Diabetic Nephropathy: a Changing Scenery
Diabetic Nephropathy: a Changing Scenery

Een wetenschappelijke proeve op het gebied van de medische wetenschappen

PROEFSCHRIFT

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The painting on the cover is made by Jan Adriaensz. van Staveren titled ‘de Dokter’ (the physician, ±1660). It depicts a physician, standing in a window inspecting a bottle of urine (uroscopy or ‘piskijken’). In the window is a metal bowl, a can and a book on human anatomy. It is a copy after the original painting of Gerard Dou (Vienna). The painting is located at the ‘Rijksmuseum’ in Amsterdam. A digital copy of the painting was kindly providend by the ‘Rijksmuseum’, Amsterdam.

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Chapter 1

Introduction and Outline
Introduction

The presence of diabetic nephropathy determines to a great extent the prognosis of patients with type 1 diabetes mellitus. In the Netherlands diabetic nephropathy is a major cause of end-stage renal disease (prevalence about 14-16%). Besides end-stage renal disease, the presence of diabetic nephropathy is also a strong prognostic factor for the development of cardiovascular morbidity and mortality. Historical data show that about 20-40% of type 1 diabetes mellitus patients will finally develop diabetic nephropathy.

Earlier studies, like the Diabetes Control and Complications Trial (DCCT), have clearly shown that improved glycaemic control reduces the development of diabetic complications. Besides, treatment of hypertension improves the degree of albuminuria, and slows the deterioration of glomerular filtration rate. A recent meta-analysis [1] showed that treatment with renin-angiotensin-system inhibitors reduces the incidence of diabetic nephropathy (primary prevention).

Despite this knowledge, the degree of glycaemic control and blood pressure regulation does not fully explain the risk of development of diabetic nephropathy: many people develop diabetic nephropathy despite tight glycaemic control, while others have poor control without any evidence of diabetic nephropathy. As such, other factors must be involved in the development of diabetic nephropathy as well. Currently, in type 1 diabetes mellitus, the presence of microalbuminuria is the strongest predictor for the development of diabetic nephropathy. However, the presence of microalbuminuria could be regarded as the first presentation of diabetic nephropathy instead of being a predictor.

In 1994 we started a prospective study in one hundred and forty-eight type 1 diabetes patients, without microalbuminuria. In this study we tried to identify other risk factors besides poor glycaemic control and hypertension, responsible for the development of microalbuminuria. Fifty control subjects, without diabetes mellitus, were also studied for reference.

This study was designed to test four main hypotheses:

Inherited hypertension A family history of hypertension predisposes to the development of diabetic nephropathy. Besides, an increased Na-Li countertransport (NaLiCT) is considered a genetic marker for essential hypertension. NaLiCT activity reflects the activity of the Na\(^+\)-H\(^+\)-transporter, which is involved in regulation of cell volume, cell-pH and cell proliferation. Also, increased NaLiCT activity is associated with insulin resistance. The percentage of patients with type 1 diabetes mellitus with an increased NaLiCT corresponds with the percentage of patients who finally develop diabetic nephropathy. Increased NaLiCT activity may be a prognostic marker for the development of diabetic nephropathy. Prospective data, however, are lacking. Therefore we hypothesised that an increased NaLiCT could be a prognostic marker for the development of diabetic nephropathy.
Introduction and outline

Atrial natriuretic peptide  Atrial natriuretic peptide (ANP) is an endogenous protein with natriuretic and vasodilating properties. In hypervolumic states like sodium overload, heart failure and increased fluid intake, ANP-concentrations are increased. In type 1 diabetes, the observed increased ANP-levels could be the consequence of increased plasma volume as insulin induces sodium-retention. Also the extracellular volume might increase due to renal cotransport of sodium during tubular glucose reabsorption. Besides systemic vasodilation, ANP also induces glomerular afferent vasodilation. This leads to increased glomerular capillary pressure and increased glomerular filtration. We hypothesised that altered baseline ANP-levels and altered physiological response to exogenous ANP could be a prognostic marker for the development of diabetic nephropathy.

Endothelial dysfunction  The endothelium plays a principal role in the regulation of vascular tone and therefore blood pressure regulation and tissue perfusion. It has been suggested that the presence of microalbuminuria reflects vascular dysfunction. Animal studies have shown that in hypertension, diabetes mellitus and hypercholesterolaemia, endothelial dependent vasodilation is diminished. Interestingly, early in the course of type 1 diabetes mellitus, basal flow seems to be increased. This could be caused by increased basal nitric oxide production. We hypothesised that altered endothelial function could be an early prognostic factor in the development of diabetic nephropathy.

Heparan-sulphate-proteoglycan  The glomerular basement membrane (GBM) is a negatively charged size and charge selective barrier. The heparan-sulphate side chains of heparan-sulphate-proteoglycans determine this negative charge and are therefore responsible for the charge selective permeability of the GBM. Decreased presence of heparan-sulphate (HS) leads to increased permeability of the GBM to negatively charged molecules like albumin. Structural changes in the heparan-sulphate-proteoglycan-complex (HSPG) may lead to increased permeability of albumin and therefore (micro-)albuminuria. Interestingly, in diabetes increased capillary permeability seems not to be confined to the glomerulus. Therefore, it has been postulated that the presence of microalbuminuria reflects widespread vascular damage (Steno-hypothesis). We hypothesised that alterations in the heparan-sulphate-proteoglycan-complex is an early marker for the development of diabetic nephropathy.

The pathophysiology of diabetic nephropathy consists of more than the four above mentioned hypothesis. We decided to confine our research project to these hypotheses.
Our study specifically addresses the following hypotheses:

- The development of diabetic nephropathy in type 1 diabetes mellitus can be predicted by the kinetic parameters of the NaLi-countertransport.
CHAPTER 1

- The development of diabetic nephropathy is preceded by increased glomerular permeability response to atrial natriuretic peptide.
- The development of diabetic nephropathy is preceded by increased basal production of endothelial derived relaxation factor (nitric oxide).
- The development of diabetic nephropathy is preceded by changes in the heparan-sulphate-proteoglycan-complex, as reflected in serum and/or urine concentrations of HS/HSPG.

We performed an extensive series of measurements and investigations in all patients and control subjects to collect the baseline characteristics necessary to test the above mentioned hypotheses. Renal function tests, before and during the infusion of ANP were performed. Endothelial function was assessed by means of forearm venous occlusion plethysmography. For this purpose N<sup>G</sup>-monomethyl-L-arginine (L-NMMA), norepinephrine and acetylcholine were infused intraarterially during these tests. Blood was taken to determine the kinetic parameters of the NaLi-countertransport. Besides many other parameters were assessed. Blood was also taken for DNA-analysis. Urine was collected for determination of HS/HSPG-concentrations. The type 1 diabetes patients were regularly screened for the development of microalbuminuria.

Outline of the thesis

In 1994 we started this prospective study in one hundred and forty-eight type 1 diabetes patients, without diabetic nephropathy. For reference, 50 control subjects without diabetes mellitus, also underwent the investigations at baseline. These subjects were not followed.

After baseline investigations, patients were followed for up to 13 years for the development of microalbuminuria. At the end of the study patients who developed microalbuminuria would be compared with those who did not develop microalbuminuria.

Chapter 2 reviews the current concepts of the pathophysiology of diabetic renal microvascular complications.

During follow up, several cross-sectional studies were performed. Results of those studies are described in chapters 3 to 8.

Renal physiology  Early in the course of diabetes, the kidneys hypertrophy, and glomerular filtration is increased. This increased glomerular filtration is (partly) held responsible for the development of diabetic nephropathy, as it can lead to increased glomerular pressure. These haemodynamic changes may cause injury to the capillary wall, resulting in increased permeability, mesangial sclerosis and glomerular basement membrane thickening. An increase in glomerular filtration
rate could result from primary actions at the glomerular/vascular level (afferent vasodilation) or could be the consequence of a primary increase in proximal tubular sodium reabsorption resulting in systemic volume expansion. Recently it was hypothesised that an increase in sodium reabsorption may lead to glomerular hyperfiltration through the tubulo-glomerular feedback mechanism (tubular-hypothesis) without volume expansion. Chapter 3 describes the results of our study in which we investigated this hypothesis.

Homocysteine has long been regarded as a (causal) factor for atherosclerosis and as an independent risk factor for cardiovascular disease. Despite the fact that type 1 diabetes mellitus is often complicated by vascular disease, plasma homocysteine levels are reduced in type 1 diabetes mellitus. Plasma homocysteine levels appear to be determined by glomerular filtration. As (early) type 1 diabetes mellitus is characterized by increased glomerular filtration, we investigated whether the increased glomerular filtration could contribute to reduced plasma homocysteine levels. Chapter 4 describes the results of an analysis in our cohort with regard to the influence of renal function on plasma homocysteine levels.

Urinary albumin excretion is not only determined by glomerular permeability, but also by tubular reabsorption. An increase of albuminuria could be the result of a diminished tubular protein reabsorption. In an earlier study [2] we investigated the effects of atrial natriuretic peptide on tubular protein handling. In this study we found that atrial natriuretic peptide markedly attenuated the proximal tubular resorption of low-molecular-weight proteins. Therefore it seemed likely that increased albuminuria after infusion of atrial natriuretic peptide results from blockade of tubular albumin reabsorption.

In chapter 5 and chapter 6, however, we describe the role of the polygeline Haemaccel® (the solvent of ANP) on tubular protein reabsorption.

**Endothelial function** Endothelial dysfunction is thought to play an important role in the development of hypertension and atherosclerosis. Nitric oxide (NO), formerly known as endothelial derived relaxation factor, plays an important role in arterial vasodilation. Alterations in NO-synthase activity could lead to decreased vasodilation and hypertension. Chapter 7 describes the role of the Glu298Asp-polyphenol polymorphism of the NO-synthase gene on basal NO production.

In the past decades, the endothelial release of nitric oxide (NO) has been shown to be one of the key regulators of baseline vascular tone. The optimal method to assess the contribution of NO to baseline vascular tone is to quantify the vasoconstrictor response to specific blockade of endothelial NO-synthase. In humans, this is almost exclusively performed by the infusion of the NO-synthase blocker NG-monomethyl-L-arginine (L-NMMA) into the brachial artery using the perfused forearm technique. However up to now, dosages did not exceed 0.4 mg·min\(^{-1}\) per dL of forearm tissue (or 4 mg·min\(^{-1}\) in total, equivalent to \(\pm 16 \mu\text{mol·min}^{-1}\)), and at this dose a maximal vasoconstrictor response to L-NMMA is not achieved. As such, an accurate assessment of the contribution of NO to
baseline vascular tone is missing in the current literature. In chapter 8 we describe the results of a pharmacodynamics study of L-NMMA using the perfused forearm technique.

**Population study** In 1994, at the start of the study, it was estimated that 25-40% of type 1 diabetes patients would develop microalbuminuria. However, after thirteen years of follow up, only 6 patients (4%) had developed microalbuminuria. Why did so few people develop diabetic nephropathy? Did we only include the patients with good metabolic control? What went wrong with the inclusion? Chapter 9 compares the cumulative incidence of patients with type 1 diabetes mellitus who participated in the study, with the eligible, but non-participating type 1 diabetes patients.

To investigate changes in the cumulative incidence of diabetic nephropathy over time, and to investigate which factors might be responsible for the presumed decline in incidence, a retrospective population study was carried out. The results of this study are described in chapter 10.

The results are summarised and discussed in chapter 11.

Een nederlands-talige samenvatting staat in hoofdstuk 12.
Chapter 2

Pathogenesis of renal microvascular complications in diabetes mellitus

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CHAPTER 2

Introduction

Microvascular disease is the main determinant in the development of late complications in diabetes mellitus. This is obvious for diabetic nephropathy [3; 4] and retinopathy [5; 6], but changes in the microcirculation may also play an important role in the pathogenesis of diabetic neuropathy [7; 8]. Although the pathogenesis of diabetic microangiopathy is incompletely understood, it is likely that it involves an interaction between metabolic and functional/haemodynamic factors. Together with genetic and environmental factors this results in the development of microvascular complications.

Epidemiology

Diabetic nephropathy is a major cause of morbidity and mortality in patients with type 1 as well as type 2 diabetes. Diabetic nephropathy is characterised by specific morphological changes including glomerular basement membrane thickening, mesangial expansion and glomerular and tubulo-interstitial sclerosis. The clinical syndrome of diabetic nephropathy exists of proteinuria, hypertension and progressive decrease of glomerular filtration rate. Various studies reveal cumulative incidence rates for diabetic nephropathy of 20-40\% [9–11], but it is suggested that the incidence is declining [12]. Although the incidence of diabetic nephropathy among diabetic patients is decreasing, the prevalence of nephropathy among the population as a whole is dramatically increasing, predominantly due to improved treatment of hypertension and coronary heart disease, and due to an increase in prevalence of type 2 diabetes mellitus. The risk of diabetic nephropathy among type 2 diabetic patients with progression to end-stage renal disease is comparable to that in type 1 diabetes mellitus [13; 14]. The peak incidence of diabetic nephropathy is between 10-17 years after the onset of diabetes [10; 15]. Thereafter, the incidence of diabetic nephropathy declines rapidly. The first clinical manifestation of diabetic nephropathy is microalbuminuria, defined as a urinary albumin-excretion rate of 20-200 µg·min\(^{-1}\). Microalbuminuria is associated with other microvascular complications as well as with cardiovascular disease suggesting some common pathophysiological mechanisms [9; 16].

Pathophysiology

The pathophysiology of renal microvascular complications in diabetes mellitus consists of an intensive interplay of metabolic and functional/haemodynamic factors that underlies the structural changes of the microvasculature.

Structural changes

These structural abnormalities in diabetic glomerulopathy include an increase in basement membrane thickness, mesangial expansion with accumulation of ex-
Renal microvascular complications

Tracellular matrix components (ECM) and glomerular fibrosis. There is an inverse correlation between heparan sulphate proteoglycan (HSPG) expression and mesangial expansion in diabetic glomerulopathy [17], stressing the importance of dysregulation of ECM synthesis what seems crucial for the development of renal microvascular complications. Furthermore, the extent of ECM accumulation correlates strongly with the degree of tubulointerstitial fibrosis and of renal failure and proteinuria [17–20]. Tubulointerstitial injury is probably a major feature of disease progression [21]. Chronic interstitial injury usually follows the onset of glomerular proteinuria. Both structural lesions are probably linked via the increase in glomerular permeability and ultrafiltration of bioactive (growth) factors which can be held responsible for inducing and/or aggravating ECM production and renal fibrosis.

Metabolic pathways

Several factors such as high glucose, intracellular polyols, non-enzymatic glycation products, hexosamines and vasoactive hormones are held responsible for the changes in regulation of the biosynthesis of matrix components composition and accumulation [22]. They stimulate the synthesis and release of growth factors and cytokines from resident renal cells, inducing cell proliferation and hypertrophy, as well as the production of extracellular matrix proteins [23]. Of these, the profibrotic cytokine, transforming growth factor-β (TGF-β) has emerged as a key factor in the development of structural abnormalities in diabetic nephropathy [24]. In vitro experiments showed that glomerular mesangial cells, epithelial cells and interstitial fibroblasts increased their TGF-β expression when exposed to high glucose [25; 26]. Also, in vivo, the expression of renal TGF-β is increased in experimental as well as in human diabetes [27; 28]. In animal models, neutralizing TGF-β antibodies prevented the increase in extracellular matrix components and the increase in mRNA encoding for type IV collagen α1 and fibronectin. Furthermore, anti-TGF-β almost completely prevented the fall in creatinine clearance in diabetic db/db mice [29]. Except for ECM accumulation, it is suggested that changes in the heparan sulphate chains of HSPG in glomerular basement membranes and ECM play a role in diabetic renal disease. A decrease in heparan sulphate, the anionic side chain of HSPG, induces proteinuria, stressing the importance of heparan sulphate for the permselectivity of the glomerular basement membrane [30]. It has been suggested that proteinuria itself plays a pathogenic role in diabetic nephropathy [31]. However, in contrast to the prevention of the decrease in glomerular filtration rate, neutralizing TGF-β antibodies did not prevent proteinuria in diabetic mice [29]. This could implicate that the detrimental effects of proteinuria on renal function are mediated via TGB-β or that proteinuria is just a consequence of permselectivity changes. Nevertheless, heparan sulphate also determines the local concentration, compartmentalisation, stability and activity of certain growth factors and proteases, thus controlling ECM expansion [32]. Heparan sulphate is probably directly involved in the inhibition of TGF-β
overexpression [33] and regulates the expression of decorin, an extracellular matrix protein that inactivates TGF-β [34].

**Hyperglycaemia**  
The link between ECM accumulation via TGF-β and other profibrotic cytokines and diabetes is met by metabolic changes characteristic for the diabetes state per se. The metabolic hypothesis suggests that microvascular complications develop as a direct consequence of hyperglycaemia. Several small, prospective, randomised intervention studies and the DCCT [35] have definitely proven that improved metabolic control achieving near-normoglycaemia can reduce the incidence of diabetic nephropathy. These studies have revealed duration and severity of hyperglycaemia as major risk factors for the development of diabetic microvascular complications. Complete normalisation of blood glucose after pancreas transplantation even shows a regression of structural renal changes [36]. The mechanisms by which hyperglycaemia give rise to microvascular complications has slowly been unravelled in the last years, supported by a large amount of experimental as well as clinical data.

**Advanced glycation end-products**  
Advanced glycation end-products (AGEs), which are formed by non-enzymatic glycation of proteins, accumulate in renal glomeruli [37]. Recent research has shown that AGE precursors (dicarbonyls like methylglyoxal) are formed intracellularly from intracellular hyperglycaemia. These precursors can react with amino groups of intra- and extracellular proteins to form AGEs. AGEs are capable of inducing increased vascular permeability, enhancing protein and lipoprotein deposition, inactivating nitric oxide and promoting matrix protein synthesis and glomerular sclerosis [38]. This latter mechanism is probably mediated by TGF-β [39; 40]. The clinical importance of AGEs in diabetic nephropathy is stressed by the role of AGE formation inhibitors like aminoguanidine and ALT-946 and the so called AGE crosslink “breakers” (phenacylthiazolium bromide; ALT-711) for treatment or prevention of diabetic (renal) complications [41; 42].

**Protein Kinase C (PKC)**  
An increase in intracellular glucose induces de novo synthesis of diacyl glycerol (DAG) [43] that activates protein kinase C (PKC). PKC is capable of phosphorylating a number of cellular proteins. Increased PKC activity modulates gene expression in mesangial cells, inducing extracellular matrix protein synthesis, especially of type IV collagen and fibronectin, which is mediated by TGF-β [24; 44]. Furthermore, PKC activation is linked to mitogen-activated protein kinase (MAPK) which is important in the intracellular signal transduction processes leading to cell proliferation and hypertrophy [45; 46]. Activation of PKC increases production of vasodilatory prostanoids leading to hyperfiltration [47]. Animal studies showed that blockade of PKC by means of LY333531 reversed renal hyperfiltration and reduced glomerular albumin permeability [48]. Treatment with a PKC-inhibitor showed a reduction of urinary albumin excre-
Renal microvascular complications

In diabetic db/db mice, possibly through attenuation of glomerular expression of TGF-β [49].

Polyol pathway Hyperglycaemia induces an increased flux through the polyol pathway. Originally it was thought that intracellular formation and accumulation of sorbitol, mediated by aldose-reductase, leads to increased intracellular osmolality and swelling of cells. However, just recently it has been shown that decreased levels of reduced glutathione (GSH), as a consequence of the reduction from glucose to sorbitol [50], are responsible for the deleterious consequences by increasing intracellular oxidative stress (see below). Clinical trials suggested the potential usefulness of aldose reductase inhibitors in preventing the progression of incipient diabetic nephropathy in patients with type 2 diabetes mellitus [51].

Hexosamine pathway In diabetes an increased flux of intracellular glucose through the hexosamine pathway results in increased N-acetylglucosamine (GlcNAc), by conversion from fructose-6-phosphate by the enzyme GFAT. Most probably, modification of transcription factors like Sp1 by GlcNAc will lead to transcription of the gene for TGF-β [51]. Furthermore, many other intracellular proteins are modified by GlcNAc like eNOS activity [51] that may contribute to the pathogenesis of renal diabetic complications.

Reactive Oxygen species as a common pathway Recently, Nishikawa et al. [52], and Du et al. [53] have shown that the formation of AGEs, activation of PKC, and activation of the polyol pathway as well as the hexosamine pathway, are mediated by the production of reactive oxygen species (ROS). They showed that elevated (intracellular) glucose levels increase the production of ROS in mitochondria. This overproduction of ROS was prevented by manganese superoxide dismutase or by an uncoupler of oxidative phosphorylation, by uncoupling protein-1, and completely prevented intracellular formation of AGEs, activation of protein kinase C, increase in polyol pathway flux and hexosamine pathway activation. They concluded that ROS-production is a common-pathway in the initiation of high-glucose mediated stimulation of the aforementioned pathways. It is hypothesised that an excess of ROS inhibits GADPH (glyceraldehyde-3-phosphate dehydrogenase), a glycolytic key-enzyme, promoting shunting of upstream glucose-metabolites into the aforementioned pathways.

Functional/haemodynamic pathway

Long-lasting poor metabolic control does not necessarily lead to diabetic microvascular disease. This means that the apparent protection of diabetic patients for nephropathy cannot solely be explained on the basis of better metabolic control. The haemodynamic hypothesis implies that due to haemodynamic alterations in
blood flow and pressure, structural changes are provoked, which will result in the development of microvascular complications.

**Flow/pressure (haemodynamic hypothesis)** Capillary hyperperfusion precedes the onset of diabetic renal microangiopathy [4]. This observation has led to the hypothesis that changes in systemic or local haemodynamics contribute to the development of diabetic nephropathy [54]. Micropuncture studies revealed a range of haemodynamic alterations in diabetes: increased intraglomerular pressure, increased singel nephron GFR and preferential afferent compared with efferent arteriolar vasodilation. These renal haemodynamic changes may be related to vasoactive hormones such as angiotensin II, endothelin, nitric oxide, locally active prostaglandins and kinins, and atrial natriuretic peptide. Furthermore, hyperglycaemia, glucagon, insulin, insulin-like growth factor and reduced sympathetic nerve activity may be involved in diabetic microvascular haemodynamic changes. These haemodynamic changes cause injury of the vascular wall, resulting in increased permeability, intima fibrosis, and vascular smooth muscle cell proliferation [55]. Therapy aimed at reversing glomerular hyperfiltration, by controlling glucose concentration early in the course of the disease, dietary protein restriction, and antihypertensive therapy, may slow the rate of progression of the renal disease. Currently many pharmacological substances are being developed which block the effect of vasoconstrictory hormones, or reduce the degradation of vasodilating hormones. Because most of the enzymes involved in production and degradation of these vasoactive hormones have considerable homology, substances are being developed which interact with more than one of these systems.

**Renin-Angiotensin System (RAS)** The therapeutic effects of angiotensin converting enzyme (ACE) inhibitors and AT1-receptor antagonists in decreasing the progression of micro- or macroalbuminuria stresses the importance of the Renin-Angiotensin System (RAS) [56–60]. The decrease in progression of diabetic nephropathy by ACE-inhibitors and AT1-receptor antagonists was originally attributed to their ability to control systemic and intraglomerular hypertension. However, despite comparable reductions in systemic blood pressure, endothelin receptor blockade was not as renoprotective as an ACE-inhibitor at least in rats [61]. Indeed, aforementioned studies showed that effective blockade of angiotensin II action had favourable renoprotective effects that go beyond the blood pressure lowering effects of these drugs. Thus, it is likely that some of these favourable effects might be related to the non-haemodynamic effects of angiotensin II. Angiotensin II induces smooth muscle cell growth, hypertrophy and proliferation of glomerular cells and stimulates the synthesis of ECM components, collagen and fibronectin. The angiotensin II induced smooth muscle cell growth and the hypertrophic and fibrogenic responses are mediated by TGF-β [62]. Neutralising anti-TGF-β antibodies are able to prevent angiotensin II stimulated production of ECM in *in vitro* studies with mesangial cells [63]. Furthermore, angiotensin II
activates PKC in glomerular cells through AT1 receptor stimulation.

**Endothelial dysfunction** The normal endothelium has important regulating properties for vascular tone and is intimately involved in the regulation of vascular and renal permeability. It regulates the composition of ECM and the proliferation of smooth muscle and mesangial cells [64]. Therefore, endothelial dysfunction has been implicated in the pathogenesis of diabetic vascular disease. In non-complicated type 1 diabetic patients acetylcholine induced endothelial-dependent vasodilatation is intact [65]. In contrast, diabetic microalbuminuria reflects widespread endothelial dysfunction. An increase in von Willebrand Factor (vWF), a component of the endothelial cell membrane and a marker of endothelial damage, precedes the occurrence of microalbuminuria [66]. The mechanisms by which diabetes causes impaired endothelial function are largely unknown. In view of the haemodynamic changes in diabetes a biphasic process is proposed. As a vasodilator, nitric oxide (NO) is a candidate for mediating the increases in blood flow and capillary permeability that are observed in the early phase of diabetes. Indeed, an increased basal endogenous NO production accounts for the renal hyperfiltration in diabetic rats, whereas in the kidney there seems to be an enhanced nitric oxide production indicated by an increased urinary nitrate and nitrite concentration [67; 68]. The cause of the increased renal nitric oxide production is not elucidated yet. There are reports of an increased expression of inducible Nitric Oxide Synthase (iNOS) [69], but animal studies showed that l-imino-ethyl-lysine, a specific inhibitor of iNOS, was unable to reduce the glomerular hyperfiltration [70]. Furthermore NO was not increased in normoalbuminuric type 1 diabetic patients using the isolated forearm technique [65]. Deficiency of nitric oxide in the vascular tree is a duration dependent process. Therefore, late in the course of diabetes, damaged endothelial cells may loose the ability to increase NO synthesis, thus favouring a proliferative and thrombogenic milieu. It is suggested that endothelial dysfunction could be the result of hyperglycaemia induced formation of free radicals, which inactivate nitric oxide (NO). In vitro, the bioavailability of NO is reduced by AGEs, which quench NO [38]. Also, hyperglycaemia interferes with the production of cyclic guanylate monophosphate (cGMP), the second messenger of NO. Hyperglycaemia is also capable of activating protein kinase C that inhibits endothelial NO-synthase [44].

Of interest, recently it was suggested that activation of poly(ADP-ribose) polymerase (PARP) is an important factor in the pathogenesis of endothelial dysfunction in diabetes. Inhibition of PARP by a novel PARP inhibitor PJ34, maintained normal vascular responsiveness, despite persisting hyperglycaemia in diabetic mice [71].

**Genetic influences**

In contrast to diabetic retinopathy and neuropathy, which develop in the majority of diabetic patients, only 20-40% of type 1 diabetic patients are at risk of develop-
ing diabetic nephropathy. In view of the observed familial clustering of diabetic nephropathy, a genetic predisposition to diabetic nephropathy has been assumed. From epidemiological studies evidence appears about genetic influences on the development of microvascular complications [72-74]. The genetic susceptibility may also explain the marked differences in incidence of microvascular complications between various races [75]. A genetic predisposition to and a parental history of hypertension are supposed to be risk factors for the development of diabetic nephropathy [76]. The likelihood of developing diabetic nephropathy was increased in patients with an elevated sodium-lithium countertransport activity, a marker of the genetic predisposition to essential hypertension [77]. As both hypertension and the development of diabetic nephropathy seem to be genetically determined, it is tempting to search for combined genetic markers. Genes involved in the RAS are promising candidates as the RAS plays a central role in both blood pressure regulation and renal function. Association studies linking these polymorphisms with the development of diabetic nephropathy reveal conflicting results. Many reports on ACE-polymorphisms suggest a contribution of the DD polymorphism in the development of diabetic nephropathy [78], although a recent thorough review by Kunz et al. [79] failed to confirm the suggested association due to methodological limitations in the original studies.

Conclusions

The pathophysiology of renal diabetic microvascular complications is slowly being unravelled now. Metabolic and haemodynamic changes interfere in which TGF-β seems to play a central role. There is also strong evidence that hereditary factors are essential in the development of microvascular complications. This suggests that the development of diabetic microvascular complications is a multifactorial process in which different mechanisms are likely to operate. Elucidating the pathophysiology of microvascular complications is important for the development of appropriate prevention and treatment of these complications.
Chapter 3

Glomerular hyperfiltration in type 1 diabetes mellitus results from primary changes in proximal tubular sodium handling without changes in volume expansion


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CHAPTER 3

Abstract

Background  Glomerular hyperfiltration plays a role in the pathophysiology of diabetic nephropathy. An increase in glomerular filtration rate (GFR) could result from primary actions at the glomerular/vascular level or could be the consequence of a primary increase in proximal tubular sodium reabsorption resulting in systemic volume expansion. Recently it was hypothesized that an increase in sodium reabsorption may lead to glomerular hyperfiltration through the tubulo-glomerular feedback mechanism (tubular-hypothesis) without volume expansion.

Methods  We have studied 54 normoalbuminuric patients with type 1 diabetes. GFR was measured by inulin clearance. Proximal and distal sodium reabsorption were calculated according to standard formulas using free water clearance technique. Plasma volume, measured by $^{125}\text{I}$-albumin method, atrial natriuretic peptide (ANP) and the second messenger cyclic guanosine-3,5-monophosphate (c-GMP) were used as markers of extracellular volume expansion.

Results  Glomerular hyperfiltration (GFR $\geq 130 \text{ mL/min}^{-1} \cdot 1.73\text{m}^{-2}$) was present in 14 out of 55 diabetes patients (25%). There were no differences in plasma volume between normo- (NF) and hyper-filtrating (HF) patients (2933±423 in NF vs 3026±562 mL in HF, NS). Also plasma ANP and c-GMP levels were not significantly different between the groups. The fractional proximal reabsorption of sodium was significantly increased in HF ($\text{fPRNa}^+$%) 90.1±2.0 vs 91.5±1.6, $p=0.02$). There were no differences in distal sodium reabsorption or distal sodium load ($\approx$ macula densa concentration of NaCl) in both groups.

Conclusions  Our data suggest that the primary event in diabetic glomerular hyperfiltration is an increase in proximal tubular sodium reabsorption. They do not give support to the hypothesis that systemic volume expansion or ANP mediate glomerular hyperfiltration in normoalbuminuric type 1 diabetes patients. As such, changes in tubular sodium handling most probably influence tubulo-glomerular feedback.
Hyperfiltration due to changed proximal sodium resorption

Introduction

Diabetic nephropathy develops in about 10-30% of patients with type 1 diabetes and accounts for the increased morbidity and mortality [10; 12]. Renal haemodynamic changes characterized by glomerular hyperfiltration occur early after onset of diabetes even before glomerular morphological changes can be found, and are likely to play a role in the pathophysiology of progressive renal disease [3; 4; 80; 81]. Glomerular hyperfiltration is attributed to vasodilatation predominantly of the afferent arteriole. Many humoral and vascular mediators have been implicated in hyperfiltration due to their primary actions at the glomerular level. Apart from glucagon [82], insulin-like growth factor [83], locally active prostaglandins [84] and kinins [85], nitric oxide [86; 87], hyperglycemia [88] and angiotensin II [87] may be involved in diabetic glomerular hyperfiltration. It has been proposed that consequences of diabetes on glomerular filtration find their origin not primarily in the glomerulus but in a primary increase in proximal tubular sodium reabsorption. In diabetes, an increase in tubular sodium reabsorption can be attributed to hyperglycemia or hyperinsulinemia [89–91]. Several studies have suggested that an increase in atrial natriuretic peptide (ANP) levels, due to increased extracellular volume may be involved in diabetic glomerular hyperfiltration [92]. In recent years it was suggested that a primary increase in proximal tubular reabsorption of sodium influences glomerular filtration not via extracellular volume expansion but through the macula densa (tubulo-glomerular feedback) [93; 94]. This is referred to as the “tubular hypothesis” of glomerular hyperfiltration (figure 3.1). In order to find out whether a primary “glomerular” or “tubular” event precedes glomerular hyperfiltration and to elucidate the role of volume expansion/ANP and tubulo-glomerular feedback in the pathogenesis of diabetic hyperfiltration, we studied baseline levels of ANP and its second messenger cyclic guanosine-3,5-monophosphate (c-GMP) in relation to extracellular volume status, tubular sodium handling and renal haemodynamics in patients with normoalbuminuric type 1 diabetes mellitus.

Methods

Study population In 1994 we initiated a large prospective study in type 1 diabetes patients, aiming at identifying factors involved in the development of microalbuminuria. For this purpose we measured renal and systemic haemodynamics including endothelial function and transcapillary escape rate of albumin in patients and matched controls. The cross-sectional results were published elsewhere [65; 95; 96]. In a large subgroup of patients we also measured plasma-volume, ANP and c-GMP levels as well as (fractional) sodium clearance. The analysis of the results of these measurements is the subject of the present study.

After approval of the study protocol by the local ethics committee, 54 patients with type 1 diabetes mellitus gave their written informed consent for participation in the study. The diabetes patients fulfilled the following inclusion criteria: dia-
Figure 3.1: Schematic view of possible tubular events involved in the pathogenesis of diabetic glomerular hyperfiltration. ANP, atrial natriuretic peptide; GFR, glomerular filtration rate; ECV, extracellular volume; MD, macula densa; TGF, tubulo-glomerular feedback.

betes onset before the age of 40, insulin treatment within 1 year after diagnosis, duration of diabetes of 5-12 years, age 18-40 years, blood pressure $<140/90$ mmHg (mercury sphygmomanometer), no anti-hypertensive medication, no clinical evidence of macrovascular disease and no evidence of nephropathy (microalbuminuria) or retinopathy (except for simple background retinopathy). Albuminuria was assessed in two timed overnight urine samples. Microalbuminuria was defined as a urinary albumin excretion $>20 \mu g\cdot min^{-1}$. The diabetes patients were recruited from the outpatient diabetes clinic of the Radboud University Nijmegen Medical Centre.

Study protocols All subjects were on an “ad libitum” diet the week before the start of the study and were instructed to abstain from alcohol and caffeine consumption for at least 24 h and to refrain from smoking 12 h before the experiments. All participants were studied in the morning after a light breakfast. At 08.00 a.m. all subjects consumed a water load of $20 mL \cdot kg^{-1}$ and two intravenous lines were inserted for constant infusion of $250 mL \cdot h^{-1}$ glucose 2.5%/NaCl 0.45% and inulin as well as for blood sampling. Inulin (Imtest®) was purchased from Laevosan-Gesellschaft m.b.H., Linz, Austria. Plasma glucose was measured using a standard glucose oxidation method. During the study blood glucose was measured every 20 min using a glucometer (Menarini Diagnostics, Utrecht, the
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Netherlands). Glucose levels were kept constant between 5 and 8 mmol·L\(^{-1}\) during the whole study period. Additional glucose or insulin was given to maintain target glucose levels if necessary. Extra tap water matching urine output was supplied for the remainder of the clearance study. The subjects were supine during the clearance study, but were allowed to stand to urinate. After 30 min a constant infusion of inulin was started, preceded by a priming dose. After an equilibration period of at least 70 min in which urine output was at least 10 mL·min\(^{-1}\), three urine samples were collected at 20 min intervals. Blood samples were taken via the intravenous cannula before and after each urine collection. Before the start of the clearance study blood pressure was recorded in supine position with a sphygmomanometer. During the clearance study blood pressure was recorded twice during each urine collection with an automatic oscillometric device (Dinamap, 1846 SX, Critikon, Tampa, Florida, USA). On a separate occasion (due to practical reasons) within 2 days of the first study, in a fasting state, plasma volume was measured using 2-4 \(\mu\)Ci \(^{125}\)I-albumin (Iodinated \(^{125}\)I Human Serum Albumin, code IM 17 P, Amersham Int., Amersham, England) given as an iv bolus injection in one arm. During the first 60 min after injection, seven arterial blood samples were collected at regular intervals from the other arm. Plasma radioactivity was measured in each sample using a scintillation detector (automatic g-counter, 1480 Wizard 3\(^{\text{TM}}\), Wallac, Turku, Finland). Plasma volume was determined from retropolation of the disappearance curve to time zero and from the injected volume of the tracer. Calculations were only done when the correlation coefficient between time points for blood sampling and the corresponding values of \(\ln\) (plasma radioactivity) exceeded 0.85 [95; 97]. Urine and blood samples were analysed for creatinine, sodium, potassium, osmolality, and inulin using standard laboratory procedures. Plasma concentrations of ANP and of its second messenger c-GMP were measured in blood samples just before the start of inulin infusion and volume loading. Plasma c-GMP was assumed to be a reflection of systemic ANP activity [98] whereas urinary c-GMP was supposed to be a marker of the renal action of ANP [99]. ANP was measured by a radioimmunoassay as described previously [100]. Urinary c-GMP was measured by an enzyme immunoassay (Biotrak c-GMP EIA, RPN 226, Amersham Life Science, Buckinghamshire, England). Plasma c-GMP concentration was measured with the same immunoassay after an additional ethanol extraction. HbA\(_{1c}\) was measured using a HPLC technique (Bio-Rad Diamat\(^{\text{TM}}\), Veenendaal, The Netherlands) with reference values of 4.8-6.2%. The renal clearance of inulin is regarded as a marker for glomerular filtration rate (GFR). The fractional excretion of sodium (FNa) is calculated as quotient of clearance of sodium and GFR. Free water clearance (\(C_{H,O}\)) is calculated as urine volume minus osmolar clearance. Fractional and distal sodium reabsorption were calculated with the free water clearance technique according to the following formula [101; 102]:
Figure 3.2: Correlation of proximal tubular sodium reabsorption calculated with either the free water clearance technique (fPRNa⁺) or lithium clearance (1-İELi⁺) in healthy volunteers after administration of triamterene (♦), acetazolamide (■), hydrochlorothiazide (▲), ethacrynic acid (○), furosemide (×) and amiloride (•).

Fractional proximal sodium reabsorption was calculated by:

\[ 100 - \left( \frac{U_{(Na+K)} \cdot V}{GFR \cdot P_{Na}} \right) \cdot 100\% - \left( \frac{C_{H_2O}}{GFR} \cdot 100\% \right) \]

Fractional distal sodium reabsorption (corrected for total tubular load [GFR x P_{Na}]) was calculated by:

\[ \left( \frac{(C_{H_2O} \cdot P_{Na})+(V \cdot U_{K})}{GFR \cdot P_{Na}} \right) \cdot 100\% \]

Fractional distal sodium reabsorption (corrected for distal load [V x P_{Na}]) was calculated by:

\[ \left( \frac{(C_{H_2O} \cdot P_{Na})+(V \cdot U_{K})}{V \cdot P_{Na}} \right) \cdot 100\% \]

These formulas were validated in a pilot experiment in which we studied the natriuretic effects of differently acting diuretics in healthy volunteers. We found a high correlation \( r^2 = 0.76 \) between proximal tubular reabsorption calculated with either free water clearance technique or lithium clearance technique as described before [103], although the free water clearance technique evidently provided a higher estimate of proximal tubular sodium and water reabsorption (figure 3.2).
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<table>
<thead>
<tr>
<th></th>
<th>NF (n=41)</th>
<th>HF (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females / Males</td>
<td>19/22</td>
<td>8/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28±7</td>
<td>28±7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72±10</td>
<td>71±8</td>
</tr>
<tr>
<td>Body mass index (kg·m⁻²)</td>
<td>23.0±3.1</td>
<td>23.6±3.0</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>116±9 / 62±6</td>
<td>117±8 / 63±6</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>8.5±3.1</td>
<td>8.3±3.0</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.3±1.4</td>
<td>8.4±1.3</td>
</tr>
<tr>
<td>Albumin excretion rate (µg·min⁻¹)</td>
<td>7.0±4.2</td>
<td>9.1±4.8</td>
</tr>
<tr>
<td>Urinary sodium excretion (mmol·24h⁻¹)</td>
<td>141±47</td>
<td>150±61</td>
</tr>
<tr>
<td>Urinary potassium excretion (mmol·24h⁻¹)</td>
<td>69±26</td>
<td>83±39</td>
</tr>
</tbody>
</table>

Values are expressed as means±SD.

Table 3.1: Baseline characteristics of the patients with diabetes. NF, patients with diabetes with normal glomerular filtration; HF, patients with diabetes with glomerular hyperfiltration (GFR ≥ 130 mL·min⁻¹·1.73m⁻²); BMI, body mass index; HbA1c, glycated haemoglobin.

Statistical analysis  Statistical analysis was performed using the SPSS 11.0.1 personal computer software package (SPSS Inc, Chicago, IL, USA). Differences in baseline levels between the two groups were analysed by unpaired Student’s t-test for normally distributed data and Mann-Whitney U test for non-parametric data. Correlation coefficient was calculated by linear regression analysis. A p-value less than 0.05 was considered to be statistically significant. Values are expressed as means±SD unless otherwise indicated.

Results

Subjects characteristics  Mean GFR was 121±11 mL·min⁻¹·1.73m⁻² in the diabetes patients. Based on studies in healthy subjects, renal hyperfiltration was defined as a GFR ≥ 130 mL·min⁻¹·1.73m⁻² [95; 96]. Patients were divided into those with a normal GFR (NF) and those with glomerular hyperfiltration (HF). The characteristics of the two groups are summarised in table 3.1. There were no demographic or anthropomorphic differences between the groups. On arrival at the hospital, mean plasma glucose level in NF diabetes patients was 13.0±4.3 mmol·L⁻¹ and in HF diabetes patients 12.9±4.4 mmol·L⁻¹ (NS). Throughout the clearance study plasma glucose was 7.2±1.9 and 7.5±2.0 mmol·L⁻¹ in NF and HF diabetes patients respectively (NS). Insulin levels at the start of the study were similar (116±71 pmol·L⁻¹ in NF vs 140±118 in HF, NS). Also, there were no differences in mean arterial blood pressure (90±7 mmHg in NF vs 88±6 in HF, NS) or in heart rate (66±8 bpm in NF vs 70±11 in HF, NS). The urinary flow, urinary osmolality, free water clearance, urinary sodium and potassium and
plasma sodium were similar in both groups (Table 3.2).

**Tubular sodium handling** The mean urinary sodium excretion was not different between the groups reflecting an average dietary salt intake of 9 grams per day (Table 3.2). The fractional proximal sodium reabsorption was significantly higher in the HF group. To appreciate this difference we calculated the effect of an increase in fractional sodium reabsorption in relation to GFR. In patients with normal GFR, an average fractional proximal sodium reabsorption of 90.1% and a GFR of 114 mL \( \text{min}^{-1} \cdot 1.73 \text{m}^2 \) mean an average proximal sodium reabsorption of 114 \( \times \) 1.44 \( \times \) 140 \( \times \) 0.901 = 20707 mmol \( \text{day}^{-1} \); in HF patients this is 143 \( \times \) 1.44 \( \times \) 140 \( \times \) 0.915 = 26378 mmol \( \text{day}^{-1} \). This reflects an increase of 27% in proximal sodium handling. Fractional distal sodium reabsorption (corrected for total tubular load...
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Figure 3.3: Correlation between fractional proximal sodium reabsorption and glomerular filtration rate

and for distal load) was not different. There were no significant differences in distal tubular load of sodium \((V \times P_{Na^+})\) in both groups, reflecting macula densa sodium exposure \((MD[NaCl])\). There was a significant and fairly high correlation between fractional proximal sodium reabsorption and GFR in the diabetes patients \((r^2=0.29, p<0.01)\) (figure 3.3).

Plasma volume, plasma ANP and c-GMP levels and urinary c-GMP excretion

Mean plasma volume was not significantly different between both groups. Plasma volume was 2933±623 mL \((2755±430 \text{ mL}\cdot\text{1.73m}^{-2})\) in NF and 3026±562 mL \((2923±360 \text{ mL}\cdot\text{1.73m}^{-2})\) in HF. Plasma ANP levels were similar in both groups (table 3.2). We found no significant correlation between plasma ANP levels and GFR or plasma volume in our study group (data not shown). Plasma c-GMP was not significantly different between groups. Also, urinary concentration of c-GMP as well as fractional excretion of c-GMP did not differ significantly. There were no significant correlations between plasma c-GMP and plasma volume or urinary c-GMP and GFR (data not shown).

Discussion

Our study indicates that glomerular hyperfiltration in normoalbuminuric type 1 diabetes mellitus is related to an increase in fractional proximal sodium reabsorption. A primary “glomerular” event resulting in an increase in GFR should be expected to lower fractional proximal sodium reabsorption due to imperfect glomerulotubular balance. As such an increase in fractional reabsorption cannot
be the consequence of a primary glomerular effect leading to glomerular hyperfiltration. Since hyperfiltration was found in the absence of extracellular volume expansion, the present findings are consistent with a primary increase in tubular reabsorption upstream to the macula densa increasing GFR via tubulo-glomerular feedback. Therefore, our data are in support of the so-called “tubular hypothesis of diabetic hyperfiltration. Normally GFR is maintained by primary glomerular/vascular events, tubulo-glomerular feedback and the glomerulotubular balance. Primary glomerular/vascular effects on GFR cause a change in tubular reabsorption through the physiological action of the glomerulotubular balance. When glomerulotubular balance functions normally, fractional reabsorption declines as GFR increases [93]. In other words, a primary increase in GFR should not increase fractional tubular sodium reabsorption as shown in our study. As such, a primary tubular event should be expected to be involved in diabetic hyperfiltration. Using lithium clearance techniques an increased proximal sodium reabsorption has been noted before in type 1 diabetes patients [104–106]. Theoretically, this increase in proximal sodium reabsorption could cause volume expansion resulting in glomerular hyperfiltration. However, an increase in extracellular fluid volume is not a uniform finding in patients with diabetes. In our study extracellular volume, assessed by measurements of plasma volume and ANP levels (and c-GMP), did not differ between NF and HF patients. Our data are in seemingly contrast with studies that have reported increased levels of ANP in diabetes patients as a reflection of increased extracellular volume [107–109]. However, in most of these studies diabetes patients were studied either during a hyperinsulinemic euglycemic clamp or without metabolic control. The expanded extracellular volume, as a result of the hyperinsulinemia or hyperglycemia could be an explanation for these raised ANP levels [90; 110]. Indeed, in diabetes patients studied under conditions similar to ours normal ANP levels were reported [111]. In recent years, it is hypothesized that the increase in proximal tubular sodium reabsorption could be the primary event in a cascade leading to glomerular hyperfiltration involving macula densa mechanisms (tubular hypothesis) [93; 94]. The increased proximal sodium reabsorption will decrease salt delivery to the macula densa (MD[NaCl]). Via tubulo-glomerular feedback this will increase GFR (hyperfiltration) thus restoring distal salt delivery. The primary increase in proximal sodium reabsorption in diabetes has been linked to tubular hypertrophy [112] and/or an increase in sodium-glucose cotransporters [111]. Indeed, sodium-glucose cotransporters were up-regulated in diabetic rat renal cortex [113]. In our study fractional proximal sodium reabsorption was increased in HF patients. Distal sodium was not different between the groups. Our findings thus are compatible with the abovementioned concept. We showed that in our study fractional proximal sodium reabsorption was indeed well correlated with GFR, although we are aware of the fact that it does not prove causality. The changes in proximal tubular salt reabsorption in diabetes patients not only explain diabetic hyperfiltration but also the so-called “paradoxical effect of dietary salt intake on GFR in diabetes [114]. Salt balance is normally maintained by changes of sodium reabsorption
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downstream of the macula densa under the control of aldosterone. In diabetes the proximal tubule is more sensitive to changes in dietary salt. As a consequence tubulo-glomerular feedback signal is strongly influenced by dietary salt such that consuming more salt will lead to an augmented activation of tubulo-glomerular feedback (and a decrease in GFR) and vice versa. Few studies indeed found such an effect of low salt intake and a concomitant increase in GFR in human diabetes [115; 116]. We could not confirm these data by plotting 24 h urinary sodium excretion (as a measure of dietary sodium intake) to GFR in the diabetes patients. However we did not put the study subjects on a low and high sodium diet, which was properly done in the aforementioned studies. Finally it can be appreciated that inhibition of proximal sodium reabsorption by acetazolamide lowers GFR. This effect is even more prominent in diabetes patients emphasizing the increased proximal sodium reabsorption in these patients [117; 118].

We conclude that in normoalbuminuric type 1 diabetes glomerular hyperfiltration seems to be related to a primary increase in proximal tubular sodium reabsorption. Since in our patients there was no evidence of expansion of plasma volume and/or an increase in ANP, changes in tubular sodium handling most probably influence tubulo-glomerular feedback.
Chapter 4

Reduced plasma total homocysteine concentrations in type 1 diabetes mellitus is determined by increased renal clearance


B.A.J. Veldman
G. Vervoort
H. Blom
P. Smits
CHAPTER 4

Abstract

Introduction  Elevated plasma levels of total homocysteine (tHcy) are related to the development of vascular complications. Patients with diabetes mellitus are particularly at risk for the development of these complications. Several factors determine tHcy including renal function.

Aims  As early type 1 diabetes is characterised by a relative glomerular hyperfiltration, increased renal clearance could contribute to decreased levels of homocysteine as observed in type 1 diabetes mellitus. Therefore we investigated the relationship between tHcy and the glomerular filtration rate (GFR).

Methods  In 92 type 1 diabetes patients and 44 control subjects we measured GFR and effective renal plasma flow (ERPF) by means of continuous infusion of inulin and p-aminohippurate. Fasting tHcy was measured using high performance liquid chromatography.

Results  GFR (121±21 resp. 104±14 mL·min$^{-1}$; $p<0.001$) and ERPF (563±127 resp. 516±121 mL·min$^{-1}$; $p=0.05$) were significantly higher in type 1 diabetes patients as compared with control subjects. tHcy was reduced in type 1 diabetes patients as compared with control subjects (11.0±4.5 resp. 13.4±7 µmol·L$^{-1}$; $p=0.01$). tHcy was strongly correlated with GFR (type 1 diabetes patients: $R= -0.43$, $p<0.001$; control subjects: $R= -0.39$, $p=0.01$).

Conclusion  GFR is a major determinant of tHcy levels in type 1 diabetes patients as well as control subjects. The reduced tHcy levels in diabetes patients can be explained by an increased GFR.
Reduced homocysteine in type 1 diabetes mellitus

Introduction

Epidemiological, experimental and clinical studies show that elevated plasma levels of the amino acid homocysteine relate to vascular complications. Several factors determine plasma total homocysteine (tHcy) including plasma levels of vitamin B\textsubscript{12}, vitamin B\textsubscript{6} and folic acid, mutations of genes involved in homocysteine metabolism (methylene-tetrahydrofolate reductase, cystathionine β-synthetase) and renal function. The importance of renal clearance of homocysteine is most clearly shown in patients with end-stage renal disease as virtually all of these patients show strongly increased tHcy levels [119–121]. These patients have a high risk for macrovascular disease. Patients with diabetes mellitus are particularly at risk for the development of macrovascular disease. However, there are some reports of reduced tHcy in type 1 diabetes mellitus [122; 123]. As glomerular hyperfiltration is highly prevalent in type 1 diabetes, increased renal clearance could contribute to decreased levels of homocysteine as observed in type 1 diabetes mellitus. Wollesen et al. [122] studied the relationship between renal clearance and tHcy in a mixed type 1 and type 2 diabetes population. They showed a significant correlation between tHcy and the glomerular filtration rate (GFR). In their study patients with microvascular complications were included as shown by the presence of microalbuminuria. Besides, many patients had hypertension. Therefore we studied the relationship between tHcy and GFR using the continuous infusion of inulin and p-aminohippurate clearance method, both gold standards to measure GFR and ERPF, in type 1 diabetes patients without renal and macrovascular complications and control subjects. We hypothesised that renal hyperfiltration is a major determinant of tHcy. To test this hypothesis we performed renal function tests and measured fasting tHcy levels in 92 type 1 diabetes patients. We also determined the GFR, effective renal plasma flow (ERPF) and tHcy in 44 control subjects to unravel whether diabetes per se or the suspected increase in GFR is responsible for the expected decrease in tHcy.

Methods

Ninety two type 1 diabetes patients and 44 control subjects participated in this study. The type 1 diabetes patients take part in a prospective study of putative prognostic factors for the development of diabetic nephropathy in which they will be prospectively followed for up to 15 years. This study describes baseline data of this study. For type 1 diabetes patients inclusion criteria were: Presence of type 1 diabetes mellitus according to the criteria of the World Health Organization 1985: presence of ketosis, low body mass index (BMI) and need for insulin therapy, age between 18 and 40 years, duration of diabetes between 5 and 12 years, normoalbuminuria (albumin excretion rate < 20 µg·min\textsuperscript{−1}) and normotension (RR < 140/90 mmHg). Presence of background retinopathy was accepted. For the type 1 diabetes patients as well as the 44 control subjects exclusion criteria were: macrovascular disease, renal failure (serum creatinine > 150
or liver function abnormalities. The study protocol was approved by the local medical ethics committee (University Medical Centre Nijmegen St. Radboud, Commissie Mensgebonden Onderzoek) and all participants gave informed consent. Prior to the renal clearance study, plasma creatinine was measured and urine was collected for 24 h to estimate GFR. Patients adhered to their usual diet in the week before the study. Consumption of caffeine containing products was not permitted in the 24 h prior to the study. All participants were studied in the morning after a light breakfast. They took a water load of 20 mL·kg⁻¹ and two intravenous catheters were inserted; one for constant infusion of glucose 2.5% / NaCl 0.45% (250 mL·h⁻¹) and blood sampling, the other for the constant infusion of p-aminohippurate and inulin adjusted for estimated GFR. After baseline blood sampling, a priming dose of inulin/p-aminohippurate was given, adjusted for weight. Thereafter, the continuous infusion of inulin/p-aminohippurate was started. At 30 minutes intervals blood samples and urine samples were collected. During the clearance study diuresis was kept above 10 mL·min⁻¹ and urinary losses over 250 mL·h⁻¹ were replaced by tap water. Glucose was measured regularly and glucose levels were kept between 5 and 10 mmol·L⁻¹ by administering glucose or insulin when appropriate. Two days after the renal function tests fasting blood samples were taken for tHcy concentration measurement. Urine and blood samples were analysed for creatinine, sodium, potassium, chloride, osmolality, inulin and p-aminohippurate using standard laboratory procedures. tHcy was measured by a high performance liquid chromatography method as described by Fiskerstrand et al. [124] with some modifications. For assessment of urinary albumin an in-house enzyme-linked immunosorbent assay was used. GFR was calculated by inulin clearance. ERPF was calculated by p-aminohippurate clearance. Inulin was purchased from Laevosan, Linz, Austria (Inutest) and p-aminohippurate from MSD, West Point, PA, USA (Amino Hippurate Sodium).

Statistics  For comparison, GFR and ERPF are expressed per 1.73 m² body surface area (BSA). Correlations between tHcy and the GFR was calculated using Spearman’s correlation. Differences between groups were calculated with Student’s t-test when appropriate, otherwise Mann-Whitney-U-test was used. Differences in gender and smoking habits between groups were calculated using the χ²-test. To test whether differences in tHcy are a consequence of the diabetic state or are solely determined by differences in GFR, stepwise multiple regression analysis was performed with sex, albumin excretion rate, HbA₁c, GFR and diabetic state as independent variables and homocysteine as the dependent variable. A second multiple regression analysis was performed that included ERPF instead of GFR.

Results  Characteristics of patients and controls are shown in table 4.1. There was no significant difference between type 1 diabetes patients and control subjects con-
Reduced homocysteine in type 1 diabetes mellitus

Table 4.1: Subject characteristics of diabetes patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Diabetes Patients (n=92)</th>
<th>Controls (n=44)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>29.1±6.6</td>
<td>28.5±6.2</td>
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<tr>
<td>Male (%) §</td>
<td>59</td>
<td>52</td>
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<tr>
<td>Smokers (%)</td>
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<td>Body mass index (kg·m$^{-2}$)</td>
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<tr>
<td>Duration of diabetes (years)</td>
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<td>Systolic blood pressure (mmHg)</td>
<td>116.3±9.9</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>65.3±10.8</td>
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<tr>
<td>HbA$_{1c}$ (%) ¶</td>
<td>8.2±1.3</td>
<td>4.9±0.4</td>
</tr>
<tr>
<td>Albumin excretion rate (µg·min$^{-1}$) ¶</td>
<td>10.1±5.1</td>
<td>7.3±3.5</td>
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<tr>
<td>Creatinine (µmol·L$^{-1}$)</td>
<td>75±10</td>
<td>76±8</td>
</tr>
<tr>
<td>GFR (mL·min$^{-1}$·1.73m$^{-2}$) ¶</td>
<td>121±21</td>
<td>104±14</td>
</tr>
<tr>
<td>ERPF (mL·min$^{-1}$·1.73m$^{-2}$) *</td>
<td>563±127</td>
<td>516±121</td>
</tr>
<tr>
<td>Plasma total homocysteine (µmol·L$^{-1}$) †</td>
<td>11.0±4.5</td>
<td>13.7±7</td>
</tr>
</tbody>
</table>

Significant difference between type 1 diabetes patients and control subjects: * $p=0.05$; † $p=0.01$; ¶ $p<0.001$; § $p=0.6$

Concerning sex, age, BMI, BSA, plasma creatinine, blood pressure and percentage of smokers, tHcy was significantly lower in type 1 diabetes patients as compared with control subjects (11.0±4.5 vs 13.4±7 µmol·L$^{-1}$; $p=0.01$; figure 4.1).

Patients with diabetes had a higher GFR and ERPF as compared with controls (121±21 vs 104±14 mL·min$^{-1}$·1.73m$^{-2}$; $p<0.001$ and 563±127 vs 516±121 mL·min$^{-1}$·1.73m$^{-2}$ $p=0.05$). Forty-two percent of type 1 diabetes patients had a GFR in the hyperfiltrating range, when upper 95 percentile of GFR in the control group was taken for reference [125]. In 5 percent of type 1 diabetes patients the GFR was above 150 mL·min$^{-1}$·1.73m$^{-2}$ [126]. Although all subjects were normoalbuminuric there was a significant difference in albumin excretion rate between type 1 diabetes patients and control subjects (10.1±5.1 vs 7.3±3.5 µmol·min$^{-1}$ respectively, $p<0.001$). As expected HbA$_{1c}$ was higher in type 1 diabetes patients compared to controls (8.2±1.3 vs 4.9±0.4%, $p<0.001$).

As shown in figure 4.2, both in type 1 diabetes patients and in control subjects there was a negative significant correlation between tHcy and GFR (type 1 diabetes patients: $R=-0.43$, $p<0.001$; control subjects: $R=-0.39$, $p=0.01$). When excluding all type 1 diabetes patients and controls with tHcy above 30 µmol·L$^{-1}$ (n=2 and n=0 respectively), tHcy remained significantly different between type 1 diabetes patients and control subjects (10.5±2.8 vs 13.4±7 µmol·L$^{-1}$; $p=0.01$). Also the significant correlation between GFR and tHcy remained virtually un-
Figure 4.1: The distribution of plasma total homocysteine for both populations

changed (type 1 diabetes patients: $R = -0.43$, $p = 0.005$; control subjects: $R = -0.39$, $p = 0.01$). GFR and ERPF were not significantly correlated with HbA$_1c$ levels, age or the duration of diabetes. In type 1 diabetes patients, GFR was significantly correlated with albumin excretion rate ($R = 0.29$, $p = 0.01$). Stepwise multiple regression analysis (table 4.2) revealed significant independent effects of sex, GFR and age on tHcy. The presence of the diabetic state was not a predictor of tHcy.

Discussion

In our large study population of patients with type 1 diabetes and healthy controls we were able to study the relationship between tHcy and GFR. We found lower plasma tHcy levels in patients with type 1 diabetes as compared with controls. In the patients with diabetes as well as the control subjects, tHcy was significantly correlated with GFR. The lower tHcy in patients with type 1 diabetes can be explained by the hyperfiltration that is often seen in these patients. Our data extent the results of previous reports that homocysteine and GFR are inversely correlated [122]). Most reports thus far are from studies in patients with end stage renal disease or subjects with a GFR within the normal range. This study is the first to report a significant correlation in patients with type 1 diabetes, including patients with relative glomerular hyperfiltration. Glomerular hyperfiltration is defined by a GFR above the upper 90 or 95 percentile of non-diabetes volunteers [125] or GFR above 150 $mL\cdot min^{-1} \cdot 1.73m^2$ [126]. The cause of increased glomerular filtration in type 1 diabetes mellitus remains unclear. There is
Figure 4.2: Scatterplots of fasting plasma total homocysteine and the glomerular filtration rate (mL·min⁻¹·1.73m²⁻¹)
Table 4.2: Stepwise multiple regression models. Effect of selected variables on plasma total homocysteine. In the left column, glomerular filtration rate is included in the model. In the right column, the glomerular filtration rate is replaced by the effective renal plasma flow.

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<td>Glomerular filtration rate</td>
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The mechanism by which increased GFR induces reduced tHcy levels is unknown. Hyperhomocysteinemia is a common finding in patients with renal insufficiency indicating the importance of the kidney in regulating tHcy levels. About seventy to eighty percent of tHcy is bound to proteins of high molecular weight. The remaining 20 to 30% non-protein bound homocysteine consists of homocysteine, the disulfide of homocysteine, and mixed disulfides of homocysteine with other aminoacids like cysteine. About 1% of homocysteine is unbound [133]. The portion of homocysteine which is bound to small peptides and amino acids and also unbound homocysteine, is subject to glomerular filtration. This fraction is freely filtrated and almost completely reabsorbed in the proximal tubules [134] since loss in the urine is negligible. Besides, inhibitors of tubular resorption like lysine and arginine seem to inhibit this resorption [135]. After tubular resorption, this fraction is metabolized into cystathionine or methionine. Studies in rats by Bostom et al. [136] demonstrated renal extraction of homocysteine by the kidney. In rats they found a 20% decrease between arterial homocysteine levels and renal vein homocysteine levels. They also found that urinary excretion of homocysteine was minimal. One human study by Van Gulderen et al. [137] in which renal artery and vein sampling was performed, was not able to show any net homocysteine...
Reduced homocysteine in type 1 diabetes mellitus

extraction by the kidney. The difference between both studies can probably be explained by higher free plasma homocysteine levels in rats compared to humans (70 vs 20%). A small renal extraction of homocysteine is hard to detect, as tHcy determination has a coefficient of variation of about 5%. In contrast to the study of Van Gulderen et al., our data support the hypothesis that the kidney is an important organ for elimination of homocysteine.

In summary, the strong correlation between tHcy and the GFR in patients with type 1 diabetes as well as in healthy controls suggests that renal filtration is a major determinant of tHcy. In patients with type 1 diabetes tHcy is reduced as compared with controls. Relative renal hyperfiltration, which is highly prevalent in patients with type 1 diabetes mellitus is responsible for decreased tHcy as compared with healthy control subjects.

Acknowledgement  H.J. Blom is established investigator of the Netherlands Heart Foundation (D 97.021)
Chapter 5

Atrial natriuretic peptide and albuminuria in diabetic patients

Eur J Clin Invest. 2003;33:1026-1027
(Letter)

B.A.J. Veldman
G. Vervoort
J.F.M. Wetzels
Sir,

In a previous study published in the *European Journal of Clinical Investigation* [2] we reported that atrial natriuretic peptide (ANP) increased albuminuria in patients with type 1 diabetes mellitus. However, the rise in albuminuria was accompanied by an increase in the urinary excretion of the low molecular weight protein β₂-microglobulin (β₂-m). These observations suggested that the albuminuria may at least partly be the result of blockade of tubular protein reabsorption. However, in our experiments ANP was dissolved in the polygeline Haemaccel® to prevent adherence of ANP to the synthetic infusion system. We recently became aware of anecdotal reports that suggested that high dosages of polygelines might inhibit tubular protein reabsorption [138]. We therefore have questioned our earlier conclusion. We have studied the effect of ANP dissolved in saline instead of Haemaccel® on urinary excretion of albumin and β₂-m in two patients with type 1 diabetes mellitus and a baseline albuminuria of 556 and 382 µg·min⁻¹ respectively. After an equilibration period of 70 minutes, two freely voided urine samples were collected at 20 minutes intervals. Subsequently, a bolus of 0.06 µg·kg⁻¹ ANP was given, followed by a continuous infusion of 0.012 µg·kg⁻¹·min⁻¹ ANP. During ANP infusion three urine samples were collected at 20 minutes intervals. Blood and urine samples were taken for measurement of creatinine clearance and urinary excretion of albumin and β₂-m. Results are presented in figure 5.1.

Infusion of ANP in saline increased urinary albumin excretion to a similar extent as ANP in Haemaccel® in our previous study. However, it is evident that ANP in saline had hardly any effect on urinary β₂-m excretion. We have further confirmed these observation by studying the effects of ANP dissolved in saline or haemaccel® on urinary albumin and β₂-m excretion in healthy volunteers (see chapter 6, [139]). These data also demonstrate that infusion of ANP does not increase urinary excretion of β₂-m. Therefore, the present study indicates that increased albuminuria caused by infusion of ANP cannot be explained by interference of ANP with tubular protein reabsorption. The conclusions of our previous paper are incorrect. Low doses of polygelines should be avoided in studies of renal physiology.
Figure 5.1: Urinary excretion of albumin (top) and β_2_-microglobulin (bottom) at baseline and after infusion of atrial natriuretic peptide (ANP). The first pair of bars represent data from the previous study [2] (ANP in Haemaccel®). The second and third pairs of bars represent the individual microalbuminuric diabetic patients (ANP in saline).
Chapter 6

Low concentrations of intravenous polygelines promote low molecular weight proteinuria

Eur J Clin Invest. 2003;33:962-968

B.A.J. Veldman
H.L.E. Schepkens
G. Vervoort
I. Klasen
J.F.M. Wetzels
Abstract

Background Previously we observed that atrial natriuretic peptide (ANP) induced albuminuria was accompanied by an increase in urinary excretion of the low molecular weight protein (LMW-protein) $\beta_2$-microglobulin ($\beta_2$-m), suggesting that the albuminuria may at least partly be the result of blockade of tubular protein reabsorption. However, in our experiments ANP was dissolved in the polygeline Haemaccel® to prevent adhesion of ANP to the infusion system. Anecdotal reports have shown that high dosages of polygelines such as Haemaccel® or Gelofusine® may influence tubular protein handling. In the present study we have evaluated the effect of a low and high dose of the polygeline Haemaccel® on proteinuria. In addition, we have reassessed the effects of ANP.

Materials and Methods We have measured urinary $\beta_2$-m and albumin excretion in healthy volunteers after infusion of a high dose pure Haemaccel® (0.04 $mL\cdot kg^{-1}\cdot min^{-1}$ for 60 minutes), a low dose Haemaccel® (0.01 $mL\cdot kg^{-1}\cdot min^{-1}$ for 60 minutes followed by infusion of 0.02 $mL\cdot kg^{-1}\cdot min^{-1}$ for 60 minutes) and a low dose Gelofusine® (dose comparable to the low dose Haemaccel®). In addition we have performed similar studies using ANP dissolved in saline and Haemaccel®.

Results Infusion of Haemaccel® caused a dose dependent increase in urinary excretion of $\beta_2$-m. There were no differences between Haemaccel® or Gelofusine®. After infusion of ANP dissolved in Haemaccel® urinary $\beta_2$-m excretion increased from 0.05±0.03 $\mu g\cdot min^{-1}$ to 27±10 $\mu g\cdot min^{-1}$ and urinary albumin excretion increased from 4.5±1.1 $\mu g\cdot min^{-1}$ to 9.7±6.3 $\mu g\cdot min^{-1}$ ($p<0.05$). During ANP+saline infusion, urinary $\beta_2$-m excretion did not change, whereas the urinary albumin excretion increased from 5.3±1.5 $\mu g\cdot min^{-1}$ to 7.9±2.4 $\mu g\cdot min^{-1}$ ($p<0.05$).

Conclusions Our study demonstrates that even low doses of the polygelines Haemaccel® or Gelofusine® profoundly attenuate the tubular reabsorption of the LMW $\beta_2$-m. ANP does not affect tubular protein reabsorption. Therefore, the rise in albuminuria during ANP infusion most likely reflects alterations in glomerular permeability.
Polygelines promote LMW-proteinuria

Introduction

Atrial natriuretic peptide (ANP) is a potent natriuretic, diuretic and vasodilating hormone which is predominantly synthetized in mammalian atria [140]. Infusion of ANP provokes albuminuria in healthy subjects, and this albuminuric response is even more prominent in patients with hypertension, diabetes or with non-diabetic renal disease and a nephrotic syndrome [141–144]. Initially, it was thought that this increase in albuminuria could completely be attributed to ANP-induced glomerular changes. These changes include an increase in capillary ultrafiltration coefficient and a characteristic rise in filtration fraction and intraglomerular pressure, the latter reflecting the preferential vasodilation of the afferent arteriole and (relative) vasoconstriction of the efferent arteriole [92; 145; 146].

We recently have suggested that blockade of tubular protein reabsorption may be an important contributing mechanism since we observed a parallel and marked increase in urinary excretion of the low molecular weight (LMW) protein β2-microglobulin (β2-m) during ANP infusion [2]. However, in our experiments ANP was dissolved in the polygeline Haemaccel® to prevent adhesion of ANP to the infusion system. We recently have confirmed the data of anecdotal reports which showed that larger amounts of polygelines may influence renal tubular handling of low molecular weight proteins [138; 147–149]. In our study we have used the polygeline Gelofusine® [138]. In the present study we have evaluated the effects of a high dose and a low dose of Haemaccel® on proteinuria. Subsequently we have compared the effects of two polygelines i.e. Haemaccel® and Gelofusine®. Finally, we have re-evaluated the effects of ANP on the tubular protein handling by comparing infusion of ANP either dissolved in saline or in Haemaccel®.

Patients and Methods

Subjects

All studies were performed in healthy volunteers recruited from the local population. Before inclusion in the study, these subjects were screened for the absence of hypertension, cardiovascular disease, renal disease and microalbuminuria. None of them used regular medication, except for the use of oral contraceptives in some female participants. The use of these oral contraceptives did not influence any of the parameters measured. The six subjects who participated in the ANP-studies were measured twice on separate occasions at least one week apart. In random order they received infusion of ANP in saline on one day and of ANP in Haemaccel® on an other. The characteristics of the participants in the separate protocols are shown in table 6.1. The experimental protocols were approved by the ethics review committee of the University Medical Centre St. Radboud and written informed consent was obtained from all participants.

Study protocols

The evening before the study, the participants ingested 4000 mg sodium bicarbonate to alkalinise (pH > 6) the urine in order to avoid degradation of urinary β2-m. They adhered to their usual diet in the days before this.
study but were instructed to abstain from alcohol, caffeine and nicotine for at least 12 h before the experiments. All participants were admitted to the clinical research centre at 8.00 a.m after they had eaten a light breakfast. Subjects did not ingest any other food until the study was completed. After arrival, they consumed an oral water load of 20 mL kg\(^{-1}\) and two plastic cannulas were inserted into an antecubital vein of each arm, one infusion and the other for blood sampling. In all studies basal infusion consisted of glucose/NaCl at a rate of 250 mL h\(^{-1}\). In order to achieve a steady-state urinary output, urinary losses (over 250 mL h\(^{-1}\)) were replaced by drinking tap water. All subjects remained supine throughout the study but were allowed to stand to urinate. After an equilibration period of 70 min, in which the urine output reached values of 10 mL min\(^{-1}\) or more, three freely voided urine samples were collected at carefully timed intervals. The average of the two last collections served as baseline. Blood samples were taken before and after each urine collection period. After obtaining baseline values, the further protocol was different between the studies. To confirm our earlier results of high dose Gelofusine\(^\circledR\) induced low molecular weight (LMW) proteinuria, we administered a high dose of Haemaccel\(^\circledR\) (Hoechst, Behring-Werke, Marburg Germany, concentration 35 g L\(^{-1}\)) intravenously (bolus: 0.1 mL kg\(^{-1}\) followed by infusion of 0.04 mL kg\(^{-1}\) min\(^{-1}\) for 60 min). Urine was collected at 20 min intervals and blood samples were again taken before and after each collection period. In a subsequent study, we investigated whether polygeline induced LMW-proteinuria was dose dependent, by infusion of a lower dose of pure Haemaccel\(^\circledR\). After a bolus injection of 0.05 mL kg\(^{-1}\), Haemaccel\(^\circledR\) was infused for 60 min at a rate of 0.01 mL kg\(^{-1}\) min\(^{-1}\), thus providing a total amount of approximately 1 g of Haemaccel\(^\circledR\) for an average 70 kg subject. Thereafter, a second bolus injection of 0.1 mL kg\(^{-1}\) was given and the infusion was continued at a rate of 0.02 mL kg\(^{-1}\) min\(^{-1}\) for 60 min. Urine was collected at 30 min intervals and blood samples were again taken before and after each collection period. To investigate whether the effect on the excretion of the LMW-protein

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</table>

Values are expressed as means±SD.

Table 6.1: Baseline characteristics of study participants. BSA, body surface area; BMI, body mass index [weight/(height\(^2\)].
Polygelines promote LMW-proteinuria

$\beta_2\text{-m}$ was polygeline specific, we administered Gelofusine® (Braun NPBI Oss, the Netherlands, concentration 40 g·L$^{-1}$), another polygeline, at a rate of 0.01 m$L\cdot kg^{-1}\cdot min^{-1}$, after a bolus injection of 0.05 m$L\cdot kg^{-1}$. Thereafter, a second bolus injection of 0.1 m$L\cdot kg^{-1}$ was given and the infusion was continued at a rate of 0.02 m$L\cdot kg^{-1}\cdot min^{-1}$ for 60 min. Urine was collected at 30 min intervals and blood samples were again taken before and after each collection period. In the ANP-study, ANP infusion followed the baseline measurements. ANP was dissolved in saline or in Haemaccel®. Taking into account an estimated loss of 20% of ANP when dissolved in saline, we adjusted the concentration of the solutions so that the effective ANP dose was equal in both experiments. The final concentration of ANP in Haemaccel® and in saline was 1 µg·m$L^{-1}$ and 1.2 µg·m$L^{-1}$ respectively. After a bolus of 0.05 m$L\cdot kg^{-1}\cdot min^{-1}$, ANP was infused at a rate of 0.01 m$L\cdot kg^{-1}\cdot min^{-1}$ for 60 min. Urine was collected at 20 min intervals and blood samples were again taken before and after each collection period. In all studies creatinine clearance was used as a marker of GFR. To allow formal comparison with our earlier study using ANP [2] in the ANP protocols we also have measured inulin and PAH clearance as markers of GFR and ERPF respectively. During all four studies blood pressure was measured at 10 min intervals during each collection period with an automatic oscillometric device (Dinamap, 1846 SX, Critikon, Tampa, Florida, USA). Urine and blood samples were analyzed for creatinine, sodium, potassium, osmolality, inulin and PAH using standard laboratory procedures. Urine samples for the determination of albumin and $\beta_2\text{-m}$ were stored at -80°C. Urinary excretion of albumin and $\beta_2\text{-m}$ were determined with an ELISA as previously described [2]. ANP-(99-126) was purchased from Clinalfa, Lauflingen, Switzerland. Inulin was purchased from Laevosan, Linz, Austria (Inutest®) and PAH from MSD, West Point, PA, USA (Amino Hippurate Sodium). In the ANP-studies, plasma concentration of ANP in blood was determined just before the start of ANP infusion (baseline) and at the end of the clearance study, and was measured by a radioimmunoassay as previously described [100].

**Calculations** Urinary clearance of a substance ($C_x$) was calculated with the standard formula:

$$U_x \times \frac{V}{P}$$

where $U =$ urinary concentration of substance $x$, $V =$ urinary flow rate and $P =$ plasma concentration. The clearances of inulin (ANP studies) and creatinine (polygeline studies) were used as markers of the glomerular filtration rate (GFR). The clearance of PAH was used as a marker of the effective renal plasma flow (ERPF) (ANP studies). The filtration fraction was calculated as GFR/ERPF. Fractional excretions were calculated as clearance of a substance divided by GFR.

**Statistical analysis** Parameters before and after the infusion of ANP were analysed by paired Student’s $t$-test for normally distributed data or Wilcoxon’s signed rank
test for non-parametric data. A $p$-value $< 0.05$ was considered to be the level of statistical significance. Unless otherwise indicated, values are expressed as means±SD.

Results

Effects of polygeline infusion The infusions were carried out without complications and data collection was complete. Infusion of Haemaccel® or Gelofusine® did not affect blood pressure, haematocrit, ECC, urinary water and sodium excretion (data not shown). The effects of Haemaccel® and Gelofusine® on the urinary excretion of albumin and $\beta_2$-m are presented in figure 6.1. During the infusion of the polygelines, a rapid and significant increase ($p$<0.001) in absolute and fractional $\beta_2$-microglobulin excretion was observed, whereas the excretion rate of albumin remained unaltered. Infusion of the higher dose of Haemaccel® resulted in a significantly higher urinary excretion of $\beta_2$-m than observed with the lower doses. We did not observe any differences between Gelofusine® or Haemaccel®.

Effects of ANP+saline and of ANP+Haemaccel® infusion No symptoms were experienced by the volunteers and the infusion of ANP had no significant influence on blood pressure or pulse rate. Plasma ANP levels rose significantly ($p$<0.001) and to the same extent during the infusion of either ANP solution (ANP+saline: 9.4±1.2 nmol·L$^{-1}$ to 47.1±11.6 nmol·L$^{-1}$; ANP+Haemaccel®: 7.6±2.0 nmol·L$^{-1}$ to 55.6±25.0 nmol·L$^{-1}$; both: $p$<0.001). The effects of ANP infusion on renal haemodynamics are shown in figure 6.2.

With both ANP solutions, the ERPF (Panel A) tended to decrease whereas the GFR (Panel B) tended to increase, although the changes did not reach statistical significance. As a result, the filtration fraction increased from 0.20±0.03 to 0.24±0.02 during ANP+saline ($p$<0.001) and from 0.19±0.01 to 0.23±0.02 during ANP+Haemaccel infusion ($p$<0.001). The infusion of ANP did not significantly alter the urinary flow rate (data not shown). Fractional sodium excretion increased from 1.36±0.56% to 2.36±0.64% during ANP+saline, and from 1.82±0.83% to 2.50±0.48% during ANP+Haemaccel®. Both studies were comparable with respect to the absolute and percentage changes of ERPF, GFR, filtration fraction, fractional sodium excretion and urinary flow rate. Major differences were observed with respect to the excretion of urinary proteins (figure 6.3).

During infusion of ANP+Haemaccel®, the urinary $\beta_2$-m excretion (Panel A) increased significantly from 0.05±0.03 µg·min$^{-1}$ to 27.1±10.4 µg·min$^{-1}$ ($p$<0.001) and urinary albumin excretion (Panel B) increased from 4.5±1.1 µg·min$^{-1}$ to 9.7±6.3 µg·min$^{-1}$ ($p$<0.05). During ANP+saline infusion, the urinary $\beta_2$-m excretion did not change significantly, whereas the urinary albumin excretion increased from 5.3±1.5 to 7.9±2.4 µg·min$^{-1}$ ($p$<0.05).
Figure 6.1: Effects of infusion of high dose Haemaccel® and low dose Haemaccel® and Gelofusine® on urinary β2-microglobulin excretion (upper panel) and albumin (lower panel). Values are given as means±SD. **p<0.001 compared with basal values.
Figure 6.2: Renal haemodynamics before (baseline) and after the infusion of atrial natriuretic peptide (ANP) in saline (○) and in Haemaccel® (■). (a) Effective renal plasma flow (ERPF). (b) Glomerular filtration rate (GFR). (c) Filtration fraction (FF). (d) Fractional excretion of sodium ($F_{\text{ENa}}$). Values are given as means±SD. The changes in ERPF, GFR and FF were similar during both infusions. Filtration fraction increased significantly during either infusion ("" $p<0.001$; " $p<0.01$).
Polygelines promote LMW-proteinuria

Figure 6.3: Effects of infusion of atrial natriuretic peptide (ANP) in Haemaccel® (■) and saline (○) on urinary excretion of (a) β2-microglobulin and (b) urinary albumin excretion. Values are given as means±SD. **p<0.001 compared with baseline values. *p<0.05 compared with baseline values.
Discussion

The results of this study clearly indicate that the infusion of a dose as low as 0.75 mL·min\(^{-1}\) (1.8 g·h\(^{-1}\)) of the polygeline Haemaccel® can substantially increase the urinary excretion of β\(_2\)-m, without affecting the renal excretion of albumin. Our data clearly show that this effect is dose dependent. Furthermore, we did not observe any difference between two preparations tested here (i.e., Haemaccel® and Gelofusine®). Taken together our study confirms and extends previous experiments which showed a profound effect of the infusion of larger amounts (10 to 17.5 g) of polygelines on the renal excretion of low molecular weight proteins [138; 147]. The increased excretion of β\(_2\)-m during Haemaccel® infusion is most likely the consequence of a competitive inhibition of tubular protein reabsorption. LMW-proteins such as β\(_2\)-m are freely filtered in the glomerulus with a sieving coefficient approximating 1.0, and effectively reabsorbed by the renal proximal tubules. Therefore, the huge increase in urinary β\(_2\)-m excretion must be explained by interference with the tubular reabsorption process. Haemaccel® is a synthetic polymer of partially degraded gelatin. Beetham et al. [147] performed a gel filtration of the preparation and demonstrated that it contains a significant proportion of degradation products of less than 35 kD molecular weight. This low molecular weight fraction rapidly undergoes glomerular filtration and appears in the urine. Even with a low dose of 1 g the concentration in the tubular fluid will be around 20-50 mg·L\(^{-1}\), much higher than the concentration of β\(_2\)-m. Therefore, Beetham et al. previously suggested that Haemaccel® inhibits tubular binding of other low molecular weight proteins simply by virtue of its higher tubular concentration. Notably, the effect is somewhat protein specific since we did not observe an increase in urinary excretion of albumin, a protein that is also reabsorbed to some extent by the proximal tubule. Polygelines like Haemaccel® and Gelofusine® are frequently used in clinical practice as plasma expanders. The inhibition of tubular reabsorption of LMW-proteins by polygelines can lead to spurious interpretation of results. LMW-proteinuria in patients who have received polygelines does not necessarily indicate tubular damage, but can be merely the result of inhibition of tubular reabsorption.

The results of our second set of experiments are also clear: ANP when dissolved in saline does not affect proximal tubular protein handling. The increased albuminuria that occurs after infusion of ANP must be the result of alterations in glomerular permeability. The increased excretion of β\(_2\)-m that we observed in our previous experiments is fully explained by the use of low concentration of Haemaccel®. Admittedly, other investigators have also reported a significant rise in urinary β\(_2\)-m excretion after ANP infusion [141; 150]. In these latter studies ANP was dissolved in saline. We feel that these apparent discrepancies in study results are most likely explained by differences in study protocol. One important issue is the effect of urinary flow rate on urinary protein excretion. This issue was addressed in a study by Jung et al. who demonstrated an increased urinary excretion rate of the low molecular weight protein β\(_2\)-m after induction
Polygelines promote LMW-proteinuria

of a firm diuresis by a fluid load of 22 $mL\cdot kg^{-1}$ body weight [151]. The authors felt supported by our finding that total protein excretion is influenced by urinary flow rate [152]. However, our results did not correspond with those of Jung et al. because we found that urinary protein is constant and independent of diuresis at urinary flow rates above 1.5 $mL\cdot min^{-1}$ [153]. We believe that the contrasting observations by Jung et al. can be explained by the fact that the investigators did not avoid excessive hydration and rapid changes in urinary volume which may have led to the washout of urinary dead space, resulting in high excretion rates of low molecular weight proteins, as has clearly been demonstrated for creatinine and for albumin [154; 155]. A similar pattern of a transient increase in the excretion of low molecular weight proteins may be involved in the studies by Semmekrot et al.[150] and by Eiskjær et al.[141]. In this context, it should be noted that in both studies very high doses (1 $\mu g\cdot kg^{-1}$ to 2 $\mu g\cdot kg^{-1}$) of ANP were administered as bolus injections, resulting in rapid changes in urinary flow rates. In addition, these high ANP dosages induced changes in systemic haemodynamics which may have interfered with tubular protein handling. The ANP dose used in our study was chosen to increase albuminuria, with minimal effects on systemic haemodynamics. Eiskjær and Pedersen [156] studied the dose-response relationship of ANP on (renal) haemodynamics. Compared to placebo, a bolus injection of 0.5 $\mu g\cdot kg^{-1}$ did not significantly alter GFR, ERPF or urinary flow rate. In a dose-dependent manner, increasing doses of ANP (up to 2 $\mu g\cdot kg^{-1}$) increased urinary sodium excretion, GFR, pulse rate and urinary flow rate reaching a plateau at the highest ANP doses. ERPF and mean arterial pressure decreased dose-dependently reaching a plateau at the highest ANP doses. In an animal study, Blaine et al. [157], reported a bell shaped dose-response relationship with regard to urinary sodium excretion in response to ANP infusion. Our cumulative ANP dose was chosen below 0.5 $\mu g\cdot kg^{-1}$. As shown the ANP dose used in our study increased albuminuria significantly. In another study by Eiskjær et al.[141], the excretion of $\beta_2$-m rose by 655% in patients with essential hypertension. It is thereby noteworthy that the abrupt changes in urinary flow after ANP injection were much more pronounced in these hypertensive patients (from 7.0 $mL\cdot min^{-1}$ to 15.3 $mL\cdot min^{-1}$) compared to the normotensive controls (from 7 $mL\cdot min^{-1}$ to 11 $mL\cdot min^{-1}$). In the latter control group $\beta_2$-m excretion increased only by 60% which is comparable to the changes in our experiments and in another study by Mc Murray et al. [142]. Nevertheless, these changes reached the level of statistical significance in the study by Eiskjær et al. whereas this was not the case in the present study and in the study by Mc Murray et al. Taken together, these observations suggest that the increased $\beta_2$-m excretion during ANP rather reflects rapid fluctuations in urinary flow rate and changes in systemic haemodynamics than alterations of proximal tubular protein reabsorption.

In conclusion, our data clearly indicate that a low dose of the polygeline Haemaccel® profoundly affects tubular reabsorption of the low molecular weight protein $\beta_2$-microglobulin. ANP when dissolved in saline increases urinary albumin excretion without affecting the urinary excretion of $\beta_2$-microglobulin. Therefore,
the rise in albuminuria during ANP infusion most likely simply reflects changes in glomerular permeability.
Chapter 7

The Glu298Asp polymorphism of the NOS3 gene as a determinant of the baseline production of nitric oxide

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P. Smits
Abstract

Background The endothelial nitric oxide synthase Glu298Asp polymorphism has been suggested to play a role in the development of hypertension, atherosclerosis and coronary artery disease.

Aim To investigate functional differences between the various genotypes with respect to basal NO production, we estimated the response to eNOS inhibition by infusion of increasing doses of L-NMMA into the brachial artery during venous occlusion plethysmography.

Methods In 41 healthy subjects forearm blood flow responses to intra-arterial infusion of increasing doses of L-NMMA (0.05, 0.1 and 0.2 mg min\(^{-1}\) dL\(^{-1}\)) and norepinephrine (10, 20 and 40 ng min\(^{-1}\) dL\(^{-1}\)) were measured. The genotype of the eNOS Glu298Asp polymorphism was assessed.

Results Nineteen subjects had the Glu/Glu genotype, 19 subjects had the Glu/Asp genotype and 3 subjects had the Asp/Asp genotype. Groups were comparable concerning demographic, haemodynamic and possible confounding factors. Subjects with the Asp allele showed a reduced response to infusion of L-NMMA as compared with subject with the Glu/Glu genotype (ANOVA, \(p=0.01\)). There was no significant difference in the response to infusion of the NO independent vasoconstrictor norepinephrine between both groups.

Conclusions The eNOS Glu298Asp polymorphism is associated with reduced basal NO production and might therefore have functional implications in the development of atherosclerosis or hypertension.
Introduction

Endothelium derived nitric oxide (NO) is an important regulator of vascular tone. Besides its potent vasodilator capacity, NO inhibits vascular smooth muscle cell migration [158] and growth [159], inhibits endothelial adhesion of leucocytes and thrombocytes [160; 161] and limits the oxidation of low-density lipoproteins [162]. Because of these properties, a reduced bioavailability of NO may play a role in the development or progression of hypertension, atherosclerosis and other vascular diseases. The importance of NO in vascular function is stressed by several studies. Knock-out mice lacking the ecNOS gene become hypertensive and inhibition of ecNOS elevates blood pressure in non-hypertensive humans [163].

As it becomes more clear that genetic factors play a role in cardiovascular disease, it is tempting to assume a role for polymorphisms of the ecNOS gene in the susceptibility to develop cardiovascular disease. NO is produced from the amino acid L-arginine by the enzyme NOS. Several iso-forms of the enzyme exist of which endothelial NO synthase (ecNOS) is expressed in the endothelium. EcNOS is encoded by the NOS 3 gene on chromosome 7. Recently, several polymorphisms of the ecNOS have been identified. Of these, the G894T nucleotide polymorphism seems clinically relevant as it leads to an amino acid substitution at the 298 location of the ecNOS gene: Glu298Asp (glutamic acid substituted by aspartic acid) [164]. Although some investigators have suggested that the Glu298Asp polymorphism is associated with an increased incidence of hypertension, functional in-vivo data on this issue are scarce [165]. We hypothesize that differences in the genotype of ecNOS may be reflected by differences in the baseline release of NO from the endothelium. To address this hypothesis, we estimated the differences in basal NO production between the different genotypes of the ecNOS Glu298Asp polymorphism in a group of healthy volunteers, by quantifying the vasoconstrictor response to ecNOS inhibition by infusion of $N^G$-monomethyl-L-arginine ($L$-NMMA) into the brachial artery.

Methods

We recruited 41 healthy subjects from the local community. All subjects fulfilled the following criteria: age between 18 and 40; blood pressure (BP) < 140/90 mmHg, no antihypertensive medication and no signs of cardiovascular disease. Since primary hypertension and atherosclerosis have been associated with an impaired vasoconstrictor response to $L$-NMMA these subjects were excluded. All subjects signed the informed consent and the study protocol was approved by the local medical ethical committee.

Subjects were instructed to abstain from alcohol and caffeine for 24 h and refrain from smoking 12 h before the study. The study was performed between 8.00-12.00 a.m. All subjects attended the clinic after an overnight fast. Fifteen minutes after cannulation of the brachial artery of the non-dominant arm, intra-arterial blood pressure was recorded for 5 min. Thirty minutes after cannulation,
arterial blood samples were collected for DNA extraction. Further, blood samples were taken for evaluation of all kind of potential confounding factors which may affect endothelial function: cholesterol, homocysteine, insulin, glucose, glycosylated haemoglobin (HbA\textsubscript{1c}), and catecholamines. Thereafter, forearm blood flow (FBF) was measured by venous occlusion strain gauge plethysmography as described before [166]. After assessment of the baseline FBF, increasing doses of the NOS inhibitor L-NMMA (0.05, 0.1 and 0.2 mg-min\textsuperscript{-1} per dL forearm volume [mg-min\textsuperscript{-1}.dL\textsuperscript{-1}]) were infused into the brachial artery. After each infusion period of five minutes for the respective doses, the effect of the infusion of L-NMMA on FBF was measured by venous occlusion plethysmography in the final two minutes. As L-NMMA blocks NO synthase, the fall in FBF can be considered as a measure of the baseline production of endothelial NO. After a 60 minute equilibration interval, FBF was returned towards baseline levels. Subsequently increasing doses of norepinephrine (10, 20, 40 ng-min\textsuperscript{-1}.dL\textsuperscript{-1}) were infused into the brachial artery. Measurements of FBF was performed as described above. Norepinephrine was used as an endothelial independent vasoconstrictor agent to rule out non-specific vascular responses in the Asp allele group.

DNA was extracted from whole blood using the QIAamp Blood Kit (Qiagen, Inc.). Genotyping of the ecNOS Glu298Asp polymorphism was performed using a multilocus genotyping assay for candidate markers of cardiovascular disease risk [167] (Roche Molecular Systems, Inc.). Briefly, each DNA sample is amplified using two multiplex polymerase chain reactions, and the alleles are genotyped simultaneously using an array of immobilised, sequence-specific oligonucleotide probes. This array of probes is blotted on plastic strips and after staining, genotypes can be scored based on blue (positive) and white (negative) bands. Each blue band, representing a specific genotype, was scored by specific software (counting the pixel intensity of each band) and manually checked. Insulin was measured with an insulin-specific double antibody RIA (interassay CV 6.2%). Plasma glucose was measured using a standard glucose oxidation method. HbA\textsubscript{1c} was measured using a HPLC technique (Bio-Rad DiaMatm, the Netherlands) with reference values of 4.8-6.2%. Plasma catecholamines were measured using a highly sensitive and specific HPLC technique [168]. Homocysteine was measured by a high performance liquid chromatography method as described by Fiskerstrand et al. [124] with some modifications. Plasma cholesterol and triglyceride concentrations were determined by commercially available enzymatic reagents. HDL-cholesterol was determined with the polyethylene glycol 6000 method [169]. Free fatty acids were measured using an enzymatic colorimetric method (ACS-ACOD method, Wako Chemicals, Neuss, Germany).

Due to expected small numbers in the Asp/Asp genotype group, this group was combined with the Glu/Asp group in the statistical analyses to form the Asp allele group (Glu/Asp and Asp/Asp). Genetic analysis was performed after the plethysmography studies, so the investigators performing these studies were blinded for the genotypes of the subjects. Results are presented as means±SD unless indicated otherwise. FBF is expressed in mL-min\textsuperscript{-1} per dL of forearm.
Figure 7.1: Mean response (±SEM) to infusion of increasing doses of L-NMMA and norepinephrine in both genotype groups (*p<0.05; **p<0.01).

tissue (mL min⁻¹ dL⁻¹). Delta FBF is calculated by subtracting the measured flow during infusion of study medication from the FBF at baseline. The percentage change in FBF (Δ%FBF) is calculated as the difference between FBF at baseline and FBF during infusion of study medication, divided by the FBF at baseline. Mann-Whitney-U test was used to test for significant differences in non-parametric variables, otherwise, Student’s t-test was used. Differences between groups in dose/response on infusion of L-NMMA and norepinephrine were tested using ANOVA for repeated measures. Differences in smoking habits and sex between the various genotypes were tested for significance using the χ² test. Differences were considered to be significant at p-values below 0.05.
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Glu/Glu Glu/Asp + Asp/Asp

<table>
<thead>
<tr>
<th>genotype</th>
<th>genotype</th>
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<tbody>
<tr>
<td>(n=19)</td>
<td>(n=22)</td>
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Demographics

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<th>Glu/Asp + Asp/Asp</th>
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<tbody>
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<td>Gender (male:female)</td>
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<td>10:12</td>
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<tr>
<td>Age (years)</td>
<td>29.7±4.9</td>
<td>28.7±7.0</td>
</tr>
<tr>
<td>Smoking (y/n)</td>
<td>4/15</td>
<td>6/16</td>
</tr>
<tr>
<td>Body mass index (kg·m⁻²)</td>
<td>22.7±2.3</td>
<td>22.8±3.7</td>
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</table>

Haemodynamics

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<td>Diastolic blood pressure (mmHg)</td>
<td>64.7±6.4</td>
<td>62.1±5.6</td>
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<tr>
<td>Pulse pressure (mmHg)</td>
<td>51.2±5.4</td>
<td>53.5±6.6</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>63.2±11.4</td>
<td>61.7±10.0</td>
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Possible confounders

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<tr>
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<td>Total serum cholesterol (mmol·L⁻¹)</td>
<td>4.4±1.1</td>
<td>4.2±1.0</td>
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<tr>
<td>LDL-cholesterol (mmol·L⁻¹)</td>
<td>2.77±1.06</td>
<td>2.49±1.10</td>
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<tr>
<td>HDL-cholesterol (mmol·L⁻¹)</td>
<td>1.23±0.31</td>
<td>1.15±0.24</td>
</tr>
<tr>
<td>Triglycerides (mmol·L⁻¹)</td>
<td>1.15±1.2</td>
<td>1.01±0.90</td>
</tr>
<tr>
<td>Free fatty acids (mmol·L⁻¹)</td>
<td>0.42±0.16</td>
<td>0.38±0.13</td>
</tr>
<tr>
<td>Homocysteine (µmol·L⁻¹)</td>
<td>11.9±3.9</td>
<td>13.5±4.8</td>
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<tr>
<td>Glucose (mmol·L⁻¹)</td>
<td>5.2±0.38</td>
<td>5.1±0.35</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>5.0±0.3</td>
<td>4.9±0.5</td>
</tr>
<tr>
<td>Insulin (pmol·L⁻¹)</td>
<td>8.0±3.8</td>
<td>7.6±2.8</td>
</tr>
<tr>
<td>Epinephrine (nmol·L⁻¹)</td>
<td>0.21±0.14</td>
<td>0.22±0.17</td>
</tr>
<tr>
<td>Norepinephrine (nmol·L⁻¹)</td>
<td>0.86±0.37</td>
<td>0.77±0.38</td>
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</table>

Values: means±SD.

Table 7.1: Clinical characteristics of the Glu/Glu genotype and the combined Glu/Asp and Asp/Asp genotype

Results

Nineteen subjects (46%) had the homozygous wild type (Glu/Glu) genotype, 19 (46%) had the heterozygote (Glu/Asp) genotype and 3 subjects (7%) had the homozygous mutant (Asp/Asp) genotype. The observed genotype frequencies were in Hardy-Weinberg equilibrium. As shown in table 7.1 there were no significant differences between the wild type group (Glu/Glu) and the Asp allele group (Glu/Asp and Asp/Asp) concerning age, sex, smoking habits, body mass index, blood pressure, heart rate and all possible confounding factors such as stated in table 7.1.

There was no significant difference in basal FBF between the two groups (Glu/Glu vs Glu/Asp and Asp/Asp: 2.1±0.7 resp. 2.1±0.6 mL·min⁻¹·dL⁻¹).
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<table>
<thead>
<tr>
<th>genotype</th>
<th>Glu/Glu (n=19)</th>
<th>Glu/Asp + Asp/Asp (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute FBF ($mL\cdot min^{-1}\cdot dL^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBF at baseline</td>
<td>2.07±0.66</td>
<td>2.06±0.63</td>
</tr>
<tr>
<td>FBF at first dose of L-NMMA</td>
<td>1.72±0.63</td>
<td>1.76±0.70</td>
</tr>
<tr>
<td>FBF at second dose of L-NMMA</td>
<td>1.47±0.52</td>
<td>1.66±0.66</td>
</tr>
<tr>
<td>FBF at third dose of L-NMMA</td>
<td>1.31±0.49</td>
<td>1.57±0.66</td>
</tr>
<tr>
<td>Change in FBF ($mL\cdot min^{-1}\cdot dL^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta$FBF at first dose of L-NMMA</td>
<td>-0.34±0.18</td>
<td>-0.30±0.33</td>
</tr>
<tr>
<td>$\Delta$FBF at second dose of L-NMMA</td>
<td>-0.60±0.23</td>
<td>-0.40±0.28*</td>
</tr>
<tr>
<td>$\Delta$FBF at third dose of L-NMMA</td>
<td>-0.76±0.25</td>
<td>-0.49±0.38*</td>
</tr>
<tr>
<td>Percentage change in FBF (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta$%FBF at first dose of L-NMMA</td>
<td>-17.7±10.1</td>
<td>-15.3±13.5</td>
</tr>
<tr>
<td>$\Delta$%FBF at second dose of L-NMMA</td>
<td>-29.3±8.0</td>
<td>-20.6±14.1*</td>
</tr>
<tr>
<td>$\Delta$%FBF at third dose of L-NMMA</td>
<td>-37.1±7.8</td>
<td>-24.9±18.6**</td>
</tr>
</tbody>
</table>

Values: means±SD. *p < 0.05, **p < 0.01.
L-NMMA, N[^G]-monomethyl-L-arginine.

Table 7.2: Results of forearm blood flow (FBF) measurement at baseline and during the infusion of increasing doses of L-NMMA.

The highest dose of L-NMMA induced an absolute fall in FBF which was less pronounced in the mutant group as compare with the wild type group (-0.49±0.38 vs -0.76±0.25 $mL\cdot min^{-1}\cdot dL^{-1}$; p=0.02) (table 7.2). Also the percentage decrease in FBF ($\Delta$%FBF) was significantly less in mutants as compared with wild type homozygotes (-25±19 vs -37±7.8%; p=0.02) (table 7.2). When testing for differences in the complete dose-response curve between mutants and wild type homozygotes, the mutant group showed a blunted response on infusion of L-NMMA as compared with the wild type group (ANOVA for repeated measures; p=0.01. See figure 7.1). The $\Delta$FBF was significantly lower in the mutant group (Glu/Asp and Asp/Asp) as compared with the wild-type homozygotes (ANOVA fore repeated measures, p=0.02. See figure 7.1). The percentage of change in FBF ($\Delta$%FBF) was also significantly lower in the mutant group as compared with wild-type homozygotes (ANOVA for repeated measures, p=0.03. See figure 7.1). Throughout the experiment the FBF in the control arm did not change significantly.

The response to intra-arterial infusion of norepinephrine was not significantly different between groups, neither for the absolute values of FBF, nor for the absolute or percentage changes in FBF (see figure 7.1).
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Discussion

In this study we investigated the functional significance of the ecNOS Glu298Asp polymorphism as far as the baseline endothelial release of NO is concerned. This study is the first to report that the presence of an Asp allele of the ecNOS Glu298Asp polymorphism is associated with a reduced basal NO production. The absolute and relative decrease in FBF during infusion of \textit{L}\textsubscript{-}NMMA was attenuated in the Asp variant of the ecNOS Glu298Asp polymorphism as compared with the Glu variant. There was no significant difference between genotype groups concerning the response to intra-arterial infusion of norepinephrine. As norepinephrine induces endothelium-independent vasoconstriction, this indicates that the observed difference between the genotypes can be attributed to differences in the NO-pathway. NO is a potent vasodilator, and the basal NO production by the endothelium is a major determinant of basal flow. As NO has a short half-life, basal NO concentrations are highly dependent on continuous synthesis from L-arginine by NOS. When blocking NO production by means of \textit{L}\textsubscript{-}NMMA, the absolute decrease in blood flow is a reflection of the basal NO production by NOS [170; 171]. In this study we report that subjects with the Asp allele of ecNOS showed an attenuated fall in FBF on infusion of \textit{L}\textsubscript{-}NMMA. This indicates that the basal NO production in subjects with the mutant variant of the ecNOS gene is reduced as compared with the wild type. In theory, this observation may underlie the reported statistical relation between ecNOS gene polymorphisms and the presence of hypertension or cardiovascular disease. Studies evaluating the association of the ecNOS Glu298Asp polymorphism in the development of hypertension reveal conflicting results. Miyamoto \textit{et al.} [172] reported an increased Asp/Asp genotype frequency in Japanese hypertensive patients. Lacolley \textit{et al.} [173] on the contrary, reported increased Glu/Glu genotype frequency in hypertensive subjects. Moreover, a study by Kato \textit{et al.} [164] was not able to find any significant difference in allele frequencies between hypertensive patients and control subjects. Hingorani \textit{et al.} [174] and Yoshimura \textit{et al.} [175] reported an increased risk for the development of coronary artery disease in Asp/Asp homozygotes. One negative linkage study of the ecNOS Glu298Asp polymorphism in hypertensive patients [164] does not rule out a functional impairment of the Asp allele, because the level of NO inhibition required to induce atherosclerosis does not necessarily cause systemic hypertension [176]. Therefore, if the Asp allele causes only slight reductions in basal NO production, this might promote the development of atherosclerosis and endothelial dysfunction without directly affecting systemic blood pressure. In our study population, no significant differences in blood pressure were found between the various genotypes. Studies on NOS polymorphism which focussed on the occurrence of atherosclerotic events as end-points are in line with this reasoning [164; 174]. Recently Schneider \textit{et al.} [165] reported that the Asp allele of the Glu298Asp polymorphism of the ecNOS gene was not associated with reduced basal NO production. Using exactly the same technique as we, they first estimated the response to infusion of acetylcholine, after which sodium
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nitroprusside (SNP) and L-NMMA were infused. They did not find any difference between the various genotypes in response to infusion of acetylcholine, SNP and L-NMMA. However, the study design of Schneider et al. was not appropriate for assessment of the baseline NO production. We have previously published that the vasoconstrictor response to L-NMMA-infusion is augmented after pre-infusion of acetylcholine as compared with baseline [177]. So, it is not possible to quantify baseline NO release appropriately after first stimulating NO release by intra-arterial infusion of acetylcholine [178]. Therefore, on the basis of the results of Schneider et al. [8], it is not possible to draw any conclusion concerning functional consequences of the Glu298Asp polymorphism of the ecNOS gene as far as the baseline production of NO is concerned. In contrast, our study only focussed on baseline production of NO since we started the experiments with infusion of L-NMMA.

The allele frequencies of the ecNOS Glu298Asp polymorphism found in this study are comparable with those reported in other studies in European subjects [173; 174; 179]. These studies report homozygosity for the Asp allele in approximately 10% of the study population. Three Japanese studies [164; 172; 175] found a combined homozygous and heterozygous frequency for the Asp allele in approximately 10-15% of healthy subjects. This marked difference in allele frequencies of the ecNOS Glu298Asp polymorphism may suggest that their study population had a genetically different background compared to the other studies.

Even though we found a marked difference in basal NO production between the various genotypes, basal blood flow itself was not different among the groups. This suggests that other mechanisms compensate for the decreased basal NO production.

In conclusion, the Glu298Asp polymorphism of the ecNOS gene seems to be functionally relevant as decreased amounts of basal NO production are found in healthy subjects that are heterozygous or homozygous for the Asp allele of this polymorphism.
Chapter 8

Pharmacodynamics of L-NMMA in type 1 diabetes patients and control subjects


B.A.J. Veldman
M. Waanders
P. Smits
CHAPTER 8

Abstract

Background  \( L\)-NMMA is widely used in venous occlusion plethysmography studies to determine baseline NO production. Studies using \( L\)-NMMA indicate that endothelial dysfunction is present early in the course of diabetic microvascular complications. However, the optimal dose to maximally inhibit NO-production is unknown.

Objective  To determine the \( L\)-NMMA-dose that maximally reduces basal forearm blood flow (FBF). To investigate whether there are any differences in the response to \( L\)-NMMA between non-complicated type 1 diabetes patients and control subjects.

Methods  In 8 non-complicated type 1 diabetes patients and 9 healthy subjects FBF-responses to intra-arterial infusion of increasing doses of \( L\)-NMMA (0.01-1.6 \( mg\cdot min^{-1}\cdot dL^{-1}\) forearm volume [FAV]) were measured using the perfused forearm technique.

Results  Infusion of 0.8 \( mg\cdot min^{-1}\cdot dL^{-1}\) maximally reduced FBF. The dose of 1.6 \( mg\cdot min^{-1}\cdot dL^{-1}\) did not additionally reduce FBF. No differences existed between non-complicated type 1 diabetes patients and controls with regard to EC\(_{50}\) (0.017±0.02 vs 0.22±0.02 mg \( L\)-NMMA-\( min^{-1}\cdot dL^{-1}\)) or maximal vasoconstrictive response (\( \Delta FBFl: 1.13±0.4\) vs 0.97±0.4 \( mL\cdot min^{-1}\cdot dL^{-1}\)). Throughout the study blood pressure increased significantly in both groups, possibly reflecting a systemic vasoconstrictive effect of \( L\)-NMMA.

Conclusions  The maximal vasoconstrictive dose was 0.8 \( mg\cdot min^{-1}\cdot dL^{-1}\) in type 1 diabetes patients as well as the control subjects. There were no significant differences between non-complicated type 1 diabetes subjects and controls with regard to the pharmacodynamics of \( L\)-NMMA. At high dosages of \( L\)-NMMA a systemic effect can not be ruled out.
Introduction

In the past decades, the endothelial release of nitric oxide (NO) has been shown to be one of the key regulators of baseline vascular tone. In patients with atherosclerosis and in those with cardiovascular risk factors like hypertension and hypercholesterolemia, dysfunction of this NO pathway is a common finding. In non-complicated diabetes mellitus, however, observations on this specific issue are still controversial. The literature varies from reports on an impaired [180; 181] an unaltered [65; 182], or even an increased release or action of NO in non-complicated diabetes mellitus [70]. The optimal method to assess the contribution of NO to baseline vascular tone is to quantify the vasoconstrictor response to specific blockade of endothelial NO-synthase. In humans, this is almost exclusively done by the infusion of the NO-synthase blocker \( \text{N}^\text{G} \)-monomethyl-L-arginine (L-NMMA) into the brachial artery using the perfused forearm technique [166; 183]. This is a very elegant technique because of its local character thereby avoiding the administration of systemic dosages. An important shortcoming in the forearm studies so far is the relative low dose of L-NMMA which is not sufficient for a complete blockade of NO-synthase. Up to now, dosages did not exceed 0.4 \( \text{mg} \cdot \text{min}^{-1} \) per dL of forearm tissue (or 4 \( \text{mg} \cdot \text{min}^{-1} \) in total, equivalent to \( \pm 16 \ \mu \text{mol} \cdot \text{min}^{-1} \)), and at this dose a maximal vasoconstrictor response to L-NMMA was not achieved [180; 182; 184; 185]. As such, an accurate assessment of the contribution of NO to baseline vascular tone is missing in the current literature, both in healthy volunteers as well as in type 1 diabetes patients. The aim of the present study is to perform complete concentration response curves for L-NMMA by use of the perfused forearm technique. Subsequent fitting of these concentration-response curves according to the sigmoid E-max model will enable us to calculate the EC\(_{50}\) and the \( E_{\text{max}} \) (maximal effect) of L-NMMA in type 1 diabetes patients and controls. This approach will answer the question as to whether the NO pathway is altered in non-complicated type 1 diabetes mellitus.

Methods

We recruited 8 non-complicated type 1 diabetes patients (DP) and 9 matched healthy control subjects (C) from the local community. All subjects fulfilled the following criteria: age between 18 and 40, blood pressure (BP) < 140/90 mmHg, no antihypertensive medication and no signs of cardiovascular disease. Since primary hypertension, atherosclerosis and smoking have been associated with an impaired vasoconstrictor response to L-NMMA these subjects were excluded. All subjects signed the informed consent and the study protocol was approved by the local medical ethics committee. Subjects were instructed to abstain from alcohol and caffeine for 24 h and refrain from smoking 12 h before the study. The study was performed between 8.00-12.00 a.m. All subjects attended the clinic after an overnight fast. Fifteen minutes after cannulation of the brachial artery of the non-dominant arm, intra-
Table 8.1: Baseline characteristics of the participants in the study

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<th>DM Subjects (n=8)</th>
<th>Controls (n=9)</th>
<th>p-value between groups</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>29±3</td>
<td>22±1</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (male:female)</td>
<td>5:3</td>
<td>4:5</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg·m⁻²)</td>
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<td>22.3±0.6</td>
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<td>Duration of diabetes (months)</td>
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<td>HbA₁c(%)</td>
<td>8.2±0.4</td>
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<td>NA</td>
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<tr>
<td>Total daily insulin dose (units)</td>
<td>63±7</td>
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<tr>
<td>Fasting glucose (mmol·L⁻¹)</td>
<td>14.2±1.2</td>
<td>4.7±0.1</td>
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<td>Baseline FBF (mL·min⁻¹·dL⁻¹)</td>
<td>2.6±0.2</td>
<td>2.1±0.2</td>
<td>NS</td>
</tr>
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Values are expressed as means±SEM.
NA, not applicable; NS, not significant.

Results

The baseline characteristics of both groups are shown in table 8.1. Besides fasting plasma glucose and body mass index, DP and C did not differ significantly with regard to any of the baseline characteristics. In DP as well as C FBF decreased significantly during infusion of the increasing doses of L-NMMA. As shown in table 8.1 DP and C did not differ significantly with regard to baseline FBF. In both populations, the maximum decrease in FBF occurred during infusion
of 0.8 mg·min\(^{-1} \cdot dL^{-1}\). The dose of 1.6 mg·min\(^{-1} \cdot dL^{-1}\) showed a significant increase with respect to 0.8 mg·min\(^{-1} \cdot dL^{-1}\) in both groups (\(p<0.001\)). During the experiment there were no significant differences between both groups with regard to the response on L-NMMA infusion. As shown in figure 8.1C, there were no significant differences between both groups regarding EC\(_{50}\) (DP: 0.017±0.001; C: 0.022±0.001; NS) and E\(_{max}\) (DP: 1.13±0.1; C: 0.97±0.1; NS). All subjects showed an increase in MAP (DP: 89±3 to 96±2 mmHg, \(p<0.005\); C: 91±2 to 99±2 mmHg, \(p<0.001\)) during the experiment (figure 8.1D). Interestingly, FBF remained constant in the control arm. The average MAP in C and DP was not significantly different between both groups throughout the experiment. At baseline there was no significant difference between FBF in the control forearm and the FBF in the experimental arm neither DP nor in C. Neither did the FBF in the control arm change significantly over time in both groups (figure 8.1A).

**Discussion**

This study is the first to compare the dose response of intra arterial infusion of L-NMMA with complete maximal blockade on venous occlusion plethysmography in type 1 diabetes patients and healthy controls. Pharmacodynamic parameters (EC\(_{50}\), E\(_{max}\)) were identical in both groups. Although there is abundant literature about altered NO metabolism in (early) type 1 diabetes [184; 186–190], we were unable to find a difference in pharmacodynamics in L-NMMA between non-complicated DP and healthy controls. Several explanations can apply for this observation. First, we studied a non-complicated diabetes population. Apparently endothelial dysfunction is not present at this early state of diabetes. Even, baseline forearm blood flow was borderline significantly higher in the diabetes patients. Some studies indicate that early type 1 diabetes mellitus is characterized by relative hyperemia [65; 187]. Second, perhaps early type 1 diabetes mellitus is not characterized by altered NO metabolism, but alterations in other regulators of vascular tone like decreased noradrenergic vasoconstrictive tone or increased vasodilator prostanoids [185]. Vervoort et al. [65] showed that early type 1 diabetes mellitus is characterized by decreased vasoconstrictive noradrenergic tone, resulting in hyperemia. They could not find any difference in the NO pathway or endothelial dependent vasodilation induced by acetylcholine.

In both populations the maximal inhibiting dose (0.8 mg·min\(^{-1} \cdot dL^{-1}\)) is higher than used in most venous occlusion plethysmography studies [181; 185; 191–198]. Most venous occlusion plethysmography studies used doses of 0.2-0.4 mg L-NMMA ·min\(^{-1} \cdot dL^{-1}\). This indicates that the NO-synthase inhibition in these studies has been sub-optimal. As it is not clear whether differences in pharmacodynamics exists between various study populations, suboptimal blockade of NO synthesis will result in spurious conclusions. Therefore, results from those studies have to be taken with caution. In our study we used increasing doses of L-NMMA to determine the concentration at which maximal blockade of nitric oxide synthase occurs. Cohen et al. [199] have clearly shown that in isolated artery
Figure 8.1: (A) Absolute forearm blood flow in the control arm of type 1 diabetes patients (■) and healthy controls (○) in response to increasing doses of L-NMMA. (B) Absolute forearm blood flow in the experimental arm of type 1 diabetes patients (■) and healthy controls (○) in response to increasing doses of L-NMMA. (C) Absolute change in forearm blood flow in the experimental arm of type 1 diabetes patients (■) and healthy controls (○) in response to increasing doses of L-NMMA. (D) Change in mean arterial pressure in type 1 diabetes patients (■) and healthy controls (○) in response to increasing doses of L-NMMA.
segments NO production is not completely abolished despite maximal blockade of nitric oxide synthase. During maximal blockade of nitric oxide synthase with two L-arginine analogs they still could determine the increase in nitric oxide production in response to acetylcholine exposure. Therefore, although maximal blockade of NOS was achieved in our study, residual nitric oxide synthase activity may be present and blockade may not have been complete. Interestingly the highest dose of L-NMMA (1.6 mg·min$^{-1}$·dL$^{-1}$) showed a smaller decrease in FBF compared to 0.8 mg·min$^{-1}$·dL$^{-1}$. Probably this can be attributed to a systemic effect of L-NMMA. As indicated in figure 8.1C a dose of 0.025 mg·min$^{-1}$·dL$^{-1}$ already induced 50% of the vasoconstrictor effect. This proves the sensitivity of the vascular endothelium for L-NMMA. The MAP showed a progressive equal increase in both study groups. This points towards systemic vasoconstriction. At the highest dose of L-NMMA, the FBF appeared to be higher than during previous dosages. This is probably the result of the increase in MAP during the highest dose.

In conclusion, non-complicated type 1 diabetes patients and control subjects show identical pharmacodynamics on infusion of L-NMMA. This suggests that at this early stage of type 1 diabetes no differences in the NO pathway exists. The maximal inhibiting concentration of L-NMMA is 0.8 mg·min$^{-1}$·dL$^{-1}$. This is higher than most commonly used L-NMMA doses. At high dosages of L-NMMA a systemic effect cannot be ruled out.
Chapter 9

Low incidence of diabetic nephropathy in a prospective study of type 1 diabetes mellitus: reality or selection bias?

B.A.J. Veldman
G. Vervoort
J.H.M. Berden
P. Smits
J.F.M. Wetzels
Abstract

Background  Renal complications determine to a great extent the morbidity and mortality in type 1 diabetes mellitus. Historical registries indicated that up to 40% of type 1 diabetes patients developed diabetic nephropathy. To investigate prognostic factors for the development of diabetic nephropathy in type 1 diabetes mellitus, a large prospective study (PROFID-study) was initiated in 1994. One hundred forty-eight normotensive type 1 diabetes patients without nephropathy were included in the study. At inclusion they were not treated with antihypertensive medication, had a diabetes duration of 5 to 12 years and their age was between 18 and 40 years. At the start of the study we anticipated an event rate (development of micro-albuminuria) of 20% after 12 years of follow-up. After 12 years of follow-up the cumulative incidence of diabetic nephropathy is 6%, far lower than expected. To elucidate whether this lower incidence of diabetic nephropathy reflects a change in the natural history of type 1 diabetes or whether the study suffered from selection bias or poor follow-up, we compared the study population with type 1 diabetes patients, who were treated in our hospital during the study period, who fulfilled the inclusion criteria but who refused to participate in the study.

Methods  From current and historical local registries 769 type 1 diabetes patients were identified. Sixty-three patients participated in the PROFID-study. One hundred sixty-four patients fulfilled the inclusion criteria for the PROFID-study but did not participate. Both groups were compared with regard to the incidence of diabetic nephropathy, glycemic control, blood pressure regulation and demographic characteristics.

Results  Both groups did not differ with regard to the cumulative incidence of diabetic nephropathy, glycemic control, blood pressure regulation, use of antihypertensive medication or demographic characteristics.

Conclusions  The observed reduced incidence of diabetic nephropathy among participants of the PROFID study is not caused by selection bias. This suggests a change in the disease course of type 1 diabetes mellitus.
Introduction

Renal complications determine to a great extent the morbidity and mortality in type 1 diabetes mellitus. Historical registries indicated that up to 40% of type 1 diabetes patients developed diabetic nephropathy [10; 15; 200]. To investigate prognostic factors for the development of diabetic nephropathy in type 1 diabetes mellitus, a large prospective study was initiated in 1994 (PROgnostic Factors In Diabetic nephropathy, the PROFID-study). The PROFID-study recruited 148 normotensive type 1 diabetes patients without nephropathy. After 12 years of follow up the cumulative incidence of diabetic nephropathy amounted 6%, lower than expected. To elucidate whether this lower incidence of diabetic nephropathy reflects a change in the disease course of type 1 diabetes or is merely explained by selection bias, we compared the study population with eligible, non-participating type 1 diabetes patients.

Methods

In the PROFID-study, 148 type 1 diabetes patients were included. At inclusion they were normoalbuminuric, not treated with antihypertensive medication, had a diabetes duration of 5 to 12 years and their age was between 18 and 40 years. Patients were recruited from the out-patient clinic of the Radboud University Medical Centre, but also from affiliated hospitals. Of the 148 PROFID-study participants, 63 were treated in our out-patient clinic. At baseline extensive studies were performed in the participants as described in Chapter 1 (Introduction and Outline). Follow-up consisted of biannual measurement of urinary albumin excretion rate (UAE) and regular blood pressure measurements.

From local historical and current registries of type 1 diabetes mellitus patients, 769 type 1 diabetes patients were identified who visited our out-patient clinic in the study period. Of these, 164 patients fulfilled the inclusion criteria, but did not participate. Of both groups, charts were reviewed and from onset of diabetes all office blood pressure measurements, all (changes in) antihypertensive medication were registered. HbA1c levels and presence of (micro) albuminuria were documented. We investigated whether there were differences in demographic characteristics, glycemic control, blood pressure regulation and incidence of diabetic nephropathy.

Definition of nephropathy All patients were tested for albuminuria at regular intervals. Urinary albumin was measured in a timed overnight urine sample using immunonephelometry. Nephropathy was diagnosed if microalbuminuria (> 20 µg·min⁻¹) was detected in two or more consecutive urine samples.

Measurement of glycosylated haemoglobin Measurement of glycosylated haemoglobin became routine in 1980. HbA1c was measured using a chromatographic method,
participants  | non-participants  
---|---
(n=63)  | (n=164)  
**Age of onset** of diabetes (years±SD)  |  |  
19±8  | 20±8  
**Male**  |  |  
29 (46%)  | 91 (55%)  
**Average duration of diabetes** until 2007 (years±SD)  |  |  
18.4±3  | 17.4±3  
**(Ever) smokers (%)**  |  |  
25 (47%)  | 56 (40%)*  
**Antihypertensive treatment (%)**  |  |  
8 (13%)  | 18 (11%)  
**Diabetic nephropathy (%)**  |  |  
4 (6%)  | 7 (4%)  
**Mortality (%)**  |  |  
2 (3%)  | 5 (3%)  

* Information on smoking habits was available for 139 subjects.

**Table 9.1:** Characteristics of participants of the PROFID-study compared with eligible, non-participating type 1 diabetes patients

with a reference value of 5.5-8.5% [201]. In 1989 the method for analyzing glycosylated haemoglobin changed from HbA1 determination to HbA1c. HbA1c was measured using a HPLC technique (Bio-Rad Diamat, the Netherlands), with reference values of 4.8-6.2%. We have evaluated the relationship between HbA1 and HbA1c. The following relationship was observed: HbA1c = 0.6476[HbA1] + 1.869. For the present study all HbA1 values were replaced by calculated HbA1c values.

**Statistical analysis** Differences between groups with regard to mortality, sex, smoking habits, antihypertensive treatment and the development of diabetic nephropathy were compared by χ² test. Differences between groups with regard to age of onset and the duration of diabetes were tested using Student’s t-test. A p-value less than 0.05 was considered statistically significant.

**Results**

Of 769 type 1 diabetes patients 227 were eligible for participation in the PROFID-study. Sixty-three patients from the Radboud University Medical Centre participated in this study whereas 164 did not participate. Table 9.1 shows the characteristics of the non-participating subjects and the participants. There were no differences in age at onset of diabetes, gender, duration of diabetes at end of follow-up, and the percentage of smokers. During follow-up 8 participants (13%) were treated with antihypertensive medication, compared to 18 non-participants (11%; p = NS). As shown in figure 9.1 there were no differences with regard to blood pressure regulation (upper panel) as well as glycemic control (lower panel). Two (3%) participants of the PROFID study died, versus five (3%) of the non-participating subjects (p = NS). At the end of follow-up four participants (6%)
patients had developed diabetic nephropathy, and seven non-participants (4%; \( p = NS \)).

**Discussion and conclusion**

The observed incidence of diabetic nephropathy in the PROFID-study population is far lower than reported in historical registries. However, the observed incidence in the study population is equal to the incidence of diabetic nephropathy in eligible, non-participating subjects followed during the same time period. There were no differences between participants and non-participants with regard to glycemic control, blood pressure, treatment with antihypertensive medication, smoking habits and sex. Therefore, the lower than expected incidence of diabetic nephropathy in the PROFID-study is not caused by selection bias, but most likely reflects a change in the disease course of type 1 diabetes mellitus.
Figure 9.1: The upper panel shows the average systolic and diastolic blood pressure (mmHg±SD) for participants of the PROFID-study (closed boxes and circles) and non-participating, but eligible type 1 diabetes patients (open boxes and circles). The lower panel shows the average HbA1c levels (%±SD) for participants of the PROFID-study (closed boxes) and non-participating, but eligible type 1 diabetes patients (open circles).
Chapter 10

Declining incidence of diabetic nephropathy in type 1 diabetes mellitus is associated with improved glycemic control

Submitted

B.A.J. Veldman
G. Vervoort
P. Smits
J.H.M. Berden
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CHAPTER 10

Abstract

Background  The development of renal complications is a major determinant of the increased morbidity and mortality in patients with type 1 diabetes mellitus. The cumulative incidence of diabetic nephropathy was 20-40%. However, recent studies suggest that the incidence may be decreasing. Both improved glycemic control and improved blood pressure regulation may be responsible.

Methods  We assessed trends in the cumulative incidence of diabetic nephropathy in a retrospective population study and evaluated treatment practice with regard to blood pressure and glycemic control. Seven hundred sixty-nine patients were identified, who visited the out-patient clinic of the Radboud University Nijmegen Medical Centre and were followed from onset of diabetes to 2007 or to death. Patients with persistent albuminuria were considered to have diabetic nephropathy. From 1980 onwards glycosylated haemoglobin was measured regularly.

Results  The cumulative incidence of diabetic nephropathy after 20 years of diabetes decreased from 17.3% among patients who developed type 1 diabetes between 1970 and 1974 to 7.7% in patients who developed diabetes between 1985 and 1989. Overall, we observed an improved glycaemic control over time, average HbA$_{1c}$ decreasing from 9.5% in 1980 to 8.0% in 2006. Although the percentage of patients treated with antihypertensive drugs increased from 9 to 25% no major change in average blood pressure was observed. In a Cox's proportional hazard regression model, year of diagnosis, time-averaged HbA$_{1c}$ and time-averaged diastolic blood pressure were independent predictors of the development of diabetic nephropathy. In a case control analysis, patients who developed diabetic nephropathy (cases) were more likely to have higher levels of glycosylated haemoglobin compared to patients who remained normoalbuminuric (controls). Prior to the development of diabetic nephropathy, cases also had higher blood pressure values, even despite being treated more often with antihypertensive medication. There was no significant difference in smoking habits between both groups.

Conclusions  In type 1 diabetes mellitus the cumulative incidence of diabetic nephropathy has decreased dramatically over the past decades. This improvement concurred with substantially improved glycemic control and intensified treatment with antihypertensive medication. Both the population study and the case-control study show that glycemic control as well as elevated blood pressure values are determinants for the development of diabetic nephropathy. As throughout the years glycemic control improved substantially while blood pressure levels remain virtually unchanged, apparently there is opportunity for intensifying antihypertensive treatment. Perhaps this may lead to a further reduction of the incidence of diabetic nephropathy.
Declining incidence of diabetic nephropathy

Introduction

Renal complications are a major determinant of morbidity and mortality in patients with type 1 diabetes mellitus. Previous studies reported a cumulative incidence of 20-40% of diabetic nephropathy \[10; 15; 200\].

Various modifiable risk factors may contribute to the development of diabetic nephropathy. The DCCT trial \[35; 202\] and the ADVANCE-study \[203\] clearly showed that improved glycemic control lowered the cumulative incidence of diabetic nephropathy. Also tight blood pressure regulation reduces the incidence and the progression of diabetic nephropathy \[1; 58; 60\]. Smoking increases the risk of diabetic nephropathy\[204\]. In recent years some \[12; 205\] but not all studies \[200; 206\] reported a decreased incidence of diabetic nephropathy.

In 1994 we initiated a prospective study to elucidate prognostic factors for the development of diabetic nephropathy in patients with type 1 diabetes mellitus \[65; 96\]. One hundred and forty-eight type 1 diabetes patients were included. All patients were normoalbuminuric, normotensive and had a diabetes duration of 5-12 years. Thus far only 6 patients (4%) developed microalbuminuria after 8-14 years of follow-up, suggesting that the natural history of diabetic nephropathy is changing. Therefore, we investigated, in a historical cohort, trends in the cumulative incidence of diabetic nephropathy. Also we evaluated the possible role of blood pressure regulation and glycemic control.

Methods

From local historical and current registries of type 1 diabetes patients 944 type 1 diabetes patients were identified. Of those 944 patients, 769 patients were normoalbuminuric and did not receive antihypertensive medication at the beginning of follow up. The charts of those 769 patients were reviewed. All office blood pressure recordings and all (changes in) antihypertensive medication were registered. Smoking habits, HbA1c levels and presence of (micro) albuminuria were documented. For the analysis of the change in the cumulative incidence of diabetic nephropathy, the patients were divided into 5-yr cohorts, according to the year in which diabetes was diagnosed (1970-1974, 1975-1979, 1980-1984, 1985-1989, 1990-1994, 1995-1999, 2000-2005).

Definition of nephropathy

All patients were tested for proteinuria at regular intervals, initially with a semi-quantitative test strip (Albustix®), since 1985 by measuring albumin in a timed overnight urine sample using immunonephelometry. Nephropathy was diagnosed if albuminuria was detected in two or more consecutive urine samples.

Measurement of glycosylated haemoglobin

Measurement of glycosylated haemoglobin became routine in 1980. HbA1c was measured using a chromatographic method,
with a reference value of 5.5-8.5% [201]. In 1989 the method for analyzing glycosylated haemoglobin changed from HbA1 determination to HbA$_{1c}$. HbA$_{1c}$ was measured using a HPLC technique (Bio-Rad Diamat®, the Netherlands), with reference values of 4.8-6.2%. We evaluated the relationship between HbA1 and HbA$_{1c}$. The following relationship was calculated: HbA$_{1c} = 0.6476 \cdot [\text{HbA1}] + 1.869$. For the present study all HbA1 values were replaced by calculated HbA$_{1c}$ values.

**Case-control analysis**  Within the cohort, for every patient that developed diabetic nephropathy (case) one matched patient without diabetic nephropathy (control) was selected. Eight patients with diabetic nephropathy could not be matched due to their high age. One hundred and seven cases and controls were matched for sex, age, age of onset of diabetes, and duration of follow-up. We compared cases and controls with regard to blood pressure regulation, glycemic control, smoking habits, blood pressure before onset of diabetic nephropathy and blood pressure before treatment of hypertension. In controls, blood pressure (treatment) and glycemic control were analyzed with regard to the date of onset of diabetic nephropathy in their matched case. Of both cases and controls the average HbA$_{1c}$, systolic blood pressure and diastolic blood pressure, prior to the development of diabetic nephropathy, were estimated by calculating the area under the curve (AUC) for all three variables. For an individual, the AUC was calculated by multiplying the average of two consecutive values by the time interval (months) for all recordings until an end point was reached (development of diabetic nephropathy, death or end of study). The sum of those calculations was divided by the total number of months prior to the development of the end points.

**Statistics**  Differences between groups were calculated with Student’s $t$-test or Mann-Whitney-$U$-test when appropriate. Differences in frequencies (like smoking habits) between groups were compared by the $\chi^2$-square-test. Univariable analysis was used to identify possible explanatory variables for the development of diabetic nephropathy. To test the relative contributions of selected variables on the development of diabetic nephropathy, a Cox’s proportional hazard regression model was used. To test the relative contributions of selected variables on the development of diabetic nephropathy, a stepwise multiple regression analysis was performed with AUC HbA$_{1c}$, AUC systolic blood pressure, AUC diastolic blood pressure, ever-smoking status and use of antihypertensive medication prior to the development of diabetic nephropathy as independent variables and the development of diabetic nephropathy as the dependent variable. A $p$-value < 0.05 was considered statistically significant. All calculations were performed with SPSS 15.0 (SPSS, Chicago, USA) or Graphpad PRISM 5.01 (Graphpad software, La Jolla, USA)
Declining incidence of diabetic nephropathy

From local historical and current registries of patients with type 1 diabetes 944 patients were identified. Of those, 769 were normoalbuminuric and did not receive antihypertensive medication at the beginning of follow up. The characteristics of this study cohort are described in table 10.1.

Figure 10.1 shows the average HbA1c (% values in type 1 diabetes mellitus patients over the years.

**Results**

From local historical and current registries of patients with type 1 diabetes 944 patients were identified. Of those, 769 were normoalbuminuric and did not receive antihypertensive medication at the beginning of follow up. The characteristics of this study cohort are described in table 10.1.

Figure 10.1 shows the average HbA1c levels from 1980 up to 2006. There was a sharp decline in HbA1c levels shortly after the introduction of HbA1c measurement in 1980. Thereafter, a gradual decrease of HbA1c occurred. The mean HbA1c level was 8.6±1.4% in the period 1985–1995 and 8.3±1.5% in the period 1996-2006.

In figure 10.2, the average systolic and diastolic blood pressures are depicted for all patients studied in the period from 1980 up to 2006. Office blood pressures
Figure 10.2: The average office systolic and diastolic blood pressure values (mmHg±SD) in patients with type 1 diabetes mellitus over the years.

did not change significantly during this observation period.

The cumulative incidence of patients who received antihypertensive treatment is depicted in figure 10.3. Only 9% of patients who developed diabetes mellitus between 1970 and 1974 received antihypertensive drugs at 20 years after onset, compared to 25% of patients who developed diabetes mellitus between 1985 and 1989. Twenty-seven patients without nephropathy and 21 patients with nephropathy died.

During the study period, 115 patients developed diabetic nephropathy. A Cox proportional hazard model was used to develop a model for predicting the development of diabetic nephropathy, using year of diagnosis, AUC HbA₁c, AUC systolic blood pressure, AUC diastolic blood pressure, ever-smoking status and sex as independent variables and the development of diabetic nephropathy as the dependent variable. Year of diagnosis, AUC HbA₁c and AUC diastolic blood pressure had a significant partial effect in the full model (table 10.2).

The cumulative incidence of nephropathy for the successive 5-yr cohorts is shown in figure 10.4. With increasing year of diagnosis the cumulative incidence of nephropathy decreased: after 20 years of diabetes, the cumulative incidence of diabetic nephropathy decreased from 17.3% for patients who developed type 1 diabetes between 1970 and 1974 to 7.7% for patients who developed diabetes between 1985 and 1989.

Onset of diabetic nephropathy for tertiles of HbA₁c levels or blood pressure levels is illustrated in figures 10.5 and 10.6. Of note, figure 10.5 indicates that
Declining incidence of diabetic nephropathy

Figure 10.3: The cumulative incidence (%) of antihypertensive treatment according to the year of onset of type 1 diabetes mellitus.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year of diagnosis</td>
<td>-0.05 (p&lt;0.05)</td>
</tr>
<tr>
<td>AUC HbA₁c*</td>
<td>0.80 (p&lt;0.001)</td>
</tr>
<tr>
<td>AUC SBP*</td>
<td>NS</td>
</tr>
<tr>
<td>AUC DBP*</td>
<td>0.05 (p&lt;0.05)</td>
</tr>
<tr>
<td>Ever smoking</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Prior to the development of diabetic nephropathy.

AUC, area under the curve; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA₁c, glycated haemoglobin A1c; NS, not significant

Table 10.2: Effects of selected variables on the probability of development of diabetic nephropathy.
CHAPTER 10

Figure 10.4: The cumulative incidence (%) of diabetic nephropathy according to the year of onset of type 1 diabetes mellitus.

Figure 10.5: Cumulative incidence of diabetic nephropathy according to AUC HbA1c levels prior to the development of diabetic nephropathy. Population divided into tertiles according to HbA1c.
Declining incidence of diabetic nephropathy

Figure 10.6: Cumulative incidence of diabetic nephropathy according to AUC diastolic blood pressure prior to the development of diabetic nephropathy. Population divided into tertiles according to AUC diastolic blood pressure.

The relation between HbA1c level and risk of diabetic nephropathy is not linear; we observed no difference in the incidence of diabetic nephropathy between patients with HbA1c levels < 7.8% and 7.8-8.6%. However, the incidence was clearly increased in patients with HbA1c levels > 8.6%. Figure 10.6 shows that with increasing diastolic blood pressure the risk for development of diabetic nephropathy increases.

Development of diabetes mellitus during childhood (age < 16 years) was associated with an increased risk for the development of diabetic nephropathy (figure 10.7). However, patients who developed diabetes mellitus before the age of 16 years had a higher AUC HbA1c compared with patients who developed diabetes mellitus at an age above 15 years (8.4±1.2% vs 8.1±1.1%, p<0.02). Thus, when age of onset of diabetes was entered into the COX-regression analysis, it was not a significant independent risk factor for the development of diabetic nephropathy.

Case-control analysis

Glycemic control In the case-control analysis, patients who developed nephropathy had a higher HbA1c in the period from onset of diabetes to development of nephropathy as compared with patients who remained normoalbuminuric (figure 10.8 panel B). Even after the development of nephropathy, HbA1c remained higher than the HbA1c levels of patients who remained normoalbuminuric (not shown).
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Figure 10.7: Cumulative incidence of diabetic nephropathy (%) in patients with onset of diabetes before the age of 16 and from the age of 16 onwards.

The average HbA1c (AUC) before the development of diabetic nephropathy in cases was 9.5±1.4% while in controls it was 8.4±0.9% ($p<0.001$).

**Blood pressure regulation**  Panel A of figure 10.8 shows the average systolic and diastolic blood pressure prior to the development of diabetic nephropathy over the years. During the months prior to the initiation of antihypertensive medication, blood pressure was higher in cases as compared with controls (systolic blood pressure: 143±2 mmHg vs 133±2 mmHg; $p=0.002$; diastolic blood pressure: 83±1 mmHg vs 75±1 mmHg; $p<0.001$). When comparing blood pressure between cases and controls in the months prior to the development of nephropathy, blood pressure was higher in cases as compared with controls (systolic blood pressure 138±2 mmHg vs 130±2 mmHg; $p=0.002$; diastolic blood pressure 80±1 mmHg vs 74±1 mmHg; $p=0.001$). Cases were more likely to be treated with antihypertensive medication prior to the development of diabetic nephropathy than controls did (cases: 55 / 107 vs controls: 20 / 107; $p<0.001$). For cases, the average systolic blood pressure (AUC) in the period from onset of diabetes until the development of diabetic nephropathy was 135±15 mmHg while in controls it was 132±13 mmHg ($p=NS$). For the average diastolic blood pressure (AUC) it was 79±9 mmHg and 76±6 mmHg respectively ($p<0.01$). In cases and controls receiving antihypertensive medication, blood pressure initially decreased from 147±18 to 143±21 after the initiation of antihypertensive medication ($p<0.05$). After two years of treatment with antihypertensive medication blood pressure levels were not significantly different from the pre-treatment values. In our study population, the first ACE-inhibitors were introduced in 1984. Treatment with ACE-inhibitors became widespread in the early nineties.
Declining incidence of diabetic nephropathy

Figure 10.8: Case-control study. Panel A shows the average blood pressure (mmHg ± SD) of cases, prior to the development of diabetic nephropathy, and the average blood pressure of the control subjects. Panel B shows the average HbA1c level (%±SD) of cases, prior to the development of diabetic nephropathy, and the HbA1c level of the control subjects.
Stepwise multiple regression model

Case-control study

<table>
<thead>
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<th>Variable</th>
<th>Beta (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC HbA$_{1c}$*</td>
<td>0.48 ($p&lt;0.001$)</td>
</tr>
<tr>
<td>AUC SBP*</td>
<td>NS</td>
</tr>
<tr>
<td>AUC DBP*</td>
<td>NS</td>
</tr>
<tr>
<td>Ever smoking</td>
<td>NS</td>
</tr>
<tr>
<td>Antihypertensive usage</td>
<td>0.21 ($p&lt;0.01$)</td>
</tr>
</tbody>
</table>

* Prior to the development of diabetic nephropathy.

AUC, area under the curve; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA$_{1c}$, glycosilated haemoglobin A1c; NS, not significant

Table 10.3: Effects of selected variables on the probability of development of diabetic nephropathy.

Among cases 61 (57%) patients were (ever) smokers while 52 (54%) controls (ever) smoked ($p=NS$).

A stepwise multiple regression analysis was used to develop a model for predicting the development of diabetic nephropathy, from AUC HbA$_{1c}$, AUC systolic blood pressure, AUC diastolic blood pressure, ever-smoking status and the use of antihypertensive medication prior to the development of diabetic nephropathy. The AUC HbA$_{1c}$ and use of antihypertensive medication prior to the development of diabetic nephropathy had a significant partial effect in the full model (table 10.3). The model was able to predict 24% of the development of diabetic nephropathy ($r^2=0.24$). AUC systolic or diastolic blood pressure did not predict the development of diabetic nephropathy.

Discussion

Our data clearly demonstrate that the natural history of type 1 diabetes mellitus is changing in our region. We observed a 10% decrease in the cumulative incidence of diabetic nephropathy when comparing patients with onset of diabetes in the period 1970-1974 and patients with onset of diabetes in the period 1985-1989. Our data suggest that the decreasing incidence of diabetic nephropathy in recent decades is caused by improvement of glucose control and earlier start of antihypertensive therapy.

To analyze risk factors for the development of diabetic nephropathy, we evaluated the data for the cohort of patients with type 1 diabetes mellitus. Cox’s proportional hazard analysis revealed year of diagnosis, average HbA$_{1c}$ and average diastolic blood pressure as independent risk factors for diabetic nephropathy. The case control study strengthened these findings. Cases had higher HbA$_{1c}$ lev-
Declining incidence of diabetic nephropathy

els, were treated more frequently with antihypertensive medication, had higher blood pressures before the onset of diabetic nephropathy and had a higher cumulative diastolic blood pressure burden (AUC diastolic blood pressure).

Poor glycemic control has been identified as a risk factor for the development of diabetic nephropathy in many observational [12; 206–209] as well as prospective studies [35; 203]. Interestingly, when dividing our study population into tertiles according to AUC HbA1c, there appears to be a threshold above which the risk of development of diabetic nephropathy increases dramatically (HbA1c > 8.6%). The risk for development of diabetic nephropathy appears not to be linear: further reductions of HbA1c below the level of 8.6% does not seem to result in a lower risk for the development of diabetic nephropathy. This is in accordance with other studies. In the United Kingdom Prospective Diabetes Study [210] reduction of HbA1c from 8 to 7% did not result in a reduced cardiovascular risk, although a metformin-treated subgroup had a lower risk of cardiovascular events. Also, the recent Action to Control Cardiovascular Risk in Diabetes (ACCORD)-trial [211] did not show a reduced cardiovascular risk when using intensive therapy to target normal HbA1c (< 6.0 vs 7.5%) levels for 3.5 years. In this study, a change in development of diabetic nephropathy was not reported. In their study of nephropathy in childhood type 1 diabetes Amin et al. [212] showed a high cumulative incidence of diabetic nephropathy. The high incidence can be explained since three quarter of their patients had an average HbA1c level above 8.5%. With HbA1c < 8.5% as the lowest quartile, the risk of development of diabetic nephropathy increased progressively with increasing quarters of HbA1c. However, even when HbA1c levels are below 8.5%, differences in HbA1c-levels might be relevant. A study in type 2 diabetes patients [203] showed a significant reduction in the development of nephropathy with intensive glucose control as compared with regular glucose control (HbA1c 6.5 vs 7.5%).

In our study HbA1c levels improved dramatically over time, especially shortly after the introduction of HbA1c. This sudden and remarkable decrease of HbA1c levels is notable. It is not clear what caused this decrease in HbA1c. In 1980 the HbA1c determination was introduced in our hospital. Within two years after introduction, HbA1c levels decreased. Perhaps the introduction of HbA1c itself made patients and their doctors aware of the actual level of glycemic control and stimulated their efforts for stricter glycemic control. An other explanation may be the introduction of self-monitoring of blood glucose. Initially, self-monitoring consisted of urine glucose measurements three times a day. In the early eighties self-monitoring of blood glucose became standard. Frequent self-monitoring of blood glucose improves glycemic control [213]. Also treatment regimens changed dramatically: while patients were treated initially with a single morning dose of long acting insulin, nowadays many patients are on treatment with multiple daily injections or even insulin pumps for continuous administration of insulin. Besides, education of patients improved, and the treatment was more adjusted to the patients life style.

In contrast to the improvement of HbA1c, average blood pressure did not
change during the study period, although patients in the most recent years were treated more often with antihypertensive agents. Thus, it is unlikely that improvement in blood pressure control contributed to the decreased incidence of diabetic nephropathy. Admittedly one might suggest that antihypertensive treatment per se, independent of blood pressure levels could protect against the development of diabetic nephropathy. However, the case-control study argues against this conclusion: in the case-control study antihypertensive treatment rather than blood pressure level per se, was an independent risk factor for development of diabetic nephropathy, suggesting that a treated blood pressure level is not comparable to the same blood pressure level untreated. Obviously, data from a retrospective study cannot be used to determine the contributory role of various risk factors in the decreased incidence of diabetic nephropathy. Meanwhile, large prospective studies have clearly shown that intensified glucose control and strict antihypertensive therapy improved renal outcome in type 1 diabetes mellitus patients.

Some studies [12; 205; 214] but not all [200; 206; 208] have reported a declining incidence of diabetic nephropathy. A study of Bojestig et al. [12], showed a decline in incidence of diabetic nephropathy. This decline was associated with improved glycemic control, and not related to antihypertensive treatment, since only a very small number of patients without diabetic nephropathy were receiving such therapy. In a study by Hovind et al. [205], the decrease in the incidence of diabetic microangiopathy with increasing calendar year of diagnosis, was associated with improved glycemic control, earlier initiation of antihypertensive medication and lower blood pressure levels. Nordwall et al. [214] also reported a decrease in incidence of diabetic nephropathy. Although blood pressure values were not investigated, they suggest that the decreased incidence of diabetic nephropathy did not result from improved blood pressure regulation, as the decrease occurred before the wide spread use of ACE inhibitors and other antihypertensive medication. Other studies failed to show a decrease in incidence of diabetic nephropathy. Tryggvason et al. [208] failed to show a decline in the incidence of diabetic nephropathy in their nationwide study in Iceland. In this study, the development of diabetic nephropathy was associated with poor glycemic control. Rossing et al. [200] also failed to show a change in incidence of diabetic nephropathy. In their study the development of microalbuminuria was associated with poor glycemic control. No relationship with blood pressure or antihypertensive use was reported. Pambianco et al. [206] also failed to demonstrate a decline in diabetic nephropathy, although in their study, the incidence of end stage renal failure was declining among type 1 diabetes patients. Glycemic control and blood pressure regulation were not investigated.

Admittedly, our study has several limitations. We tried to select all current and historic type 1 diabetes patients controlled at the outpatient clinic of the Radboud University Nijmegen Medical Centre. As we selected our patients retrospectively there is a change that patients died prematurely, with the risk of selection bias. The patients with early diabetic nephropathy or those most at risk for developing diabetic nephropathy may have died prematurely, influencing the
results. If this bias is applicable, it would underestimate the improvement in the change in natural history of diabetic nephropathy, as it is likely that most selection bias occurred in the eldest cohorts. To correct for a possible effect of selection bias we analyzed the cohort in a case-control manner. In our case-control analysis, eleven control subjects died during follow-up after their reciprocal case developed diabetic nephropathy. These patients could have developed diabetic nephropathy. This can have weakened the results of this analysis. We defined nephropathy as two or more consecutive positive tests for urinary albumin (albustix® positive or timed urine collection). As the former is less sensitive than the latter, this tends to underestimate the cumulative proportion of diabetic nephropathy in the older cohorts compared with the younger cohorts. Despite this possible underestimation, the older cohorts showed an increased incidence of diabetic nephropathy.

Conclusions

Our study demonstrates a decrease in the cumulative incidence of diabetic nephropathy in type 1 diabetes mellitus over the past decades. This improved outcome is largely explained by the parallel improvement in glucose control. Our analysis revealed that blood pressure was also a risk factor for the development of diabetic nephropathy. Since average blood pressure levels did not decrease over the past decades, there may be opportunities to further reduce the risk of diabetic nephropathy by more aggressive lowering of blood pressure.
Chapter 11

Summary
Summary of the thesis

The presence of micro- and macrovascular complications determines to a great extent the prognosis of patients with type 1 diabetes mellitus. Besides being a prognostic factor for the development of end stage renal disease, the presence of diabetic nephropathy is also associated with an increased risk for the development of cardiovascular disease. Microalbuminuria, the presence of slightly elevated amounts of albumin in the urine, is currently the strongest predictor for the development of diabetic nephropathy.

To better understand the factors underlying the development of diabetic nephropathy, we have initiated a prospective study in 1994. One-hundred-and-forty-eight patients with type 1 diabetes mellitus, with a duration of diabetes of 5 to 12 years at inclusion, without micro- or macrovascular disease, were followed up to 13 years for the development of diabetic nephropathy. At entry, these patients underwent an extensive study protocol. This protocol was designed to investigate mechanisms possibly involved in the development of diabetic nephropathy.

The rationale for the study is described in chapter 1.

While awaiting the results of the prospective study, several descriptive and physiological studies were performed which are described in this thesis. In this summary, the results of these studies and their clinical implications will be discussed and put into perspective.

Descriptive and physiological studies

Chapter 2 describes an overview of the literature (2002) with regard to factors which are held responsible for the development of diabetic nephropathy.

Several hypotheses exist about the pathophysiology of diabetic nephropathy. Metabolic and haemodynamic changes both seem to play important roles in the development of diabetic nephropathy. Hyperglycemia seems crucial for the induction of metabolic changes, like, among others, the formation of advanced glycation end products, and the activation of protein kinase C and transforming growth factor-β. Others put forward the haemodynamic hypothesis, in which haemodynamic alterations provoke structural changes. Thusfar no single mechanism has been presented which can be held responsible for the development of diabetic nephropathy.

Increased glomerular filtration Early in the course of diabetes, the kidneys hypertrophy, and glomerular filtration is increased. This increased glomerular filtration is (partly) held responsible for the development of diabetic nephropathy, as it can lead to increased glomerular pressure. These haemodynamic changes may cause injury to the capillary wall, resulting in increased permeability, mesangial sclerosis and glomerular basement membrane thickening.

The cause of the glomerular hyperfiltration is unknown. Glomerular hyperfiltration is attributed to vasodilation predominantly at the afferent arteriole. Many
Summary

Humoral factors have been implicated in glomerular hyperfiltration. Recently, it has been proposed that the glomerular hyperfiltration finds its origin not primarily in the glomerulus, but in a primary increase in proximal tubular sodium reabsorption. This than leads to a systemic volume expansion. We investigated whether the primary event leading to systemic volume expansion is caused by a glomerular or a tubular event. Chapter 3 describes that increased proximal tubular sodium reabsorption contributes to the increased glomerular hyperfiltration. This study does not support the hypothesis that the increase in glomerular filtration results from systemic volume expansion or an increase in atrial natriuretic peptide.

Homocysteine Homocysteine has long been seen as a (causal) factor for atherosclerosis and as an independent risk factor for cardio-vascular disease. Despite the fact that type 1 diabetes mellitus is often complicated by vascular disease, plasma homocysteine levels are reduced in type 1 diabetes mellitus. Plasma homocysteine levels appear to be determined by glomerular filtration. As (early) type 1 diabetes mellitus is characterized by increased glomerular filtration, we investigated whether the increased glomerular filtration could contribute to reduced plasma homocysteine levels. Chapter 4 describes the results of an analysis in our cohort with regard to the influence of renal function on plasma homocysteine levels. In our study cohort, there was a negative correlation between glomerular filtration rate and plasma homocysteine levels, suggesting that plasma homocysteine levels are determined to a great extent by glomerular filtration. Recent interventional trials have shown that lowering plasma homocysteine by means of vitamin supplementation does not result in reduction of cardiovascular disease.

Atrial natriuretic peptide Administration of atrial natriuretic peptide (ANP) increases albuminuria by increasing glomerular pressure by vasodilation of the afferent glomerular arteriole. In our earlier experiments, the rise in albuminuria was accompanied by an increase in the urinary excretion of the low-molecular weight protein $\beta_2$-microglobulin. This led us to hypothesize that ANP might increase urinary albumin excretion not only by increasing glomerular pressure but also by attenuating tubular protein reabsorption. However, in chapters 5 and 6 we show that the abovementioned increase in low molecular weight proteinuria, after administration of ANP, is not the consequence of ANP itself, but of polygelines (Haemaccel®), the solvent used to prevent adherence of ANP to the infusion system. We found that even low dosages of Haemaccel® were able to block tubular reabsorption of low molecular weight proteins. The reabsorption of $\beta_2$-microglobulin is mediated by the megalin-cubulin complex, the system also used for tubular reabsorption of tubulo-toxic medication like aminoglycosides. Possibly, blockade of the megalin-cubulin complex by means of administration of low dosages of polygelines can result in increased therapeutic application of aminoglycosides, when administered together. Interestingly, the megalin-cubulin complex
is also found in the inner ear, possibly explaining the occurrence of ototoxicity of aminoglycosides. Therapeutic application of polyglylines for the prevention of aminoglycoside-induced ototoxicity and renal tubular toxicity has not been performed. This needs further investigation.

**Endothelial function** Endothelial dysfunction is thought to play an important role in the development of hypertension and atherosclerosis. Nitric oxide (NO), formerly known as endothelial derived relaxation factor, plays an important role in arteriolar vasodilation. Alterations in NO-synthase activity could lead to decreased vasodilation and hypertension. Chapter 7 describes the role of the Glu298Asp-polymorphism of the NO-synthase gene on basal NO production. The Asp-allele of the Glu298Asp-polymorphism is associated with reduced NO-production. Although marked differences in basal NO production were observed between the genotypes, no differences in basal forearm blood flow was observed. Apparently, other mechanisms compensate for the decreased basal NO production.

\[ \text{\textit{N}G\text{-monomethyl-L-arginine (L-NMMA)}} \] is widely used in venous occlusion plethysmography studies to determine basal NO production. However, the optimal dose to maximally inhibit NO production was unknown. Chapter 8 describes the results of a dose-response study of L-NMMA infusion to determine the L-NMMA dose that maximally reduces basal forearm blood flow. The maximal inhibiting dose of L-NMMA was 0.8 \( \text{mg} \cdot \text{min}^{-1} \cdot \text{dL}^{-1} \), a dose higher than most commonly used. At higher dosages of L-NMMA a systemic effect cannot be ruled out.

**Follow-up results of the PROFID-study**

After 13 years of follow up the cumulative incidence of diabetic nephropathy was 4% (6 out of 148 participants), while based on data from historical cohorts the expected incidence was 25-40% (30-60 participants). The hypotheses formulated at the beginning of the prospective study could therefore not be tested. Why did so few people develop diabetic nephropathy? Did we only include the patients with good metabolic control? What went wrong with the inclusion?

To elucidate whether this lower incidence of diabetic nephropathy reflects a change in the disease course of type 1 diabetes or is merely explained by selection bias or poor follow-up, we compared the study population with eligible, non-participating type 1 diabetes patients. The results of this evaluation are described in chapter 9. The data show a similar incidence of diabetic nephropathy in the PROFID-study population and in the eligible, type 1 diabetes patients who did not participate. Both populations were comparable with regard to age at onset of diabetes, gender, duration of diabetes at end of follow-up, and the percentage of smokers. The reduced incidence of diabetic nephropathy therefore does not reflect selection bias or poor follow-up, but suggests a real change in the disease course of diabetic nephropathy.
Summary

To investigate which factors might have contributed to this change in disease course of diabetic nephropathy, a study of the total historic and current type 1 diabetes mellitus population of the outpatient clinic of the Radboud University Medical Centre was carried out. Of the charts of 944 identified patients, 769 were eligible for this study. Chapter 10 describes the results of this study. In type 1 diabetes mellitus the cumulative incidence of diabetic nephropathy has decreased dramatically over the past decades. This improvement concurred with substantially improved glycemic control and intensified treatment with antihypertensive medication. Despite intensified treatment with antihypertensive medication, actual blood pressure levels remained virtually unchanged throughout the years. In the population study as well as a case-control analysis, elevated blood pressure levels are determinants for the development of diabetic nephropathy. Apparently there is opportunity for intensifying antihypertensive treatment. Perhaps this may lead to a further reduction of the incidence of diabetic nephropathy.

Conclusions

Over the years, the incidence of diabetic nephropathy has decreased dramatically. The PROFID-study is not able to answer the question which factors determine the development of diabetic nephropathy. From the population study we can conclude that improved glycemic control is an important factor in the decreased incidence of diabetic nephropathy. Although hypertension is an important factor in the development and progression of diabetic nephropathy, the average blood pressure remained virtually unchanged during the last decades despite intensified treatment. Apparently there is opportunity for intensifying antihypertensive treatment. Perhaps this may lead to a further reduction of the incidence of diabetic nephropathy.
Chapter 12

Samenvatting
Samenvatting

Niercomplicaties komen bij tot 40% van de mensen met type 1 diabetes mellitus (suikerziekte) voor. In de Verenigde Staten is diabetische nefropathie zelfs de belangrijkste reden voor het ondergaan van nierfuntievervangende behandeling (hemodialyse, peritoneaal dialyse). Eerdere onderzoeken hebben aangetoond dat goede instelling van de diabetes (metabole controle) het optreden van complicaties kan voorkomen of in ieder geval uitstellen. Niet alleen een goede instelling van de diabetes maar ook goede bloeddrukbehandeling kan het optreden van niercomplicaties voorkomen.

Er zijn echter vele patiënten die ondanks een goede metabole controle en goede behandeling van de bloeddruk toch niercomplicaties ontwikkelen. Ook zijn er patiënten die geen niercomplicaties krijgen ondanks matige instelling van de diabetes en slechte regulatie van de bloeddruk: andere factoren dan diabetesinstelling en bloeddrukbehandeling alleen moeten een rol spelen.

In de onderzoeken beschreven in dit proefschrift probeerden we de vraag te beantwoorden, welke factoren, naast bloedsuikerregulatie en bloeddruk, een rol spelen bij de ontwikkeling van niercomplicaties (diabetische nefropathie) bij type 1 diabetes mellitus.

Dankzij een subsidie van het Diabetesfonds Nederland, is hiertoe in 1994 een onderzoek gestart, waarbij honderdachtenveertig mensen met type 1 diabetes mellitus, zonder niercomplicaties, gedurende lange tijd (tot 13 jaar) gevolgd werden op de ontwikkeling van niercomplicaties. De studie heeft het acroniem PROFID-studie mee gekregen (PROgnostic Factors In Diabetic nephropathy). Bij deze mensen werd bij aanvang van het onderzoek een grote serie onderzoeken uitgevoerd, waarna deze deelnemers gedurende lange tijd gevolgd werden op de ontwikkeling van niercomplicaties. Aan het einde van het onderzoek zou nagekeken worden waarin de deelnemers die wel complicaties zouden ontwikkelen zouden verschillen van de deelnemers die na al die jaren geen complicaties ontwikkeld zouden hebben.

Bij aanvang van het onderzoek ondergingen de deelnemers een uitvoerige serie onderzoeken in een poging de volgende vraagstellingen te beantwoorden:

1. Kan de ontwikkeling van nierschade worden voorspeld door het meten van veranderingen in de nierdoorbloeding en de albumine-uitscheiding in de urine na toediening van atriaal natriuretisch peptide (ANP)? Hiertoe ondergingen de deelnemers een invasieve nierfuntie meting. Voor en na toediening van ANP werden filtratie, nierdoorbloeding en albuminerie gemeten.

2. Kan de ontwikkeling van nierschade worden voorspeld door het meten van de endotheelfunctie? De endotheelfunctie werd gemeten aan de hand van veranderingen in de doorbloeding van de onderarm tijdens infusie van diverse geneesmiddelen in de slagader van de onderarm.

3. Kan de ontwikkeling van nierschade worden voorspeld door het meten van de activiteit van het natrium-lithium countertransport, welke mogelijk een
Samenvatting

maat is voor erfelijke hoge bloeddruk? Hoge bloeddruk (hypertensie) blijkt vaak familair voor te komen. Toegenomen Na-Li-countertransport is gerasocieerd met hypertensie. Mogelijk dat een toegenomen activiteit van het Na-Li-countertransport in de erytrocyt (rode bloedcel) een vroege voor-speller is voor het krijgen van verhoogde bloeddruk.


In hoofdstuk 2 wordt een overzicht gegeven van de factoren die bijdragen aan de ontwikkeling van diabetische nefropathie. Zowel metabole als hemodynamische veranderingen spelen een rol.

Vaatverwijding speelt mogelijk een belangrijke rol in de ontwikkeling van nier-complicaties, omdat het aanleiding kan geven tot verhoogde druk in het microscopische vaatbed. Dit kan aanleiding geven tot een toegenomen lekkage van eiwit door de vaatwand. Inderdaad kan bij patiënten met type 1 diabetes mellitus (vaak) een toegenomen nierdoorbloeding worden vastgesteld. De oorzaak van deze toegenomen doorbloeding is onbekend. De toegenomen glomerulaire filtratie wordt toegeschreven aan een vaatverwijding voor het nierfilter (glomerulus). Dit leidt tot een toegenomen filtratie door het filter. Recent is echter de hypothese geopperd dat een verhoogde tubulaire natriumreabsorptie aanleiding geeft tot toegenomen glomerulaire filtratie. Hoofdstuk 3 beschrijft een mogelijke verklaring voor de toegenomen filtratie bij mensen met type 1 diabetes mellitus. Wij vonden aanwijzingen dat de toegenomen natriumreabsorptie leidt tot een toename van de nierdoorbloeding door activering van de tubulo-glomerulaire feedback. De toegenomen glomerulaire filtratie lijkt niet het gevolg te zijn van een systemische volumetoename of van een verhoogd atriaal natriuretisch peptide.

Van homocysteine is lange tijd gedacht dat het bijdraagt aan aderverkalking en dat het een onafhankeelijke risicofactor is voor het krijgen van hart- en vaatziekten. Ondanks dat type 1 diabetes mellitus ook gepaard gaat met een toegenomen incidentie van hart- en vaatziekten, blijkt bij mensen met type 1 diabetes mellitus de homocysteine-spiegel juist verlaagd. Hoofdstuk 4 beschrijft dat het lagere homocysteine-gehalte een gevolg is van de toegenomen nierfiltratie.

Voorheen werd er vanuit gegaan dat de primaire functie van ANP het veroorzaken van drukverhoging in het nierfilter was. ANP doet dit door het veroorzaken van vaatverwijding voor het filter en vaatvernauwing na het filter. In onze eerste studies werd echter aangetoond dat toediening van ANP leidde tot een verminderde opname van eiwit in de nertubuli. In hoofdstuk 5 en 6 wordt beschreven dat dit niet het gevolg is van ANP, maar van het gebruikte middel waarin ANP werd opgelost (Haemaccel). Haemaccel blijkt dus de tubulaire opname van stoffen,
zoals kleine eiwitten, te blokkeren. Dit zou zowel therapeutische als diagnostische doelen kunnen dienen.

Endotheeldysfunctie lijkt een belangrijke rol te spelen in de ontwikkeling van hypertensie en atherosclerose. Hoofdstuk 7 beschrijft de rol van een genetische variant van het stikstofmonoxide-synthetase (NO-synthase). Het blijkt dat mensen met een of meer Asp-allelen van het Glu298Asp-polymorfisme van het NO-synthasegene een vermindere basale NO-productie hebben. Toekomstig onderzoek moet uitwijzen of deze mensen ook een verhoogde kans hebben op het optreden van hypertensie of atherosclerose.

In eerdere studies is vaak gebruik gemaakt van \( \text{L-NMMA} \) (L-N-monomethyl-L-arginine) om de basale stikstooxide (NO) productie, hetgeen vaatverwijding veroorzaakt, te meten. Hiertoe is in vele onderzoeken gebruik gemaakt van het toedienen van opklimmende doseringen L-NMMA. Ook in de PROFID-studie werd L-NMMA gebruikt omde basale NO-productie te meten. De dosering waarbij de maximale blokkade van NO-productie plaatsvindt was echter onbekend. Hoofdstuk 8 beschrijft het onderzoek naar de dosis welke de maximale blokkade geeft van NO-productie. Hieruit blijkt dat alle tot dan toe uitgevoerde studies een te lage dosering L-NMMA hebben gebruikt. Wat verder opvalt is dat bij hogere doseringen een systemisch effect van L-NMMA niet kan worden uitgesloten.

Bij aanvang van de studie werd op grond van historische gegevens verwacht dat ongeveer 25-40 % van de deelnemers nefropathie zou ontwikkelen. De PROFID-studie heeft van 1994 tot 2007 gelopen. Gedurende die periode hebben zes mensen diabetische nefropathie ontwikkeld terwijl verwacht werd dat tussen de 30 en 60 deelnemers nefropathie zouden ontwikkelen. Hierdoor kunnen de uitgangsvragen vooralsnog niet worden beantwoord. Het is dus de vraag wat de oorzaak is van dit veel lagere aantal mensen met nefropathie in vergelijking met de historische gegevens. Hebben we alleen de mensen met een goede instelling weten in te sluiten in het onderzoek? Is er een probleem met de follow-up? Is het onderzoek nu mislukt?

Hoofdstuk 9 beschrijft een populatie-onderzoek waarbij de deelnemers aan de PROFID-studie worden vergeleken met mensen met type 1 diabetes mellitus die wel met het onderzoek mee konden doen, maar desondanks niet met het onderzoek hebben mee gedaan. Het blijkt dat beide groepen (deelnemers en niet-deelnemers) volledig vergelijkbaar waren ten aanzien van aantal mensen dat nefropathie heeft ontwikkeld, bloedsuikerregulatie, bloeddrukregulatie en rookgedrag. Ook was er geen verschil in sexe-verdeling. Het lijkt er dus op dat de deelnemers een goede representatie zijn van de populatie in zijn geheel. Daarnaast blijkt er sprake te zijn van een veel minder voorkomen van nefropathie dan in historische populaties beschreven is. Het in de onderzoekspopulatie waargenomen lage aantal complicaties lijkt dus reëel.

Om te onderzoeken welke factoren het verminderd optreden van nefropathie verklaren werden alle data geanalyseerd van alle bekende type 1 diabetes-patiënten in het UMC St Radboud. Wanneer deze patiëntengroep opgedeeld wordt in groepen naar jaar van ontstaan van de diabetes mellitus, dan blijkt er over de
jaren inderdaad een significante daling van het aantal gevallen van nefropathie te zijn. Verder valt op dat over de jaren de bloedsuikerinstelling sterk verbeterd is. Opmerkelijk genoeg blijkt de gemiddelde bloeddruk over de jaren nagenoeg onveranderd te zijn. Wel worden patiënten vaker behandeld met bloeddrukverlagende medicijnen. Deze populatie is ook nog op een andere manier geanalyseerd: voor iedere patiënt die nefropathie ontwikkeld heeft (Case), is er een gematchte patiënt, zonder nefropathie, bijgezocht (Controle). Wanneer beide groepen met elkaar vergeleken worden, dan blijkt dat de cases een slechtere bloedsuikerregulatie te hebben dan de controles. De bloeddrukwaarden van de cases bleek hoger te zijn dan die van controles. Ook werden cases vaker behandeld met bloeddrukverlagende medicatie. Uit een multi-variate analyse blijkt dat bloedsuikerregulatie en het krijgen van bloeddrukverlagende medicatie de belangrijkste voorspellers zijn voor de ontwikkeling van diabetische nefropathie. Deze resultaten staan beschreven in hoofdstuk 10.

Conclusie

Over de jaren is het aantal mensen met type 1 diabetes mellitus dat nefropathie ontwikkelt, aanzienlijk gedaald. De PROFID-studie kan geen uitspraak doen over de mogelijke oorzaken van diabetische nefropathie. Uit het populatie-onderzoek blijkt dat betere bloedsuikerregulatie een belangrijke oorzaak is van de afname in de incidentie van diabetische nefropathie. Hoewel een verhoogde bloeddruk bijdraagt aan het ontstaan van diabetische nefropathie, is de gemiddelde bloeddruk de afgelopen jaren niet gedaald. Door betere behandeling van verhoogde bloeddruk kan het aantal gevallen van diabetische nefropathie mogelijk nog verder afnemen.
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Dankwoord

Er staat slechts één naam voor op dit proefschrift. Het is echter tot stand gekomen door de bewuste, maar vaak ook onbewuste medewerking en steun van velen. Zonder iemand te kort te doen wil ik enkelen noemen. Uitputtend kan de opsomming niet zijn. Te veel mensen hebben bijgedragen: Honderden, nee, de meer dan duizend patiënten die hebben bijgedragen aan de totstandkoming van dit proefschrift. Velen hebben kostbare tijd geïnvesteerd in de resultaten zoals beschreven in het proefschrift. Het aantal infusen dat zij hebben gekregen, de hoeveelheid bloed en urine dat van hen is nagekeken, gaat de verbeelding te boven. De persoonlijke contacten tijdens de vaak langdurige metingen waren altijd weer interessant.

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ook gezellige babbels op de klinische fysiologie hebben de onderzoekstijd tot een mooie gemaakt. Hans Schepkens gaf het onderzoek een zuidelijk, internationaal karakter. Marloes, je hebt me ingehaald.


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Dankwoord

Nynke, Mendel en Carlijn, doorploeteren tot in de kleine uurtjes is er nu wel vanaf, en dat is maar goed ook.

Ingeborg, mijn rots in de branding: later is nu (echt?) begonnen.
Curriculum Vitae


De opleiding tot internist werd op 1 januari 2007 afgerond. Hierna volgde de opleiding in het aandachtsgebied Nefrologie, gedeeltelijk in het Universitair Medisch Centrum St. Radboud (Opleider Prof. Dr. J.H.M. Berden), gedeeltelijk in het Canisius Wilhelmina Ziekenhuis (Opleider Dr. M. ten Dam). Sinds 1 maart 2008 is hij werkzaam als internist-nefroloog in het Canisius Wilhelmina Ziekenhuis te Nijmegen.
