tr>
<td>IGFBP</td>
<td>Insulin growth factor-binding protein</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan-binding lectin</td>
</tr>
<tr>
<td>MDRD study</td>
<td>Modification of Diet in Renal Disease Study</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factorβ system</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist-to-hip ratio</td>
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Abstract

Introduction: The pathogenesis of diabetic nephropathy remains a matter of debate, although strong evidence suggests that it results from the interaction between susceptibility genes and the diabetic milieu. The true pathogenetic mechanism remains unknown, but a common denominator of micro- and macrovascular complications may exist. Some have suggested that the renal analogue to the inflammatory process observed in atherosclerosis is glomerulosclerosis. Others have also suggested that activation of the complement system contributes to the cascade of inflammation. Defensins, as part of the innate immune system, may play a regulatory role in the complement cascade and augment the production of proinflammatory cytokines. Adiponectin, a hormone secreted by the adipocytes, has been associated with both insulin-sensitizing and anti-inflammatory properties, and the concentration of adiponectin has proved to be consistently higher in patients with non-diabetic renal disease than in healthy control subjects, even if such patients display insulin resistance and an increased risk for cardiovascular disease.

Aims of the study: The present studies were undertaken to investigate whether low-grade inflammation, mannan-binding lectin (MBL) and α-defensin play a role, together with adiponectin, in patients with type 1 diabetes and diabetic nephropathy.

Subjects and methods: This study is part of the ongoing Finnish Diabetic Nephropathy Study (FinnDiane). The first four cross-sectional substudies of this thesis comprised 194 patients with type 1 diabetes divided into three groups (normo-, micro-, and macroalbuminuria) according to their albumin excretion rate (AER) in two of three consecutive overnight or 24-hour urine collections. The fifth substudy aimed to determine whether baseline serum adiponectin plays a role in the development and progression of diabetic nephropathy. This follow-up study included 1330 patients with type 1 diabetes and a mean follow-up period of five years. The patients were divided into three groups depending on their AER at baseline. As a measure of low-grade inflammation, highly sensitive CRP (hsCRP) and α-defensin were measured with radio-immunoassay, and interleukin-6 (IL-6) with high- sensitivity enzyme immuno-assay. Mannan-binding lectin and adiponectin were determined with time-resolved immunofluorometric assays. The progression of albuminuria from one stage to the other served as a measure of the progression of diabetic nephropathy.

Results: Low-grade inflammatory markers, MBL, adiponectin, and α-defensin were all associated with diabetic nephropathy, whereas MBL, adiponectin, and α-defensin per se were unassociated with low-grade inflammatory markers. hsCRP was higher in patients with micro- or macroalbuminuria than in those with normoalbuminuria. AER was the only clinical variable independently associated with hsCRP. IL-6 increased in parallel with the severity of renal disease, whereas AER, HDL-cholesterol and the duration of diabetes were independently associated with IL-6. MBL was higher in patients with micro- or macroalbuminuria than in those with normoalbuminuria, but no difference was observed between those with micro- and macroalbuminuria. HbA1c was the only variable independently associated with MBL. Adiponectin increased in parallel with the severity of diabetic nephropathy. The estimated glomerular filtration rate (eGFR), AER, and waist-to-hip ratio were independently associated with adiponectin. α-defensin was lower in patients with normo- and microalbuminuria than in those with macroalbuminuria, and systolic blood pressure, HDL-cholesterol, total cholesterol, age, and eGFR were
all independently associated with α-defensin. In patients with macroalbuminuria, progression to end-stage renal disease (ESRD) was associated with higher baseline adiponectin concentrations, but no differences were observed between progressors and non-progressors in patients with normo- or microalbuminuria. In addition to adiponectin, progression to ESRD was also associated with HbA1c, triglycerides, and eGFR.

Discussion and conclusions: Low-grade inflammation, MBL, adiponectin, and defensin were all associated with diabetic nephropathy in these cross-sectional studies. In contrast however, MBL, adiponectin, and defensin were not associated with low-grade inflammatory markers per se. Nor was defensin associated with MBL, which may suggest that during the acute phase response, these different players function in a coordinated fashion during the deleterious process of diabetic nephropathy.

The question of what causes low-grade inflammation in patients with type 1 diabetes and diabetic nephropathy, however, remains unanswered. Potential causative factors may include susceptibility genes, obesity, hyperglycemia, hyperlipidemia, smoking, and a low level of physical activity. To support this suggestion we could in our study observe that glycemic control, an atherosclerotic lipid profile, and waist-to-hip ratio (WHR) were associated with low-grade inflammation in the univariate analysis, although in the multivariate analysis, only AER, HDL-cholesterol, and the duration of diabetes, as a measure of glycemic load, proved to be independently associated with inflammation. Notably, all these factors, except the genes, are modifiable with changes in lifestyle or with a targeted medication or both. In the follow-up study, elevated serum adiponectin levels at baseline predicted the progression from macroalbuminuria to ESRD independently of renal function at baseline. This observation does not preclude adiponectin as a favorable factor during the process of diabetic nephropathy, since the rise in serum adiponectin concentrations may remain a mechanism by which the body compensates for the demands created by the diabetic milieu.
1. Introduction

The discovery of insulin in 1921 and its industrialized production made it possible to save the lives of thousands of patients with type 1 diabetes already during the first years of injectable insulin (1). At that time, knowledge of future secondary complications in the eyes and kidneys as well as their association with long-lasting high blood glucose was scarce. Even so, during the first two decades of the insulin era, people with type 1 diabetes lived long enough to develop complications.

As early as 1936, Paul Kimmelstiel and Clifford Wilson described structural changes in the kidneys and the clinical picture of diabetic kidney disease (diabetic nephropathy) (2). Evidence-based knowledge has since accumulated and has highlighted the importance of strict glycemic and blood pressure control in the avoidance and treatment of diabetic nephropathy. The goals of such treatment have changed in parallel with emerging new evidence, and fortunately treatment options have also improved in parallel with stricter targets for glycemic and blood pressure control. Despite all this positive development, epidemiological studies have demonstrated that during the past three decades, diabetic nephropathy continues to occur in 15-40% of patients with type 1 diabetes with a peak incidence after 15 to 20 years of diabetes (3, 4, 5). Diabetic nephropathy is the most common cause of renal failure in the industrialized world (6, 7). In addition, diabetic nephropathy is also strongly associated with premature cardiovascular mortality (8, 9, 10).

The pathogenesis of diabetic nephropathy remains somewhat unclear, but evidence suggests that it results from an interaction between susceptibility genes and the diabetic milieu.

Due to the strong association between diabetic nephropathy and macrovascular disease, some researchers have suggested a common denominator may link micro- and macrovascular complications. One such factor could be chronic low-grade inflammation (11, 12).

The present studies were therefore undertaken to explore the possible role of inflammation, mannan-binding lectin, adiponectin, and defensin in the deleterious process leading to diabetic nephropathy.
2. Review of the literature

2.1 Definition, diagnosis and classification of diabetes

2.1.1 Definition of diabetes

Diabetes is a systemic disease characterized by chronic hyperglycemia and disturbances in carbohydrate, lipid, and protein metabolism. Diabetes is the consequence of a decrease in insulin secretion or in the activity of insulin, or both. The diabetic syndromes represent a diverse clinical spectrum. Diabetes may present with characteristic symptoms such as thirst, polyuria, and weight loss, but its most severe manifestations are ketoacidosis or nonketotic hyperosmolaric coma. The symptoms are often vague or may even be absent altogether (13).

Diabetes is a universal chronic disease with widely varying prevalence rates across different populations. All over the world, the prevalence rates of diabetes are increasing, and in the latest IDF ATLAS, northern Europe had a 7% prevalence of diabetes in the adult population, while corresponding rates were already 8% in the US and 9% in China (14).

2.1.2 Diagnosis of diabetes

The WHO has established criteria for the diagnosis of diabetes mellitus (15). If a patient presents symptoms such as thirst, polyuria and weight loss, the diagnosis can be established by demonstrating fasting hyperglycemia. If the fasting plasma glucose falls within the diagnostic range for diabetes (> 6.9 mmol/l), no oral glucose tolerance test (OGTT) is required for the diagnosis. On the other hand, if the patient presents only minimal symptoms or the fasting plasma glucose concentration is within the normal range, an OGTT is required to confirm the diagnosis of diabetes.

2.1.3 Classification of diabetes

The classification of diabetes is based on the etiology of the disease, even if the true etiology and pathogenesis of the two most common remains only partially understood (15). The clear majority of cases falls into two broad etiopathogenetic categories, called type 1 and type 2 diabetes, although the extent of heterogeneity among these two types remains uncertain. The third category of diabetes includes nongenetic forms secondary to pancreatitis,
pancreas cancer, or a number of endocrine entities on the one hand, and monogenic forms of diabetes on the other. Monogenic diabetes comprises genetic defects in beta cell function, the most common form of which is known as MODY, or maturity onset diabetes of the young. The fourth category of diabetes is called gestational diabetes, and is characterized by carbohydrate intolerance and hyperglycemia of variable severity with onset during pregnancy.

2.2 Diabetic complications

Diabetes has wide-ranging effects on metabolism, and a long-term disease such as diabetes may have several potential mediators of tissue damage. The consequences of such tissue damage may include the dysfunction and total failure of various organs, especially the eyes, kidneys, heart, feet, and blood vessels. One explanation for the development of long-term complications of diabetes is the failure of antidiabetic therapy to normalize metabolism completely.

The prevalence of microangiopathic complications such as retinopathy, neuropathy, and nephropathy was the highest in patients with poor glycemic control in a 25-year follow-up study in a large cohort of patients with both type 1 and type 2 diabetes (16). This observation later proved to be true also despite more modern treatment options in the DCCT study in patients with type 1 diabetes and in the UKPDS in patients with type 2 diabetes (17, 18).

It is worth noting that in these studies, some patients presented no complications even though they had chronically poor metabolic control. Regarding nephropathy, this interesting escape phenomenon was associated with low blood pressure, thus supporting the role of concomitant poor glycemic control and elevated blood pressure for the development of diabetic nephropathy and possibly also for retinopathy (19, 20).

Patients with either type 1 or type 2 diabetes are at increased risk for atherosclerotic, macrovascular disease. Macroangiopathy and cardiovascular disease account for 70-75% of deaths in people with diabetes (21). The clinical picture related to the etiopathogenesis of macrovascular disease includes several abnormalities such as hyperlipidemia, hypertension, hyperglycemia and insulin resistance.

Patients with diabetic nephropathy and non-diabetic patients with already diagnosed macroangiopathy share the same risk factors for cardiovascular disease, as as well as the risk of early death from cardiovascular disease is also greatly increased (8, 9).
2.2.1 Diabetic nephropathy

2.2.1.1 Definitions
Diabetic nephropathy is defined as a progressive increase in the urinary albumin excretion rate accompanied by increasing blood pressure and a relentless decline in the glomerular filtration rate with end-stage renal failure as the final endpoint (22). Diabetic nephropathy is typically accompanied by retinopathy. Many people with diabetes do not necessarily progress to end-stage renal disease (ESRD), as they may die before then from cardiovascular disease (8, 9).

The different stages of diabetic nephropathy are classified according to the increase in the urinary albumin excretion rate in timed urine collections either overnight or during a 24-h period. Microalbuminuria is defined as an increase in the AER above normal (i.e. ≥ 20 µg/min or ≥ 30 mg/24 h). Proteinuria represents an increase in albuminuria of ≥ 200 µg/min or ≥ 300 mg/24 h. When daily proteinuria exceeds 3 g, the patient is deemed to have nephrotic syndrome. The final stage of diabetic nephropathy is ESRD. To be classified as microalbuminuric or proteinuric, the patient’s AER must exceed the upper limit in at least two of three urine collections.

2.2.1.2 Natural history of diabetic nephropathy in patients with type 1 diabetes

Albumin excretion rate
At diagnosis of type 1 diabetes, patients typically exhibit an elevated AER and display glomerular hyperfiltration. However, the AER returns to normal after the initiation of insulin treatment, and the same change occurs regarding the glomerular filtration rate (GFR) in most patients (23-27).

The transition from normoalbuminuria to microalbuminuria has been associated with the baseline AER, blood glucose control, blood pressure, and the presence of retinopathy (22).

Some studies have suggested that the prevalence of microalbuminuria, incipient diabetic nephropathy, has been relatively high, even as high as 19% of patients, during the first five years of type 1 diabetes (28, 29), whereas other studies, show that the AER remained normal during these initial years (26, 27).

The proportion of patients with microalbuminuria has been reported to increase during the first decades of diabetes. In a large-scale European study, the prevalence of microalbuminuria was 31% after 15 years (28), and 27% after 15-29 years of type 1 diabetes in Northern Wales (30). In Sweden, the prevalence rates for microalbuminuria have been considerably lower: only 6% in patients diagnosed at the beginning of the 1970s and followed for 20 years (31). The reason for the discrepancy between the Swedish and the other studies may be strongly associated with the significantly better
glycemic control in the Swedish patients than in the patients in the other studies.

In previous studies approximately 80% of the patients with microalbuminuria developed proteinuria (32, 33). In more recent studies, however, only about 20% of microalbuminuric patients progress to overt proteinuria over a period of ten years, whereas 50% remain microalbuminuric, and 30% regress to normoalbuminuria (27, 34). The risk for progression from microalbuminuria to overt nephropathy is strongly associated with blood pressure (34).

When a patient has progressed to persistent proteinuria, his/her urinary protein excretion rate rises continuously and, as in the stage of microalbuminuria, the blood pressure becomes a particularly important determinant of this process (35).

**Renal function**
At the moment the patient presents with persistent proteinuria, the GFR will begin to decline. Because blood pressure is the key determinant of the progression of the disease the average fall in the GFR will be approximately 10-12 ml/min annually, if the hypertension goes untreated. Thus the progression of the disease from the onset of proteinuria to the inevitable ESRD will take roughly 8-10 years (36-38). However, efficient treatment and the normalization of blood pressure will retard the disease process and postpone the development of ESRD (39-41).

**Blood pressure**
Blood pressure rises in parallel with the increase in the urinary albumin excretion rate, and if blood pressure goes untreated, over 80% of the patients with proteinuria will have blood pressure exceeding 140/90 mmHg. It thus comes as no surprise that hypertension is an essential component of the clinical picture in patients with ESRD (22).

### 2.2.1.3 Prevention of diabetic nephropathy

**Primary prevention**
The landmark DCCT study demonstrated that good glycemic control can considerably reduce the risk for micro- and macroalbuminuria. In patients with normoalbuminuria at baseline, the reduction in the relative risk for developing microalbuminuria was 39%, and that for developing proteinuria, 54% in those patients with an HbA1c of 7% compared to those with an HbA1c of 9%. The study showed that the lower the HbA1c, the lower the risk (42).

**Secondary prevention**
Once microalbuminuria or proteinuria has become manifest, there is no solid evidence of a benefit of good glycemic control; there is, however, abundant
evidence that the reduction of blood pressure can slow the progression of micro- and macroalbuminuria to ESRD (43, 44). Angiotensin-converting enzyme (ACE) inhibitors are preferred as the first-line treatment in patients with type 1 diabetes and nephropathy (45), since ACE inhibitors reduce the albumin excretion rate more than do other classes of antihypertensive agents (46). Furthermore, this renoprotective effect is independent of blood pressure reduction and may be related to reduced intraglomerular pressure and the passage of proteins into the proximal tubule (47, 48). The reduction of blood pressure to < 140/80 mmHg, which is still a rather conservative level, has proved capable of reducing the decline in GFR from 10-12 ml/min per year without treatment to 1 ml/min per year in patients with reasonably good glycemic control and low cholesterol (44). However, most patients with proteinuria require multiple agents in addition to ACE inhibitors to achieve a new blood pressure target of < 130/80 mmHg (49).

2.2.1.4 Pathogenesis of diabetic nephropathy
The specific pathology of diabetic nephropathy is restricted mainly to the renal glomeruli and the tubular interstitium (50). Histologically, the hallmarks of diabetic nephropathy include thickening of the glomerular basement membrane and an increase in the fractional volume of the mesangium (51). Expansion of the glomerular mesangium correlates closely with a reduced renal function and the development of proteinuria (52).

During the progression of the disease, mesangial expansion typically presents as nodular glomerular lesions. For diabetic nephropathy, these pathognomonic lesions bear the name Kimmelstiel-Wilson nodules according to their first description by Kimmelstiel and Wilson (2).

The tubulointerstitial injury appears to be closely associated to the glomerular pathology. Interstitial expansion is related to renal dysfunction, proteinuria, and mesangial expansion (53).

Patients with type 1 diabetes and microalbuminuria typically exhibit an increased fractional volume of the mesangium, an observation which maybe associated with a rise in blood pressure and a small reduction in creatinine clearance (54, 55). In patients with macroalbuminuria, the expansion of the mesangium and the interstitium, together with glomerular occlusions, closely correlate with a reduced renal function (50).

2.2.1.5 Pathogenic mechanisms of diabetic nephropathy

Hyperglycemia
As described above, poor glycemic control has been shown to contribute to the development of micro- and macroalbuminuria. Intensive glucose control, HbA1c 7% vs 9%, reduces the risk for progression from normo- to microalbuminuria in patients with type 1 diabetes (42).

Hyperglycemia has also been linked to many deleterious processes in renal tissue. High glucose in the mesangial cells induces cell hypertrophy
and increases the extracellular matrix deposits by stimulating the expression of various genes and protein secretion, such as collagen and fibronectin (56, 57). Furthermore, hyperglycemia has been shown to stimulate the transforming growth factor (TGF)-β system, and the induction of this system is considered to be one of the main determinants of hypertrophy of the mesangial and tubular cells in diabetic nephropathy (58).

Sustained hyperglycemia leads to enhanced non-enzymatic protein glycation, which represents the increased covalent binding of glucose to proteins. The process of glycation progresses via relatively stable ketoamines, products of Amadori, to stable advanced glycation end-products (AGEs). AGEs accumulate in the tissues over the lifetime of the protein (59). In patients with diabetes, AGEs accumulate in renal glomeruli and tubuli (60). AGEs have been shown to affect properties of extracellular matrix proteins leading to matrix rigidity and mesangial expansion (61).

The enzyme aldose reductase via the polyol pathway, reduces glucose to sorbitol. In chronic hyperglycemia sorbitol accumulates in many tissues, including the renal glomeruli and tubuli. Some have suggested that this accumulation of sorbitol is deleterious to the renal tissue by disturbing cellular osmoregulation and by changing the cellular redox potential (62, 63). In addition, inhibition of the enzyme aldose reductase has been shown to prevent a glucose-induced increase in TGF-β1 production and protein kinase C (PKC) activity in human mesangial cells (64). Aldose reductase inhibitors have not yet been introduced as the treatment of choice for human diabetic nephropathy, since clinical trials have not only proved ineffective, but also caused adverse effects on many of the potential inhibitors tested in recent years (65).

The hexosamine pathway, another of the intracellular pathways of glucose metabolism, also appears to be related to diabetic complications (66). Activation of this pathway during hyperglycemia has been linked to diabetic nephropathy through its end-product, N-acetylglucosamine, which in turn is associated with increased TGF-β1 expression (67).

Hyperglycemia is also associated with oxidative stress through the increased production of reactive oxygen species (68-70). Oxidative stress and its concomitant, reactive oxygen species, are not only recognized as one of the most important components in the pathogenesis of diabetic microvascular complications, but also as a possible unifying mechanism in the pathogenesis of both microvascular and macrovascular complications (71, 72). High glucose in diabetic cells induces the production of superoxide, which is deleterious to the cells by activation of the polyol and hexosamine pathways, the formation of AGEs, and the activation of PKC (72).

**Hypertension**

In patients with diabetes, the development of proteinuria is typically paralleled by an increase in systemic blood pressure, and blood pressure is further closely related to a decline in the glomerular filtration rate (73). In
patients with type 1 diabetes and normoalbuminuria, blood pressure has been shown to be already higher in those who progress to microalbuminuria than in patients with stable normoalbuminuria, even if the mean systemic blood pressure in the progressors was no higher than a mean of 138/82 mmHg (74).

Glycemic control is also linked to high blood pressure, since vasodilatation, induced by hyperglycemia, reduces afferent arteriolar resistance in the glomerulus proportionally more than efferent arteriolar resistance. The net effect is an increase in glomerular capillary pressure level. In the presence of hyperglycemia, even a small increase in systemic blood pressure may thus be deleterious to the hemodynamics of the glomerulus (75).

Increased glomerular capillary pressure and concomitant glomerular expansion are associated with a stretching of its components. Stretch in the mesangial cells leads in turn to stimulation of the synthesis and deposition of matrix components (76).

**Proteinuria**

Proteinuria is a key factor in diabetic nephropathy and a predictor of progression to ESRD, and has even been suggested as an important factor per se in promoting the progression of diabetic nephropathy (77, 78). Excessive protein overload leads to tubulointerstitial damage by inducing the release of chemokines and endothelin (79-81). The beneficial effect of ACE-inhibition in diabetic nephropathy has at least partly been associated with its effect on proteinuria (46).

### 2.2.1.6 Signaling pathways and mediators of diabetic nephropathy

Several cytokines have been associated with the pathophysiology of diabetic nephropathy, particularly TGF-β1 (82). Increased TGF-β1 expression has been demonstrated in an animal model of diabetic kidney disease (rat model) and in patients with type 2 diabetes and diabetic nephropathy (83, 84). Hyperglycemia stimulates TGF-β1 expression in a variety of renal cells, such as glomerular mesangial cells and renal interstitial fibroblasts (85). TGF-β1 increases matrix synthesis as well as inhibits its degradation, and has also been associated with the upregulation of adhesion molecules and enhanced chemotraction (86, 87).

Connective tissue growth factor (CTGF) is another cytokine that has been associated with diabetic nephropathy (88, 89). High glucose and TGF-β1 induce CTGF expression in mesangial cells, and CTGF in turn mediates TGF-β1-induced fibroblast collagen synthesis (88-90).

Circulating and local vascular endothelial growth factor (VEGF) levels are high in diabetes, and an excess of VEGF plays a role in mediating glomerular hypertrophy and proteinuria (91, 92). Excessive angiogenesis, induced by VEGF, has even been linked to the progression of diabetic nephropathy (93, 94).

In the kidney high glucose has been associated with an activation of a
local renin-angiotensin system in the mesangial cells, the proximal tubular cells and the podocytes (95-97). Angiotensin-2 stimulates the expression of TGF-β1 in the kidney. Thus hemodynamic as well as structural changes in diabetic nephropathy are suggested to result from the interplay between angiotensin-2 and TGF-β1, and angiotensin-2 even plays a role in the progression of diabetic nephropathy (98).

The growth hormone-insulin-like growth factor-insulin growth factor-binding protein (GH-IGF-IGFBP) axis has been suggested both to maintain normal renal function and to play an important role in the development of DN (99). These growth factors are linked to the earliest detectable renal changes associated with hyperglycemia (100, 101). The kidney is a site for the production of IGF-1, which normally mediates its effects on renal growth and function (102, 103). In diabetes the expression of IGF-1 in the kidneys increases, and furthermore, the upregulation of IGF-binding proteins has been implicated in IGF-1 trapping in the kidney (104). In cell cultures, IGF-1 has been shown to induce mesangial proliferation and the secretion of collagen (105). Recently IGFBP-3, one of the IGF-binding proteins, has been associated with podocyte apoptosis (99).

Protein kinase C, PKC, a family of many serine-threonine kinases, is activated by diacylglycerol (DAG) (106). Concentrations of DAG typically increase in diabetes due to hyperglycemia (107-109). The activation of PKC is present in many different tissues, such as the glomeruli, and a variety of functional abnormalities may result from the activation of the DAG-PKC pathway in the renal tissue also (110, 111). PKC has been shown to mediate the intracellular signals of TGF-β1, VEGF, and angiotensin-2, and PKC is further suggested to be a major signaling pathway for TGF-β in inducing extracellular matrix production in diabetic nephropathy (111-113). The activation of PKC further activates mitogen-activated protein kinase (MAPK) to form the DAG-PKC-MAPK pathway, a transduction system of signals from hyperglycemic plasma to the glomerular cell nucleus in patients with diabetic nephropathy. The high expression of the DAG-PKC-MAPK mRNA has accordingly been suggested to play an important role in the pathogenesis of diabetic nephropathy (113).

2.2.1.7 Genetics of diabetic nephropathy in type 1 diabetes
The familial clustering of diabetic nephropathy suggests that genetic factors are important in determining susceptibility (114, 115). The cumulative incidence of diabetic nephropathy among siblings with type 1 diabetes who have a proband with type 1 diabetes and diabetic nephropathy is approximately 70% compared to 25% among diabetic siblings with a proband unaffected by diabetic nephropathy (116). A family history of hypertension has also been associated with increased predisposition to diabetic nephropathy (117). In Finland, a family history of type 2 diabetes was also associated with the risk for diabetic nephropathy in family members with type 1 diabetes (118).
2.2.1.8 Birth weight and diabetic nephropathy in patients with type 1 diabetes

Low birth weight has been suggested to confer increased risk for diabetic nephropathy in patients with both type 1 diabetes (119) and type 2 diabetes (120). The association between low birth weight and diabetic nephropathy in patients with type 1 was observed only in females (119), but in the Pima Indian population with type 2 diabetes, there was no sex-specific effect (120). In contrast, cross-sectional data from Finland in a Caucasian population of 1543 patients with type 1 diabetes, suggested no role for low birth weight in the development of diabetic nephropathy (121).

2.3 Immune defense systems

2.3.1 Adaptive and innate immunity

The antimicrobial defense system is generally divided into acquired (adaptive) immunity and innate immunity (122). The acquired immune system uses B- and T-lymphocytes to mediate antigen-specific humoral and cellular responses. These responses require a relatively long period of time, from days to weeks, for maximal activity and also result in immunologic memory. The function of the system is intimately tied to the innate immune system (123-125).

The innate immune system depends on non-lymphoid tissue and is phylogenetically older than acquired immunity. The innate immune system is a first-line defense system which uses soluble and cellular sensing mechanisms to recognise potentially harmful substances (122, 126). The innate immune system is fast and immediately inducible in comparison to the slower acquired immune system. The various elements of innate immunity do not function in isolation, but interact to ensure that the magnitude of the host response reflects the severity of the foreign threat (125).

A reaction to inflammation, infection, or trauma is a change in the concentration of certain plasma proteins, such as fibrinogen, haptoglobin, C-reactive protein (CRP), and serum amyloid A (127). These acute phase proteins are synthesized in the liver, and the process is stimulated by pro-inflammatory cytokines, especially interleukin 1 and 6 (IL-6) as well as tumour necrosis factor-α (128). The purpose of the acute-phase response is to neutralize the “enemy” and to restore homeostasis.

2.3.2 Chronic inflammation and C-reactive protein

The pathogenesis of vascular complications in diabetes involves inflammation...
and endothelial dysfunction (129-131), which are also part of the metabolic syndrome and of insulin resistance (129). Low-grade inflammation has itself been suggested to be a common ground for endothelial dysfunction and insulin resistance (129). Low-grade inflammation facilitates the invasion of monocytes into the vascular wall, which contributes to the formation of atherosclerosis and cardiovascular disease (132).

CRP is a sensitive marker of inflammation and increases rapidly in response to several disease conditions. The generally held clinical cutoff for significant inflammatory disease is 10.0 mg/l (133), where as the introduction of a reproducible high sensitivity assay to measure CRP made it possible to obtain further information from values between 3-10 mg/l and even below 3 mg/l (134). The median value in healthy people, after exclusion of smokers and users of oral contraceptives, is between 0.6 and 1.7 mg/l. The median increases to 2.2 in men and to 2.4 in women among smokers and if contraceptives are used. The upper end (97.5th percentile) of the reference interval is between 3 and 6 mg/l (134-137).

In prospective studies, high concentrations of CRP have been associated with increased risk for coronary heart disease and myocardial infarction (MI) (138-145). Interestingly, even CRP concentrations below 1 mg/l have been associated with increased risk for MI, coronary heart disease mortality, and ischemic stroke (140-142, 146).

2.3.3 Determinants of C-reactive protein

The degree of adiposity is a major determinant of CRP in the general population (147-149). Waist circumference is also an important determinant and source of variation in the CRP concentrations (149). A weight loss program which includes a Mediterranean diet and moderate physical activity proved to have a favorable effect on CRP, proinflammatory cytokines, and endothelial function in obese premenopausal women (150, 151). Weight loss achieved by a caloric restriction diet alone also decreased CRP in obese postmenopausal women (152).

The role of physical activity in CRP levels remains unclear, since regular physical activity is also generally associated with a lower degree of body fat. Regular and nearly daily vigorous physical activity was associated with reduced risk for elevated CRP when compared to sedentary individuals in the NHANES study (153, 154). Cardiorespiratory fitness, measured with the maximal treadmill test, was associated with lower CRP concentrations in men and women (155, 156). In the Physicians Health Study, however, physical activity showed no association with CRP after adjustment for BMI (157).

Smoking was associated with high CRP when smokers were compared to those who had never smoked, and based on the fact that former smokers are at lower risk for high CRP than are current smokers. This association represents further evidence of the benefit of stopping smoking (158).
Pharmacologic interventions also affect serum CRP concentrations. Both statin treatment and antihypertensive medication with ACE inhibitors were associated with decreased serum CRP (159, 160).

### 2.3.4 Chronic inflammation and diabetic nephropathy

In patients with diabetic nephropathy and those with incipient nephropathy (microalbuminuria), studies have reported increased CRP concentrations, suggesting that inflammation may play a role in the process of failing kidney function (161-163). However, these studies did not measure IL-6, a proinflammatory cytokine produced by many cells such as adipocytes, activated leucocytes, myocytes and endothelial cells (145, 164-167). The measurement of IL-6 is crucial, since studies show it is the main stimulus for the hepatic production of CRP (168, 169), and that its gene transcripts are expressed in human atheromatous lesions (170). Expression studies have further shown that IL-6 mRNA is present in the mesangium of renal specimens from diabetic subjects (171). Interestingly, CRP itself has been shown to stimulate monocyte release of IL-6 (164).

The role of chronic inflammation in patients with type 1 diabetes and diabetic nephropathy obviously remains somewhat unclear as available studies have shown rather contradictory results (161, 172). Myrup et al. failed to detect any increase in CRP in patients with type 1 diabetes, even in the macroalbuminuria stage, when compared to healthy controls. However, IL-6 was elevated in those patients with diabetes, and diabetic patients with normoalbuminuria already differed from healthy controls (172). In contrast, Schalkwijk et al. observed an association between CRP and DN, and even patients with normoalbuminuria had higher CRP than did healthy control subjects (161). They presented no data on IL-6, however, and given the close relationship between IL-6 as the stimulus and CRP as the product, showing a simultaneous increase in both markers as a proof of the presence of inflammation is important.

Declining renal function has been associated with increased serum cytokine levels, such as those of IL-6, IL-8, and TNF-α (173). Possible causes of chronic inflammation in chronic kidney disease in patients with diabetes are most likely multifactorial (174, 175). It is noteworthy that AGEs and oxidative stress are enhanced in chronic kidney disease, and that both presumably play a role in the activation of mononuclear cells and in the inflammatory response (176, 177). Diabetes is in itself a state of chronic hyperglycemia associated with oxidative stress and presents from the onset of disease (178). One can thus hypothesize that in diabetes, not only diabetes per se, but also its consequences (advanced glycation end-products and oxidative stress) promote chronic inflammation in susceptible individuals.

Increased concentrations of IL-6 and CRP have been observed in patients with type 2 diabetes, a finding that suggests the presence of chronic low-
grade inflammation. High IL-6 and CRP concentrations have even been shown to predict the development of type 2 diabetes (179). Furthermore, IL-6 is associated with visceral obesity and insulin resistance, both of which are key features of microalbuminuria and macrovascular complications in patients with type 2 diabetes (180). Figure 1 shows known associations between low-grade inflammation, insulin resistance, atherosclerosis, and diabetic nephropathy. Interestingly, in patients with type 1 diabetes, insulin resistance has also been shown to play a central role in the pathogenesis of diabetic nephropathy (181).

**Figure 1.** Chronic inflammation and its associations with insulin resistance, atherosclerosis and diabetic nephropathy (+ = an association shown). AMI, acute myocardial infarction; CRP, C-reactive protein; IL-6, interleukin-6; T1 DM, type 1 diabetes; T2 DM, type 2 diabetes

Although evidence suggests that low-grade inflammation may be associated with diabetic nephropathy, whether this is a true relationship remains unknown. Furthermore, whether chronic inflammation is associated with renal function, blood pressure, waist-to-hip ratio, and insulin resistance in patients with type 1 diabetes also remains unknown.

### 2.4 The complement system

#### 2.4.1 The complement system and lectin pathway

The complement system can be activated by three different pathways (Figure 2) of which the lectin pathway most probably predates the classical and the alternative pathways (182).
Mannan-binding lectin (MBL), a key molecule of the innate immune system, is synthesized by the liver and secreted into the bloodstream (183). MBL belongs to the C-type lectins and features binding sites for carbohydrates. MBL can bind to common carbohydrate structures such as mannose and N-acetylglucosamine (184, 185). If carbohydrates are present in the correct pattern (e.g. on the surface of micro-organisms), the binding of MBL will result in direct opsonophagocytosis and activation of the complement by MBL-associated proteases via the lectin pathway (183, 185-187).

The median serum MBL concentration is 800 to 1000 µg/l in healthy Caucasians (188, 189), but it is worth noting that serum concentration varies considerably in humans. This variability is due largely to genetic diversity (i.e. polymorphisms that lead to amino acid replacements in the collagen-like region of the MBL). These polymorphisms in the coding region will affect MBL assembly and stability, but the final MBL is also under the influence of polymorphisms in the promoter region (190). Interestingly, a low level of MBL negatively affects the outcome of infectious diseases, which was observed in very young children with acute respiratory tract infections and in patients with severe infections after chemotherapy (191, 192). Patients with cystic fibrosis and low MBL concentrations have even been shown to have a shortened life expectancy (193).

A high level of MBL, however, in spite of offering protection against invading organisms, may also be deleterious to the host in some disease states through exaggerated complement activation (194, 195). Thus, a high MBL was associated with the aggravation of ischemic injury in an animal model of an acute myocardial infarction (MI) (196). In humans,
MBL has been suggested as the main mediator of complement activation during thoraco-abdominal aortic aneurysm repair, an operation of extensive ischemia reperfusion and systemic inflammation (197).

### 2.4.2 The complement system and diabetic nephropathy

As previously highlighted, diabetic nephropathy may be associated with low-grade inflammation, and activation of the complement system may contribute to this inflammatory process (198). Patients with type 1 diabetes and normal AER show higher levels of MBL than do healthy subjects. Although the MBL concentration was associated with AER, no correlation was observed evident between MBL and CRP in these patients (198). On the other hand, patients with type 1 diabetes and diabetic nephropathy seem to have even higher levels of MBL than do patients with normalalbuminuria. A history of cardiovascular disease was associated with high MBL in these patients (199). Although patients with diabetic nephropathy have higher CRP concentrations than do patients with an AER in the normoalbuminuric range, no association was observed between MBL and CRP in this patient population (199).

Reasons for the differences in MBL concentrations between patients with diabetes and healthy controls remains unknown. Genetic differences failed to explain the higher MBL concentrations in patients with type 1 diabetes, however (199, 200). One possible explanation could be that hypoinsulinemia in the portal vein upregulates MBL expression in the liver in patients with subcutaneous insulin injections (199). In patients with diabetic nephropathy, high-expression MBL genotypes were more prevalent and could in part explain why these patients showed higher MBL concentrations than did patients without nephropathy (199).

The mechanism behind the potentially deleterious effect of high MBL in patients with diabetic nephropathy is also unknown. In other chronic renal diseases, such as IgA nephropathy and Henoch-Schönlein purpura nephritis, upregulation of MBL and activation of the complement system have been implicated in the disease process (201, 202). The IgA molecule is a heavily glycosylated molecule with mannose-type N-linked glycan chains that can be recognized and bound by MBL (185).

In patients with type 1 diabetes, chronic hyperglycemia and an abundance of AGEs are typical features that have been suggested to alter the autoreactivity of MBL (199). However, thus far remains unknown, whether MBL increases in parallel with the severity of diabetic nephropathy.
2.5 Adiponectin

2.5.1 Adiponectin

Adiponectin, a hormone with a M_r of 30000 is structurally similar to complement factor C1q (203). Adiponectin is secreted exclusively from adipocytes and, compared to many other important hormones, is abundantly released into circulation (203, 204). Adiponectin accounts for up to 0.05% of total serum protein (203), and several isoforms have been characterised (205, 206). The ability of adiponectin to polymerize, resulting in trimers and higher-order polymers, is suggested to be crucial for its biological activity (205, 206). The collagenous domain of the molecule has four conserved lysines that can be hydroxylated and glycosylated, both of which are processes proposed to be critical for the three-dimensional structure of the biologically active adiponectin molecule (207). In fact, glycosylation likely represents one of the major posttranslational modifications of adiponectin (207).

![Adiponectin Regulation, Target, and Action](image)

Adiponectin is a hormone that plays a role in the regulation of glucose and lipid metabolism (Figure 3) (208, 209). Two cell-surface adiponectin receptors have been characterized and cloned, and the liver and the muscle show the most prominent expression (210). The binding of adiponectin to its receptor leads to the stimulation of adenosine monophosphate (AMP)-activated protein kinase and the activation of peroxisome proliferator-activated receptor-α, which in turn positively affects glucose uptake and fatty acid oxidation in the muscle as well as the reduction of molecules involved in hepatic gluconeogenesis (207, 211). The activation of AMP kinase has also been linked to proliferator-activated receptor-γ co-activator-1, and thus to mitochondrial oxidation and glucose uptake (212).

Adiponectin has also been shown to exert anti-atherosclerotic effects by inhibiting neointimal thickening and vascular smooth muscle cell proliferation in mechanically injured arteries (213, 214).
In contrast to observed increases in the plasma levels of several adipokines (leptin, resistin), the plasma levels of adiponectin are markedly reduced in individuals with visceral adiposity (Figure 4). Low plasma adiponectin concentrations thus occurs in obesity (204, 215) and type 2 diabetes (215, 216), but also in patients with coronary artery disease (216, 217).

Circulating levels of adiponectin thus negatively correlate with insulin resistance, fasting serum insulin, serum triglycerides, and fasting plasma glucose concentrations (216, 218-220).

Adiponectin is also generally lower in males than in females (215, 219), a gender difference attributed to the effect of testosterone (221).

Importantly, high basal plasma adiponectin levels were associated with a reduced risk for MI in men in a nested case-control study with a follow-up of six years (222).

Besides its anti-atherosclerotic effect, adiponectin also has an anti-inflammatory effect. Thus, in the early stage of atherosclerosis, physiological concentrations of adiponectin have been shown to inhibit tumor necrosis factor (TNF)-α-induced monocyte adhesion and the expression of adhesion molecules in vascular endothelial cells (217). Adiponectin furthermore negatively correlates with CRP in patients with coronary atherosclerosis (223), and both plasma IL-6 and CRP concentrations appear inversely associated with adiponectin in obese women (224). IL-6 has also been shown to downregulate adiponectin gene expression in adipocytes (225).

Weight reduction increases adiponectin not only in patients with diabetes, but also in non-diabetic subjects (216). Interestingly, the insulin sensitizers thiazolidinediones, used as hypoglycemic agents, both improve
insulin sensitivity in patients with type 2 diabetes and stimulate the synthesis of adiponectin (226, 227).

2.5.2 Adiponectin in patients with type 1 diabetes and diabetic nephropathy

Adiponectin concentrations are higher in patients with short duration of type 1 diabetes than in weight-matched healthy volunteers, observation also replicated in patients with a long duration of type 1 diabetes and normal kidney function (228-230). The reason for this finding remains unknown, although some have speculated that this finding may be related to the glycosylation of the adiponectin molecule (207).

Notably, adiponectin concentrations have consistently proved higher in patients with renal disease than in healthy control subjects, even if the same patients also display insulin resistance and increased risk for cardiovascular disease (231, 232). In patients with advanced ESRD, high adiponectin concentrations were associated with type 1 diabetes, low visceral fat mass and low CRP (231). However, these studies included only a small number of patients with type 1 diabetes, and thus, the rather unexpected finding of high adiponectin concentrations when one would expect low concentrations must be replicated in larger patient cohorts.

The mechanism responsible for the increase of adiponectin consistently observed in ESRD remains unclear. Because high plasma adiponectin concentrations decrease after renal transplantation, renal insufficiency may either affect the clearance of adiponectin or stimulate its production or both (233).

A number of questions still await answers. For instance, whether adiponectin increases in parallel with the severity of diabetic nephropathy or whether adiponectin is associated with inflammation and metabolic control in patients with type 1 diabetes at various stages of renal function remains unknown. Furthermore, whether baseline adiponectin predicts the progression of diabetic nephropathy also remains unknown.

2.6 Defensins

2.6.1 The defensin family

Defensins belong to an antimicrobial peptide family, consisting of polypeptides with fewer than 100 amino acids. They function as a part of the innate local host response of multicellular organisms. Antimicrobial peptides have been found in both non-vertebrates, such as plants and insects, and vertebrates ranging from amphibians to humans, which suggests that these
defense molecules predate the evolutionary divergence of animals and plants (123, 234-237).

The defensin family itself consists of small cystein-rich peptides, 30 to 40 aminoacids in mammalians, with broad cytotoxic activity against bacteria, fungi, parasites, viruses, and host cells (123, 237, 238). Mammalian defensins are further organised into three classes: α-, β- and θ-defensins (238).

The role of the defensin family in the human innate immune system is based on studies of α- and β-defensins, since the peptide production of known human θ-defensin genes remains to be shown (239).

### 2.6.2 α-defensins

Human α-defensins, a group of six peptides, are predominantly found in neutrophils and in intestinal Paneth cells (237-239). Neutrophil-originated α-defensins, α-defensins-1 to -4, are also called human neutrophil peptides (HNP). These defensins are synthesized constitutively in the bone marrow during specific differentiation stages of neutrophil development in promyelocytes and early myelocytes. They are stored in the granules of phagocytes and released on demand from these cytoplasmic granules (237, 240, 241). The first three (α-defensin-1, -2 and -3) have a similar structure, with a difference of only one amino acid between each other (FIGURE 5) (123, 237).

![FIGURE 5. The amino acid structure of α-defensin-1, -2, and -3 (Mod. from Ref 123)](image)

It is worth noting that even if the production of α-defensin has not yet been shown to be inducible by inflammatory mediators, such as β-defensin-2 and -3 (242), the expression of α-defensin in the neutrophils can be increased by granulocyte colony-stimulating factor (GC-SF) (243).

α-defensins are involved in the intracellular destruction of foreign pathogens but can also be released into the extracellular environment by neutrophil degranulation and thereby contribute to the innate host defence against microbial invasion (243). In addition, α-defensins-1 to -3 have been observed to be chemotactic for monocytes and naive T cells (244, 245).

Based on in vitro studies, however, human defensins have also been suggested to have a deleterious effect on host cells (242). In addition,
some defensins have been shown to be cytotoxic to mammalian cells in higher concentrations (245-248). High concentrations of defensins are associated with the generation of pro-inflammatory signals (249), which is a phenomenon suggested to contribute to tissue injury in the lungs (250).

Plasma and serum α-defensin levels may decrease with activated alpha2–macroglobulin, a protease inhibitor able to bind to defensin peptides (251). It is noteworthy that patients with diabetes have higher serum alpha2-macroglobulin levels than do healthy controls (252). Whether interaction between these molecules plays a physiological role remains unknown.

α-defensins, shown to inhibit or to enhance inflammation in many ways, can stimulate the cytokine production of bronchial epithelial cells and modify inflammation through regulation of cytokine production in human monocytes and adhesion molecule expression in endothelial cells (249, 253). Regarding interference with complement system, α-defensin has been shown to either stimulate or suppress activation of the classical complement pathway (254, 255). In addition, α-defensin can reportedly inhibit the fibrinolytic system and to stimulate the binding of lipoprotein (a) and low-density lipoprotein to vascular cells. Thus, α-defensin may be a true link between inflammation and atherosclerosis (256-258).

2.6.2 α-defensin and diabetic nephropathy

Defensins, as a part of the innate immune system, appear to play a role in the regulation of the complement system and to augment the production of pro-inflammatory cytokines (240, 259).

Whether α-defensin is also associated with diabetic nephropathy and with low-grade inflammation and blood lipid values in patients with nephropathy remains unknown.
3. Aims Of The Study

One-third of patients with type 1 diabetes still develop diabetic nephropathy despite modern treatment options, and diabetic nephropathy is the most important cause of renal failure in the industrialized world. Because diabetic nephropathy and macrovascular disease are strongly associated, some have suggested that micro-and macrovascular complications may share a common origin, such as inflammation and a generally over-active innate immune system. The present studies were therefore undertaken to explore the possible role of inflammation, mannan-binding lectin, adiponectin, and defensin as a part of the deleterious process of diabetic nephropathy in patients with type 1 diabetes.

The main objectives were to answer the following questions:

1. Is low-grade inflammation associated with diabetic nephropathy in patients with type 1 diabetes? (I)

2. Is mannan-binding lectin (MBL) associated with diabetic nephropathy in patients with type 1 diabetes, and is there an association between MBL and low-grade inflammatory markers or insulin resistance? (II)

3. Is serum adiponectin associated with renal function, low-grade inflammatory markers, metabolic control, and insulin resistance in patients with type 1 diabetes? (III)

4. Is α-defensin (-1, -2, and -3) associated with renal function, low-grade inflammatory markers, and the blood lipid profile of patients with type 1 diabetes? (IV)

5. Does adiponectin play a role in the development and progression of diabetic nephropathy in patients with type 1 diabetes? (V)
4. Subjects

4.1 Cross-sectional studies (I-IV)

This study was part of the ongoing Finnish Diabetic Nephropathy Study (FinnDiane). The first four substudies (I-IV) were cross-sectional, and the last (V) was a follow-up study. The studies were conducted in accordance with the Declaration of Helsinki, and the ethical committees of all participating centers approved the study protocol. Each subject provided his or her written informed consent.

To minimize the effect of potential confounding factors, we carefully selected patients in order to achieve representative phenotypes of patients with normo-, micro-, or macroalbuminuria. Patients for the cross-sectional studies (I-IV) were selected from the entire study population of 1616 patients with complete information available about their histories of hypertension, diabetes, cardiovascular disease and the mortality of both of their parents. The patients were required to have a duration of diabetes of 10 to 30 years, which reduced the number of eligible patients to 882. To assure renal status, three complete urine collections were required, which reduced the number of eligible patients to 577. Those patients with normal AER were further required to take neither antihypertensive medication nor show any signs of cardiovascular disease, whereas those patients with microalbuminuria or macroalbuminuria were required to be undergoing ACE inhibitor treatment.

In all substudies, the patients were divided into three groups (normo-, micro-, or macroalbuminuria) according to their AER in three consecutive overnight or 24-h urine collections. Normal AER (normoalbuminuria) was defined as an AER persistently < 20 µg/min or < 30 mg/24 h, microalbuminuria as AER ≥ 20 < 200 µg/min or ≥ 30 < 300 mg/24 h, and macroalbuminuria as AER ≥ 200 µg/min or ≥ 300 mg/24 h in at least two of three urine collections. Type 1 diabetes was defined as the onset of diabetes before the age of 35 years and the initiation of permanent insulin treatment within one year of diagnosis.

A total of 401 patients with type 1 diabetes met all these selection criteria. Thereafter, the patient groups were matched for duration of diabetes. Because the shortest disease duration in the macroalbuminuric group was 13 years, this cut-off point was chosen for all patients. Finally, the patients were matched for sex, which resulted in 194 patients representative of a wide range of AER. At inclusion, five microalbuminuric and eight macroalbuminuric patients were treated with statins. None of the patients used acetosalicylic acid.

In study I, 194 patients were divided into three groups based upon their AER. Patients with normoalbuminuria (n = 67) received no antihypertensive
medication or showed no signs of cardiovascular disease, whereas all patients with microalbuminuria (n = 64) or macroalbuminuria (n = 63) were treated with an ACE inhibitor. A total of 66 healthy volunteers comprising Finnish research scientists and laboratory personnel working at the Folkhälso Research Center in Biomedicum Helsinki served as a control group.

Study II included a total of 191 patients, which was three less than in the first study. This was due to the fact that insufficient serum remained for the analyses in two patients in the microalbuminuria group and in one patient in the macroalbuminuria group.

Studies III and IV comprised a total of 189 patients, two less than in the second study for the same reason: due to a lack of serum samples for two patients with microalbuminuria.

### 4.2 Follow-up study (V)

In the prospective follow-up study (V), the main outcome was the progression of albuminuria. At baseline, all patients participating in the FinnDiane Study underwent a thorough clinical investigation that took place in conjunction with a regular visit to the attending physician. The medical file of every patient included in the analysis was reviewed, and any changes in renal status or the occurrence of cardiovascular events was verified. All the patients were re-examined at their local medical center according to the same protocol used during the baseline visit. Based on these procedures, 1330 type 1 diabetic patients were included in the study with a mean follow-up of 5.0 ± 2.2 years. Patients were divided at baseline into three groups according to their AER: 818 patients with normoalbuminuria, 216 patients with microalbuminuria, and 296 patients with macroalbuminuria. A total of 204 non-related healthy control subjects of Finnish origin, recruited from the personnel of Biomedicum Helsinki and their spouses (108 female and 96 male), with a mean age of 36.1 years and a BMI of 23.9 kg/m², were also included to compare adiponectin concentrations between healthy subjects and patients with type 1 diabetes.

### 4.3 Study design

In the first cross-sectional study, low-grade inflammatory markers IL-6 and CRP were measured and correlated with AER and estimated GFR (eGFR). Furthermore, we assessed a possible association of low-grade inflammation with clinical variables and the estimated glucose disposal rate (eGDR) [as a measure of insulin sensitivity].

In the second study, MBL was measured and correlated with AER and eGFR. In addition, we assessed a possible association of MBL with low-grade inflammatory markers and eGDR.
In the third study, adiponectin was measured and correlated to AER and eGFR. Furthermore, we assessed a possible association of adiponectin with low-grade inflammatory markers, eGDR, and clinical variables/metabolic control.

In the fourth cross-sectional study α-defensin (-1, -2, and -3) was measured and correlated with AER and renal function. Furthermore, we assessed a possible association of α-defensin (-1, -2, and -3) with low-grade inflammatory markers, serum lipids, and clinical variables.

In the fifth study, the follow-up study, the main outcome was the progression of diabetic nephropathy, a change in the albumin excretion rate from one level to a higher level (normo- → microalbuminuria or micro- → macroalbuminuria) or the development of ESRD. Adiponectin was measured at baseline and correlated with the progression of diabetic nephropathy during the follow-up of the entire study population as well as also separately in men and women.
5. Methods

5.1 Medical history

In the cross-sectional studies (I-IV), data on medication, smoking, cardiovascular status, diabetic complications, and parental history of diabetes, hypertension, and cardiovascular disease were registered with a standardized questionnaire completed by the patient’s attending physician based upon medical files.

In the follow-up study (V), two different approaches complementing each other were used. First, the medical files were reviewed, and any changes in renal status or the occurrence of cardiovascular events was verified. Second, patients were re-examined at their local medical center according to the same protocol as during the baseline visit.

5.2 Blood pressure and demographics

Blood pressure was measured twice in the sitting position with a mercury sphygmomanometer after a 10-min rest. The mean of these two measurements served in the analyses. Height, weight, as well as waist and hip circumferences were recorded, and the body-mass index (BMI=weight [kg]/height$^2$ [m$^2$]) and the waist-to-hip ratio (WHR) was calculated.

5.3.1 Albumin excretion rate and renal function

Urine samples were collected in one of two ways: by a timed overnight or 24-h urine collection. Urinary AER was determined by radioimmunoassay until November 2002 (Pharmacia, Uppsala, Sweden), and thereafter by immunoturbidimetry with a correlation coefficient of 0.96 between these two methods. The patients were divided into three groups according to their AER in three consecutive overnight or 24-h urine collections. Normal AER (normoalbuminuria) was defined as an AER persistently < 20 µg/min or < 30 mg/24 h, microalbuminuria as AER ≤ 20 < 200 µg/min or ≤ 30 < 300 mg/24h, and macroalbuminuria as AER ≥ 200 µg/min or ≥ 300 mg/24 h in at least two of three urine collections. Serum creatinine was assessed with enzymatic methods at a central laboratory. To determine renal function, we estimated GFR with an equation derived from the Modification of Diet in Renal Disease (MDRD) Study (260, 261) as well as with the Cockroft-Gault formula (262).
5.3.2 Definition of progression of diabetic nephropathy (Study V)

All data on AER obtained between baseline and the follow-up visit were reviewed and based on the result in two of the last three consecutive urine collections, the renal status of the patients was determined using the same cut-off values as in the baseline examination. Progression was defined as a change from one level to a higher level of AER or the development of ESRD. Patients with no progression of renal disease were classified as non-progressors.

5.4 Glucose control, lipids, and insulin sensitivity

HbA$_1c$ was determined locally by standardized assays at each center. Serum lipid and lipoprotein concentrations were measured centrally at the research laboratory of the Helsinki University Central Hospital, Division of Cardiology, Helsinki, Finland, with automated enzymatic methods that use the Cobas Mira analyzer (HoffmanLa Roche, Basel, Switzerland). As a measure of insulin sensitivity, we calculated the estimated glucose disposal rate (eGDR) with an equation modified for HbA$_1c$, as previously described (263).

5.5 Low-grade inflammatory markers CRP and IL-6

CRP was measured with radioimmunoassay; CRP standards (Orion Diagnostica, Espoo, Finland) and patients’ sera were incubated with sheep CRP antiserum (Code C 4063, Sigma Chemical Co, St. Louis, Mo., USA) and with Sepharose anti-sheep IgG (Pharmacia, Uppsala, Sweden) for 1 h at room temperature, and were then centrifugated. The radioactivity of the pellets was measured, and the standard curve was used for the calculations (264). Within- and between-assay CVs were, respectively, 4.0% and 6.5%. The detection limit of the assay was 0.01 mg/l.

IL-6 was determined with a quantitative sandwich enzyme immunoassay technique, that used a monoclonal and an enzyme-linked polyclonal antibody specific for IL-6 (Quantikine HS human IL-6 immunoassay, R&D Systems, Minneapolis, MN, USA). Within- and between-assay CVs were, respectively 2.6% and 4.5%. The detection limit for IL-6 was 0.1 ng/l.

CRP and IL-6 were also measured in sera from 66 healthy control subjects (36 women and 30 men with a mean age of 37.6 years [range 19–63]). In these control subjects, the concentration of CRP varied from 0.2 to 4.8 mg/l (means ± SD; 1.2 ± 1.0 mg/l), and the IL-6 concentration varied from 0.13 to 5.20 ng/l (1.25 ± 1.07 ng/l) (264).
5.6 Mannan-binding lectin (MBL)

Serum MBL concentrations were determined with an in-house time-resolved immunofluorometric assay at the University of Aarhus, Denmark. The measurement was based on monoclonal antibody (anti-MBL-antibody, Immunolex, Denmark, labeled with reagents from Perkin Elmer, USA). The intra- and interassay CVs were below 5% and 10%, respectively. The detection limit for MBL was 10 ng/ml (265).

5.7 Adiponectin

Serum adiponectin was determined with an in-house time-resolved immunofluorometric assay at the University of Aarhus, Denmark. The measurement was based on two monoclonal antibodies and recombinant human adiponectin (R&D Systems, Abingdon, UK). The within-assay CVs of standards and unknown samples averaged less than 5%. The between-assay CV was 12% at a final dilution of 1:2500 and 8% at a final dilution of 1:50 (266). The detection limit for adiponectin was less than 1.5 mg/l.

5.8 α-defensin

The plasma level of α-defensin was determined with a novel, validated, in-house, solid-phase radioimmunoassay (RIA) technique at the University of Aarhus, Denmark. The measurement was based on a monoclonal antibody, which recognizes α-defensin isoforms 1-3. The mean within-assay CVs of standards and samples was less than 6%. The in-between assay CV was 9% (267). The detection limit of α-defensin was less than 1.95 µg/l.

5.9 Statistical analysis

Data are expressed as means ± SD for normally distributed values, as median with a range for non-normally distributed values, and as percentages. Differences between groups for normally distributed variables were tested using ANOVA and non-parametric data with the Mann-Whitney or Kruskal-Wallis tests. Frequencies were tested with Pearson's Chi-squared test or two-tailed Fisher's exact test when appropriate. Correlations were calculated using simple and multiple linear regression analysis. In study V, risk factors for the progression of diabetic nephropathy were assessed using Cox regression analysis. Calculations in studies I-III were carried out with a BMDP statistical package (BMDP Statistical Software, Los Angeles, CA, USA). In studies IV and V calculations were performed with SPSS 12.01 software (SPSS Inc., Chicago, Illinois, USA). In all studies, a P value of less than 0.05 was considered statistically significant.
6. Results

6.1 Inflammatory markers at various stages of diabetic nephropathy (I)

The concentrations of serum low-grade inflammatory markers, C-reactive protein (CRP), and interleukin-6 (IL-6) appear in Figure 6.

![Figure 6. CRP and IL-6 concentrations in 194 Type 1 diabetic patients with and without diabetic nephropathy, as well as in 66 healthy control subjects. NORMO, normoalbuminuria; MICRO, microalbuminuria, MACRO, macroalbuminuria. Data are medians.](image)

CRP was higher in patients with incipient (microalbuminuria) and overt (macroalbuminuria) diabetic nephropathy than in normoalbuminuric patients, whereas interleukin-6 increased in parallel with the severity of the renal disease. No difference was observed in CRP concentrations between the micro- or macroalbuminuria groups of patients. Healthy controls had lower CRP and IL-6 than did patients with diabetes, either with or without diabetic nephropathy.

IL-6 and CRP correlated positively with each other ($r = 0.3291, P < 0.0001$). In the univariate analysis, CRP correlated positively with diastolic blood pressure, as did triglycerides and AER; CRP and eGDR showed a negative correlation. IL-6 correlated positively with the duration of diabetes, WHR, HbA$_{1c}$, creatinine, triglycerides, and AER, and inversely with eGDR, HDL-cholesterol, and eGFR (MDRD).

In a multiple linear regression analysis with diastolic blood pressure, eGDR, triglycerides, and AER in the model, an independent relationship emerged between CRP and AER ($\beta$-coefficient $\pm$ SE: $0.10 \pm 0.05$, $P = 0.030$).
IL-6 was independently associated with the duration of diabetes (0.03 ± 0.1, P = 0.0176), HDL-cholesterol (-0.29 ± 0.12, P = 0.0135), and AER (0.10 ± 0.03, P = 0.0003) in a model that also included WHR, eGDR, HbA1c, creatinine, eGFR, and triglycerides.

### 6.2 MBL at various stages of diabetic nephropathy (II)

The MBL concentrations appear in Figure 7. MBL was higher in microalbuminuric patients than in normoalbuminuric patients, but as with CRP, no difference was observed in the MBL concentrations between patients with microalbuminuria and those with macroalbuminuria.

![Figure 7. MBL levels (median with interquartile range) in 191 type 1 diabetic patients with and without diabetic nephropathy. NORMO = normoalbuminuria, MICRO = microalbuminuria, MACRO = macroalbuminuria.](image)

MBL correlated with HbA1c, HDL-cholesterol, and eGDR. However, no significant correlations were observed between MBL and inflammatory markers CRP or IL-6 or between any of the other variables tested.

In a multiple regression analysis with HbA1c, eGDR, and HDL-cholesterol in the model, HbA1c was the only variable independently associated with MBL ($\beta \pm \text{SEM}: 0.26 \pm 0.08; P = 0.003$).

### 6.3 Adiponectin in patients with type 1 diabetes (III)

Adiponectin concentrations were clearly higher in females than in males, but because no significant differences in gender distribution between the groups were observed, the data were pooled in the analyses. Adiponectin concentrations were higher in patients with macroalbuminuria (19.8 ± 12.0 mg/l) than in patients with microalbuminuria (13.1 ± 4.8 mg/l) or normoalbuminuria (11.8 ± 4.2 mg/l). However, no difference was observed between patients with normo- and microalbuminuria (Figure 8).
In univariate analysis, adiponectin was positively associated with creatinine, AER, interleukin-6, systolic blood pressure, HbA₁c, total cholesterol, and HDL-cholesterol, and inversely with eGFR and WHR.

In a multiple linear regression analysis, including the above mentioned variables, eGFR (β ± SE: -0.47 ± 0.10; P < 0.0001), AER (0.07 ± 0.02; P < 0.0001), and WHR (-1.30 ± 0.38; P < 0.001) were independently associated with serum adiponectin concentrations (R² = 0.32).

6.4 α-defensin (-1, -2, and -3) at various stages of diabetic nephropathy (IV)

The total serum concentration of α-defensin (-1, -2, and -3) appears in Figure 9. Compared to micro- and normoalbuminuria, serum α-defensin concentrations were higher in diabetic patients with macroalbuminuria, but no difference was observed between patients with normo- and microalbuminuria.
Since the distribution of α-defensin was not normally distributed, the data were logarithmically transformed before inclusion in the regression analyses. In univariate analysis, α-defensin correlated positively with WHR, systolic blood pressure and diastolic blood pressure, HbA1c, cholesterol, triglycerides, creatinine, AER, CRP, and IL-6, and inversely with age, age at onset of diabetes, HDL-cholesterol, eGDR, and eGFR.

According to the multiple regression analysis, an independent relationship existed between α-defensin and age, systolic blood pressure, cholesterol, HDL-cholesterol, and eGFR (Table 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>B ± SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>0.003 ± 0.001</td>
<td>0.032</td>
</tr>
<tr>
<td>ln(HDL-cholesterol)</td>
<td>-0.198 ± 0.079</td>
<td>0.013</td>
</tr>
<tr>
<td>ln(cholesterol)</td>
<td>0.334 ± 0.124</td>
<td>0.008</td>
</tr>
<tr>
<td>Age</td>
<td>-0.010 ± 0.003</td>
<td>0.001</td>
</tr>
<tr>
<td>eGFR(Cockroft-Gault)</td>
<td>-0.264 ± 0.077</td>
<td>0.001</td>
</tr>
</tbody>
</table>

SBP = systolic blood pressure; eGFR = estimated glomerular filtration rate. Model also included WHR, HbA1c, lnAER, lnIL-6, lnCRP, and lnTG. R² = 0.438.

6.5 Adiponectin and the progression of diabetic nephropathy (V)

The data on adiponectin in study V, including a substantially larger number (n = 1330) of patients than in study III (n = 189) were similar and thus adequately replicated. Again, serum adiponectin concentrations were higher in patients with macroalbuminuria and similar in those with normo- and microalbuminuria (Figure 10).

![Figure 10. Serum adiponectin concentrations (mg/l) in type 1 diabetic patients. Data are means ± SD. NORMO = normoalbuminuria (n = 818), MICRO = microalbuminuria (n = 216), MACRO = macroalbuminuria (n = 296).](image-url)
A total of 73 (9%) of the 818 patients with normoalbuminuria progressed to microalbuminuria, 37 (17%) of 216 progressed from micro- to macroalbuminuria, and 83 (28%) of 296 from macroalbuminuria to ESRD.

In patients with normo- or microalbuminuria, no differences in the baseline adiponectin concentrations were observed between progressors and non-progressors (12.0 ± 6.1 vs. 12.1 ± 5.8 mg/l; P = NS), not even when analyzing men and women separately. In the macroalbuminuric group, however, progressors had significantly higher serum adiponectin concentrations than did those who did not progress (Table 2).

Table 2. Baseline adiponectin concentrations (mg/l) and the progression from one stage to the next in diabetic nephropathy.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Progressor</th>
<th>Non-progressor</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroalbuminuria (all)</td>
<td>23.4 ± 17.1 (83)</td>
<td>16.0 ± 8.5 (213)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macroalbuminuria (men)</td>
<td>19.1 ± 9.9 (56)</td>
<td>13.5 ± 7.0 (119)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macroalbuminuria (women)</td>
<td>32.2 ± 24.5 (27)</td>
<td>19.1 ± 9.3 (94)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are means ± SD (n).

In univariate analysis in patients with macroalbuminuria, a significant relationship emerged between progression and systolic blood pressure (P = 0.022), insulin dose (P = 0.001), HbA1c (P = 0.040), cholesterol (P = 0.007), triglycerides (P < 0.001), creatinine (P < 0.001), AER (P < 0.001), and eGFR (P < 0.001).

Notably, adiponectin remained an independent predictor of progression from macroalbuminuria to ESRD when adjusted for the above variables in the Cox-regression model (Table 3).

Table 3. Cox regression analysis of risk factors for progression from macroalbuminuria to ESRD

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>B ± SE</th>
<th>Hazard ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR 1 ml/min per 1.73 m²</td>
<td>-0.065 ± 0.008</td>
<td>0.937 (0.923 - 0.952)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>SBP 1 mmHg</td>
<td>0.009 ± 0.006</td>
<td>1.009 (0.997 - 1.021)</td>
<td>0.158</td>
</tr>
<tr>
<td>HbA1C 1%</td>
<td>0.203 ± 0.076</td>
<td>1.225 (1.055 - 1.422)</td>
<td>0.008</td>
</tr>
<tr>
<td>TG 1 mmol/l</td>
<td>0.355 ± 0.098</td>
<td>1.426 (1.176 - 1.728)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Adiponectin 1 mg/l</td>
<td>0.021 ± 0.008</td>
<td>1.022 (1.005 - 1.039)</td>
<td>0.011</td>
</tr>
<tr>
<td>Insulin 1 IU per kg</td>
<td>-0.473 ± 0.681</td>
<td>0.623 (0.164 - 2.366)</td>
<td>0.487</td>
</tr>
<tr>
<td>Total cholesterol 1 mmol/l</td>
<td>-0.110 ± 0.128</td>
<td>0.896 (0.697 - 1.152)</td>
<td>0.391</td>
</tr>
</tbody>
</table>

eGFR = estimated glomerular filtration rate determined with the Cockroft-Gault formula; SBP = systolic blood pressure; TG = triglycerides
7. Discussion

7.1 Subjects and methods

7.1.1 Cross-sectional studies (I-IV)

In the cross-sectional substudies (I-IV), all patients were recruited from the nationwide multi-center FinnDiane study and matched for sex and duration of diabetes. Patients without diabetic nephropathy (normoalbuminuria) were required to take no antihypertensive medication, to have no signs of cardiovascular disease, and to have a relatively long duration of diabetes of at least 13 years. The cut-off of 13 years was chosen as the cut-off point for all patients, since that was the shortest duration for a patient in the macroalbuminuric group. Furthermore, long disease duration indicates that these patients with normoalbuminuria are at rather low risk for diabetic nephropathy and would thus represent true normoalbuminuric subjects.

Patients with incipient or overt diabetic nephropathy, micro-, or macroalbuminuria were required to undergo treatment with an ACE inhibitor in order to be representative of typical patients with type 1 diabetes and increased AER in Finland. Nearly all patients with type 1 diabetes and with signs of incipient or macroalbuminuria in the FinnDiane were, at the time of the study and according to the recommendations, treated with an ACE inhibitor. This approach differed from those of previous studies, where the patients studied had either no medication, despite the presence of microalbuminuria, or no data were available regarding blood pressure medication in those patients with diabetic nephropathy (172, 161). This important matter is further discussed in section 7.2.1.

Low-grade inflammation was estimated by measuring both CRP and IL-6. CRP is an acute-phase protein produced by the liver in response to various stimuli, and the synthesis rate has been shown to be the only significant determinant of its plasma level (268). On the other hand, CRP is also a downstream marker of pro-inflammatory processes. It is worth noting that CRP has been suggested to be the “best” inflammatory marker and clinical tool in identifying those at risk for cardiovascular disease (145, 164), a complication typical for patients with diabetic nephropathy (269, 270). IL-6 is a proinflammatory cytokine which has been shown to be the main stimulus for the hepatic production of CRP (168, 169). In addition, IL-6 has been linked not only to atherosclerosis, but also to insulin resistance (180), which is also a typical feature of diabetic nephropathy, even in patients with type 1 diabetes (181). In clinical settings and in our studies as well, another strong candidate marker could have been TNF-α, but it has a short half-life.
of less than ten minutes (271) when compared to CRP, with a half-life of 18 hours. It is also worth noting that the CRP plasma concentrations have been shown to be relatively stable over several years (164).

7.1.2 Follow-up study (V)

Two different complimentary approaches were implemented for the follow-up of the patients. First, their medical files were reviewed, and any changes in renal status or in the occurrence of any cardiovascular events were recorded. Second, the patients were re-examined according to the same protocol used during the baseline visit.

Progression of diabetic nephropathy was defined as a change from one level of AER to a higher level or as the development of ESRD. Only those re-examined patients with available follow-up data on AER, and who were therefore classifiable as either progressors or non-progressors, were included in this follow-up study. One could argue whether the approach to define progression from one category to the other based on the AER was better or worse than using an increment of the AER (ΔAER). We chose this approach, however, since this is still the clinical basis for categorizing patients in different stages of diabetic nephropathy based on the staging recommendations of the ADA (272).

7.2.1 Low-grade inflammation and diabetic nephropathy in patients with type 1 diabetes (I)

The concept that an inflammatory component could exist behind diabetic nephropathy in patients with type 1 diabetes originated from two rather contradictory studies. The first showed that IL-6 was already higher in patients with normoalbuminuria than in healthy control subjects, although CRP showed no differences between healthy control subjects and patients with type 1 diabetes at any stage of albuminuria (172). The second study, however, showed higher CRP concentrations in patients with normoalbuminuria (161). Although these two studies failed to provide a definite answer, they did hint that chronic inflammation may be linked to elevated albuminuria.

We were able to confirm the association of low-grade inflammation with diabetic nephropathy in patients with type 1 diabetes and to extend the finding to include not only CRP, but also IL-6. Low-grade inflammation was already present in the early stage of microalbuminuria. The reason why no significant increase in CRP concentrations was observed in the previous study in patients with type 1 diabetes and microalbuminuria may be that we included a much larger patient population (273).

We not only confirmed the previous finding that patients with type 1
diabetes and albuminuria have elevated CRP and IL-6 concentrations, but also that, CRP and IL-6 were positively correlated with each other. These observations are both important, since IL-6 is considered to be the main stimulus for the hepatic production of CRP (168, 169).

IL-6 concentrations rose in parallel with renal engagement, and this difference between the groups remained evident even after adjustment for WHR, suggesting that low-grade inflammation is truly a part of the deleterious process of diabetic nephropathy, at least in patients with type 1 diabetes. In a rather recent study in patients with type 2 diabetes, an increase in IL-6 was associated not only with diabetic nephropathy, but also with glomerular basement membrane thickening, a crucial lesion of diabetic nephropathy and a strong predictor of renal progression (274).

Patients with microalbuminuria or macroalbuminuria were treated with an ACE inhibitor (see section 7.1.1). Since ACE inhibition has been associated with reduced CRP and IL-6, and is thus suggested to inhibit inflammation (275, 276), ACE inhibition could even have diminished the true difference between the groups. This further strengthens our finding and suggestion that diabetic nephropathy is a state of low-grade inflammation.

Duration of diabetes was independently related to IL-6 in our study, which suggests that chronic exposure to glucose and possibly to AGEs could stimulate the production of IL-6. In this respect the finding that serum-free pentosidine and the monocyte activation marker neopterin correlates with the rate of progression of diabetic nephropathy (277) supports our hypothesis that AGEs could activate monocytes to produce IL-6. Furthermore, given the fact that low-grade inflammation has been linked to markers of endothelial dysfunction in patients with type 1 diabetes and macroalbuminuria (161, 278), the source of IL-6 could also reside in the smooth muscle cells of the vasculature and result from chronic exposure to AGEs.

Increased concentrations of IL-6 and CRP were both associated with lower insulin sensitivity, shown as a decrease in the eGDR, calculated by using a previously described modified formula (263). It is worth noting that by using the eGDR, we could show that insulin sensitivity worsened in parallel with the severity of renal disease in our patients, just as with IL-6. The positive correlation observed between IL-6 and triglycerides, and the negative correlation between IL-6 and HDL-cholesterol, further supports this observation. Thus, low-grade inflammation in patients with type 1 diabetes seems to be connected not only to the severity of proteinuria, but also to insulin sensitivity, as in type 2 diabetic patients with metabolic syndrome (180). Furthermore, these results were also in line with the data from the Pittsburgh Epidemiology of Diabetes Complication Study, showing that insulin resistance is indeed associated with overt nephropathy, although that study failed to include measurements of inflammatory markers (181).

During resting conditions, roughly 15% to 35% of IL-6 derives from adipose tissue, and the majority derives from visceral adipose tissue (279-
Given the strong relationship between IL-6 and WHR, we adjusted our results for WHR, but the adjustment failed to change the results. Thus, low-grade inflammation seems to be a key finding of diabetic nephropathy and could even be involved in the pathogenesis of insulin resistance in these patients. Such a view is supported by experimental data showing that IL-6 is capable of inducing insulin resistance in mouse hepatocytes and human hepatocarcinoma cell lines by interfering with insulin receptor signal transduction in these cells (282). IL-6 has also been shown to induce insulin resistance in adipocytes, and the expression of IL-6, like that of TNF-α, has been shown to be markedly elevated in the fat cells of insulin-resistant humans (283). Furthermore, regarding this induction of insulin resistance in adipocytes, IL-6 has been suggested to act in concert with TNF-α (283).

Thus, low-grade inflammatory markers are associated with diabetic nephropathy in patients with type 1 diabetes. Whether low-grade inflammatory markers could serve in predicting initiation of the disease, and the progression of diabetic nephropathy needs to be assessed.

7.2.2 Mannan-binding lectin in patients with type 1 diabetes and incipient or overt diabetic nephropathy (II)

In study II, we reported increased circulating concentrations of serum MBL in patients with type 1 diabetes and diabetic nephropathy. In contrast, we could observe no correlation between MBL and AER in patients with micro- or macroalbuminuria in this cross-sectional study, although this result could have been expected since such a correlation has been observed in patients with normal AER (198). However, HbA1c, a known risk factor for diabetic nephropathy, was independently related to the MBL levels. One possible explanation for these conflicting results could be the differences in glycemic control between our patient groups.

The fact that MBL was unassociated with AER was in line with our finding that no relationship was observed between MBL and low-grade inflammatory markers IL-6 and CRP in this study, since we had previously shown an association between AER and these inflammatory markers in diabetic patients with incipient or overt nephropathy (273). One reason for the lack of correlation between MBL and inflammatory markers could be the simple fact that CRP may inhibit the production of MBL (284, 285). CRP has also been shown to modulate the cascade in which the complement regulatory protein H regulates MBL-initiated cytolysis, which may suggest some kind of coordination between MBL and CRP during the acute phase response (285).

Even if we could confirm no independent relationship between MBL and insulin sensitivity as measured by using the eGDR in the multivariate analysis, there was a negative correlation between MBL and eGDR as well as between MBL and HDL-cholesterol in the univariate regression analysis,
suggesting that MBL may still be a marker of insulin resistance.

In diabetic nephropathy, whether MBL plays an active role in the initial pathogenesis or acts as a progression promoter of an already existing disease remains unknown. An active role was suggested in an inception cohort study in which high serum MBL concentrations, measured three years after the diagnosis of diabetes, were associated with the development of incipient or overt diabetic nephropathy during a median follow-up of 18 years (286). Furthermore, patients with diabetic nephropathy and high serum concentrations of MBL have been shown to carry genetic variants of the MBL gene that promote high MBL production (199).

On the other hand, MBL has been suggested to play an unfavourable role in other chronic kidney diseases such as IgA nephropathy and Henoch-Schönlein purpura nephritis. MBL binding to the IgA complex results in complement activation, and the deposition of MBL in association with IgA nephropathy was found in the mesangial area of the kidneys in a subpopulation of patients with this disease (201, 287-289). Furthermore, activation of the lectin pathway of the complement has, in some studies (201, 290), though not all (287, 288), been associated with more severe renal damage in IgA nephropathy. In Henoch-Schönlein purpura nephritis, MBL deposition has also been observed and has even been associated with increased progression of renal disease (291).

IgA1, the isoform of IgA deposited in the mesangium, is a heavily glycosylated molecule (292). Some have suggested that the difference between patients who have IgA with and without mesangial MBL deposition is based on differences in IgA glycosylation (290).

Whether MBL is also found in the diabetic kidney remains unknown, but due to chronic hyperglycemia, one can speculate that MBL ligands are present in the diabetic glomeruli, leading to mesangial deposition of MBL with deleterious effects.

The mechanism could be that hyperglycemia stimulates the production of N-acetylglucosamine through the hexosamine pathway, and that as a consequence, an abundance of various secretory and cell membrane glycoproteins would be modified by N-linked glycosylation, thus enabling these proteins to become targets for MBL in the kidneys and in other target organs.

In conclusion, MBL concentrations are elevated in patients with type 1 diabetes and diabetic nephropathy. Whether MBL also plays a pathogenetic role remains unknown, but its independent association with glycemic control raises the possibility that it may have deleterious effects.

7.2.3 Adiponectin in patients with type 1 diabetes (III)

In healthy people, a high concentration of adiponectin was, at the time we planned this study, associated with lower risk for myocardial infarction
in men (222) and with lower incidence of type 2 diabetes in both sexes (293, 294). Given these observations, we expected to find a decrease in adiponectin concentrations in patients with diabetic nephropathy, who usually present with insulin resistance and an aberrant lipid profile. The demonstration of markedly increased serum concentrations of adiponectin in patients with type 1 diabetes and overt nephropathy and, further, that adiponectin was associated with renal insufficiency were thus novel findings in our study. We could observe no independent association between adiponectin and inflammatory markers in this patient population, which was in line with recent findings among ESRD patients on dialysis (231), but in contrast to findings among predialytic patients with ESRD (232). These contrasting observations may be due to the different patient populations. Patients with ESRD are presumably living in a different metabolic milieu than are patients with mildly impaired renal function, and they have presumably more interfering factors, such as polypharmacy and uremic toxins, which can impact adiponectin and inflammatory markers. Notably, in a cross-sectional analysis of Eurodiab Prospective Complications Study data, which included a reasonable number of patients with type 1 diabetes from normoalbuminuria to macroalbuminuria, adiponectin showed no association with CRP (295). Furthermore, a study in patients in the early stages of chronic nondiabetic kidney disease, showed no relationship between adiponectin and CRP (296).

We found an association between adiponectin and WHR, but not between adiponectin and more direct indices of insulin resistance. Research has established that not all fat is equal. WHR has been widely used to investigate the relationship between regional adipose tissue distribution and metabolic profile, and has been associated with the amount of abdominal visceral adipose tissue measured by CT (297-299). Adipose tissue in the abdominal or visceral versus subcutaneous depot differs in cell size, metabolic activity, and potential role in insulin resistance. Visceral fat and large adipocytes, prominent features in obesity, are more pathogenic and are associated with low adiponectin levels (300-306).

Some (307, 308), but not all (266, 309, 310) studies have suggested that antihypertensive medication, ACE inhibition, or angiotensin receptor blocker (ARB) treatment positively effect levels of adiponectin. In our study, all the patients with microalbuminuria underwent ACE inhibition, but even so, no significant difference in adiponectin concentrations was observed between patients with microalbuminuria and those with normoalbuminuria. If the increase in adiponectin was due to ACE inhibition, a significant rise in adiponectin concentrations would have been expected in patients with microalbuminuria also.

The collagenous domain of the adiponectin molecule has four conserved lysines that can be modified by both hydroxylation and glycosylation. Hydroxylation and glycosylation are believed to be critical for the three-dimensional structure of the adiponectin molecule by changing its configuration to a more biologically active form (207). Furthermore,
glycosylation is believed to be one of the major post-translational modifications of adiponectin (207). In patients with diabetes and constant hyperglycemia, the glycosylation process is probably altered, which could further modify the function of the adiponectin molecule. Consequently, a modified adiponectin molecule could result in a functional change in the essential hormonal feedback systems of the body (i.e. in the form of diminished negative feedback) and thereby increased adiponectin concentrations in diabetes.

Insulin resistance, a typical feature of nephropathy, is in patients with type 2 diabetes associated with suppressed/low serum adiponectin concentrations (215). Therefore, we would also have expected to find low serum adiponectin levels in patients with macroalbuminuria, an insulin-resistant state in patients with type 1 diabetes also (181, 273, 311). One potential explanation for this finding would be renal insufficiency per se, which could stimulate adiponectin production or alternatively lead to a defect in the clearance of adiponectin. Both alternatives, either separately or together, would raise adiponectin concentrations. A defect in clearance is supported by the finding that plasma adiponectin concentrations are lower after successful kidney transplantation than they are before (233). Although the true mechanisms responsible for such an increase in circulating adiponectin in diabetic nephropathy remain unclear, one can speculate that adiponectin itself may play a role in mitigating the burden of vascular complications in diabetic nephropathy, as has been suggested in ESRD (312). Notably, adiponectin has been shown to play a role in endothelium-dependent vasodilatation (313). Furthermore, some have suggested that adiponectin participates in the defence mechanisms of the endothelium by inhibiting the deleterious effects of tumor necrosis factor-α (217).

In our study, both AER and estimated GFR were independently associated with increased adiponectin concentrations in type 1 diabetic patients with nephropathy. Our results differ from those of previous studies, which failed to detect any association between GFR and adiponectin, even if the degree of proteinuria was positively associated with adiponectin in the first study (231), though not in the following study (232). The most plausible explanation is the inclusion of different patient populations as well as the number of patients with type 1 diabetes, which was significantly higher in our study than in the previous two studies. Notably, in a study of type 2 diabetic Pima Indians, adiponectin was associated with elevated serum creatinine and macroalbuminuria (314). In conclusion, serum adiponectin concentrations are elevated in patients with type 1 diabetes and nephropathy, and these levels are further associated with renal insufficiency. The reason why circulating adiponectin levels are high in these insulin-resistant conditions, such as proteinuria and advanced nephropathy, remain unclear.
7.2.4 α-defensin (-1, -2, and -3) in patients with type 1 diabetes and nephropathy

α-defensin (-1, -2, and -3) was significantly higher in patients with macroalbuminuria than in those with either normo- or microalbuminuria. No difference in α-defensin serum concentrations was observed between the latter two groups. In addition, there was an independent relationship between kidney function, estimated by the Cockcroft–Gault formula, and α-defensin, suggesting an impact of renal insufficiency on circulating α-defensin levels. This suggestion is supported by earlier observations of peptide hormone metabolism in kidneys (315), and by observations that patients with diabetic nephropathy have a reduced capacity to degrade peptides (316-318). The production of α-defensin has until now proved uninducible, as is the production of β-defensin-2 and -3, by inflammatory mediators (242), which further supports the possible role of decreased renal degradation as the cause for increased serum concentrations of α-defensin in advanced diabetic nephropathy.

Our finding of an independent relationship between age and α-defensin was in line with the known age-related decline in GFR.

We observed an association between α-defensin and inflammatory markers (CRP and IL-6) in the univariate association, but not in the multivariate analysis. Nor was there any association between α-defensin and MBL, even though chronic low-grade inflammation and the lectin pathway have both been associated with diabetic nephropathy, and are believed to play a role in the pathogenesis of diabetic nephropathy (273, 286, 319, 320). Thus, α-defensin could be an independent novel marker of diabetic nephropathy.

Notably, α-defensin was positively associated with cholesterol and negatively associated with HDL cholesterol, suggesting that α-defensin may play a role in the pathogenetic processes of atherosclerosis, a common phenomenon in patients with diabetic nephropathy. This suggestion is in line with observations showing that α-defensin stimulates the binding of lipoprotein (a) and low-density lipoprotein to vascular cells (256, 257); it is worth noting that α-defensin is abundantly present in the walls of human coronary arteries (321). In a recent prospective study of Danish patients with long-lasting (mean duration of diabetes 27 years) type 1 diabetes, α-defensin was associated with increased cardiovascular mortality during the ten-year follow-up (322). Notably, in that study, α-defensin levels were higher in patients with diabetic nephropathy than in those with normoalbuminuria, as in our study.

In conclusion, serum α-defensin (-1, -2, and -3) concentrations are higher in patients with type 1 diabetes and diabetic nephropathy than in patients with either normo- or microalbuminuria. Whether the elevation in serum α-defensin plays a role in the pathogenesis of diabetic nephropathy or simply reflects changes in renal function must be resolved in further studies.
7.2.5 **Adiponectin and the progression of diabetic nephropathy (V)**

Our observation that an elevated serum concentration of adiponectin at baseline may predict the progression from overt diabetic nephropathy to ESRD in patients with type 1 diabetes is a new finding. In contrast, we observed no differences in the serum adiponectin concentrations between progressors and non-progressors in patients with normo- and microalbuminuria.

Our data thus contrast with the results of a previous study in which those patients who progressed from normo- to microalbuminuria exhibited higher adiponectin concentrations than did those who showed no progress (323). It is worth noting that we studied a substantially larger patient population (818 vs. 126 patients) with type 1 diabetes and normoalbuminuria, which may explain the observed difference. Furthermore, our patients were younger and had better glycemic control, but a higher BMI.

Notably, our observation also contrasted with an observation from the Modification of Diet in Renal Disease (MDRD) study (324). The MDRD study found no association between adiponectin and the progression of kidney disease, even when including those patients with moderate to advanced kidney disease. A plausible explanation for this discrepancy is probably the different patient populations. The patients in the MDRD study were substantially older and had a higher BMI, only 42 patients actually had diabetes, and none of them had type 1 diabetes.

As expected, most of our patients with type 1 diabetes and diabetic nephropathy were treated with an ACE inhibitor or an ARB, agents known to increase serum adiponectin in non-diabetic patients with essential hypertension (307). The great majority of the patients in the macroalbuminuric group were receiving ACE inhibitor treatment (n = 217), and only 25 patients were receiving ARB treatment. However, we observed no differences in adiponectin concentrations regardless of whether the patients were taking ACE inhibitors, an observation that is in line with the results of a recent study in which the authors observed no effects on the adiponectin concentration after adding the ACE inhibitor ramipril to the treatment of patients with type 2 diabetes and hypertension (309). Notably, in patients with type 1 diabetes, treatment with an ARB has been shown to increase the adiponectin concentration in a short-term study in nine normotensive male patients with type 1 diabetes and an AER in normal range (308). However, two other recent studies with substantially larger patient populations and patients of both genders showed no independent effect of an ACE inhibitor or an ARB on adiponectin levels. In the first of these studies (266), the patients receiving ACE inhibition had a slightly higher serum adiponectin concentration, but the difference was insignificant, and in the second study, treatment with an ACE inhibitor or an ARB correlated with adiponectin levels in the univariate, but not in the multivariate analysis (310). In our study, 75% of the progressors received ACE inhibition/ARB treatment in comparison with 84% of the non-progressors. This
small difference was statistically insignificant.

Taken together, these results indicate that at least ACE inhibitors have no dramatic effect on adiponectin levels in patients with type 1 diabetes. Whether this is also true for ARBs remains to be seen. Only 25 patients with macroalbuminuria received ARB treatment compared to 217 who received ACE inhibition.

The reason for the elevated concentrations of adiponectin in the progressors from macroalbuminuria to ESRD remains unknown. Potential hypotheses include a compensatory response to vascular injury (231, 266, 295, 312, 325, 326), the effects of subcutaneous insulin treatment (209), decreased clearance of adiponectin due to renal insufficiency (231, 233), and post-translational glycosylation modifications (327, 328).

Importantly, glycosylation represents one of the major post-translational modifications of adiponectin, which, together with hydroxylation, is critical for the construction of the three-dimensional structure of the biologically active adiponectin molecule that regulates the formation of the high-molecular-weight oligomeric adiponectin complex (207, 327, 328). Notably, even a relatively short period (72h) of high plasma glucose (15 mmol/l) reportedly increased production of the biologically highly active high–molecular-weight adiponectin when compared to normoglycemic conditions (5 mmol/l) (328). Whether this experimental observation in human adipose tissue culture also occurs in the clinical setting of patients with type 1 diabetes, macroalbuminuria, and chronic hyperglycemia remains unknown. However, some support for the hypothesis comes from the observation that our patients with type 1 diabetes and macroalbuminuria had “by definition” worse glycemic control than did the patients with normo- or microalbuminuria. In addition, adiponectin and glycemic control, as measured by HbA1c, were both associated with the progression of diabetic nephropathy.

Whether decreased clearance of adiponectin due to renal insufficiency per se could trigger an increase in adiponectin concentrations is another possibility. A high concentration of plasma adiponectin has been shown to decrease after renal transplantation, which supports the role of adiponectin clearance for the high serum adiponectin concentrations observed in patients with macroalbuminuria (233). In a study of patients with type 2 diabetes and macroalbuminuria, however, enhanced production of adiponectin was considered a stronger determinant of serum adiponectin concentrations than a reduction in the renal clearance of adiponectin. This was based on a marked increase in both the serum and the urinary adiponectin levels in patients with overt diabetic nephropathy and on a positive correlation between serum adiponectin concentrations and urinary adiponectin excretion in all diabetic patients (329). Thus, renal insufficiency may not only affect the clearance of adiponectin, but also stimulate adiponectin production.

Insulin has been shown either to stimulate (330, 331) adiponectin synthesis or to reduce it (225). Insulin has also been shown to stimulate adiponectin secretion from adipocytes (203, 332). During long-standing insulin treatment,
Discussion

However, such as in patients with type 1 diabetes, insulin has been inversely associated with adiponectin levels (266, 295, 310, 333), a finding which is also enjoys the support of observations during a 5-h hyperinsulinemic-euglycemic clamp study in patients with type 2 diabetes (226). In the clamp study, adiponectin levels decreased 20% after insulin administration in both lean and obese people as well as in patients with type 2 diabetes (226). In our study, surrogate measures of insulin sensitivity, such as the total insulin dose or the dose of insulin per kilogram of body weight, were both inversely associated with adiponectin concentrations, similar to what was observed in the prospective MDRD study (324).

The effect of insulin on the level of adiponectin is also believed to be modified by hyperglycemia, since hyperglycemia can neutralize the negative effect of insulin on adiponectin concentrations (334). In this respect, it is noteworthy that in our study, the progressors from macroalbuminuria also had the worst glycemic control.

In patients with type 1 diabetes and diabetic nephropathy, an increased concentration of adiponectin may in fact mitigate the micro- and macrovascular burden of diabetic nephropathy, thus acting as a compensatory response to the demands of the metabolic milieu and vascular injury (266, 295, 326). In patients with type 1 diabetes and nephropathy and hyperglycemia, an increase in adiponectin could also serve as a mechanism to reduce hepatic gluconeogenesis. This is supported by the fact that the increase in adiponectin in patients with type 1 diabetes, and even more so in those with diabetic nephropathy, is due mainly to an increase in high-molecular-weight adiponectin (335). High-molecular-weight adiponectin is associated with the inhibition of liver gluconeogenesis, and further, the observed positive effect of adiponectin on glucose tolerance and insulin sensitivity is especially related to this isoform of adiponectin (205, 336-338).

An increase of adiponectin in patients with type 1 diabetes and diabetic nephropathy could also be a counter-regulatory response to adiponectin resistance (339, 340). Such adiponectin resistance could result from altered adiponectin function due to glycosylation (207, 329). Consequently, a modified adiponectin molecule could lead to reduced negative feedback, and thus to higher adiponectin concentrations. An increase in serum adiponectin concentrations has also been suggested to reflect a dysfunction of the adiponectin receptors or to stem from a reduction in adiponectin receptor expression. This would decrease tissue adiponectin sensitivity, thus resembling situations in patients with low adiponectin concentrations, such as in type 2 diabetes (341, 342).

In conclusion, an increased adiponectin concentration is prognostic regarding the progression from overt diabetic nephropathy to ESRD in patients with type 1 diabetes.
8. Summary and Conclusions

8.1 Summary

1. Low-grade inflammation is associated with diabetic nephropathy in patients with type 1 diabetes.

2. Mannan-binding lectin (MBL) is associated with diabetic nephropathy in patients with type 1 diabetes, but there is no association between MBL and low-grade inflammatory markers, nor any independent association between MBL and insulin resistance.

3. Serum adiponectin is associated with renal function. Adiponectin is also associated with WHR as a marker of insulin resistance, but not independently with low-grade inflammatory markers or metabolic control.

4. \( \alpha \)-defensin (-1, -2, and -3) is associated with renal function, total and HDL cholesterol, but not independently with low-grade inflammatory markers in patients with type 1 diabetes.

5. In patients with normo- or microalbuminuria, progressors and non-progressors show no differences in baseline adiponectin concentrations, but, in contrast to this finding, adiponectin is an independent predictor of progression from macroalbuminuria to ESRD in patients with type 1 diabetes.

8.2 Conclusions

All studied variables – low-grade inflammation, MBL, adiponectin, and \( \alpha \)-defensin – were associated with diabetic nephropathy in our cross-sectional studies. In contrast, however, MBL, adiponectin, and \( \alpha \)-defensin on their own were unassociated with low-grade inflammatory markers. Further, \( \alpha \)-defensin was unassociated with MBL, which may suggest that during the acute phase response, these different factors function in a coordinated fashion during the deleterious process of diabetic nephropathy.

Which factors cause low-grade inflammation and increased serum MBL, adiponectin, and \( \alpha \)-defensin concentrations in patients with type 1 diabetes and diabetic nephropathy remains unknown. Potential factors may include inheritance (genes), smoking, obesity, hyperglycemia, hyperlipidemia, and
a low level of physical activity. To support this suggestion, we could observe in our study that glycemic control, an atherosclerotic lipid profile, and WHR were associated with low-grade inflammation in a univariate analysis. In a multivariate analysis, however, only the duration of diabetes (as a measure of glycemic load), HDL-cholesterol, and AER proved to be independently associated with inflammation. Glycemic control, as measured by HbA1c, was the only variable independently associated with MBL, although in a univariate analysis, eGDR and HDL cholesterol were also associated with MBL. In addition to renal function, AER and WHR were independently associated with adiponectin, whereas age, systolic blood pressure, HDL- and total cholesterol were independently associated with α-defensin in our cross-sectional study. Notably, all these factors, except genes and age, are modifiable by changes in lifestyle or a targeted medication or both. In our follow-up study, elevated serum adiponectin levels at baseline predicted progression from macroalbuminuria to ESRD independently of renal function at baseline. This observation does not preclude adiponectin as a positive factor during the process of diabetic nephropathy, however, since the increase in serum adiponectin concentrations may still be a mechanism by which the body compensates for the demands created by the diabetic milieu.

In order to be able to prevent diabetic nephropathy and to improve the prognosis of our patients, detecting any signs of problems as early as possible is vital. Clearly, this will pose a real challenge not only for the patients, but also for the health care personnel. Markers of inflammation may represent early warning signs if combined with other relevant characteristic features associated with the development of diabetic nephropathy. Consequently, future studies should combine different markers of inflammation with a variety of clinical variables, such as various components of the metabolic syndrome for instance, to possibly enable more accurate and sufficiently early risk detection for our patients with diabetes. We foresee that a new risk score, similar to what has already been available for many years for the prevention of myocardial infarction, could serve as a call to action both in the sense of prevention and in the treatment of diabetic nephropathy.
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Appendix

The Finnish Diabetic Nephropathy Study Centers

Anjalankoski Health Center: S. Koivula, T. Uggeldahl
Central Hospital of Åland Islands, Mariehamn: M. Forsen, H. Granlund, A.-C. Jonsson, B. Nyroos
Central Hospital of Kanta-Häme, Hämeenlinna: P. Kinnunen, A. Orvola, T. Salonen, A. Vähänen
Central Hospital of Kymenlaakso, Kotka: R. Paldanius, M. Riihelä, L. Ryysy
Central Hospital of Länsi-Pohja, Kemi: H. Laukkanen, P. Nyländen, A. Sademies
Central Ostrobothnian Hospital District, Kokkola: S. Anderson, B. Asplund, U. Bysskata, P. Liedes, M. Kuusela, T. Virkkala
City of Hyvinkää Health Center: S. Klemetti, T. Nyandoto, E. Rontu, S. Satuli-Autere
Heinola Health Center: P. Hentunen, J. Lagerstam
Helsinki University Central Hospital, Department of Herttoniemi Hospital, Helsinki: V. Sipilä
Hospital of Lounais-Häme, Forssa: T. Kalliomäki, J. Koskelainen, R. Nikkanen, N. Savolainen, H. Sulonen, E. Valtonen
Ilisalmi Hospital: E. Toivanen
Jokilaakso Hospital, Jämsä: A. Parta, I. Pirttiniemi
Jorvi Hospital, Helsinki University Central Hospital: S. Aranko, S. Ervasti, R. Kauppinen-Mäkelin, A. Kuusisto, T. Leppälä, K. Nikkila, L. Pekkonen
Jyväskylä Health Center, Kylö: K. Nuorva, M. Tiilinen
Kainuu Central Hospital, Kajaani: S. Jokelainen, P. Kemppainen, A-M. Mankinen, M. Sankari
Kerava Health Center: H. Stuckey, P. Suominen
Kirkkonummi Health Center: A. Lappalainen, M. Liimatainen, J. Santaholma
Kivelä Hospital, Helsinki: A. Aimolahti, E. Huovinen
Koskela Hospital, Helsinki: V. Ilkka, M. Lehtimäki
Kotka Health Center: E. Pälikkö-Kontinen, A. Vanhanen

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Kouvola Health Center: E. Koskinen, T. Siitonen
Kuopio University Hospital: E. Huttunen, R. Iläheimo, P. Karhapää, P. Kekäläinen,
M. Laakso, T. Lakka, E. Lampainen, L. Moilanen, L. Niskanen, U. Tuovinen,
I. Vauhkonen, E. Voutilainen
Kuusamo Health Center: T. Kääriäinen, E. Isopoussu
Kuusankoski Hospital: E. Kilkki, I. Koskinen, L. Riihelä
Laakso Hospital, Helsinki: T. Meriläinen, P. Poukka, R. Savolainen, N. Uhlenius
Lahti City Hospital: A. Mäkelä, M. Tanner
Lapland Central Hospital, Rovaniemi: L. Hyvärinen, S. Severinkangas, T. Tulokas
Lappeenranta Health Center: P. Linkola, I. Pulli
Lohja Hospital: T. Granlund, M. Saari, T. Salonen
Länsi-Uusimaa Hospital, Tammisaari: I.-M. Jousmaa, J. Rinne
Loimaa Health Center: A. Mäkelä, P. Eloranta
Malmi Hospital, Helsinki: H. Lanki, S. Moilanen, M. Tilly-Kiesi
Mikkeli Central Hospital: A. Gynther, R. Manninen, P. Nironen, M. Salminen,, T. Vänttinen
Mänttä Regional Hospital: I. Pirttiniemi, A-M. Hänninen
North Karelian Hospital, Joensuu: U-M. Henttula, P. Kekäläinen, M. Pietarinen,
A. Rissanen, M. Voutilainen
Nurmijärvi Health Center: A. Burgos, K. Urgamo
Oulankangas Hospital, Oulainen: E. Jokelainen, P.-L. Jylkkä, E. Kaarlela, J. Vuolaspuro
Oulu Health Center: L. Hiltunen, R. Härkkinen, S. Keinänen-Kiukaanniemi
Oulu University Hospital: R. Iläheimo
Päijät-Hame Central Hospital: H. Haapamäki, A. Helanterä, S. Hämäläinen,
V. Ilvesmäki, H. Miettinen
Palokka Health Center: P. Sopanen, L. Welling
Pieksämäki Hospital: V. Javtsenko, M. Tamminen
Pietarsaari Hospital: M-L. Holmbäck, B. Isomaa, L. Sarelin
Pori City Hospital: P. Ahonen, P. Merensalo, K. Sävelä
Porvoo Hospital: M. Kallio, B. Rask, S. Rämö
Raahe Hospital: A. Holma, M. Honkala, A. Tuomivaara, R. Vainionpää
Rauma Hospital: K. Laine, K. Saarinen, T. Salminen
Riihimäki Hospital: P. Aalto, E. Immonen, L. Juurinen
Salo Hospital: A. Alanko, J. Lapinleimu, P. Rautio, M. Virtanen
Satakunta Central Hospital, Pori: M. Asola, M. Juhola, P. Kunelius, M.-L. Lahdenmäki,
P.Pääkkönen, M. Rautavirta
Savonlinna Central Hospital: T. Pulli, P. Sallinen, M. Taskinen, E. Tolvanen,
H. Valtonen, A. Vartia
Seinäjoki Central Hospital: E. Korpi-Hyövätli, T. Latvala, E. Leijala
South Karelia Central Hospital, Lappeenranta: T. Ensala, E. Hussi, R. Härkönen,
U. Nyholm, J. Toivanen
Tampere Health Center: A. Vaden, P. Alarotu, E. Kujansuu, H. Kirkkopalto-Jokinen,
M. Helin, S. Gummerus, L. Calonius, T. Niskanen, T. Kaitala, T. Vatanen
Tampere University Hospital: I. Ala-Houhala, T. Kuningas, P. Lampinen, M. Määttä,
H. Oksala, T. Oksanen, K. Salonen, H. Tauriainen, S. Tulokas
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