Novel approaches in diabetic nephropathy

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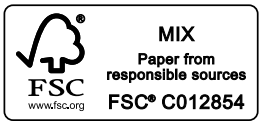

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CONTENTS

Chapter 1

Introduction

The prevalence of type 2 diabetes mellitus is increasing due to the combination of ageing of the population and the pandemic increase in overweight and obesity [1, 2]. About one third of patients with diabetes develops diabetic nephropathy, which is the most common cause for chronic kidney disease [3]. This is associated with a huge burden of disease, in terms of human suffering as well as health care expenditure [4, 5]. A better knowledge of risk factors for diabetic nephropathy and early detection of patients at risk for diabetic renal disease, as well as early initiation of appropriate treatment could reduce the morbidity and mortality associated with this major health problem.

Pathogenesis of diabetic nephropathy

The pathogenesis of diabetic nephropathy is complex, and essentially driven by exposure of the kidneys to an altered internal milieu, that triggers multiple pathways of disease [6, 7]. Within the kidney, hyperglycemia induces glomerular hyperfiltration and endothelial dysfunction, leading to changes in basement membrane properties, intraglomerular cellular hypertrophy and hyperplasia, nodular intercapillary glomerulosclerose (Kimmelstiel-Wilson lesion), and glomerular matrix changes, clinically often detectable by albuminuria [8, 9]. This is usually associated with volume expansion and hypertension, due to increased tubular sodium reabsorption. The increased tubular protein load, by uptake in proximal tubular cells, triggers a pro-inflammatory tubulo-interstitial response leading to progressive tubulo-interstitial inflammation and fibrosis, which is also accelerated by hyperglycemia itself [10, 11]. In patients with diabetes, an inappropriately elevated activity of the renin-angiotensin-aldosterone system (RAAS) also plays a main role in these processes by its effect on systemic and glomerular pressure, its effect on sodium handling, and by direct pro-fibrotic effects of angiotensin II and aldosterone [12, 13].

Futhermore, glycation of tissue proteins is thought to contribute to development of diabetic nephropathy and other microvascular complications. In hyperglycemic states, excess glucose binds to free amino acids on circulating or tissue proteins, leading to a nonenzymatic reactions resulting in reversible glycation products, and in a later stage in advanced glycation endproducts (AGEs), stable and often long living molecules, which can be found not only in the circulation, but also in many

tissues, including the kidney [14]. Interaction between AGEs and AGE-receptors plays a role in induction of inflammation and endothelial dysfunction [15]. Thus, tissue accumulation of AGEs is not just a measure of prevailing hyperglycemia, but also represents cumulative metabolic burden, oxidative stress (overload of superoxides and cellular pseudohypoxia) and inflammation [16]. Interestingly, AGE accumulation has been shown to precede and correlate with early manifestation of renal disease in diabetes [17, 18].

Genetic risk factors for diabetic nephropathy

In addition to the well-known risk factors of diabetic nephropathy (age, obesity, smoking, hypertension and duration of diabetes), genetic factors have been suggested to be important determinants of the predisposition to diabetic nephropathy [19, 20]. Evidence from family-based linkage studies showed that diabetic nephropathy is more prevalent in some families while other families seem protected and led to identify susceptibility loci and map them to different chromosomes [21, 22]. A few candidate genes, such as Angiotensin-converting enzyme (ACE) gene, endothelial nitric oxide synthase (NOS3) gene, Erythropoietin (EPO) gene, and Apolipoprotein L1 (APOL1) gene have been reproducibly associated with diabetic nephropathy and recent genome-wide linkage studies have also identified chromosomal loci for susceptibility genes, including 3q, 7q, 10p, 14q and 18q [23-26]. Although the individual contribution of these genes to the variability in incidence of diabetic nephropathy appears to be small, genetic studies may still provide valuable information regarding the pathophysiology of nephropathy and potential targets for its treatment [27].

CNDP1

Several candidate genes provide promising prospects. Recently, an allelic variant within the leader peptide of carnosinase gene (CNDP1) was suggested to be associated with diabetic nephropathy by influencing the carnosine pathway [28]. This gene encodes the enzyme serum carnosinase, which hydrolyses the substrate L-carnosine (β-alanyl-L-histidine). Carnosine is a dipeptide that is present intracellularly in most tissues, with the highest levels found in muscles, where it is released into the serum. L-Carnosine can also be derived from food intake [29]. Carnosine was

first described to function as a natural inhibitor of angiotensin-converting enzyme (ACE) and advanced glycation endproducts (AGEs), as well as a scavenger of radical oxygen species (ROS) [30, 31]. Cell culture and animal experiments have shown that L-Carnosine can influence glucose metabolism and inhibit effects of high glucose on mesangial cells, and could thereby play a role in the cascades of inflammation and oxidative stress which are supposed to contribute to the development of microvascular complications in diabetes [28, 32]. The gene variant with the lowest number of leucine repeat (5L-5L) in CNDP1 gene has been found to be associated with a lower prevalence of diabetic nephropathy compared to variants with more leucine repeats (i.e., 5L-6L, 6L-6L, 6L-7L, 5L-7L or 7L-7L) [28, 33]. However, data are conflicting and prospective, longitudinal studies on this gene are scarce [34].

CCR2

Genetic variation in chemokine pathways has also been shown to modulate the outcome in inflammation-driven vascular disease, including diabetic nephropathy, as demonstrated for gene of the chemokine receptor-5 (CCR5) [35]. CCR2 is another chemokine receptor which may act as coreceptor for macrophage [36]. Genetic variation in CCR2, i.e CCR-V64I is suggested to have a role in the inflammatory pathway by modulating monocyte chemoattractant protein-1 (MCP-1)-receptors, hence modifying cardiovascular outcome in several populations [37, 38]. This SNP has however not yet been studied in patients with diabetes.

Prediction and diagnosis of diabetic nephropathy

Although many risk factors for developing diabetic nephropathy have been identified, none is yet sufficiently predictive in the individual patient. Microalbuminuria, defined as urinary albumin excretion between 30 and 300 mg/day, or albumin creatinine ratio of 2.5 to 25 mg/mmol in males and 3.5 to 35 mg/mmol in females in a random urine sample, is the earliest detectable sign of diabetic nephropathy and is also a predictor of risk of progressive renal disease and mortality in patients with type 1 and type 2 diabetes [39, 40]. Although guidelines recommend the use of albuminuria as a screening tool for renal disease in diabetes, its specificity is still a subject of debate [41], since especially in subjects with type 2 diabetes, (micro)albuminuria

is also associated with general vascular damage. Thus, when albuminuria is already established, it is a sign of substantial organ damage, not only of the kidneys, but also of other organs such as the heart and the arterial vascular system.

Alternative non-invasive methods that may detect diabetic nephropathy at earlier stages would be beneficial to start early treatment and thereby to delay or prevent progression of chronic kidney disease. In the last decades, novel urinary markers of renal damage such as kidney injury molecule-1 (KIM-1), α-1 microglobulin and neutrophil gelatinase-associated lipocalin (NGAL), have been discovered and investigated as potential predictors of acute and chronic kidney disease [42-44].

Additionally, urinary proteome analysis, as measured by Capillary Electrophoresis coupled Mass Spectrometry (CE-MS), is a non invasive means to identify biomarkers of early damage in many conditions, including renal disease [45, 46]. The urinary proteome contains an array of stable low molecular proteins and peptides, which could function as a powerful diagnostic and prognostic tool in chronic kidney disease and diabetic nephropathy [47, 48]. In a recent study, 65 biomarkers of diabetic nephropathy were identified with urinary proteome analysis using CE-MS. This model of 65 biomarkers, independently validated in another cohort (n=70), correctly identified patients with diabetic nephropathy with 97% sensitivity and specificity [49]. Confirmation of the diagnostic value of the identified urine biomarkers is however necessary before the assessment of urinary proteome can be used in clinical practice [50].

Treatment of diabetic nephropathy

Tight glycaemic control has been shown to reduce the risk for development of albuminuria in patients with diabetes, as shown in several controlled trials in type 1 and type 2 diabetes [51, 52]. However, once established, glomerular lesions and albuminuria are poorly reversible and glycaemic control has, therefore, only limited influence on progression to end-stage renal disease (ESRD) once albuminuria is present [53].

Blockade of the RAAS by angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin receptor blockers (ARBs) is currently the cornerstone of preventing the progression of overt diabetic nephropathy to ESRD [54-56]. Reductions in relative

risk for decline in renal function were approximately 50% with ACE-I treatment in patients with type 1 diabetes (25/207 in the Captopril group compared to 43/202 in the placebo group) [55]. These risk reductions were considerably less – approximately 15% – for the end-point of doubling of serum creatinine or reaching ESRD in type 2 diabetic patients with diabetic nephropathy (327/751 in the Losartan group compared to 359/762 in the placebo group) [57].

Several strategies to improve the efficacy of RAAS-blockade-based regimens have been proposed, including higher dosing, and dual or even triple RAAS-blockade [58-60]. However, the long term effects of such combinations on renal or cardiovascular outcomes have not yet been evaluated in clinical trials and they are associated with increased risk for hyperkalemia. Taken together, the current data seem to indicate that intensification of reduction of blood pressure and albuminuria has its limits, and accordingly, there is a need for further adjunctive treatments, interfering with pathways of disease not targeted with current therapy, that can prevent progressive decline in renal function in patients with diabetic nephropathy, in type 2 diabetes in particular.

In animal and experimental studies, benfotiamine, a lipid-soluble thiamine derivative, has been shown to be able to block three major pathways of hyperglycemic damage: the hexosamine pathway, AGE formation pathway and the diacylglycerol (DAG)-protein kinase C (PKC) pathway. Benfotiamine has also been shown to block hyperglycemia-associated pro-inflammatory transcription factor NF-κB activation [61]. These pathways, when activated by hyperglycemia, result in accumulation of triphosphates and overproduction of superoxides, inducing metabolic pseudohypoxia, mitochondrial dysfunction and oxidative stress in renal and endothelial cells, finally leading to vascular damage [7]. Thiamine and benfotiamine inhibited these pathways by activating the enzyme transketolase (TK), which converts glyceraldehydes-3-phospate and fructose-6-phosphate into pentose-5-phosphates and other sugars. It has therefore been suggested that thiamine and benfotiamine are able to oppose diabetic vascular complications, including diabetic nephropathy [61, 62]. An animal study performed in 2003 has shown that high dose of thiamine and benfotiamine prevented microalbuminuria and proteinuria [62]. A recent pilot study has suggested a beneficial effect of a three-month treatment with thiamine on urinary albumin

excretion in Pakistani patients with type 2 diabetes and microalbuminuria [63]. Randomized controlled trials in patients with diabetes are however necessary to answer the question whether benfotiamine will decrease albuminuria or ameliorate the effects of both hyperglycemia and albuminuria on AGE accumulation, and thereby decrease inflammatory and fibrotic responses, whereby it could potentially slow down the progression to ESRD.

Aim of the thesis

In this thesis, several novel risk prediction and intervention aspects in diabetic nephropathy are evaluated. First, genetic variations in CNDP1 and CCR2 genes and their associations with progression of diabetic renal disease and/or mortality in patients with diabetes were investigated. Second, the value of urinary proteomic biomarkers as a diagnostic tool for identifying patients with diabetic nephropathy was investigated. Third, a randomized controlled trial was performed to investigate whether treatment with benfotiamine could be a beneficial intervention for diabetic nephropathy in patients with type 2 diabetes.

Outline of the thesis

In **Chapters 2 and 3**, two prospective studies on CNDP1 gene were performed in order to investigate the associations of this polymorphism with progression of diabetic renal disease (progression of decline in renal function or development of ESRD) and/or mortality in patients with diabetes. It was also explored whether the association between 5L-5L genotype of CNDP1 gene and diabetic nephropathy is sex-specific, as suggested by a recent cross-sectional study [64].

In **Chapter 2**, these questions were investigated in a multi-center cohort of European patients with type 1 diabetes from Denmark, Finland and France (EURAGEDIC study). In this study, 2086 patients were included. 916 patients had diabetic nephropathy (cases) and 1170 patients had diabetes without nephropathy (controls). These patients were prospectively followed for a median period of 8.8 years. In **Chapter 3**, the same questions were investigated in patients with type 2 diabetes from the Zwolle Outpatient Diabetes Integrating Available Care (ZODIAC) study. This study

was initiated in 1998 as a large shared-care diabetes project in which hospital-based nurses specialized in diabetes assist general practitioners in caring for patients with type 2 diabetes. From a total of 1143 patients, blood samples from 913 patients were available for future research, including genetic studies. Data on mortality (total and cardiovascular mortality) were gathered in 2008 after a median follow-up period of 9.6 years.

In **Chapter 4,** the association between the CCR2-V64I polymorphism and the development of diabetic nephropathy was investigated. It was also investigated to which extent this SNP adds to the risk carried by the well known ACE I/D polymorphism. For this purpose, we performed a prospective analysis in 1128 patients from the BErgamo NEphrologic Diabetic Complications Trial (BENEDICT) – a prospective randomized trial evaluating the effect of ACE-I on new-onset microalbuminuria (albuminuria 20-200 µg/min) in hypertensive type 2 diabetes patients without albuminuria (<20 µg/min) at inclusion. In total, 1124 patients were genotyped according to ACE I/D and CCR2-V64I polymorphisms and followed until the development of new-onset microalbuminuria during a median period of 42 months.

In **Chapter 5**, the diagnostic value of urinary proteomic analysis in diabetic nephropathy was validated by investigating the diabetic nephropathy biomarker classifier derived from 65 biomarkers, which were identified in a previous study [49]. This was performed in urine of 148 Caucasian patients with type 2 diabetes from Zwolle (The Netherlands), Graz (Austria) and Prague (Czech Republic), who participated in a case-control study. For the diagnosis of diabetic nephropathy, urinary proteome analysis was successfully applied in a pilot study. We therefore aimed to confirm whether this tool could adequately identify subjects with diabetes nephropathy.

In **Chapters 6 and 7**, It was investigated whether a three-month treatment with benfotiamine would be able to prevent progression of diabetic nephropathy in patients with type 2 diabetes. For this purpose, a randomized, double-blind, placebo-

controlled clinical trial was performed in patients with type 2 diabetes and albuminuria to examine the effects of benfotiamine on urinary albumin excretion and excretion of urinary markers of tubular damage **(Chapter 6)**. It was also investigated whether benfotiamine treatment reduced the formation of AGEs, markers of endothelial damage and inflammation **(Chapter 7)**.

In **Chapter 8**, I summarize the results of all performed studies. I also discuss the implications, strengths and limitations, as well as future perspectives of the studies presented in this thesis.

In **Chapter 9**, a summary of all chapters is presented in Dutch.

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Chapter 2

A polymorphism in the gene encoding carnosinase (CNDP1) as a predictor of mortality and progression from nephropathy to end-stage renal disease in type 1 diabetes

Alkhalaf A, Bakker SJL, Bilo HJG, Gans ROB, Navis GJ, Postmus D, Forsblom D, Groop PH, Vionnet N, Hadjadj S, Marre M, Parving HH, Rossing P, Tarnow L

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ABSTRACT

Background

Homozygosity for a 5-leucine repeat (5L–5L) in the carnosinase gene (*CNDP1*) was found to be cross-sectionally associated with a low frequency of diabetic nephropathy (DN), mainly in type 2 diabetes. We prospectively investigated in patients with type 1 diabetes whether: (1) 5L–5L is associated with mortality; (2) there is an interaction of 5L–5L with DN or sex for prediction of mortality; and (3) 5L–5L is associated with progression to end-stage renal disease (ESRD).

Methods

In this prospective study in white European patients with type 1 diabetes, individuals with DN were defined by persistent albuminuria ≥ 300 mg/24 h. Controls without nephropathy were defined by persistent (>15 years) normoalbuminuria <30 mg/24 h. Leucine repeats were assessed with a fluorescent DNA analysis system. Onset of ESRD was defined by need to start chronic dialysis or kidney transplantation.

Results

The study involved 916 patients with DN and 1170 controls. During follow-up for 8.8 years, 107 patients (14%) with 5L–5L died compared with 182 patients (13.8%) with other genotypes (p=0.99). There was no significant interaction of 5L–5L with DN for prediction of mortality (p=0.57), but a trend towards interaction with sex (p=0.08). In cases with DN, HR for ESRD in 5L–5L vs other genotypes was not constant over time, with increased risk for 5L–5L beyond 8 years of follow-up (p=0.03).

Conclusions

CNDP1 polymorphism was not associated with mortality, and nor was there an interaction of this polymorphism with DN for prediction of mortality in patients with type 1 diabetes. *CNDP1* polymorphism predicts progression to ESRD in patients with DN, but only late after baseline measurements.

INTRODUCTION

Diabetes is the leading cause of end-stage renal disease (ESRD) in the western world. In the last decade, diabetic patients in the USA and Europe accounted for 40% and 26% of the populations receiving dialysis, respectively [1-3]. Poor glycaemic control and high blood pressure are acknowledged contributors to the pathophysiology of diabetic nephropathy (DN) [4], but epidemiological and familial studies have suggested genetic predisposition to be involved as well [5-7]. Analyses of family-based studies in Turkish, Pima Indian and African-American patients with type 2 diabetes have mapped a major susceptibility locus to chromosome 18 [8, 9]. In a cross-sectional study in patients with type 1 and type 2 diabetes, this locus was identified as the carnosinase gene 1 (*CNDP1*); homozygosity for five copies of a trinucleotide repeat encoding leucine (5L–5L) in this gene was found to be more common in patients without DN than in those with DN [10]. This finding was confirmed by findings from other cross-sectional studies in patients with type 2 diabetes [11, 12] and has been suggested to be consistent with a protective role of the 5L–5L genotype against DN, particularly in women [13]. However, these findings from cross-sectional studies could be subject to selection bias. They could, for instance, be explained by a possible interaction of 5L–5L genotype with DN, sex or both for prediction of mortality. Survival (dis)advantages in subgroups may induce false associations in cross-sectional studies [14].

In this observational study in a white European cohort of patients with type 1 diabetes, we therefore aimed to investigate prospectively: (1) the association of *CNDP1* gene polymorphism with mortality; (2) a potential interaction of this gene polymorphism with DN or sex for prediction of mortality; and (3) an association of this gene polymorphism with development of ESRD.

METHODS

Patients

The present study included patients from three European coordinating centres in Denmark, Finland and France. All the individuals included were of European descent. In Denmark between 1993 and 2000, all Danish patients with type 1 diabetes attending the outpatient clinic at Steno Diabetes Center were invited to participate in a study of genetic risk factors for micro- and macrovascular complications of diabetes [15]. In France and Belgium, 17 diabetes clinics participated in a study of the genetic risk factors for diabetes complications between 1994 and 2001, which included eligible patients with type 1 diabetes [16, 17]. In Finland, between 1994 and 2002, patients with type 1 diabetes were recruited from 56 referral centres to participate in the prospective Finnish Diabetic Nephropathy Study (FinnDiane).

Type 1 diabetes was considered present if the age at onset of diabetes was ≤ 35 years and the time to definitive insulin therapy ≤ 1 year. Established diabetic nephropathy (cases) was defined by persistent albuminuria (>300mg/24h or >200µg/min or >200mg/l) in two out of three consecutive measurements made on sterile urine samples, after >5 years' diabetes duration. In patients using ongoing regimens of angiotensin-converting enzyme inhibitor (ACE-I) or angiotensin-receptor blocker (ARB), the last measurements of urinary albumin excretion before treatment initiation were used for classification. Patients with clinical suspicion of non-diabetic renal or urinary tract disease were excluded. Absence of diabetic nephropathy (controls) was defined as persistent normoalbuminuria (urinary AER [uAER] <30 mg/24 h or <20 µg/min or <20mg/l) after at least 15 years of diabetes in patients not treated with ACE-Is or ARBs.

The study was performed in accordance with the Helsinki Declaration. The local ethics committee approved the study and all patients gave their informed consent.

Baseline clinical laboratory investigations

All patients had blood samples and phenotypic characteristics collected as part of the European Rational Approach for the Genetics of Diabetic Complications (EURAGEDIC) project [18]. Blood pressure was measured twice in the resting state. HbA_{1c} was

determined by standard HPLC techniques with normal values in the range 4.1% to 6.4%. Plasma creatinine concentration was determined by modified Jaffe's method. Timed urine collections were used to obtain uAER. Diabetic retinopathy was assessed by fundus photography or direct ophthalmoscopy carried out by an experienced ophthalmologist. Based on standardised questionnaires, former or current smokers of one or more cigarettes/cigars/pipes per day were classified as smokers and all others as non-smokers. Non-fatal cardiovascular disease was considered present in patients with a history of admission for stroke, myocardial infarction or vascular amputations. DNA material was genotyped as part of the EURAGEDIC project as follows. Genomic DNA was isolated from human leucocytes using standard methods. D18S880 marker genotyping was performed at the French National Genotyping Centre using an automated high-throughput method. All liquid handling was performed robotically in 384 well plates with a BasePlate Robot (The Automation Partnership, Royston, UK). PCR amplification was carried out in a 5µl volume with 5 ng of genomic DNA and primers AGGCAGCTGTGTGAGGTAAC (forward) labelled with the fluorescent dye Fam and GGGTGAGGAGAACATGCC (reverse) using a standard protocol. The annealing temperature was 55°C. Fluorescent PCR products were analysed on a MegaBace TM 1000 Sequencer (Amersham Biosciences, Buckinghamshire, UK) using appropriate software. Automatic genotyping was performed using Genetic Profiler software (version 3.1). Before statistical analysis, rigorous genotype quality assurance was performed to ensure accurate binning of alleles. Three alleles were observed with fragment sizes of 167, 170 and 173 base pairs corresponding to 5-, 6- and 7-leucine repeats respectively. The 5L–5L homozygous genotype was compared with all other genotypes (i.e. genotypes with six or more leucine repeats).

Follow-up

In this prospective observational study, patients were followed: until an endpoint was reached; to the last visit at the outpatient department; or until 1 September 2006 for the Danish population, 1 December 2009 for the Finnish population or 1 February 2007 for the French population. The endpoints were all-cause mortality and ESRD, defined as need to start chronic dialysis or kidney transplantation. All patients were traced through the national register. If a patient died before the last update, the date

of death was recorded. Information about date of ESRD was obtained from patient records or discharge letters from other hospitals.

Statistical analysis

Variables with normal distribution are presented as mean ± SD and variables with skewed distribution were log-transformed before analysis and presented as median and interquartile range (IQR). For variables with normal distribution, comparisons between groups were performed using unpaired Students t tests, whereas for variables with skewed distribution Mann–Whitney *U* tests were used. χ^2 tests were used to compare non-continuous variables. Time-to-event analyses were performed using Kaplan–Meier plots and logrank testing. Tests for a non-zero slope of scaled Schoenfeld residuals on functions of time were performed to explore the proportional hazards (PH) assumption in Cox regression models [19]. If the PH assumption was met (test based on Schoenfeld residuals $p > 0.05$), Cox regression models were used to estimate unadjusted and adjusted hazard ratios with 95% confidence intervals. Otherwise, Cox models with time-dependent covariates were used to calculate HRs over time [20]. After an initial crude analysis, two subsequent models were constructed in which the associations were adjusted for potential confounders and covariates defined a priori. In the first multivariate model, adjustment was performed for age, sex and centre of inclusion. In the other model, further adjustment was performed for duration of diabetes, HbA_{1c}, blood pressure, plasma creatinine and uAER. In this study, we were able to detect a genotype relative risk for mortality of 1.2 with a power of 95% at the level of significance $p = 0.05$. In those with DN, we were able to detect a genotype RR for ESRD of 1.3 with a power of 95% at $p= 0.05$. The PS program of Dupont and Plummer [21] was used to calculate power. Analysis of the Danish, Finnish and French populations separately gave comparable results, and thus pooled data are presented.

A two-tailed p value of 0.05 or less was considered statistically significant. Statistical analyses were done by using a commercially available program (SPSS for Windows, version 16.0, Chicago, IL, USA).

RESULTS

Baseline characteristics

In total, 2487 patients (900 from Denmark, 687 from France and 900 from Finland) were included. The *CNDP1* genotype could not be determined in 401 patients: DNA samples from 74 patients from France were missing, and poor-quality DNA samples were obtained from 65 patients from Denmark, 88 patients from France and 174 from Finland. The baseline characteristics of these patients were not significantly different from those who were genotyped. Table 1 shows the baseline characteristics of the study population (n = 2086). Patients with DN were younger, more commonly men, and had higher HbA_{1c}, blood pressure and serum creatinine levels than controls. The frequency of the 5L–5L genotype was not significantly different between patients with DN and patients without DN [22] and also not between men and women. The OR for the presence of DN according to 5L–5L genotype was 0.99 (95% CI 0.82-1.18) for the whole population, with an OR of 1.05 (95% CI 0.83-1.34) for men and 0.90 (95% CI 0.69-1.18) for women (p = 0.40 for interaction).

Prospective analyses for mortality

Median (IQR) follow-up was 8.8 (6.1-10.5) years. Of patients with DN, 81 of 334 patients (24.3%) with 5L–5L died during the follow-up vs 142 of 582 patients (24.4%) with other genotypes (logrank test p =0.73). In controls without DN, these numbers were 26 of 430 (6.0%) and 40 of 740 (5.4%), respectively (logrank test p = 0.66). The PH assumption for Cox regression was met (test based on Schoenfeld residuals, p = 0.17). After adjustment for potential confounders, including age, sex, blood pressure and history of cardiovascular events in Cox regression analyses, HR for mortality in 5L–5L vs other genotypes was 0.95 (95% CI 0.73-1.22, p = 0.67).

There was no significant interaction between 5L–5L and presence of DN for prediction of mortality (p = 0.57). However, there was a trend towards interaction between 5L–5L and sex for prediction of mortality (p = 0.08). After stratification for sex, adjusted HR for mortality in 5L–5L vs other genotypes was 0.87 (95% CI 0.62-1.21, p = 0.41) in men and 1.31 (95% CI 0.87-1.98, p=0.31) in women.

Table 1: Baseline characteristics of the study population

Characteristic	Denmark (*n*= 835)		France (*n*= 525)		Finland (*n*=726)		Combined population (*n*=2086)		*p* value
	Cases *n*= 426	Controls *n*= 409	Cases *n*= 226	Controls *n*= 299	Cases *n*= 264	Controls *n*= 462	Cases *n*= 916	Controls *n*= 1170	
Sex (men/women)	257/169	213/196	131/95	151/148	156/108	184/278	544/372	548/622	<0.001
Age (years)	42.0 ± 10.2	45.3 ± 11.6	42.5 ± 11.3	45.1 ± 12.1	39.8 ± 9.4	42.9 ± 10.3	41.5 ± 10.3	44.3 ± 11.3	<0.001
Diabetes duration (years)	28.1 ± 8.7	27.8 ± 10.1	26.7 ± 8.7	29.3 ± 8.8	28.1 ± 7.9	29.5 ± 8.1	27.7 ± 8.5	28.9 ± 9.1	<0.01
BMI (kg/m^2)	24.2 ± 3.3	24.1 ± 3.1	24.3 ± 3.7	24.1 ± 3.0	25.9 ± 3.8	25.1 ± 3.1	24.7 ± 3.6	24.5 ± 3.1	0.19
Smokers *n* (%)	169 (39.7)	147 (35.9)	114 (50.4)	107 (35.8)	148 (56.1)	163 (35.3)	431 (47.1)	417 (35.6)	<0.001
Cardiovascular disease *n* (%)	42 (9.9)	15 (3.7)	28 (12.4)	17 (5.7)	42 (15.9)	30 (6.5)	112 (12.2)	62 (5.3)	<0.001
Systolic BP (mmHg)	145 ± 22	134 ± 19	144 ± 21	128 ± 15	143 ± 19	133 ± 17	144 ± 21	132 ± 17	<0.001
Diastolic BP (mmHg)	83 ± 12	76 ± 9	82 ± 10	72 ± 9	83 ± 10	78 ± 8	83 ± 11	76 ± 9	<0.001
Anti-hypertensive medication *n* (%)	295 (69.2)	66 (16.1)	177 (78.3)	56 (18.7)	247 (93.6)	93 (20.1)	719 (78.5)	215 (18.4)	<0.001
RAAS-blockade *n* (%)	259 (60.8)	36 (8.8)	156 (69.0)	50 (16.7)	225 (85.2)	67 (14.5)	640 (69.9)	153 (13.1)	<0.001
HbA_{1c} (%)	9.4 ± 1.5	8.4 ± 1.1	8.8 ± 1.6	8.4 ± 1.2	9.4 ± 1.6	8.1 ± 1.2	9.2 ± 1.6	8.2 ± 1.2	<0.001
Plasma creatinine (µmol/l)	102 (82–135)	79 (71–87)	127 (97–204)	79 (62–89)	127 (97–181)	84 (77–94)	114 (89–165)	81 (72–91)	<0.001
eGFR-MDRD (ml min^{-1} 1.73m^{-2})	65 (47–88)	86 (76–96)	48 (29–70)	87 (74–109)	55 (34–71)	77 (67–88)	58 (37–79)	82 (72–95)	<0.001
uAER (mg/24 h)	593 (253–1524)[a]	7 (4–12)	551 (175–1452)[a]	6 (3–12)	564 (193–1399)[a]	8 (5–14)	571 (221–1498)	7 (4–12)	<0.001
5L–5L genotype *n* (%)	160 (37.6)	139 (34)	86 (38.1)	122 (40.8)	88 (33.3)	169 (36.6)	334 (36.5)	430 (36.8)	0.89

Data are presented as mean±SD or median (IQR) unless otherwise indicated

With the exception of BMI, smoking habits, HbA_{1c}, cardiovascular disease and anti-hypertensive medication, all covariates show significant heterogeneity across populations, $p \leq 0.01$

[a]Some patients with previously persistent macroalbuminuria receiving treatment with anti-hypertensive agents had values <300 mg/24 h at the time of inclusion

eGFR-MDRD, estimated glomerular filtration rate according to Modification of Diet in Renal Disease formula; RAAS, renin–angiotensin–aldosterone system

Prospective analyses for end-stage renal disease

No events occurred in controls without nephropathy. We therefore limited further analyses to patients with DN (*n* = 916). At baseline, 52 patients were already diagnosed with ESRD and were not included in the prospective analyses.

During follow-up, 77 out of 312 patients (24.7%) with 5L–5L developed ESRD vs 121 of 552 (21.9%) patients with other genotypes. Figure 1 shows a Kaplan–Meier plot of development of ESRD according to *CNDP1* genotype (logrank test p =0.57). The PH assumption for Cox regression was not met (p = 0.02). Further Cox-regression analysis with a time-dependent covariate showed that the hazard ratio for ESRD was not constant over time (p = 0.03).

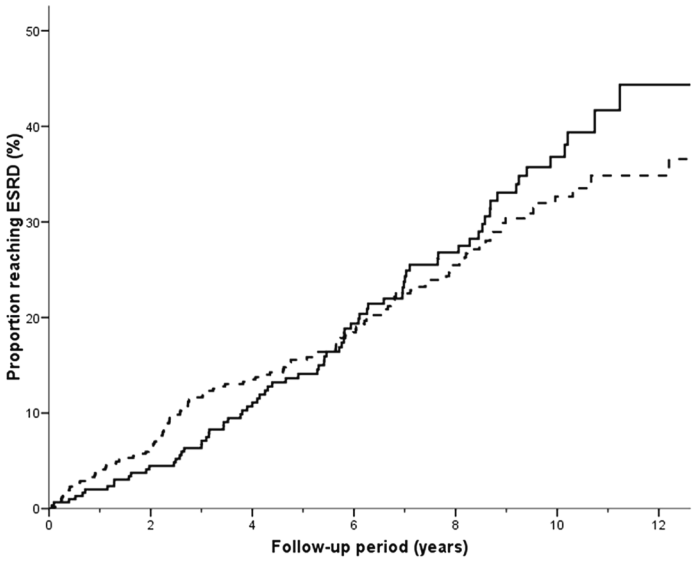

Figure 1: Kaplan–Meier curves of ESRD in 864 patients with type 1 diabetes and diabetes nephropathy according to *CNDP1* genotype: 5-leucine repeat homozygous (5L–5L) vs all other genotypes (logrank test p=0.57). Solid line, 5L–5L genotype; dotted line, all other genotypes

As shown in Table 2, within the first 6 years, the hazards were not significantly different between patients with 5L–5L genotype and those with other genotypes. For example, the HR for ESRD in patients with 5L–5L genotype compared with other

genotypes was 0.82 (95% CI 0.55-1.22) at 2 years of follow-up. However, after 8 years of follow-up, patients with the 5L–5L genotype appeared to have an increased risk of ESRD compared with those with other genotypes, with an HR of 1.53 (95% CI 1.01-2.34) at 8 years of follow-up and an HR of 1.89 (95% CI 1.07-3.36) at 10 years of follow-up. Adjustment for possible confounders, including duration of diabetes, baseline glycated haemoglobin, blood pressure, plasma creatinine and urinary albumin excretion did not materially change the results of the analyses for 5L–5L: HR at 8 years, 1.71 (95% CI 1.11-2.65); and at 10 years, 2.19 (95% CI 1.21-4.01).

Table 2: Hazard ratios for ESRD from Cox models with time-dependent variables

Model/genotypes	HR (95% CI)
Crude model	
Other genotypes	1.0 (Ref.)
5L–5L genotypes	
At 1 year	0.73 (0.46-1.17)
At 2 years	0.82 (0.55-1.22)
At 4 years	1.01 (0.75-1.36)
At 6 years	1.24 (0.91-1.69)
At 8 years	1.53 (1.01-2.34)
At 10 years	1.89 (1.07-3.36)
Adjusted for age, sex and centre	
Other genotypes	1.0 (Ref.)
5L–5L genotypes	
At 1 year	0.75 (0.74-1.21)
At 2 years	0.84 (0.56-1.25)
At 4 years	1.04 (0.76-1.40)
At 6 years	1.28 (0.94-1.75)
At 8 years	1.59 (1.04-2.41)
At 10 years	1.96 (1.11-3.49)
Further adjusted for duration of diabetes, HbA_{1c}, blood pressure, plasma creatinine and uAER	
Other genotypes	1.0 (Ref.)
5L–5L genotypes	
At 1 year	0.71 (0.41-1.27)
At 2 years	0.81 (0.51-1.32)
At 4 years	1.04 (0.73-1.49)
At 6 years	1.34 (0.96-1.87)
At 8 years	1.71 (1.11-2.65)
At 10 years	2.19 (1.21-4.01)

Crude model: multivariate Cox model with time-dependent covariate (time × group)

DISCUSSION

In this study of a large cohort of white European patients with type 1 diabetes, the prevalence of homozygosity for 5-leucine repeats in the *CNDP1* gene was not significantly different between patients with DN and patients without DN at study entry. In prospective analyses, we found neither an association of 5L–5L with mortality nor an interaction of 5L–5L with DN for prediction of mortality. Patients with 5L–5L have shown a trend towards a sex-dependent association with risk for mortality, with higher risk associated with 5L–5L in women than in men, but no significant associations with mortality were present in the sexes separately. Patients with DN and 5L–5L were at increased risk of progression to ESRD compared with those with other allelic variations, but this increase in risk became apparent only after 8 years of follow-up. The increase in risk was independent of potential confounders.

In an earlier study in a large cohort of patients with type 1 diabetes and DN (*n*= 445), Wanic et al. [23]found no significant association between 5L–5L and susceptibility for progression to ESRD after a mean follow-up of 5.6 years. In that study, it was not investigated whether the proportional hazards assumption of Cox-regression analyses was violated. If we performed similar analyses as performed in the study of Wanic et al., we would have found similar results. However, taking into account the change in hazards over time unmasked an increased risk associated with 5L–5L after prolonged follow-up.

The *CNDP1* gene, which is located on chromosome 18, encodes a secreted serum carnosinase that degrades carnosine specifically, whereas *CNDP2* encodes tissue carnosinase [24]. Carnosine is a naturally occurring dipeptide that has been shown to have beneficial actions as, for example, a scavenger of free oxygen radicals and an inhibitor of formation of advanced glycation endproducts [25-27]. Individuals with genotypes containing a higher number of leucine repeats (six or seven repeats) in their *CNDP1* gene were found to have higher serum carnosinase activity compared with those with five leucine repeats [28]. High serum carnosine levels resulting from low carnosinase activity were considered to underlie findings from cross-sectional studies that suggested a protective role of 5L–5L genotype against DN, mainly in type 2 diabetes [10, 11].

Findings from cross-sectional studies should be interpreted carefully. For instance, interaction between 5L–5L and DN resulting in a survival disadvantage in these patients could falsely suggest a protective effect of 5L–5L for DN in a cross-sectional study [14]. Our study is the first to investigate such a potential interaction prospectively. We found no evidence for an interaction between 5L–5L and DN for mortality. Another interaction that we considered was an interaction by sex, given a recent finding from cross-sectional data that in patients with type 2 diabetes the association between *CNDP1* and DN is sex specific, with a decreased risk in women with the 5L–5L genotype [13]. In the current study, we found a trend towards different risk of mortality between men and women, with a trend toward increased risk in women with 5L–5L genotype compared with men. In contrast to these findings, however, our study did not indicate an interaction by sex for the prospective relation of 5L–5L genotype with ESRD. Furthermore, a protective role of 5L–5L for progression to ESRD in patients with DN could not be confirmed in our large study nor in a previous study in type 1 diabetes [23]. Differences in factors or genes predisposing for DN or mortality between type 1 and type 2 diabetes may underlie discrepant findings of cross-sectional analyses of associations of 5L–5L with DN in patients with type 1 and type 2 diabetes [29].

Although *CNDP1* polymorphism was not associated with DN in our population, it should be noted that once DN is established, the 5L–5L genotype appeared to be significantly associated with increased rather than decreased risk of progression to ESRD beyond 6 years of follow-up. A possible explanation for this discrepancy from previous findings that suggested a protective role of 5L–5L could be that other unidentified risk alleles may be involved in deterioration of renal function in diabetic patients. For example, McDonough et al. [30] have shown that in African-Americans, a population in which there is no association of *CNDP1* and *CNDP2* with ESRD, other variants in this region of chromosome 18 are contributing to risk of DN. These other variants may mask or modify the effect of 5L–5L. Another potential explanation for an inverse association between *CNDP1* and ESRD to only appear after prolonged follow-up could lie in environmental factors that alter over time, such as glycaemic control. Indeed, Riedl et al. [31] have shown that hyperglycaemia enhances secretion of carnosinase and its activity. Additionally, tissue carnosinase could have an important

role in susceptibility to DN and ESRD. Whether 5L–5L genotype also determines the consequent availability of the intracellular precursor of carnosinase and whether this may have consequences for intracellular carnosine concentrations remains to be investigated.

The strength of our study is its prospective design. It was also performed in three independent, but relatively homogeneous, populations of white patients with type 1 diabetes mellitus, and it included more cases and controls than all previous studies on the *CNDP1* polymorphism.

In conclusion, this study provides evidence that *CNDP1* polymorphism is neither related to risk for mortality nor interacts with DN regarding survival in white Europeans with type 1 diabetes. Homozygosity for the 5-leucine repeat was associated with increased risk of progression from nephropathy to ESRD after prolonged follow-up. Based on these results, we suggest that possible interaction between *CNDP1* polymorphism and other candidate genes or environmental factors in different populations should be further investigated, preferably in prospective studies, in order to elucidate the role of carnosine and its gene in DN.

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Chapter 3

Sex specific association between carnosinase gene (CNDP1) and cardiovascular mortality in patients with type 2 diabetes

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Submitted

ABSTRACT

Background

Homozygosity for a 5-leucine repeat (5L-5L) in the carnosinase gene (*CNDP1*) has been found to be associated with a reduced prevalence of diabetic nephropathy in cross-sectional studies in type 2 diabetes, particularly in women. Data on mortality are not available. We prospectively investigated whether 5L-5L is associated with mortality or progression of renal function loss and to what extent this effect is modified by sex.

Methods

Prospective study in patients with type 2 diabetes. A Cox proportional hazard model was used to compare 5L-5L with other genotypes regarding (cardiovascular) mortality. Renal function slopes were obtained by within-individual linear regression of the estimated glomerular filtration rate (eGFR) using Modification of Diet in Renal Disease equation (MDRD) and compared between 5L-5L and other genotypes.

Results

871 patients were included (330 (38%) with 5L-5L). After 9.5 years of follow-up, hazards ratios (HR) for all-cause and cardiovascular mortality in 5L-5L versus other genotypes were 1.09 (95%CI 0.88-1.36) and 1.12 (95%CI 0.79-1.58), respectively. After adjustment for relevant confounders, there was a significant interaction between *CNDP1* and sex for the association with cardiovascular mortality (*P*=0.01), but not for all-cause mortality (*P*=0.32). Adjusted HR in 5L-5L for cardiovascular mortality was 0.69 (95%CI 0.39-1.23) in males and 1.77 (95%CI 1.12-2.81) in females. Regarding slopes of eGFR-MDRD, neither significant difference between 5L-5L and other genotypes nor interaction between *CNDP1* and sex was found.

Conclusions

The association between *CNDP1* and cardiovascular mortality was sex-specific, with a higher risk in women with 5L-5L genotype. *CNDP1* was not associated with change in eGFR-MDRD.

INTRODUCTION

The epidemic of type 2 diabetes is associated with an increasing number of patients with micro- and macrovascular complications, leading to substantial mortality. In addition to traditional cardiovascular risk factors, such as hypertension and smoking, genetic predisposition has been suggested to play a role in susceptibility for diabetic complications, including diabetic nephropathy (DN) [1, 2].

Recently, a locus on chromosome 18q, which contains two carnosinase dipeptidase genes (*CNDP1* and *CNDP2*), was found to be associated with DN [3, 4]. *CNDP1* encodes the enzyme serum carnosinase which hydrolyses and breaks down histidine-containing dipeptides such as L-carnosine while *CNDP2* encodes a non-specific dipeptidase [5]. The addition of L-carnosine to human podocytes and mesangial cells *in vitro* blocked glucose-induced, TGF-dependent extracellular matrix production in these cells, suggesting a protective role of carnosine against diabetic complications [6]. Interestingly, genetic variation in *CNDP1*, determined by a trinucleotide repeat polymorphism (D18S880, coding for five to seven leucine repeats), was correlated with serum carnosinase activity in healthy volunteers [7, 8]. Moreover, homozygosity for 5 leucine repeats (5L-5L) encodes for the lowest serum carnosinase activity in patients with type 2 diabetes [7]. Several cross-sectional studies have shown the frequency of 5L-5L genotype to be higher in patients without DN compared to those with DN, suggesting a protective effect of 5L-5L genotype on DN [7-9]. A recent cross sectional study in patients with type 2 diabetes has suggested the correlation between *CNDP1* and DN to be sex-specific, with protection by the 5L-5L homozygous genotype restricted to women [10]. It was assumed that this could be attributed to that fact that men have higher carnosine levels in their muscle tissue and women have slightly higher serum carnosinase levels [11], and that differences in carnosinase activity due to the different *CNDP1* polymorphism may have a stronger impact in women.

Although many cross-sectional studies suggested *CNDP1* polymorphism as a protective factor against DN, longitudinal studies in patients with type 2 diabetes on *CNDP1* polymorphism (leucine repeat polymorphism D18S880) and its relationship with cardiovascular mortality or progression of renal function loss are lacking.

Therefore, we aimed to investigate in a prospectively designed cohort study of patients with type 2 diabetes, whether *CNDP1* is associated with the risk of (cardiovascular) mortality or progression of renal function loss over time, and to test whether these associations are sex-specific.

METHODS

Patients

In 1998, a large shared-care diabetes project was initiated: the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) [12]. In brief, hospital-based specialist diabetes nurses assist general practitioners in caring for patients with type 2 diabetes. Of all the patients in primary care in the Zwolle region, 90% participated in this study and 10% were excluded or refused to participate. Nearly all patients (99%) are Caucasian. Patients with a very short life expectancy or with insufficient cognitive abilities were excluded, as well as patients being treated by an internist. At the end of the project's first year (1998) and the start of the second year (1999), blood was taken from 913 consecutive patients and stored for future research. In this prospective study, patients were followed until 2009. Life status and cause of death were obtained from records maintained by the hospital and general practitioners. Causes of death were coded according to the International Classification of Diseases, ninth revision (ICD-9). The ZODIAC study was approved by the medical ethics committee of the Isala Clinics in Zwolle, the Netherlands, and all patients signed informed consent.

Baseline clinical laboratory investigations

Baseline data were collected in 1998 and 1999. Physical assessments and laboratory measurements were performed annually. HbA1c was determined by standard HPLC techniques with normal values in the range 4.1% to 6.4%. The kinetic colorimetric Jaffé method [13] was used to measure creatinine in serum and urine (Modular P800 Analyzer; Roche, Almere, the Netherlands) until march 2007, thereafter an enzymatic technique (Modular P Analyzer from Roche, Creatinine Plus assay, Roche Diagnostics, Mannheim, Germany) was used. Based on a measured difference between the Jaffé method en the enzymatic method at our laboratory, a constant of 17 was added

to the plasma creatinine values measured after March 2007 in order to adjust for the measured difference in plasma creatinine. Renal function was estimated by the 4-variable Modification of Diet in Renal Disease equation (MDRD) [14]. Urinary albumin-creatinine ratio (UACR) was measured in spot morning urine samples. Blood samples to isolate DNA material were collected at baseline. Genotyping was performed as described previously (5). In brief, after PCR amplification, fragment analysis was performed on the ABI-3130 analyzer to determine the number of trinucleotide repeats of exon 2 in each allele. The success rate was 95%, and no errors were detected. Genotyping was performed in Leiden and Mannheim.

Statistical analysis

Variables with normal distribution are presented as mean and standard deviation (SD) and variables with skewed distribution are log-transformed before analysis and presented as median and interquartile range (IQR). For variables with normal distribution, comparisons between groups were performed by using unpaired Student's t-tests, whereas for variables with skewed distribution Mann-Whitney-U tests were used. Chi-square tests were used to compare non-continuous variables.

The 5L-5L homozygous genotype was compared to genotypes with six or more leucine repeats (i.e. recessive model), in accordance with previous studies [10, 15, 16]. Time-to-event analyses were performed using Kaplan–Meier plots and log-rank testing. Tests for a non-zero slope of scaled Schoenfeld residuals on functions of time were performed to explore the proportional hazards (PH) assumption in Cox regression models [17]. Cox regression models were used to estimate unadjusted and adjusted hazard ratios with 95% confidence intervals (CI) for all-cause mortality and cardiovascular mortality. Three models were constructed: The crude model, a model after adjustment for age and gender, and a model after adjustment for additional confounders, including body mass index, smoking status, duration of diabetes, history of macrovascular complications (patients were considered to have macrovascular complications when they had a history of angina pectoris, myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, stroke or transient ischemic attack), systolic blood pressure, HbA1c, Cholesterol to HDL-cholesterol ratio, plasma creatinine, and urinary albumin

excretion. Subsequently, in order to investigate whether the correlation between *CNDP1* polymorphism and mortality was gender-specific, an interaction term (*CNDP1* polymorphism*gender) was used in all models. In case the interaction term was significant, stratification according to sex was performed to calculate HR for mortality in males and females separately. We also tested for interaction between genotype and HbA1c because a recent study suggested that the effect of the genotype on plasma carnosinase activity is modified by levels of glycaemia [18]. Following the suggestion of Mathew et al. [19], the individual change in eGFR-MDRD during the follow-up time was modelled as a linear function of time with an individual slope and intercept. For this analysis, patients with at least 3 known values of eGFR-MDRD were included. A negative slope function indicates a decrease in eGFR-MDRD over time and a positive slope function indicates an increase in eGFR-MDRD over time. The bivariate correlations procedure was used to evaluate the correlation between the eGFR-MDRD slope functions and different baseline parameters. To compare the slopes of 5L-5L carriers with the slopes of patients with other genotypes, the Mann-whitney U test was used. In order to investigate whether the correlation between *CNDP1* polymorphism and eGFR-MDRD slopes is gender-specific, general linear model (GLM) univariate analysis was used to adjust for baseline variables and to build a model with an interaction term (*CNDP1* polymorphism*gender).

A two-tailed p-value of 0.05 or less was considered statistically significant. Statistical analyses were done by using a commercially available program (SPSS for Windows, version 16.0., Chicago, IL, USA).

RESULTS

Baseline characteristics

A total of 913 patients were included in this study. The genotype from 42 patients could not be determined due to poor quality DNA. The baseline characteristics of these patients were not different compared to the patients with known genotype (general linear model (GLM) statistics F= 1.225, *P*= 0.25 (data not shown)). Table 1 shows the baseline characteristics of the study population (*n* = 871) according to genotype. 5L-5L was found in 330 patients (38%; for men this was 39%; for women 37%). The study population was in Hardy-Weinberg equilibrium (χ^2 = 4.479, *P*=0.21).

Table 1: Baseline characteristics of the study population

	Total	*CNDP1*		
		5L-5L	*Other genotypes*	*P value*
N	871	330 (38)	541 (62)	
Males	366 (42)	143 (43)	223 (41)	0.56
Age (years)	68.0 ± 11.2	68.2 ± 11.2	67.4 ± 11.2	0.61
BMI (kg/m^2)	28.8 ± 4.5	28.7 ± 4.5	28.9 ± 4.5	0.55
Smokers	156 (18)	55 (17)	101 (19)	0.47
Diabetes duration (years)	5 [2; 11]	5 [2; 11]	5 [3; 10]	0.42
History of macrovascular complications	268 (31)	96 (29)	172 (32)	0.39
Medication use				
Insulin	132 (15)	51 (15)	81 (15)	0.80
Oral hypoglycaemic agents	563 (65)	206 (62%)	357 (66%)	0.30
Antiplatelet agents	95 (11%)	31 (9%)	64 (12%)	0.26
Anti-hypertensive agents (any)	327 (37%)	118 (36%)	209 (39%)	0.37
Beta blockers	113 (13)	43 (17)	70 (13)	0.99
ACE-Is or ARBs	119 (14)	38 (11)	81 (15)	0.13
Lipid-lowering agents	65 (7)	28 (8)	37 (7)	0.38
SBP (mmHg)	152 ± 25	153 ± 24	152 ± 26	0.38
DBP (mmHg)	83 ± 11	83 ± 11	83 ± 11	0.88
HbA1c (%)	7.35 ± 1.15	7.38 ± 1.13	7.32 ± 1.13	0.53
Total cholesterol (mg/dl)	5.6 ± 1.1	5.6 ± 1.1	5.6 ± 1.1	0.51
HDL (mg/dl)	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	0.45
Triglycerides (mg/dl)	2.4 ± 1.4	2.3 ± 1.3	2.5 ± 1.5	0.08
Plasma creatinine (mg/dl)	95 ± 21	93 ± 22	96 ± 21	0.01
eGFR-MDRD (ml/min)	65.3 ± 20.9	67.5 ± 27.4	63.9 ± 15.4	0.01
UAER (µg/min)	1.94 [0.95; 6.20]	1.82 [0.96; 6.53]	2.00 [0.95; 6.13]	0.87
Albuminuria	336 (39)	119 (36)	217 (40)	0.22

Data represent mean ± standard deviation, median [interquartile range] or absolute number (proportion). P values are based on t-test, mann-whitney U test, or χ^2 test.BMI, body mass index; ACE-Is, Angiotensin-converting enzyme inhibitor; ARBs, Angiotensin-receptor blockers; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, Glycated haemoglobin; HDL, high-density cholesterol; eGFR-MDRD, estimated glomerular filtration rate using Modification of Diet in Renal Disease equation; UAER, urinary albumin excretion rate.

Except for slightly lower plasma creatinine and correspondingly higher eGFR in patients with 5L-5L, no significant differences at baseline were found between 5L-5L and other genotypes.

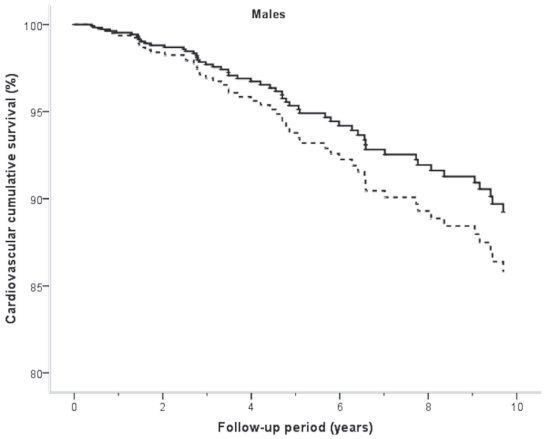
Follow-up: mortality

After 9.5 (IQ 5.9-10.3) years of follow-up, 136 (41%) patients with 5L-5L and 205 (38%) patients with other genotypes had died (log-rank test *P*= 0.39). Regarding cardiovascular mortality, these numbers were 55 (17%) and 81 (15%), respectively (log-rank test *P*= 0.51). The PH assumption was met in all Cox regression models (test based on Schoenfeld residuals p>0.05). The hazard ratio (HR) for all-cause mortality in patients with 5L-5L compared to other genotypes was 1.09 (95% (CI) 0.88-1.36). Regarding cardiovascular mortality, the HR was 1.12 (95%CI 0.79-1.58). Adjusted hazard ratios for all-cause mortality and cardiovascular mortality are presented in table 2. In model 2 (adjusted for age and gender) and model 3 (fully-adjusted model), there was a significant interaction between *CNDP1* and sex for prediction of cardiovascular mortality (*P* for interaction 0.05 and 0.01, respectively), but not for all-cause mortality. After stratification for sex, fully adjusted HRs of 5L-5L for cardiovascular mortality were 0.66 (0.37-1.18) in males and 1.81 (95%CI 1.14-2.86) in females (table 2 and figure 1). Analyses on interaction between *CNDP1* and A1c for prediction of all-cause mortality and cardiovascular mortality did not reveal significant results (data not shown).

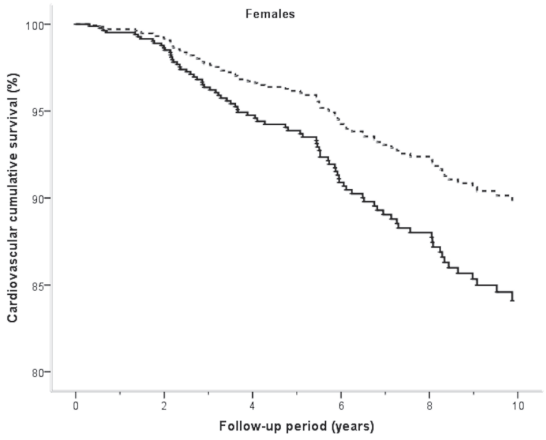
Table 2: Hazards ratios (95%CI) for all-cause and cardiovascular mortality according to *CNDP1* genotype

	Total *n* = 871	*P* for interaction	Men *n* = 366	Women *n* = 505
Model 1: Crude model				
All-cause mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L genotype	1.09 (0.88-1.36) *P*=0.40	0.73	1.14 (0.82-1.60) *P*=0.43	1.06 (0.80-1.41) *P*=0.68
Cardiovascular mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L genotype	1.12 (0.80-1.58) *P*=0.51	0.13	0.80 (0.45-1.41) *P*=0.44	1.40 (0.90-2.15) *P*=0.14
Model 2: Adjusted for age and gender				
All-cause mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L genotype	1.03 (0.82-1.28) *P*=0.79	0.63	0.94 (0.68-1.32) *P*=0.74	1.05 (0.79-1.40) *P*=0.75
Cardiovascular mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L genotype	1.03 (0.73-1.46) *P*=0.85	0.05	0.67 (0.39-1.19) *P*=0.17	1.38 (0.89-2.13) *P*=0.15
Model 3: adjusted for other covariates				
All-cause mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L	1.12 (0.90-1.40) *P*=0.32	0.38	1.01 (0.72-1.41) *P*=0.95	1.27 (0.94-1.71) *P*=0.12
Cardiovascular mortality				
Other genotype	1.0 (reference)		1.0 (reference)	1.0 (reference)
5L-5L genotype	1.19 (0.84-1.69) *P*=0.33	0.01	0.66 (0.37-1.18) *P*=0.16	1.81 (1.14-2.86) *P*=0.01

Model 1: crude model;
Model 2, a model after adjustment for age and gender;
Model 3, further adjustment for body mass index, smoking status, duration of diabetes, history of macrovascular complications, systolic blood pressure, A1c, Cholesterol to HDL-Cholesterol ratio, plasma creatinine, and urinary albumin excretion.



Adjusted hazard ratio: 0.69 (95%CI 0.39-1.23)



Adjusted hazard ratio: 1.77 (95%CI 1.12-2.81)

Figure 1: Adjusted survival curves for cardiovascular mortality in males en females according to *CNDP1* genotype (5L-5L vs. other genotypes). Solid line, 5L–5L genotype; dotted line, all other genotypes

Follow-up: progression of renal function loss

The individual slopes of eGFR-MDRD were calculated in 779 patients (89%). The median [IQR] of the slopes was -1.00 [-2.12; -0.08] mL/min/1.73m^2/year. As shown in table 3, baseline eGFR and urinary albumin excretion rate (UAER) were negatively

correlated with eGFR-MDRD slopes. In patients with 5L-5L genotype, eGFR-MDRD slopes were not significantly different from the slopes in patients with other genotypes (-1.10 [-2.18; -0.15] vs. -0.95 [-2.07; -0.06], respectively, *P* = 0.36). Adjustment for baseline variables in GLM analysis revealed similar results (data not shown). GLM analysis showed no significant interaction between *CNDP1* polymorphism and gender regarding slopes of eGFR-MDRD (*P* for interaction 0.99).

Table 3: Pearson correlation coefficients for the correlation between eGFR-MDRD slopes and baseline parameters.

	Pearson Correlation	*P value*
Parameter		
Age	-0.35	0.33
Gender	0.06	0.11
BMI	0.06	0.08
Smoking status	0.01	0.76
Diabetes duration	0.06	0.12
History of macrovascular complications	-0.07	0.05
SBP	-0.03	0.36
HbA1c	-0.04	0.30
Total cholesterol/HDL	0.07	0.03
eGFR-MDRD	-0.11	0.001
UAER	-0.14	<0.001
CNDP1 polymorphism	0.02	0.58

BMI, body mass index; SBP, systolic blood pressure; A1c, Glycated haemoglobin; HDL, high-density cholesterol; eGFR-MDRD, estimated glomerular filtration rate using Modification of Diet in Renal Disease equation; UAER, urinary albumin excretion rate

DISCUSSION

For the first time in a prospective cohort of patients with type 2 diabetes, we found that *CNDP1* gene was associated with cardiovascular mortality in a sex-specific manner. In female patients, a higher risk for cardiovascular mortality was found in carriers of 5L-5L genotype compared to other genotypes. Neither an association nor an interaction with gender was found concerning all-cause mortality or progression of renal function loss over time. Data from previous cross-sectional studies on *CNDP1* are conflicting. In a case-control study in European Caucasians and Arabs with type

1 and type 2 diabetes (135 cases and 107 controls), a protective effect of 5L-5L was suggested, based on the finding that patients without DN were frequent carriers of the 5L-5L genotype, compared to those with other genotypes [7]. In the same study, the 5L-5L genotype (*CNDP1* variant with the lowest number of leucine repeats) was shown to be associated with the lowest serum carnosinase activity compared to other genotypes (5L-6L, 6L-6L, 6L-7L, 7L-7L) [7]. This finding was supported by a larger cross-sectional study in 552 Caucasian patients (European American) with type 2 diabetes, that found subjects lacking nephropathy to be more frequently carriers of 5L-5L compared to those with diabetes-related ESRD [9]. On the other hand, large cross-sectional studies in African Americans [20] and Scandinavians [21] with type 2 diabetes did not replicate the finding of an association between the polymorphism in *CNDP1* gene and DN or ESRD. Interestingly, a recent study suggested that the association between *CNDP1* and DN in type 2 diabetes was limited to women, with a lower frequency of 5L-5L in patients with DN compared to those without DN [10].
Based on these findings from cross sectional studies that suggest 5L-5L genotype of *CNDP1* polymorphism as a protective factor against DN, we aimed to investigate whether 5L-5L genotype is associated with risk of (cardiovascular) mortality or progression of renal function loss in patients with type 2 diabetes. In this prospective study, while no association was found between *CNDP1* polymorphism and progression of renal function loss, we found a sex-specific association of *CNDP1* polymorphism and cardiovascular mortality. In our study, women with 5L-5L were at higher risk for cardiovascular mortality compared to other genotypes. At present, there is no clear explanation for the difference between findings from our prospective study and findings from previous cross-sectional studies. Prospective studies in patients with type 1 diabetes also failed to confirm findings from cross sectional studies on *CNDP1* gene [15, 16]. Rather, one study found an opposite effect, with increased risk of development of end-stage renal disease in patients with 5L-5L beyond a certain time of follow-up [16], which is in line with our current finding of 5L-5L being a risk factor for cardiovascular mortality in women. The exact cardiac cause of death in women with type 2 diabetes and 5L-5L genotype has not been documented. However, a previous study has failed to demonstrate a correlation between *CNDP1* genotype and coronary heart disease [22], so it is tempting to speculate that the genetic

polymorphism of *CNDP1* affects susceptibility to develop diabetic cardiomyopathy and not coronary atherosclerosis. To which extent carnosine metabolism in the myocard is different from skeletal muscle and the kidney remains to be investigated.

Several factors may underlie the discrepancy between findings from cross-sectional and prospective studies. Firstly, if women with 5L-5L truly have an increased risk for cardiovascular mortality, many women with 5L-5L and type 2 diabetes could have died before being included in cross-sectional analyses, thereby leading to a bias with underestimation of the true effect associated with 5L-5L. Our prospective study was part of one of the cross-sectional analyses previously performed [10], increasing the likelihood of the above mentioned potential explanation. Secondly, other polymorphisms in *CNDP1* and *CNDP2* genes were found to play a role in the risk of DN and ESRD [20, 21] and interaction between genotype and glycemic status was found to influence the activity of *CNDP1* gene [18]. Thus, it is still to be investigated if other polymorphisms in *CNDP1* and *CNDP2*, in addition to leucine repeats polymorphism in *CNDP1*, affect serum carnosinase activity. The exact mechanism by which *CNDP1* polymorphism may exert its effects in diabetes is still to be investigated. While 5L-5L was found to result in the lowest activity of serum carnosinase and thereby suggested to be associated with high levels of serum carnosine [7, 23], intracellular carnosine may not necessarily be influenced by *CNDP1* polymorphism. For instance, a recent study has shown muscle carnosine to be independent of *CNDP1* polymorphism [24]. Besides, a physiologic effect of leucine repeat polymorphism in *CNDP1* on serum carnosine has not yet been fully clarified, since serum carnosine levels appeared to be undetectable in humans due to the presence of highly active serum carnosinase [25]. Strengths of our study are the prospective design, the large number of patients and the long duration of follow-up. Limitations are that we did not have data on carnosinase activity, which could have given us additional information on the actual effect of *CNDP1* gene on carnosine metabolism. Besides, caution is warranted when interpreting the results of slopes of eGFR-MDRD, taking the limitations of the MDRD Study equation into consideration [26]. Long-term estimation of renal function using slopes of eGFR-MDRD have been shown to slightly underestimate the renal function in patients with progressive renal function loss [27]. It is also important to realise

that absence of significant association between *CNDP1* polymorphism and slopes of eGFR-MDRD in this study does not exclude the role of CNDP1 in risk of development of DN. Prospective data on albuminuria and use of renin-angiotensin-aldosterone-system blockade are necessary to investigate this issue.

In conclusion, *CNDP1* polymorphism was not associated with all-cause mortality or progression of renal function loss in patients with type 2 diabetes. Only in female patients, 5L-5L genotype was associated with higher risk of cardiovascular mortality compared to those with other genotypes. Further prospective studies that preferably measure carnosinase activity are necessary to understand the mechanism by which *CNDP1* influences carnosine metabolism and vascular complications of diabetes. Replication of our findings will increase the probability of the sex-specific association found. With the prospective nature of our study, it is more likely that in women with type 2 diabetes, 5L-5L genotype is associated with increased cardiovascular mortality instead of being protective as suggested in previous cross-sectional studies.

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Chapter 4

Impact of ACE I/D and CCR2 V64I polymorphisms on new-onset microalbuminuria in type 2 diabetes; evidence from the BENEDICT trial

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Ready for submission

ABSTRACT

Background

Beside activation of Renin-angiotensin-aldosterone system (RAAS), monocyte chemoattractant protein-1 and its receptor (CCR2) are also suggested to play an important role in renal disease, including diabetic nephropathy. We prospectively investigated whether genetic variations in ACE gene (ACE I/D polymorphism) predicts new-onset microalbuminuria, (2) CCR2 V64I polymorphism has additional value in addition to ACE I/D polymorphism, and (3) there is an interaction between either of these polymorphisms and treatment with ACE-I for prediction of new-onset microalbuminuria.

Methods

ACE I/D and CCR2 V64I was genotyped by agarose gel electrophoresis and sequences analysis respectively in 1128 patients from the BErgamo NEphrologic Diabetic Complications Trial (BENEDICT) – a prospective randomized trial evaluating ACE inhibition effect on new-onset microalbuminuria (albuminuria 20-200 μg/min) in hypertensive type 2 diabetes patients without albuminuria (<20 μg/min) at inclusion.

Results

During a median follow-up of 42.3 months, ACE I/D was significantly associated with increased new-onset microalbuminuria; unadjusted hazards ratio (HR) for microalbuminuria in DD compared to II/ID was 1.56 (95%CI 1.05-2.32, *P* = 0.03). Adjusted HR was 1.21 (95%CI 0.81-1.82, *P* = 0.35). CCR2 V64I was not significantly associated with new-onset microalbuminuria; Unadjusted HR for microalbuminuria in VI/II compared to VV was 1.35, 95%CI 0.85-2.14, *P* = 0.198, adjusted HR was 1.42 (95%CI 0.89-2.25, *P* = 0.14). The unadjusted HR for development of microalbuminuria in patients with both mutant genotypes, i.e. DD from ACE I/D and VI/II from CCR2, compared to carriers of neither of these genotypes, i.e. ID/DD and VV, was 2.17 (95%CI 1.14-4.13, *P*=0.02), adjusted HR 1.58 (95%CI 0.82-3.04, *P* = 0.17). No significant interaction was found between ACE-I treatment and ACE I/D or CCR2 V64I.

Conclusions

DD carriers of ACE I/D SNP had significantly increased risk of microalbuminuria compared to II/ID carriers, even after adjustment for ACE-I treatment, but not after further adjustment for other risk factors. CCR2 V64I did not predict new-onset microalbuminuria. Carriers of both “risk” genotypes DD and VI/II have increased risk of developing microalbuminuria compared to carriers of both “protective” genotypes, suggesting additional value of CCR2 V64I polymorphism on renal outcome.

INTRODUCTION

Inhibiting of renin-angiotensin-aldosterone system (RAAS) by use of angiotensin-converting enzyme inhibitors (ACE-I) and/or angiotensin-receptor blockers (ARB) is currently the first-line therapy for prevention and treatment of DN, due to their important hemodynamic effects on blood pressure and their antiproteinuric action [1-3]. Interestingly, the circulating and renal activity of the RAAS has been shown to be influenced by genetic factors, such as an intronic 287 bp Alu repeat sequence insertion (I) /deletion (D) polymorphism of the angiotensin-converting enzyme (ACE) gene. Subjects with DD genotype of the ACE gene had a worse renal function prognosis compared with those carrying the II genotype [4, 5]. In patients with diabetes, several studies supported the genetic association of ACE I/D polymorphism with DN, as confirmed in a recent meta-analysis [6]. However, prospective data on the impact of ACE I/D polymorphism on the incidence of microalbuminuria and the possible interaction of ACE I/D with RAAS-blockade to prevent DN are not available.
On the other hand, beyond the various hemodynamic effects of RAAS on glomeruli and filtration rate, angiotensin II has been shown to stimulate tubulointerstitial inflammation and fibrosis by release of cytokines, growth factors, and chemokines that induce recruitment of macrophages and lymphocytes in arterial wall [7, 8]. The prototype chemokine, monocyte chemoattractant protein-1 (MCP-1), which is activated by its C-C chemokine receptor (CCR2), is implicated in chronic monocyte-mediated inflammation and endothelial damage [9, 10]. In animal experiments, CCR2 inhibition has been shown to protect the kidney in hypertensive states by reducing inflammation and delaying the progression of hypertension [11]. A single nucleotide polymorphism (SNP) causing replacement of valine by isoleucine at position 64 in the CCR2 gene on chromosome 3 (CCR2-V64I) has been reported to play a modulating role in the MCP-1 pathway. CCR2-V64I polymorphism, which occurs with an allele frequency of 10-25% depending on ethnic populations (NCBI, SNP rs1799864), has been associated with inflammatory vascular diseases including myocardial infarction [12], carotid atherosclerosis [13] and preeclampsia [14]. Based on this, we hypothesized that genetic variations in CCR2, could synergize with the ACE I/D polymorphism in influencing the risk of

DN and the response to renoprotective therapies in patients with type 2 diabetes. Thus, in a large cohort of hypertensive normoalbuminuric patients with type 2 diabetes who participated in phase A of the BErgamo NEphrologic Diabetic Complications Trial (BENEDICT), we prospectively investigated whether: (1) ACE I/D polymorphism predicts new-onset microalbuminuria, (2) CCR2 V64I polymorphism synergizes with the ACE I/D polymorphism in determining the risk of DN, and (3) there is an interaction between either of these polymorphisms and treatment with ACE-I for prevention of new-onset microalbuminuria.

METHODS

Patients

BENEDICT was a double-blind, randomized, placebo-controlled and prospective trial aimed to assess whether ACE-I and nondehydropyridine calcium-channel blockers, alone or in combination, prevent microalbuminuria in subjects with type 2 diabetes, hypertension and normal urinary albumin excretion (UAE) at baseline [2]. Patients (*n*=1204) who fulfilled inclusion criteria for the clinical study (age >40 years, type 2 diabetes duration ≤ 25 years, serum creatinine <1.5 mg/dl, glycated haemoglobin A1c <11%, blood pressure higher than 140/90 mmHg after 3 weeks without antihypertensive treatment or lower than 140/90 mmHg with antihypertensive treatment and urinary albumine excretion<20 μg/min), were randomly allocated to at least 3-years treatment with ACE-I trandolapril, nondehydropyridine calcium-channel blocker verapamil, combination of trandolapril plus verapamil or placebo [2]. Primary endpoint was new-onset persistent microalbuminuria (UAE ≥20 μg/min and <200 μg/min in at least two of three consecutive onvernight urine collections on two consecutive visits 2 months apart). UAE was measured on fresh urine at the coordinating centre by nephelometry (Beckman Array System; Beckman Coulter) at randomization and every 6 months thereafter.

Trandolapril plus verapamil and trandolapril alone significantly delayed the onset of microalbuminuria by factors of 2.6 and 2.1, respectively, compared with placebo ($P < 0.01$ for both). The incidence of microalbuminuria was reduced to a similar extent by both treatments, whereas verapamil had an effect on the incidence for

microalbuminuria that was similar to that of placebo [2]. Thus, patients were pooled in two cohorts according to their original allocation to ACE or non-ACE inhibitor therapy regardless of concomitant therapy with verapamil or placebo. Gene by treatment interactions were tested according to ACE inhibitor therapy (yes or no).
The study protocol was approved by the local ethics committees, and only samples from patients who provided written informed consents according to the Helsinki Declaration guidelines were considered for genetic analyses. All data were handled in respect of patient confidentiality and anonymity.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by standard methods. Genotyping for ACE I/D polymorphism was determined according to the method described by Rigat et al. [15] while the CCR2 V64I polymorphism (CCR2 G190A), coding Val64Ile, was performed using direct sequencing analysis. Genotyping success rate was >99%. As a quality-control procedure, we doubled genotyped for ACEI/D a subset of 200 individuals by direct sequencing. The concordance rate between the two genotyping methods was 100%.

Statistical analysis

Variables with normal distribution are presented as mean ± standard deviation (SD) and variables with skewed distribution were log-transformed before analysis and presented as median and interquartile range (IQR). For variables with normal distribution, comparisons between groups were performed by using unpaired Student's t-tests, whereas for variables with skewed distribution Mann-Whitney-U tests were used. χ^2-tests were used to compare non-continuous variables. Time to onset of microalbuminuria was considered as the main endpoint. For patients who did not reach the main endpoint, time was censored for the last follow-up visit. Analyses of time to main endpoint were performed using Kaplan-Meier plots and log-rank testing. Cox regression models were used to estimate unadjusted and adjusted hazard ratios (HR) with 95% confidence interval (CI). To test the difference between different genotypes on outcome, four statistical models were constructed: the first one is a crude model, the second is a model adjusted for treatment with ACE-I, the

third is a model further adjusted for baseline urinary albumin excretion rate and the fourth model is further adjusted for other potential confounders which were a priori defined: age, sex, smoking status duration of diabetes, baseline glycated haemoglobin (A1c) and baseline systolic blood pressure. For assessment of response to treatment with ACE-I according to genotype, two models were used: a crude model and a model after adjustment for potential confounders. To test whether there is an interaction between genotype (ACE I/D or CCR2 V64I) or treatment with ACE-I, an interaction term (genotype*treatment) was used in the Cox-regression model. Besides, incidences of new-onset microalbuminuria in each subgroup were calculated and compared with each other by Pearson χ^2-tests. A two-tailed *P*-value of 0.05 or less was considered statistically significant. Statistical analyses were done by using a commercially available program (SPSS for Windows, version 16.0., Chicago, IL, USA).

RESULTS

Baseline characteristics

Of the 1,204 BENEDICT patients, genomic DNA was available from 1128 patients. Baseline characteristics of those from whom no genotype is known (*n*=76) were not significantly different from those who were genotyped. Of the 1128 patients, 429 (38%) were homozygous for the D allele of the ACE gene, from now DD carriers, while 526 (46.6%) and 173 (15.3%) were heterozygous and homozygous for the I allele, respectively, from now on II/ID carriers (figure 1). Genotype frequencies for the ACE gene are in Hardy-Weinberg equilibrium (χ2= 0.3, FD=1, *P*=0.58). Regarding CCR2 gene, 226 patients (20%) were homozygous or heterozygous carriers of the 64I variant, from now on VI/II carriers, and 902 patients (80%) were homozygous for the V allele (VV) of CCR2 gene, from now on VV carriers. (figure 1). Genotype frequencies for the CCR2 gene are also in Hardy-Weinberg equilibrium (χ2= 2.63, FD=1, *P*=0.10). Baseline characteristics of the study population are shown in table 1.

Table 1: Baseline characteristics of study population

	ACE I/D		CCR2 V64I		Total
	II/ID	DD	VV	VI/II	
N	699	429	902	226	1128
Males (*n*, (%))	356 (51.1)	243 (56.6)	438 (53.3)	117 (51.8)	600 (53.2)
Age (years)	62.4 ± 8.3	62.0 ± 7.8	62.4 ± 7.9	61.5 ± 8.7	62.2 ± 8.1
BMI (kg/m^2)	29.1 ± 4.6	29.0 ± 4.7	29.2 ± 4.8	28.5 ± 4.1	29.1 ± 4.6
Smokers (*n*, (%))					
Current	84 (12.0)	54 (12.6)	111 (12.3)	27 (11.9)	138 (12.2)
Never	412 (58.9)	244 (56.9)	518 (57.4)	138 (61.1)	656 (58.2)
Former	203 (29.0)	131 (30.5)	273 (30.3)	61 (27.0)	334 (29.6)
Diabetes duration (years)	7.9 ± 6.7	7.6 ± 6.5	7.8 ± 6.6	7.9 ± 6.7	7.8 ± 6.6
Hypertenstion duration (years)	5.1 ± 7.0	4.8 ± 6.1	5.0 ± 6.5	5.0 ± 7.1	4.9 ± 6.6
HbA1c (%)	5.82 ± 1.35	5.82 ± 1.43	5.81 ± 1.37	5.86 ± 1.43	5.82 ± 1.38
SBP (mmHg)	151 ± 15	150 ± 14	151 ± 15	149 ± 12	151 ± 14
DBP (mmHg)	88 ± 8	87 ± 7	88 ± 8	87 ± 7	88 ± 8
Plasma creatinine (mg/dl)	0.90 ± 0.15	0.91 ± 0.17	0.91 ± 0.16	0.90 ± 0.16	0.91 ± 0.16
Triglycerides (mg/dl)	125 [92 -183]	122 [89 - 179]	125 [91 -183]	123 [92 -175]	125 [91 -180]
HDL (mg/dl)	47.2 ± 12.1	46.5 ± 11.8	46.8 ± 11.7	47.3 ± 12.9	46.9 ± 11.9
LDL (mg/dl)	163.9 ± 35.1	160.2± 36.3	163.1 ± 34.8	159.9 ± 38.3	162.5 ± 35.6
UAER (µg/min)	5.15 [3.56 - 8.95]	5.63 [3.70 - 10.22]	5.47 [3.66 - 9.66]	4.78 [3.49 - 8.87]	5.33 [3.61 - 9.44]
Randomised to ACE-I (*n*, (%))	353 (50.5)	208 (48.5)	449 (49.8)	112 (49.6)	561 (49.7)

Data are presented as mean ± standard deviation or median [interquartile range].
BMI, body mass index; HbA1c, glycated haemoglobin; SBP, systolic blood pressure; DBP, diastolic blood pressure; UAER, urinary albumin excretion rate.
Except for significant difference in BMI between VV and VI/II ($P \leq 0.05$), no significant differences exist between subgroups.

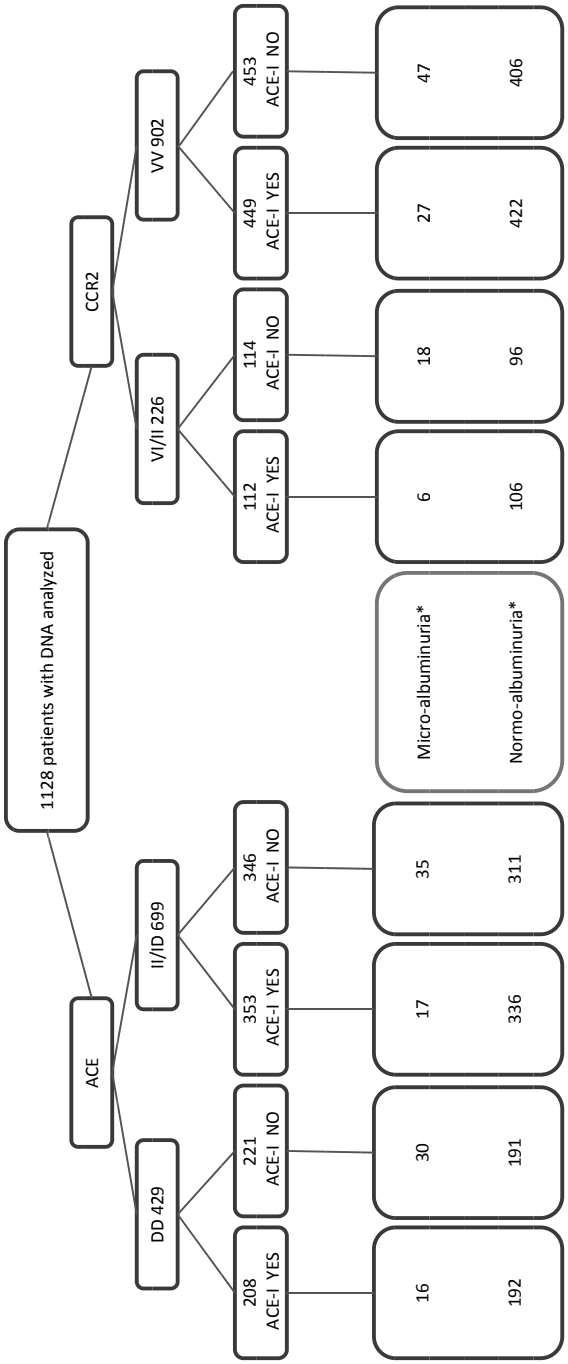


Figure 1: Schematic diagram of the study.
ACE-I, ACE inhibitor therapy; ACE, I/D polymorphism; CCR2, CCR2 V64I SNP.
* At the end of follow-up

Outcome according to ACE I/D genotype

During a follow-up of 42.3 (12.1-51.9) months, 46 out of 429 patients (10.7%) with DD developed microalbuminuria versus 52 out of 699 patients (7.4%) with II/ID (log rank test *P* = 0.03, figure 2A). As shown in table 2, DD carriers had significantly increased risk of microalbuminuria compared to II/ID carriers, even after adjustment for ACE-I treatment, but not after further adjustment for other risk factors.

Response to ACE-I according to ACE I/D genotype

Treatment with ACE-I significantly reduced the incidence of microalbuminuria compared with placebo with a reduction of incidence by factors 2.6 and 2.1 for treatment with ACE-I plus verapamil and ACE-I alone, respectively, (*P* < 0.01 for both) [2]. Among DD carriers, 16 of the 208 patients (7.7%) on ACE-I and 30 of the 221 patients (13.58 %) that did not receive ACE-I developed microalbuminuria; log rank test *P*= 0.038. Similarly, in II/ID carriers incidence of microalbuminuria was significantly lower in those on ACE (17/353, 4.8%) compared to those on non-ACE inhibitor therapy (35/346, 10.1%); log rank test *P*= 0.002. As shown in table 3, ACE-I significantly protected from development of microalbuminuria in both DD and II/ID genotype carriers. No significant interaction was found between ACE-I treatment and ACE I/D (*P* for treatment*genotype 0.54).

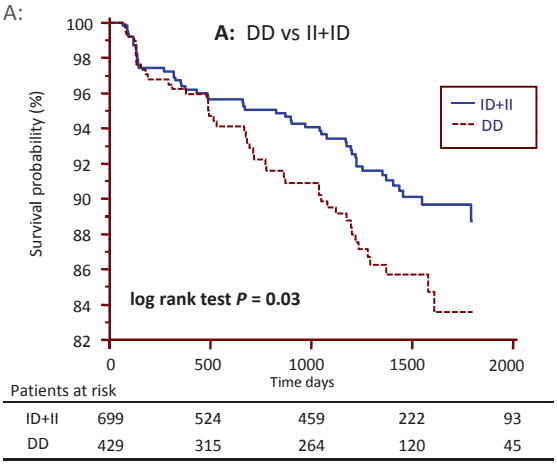
Outcome according to CCR2V64I genotype

Among VI/II carriers, 24 out of 226 patients (10.6%) developed microalbuminuria versus 74 out of 902 patients (8.2%) with the VV genotype (log rank test *P*= 0.21, figure 2B). As shown in table 2, incidence of microalbuminuria was not significantly different in VI/II carriers compared to VV carriers.

Table 2: Cox-regression models for development of microalbuminuria in 1128 patients with type 2 diabetes

	ACE I/D			CCR2 V64I			ACE I/D and CCR2 V64I		
Cox model	Genotype	HR (95%CI)	*P*-value	Genotype	HR (95%CI)	*P*-value	Genotype	HR (95%CI)	*P*-value
Model 1									
	II/ID	1 (ref.)		VV	1 (ref.)		II/ID and VV	1 (ref.)	
	DD	1.56 (1.05-2.32)	0.028	VI/II	1.35 (0.85-2.14)	0.198	II/ID and VI/II	1.27 (0.67-2.43)	0.462
							DD and VV	1.51 (0.96-2.39)	0.075
							DD and VI/II	2.17 (1.14-4.13)	0.019
Model 2									
	II/ID	1 (ref.)		VV	1 (ref.)		II/ID and VV	1 (ref.)	
	DD	1.51 (1.01-2.24)	0.043	VI/II	1.34 (0.84-2.12)	0.213	II/ID and VI/II	1.26 (0.66-2.41)	0.479
							DD and VV	1.46 (0.92-2.31)	0.104
							DD and VI/II	2.07 (1.08-3.94)	0.028
Model 3									
	II/ID	1 (ref.)		VV	1 (ref.)		II/ID and VV	1 (ref.)	
	DD	1.36 (0.91-2.02)	0.132	VI/II	1.36 (0.86-2.16)	0.186	II/ID and VI/II	1.46 (0.77-2.79)	0.246
							DD and VV	1.40 (0.88-2.21)	0.152
							DD and VI/II	1.69 (0.89-3.23)	0.111
Model 4									
	II/ID	1 (ref.)		VV	1 (ref.)		II/ID and VV	1 (ref.)	
	DD	1.21 (0.81-1.82)	0.347	VI/II	1.42 (0.89-2.25)	0.141	II/ID and VI/II	1.53 (0.80-2.93)	0.199
							DD and VV	1.24 (0.78-1.99)	0.357
							DD and VI/II	1.58 (0.82-3.04)	0.167

Model 1: Crude model
Model 2: Adjusted for ACE-I treatment
Model 3: Further adjusted for baseline urinary albumin excretion rate
Model 4: Further adjusted for, age, sex, smoking status, duration of diabetes, baseline A1c, and baseline systolic blood pressure.



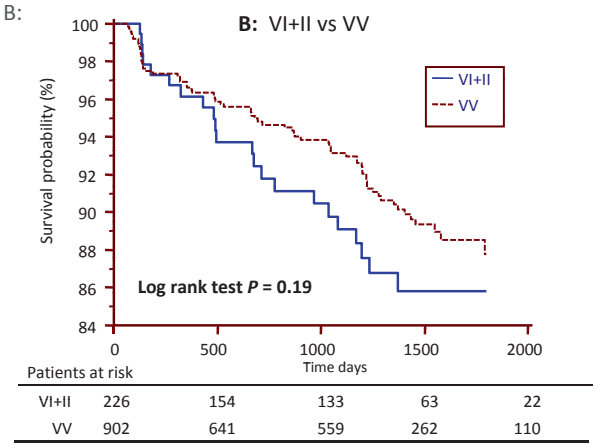


Figure 2: Kaplan–Meier curves of albuminuria in patients with type 2 diabetes according to A: ACE I/D and B: CCR2 V64I genotypes

Response to ACE-I according to CCR2 V64I genotype

Among VI/II carriers, 6 of the 112 patients (5.4%) on ACE-I and 18 of the 114 patients (15.8%) that did not receive ACE-I developed microalbuminuria; log rank test *P*= 0.013. Similarly in VV carriers, incidence of microalbuminuria was significantly lower in those on ACE-I than in those who did not receive ACE-I (27/449, 6% vs.

47/453, 10.4%, respectively); log rank test *P*= 0.04. As shown in table 3, ACE-I significantly protected from development of microalbuminuria in both VV and VI/II genotype carriers. Althoughthe Mantel-Haenszel chisquared test confirmed the different ACE-1 effect in the 2 genotype groups (Chi-square MH = 11.05, df = 1 and p-value = 0.0009), no significant interaction was found between ACE-I treatment and CCR2 V64I (*P* for interaction term treatment * genotype 0.37)

Outcome according to combined genotypes ACE I/D and CCR2 V64I

The risk for developing microalbuminuria progressively increased moving from carriers of protective genotypes for both ACE and CCR2 genes (i.e., II/ID and VV) to carriers of either CCR2 (VI/II and II/ID) or ACE (DD and VV) "risk" genotypes alone to carriers of both risk genotypes (DD and VI/II). As shown in table 2, the unadjusted HR for development of microalbuminuria in patients with both "risk" genotypic variants, i.e. DD from ACE I/D and VI/II from CCR2, compared to carriers of neither of these genotypes, i.e. ID/II and VV, was 2.17 (95%CI 1.14-4.13, *P*=0.02). This increased risk of microalbuminuria in carriers of both DD and VI/II was significant after adjustment for ACE-I, but not after further adjustment for other risk factors (table 2).

Response to ACE-I according to combined ACE I/D and CCR2 V64I genotypes

As shown in table 3 and table 4, among carriers of "risk" genotypes for both ACE and CCR2 genes (i.e., DD and VI/II), treatment with ACE-I did not significantly protect from microalbuminuria. Of notethere was a trend toward protective effect of ACE-I in carriers of both II/ID and VV, while the highest efficacy of ACE-I was observed in carriers of VI/II plus ID/II genotypes (table 4). No significant interaction was found between ACE-I treatment and combined genotypes (ACE I/D and CCR2 V64I) (*P* for interaction term treatment * combined genotypes 0.17).

Table 3: Hazards ratio (95% confidence interval) of response to ACE-I treatment according to ACE I/D, CCR2 V64I, and the combination of both genotypes

			CCR2 V64I				
				HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
				VI/II		*VV*	
			Model 1	0.33 (0.13-0.83)	0.019	0.51 (0.31-0.81)	0.005
			Model 2	0.22 (0.08-0.61)	0.004	0.49 (0.31-0.80)	0.004
ACE I/D							
	HR (95%CI)	*P*-value					
	DD			*DD and VI/II*		*DD and VV*	
Model 1	0.53 (0.29-0.98)	0.042	Model 1	0.76 (0.24-2.38)	0.633	0.47 (0.23-0.97)	0.040
Model 2	0.47 (0.25-0.88)	0.018	Model 2	0.66 (0.17-2.59)	0.554	0.39 (0.18-0.83)	0.014
	II/ID			*II/ID and VI/II*		*II/ID and VV*	
Model 1	0.41 (0.23-0.73)	0.003	Model 1	0.09 (0.01-0.67)	0.019	0.55 (0.29-1.04)	0.067
Model 2	0.44 (0.25-0.80)	0.007	Model 2	0.05 (0.004-0.76)	0.030	0.61 (0.32-1.17)	0.142

Model 1: crude model
Model 2: adjusted for age, sex, smoking status, duration of diabetes, baseline A1c, baseline systolic blood pressure, and baseline urinary albumin excretion rate.

Table 4: Effect of ACE-I on incidence of microalbuminuria according to genotype

Genotype	*n*	Incidence of microalbuminuria (%)		Log rank test *P*	Absolute reduction of risk	Percentage reduction of risk
		No ACE-I	ACE-I			
DD + VI/II	86	16.28	11.63	0.63	4.65	29
II/ID + VV	559	8.73	5.63	0.06	3.10	36
DD + VV	343	12.92	6.67	0.04	6.25	48
II/ID + VI/II	140	15.5	1.45	0.003	14.05	91
OVERALL	1128	11.46	5.88	<0.001	5.58	49

DISCUSSION

In this prospective study we found that ACE I/D polymorphism predicts new-onset microalbuminuria in patients with type 2 diabetes. The incidence of microalbuminuria was found to be higher in carriers of the deletion allele (DD carriers) compared to II/ID carriers. We also prospectively investigated the possible impact of CCR2 V64I polymorphism on incidence of microalbuminuria. Although we found that the CCR2 V64I alone did not significantly predict new-onset microalbuminuria, combined analyses of ACE I/D and CCR2 V64I revealed increased risk of reaching the endpoint microalbuminuria in carriers of both the "risk genotypes" DD and VI/II compared to carriers of both the "protective genotypes" II/ID and VV (figure 3).

Regarding response to therapy, all patients appeared to benefit from ACE-I treatment in terms of new-onset microalbuminuria, with lack of interaction between ACE I/D and CCR2 V64I on one hand, and treatment with ACE-I on the other hand. Of note, combined analysis of both polymorphism showed carriers of both II/ID and VI/II to benefit the most from ACE-I treatment with 91% reduction of risk.

This is the first study that examines the potential impact of ACE I/D polymorphism on new-onset microalbuminuria in patients with diabetes. Genotypes of ACE I/D polymorphism have been evaluated in many studies in diabetic and nondiabetic nephropathies. In the REIN study [16], ACE/ID polymorphism did not predict progression in GFR or risk of ESRD in 352 patients with nondiabetic proteinuric chronic nephropathy. In the same trial, ACE-I treatment is renoprotective in women regardless of the ACE I/D polymorphism. On the other hand an interaction between

ACE I/D genotypes and response to treatment was evidenced in male patients, indeed ACE-I protected from renal disease progression male patients with the DD genotype, but lacked beneficial effect in men with II or ID genotypes [17].

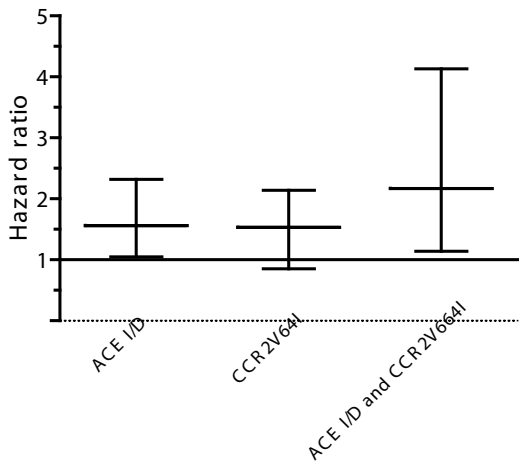


Figure 3: Hazard ratio for each genotype.
ACE I/D: II/ID vs. DD
CCR2 V64I: VV vs. VI/II
Combined analysis: II/ID and VV vs. DD and VI/II

The impact of ACE I/D polymorphism on renal outcome in diabetes has been investigated in the RENAAL study [18]. In this randomized, double-blind, placebo-controlled study in 1443 patients with type 2 diabetes and nephropathy, homozygous carriers of the D allele had the worse prognosis regarding the composite endpoint of doubling serum creatinine, ESRD, and death. Furthermore, treatment with Losartan gave the greatest benefit in carriers of the D allele.

In our study, we found that patients with diabetes who were homozygous carriers of D allele have increased risk of developing microalbuminuria. However in contrast to findings from the RENAAL study, we found that treatment with ACE-I trandolapril was uniformly renoprotective in all subgroups of the BENEDICT study, regardless of genotype or gender.

This discrepancy could be explained by differences in the stage of nephropathy at initiation of treatment. In the RENAAL study [18], patients had advanced stage of diabetic nephropathy (median urinary albumin/creatinine ratio 1166 mg/g) at randomisation, whereas the BENEDICT trial investigated the effect of RAAS blockade given as prophylactic intervention in diabetic patients without albuminuria at baseline. It could therefore be possible that potential impact of the D allele is more pronounced in advanced stages of nephropathy. In situations of overt nephropathy and macroalbuminuria, blockade of RAAS is expected to give additional benefit in DD carriers who have higher levels of ACE [19]

Interestingly, this harmful impact of D allele in our study remained significant after adjustment for use of ACE-I, but not after adjustment for other risk factors of microalbuminuria in the multivariate model. This could be explained by the fact that these risk factors are part of the causal pathway between the genetic risk and the clinical outcome.

Furthermore, in this study we evaluated the impact of CCR2 V64I polymorphism on new-onset microalbuminuria in diabetic patients. CCR2 is a seven-transmembrane, protein-coupled receptor of MCP-1, which plays an important role in the pathophysiology of renal damage in diabetes by recruitment of monocytes and other inflammatory cells [20, 21]. MCP-1 inhibition has been shown to prevent glomerulosclerosis and improve glomerular filtration rate in experimental diabetes [22]. Recently, Kang et al. [23] have shown that treatment with a CCR2-antagonist in diabetic mice resulted in decrease of albuminuria and improvement in mesangial expansion by suppression of synthesis of pro-inflammatory molecules in renal tissues, including TGF-β1 and type IV collagen. In our study, CCR2 V64I polymorphism alone did not predict new-onset microalbuminuria, however combined analyses of CCR2 V64I and ACE I/D showed that carriers of VI/II variants from CCR2 V64I who also had the DD genotype of ACE I/D were at increased risk of developing new-onset microalbuminuria. While this finding could suggest some impact of genetic variation in CCR2 in subjects with activated RAAS, our data should be interpreted with caution, since the increased risk of microalbuminuria in carriers of the combined DD and VI/II genotypes was not significant after adjustment for baseline microalbuminuria and other risk factors.

In conclusion, ACE I/D polymorphism predicts new-onset microalbuminuria in patients with type 2 diabetes without nephropathy, with an increased incidence of microalbuminuria inDD carrierscompared to II/ID carriers. Carriers of both “risk” genotypes DD and VI/II have increased risk of developing microalbuminuria compared to carriers of both “protective” genotypes. Large prospective clinical studies are needed to investigate the potential role of CCR2 V64I gene variant in renal inflammation and clinical outcome in patients with diabetes.

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Chapter 5

Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy

Alkhalaf A, Zürbig P, Bakker SJL, Bilo HJG, Cerna M, Fischer C, Fuchs S, Janssen B, Medek K, Mischak H, Roob JM, Rossing K, Rossing P, Rychlík I, Sourij H, Tiran B, Winklhofer-Roob BM, Navis GJ

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ABSTRACT

Background

Urine proteome analysis is rapidly emerging as a tool for diagnosis and prognosis in disease states. For diagnosis of diabetic nephropathy (DN), urinary proteome analysis was successfully applied in a pilot study. The validity of the previously established proteomic biomarkers with respect to the diagnostic and prognostic potential was assessed on a separate set of patients recruited at three different European centers. In this case-control study of 148 Caucasian patients with diabetes mellitus type 2 and duration ≥5 years, cases of DN were defined as albuminuria >300 mg/d and diabetic retinopathy (n=66). Controls were matched for gender and diabetes duration (n=82).

Methods

Proteome analysis was performed blinded using high-resolution capillary electrophoresis coupled with mass spectrometry (CE-MS). Data were evaluated employing the previously developed model for DN.

Results

Upon unblinding, the model for DN showed 93.8% sensitivity and 91.4% specificity, with an AUC of 0.948 (95% CI 0.898-0.978). Of 65 previously identified peptides, 60 were significantly different between cases and controls of this study. In <10% of cases and controls classification by proteome analysis not entirely resulted in the expected clinical outcome. Analysis of patient's subsequent clinical course revealed later progression to DN in some of the false positive classified DN control patients.

Conclusions

These data provide the first independent confirmation that profiling of the urinary proteome by CE-MS can adequately identify subjects with DN, supporting the generalizability of this approach. The data further establish urinary collagen fragments as biomarkers for diabetes-induced renal damage that may serve as earlier and more specific biomarkers than the currently used urinary albumin.

INTRODUCTION

Diabetic nephropathy (DN) is a leading cause of morbidity and mortality in patients with diabetes mellitus [1]. Accurate diagnostic tools are important, not only for the allocation of preventive measures but also to better unravel the complex pathogenesis of DN. Current clinical biomarkers used to diagnose diabetic kidney disease, urinary albumin excretion and glomerular filtration rate, are subject to considerable measurement variability [2], and are heterogeneous as to prognostic impact [3]. Whereas albuminuria is broadly used as a renal biomarker, its specificity is still subject of debate [4]. Moreover, urinary albumin excretion and glomerular filtration rate (GFR) are also affected in non-diabetic renal disease, and accordingly not specific for diabetic nephropathy [5]. As such their potential to detect and monitor the specific pathogenetic processes involved in diabetic nephropathy is limited. Furthermore, especially GFR, but also albuminuria are late stage biomarkers, only indicative after substantial organ damage [6]. Alternative non-invasive diagnostic methods, that may enable detection of DN at an earlier stage, and/or with higher accuracy, would be beneficial for clinical management of diabetic patients, as well as for pathogenetic studies aimed at further deciphering pathophysiology, and identifying targets for intervention. Potential sources for such biomarkers may be urinary proteins and/or peptides, as these should display significant changes at an early state of disease, displaying initial pathophysiological changes in the kidney [7].

Proteome analysis using capillary electrophoresis coupled mass spectrometry (CE-MS) has recently emerged as a powerful tool to define biomarkers that enable diagnosis [8, 9], prognosis [10], assessment of therapeutic intervention [11], and monitoring of specific pathogenetic pathways. The different technological considerations, both with respect to samples and technological platform have recently been discussed and reviewed [12-15]. We have focused on urinary proteome analysis as the urinary proteome has been found to be quite stable [16, 17] and contains an array of low molecular weight proteins and peptides that can be analyzed without the need for additional manipulation such as proteolytic digests [18].

Recent studies demonstrated that urinary proteome analysis enables the definition of biomarkers specific for chronic kidney disease (CKD) [12, 19] and for DN [1, 20].

These might prove valuable in clinical practice. As a first step, however, confirmation of the diagnostic value of these markers in a controlled study in independent clinical centers, different from the ones that were involved in the identification of the biomarkers, has to be obtained. Such rigid independent confirmation is required prior to any further development, investigating e.g. prognostic value, to clearly support the validity and reliability of the biomarkers and biomarker-based models [21]. In the past, confirmation of potential disease-associated biomarkers has often failed (e.g. [22, 23]), hence this step is of the outmost importance. Therefore, we aimed to validate identified biomarkers and a biomarker-based model for DN that was described previously in an independent blinded set of samples [1], collected prospectively in multiple centers not involved in the original identification of biomarkers to rule out any center-based bias.

To ease data interpretation, a case-control set-up was chosen. The low molecular proteome of diabetes mellitus type 2 patients with normoalbuminuria (controls) and matched diabetes patients with diabetic nephropathy (cases) was analyzed in a blinded study (PREDICTIONS study) by capillary electrophoresis-mass spectrometry (CE-MS), and samples were successful classified using the previously defined biomarker model.

METHODS

Ethics Statement

The study was conducted according to the requirements of the Declaration of Helsinki, the protocol was approved by the respective Ethical review boards of the participating centers (Medical Ethics Committees of the Isala Clinics in Zwolle and of the University Medical Center in Groningen, the Ethics Committee of Third Faculty of Medicine, Charles University at Prague, and the Ethics Committee of the Medical University of Graz), and written informed consent was obtained from all patients.

Settings and participants

The study was set up as a cross-sectional case-control study, cases being type 2 diabetes patients with nephropathy, controls being type 2 diabetes patients without nephropathy. Patients aged 35-75 with type 2 diabetes with a documented duration of

diabetes of ≥5 years were eligible. Diagnosis of diabetes was established in accordance with the WHO criteria, by the following: fasting plasma glucose ≥7.0 mmol/l, a two-hour value in an oral glucose tolerance test ≥11.1 mmol/l, or random plasma glucose ≥11.1 mmol/l in the presence of symptoms. Type 2 diabetes was diagnosed by lack of criteria for type 1 diabetes. Inclusion criteria for cases were: albuminuria >300 mg/d and known overt diabetic retinopathy. Retinopathy is requested to be present is to ensure that albuminuria is the consequence of diabetic nephropathy rather than a non-diabetic glomerulopathy. A renal biopsy would be the gold standard to discriminate between diabetic nephropathy and a non-diabetic glomerulopathy, but a renal biopsy is nearly never taken in diabetic patients and several studies have indicated that the request for retinopathy being present is a good alternative for discrimination between diabetic nephropathy and non-diabetic glomerulopathy in type 2 diabetic patients with albuminuria [24-26]. Exclusion criteria were end stage renal failure, known causes of renal failure other than diabetes and non-Caucasian ethnic origin. Controls were matched within center for gender and diabetes duration. Exclusion criteria for controls were micro-albuminuria, non-Caucausian ethnic origin, and in case of use of RAAS-blocking medication, unknown albuminuria status prior to start of treatment. Patients were prospectively recruited from the outpatient clinics for diabetes and nephrology in three participating centers, located in Zwolle (The Netherlands), Graz (Austria), and Prague (Czech Republic), respectively.

Sample collection and preparation

The second urine of the morning was collected as described [27] and stored frozen below -20°C. A 0.7 mL aliquot was thawed immediately before use and diluted with 0.7 mL 2 M urea, 10 mM NH_4OH containing 0.02 % SDS. In order to remove high molecular weight polypeptides, samples were filtered using Centrisart ultracentrifugation filter devices (20 kDa molecular weight cut-off; Sartorius, Goettingen, Germany) at 3,000 g until 1.1 mL of filtrate was obtained. Subsequently, filtrate was desalted using PD-10 column (GE Healthcare, Sweden) equilibrated in 0.01% NH_4OH in HPLC-grade water. Finally, samples were lyophilized and stored at 4°C. Shortly before CE-MS analysis, lyophilisates were resuspended in HPLC-grade water to a final protein concentration of 0.8 µg/µL checked by BCA assay (Interchim, Montlucon, France).

CE-MS analysis

CE-MS analysis was performed as described [27], using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) on-line coupled to a Micro-TOF MS (Bruker Daltonic, Bremen, Germany). Data acquisition and MS acquisition methods were automatically controlled by the CE *via* contact-close-relays. Spectra were accumulated every 3 s, over a range of *m/z* 350 to 3000 Th. Accuracy, precision, selectivity, sensitivity, reproducibility, and stability are described in detail elsewhere [27, 28]. The average recovery of sample in the preparation procedure was ~85% and the limit of detection was ~1 fmol. Mass resolution was above 8,000 enabling resolution of monoisotopic mass signals for $z \leq 6$. After charge deconvolution, mass accuracy was <25 ppm for monoisotopic resolution and <100 ppm for unresolved peaks ($z > 6$). The analytical precision of the set-up was assessed by (a) reproducibility achieved for repeated measurement of the same replicate and (b) by the reproducibility achieved for repeated preparation and measurement of the same urine sample. To ensure high data consistency, a minimum of 950 peptides/proteins had to be detected with a minimal MS resolution of 8,000 in a minimal migration time interval of 10 minutes.

Data processing

Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software [29]. Both CE-migration time and ion signal intensity (amplitude) show variability, mostly due to different amounts of salt and peptides in the sample and are consequently normalized. Reference signals of 1770 urinary polypeptides are used for CE-time calibration by local regression. For normalization of analytical and urine dilution variances, MS signal intensities are normalized relative to 29 “housekeeping” peptides generally present in at least 90% of all urine samples with small relative standard deviation, as described in detail recently [28]. For calibration, local regression is performed. The obtained peak lists characterize each polypeptide by its molecular mass [Da], normalized CE migration time [min] and normalized signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Classification model of DN

Data of the current samples were tested against the previously developed biomarker model for DN [1]. Rossing et al. defined and validated models for the differentiation of diabetic patients type 1 with macroalbuminuria and normoalbuminuria after CE-MS analysis. Among these diabetes patients, 102 urinary biomarkers differed significantly between patients with normoalbuminuria and DN. For reduction of the number of variables, a "take-one-out" procedure was used, decreasing the number of biomarkers to 65 without losing performance in the classification. A support vector machine (SVM) biomarker model with these 65 polypeptides identified patients with DN in blinded data set of 70 individuals (35 cases and 35 controls) with 100% sensitivity and 97% specificity (AUC=0.994).

SVM-based classification on the urinary peptidome was performed using MosaCluster software (version 1.7.0) [30]. This software tool allows the classification of samples in the high-dimensional parameter space by using support vector machine (SVM) learning. For this purpose, MosaCluster generates polypeptide models, which rely on polypeptides displaying statistically significant differences when comparing data from patients with a specific disease to controls or other diseases, respectively. Each of these polypeptides represents one dimension in the n-dimensional parameter space [9, 31-33]. SVM views a data point (probands plasma sample) as a p-dimensional vector (p numbers of protein used), and it attempts to separate them with a (p-1) dimensional hyperplane. There are many hyperplanes that might classify the data. However, maximum separation (margin) between the two classes is of additional interest, and therefore, the hyperplane with the maximal distance from the hyperplane to the nearest data point is selected. All marker proteins are used without any weighting to build up the n-dimensional classification space and to display the data set in the classification space. Classification is performed by determining the Euclidian distance of the data set to the n-1 dimensional maximal margin hyperplane (absolute value of the normal vector) and the direction of the vector (class 1 or class 2).

Statistical analysis

Sensitivity and specificity of the previously defined biomarker models, and 95% confidence intervals (95% CI) were calculated using receiver operating characteristic (ROC) plots (MedCalc version 8.1.1.0, MedCalc Software, Belgium, www.medcalc.be) [34]. Furthermore, Mann-Whitney test (for independent samples) was performed to receive Box-Whisker-Plots with this software. Statistical significance was assumed at $p<0.05$. For analysis of differences of individual peaks between cases and controls, statistical significance was assumed at $p<0.001$ to account for multiple testing. For the correlation analysis of each peptide biomarker, Rank correlation was used with Spearman's rank correlation coefficient (Spearman's ρ) (MedCalc version 8.1.1.0, MedCalc Software, Belgium, www.medcalc.be). For biomarker definition, polypeptides that were found in more than 70 % of the samples in at least one of the two groups (DN or non-DN) were considered. This pre-defined set of polypeptides was further validated by randomly excluding 30 % of available samples. This bootstrapping procedure was repeated up to 10 times. Further on, mutilvariate statistic methods (e.g., Benjamini-Hochberg) were applied for selection refinement.

Sequencing of peptides

Candidate biomarkers from urine were sequenced using CE-MS/MS or LC-MS/MS as recently described [35].

Raw data files were either converted into dta-files (RAW files generated by ion traps from Thermo Fisher Scientific) with the use of DTA Generator [36, 37] or into mgf-files (data derived from MALDI-TOF and Q-TOF analyses) with the use of DataAnalysis (version 4.0; Bruker Daltonik). All resultant MS/MS data were submitted to MASCOT (www.matrixscience.com; release number: 2.3.01) for a search against human entries (20,295 sequences) in the Swiss-Prot database (Swiss-Prot number 2010.06) without any enzyme specificity and with up to one missed cleavage. No fixed modification was selected, and oxidation products of methionine, proline, and lysine residues were set as variable modifications. Accepted parent ion mass deviation was 0.5 Da (20 ppm for all Orbitrap spectra); accepted fragment ion mass deviation was 0.7 Da. Only search results with a MASCOT peptide score equally or higher as the MASCOT score threshold were included. Additionally, ion coverage was

controlled to be related to main spectral fragment features (b/y or c/z ion series). For further validation of obtained peptide identifications, the strict correlation between peptide charge at pH 2 and CE-migration time was utilized to minimize false-positive identification rates [38, 39]. As depicted in figure 1, the polypeptides are arranged in four to five lines. The members of each line are characterized by the numbers of basic amino acids (arginine; histidine; lysine) included in the peptide sequence. Specifically, the peptides in the right line contain no basic amino acids, only the N-terminus of the peptide is positively charged at pH 2. In contrast, peptides in the other lines (from right to left) show increasing number of basic amino acids in addition to their N-terminal ammonium group [39]. Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time. A peptide was accepted only if it had a mass deviation below ±50 ppm and a CE-migration time deviations less than ±2 min.

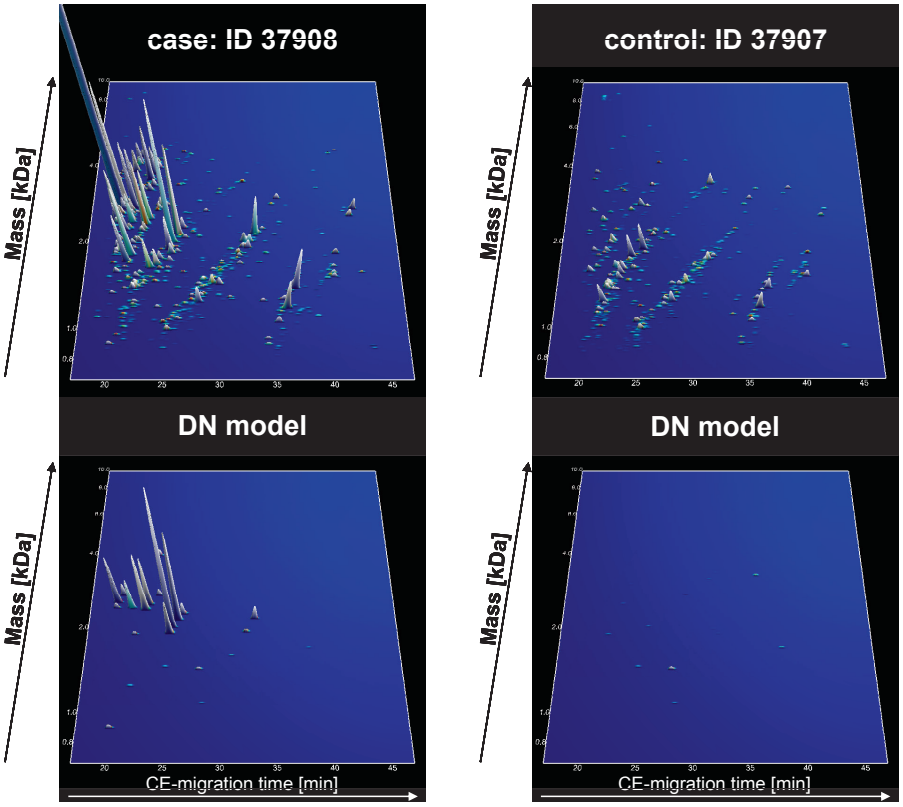

Figure 1: Polypeptide patterns of exemplarily urine samples.
The upper panel shows polypeptide patterns of all peptides, which are in the urinary proteome from one patient with (ID: 37908) and one patient without DN (ID: 37907). The lower panel shows distinct peptides of the DN model of these patients urine sample. Each polypeptide is defined by its CE-migration time (x-axis, minutes), mass (y-axis, kDa), and signal intensity (z-axis). The molecular mass is indicated on the left, the normalized migration time is indicated on the bottom.

RESULTS

The case/control study was composed of 148 diabetes mellitus type 2 patients, including 65 cases and 83 controls. The patients were well-matched for age, gender and diabetes duration. Blood pressure was significantly higher, and creatinine clearance significantly lower in cases (all $p<0.05$). Albuminuria was by definition present in cases, and absent in control. All samples were analyzed with CE-MS. For 145 urine samples (64 cases and 81 controls) of this study population high quality CE-

MS data sets were obtained. The data obtained from 3 samples did not pass quality control and were excluded from the subsequent analysis. Patient characteristics and classification scores of the proteome analysis are presented in table 1.

Table 1: Patient characteristics (means ± SD) of the PREDICTIONS cohort

	n	M/F	Age [year]	Duration DM [year]	SBP [mmHg]	DBP [mmHg]	UAE [mg/L]	CrCl [ml/ min/1.73m^2]
cases	64	44/20	64±10	17±8	143±21	78±12	953±931	72±40
controls	81	47/34	62±11	16±6	133±15	74±11	6±4	94±32

M/F: male/female ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; UAE: urinary albumin excretion; CrCl: creatinine clearance estimated by Cockroft-Gault equation.

Rossing et al. defined and validated models for the differentiation of diabetic patients type 1 with macroalbuminuria and normoalbuminuria after CE-MS analysis [1]. A support vector machine biomarker model (SVM-BM) composed of 65 of these biomarkers identified DN in blinded data set with 100% sensitivity and 97% specificity. This 'DN model', was applied to the collected 'case and control' samples of the PREDICTIONS study cohort. After evaluation of the blinded samples, all data were reported to the central study database for further evaluation and subsequent unblinding. After unblinding, accuracy of prediction was assessed. The complete polypeptide profiles and the DN-specific panels are depicted in figure 1 exemplarily for one patient with and one without DN.

Classification of the 'case/control' urine samples with this 'DN biomarker model' was accomplished with sensitivity of 93.8% and specificity of 91.4%. The AUC value in the ROC-analysis was 0.948 [95% CI: 0.898 to 0.978] (see figure 2A). As depicted in the Box-and-Whisker plot in figure 2B, this classification resulted in a significant ($P<0.0001$) difference of the median classification factor between patients with DN (0.889 [95% CI: 0.843 to 0.924]) and patients without DN (-0.461 [95%°CI: -0.592 to -0.255]). The classification results are shown in table S2 ('DN model').

Of the 65 previously defined differentially expressed peptides [1], in the current study 92.3% (60 markers) could be confirmed as being significantly different in this PREDICTIONS cohort between diabetic patients with DN and diabetic controls at $p<0.05$ and 50/60 were significant at $p<0.0001$.

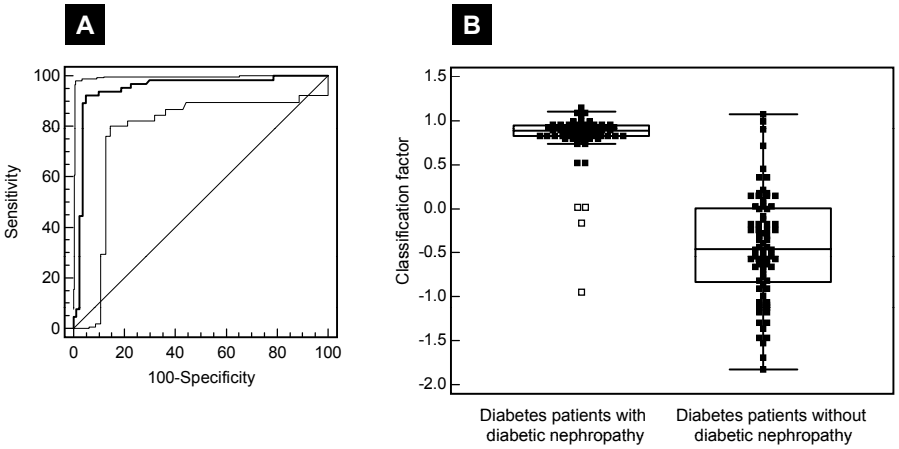

Figure 2: Statistical analysis of the classification results.
(A) ROC curve and (**B)** Box-Whisker-plot for classification of the 'case and control' patient collective with the 'DN' pattern are shown.

A correlation analysis of these classification factors and the clinical parameters was performed (see figure 3). In figure 3A the correlation with log urinary albumin excretion (UAE) is demonstrated with a positive correlation coefficient r=0.701 [95% CI: 0.607 to 0.775] and a significance level of P<0.0001. Here, most of the urine samples from patients without DN (<20mg/L) have classification factors below 0.3 and from patients with DN (<200mg/L) have classification factors above 0.5. The correlation analysis of the proteomic results with the creatinine clearance (CrCl) (see figure 3B) resulted in a negative correlation (r=-0.368 [95% CI: -0.501 to -0.218] (P<0.0001)), which is lower than the correlation with urinary albumin excretion. Many patients with a classification factor above 0.5 also show a CrCl <90 ml/min/1.73m^2. Otherwise, patients with classification factors lower than 0.5 differ in their CrCl between 50-200 ml/min/1.73m^2.

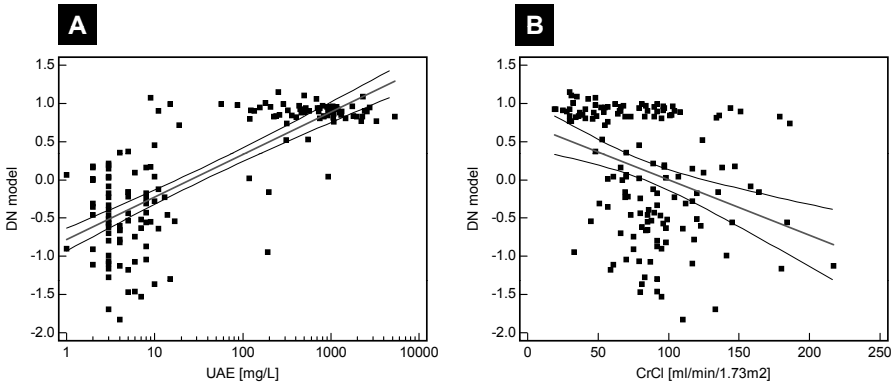

Figure 3: Correlation analysis.
Scatter diagrams of correlation from proteomic biomarker pattern with urinary albumin excretion (UAE) **(A)** and creatinine clearance **(B)**. The red line shows the regression line with 95% confidence interval (dashed line).

34 of the 65 biomarkers could be sequenced until today. We have identified 8 more peptides in comparison to in the previous study [1], where the used biomarker pattern was generated. For the sequenced peptides the direction of regulation is illustrated in figure 4 and 5. Up-regulated markers in urine samples of patients with DN (see figure 4) are fragments of blood components, like alpha-1-antitrypsin, albumin, transthyretin, alpha-2-HS-glycoprotein, and beta-2-microglobulin. In figure 5 the regulation of CD99 antigen fragment, collagen fragments, membrane associated progesterone receptor component 1 fragment, and uromodulin fragment is shown. Only one collagen fragment is up-regulated in urine samples of DN patients. This peptide belongs to the five biomarkers, which are not significant different between cases and controls.

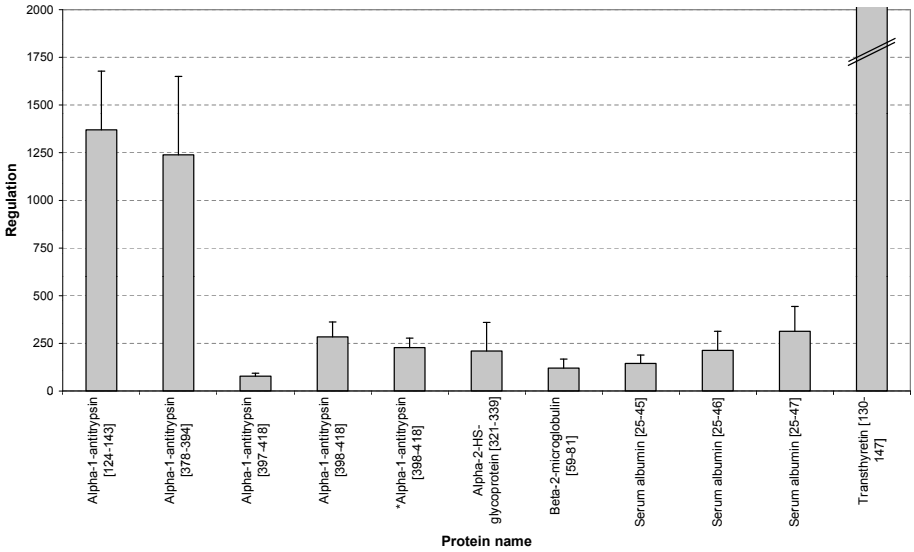

Figure 4: Up-regulation of blood derived protein fragments in urine samples of the PREDICTIONS cohort.
Displayed is the regulation of alpha-1-antitrypsin fragments, an alpha-2-HS glycoprotein fragment, a beta-2-microglobulin fragment, serum albumin fragments, and a transthyretin fragment. The asterisk (*) indicate same peptide with one more modification (oxidation).

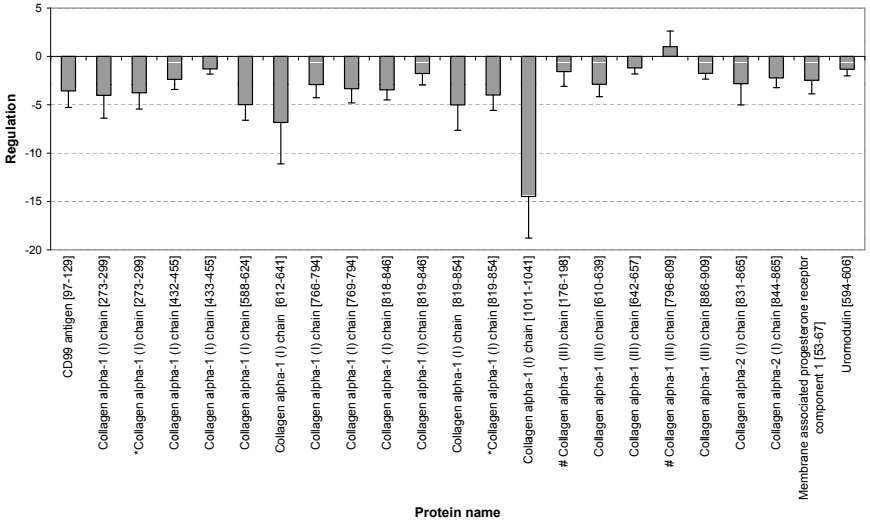

Figure 5: (Down-) Regulation of further peptide markers in urine samples of the PREDICTIONS cohort.
Displayed is the regulation of a CD99 antigen fragment, different collagen fragments, membrane associated progesterone receptor component 1 fragment, and uromodulin fragment. The hash (#) depicts peptide fragments, which are not significant in this cohort. The asterisk (*) indicate same peptide with one more modification (hydroxylation).

In addition, a Rank correlation of each of the 65 biomarker was performed. All biomarkers, which presented a significant correlation, have reciprocal correlation coefficients for UAE and CrCl. As expected the correlation between albumin excretion and known albumin and other blood protein fragments is positive, in contrast to the correlation of albumin excretion with the collagen fragments, CD99 antigen, membrane-associated progesterone receptor component 1, and uromodulin fragments. Furthermore, the correlation of UAE with blood protein fragments is stronger than the correlation with collagen fragments. The five biomarkers, which are not significant in the U-test, also show no significant correlation to urinary albumin excretion. The Rank correlation of creatinine clearance with collagen fragments and blood protein fragments is not very high in both cases.

Investigation of the false positive classified patients indicated that in several cases the classification "diabetic renal damage" may in fact be correct, but albuminuria may be under the respective criteria (see method section: 'Methods'). Seven 'control' patients were classified as cases. All of them show GFR values below 90 (stage 2), three of them even have a GFR<60 (stage 3), and two of them have an increasing urinary albumin excretion at a later visit (approximately 1 year later). These data may indicate the utility of the biomarkers not only for detection of overt nephropathy, but also prediction of its development in patients with diabetes and normoalbuminuria.

For the generation of a new model for DN in diabetic type 2 patients (using the PREDICTIONS cohort), urinary polypeptides of the control group were compared with those of patients with diabetic nephropathy. This analysis identified 103 peptides of statistical significance using multivariate statistic analysis like Benjamini-Hochberg [40] (p=0.05). A support vector machine-based model with these biomarkers discriminated controls from cases with 98% sensitivity and 99% specificity. The distribution of the polypeptides in the two groups is shown in figure 6A. The validity of the 'DN type 2' biomarkers was further evaluated in a diabetes type 1 test-set cohort (trainingset of Rossing et al. [1]) and resulted in 86% sensitivity and 100% specificity with an AUC value of 0.948 (see figure 6B). Of the 103 defined differentially expressed peptides, 65% (67 markers) could be confirmed as also being significantly different in the 'Rossing' cohort between diabetic patients with DN and diabetes controls. Of note, most biomarkers with high significance were found to be significantly different in both cohorts.

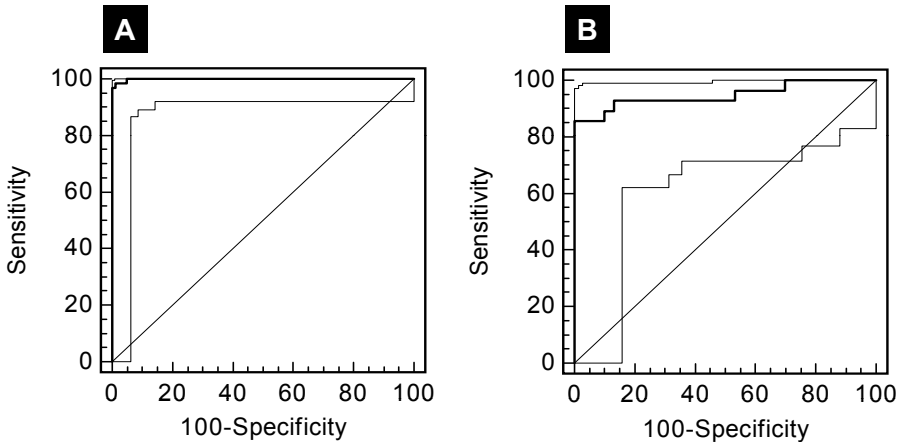

Figure 6: ROC curves for classification of the patient collectives with the 'DN type 2' pattern. ROC analysis for CKD diagnosis of the training set **(A)** and the test set **(B)**.

DISCUSSION

This study provides independent confirmation of the performance of a previously developed biomarker model for diabetic nephropathy using proteomic analysis with high-resolution CE-MS of the urinary proteome [1, 41]. The model for DN has high specificity and sensitivity, notwithstanding the fact that the current study was done in subjects with type 2 diabetes, whereas the population used for development of the model for diagnosis of diabetic nephropathy had diabetic mellitus type 1. These findings clearly indicate that the applied peptide pattern allowed diabetes-type independent classification of diabetic nephropathy.

For most of the peptides in the 'DN model', the difference between the cases and controls reached a high level of statistical significance, with p-values <0.0001, demonstrating the high selectivity of the urinary proteome analysis. The fact that 92% of the markers included in the 'DN model' were also significant in the PREDICTIONS cohort supports a valid strategy of marker selection in the Rossing study [1].

When distinguishing patients with DN from normoalbuminuric diabetic patients, the distribution of the classification factors in the control group (patients without DN) was broader than in the case group (see figures 2B and 3A). This may be explained by the existence of early stages of DN, in the absence of any clinical symptoms yet. The

arrangement of the case and control group was performed based on classical urine analyses (urinary albumin excretion rate).

Identification of the specific peptides in the biomarker model may allow better insights in patho-physiological pathways involved in renal damage in general, and specific pathways for renal damage in diabetes. The regulation of the sequenced biomarkers in the 'DN model', as reported here, shows a consistent pattern that is apparently specific for DN. The up-regulation of the serum protein fragments and the down-regulation of the collagen fragments in the urine is a consistent feature of DN, as also discussed in detail in the literature (see [6]). Furthermore, the correlation analysis confirms these findings. As expected, the correlation of the biomarkers with UAE and with CrCl resulted in reciprocal values.

Thousand-fold up-regulation of blood-derived protein fragments in urine (see figure 3B) is expected in the light of substantial glomerular damage that results in albuminuria. Hence, this likely does not reflect better or earlier markers. The presence of high amounts of these proteins likely indicates an insufficiency of readsorption or altered glomerular permeability of the kidney, implying an existing damage. In contrast, changes in the collagen metabolism may be closely linked to early renal damage in patients with diabetes and may help to provide information for the prognosis and monitoring of DN [1]. Type I and III collagens alpha-1 are main components of renal interstitial fibrosis [42]. It is tempting to speculate that the decrease in urinary collagen fragments reflects decreased collagen breakdown, and hence a propensity to progressive fibrotic lesions. However, the origin of the urinary collagen fragments cannot be determined from the current data and must be investigated in further studies. The differential excretion of uromodulin fragments gains additional interest from the recently reported association between genetic variation in the coding for uromodulin with susceptibility to CKD [43]. Finally, altered collagen metabolism appears to be involved in non-diabetic CKD as well, albeit with a differential excretory pattern. The patho-physiological impact of this finding deserves further exploration.

Investigation of the few cases where the classification factors of the DN model did not coincide with the clinical diagnosis suggests that several of these may in fact not be incorrectly classified by the DN model, but the clinical assessment of the patient

was not correct (see also figure 3A: classification factor >0.3 and UAE <10mg/L). Of the 7 controls that were classified as cases, all patients had at least GFR below 90, indicating the presence or the possible onset of renal disease. It is tempting to speculate that these patients may well have developed a diabetic renal disease that does not exactly resemble “classical DN”, hence is undetected by assessing albuminuria only.

The generation of a new model for DN with type 2 diabetic patients (PREDICTINS cohort) and the validation of this model with type 1 diabetic patients (Rossing cohort), resulted in the same AUC value as the validation of the previously defined ‘DN type 1’ model with the PREDICTIONS cohort. The differences in the biomarker selection/identification in type 1 and 2 diabetic patient urine samples may be caused by different patho-physiology of the DN, but also by the differences in both cohorts in general. The groups differ in age (~10 years), diabetes duration (~20 years), and medication (e.g. insulin). Therefore, this study is not suited for the analysis of differences of DN derived from type 1 or 2 diabetes. Also, the investigation of such potential differences was not a focus of this study. This will require greater population sizes and external validation of training profiles and components of profiles in independent populations of subjects with type 1 and type 2 diabetes and should be the focus of a further study.

In conclusion, urinary profiling using CE-MS was successfully applied to urine samples of an independent population of diabetic patients with or without existing DN. A biomarker model for the identification of patients with DN was validated with this multicenter blinded test set and allowed diagnosis of DN with high accuracy. These results provide clear independent confirmation for the accuracy of urinary proteome analysis for detection of DN. As these biomarkers have now been validated in independent clinical centers, we will in the next step investigate their prognostic value. Albumin excretion does reflect late pathological changes. However, we are tempted by the data presented to speculate that the assessment of urinary collagen fragments may result in a substantial improvement, enabling detection of diabetic nephropathy at earlier stages. This is also indicated by preliminary data on small populations ([1] and unpublished). As a next step, these promising data have to be verified in longitudinal studies of sufficient statistical power to prove, or disprove, the prognostic value of the urinary collagen fragments.

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Chapter 6

A double-blind randomized placebo-controlled clinical trial on benfotiamine treatment in patients with diabetic nephropathy

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ABSTRACT

Background

Benfotiamine, a lipid-soluble thiamine derivative, have been suggested as an agent that can prevent occurrence and deterioration of diabetic complications, including diabetic nephropathy. We aimed to investigate the effect of benfotiamine on urinary excretion of albumin (UAE) and the tubular damage marker kidney injury molecule 1 (KIM-1) in patients with type 2 diabetes and nephropathy.

Methods

In this double-blind, placebo-controlled trial, patients with type 2 diabetes and high-normal to micro-albuminuria (UAE 15-300 mg/24h) despite use of angiotensin-converting enzyme inhibitors (ACE-I) or angiotensin-receptor blockers (ARB), were randomly assigned to receive 12-week treatment with benfotiamine (900mg/day) or placebo. Thiamine status was assessed by whole blood thiamine concentrations, erythrocyte transketolase activity, and thiamine pyrophosphate effect. Primary outcome measures were 24h-UAE and 24h urinary KIM-1 excretion.

Results

In 39 patients assigned to benfotiamine and 43 patients assigned to placebo, median [interquartile range] baseline 24h-UAE was 90 [38; 267] vs 97 [48; 177] mg/24h, respectively, and 24h-KIM-1 was 1.67 [0.9; 2.4] vs 1.56 [1.1; 1.9] µg/24h respectively. Benfotiamine treatment resulted in significant improvement in all three domains of thiamine status (P <0.001). After 12 weeks of treatment with benfotiamine, there were no significant reductions in 24h-UAE and 24h-KIM-1 compared to placebo (ΔUAE: -9 [-53; 34] vs -7 [-56; 65] mg/24h respectively, P=0.36; ΔKIM-1: -0.014 [-0.23; 0.56] vs -0.043 [-0.36; 0.19] µg/24h respectively, P=0.09).

Conclusions

In patients with type 2 diabetes and nephropathy, high-dose benfotiamine treatment for 12 weeks as add-on therapy to ACE-I or ARB did not reduce urinary excretion of albumin or KIM-1 despite improving thiamine status.

INTRODUCTION

The incidence of diabetes related complications, like diabetic nephropathy (DN), increases, also in the perspective of the worldwide increase in prevalence of type 2 diabetes mellitus [1]. Diabetes has become the leading cause of end-stage renal disease (ESRD), with in some countries more than 40% of all new cases of ESRD occurring in patients with diabetes [2].

ESRD caused by diabetes can be explained by different pathophysiological mechanisms, including induction of glomerular endothelial damage, which in turn leads to albuminuria [3]. Albuminuria as such also plays an etiological role inducing tubulointerstitial inflammation and fibrosis with increasing albuminuria [4, 5].

Improving glycaemic control has shown to reduce the risk of the development of microalbuminuria [6, 7]. Once established, reduction of albuminuria, in particular by using angiotensin-converting enzyme inhibitors (ACE-Is) and angiotensin-receptor blockers (ARBs), is the cornerstone in preventing or retarding the occurrence of ESRD [8, 9]. Despite this successful therapy, there are still people with diabetes progressing to ESRD. Therefore, there is a great need for further adjunctive treatments which can help to prevent ESRD.

Recently, patients with type 1 and type 2 diabetes were found to have low plasma thiamine concentrations, due to increased thiamine loss with urine [10]. Additionally, thiamine and benfotiamine have recently been proposed as agents that can prevent occurrence and deterioration of diabetic complications [11, 12]. Benfotiamine is a lipophilic thiamine derivative with higher bioavailability compared to thiamine [13]. In animal experimental studies, benfotiamine has a beneficial effect on microvascular complications [12, 14].

We aimed to investigate whether additional treatment with benfotiamine in patients with type 2 diabetes and increased urinary albumin excretion rate on ACE-Is or ARBs results in reduction in urinary excretion of albumin or tubulointerstitial damage markers.

METHODS

Patients

Participants were recruited from the outpatient department population of the Isala Clinics in Zwolle, The Netherlands. Inclusion criteria were diagnosis of type 2 diabetes according to American Diabetes Association criteria [15], age between 40 and 75 years, active DN as indicated by urinary albumin excretion (UAE) in the high-normal or microalbuminuric range (UAE 15-300 mg/24-hour urine, or spot urine albumin/creatinine 1.25-25 mg/mmol in males and 1.75-35 mg/mmol in females) in at least two out of three samples within 2-6 weeks in advance of inclusion in the trial despite treatment with ACE-Is and/or ARBs in an unchanged dose for at least 3 months, glycated hemoglobin (HbA_{1c}) < 8.5% and estimated glomerular filtration rate calculated using the Modification of Diet in Renal Disease formula [16] (eGFR-MDRD) > 30 ml/min. Exclusion criteria were participation in another study ≤ one month before joining this study, renal impairment by causes other than diabetes, liver enzymes (AST and ALT) ≥ three times upper limit of normal values (normal values: AST < 40 IU/L, ALT < 45 IU/L), hyper-/hypothyroidism, a blood pressure > 160/90 mmHg, severe cardiac function disturbances or heart rhythm disturbances, neoplasm, severe general diseases or mental disorders, drug abuse, pregnancy or lactation, active menses during the past year, hypersensitivity to benfotiamine or other constituents of the study medication, use of vitamin B-containing supplements during the last 3 months and use of nonsteroidal anti-inflammatory drugs more than 3 times per week. Additionally, it was required that no changes had been made in prescription of cholesterol lowering medication, blood pressure lowering drugs and oral hypoglycaemic agents during 3 months prior the study. In total 2711 patients registered in our local Diabetes Electronic Management System (DEMS) were screened for eligibility [17]. Those who fulfilled inclusion criteria were informed about the study by sending information per mail. Patients who accepted were included after written informed consent was obtained. This trial was conducted in accordance with the Helsinki Declaration, approved by the medical ethics committee of the Isala Clinics, and registered in the clinical trial registry (ClinicalTrial.gov) under number (NCT00565318).

Procedures

Patients were randomised to benfotiamine 300 mg t.i.d. (daily dose 900mg) or placebo for 12 weeks. Benfotiamine and placebo tablets were prepared by Wörwag pharma (Böblingen, Germany) and packed in numbered boxes, unrecognized from each other, according to a computer-generated randomisation list which was prepared by an independent statistician. Independent pharmacists dispensed the medication box with the lowest available number to each patient. Neither the researchers nor the patients knew into which group they had been allocated.

During a run-in phase, patients were instructed how to collect 24-hour urine and asked not to change their usual diet or daily activity during the study, particularly during the week preceding their clinical visits. Patients were instructed to take one tablet after the three main meals, every day. In case of suspected side effects, patients were asked to contact the study physician who instructed them to stop the study medication until disappearance of complaints for a maximum of one week. When complaints disappeared, study medication was resumed once again. All participants were evaluated at baseline, after 6 weeks, and after 12 weeks of treatment. Patients were asked to deliver a 24-hour urine collection to the laboratory on each visit. At the laboratory, additional morning spot-urine sample and blood samples were taken. On the last visit, tablets were counted to assess compliance. Non-compliance was considered if less than 80% of the study medication had been taken. At the end of the study, after data collection and laboratory analyses had been completed, the randomisation list was provided to the researchers for unblinding.

Laboratory analyses

Thiamine concentration was measured in whole blood by HPLC, reference range 90-200 nmol/l, lower limit of detection 10 nmol/L, upper limit of detection 300 nmol/l [18]. Erythrocyte transketolase (TK)-activity (expressed in mU/mgHb) and thiamine pyrophosphate (TPP) effect (expressed as %) were measured according to the kinetic method of Chamberlain et al [19] in washed erythrocyte samples after being haemolysed by mixing with Aqua Purificata. Reagents were purchased from Sigma Aldrich® (Gillingham, United Kingdom). Thiamine deficiency was considered present if TPP effect >15% [20]. All these results were left unrevealed until all patients had

completed the study. Urinary albumin was measured by immunonephelometry (Behring Nephelometer, Mannheim, Germany) with a threshold of 1.8-2.3 mg/l and intra- and inter-assay coefficients of variation of less than 2.2 and 2.6%, respectively. Urinary kidney injury molecule-1 (KIM-1) was measured by ELISA, lowest limit of detection: 0.12 ng/ml, intra- and inter-assay coefficients of variation: 7.9% and 14.4%, respectively [21]. The other tubular markers, neutrophil gelatinase-associated lipocalin (Ngal, R&D Systems, Abingdon, UK) and α1-microglobulin (α1-m, Fitzgerald, Concord MA, USA; ICL Inc, Newberg, OR, USA), were measured using routine ELISA and competitive EIA assays, respectively. Cystatin C was measured by immunoassay (Gentian-AS, Moss, Norway). HbA1c was measured with Primus Ultra2 system using high-performance liquid chromatography. Other laboratory measurements were performed according to standard hospital procedures. Creatinine clearance was calculated from 24-hour urinary creatinine excretion and plasma creatinine.

Statistical analyses

Normally distributed variables are presented as means ± standard deviations (SD) and variables with a skewed distribution as medians and interquartile ranges (IQR). Q-Q plot was used to assess whether variables were distributed normally or skewed. $\chi 2$ test was used to compare non-continuous variables. Changes were analyzed by ANOVA for repeated measurements. P-values for change over time are presented. Additionally, changes in outcome measures from baseline to 6 weeks (Δ 6 weeks) and from baseline to 12 weeks (Δ 12 weeks) were computed. Positive changes indicate increase over time and negative changes indicate decrease over time. Comparisons of changes in primary and secondary parameters between groups were performed by an unpaired Student's t-test (in case of normal distribution) or Mann-Whitney-U test (in case of skewed distribution). Multivariate regression analysis was used to adjust for baseline differences between groups.

To test our hypothesis that benfotiamine reduces 24-hour urinary excretion of albumin and KIM-1 (primary outcome measures), 38 evaluable patients per group were required to detect an effect of size 0.65 (power 80%, $\alpha = 0.05$, one-sided test). To compensate for possible drop-out, we planned to enroll 43 patients per group. Secondary outcome parameters were ratio of albumin over creatinine in 24-hour

urine and spot morning urine samples, 24-hour urinary excretion of tubulointerstitial damage markers (α1-m and Ngal), and ratios of these markers and KIM-1 over creatinine concentration in 24h urine collections. A *P*-value of 0.05 or less was considered statistically significant. One-sided *P*-values were calculated for primary outcome measures and two-sided *P*-values were calculated for the other outcome measures. Statistical analyses were done by using a commercially available program (SPSS for Windows, version 16.0., Chicago, IL, USA).

Intention-to-treat analysis and per-protocol analysis were planned. In case of drop-out, data was not replaced and these patients had then to be excluded from analysis. Non-compliance and change in concomitant medications (including ACE-I or ARB) were considered as deviations from study protocol that lead to exclusion from per-protocol analysis.

RESULTS

Patient flow and baseline characteristics at randomization

Participants were recruited from February 2008 till February 2009. A CONSORT diagram of the study is shown in figure 1.

43 patients were randomized to benfotiamine and 43 to placebo. In the benfotiamine group, 2 patients did not complete the study because of newly diagnosed malignancy (lung cancer and stomach cancer, both were considered not causally related to study medication) and 2 others withdrew informed consent (one complained of dizziness and the other complained of urticaria and dry mouth). Because of this missing follow-up data, 39 patients out of 43 were analyzed in this group. In the placebo group, all patients finished the study and were analyzed. Baseline characteristics of the two groups are shown in table 1.

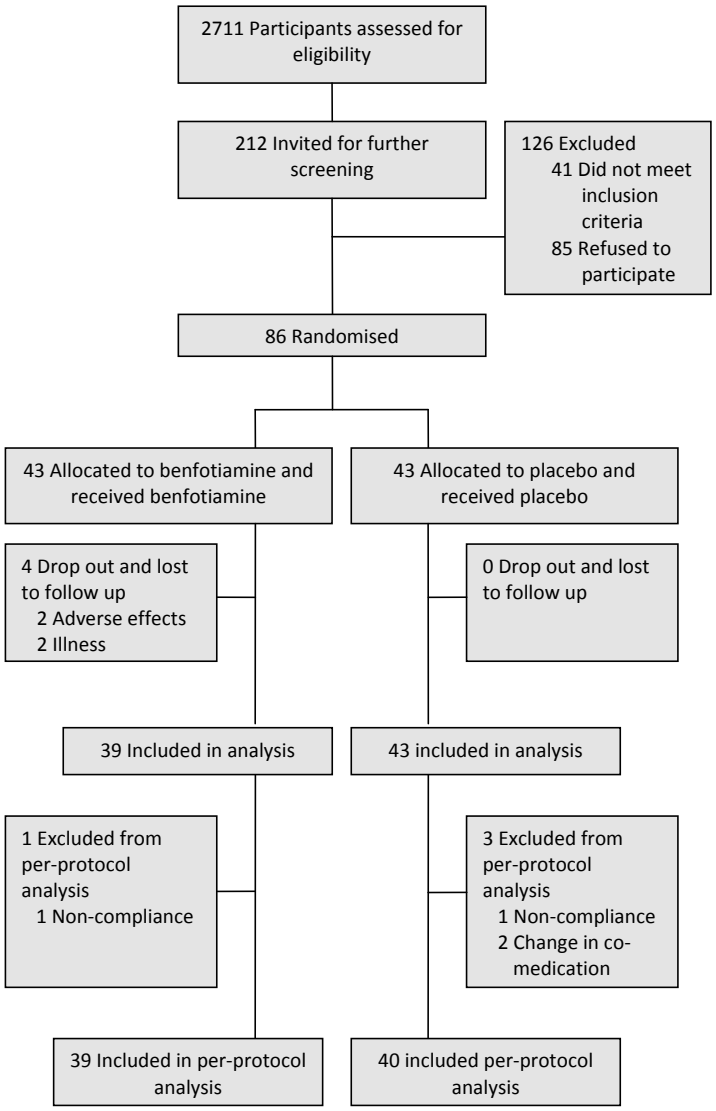


Figure 1: *CONSORT* flow diagram of the study.

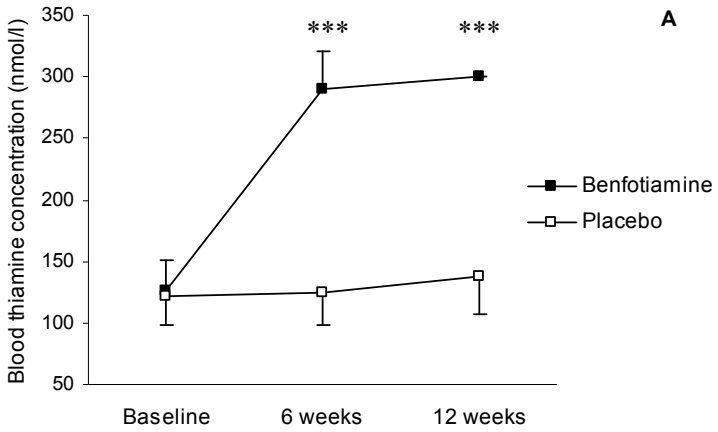
Table 1: Baseline characteristics of study population at baseline according to randomisation group

	Benfotiamine	Placebo	*P-value*
General			
n	39	43	
Age (years)	65.3 ± 5.9	64.6 ± 6.1	0.63
Males	30 (76.9)	33 (76.7)	0.98
BMI (kg/m^2)	32.1 ± 5.1	31.9 ± 5.9	0.93
Duration of diabetes (years)	12 [9; 18]	10 [7; 18]	0.41
Systolic blood pressure (mmHg)	140 ± 16	137 ± 20	0.48
Diastolic blood pressure (mmHg)	76 ± 8	76 ± 10	0.91
Smoking	10 (26)	10 (24)	0.72
Insulin treatment	31 (79)	29 (67)	0.22
Oral hypoglycemic agents	19 (49)	29 (67)	0.05
HbA_{1c} (%)	7.3 ± 0.9	7.4 ± 0.9	0.55
LDL-cholesterol (mmol/L)	1.9 ± 0.7	1.8 ± 0.9	0.37
HDL-cholesterol (mmol/L)	1.2 ± 0.3	1.1 ± 0.3	0.18
Triglycerides (mmol/L)	1.8 [1.4; 2.6]	2.1 [1.4; 3.4]	0.11
Serum creatinine (µmol/L)	84 ± 19	87 ± 23	0.51
Creatinine clearance (mL/min)	135 ± 51	130 ± 58	0.69
Cystatin C (mg/l)	1.01 ± 0.21	1.03 ± 0.23	0.66
Thiamine status			
Thiamine (nmol/L)	126 ± 23	120 ± 23	0.39
Transketolase activity (mU/mgHb)	0.41 ± 0.10	0.38 ± 0.11	0.69
TPP effect (%)	6.2 [1.0; 11.6]	9.1 [4.6; 15.5]	0.15
TPP effect > 15%	6 (15)	10 (23)	0.37
Primary outcome parameters			
UAE (mg/24 hours)	90 [38; 267]	97 [48; 177]	0.70
KIM-1 (µg/24 hours)	1.67 [0.9; 2.4]	1.56 [1.1; 1.9]	0.73
Secondary outcome parameters			
Spot urine UACR (mg/mmol)	10.3 [3.7; 23.4]	7.6 [4.3; 13.3]	0.60
24h UACR (mg/mmol)	9.3 [2.4; 16.8]	6.2 [3.4; 10.5]	0.47
KIM-1/creatinine (ng/mmol)	103 [63; 158]	99 [79; 141]	0.96
Urinary α1-m (mg/24 hours)	9.4 [4.3; 24.4]	8.2 [4.3; 20.3]	0.96
Urinary α1-m/creatinine (mg/mmol)	0.57 [0.28; 1.38]	0.64 [0.30; 1.35]	0.78
Urinary Ngal (mg/24 hours)	131.5 [66.8; 226.8]	122.2 [53.5; 224.2]	0.73
Urinary Ngal/creatinine (mg/mmol)	6.68 [4.25; 13.91]	7.68 [4.22; 18.86]	0.93

Data are *n* (%), mean ± standard deviation, or median [interquartile range]. $HbA_{1c,}$, glycated hemoglobin; TPP, thiamine pyrophosphate; UAE, urinary albumin excretion; KIM-1, kidney injury molecule-1; UACR, urinary albumin-creatinine ratio; α1-m, α1-microglobuline; Ngal, neutrophil gelatinase associated lipocalin.

Thiamine status

Effects of intervention on thiamine status are shown in figure 2. In patients receiving benfotiamine, whole blood thiamine concentrations increased, reaching the upper limit of detection (300 nmol/l) in all patients at 12 weeks. Erythrocyte TK-activity also significantly increased after 12 weeks of treatment in the benfotiamine group compared to placebo (median [IQR] change after 12 week 0.13 [0.05; 0.18] versus 0.04 [-0.03; 0.06] mU/mgHb in benfotiamine and placebo, respectively, $P<0.001$). Concomitantly, there was a significant decrease in TPP-effect in the benfotiamine group (median [IQR] change after 12 week -9.9 [-14.1; -3.6] % versus -1.4 [-9.9; 3.6] % in benfotiamine and placebo, respectively, $P= 0.002$). At 12 weeks, no patients in the benfotiamine group and 2 patients (5%) in the placebo group had thiamine deficiency defined as TPP effect > 15%.



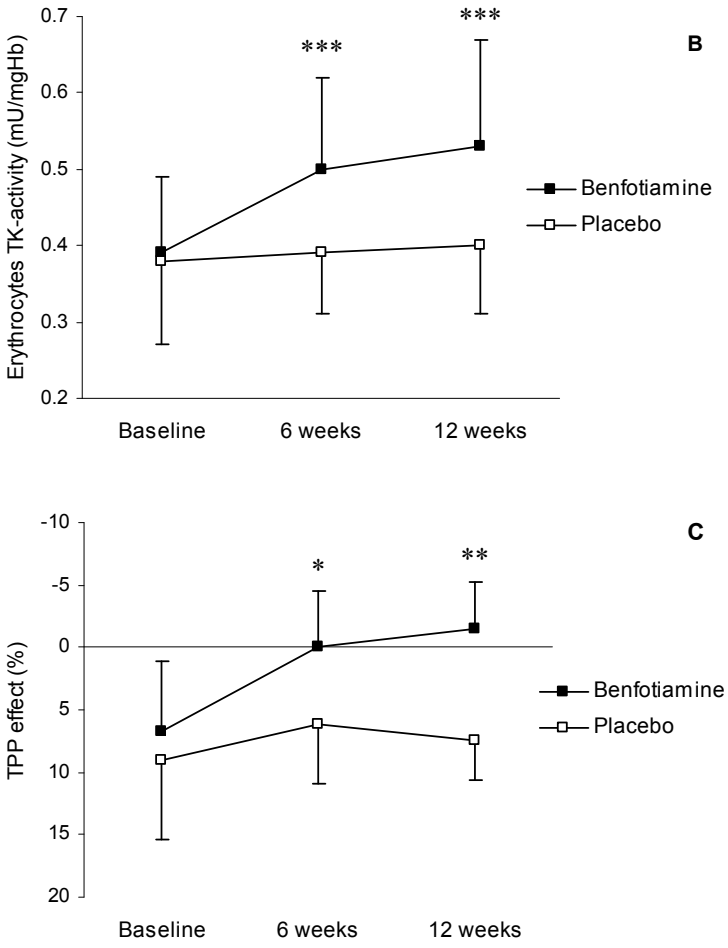


Figure 2: Effects of intervention on thiamine status parameters after 6 and 12 weeks according to group (benfotiamine vs placebo)
A. mean values and standard deviations of blood thiamine concentration,
B. mean values and standard deviations of erythrocyte transketolase activity,
C. median values and interquartile ranges of TPP effect.

TK, transketolase; TPP, thiamine pyrophosphate. *P<0.05 **P<0.01; ***P<0.001, compared with changes from baseline in placebo group.

Table 2: Summary of absolute changes in primary outcome, secondary outcome, and clinical characteristics after 6 and 12 weeks of intervention (Benfotiamine vs Placebo)

	Benfotiamine	Placebo	*P-value*
Primary outcome parameters			
UAE (mg/24h)			
Δ 6 weeks	-3 [-57; 51]	12 [-61; 40]	0.37
Δ 12 weeks	-9 [-53; 34]	-7 [-56; 65]	0.36
Urinary KIM-1 excretion (µg/24h)			
Δ 6 weeks	0.084 [-0.16; 0.36]	-0.100 [-0.35; 0.17]	0.02
Δ 12 weeks	-0.014 [-0.23; 0.56]	-0.043 [-0.36; 0.19]	0.09
Secondary outcome parameters			
24h UACR (mg/mmol)			
Δ 6 weeks	-0.2 [-7.0; 3.4]	-0.1 [-2.6; 1.6]	0.74
Δ 12 weeks	-0.1 [-4.3; 2.1]	-0.1 [-3.3; 4.6]	0.40
Spot urine UACR (mg/mmol)			
Δ 6 weeks	-0.7 [-4.9; 1.7]	-1.4 [-4.6; 2.2]	0.94
Δ 12 weeks	-0.4 [-8.2; 2.4]	1.1 [-3.4; 3.5]	0.21
Urinary KIM-1/creatinine (ng/mmol)			
Δ 6 weeks	-4 [-22; 40]	11 [-41; 9]	0.14
Δ 12 weeks	2 [-26; 27]	4 [-37; 14]	0.55
Urinary α1-m excretion (mg/24h)			
Δ 6 weeks	0.4 [-3.9; 7.1]	0.8 [-7.5; 5.9]	0.94
Δ 12 weeks	0.4 [-5.2; 5.3]	-0.2 [-7.8; 5.6]	0.63
Urinary α1-m/creatinine (mg/mmol)			
Δ 6 weeks	0.05 [-0.42; 0.52]	-0.01 [-0.65; 0.37]	0.60
Δ 12 weeks	0.05 [-0.17; 0.32]	-0.02 [-0.70; 0.39]	0.54
Urinary Ngal excretion (mg/24h)			
Δ 6 weeks	-0.8 [-19; 23]	-0.9 [-18; 17]	0.97
Δ 12 weeks	3.8 [-17; 31]	-4.2 [-19; 2]	0.08
Urinary Ngal/creatinine (mg/mmol)			
Δ 6 weeks	0.54 [-3.45; 3.61]	-0.73 [-4.24; 1.22]	0.33
Δ 12 weeks	-1.23 [-3.64; 1.40]	0.37 [-3.08; 2.39]	0.23
Clinical characteristics			
Systolic blood pressure (mmHg)			
Δ 6 weeks	-1 [-9; 9]	6 [-9; 16]	0.25
Δ 12 weeks	0 [-6; 11]	4 [-7; 14]	0.98
Diastolic blood pressure (mmHg)			
Δ 6 weeks	1 [-5; 6]	-1 [-6; 5]	0.59
Δ 12 weeks	1 [-5; 6]	0 [-5; 6]	0.95
HbA1c (%)			
Δ 6 weeks	-0.1 [-0.4; 0.1]	-0.1 [-0.4; 0.1]	0.78
Δ 12 weeks	-0.1 [-0.4; 0.3]	0.1 [-0.3; 0.1]	0.92

Serum creatinine (µmol/l)			
Δ 6 weeks	6 [0; 9]	2 [-4; 5]	0.08
Δ 12 weeks	4 [0; 8]	0 [-3; 4]	<0.001
Creatinine Clearance (ml/min)			
Δ 6 weeks	0 [-21; 21]	5 [-24 ; 40]	0.35
Δ 12 weeks	3 [-29; 34]	4 [-34; 24]	0.92
Cystatine C			
Δ 6 weeks	-0.05 [-0.30; 0.14]	0.13 [-0.20; 0.36]	0.10
Δ 12 weeks	0.04 [-0.22; 0.34]	0.05 [-0.28; 0.46]	0.72
LDL-cholesterol (mmol/l)			
Δ 6 weeks	-0.05 [-0.30; 0.14]	0.13 [-0.20; 0.36]	0.10
Δ 12 weeks	0.04 [-0.22; 0.34]	0.05 [-0.28; 0.46]	0.72
HDL-cholesterol (mmol/l)			
Δ 6 weeks	-0.06 [-0.12; 0.06]	0.02 [-0.05; 0.10]	0.02
Δ 12 weeks	-0.01 [-0.07; 0.13]	0.02 [-0.07; 0.10]	0.66
Triglycerides (mmol/l)			
Δ 6 weeks	0.17 [-0.16; 0.67]	-0.13 [-0.66; 0.20]	0.01
Δ 12 weeks	-0.13 [-0.52; 0.60]	-0.28 [-0.55; 0.11]	0.18

Data are median [interquartile range]; Δ 6 weeks/12 weeks, change from baseline to 6 weeks/12weeks; UAE, urinary albumin excretion; KIM-1, kidney injury molecule-1; UACR, urinary albumin-creatinine ratio; α1-m, α1-microglobuline; Ngal, neutrophil gelatinase associated lipocalin; HbA_{1c}, glycated hemoglobin; eGFR-MDRD, estimated glomerular filtration rate calculated using Modification of Diet in Renal Disease formula

Table 3: Baseline characteristics and changes in thiamine status parameters, primary outcome measures, secondary outcome measures, and clinical characteristics over time

	Benfotiamine (*n* = 39)		
	Baseline	6 weeks	12 weeks
Baseline characteristics			
Males (*n* (%))	30		
Age (years)	65.3 ± 5.9		
BMI (kg/m^2)	32.1 ± 5.1		
Duration of diabetes (years)	12 [9; 18]		
Insulin treatment (*n* (%))	31 (79)		
Oral hypoglycaemic agents (*n* (%))	19 (49)		
Plasma thiamine (nmol/l)	31.8 ± 7.7		
Thiamine status			
Thiamine (nmol/L)	126 ± 23	290 ± 31	300 ± 0
TK-activity (mU/mgHb)	0.41 ± 0.10	0.51 ± 0.12	0.53 ± 0.15
Primary outcome parameters			
UAE (mg/24h)	90 [38; 267]	75 [49; 280]	72 [38; 199]
U-KIM-1 (µg/24h)	1.67 [0.95; 2.47]	1.51 [0.86; 2.59]	1.68 [1.06; 2.40]
Secondary outcome parameters			
24h UACR (mg/mmol)	10.3 [3.7; 23.4]	6.1 [3.0; 17.7]	4.9 [2.5; 18.4]
Spot urine UACR (mg/mmol)	9.3 [2.4; 16.8]	5.8 [3.7; 17.9]	7.1 [3.6; 17.8]
U-KIM-1/creatinine (ng/mmol)	103 [63; 158]	95 [66; 170]	96 [77; 148]
U-α1m (mg/24h)	9.4 [4.3; 24.4]	11.9 [4.4; 20.2]	11.2 [4.1; 18.8]
U-α1m/creatinine (mg/mmol)	0.6 [0.3; 1.4]	0.7 [0.3; 1.3]	0.6 [0.3; 1.2]
U-Ngal (mg/24h)	131 [67; 227]	118 [77; 229]	115 [73; 284]
U-Ngal/creatinine (mg/mmol)	6.7 [4.3; 13.9]	6.2 [3.4; 15.9]	5.1 [3.2; 12.9]
Clinical characteristics			
SBP (mmHg)	140 ± 16	139 ± 14	143 ± 17
DBP (mmHg)	76 ± 8	77 ± 10	76 ± 9
HbA_{1c} (%)	7.3 ± 0.9	7.1 ± 0.9	7.3 ± 1.0
Plasma creatinine (µmol/l)	84 ± 19	89 ± 19	88 ± 20
Creatinine Clearance (ml/min)	135 ± 51	129 ± 53	133 ± 45
Cystatin C (mg/l)	1.01 ± 0.21	1.06 ± 0.22	1.09 ± 0.23
LDL-cholesterol (mmol/l)	1.9 ± 0.7	1.9 ± 0.8	2.1 ± 0.8
HDL-cholesterol (mmol/l)	1.2 ± 0.3	1.1 ± 0.3	1.2 ± 0.3
Triglycerides (mmol/l)	1.8 [1.4; 2.6]	1.9 [1.4; 2.8]	1.7 [1.2; 2.6]

Data are mean ± standard deviation or median [interquartile range]. BMI, body mass index; TK, transketolase; UAE, urinary albumin excretion; U-KIM-1, urinray excretion of kidney injury molecule-1; UACR, urinary albumin-creatinine ratio; U-α1m, urinary excretion of α1-microglobulin; U-Ngal, urinary excretion of neutrophil gelatinase-associated lipocalin; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated hemoglobin. Comparison of baseline characteristics was performed by unpaired Student's t-test (for normally distributed variables) or Mann-Whitney-U test (for non-normally distributed variables). x^2-test was used to compare non-continuous variables. Changes in thiamine status parameters, primary outcome measures, secondary outcome measures, and clinical characteristics over time were analyzed by ANOVA for repeated measures, with log-transformation of variables with skewed distribution prior to analysis.

Placebo (*n* = 43)			*P values*
Baseline	6 weeks	12 weeks	
33			0.98
64.6 ± 6.1			0.63
31.9 ± 5.9			0.93
10 [7; 18]			0.41
29 (67)			0.22
29 (67)			0.05
31.6 ± 9.8			0.92
122 ± 23	124 ± 25	138 ± 30	<0.001
0.38 ± 0.11	0.39 ± 0.08	0.41 ± 0.10	<0.001
97 [48; 177]	99 [43; 200]	96 [45; 200]	0.36
1.56 [1.06; 1.83]	1.56 [1.06; 1.83]	1.39 [1.02; 2.01]	0.12
7.6 [4.3; 13.3]	7.4 [2.8; 11.0]	7.1 [4.0; 12.5]	0.37
6.2 [3.4; 10.5]	8.2 [3.9; 14.2]	8.1 [4.6; 15.9]	0.58
99 [79; 141]	89 [58; 130]	81 [66; 150]	0.37
8.2 [4.3; 20.3]	9.0 [5.7; 21.1]	10.2 [2.5; 19.7]	0.33
0.6 [0.3; 1.3]	0.6 [0.3; 1.4]	0.7 [0.2; 1.1]	0.47
122 [53; 224]	112 [52; 218]	99 [52; 222]	0.17
7.7 [4.2; 18.9]	6.4 [3.2; 15.1]	8.5 [3.3; 13.1]	0.18
137 ± 20	140 ± 20	140 ± 17	0.60
76 ± 10	76 ± 9	76 ± 10	0.68
7.4 ± 0.9	7.2 ± 0.9	7.2 ± 0.9	0.33
87 ± 23	89 ± 25	87 ± 21	0.04
130 ± 58	139 ± 58	131 ± 64	0.57
1.03 ± 0.23	1.10 ± 0.26	1.11 ± 0.23	0.53
1.8 ± 0.9	1.8 ± 0.9	1.9 ± 0.9	0.55
1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.25
2.1 [1.4; 3.4]	2.2 [1.4; 2.9]	2.0 [1.2; 2.9]	0.06

Primary and secondary outcome parameters

Changes in primary outcome parameters, secondary outcome parameters and clinical characteristics are shown in table 2 and table 3. Significant differences in primary or secondary outcome parameters were neither found after 6 nor after 12 weeks of treatment between the benfotiamine group and the placebo group. Change in UAE between baseline and 12 weeks was -18mg/24h in the benfotiamine and -1mg/24h in the placebo group. For individual differences, respective changes were -9 [-53; 34] mg/24h and -7 [-56; 65]mg/24h. Adjustment for differences in baseline use of oral hypoglycemic agents, and prevalence of TPP>15% in multivariate regression analyses did not reveal any relevant different results (data not shown).

With respect to clinical characteristics, there was a trend towards increase in plasma creatinine in the benfotiamine group compared to placebo, which reached significance after 12 weeks of treatment, but this was not accompanied by changes in creatinine clearance or cystatin C In addition, there was a significant increase in serum triglycerides (TG) and a significant decrease in HDL-cholesterol in the benfotiamine group compared to placebo group at 6 weeks, which were not significant any more at 12 weeks.

Side effects

During the study, no serious adverse effects occurred. In the benfotiamine group one patient contacted the study physician because of nausea and heartburn. Attempt to stop the medication for one week and to retry resulted in symptoms to reappear. This patient continued the study with a reduced dose of 1 tablet/day (300mg) and was therefore categorised as non-compliant. In the placebo group, one patient was non-compliant, as concluded by more than 50 tablets (>20% of the total amount) not being taken at the end of the study. Besides, two protocol deviations occurred: ACE-I was stopped and antibiotic treatment was initiated for prostatitis in one patient and another patient suffered from ACE-I-induced angioedema and was then switched to ARB. As a consequence, 38 in the benfotiamine group and 40 in the placebo group were available for the per-protocol analysis. The results of these per-protocol analyses (data not shown) were not materially different from the presented analyses.

DISCUSSION

In this double-blind placebo-controlled trial, we found that 12 weeks of treatment with high-dose benfotiamine did not result in a decrease in urinary excretion of albumin or tubulointerstitial damage markers, such as KIM-1, Ngal, and α1-m. On the other hand, high-dose benfotiamine did result in improvement in thiamine status, as reflected by whole blood thiamine concentrations, erythrocyte TK-activity and TPP effect.

Our findings contrast with earlier findings from studies with low dose (7mg/kg) and high dose (70 mg/kg) of thiamine and benfotiamine treatment in animal models with streptozotocin-induced diabetes, in which 24 weeks of treatment protected against a further increase in urinary albumin excretion already after 6 weeks of treatment [12, 14]. Although no clear evidence of dose-response relationship regarding albuminuria was found in these studies, only high-dose benfotiamine suppressed oxidative stress. The relatively high dose of benfotiamine (900 mg/day) in our study is still less than 70mg/kg and might be insufficient to achieve all therapeutic goals in humans.

Our results also contrast with results of a randomised, double-blind placebo-controlled pilot study of 12 weeks of high dose (300 mg/day) oral thiamine supplementation in 40 Pakistani patients with type 2 diabetes [22]. In this study, a median decrease of 17.7 mg/24h (33%) in UAE within the thiamine treated group was observed after 12 weeks of treatment.

We investigated the effect of benfotiamine instead of thiamine in addition to existing ACE-I or ARB treatment, while in the study by Rabbani et al, more than 50% of the patients was not on such treatment [22]. Nevertheless, in a separate analysis of that study it was reported that presence or absence of ACE-I/ARB treatment made no difference in outcome [23]. Another point is that we investigated Caucasian patients, where only about 20% had thiamine deficiency at baseline by the “thiamine effect” criterion, while Rabbani et al. investigated Pakistani patients with low plasma thiamine concentration. Thus, a difference in basal thiamine status, background diet or genetic susceptibility for the effects of benfotiamine/thiamine supplementation might also play a role.

In their study in rats, Babaie-Jadidi et al. suggested that benfotiamine has a renal hemodynamic effect, antagonizing renal hyperfiltration [12], similar to ACE-I and ARB treatment [24]. Consistent with this putative mechanism, we found a small but significant increase in plasma creatinine, but this was not paralleled by changes in cystatin C or creatinine clearance. Yet, measured glomerular filtration rate would have been necessary in order to elucidate renal effects of benfotiamine.

Finding no effect on urinary albumin excretion and tubulointerstitial damage markers in patients with type 2 diabetes and DN, it is important to realise that thiamine and benfotiamine are supposed to antagonise the detrimental effects of hyperglycaemia. In line with this, it has been shown that these agents interfere with at least three biochemical pathways by which hyperglycaemia otherwise exerts its detrimental effects, including formation of advanced glycation end-products [14]. Still, changes may take much time. For example, it took 4-5 years of lowering of HbA1c in patients with type 1 diabetes in the Diabetes Control and Complications Trial before a difference could be discerned between subjects with strict metabolic control and standard therapy regarding urinary albumin excretion rate [6]. Likewise, a similar delay in separation of curves was reported in patients with type 2 diabetes in the United Kingdom Prospective Diabetes Study [25]. Therefore, given the biology of DN and the long duration of detrimental effects of hyperglycaemia to be reversed, it is more likely that our study was too short to demonstrate an effect of an agent interfering with glucotoxicity. A larger trial with longer follow-up of at least 4 years is necessary to investigate possible preventive effects of benfotiamine in DN.

Few adverse events were observed in the benfotiamine group, but none of these was serious. Two premature terminations of the study were caused by newly diagnosed malignancies; a lung cancer and a stomach cancer. These two cases were assessed by the treating physician as not causally related to the study medication and not unusual considering the demographic background of the study population.

One limitation of our study is that it was not powered to detect a small effect of benfotiamine on urinary albumin excretion as an add-on therapy besides existing ACE-I or ARB treatment. Our study was explorative and used a one-sided hypothesis based on the likelihood of improvement in outcome measures. However, after 12 weeks of treatment, the median change in UAE was -9 mg/24h in the benfotiamine

group versus -7 mg/24h in the placebo group. It is questionable whether such a difference would be considered clinically relevant and a much larger study would have been necessary to find this difference statistically significant.

In conclusion, 12-week treatment with benfotiamine in patients with type 2 diabetes and mild diabetic nephropathy did not reduce urinary excretion of albumin or tubulointerstitial damage markers. Long-term intervention studies are likely to be necessary to discern whether benfotiamine treatment has an effect on development and course of diabetic nephropathy.

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Chapter 7

Effects of benfotiamine treatment on advanced glycation endproducts and markers of endothelial dysfunction and inflammation in diabetic nephropathy

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ABSTRACT

Background

Formation of advanced glycation endproducts (AGEs), endothelial dysfunction, and low-grade inflammation are intermediate pathways of hyperglycemia-induced vascular complications. We investigated the effect of benfotiamine on markers of these pathways in patients with type 2 diabetes and nephropathy.

Methods

Patients with type 2 diabetes and urinary albumin excretion in the high-normal and microalbuminuric range (15-300 mg/24h) were randomized to receive benfotiamine (*n*=39) or placebo (*n*=43). Plasma and urinary AGEs (N^{ε}-(carboxymethyl)lysine [CML], N^{ε}-(Carboxyethyl)lysine [CEL], and 5-hydro-5-methylimidazolone [MG-H1]) and plasma markers of endothelial dysfunction (soluble vascular cell adhesion molecule-1 [sVCAM-1], soluble intercellular adhesion molecule-1 [sICAM-1], soluble E-selectin) and low-grade inflammation (high-sensitivity C-reactive protein [hs-CRP], serum amyloid-A [SAA], myeloperoxidase [MPO]) were measured at baseline and after 6 and 12 weeks.

Results

Compared to placebo, benfotiamine did not result in significant reductions in plasma or urinary AGEs or plasma markers of endothelial dysfunction and low-grade inflammation.

Conclusions

Benfotiamine for 12 weeks did not significantly affect intermediate pathways of hyperglycemia-induced vascular complications.

INTRODUCTION

Diabetic nephropathy (DN) is a serious complication of diabetes and a leading cause of end-stage renal disease [1]. Thiamine and benfotiamine, a lipophilic thiamine-derivative, have been suggested as novel therapies for diabetic complications, including DN [2]. These agents would not exert their beneficial effects by improvement of hyperglycemia itself, but rather by activation of transketolase [2, 3]. This leads to a decrease in triosephospates and methylglyoxal; i.e. the major precursors of advanced glycation endproducts (AGEs), and subsequently inhibition of endothelial dysfunction and chronic low-grade inflammation [4, 5]. However, in a recent 12-week double-blind placebo-controlled trial in patients with type 2 diabetes, we found no effect of benfotiamine on urinary albumin excretion (UAE) or renal tubular damage markers [6]. One possibility is that our choice for these primary endpoints is too late in the sequence of events, because even the reduction in AGEs that occurs after pancreas transplantation has been reported to take years to translate into an effect on urinary albumin excretion [7]. We now aimed to evaluate the effect of benfotiamine on AGEs and markers of endothelial dysfunction and chronic low-grade inflammation, and to find ground to set up a study of longer duration.

METHODS

Patients and study design

A detailed description of the study has been published [6]. We included patients from the outpatients department in the Isala Clinics, Zwolle, the Netherlands, in the period from January 2008 till June 2009. Included subjects were patients with type 2 diabetes, aged 40 to 75 years, with UAE between 15-300 mg/24h despite treatment with ACE inhibitors (ACE-Is) and/or angiotensin receptor blockers (ARBs). Patients (*n*=86) were randomized to receive either benfotiamine (Wörwag pharma, Böblingen, Germany) 300mg t.i.d. (total daily dose 900mg) or placebo during 12 weeks. On each visit (baseline, 6 weeks, and 12 weeks), patients delivered 24-h urine collection, and additional morning spot-urine and blood samples were taken. All patients signed informed consent. This trial was conducted in accordance with

the Helsinki Declaration and approved by the Medical Ethics Committee of the Isala Clinics, Zwolle, the Netherlands.

Clinical laboratory investigations

For the current report, urine and plasma samples were stored frozen at -80°C until assessment. Detection of plasma AGEs N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(Carboxyethyl)lysine (CEL) and urine AGEs (CML, CEL and the methylglyoxal-arginine-adduct 5-hydro-5-methylimidazolone (MG-H1)) in 24-h urine samples was performed by means of a stable-isotope-dilution tandem mass spectrometry method [8, 9]. Soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), high sensitivity C-reactive protein (hs-CRP) and serum amyloid-A (SAA) were assessed by multi-array detection system (SECTOR-Imager 2400, Mesoscale Discovery, Maryland). Soluble E-selectin and myeloperoxidase (MPO) were measured by commercially available multiplex assays (Millipore, Massachusetts). Other measurements were performed according to standard hospital procedures.

Statistical analysis

Variables with a normal distribution are presented as mean and standard deviation and variables with a skewed distribution are presented as median and interquartile range. Intention-to-treat analysis and per-protocol analysis were planned. After randomization, four patients from the benfotiamine group withdrew from consent. Therefore, these subjects were not available for follow-up visits and no samples could be obtained from these subjects. All remaining patients (39 patients in the benfotiamine group and 43 patients in the placebo group) were available for analyses. In per-protocol analyses, patients who deviated from the study protocol (non-compliance or change in concomitant medications, n = 1 in the benfotiamine group and n = 3 in the placebo group [6]) were excluded. In the previous report [6], analyses of the primary endpoints (UAE and KIM-1) were presented. In this report, we present predefined analyses of secondary endpoints: plasma and urinary AGEs and plasma levels of biomarkers of endothelial dysfunction and low-grade inflammation. Using ANOVA for repeated measures (Mixed Model Analysis), within-subjects

factors (effect of time; disease modifying model), effects of the between-subjects factors (difference between groups; symptomatic relief), and interaction between time of visit (0, 6, and 12 weeks) and group (benfotiamine versus placebo) were evaluated. Q-Q plots were used to assess whether the residuals of the dependent variables in the model had normal distribution. Because of skewed distribution of the residuals, logarithmic transformation (natural logarithm) of the data was performed before analysis. The results are summarized in terms of estimated means with 95% confidence intervals (CI). Differences in mean change between benfotiamine and placebo at 6 weeks and 12 weeks are presented as mean difference with 95% CI. *P*-values for time, group, and time*group interaction are presented. A two-sided *P*-value <0.05 was considered statistically significant. Statistics were done with SPSS, version 16.0 (Chicago, IL).

RESULTS

Baseline characteristics are presented in table 1. At baseline, blood thiamine was correlated with urinary excretion of CML (r=0.26, P=0.02) and CEL (r=0.25, P=0.02). No significant correlations of thiamine status with other AGEs or biomarkers of endothelial dysfunction and low-grade inflammation were found. Baseline characteristics were not materially different between groups, except for a more frequent use of oral hypoglycaemic agents and a slightly higher plasma creatinine in the placebo group. As shown in table 2, benfotiamine treatment had neither a significant effect on plasma or urinary AGEs nor on markers of endothelial dysfunction or chronic low-grade inflammation. Adjustment for baseline differences gave similar results. Results of per-protocol analysis (not shown) were not different from presented intention-to-treat analyses. Subgroup analyses in patients with low range UAE (<100 mg/24u) and high range UAE (>100mg/24h) did not reveal differences in response to benfotiamine compared to placebo.

Table 1: Baseline characteristics of study population

	Benfotiamine (*n* = 39)	Placebo (*n* = 43)
Males (*n* (%))	30 (77%)	33 (82%)
Age (years)	65.3 ± 5.9	64.6 ± 6.1
BMI (kg/m^2)	32.1 ± 5.1	31.9 ± 5.9
Duration of diabetes (years)	12 [9; 18]	10 [7; 18]
SBP (mmHg)	140 ± 16	137 ± 20
DBP (mmHg)	67 ± 8	76 ± 10
A1c (%)	7.3 ± 0.9	7.4 ± 0.9
Plasma creatinine (µmol/l)	84 ± 19	87 ± 23
UAE (mg/24h)	90 [38; 267]	97 [48; 177]
Thiamine (nmol/l)	126 ± 23	122 ± 23
Plasma AGEs		
CML (nmol/mmol lysine)	64.48 [58.21; 69.69]	62.51 [54.88; 71.20]
CEL (nmol/mmol lysine)	51.14 [44.78; 59.25]	56.99 [43.71; 62.10]
Urine AGEs		
CML excretion (nmol/24h)	7630 [6761; 10576]	8879 [6476; 11769]
CML/creatinine (nmol/mmol)	572 [416; 731]	596 [483; 788]
CEL excretion (nmol/24h)	12405 [9105; 15240]	11204 [8922; 16384]
CEL/creatinine (nmol/mmol)	763 [602; 1061]	871 [648; 1034]
MG-H1 excretion (nmol/24h)	479122 [34431; 69775]	44930 [32095; 58614]
MG-H1/creatinine (nmol/mmol)	3459 [2196; 4856]	2999 [2260; 4563]
Endothelial dysfunction markers		
s-ICAM (ng/ml)	257.3 [222.0; 281.1]	241.7 [213.2; 308.0]
s-VCAM (ng/ml)	399.1 [362.7; 431.7]	388.3 [335.8; 461.9]
s-E-Selectin (ng/ml)	45.3 [29.8; 54.6]	39.5 [26.8; 51.3]
Low-grade inflammation markers		
Hs-CRP (ng/ml)	1395 [754; 2891]	1738 [824; 4097]
SAA (ng/ml)	1356 [927; 2028]	1162 [694; 2328]
MPO (ng/ml)	20.4 [9.9; 28.3]	20.4 [6.2; 27.2]

Data are mean ± standard deviation or median [interquartile range].
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; A1c, Glycated haemoglobin; UAE, urinary albumin excretion; AGEs, advanced glycation endproducts; CML, N^{ε}-(Carboxymethyl)lysine; CEL, N^{ε}-(Carboxyethyl)lysine; MG-H1, 5-hydro-5-methylimidazolone; s-ICAM, serum inter-cellular adhesion molecule-1; s-VCAM, serum vascular cell adhesion molecule-1; Hs-CRP, high sensitive C-reactive protein; SAA, serum amyloid A; MPO, myeloperoxidase.

Table 2: Estimated means of the log-transformed data at each visit, changes within group compared to baseline, and mean differences of change between groups compared to baseline

	Benfotiamine (n = 39)	Placebo (n = 43)	Difference of change between groups	*P*		
				Time	Group	Time x Group
Plasma AGEs						
CML (nmol/mmol lysine)						
Baseline	4.17 (4.11 to 4.23)	4.15 (4.09 to 4.20)				
Week 6	4.15 (4.09 to 4.21)	4.14 (4.09 to 4.20)				
Week 12	4.15 (4.09 to 4.21)	4.12 (4.07 to 4.18)				
At 6 weeks	-0.02 (-0.12 to 0.08)	0.00 (-0.10 to 0.09)	-0.01 (-0.13 to 0.10)			
At 12 weeks	-0.02 (-0.12 to 0.08)	-0.02 (-0.12 to 0.07)	0.01 (-0.11 to 0.12)	0.77	0.50	0.95
CEL (nmol/mmol lysine)						
Baseline	3.94 (3.86 to 4.02)	3.97 (3.90 to 4.05)				
Week 6	3.88 (3.81 to 3.96)	3.94 (3.87 to 4.01)				
Week 12	3.90 (3.83 to 3.97)	3.98 (3.92 to 4.04)				
At 6 weeks	-0.06 (-0.18 to 0.07)	-0.03 (-0.16 to 0.09)	-0.02 (-0.17 to 0.12)			
At 12 weeks	-0.04 (-0.17 to 0.08)	0.01 (-0.11 to 0.13)	-0.05 (-0.19 to 0.09)	0.45	0.06	0.78
Urine AGEs						
U-CML excretion (nmol/24h)						
Baseline	8.98 (8.83 to 9.13)	9.08 (8.93 to 9.22)				
Week 6	8.99 (8.85 to 9.13)	9.08 (8.94 to 9.21)				
Week 12	9.04 (8.91 to 9.17)	9.02 (8.90 to 9.15)				
At 6 weeks	0.01 (-0.23 to 0.26)	0.00 (-0.23 to 0.24)	0.01 (-0.27 to 0.29)			
At 12 weeks	0.06 (-0.18 to 0.30)	-0.05 (-0.27 to 0.17)	0.11 (-0.16 to 0.38)	0.99	0.31	0.67
U-CML/creatinine (nmol/mmol)						
Baseline	6.29 (6.14 to 6.44)	6.40 (6.26 to 6.55)				
Week 6	6.30 (6.16 to 6.45)	6.31 (6.17 to 6.44)				
Week 12	6.30 (6.13 to 6.47)	6.38 (6.22 to 6.54)				
At 6 weeks	0.01 (-0.24 to 0.27)	-0.10 (-0.34 to 0.15)	0.11 (-0.18 to 0.40)			
At 12 weeks	0.01 (-0.27 to 0.29)	-0.02 (-0.28 to 0.24)	0.03 (-0.28 to 0.34)	0.82	0.29	0.74

Table 2: Continued

U-CEL excretion (nmol/24h)						
Baseline	8.98 (8.83 to 9.13)	9.08 (8.93 to 9.22)				
Week 6	8.99 (8.85 to 9.13)	9.08 (8.94 to 9.21)				
Week 12	9.04 (8.91 to 9.17)	9.02 (8.90 to 9.15)				
At 6 weeks	0.01 (-0.23 to 0.26)	0.00 (-0.23 to 0.24)	0.01 (-0.27 to 0.29)			
At 12 weeks	0.06 (-0.18 to 0.30)	-0.05 (-0.28 to 0.18)	0.11 (-0.16 to 0.38)	0.99	0.31	0.67
U-CEL/creatinine (nmol/mmol)						
Baseline	6.29 (6.14 to 6.44)	6.40 (6.26 to 6.55)				
Week 6	6.30 (6.16 to 6.45)	6.31 (6.17 to 6.44)				
Week 12	6.30 (6.13 to 6.47)	6.38 (6.22 to 6.54)				
At 6 weeks	0.01 (-0.24 to 0.27)	-0.10 (-0.34 to 0.15)	0.11 (-0.18 to 0.40)			
At 12 weeks	0.01 (-0.27 to 0.29)	-0.02 (-0.28 to 0.24)	0.03 (-0.28 to 0.35)	0.82	0.29	0.74
MG-H1 excretion (nmol/24h)						
Baseline	10.74 (10.56 to 10.91)	10.72 (10.55 to 10.89)				
Week 6	10.63 (10.46 to 10.80)	10.72 (10.55 to 10.88)				
Week 12	10.63 (10.46 to 10.81)	10.58 (10.42 to 10.75)				
At 6 weeks	-0.10 (-0.40 to 0.19)	0.00 (-0.28 to 0.28)	-0.10 (-0.44 to 0.24)			
At 12 weeks	-0.10 (-0.40 to 0.20)	-0.14 (-0.42 to 0.15)	0.04 (-0.30 to 0.38)	0.38	0.94	0.69
U-MG-H1/creatinine (nmol/mmol)						
Baseline	8.05 (7.85 to 8.24)	8.05 (7.86 to 8.23)				
Week 6	7.94 (7.75 to 8.13)	7.94 (7.78 to 8.14)				
Week 12	7.90 (7.72 to 8.08)	7.90 (7.77 to 8.11)				
At 6 weeks	-0.10 (-0.44 to 0.23)	-0.08 (-0.40 to 0.23)	-0.02 (-0.40 to 0.36)			
At 12 weeks	-0.15 (-0.47 to 0.17)	-0.11 (-0.41 to 0.20)	-0.04 (-0.41 to 0.33)	0.36	0.80	0.98

Endothelial dysfunction markers						
s-ICAM (ng/ml)						
Baseline	5.53 (5.44 to 5.61)	5.55 (5.47 to 5.63)				
Week 6	5.55 (5.47 to 5.64)	5.53 (5.45 to 5.62)				
Week 12	5.56 (5.48 to 5.63)	5.52 (5.44 to 5.59)				
At 6 weeks	0.03 (-0.12 to 0.18)	-0.02 (-0.16 to 0.12)	0.05 (-0.12 to 0.22)			
At 12 weeks	0.03 (-0.11 to 0.17)	-0.03 (-0.17 to 0.10)	0.06 (-0.10 to 0.22)	0.99	0.74	0.74
s-VCAM (ng/ml)						
Baseline	5.98 (5.92 to 6.05)	5.98 (5.92 to 6.04)				
Week 6	5.99 (5.93 to 6.05)	5.96 (5.90 to 6.02)				
Week 12	6.02 (5.96 to 6.09)	5.96 (5.89 to 6.02)				
At 6 weeks	0.01 (-0.10 to 0.11)	-0.02 (-0.12 to 0.09)	0.02 (-0.10 to 0.14)			
At 12 weeks	0.04 (-0.07 to 0.15)	-0.02 (-0.12 to 0.08)	0.06 (-0.07 to 0.18)	0.91	0.19	0.64
s-E-selectin (ng/ml)						
Baseline	3.66 (3.46 to 3.85)	3.57 (3.39 to 3.75)				
Week 6	3.84 (3.70 to 3.97)	3.71 (3.59 to 3.84)				
Week 12	3.79 (3.64 to 3.95)	3.69 (3.54 to 3.84)				
At 6 weeks	0.18 (-0.10 to 0.46)	0.14 (-0.13 to 0.41)	0.04 (-0.28 to 0.37)			
At 12 weeks	0.14 (-0.16 to 0.44)	0.12 (-0.17 to 0.40)	0.02 (-0.32 to 0.36)	0.14	0.10	0.96
Low-grade inflammation markers						
Hs-CRP (ng/ml)						
Baseline	7.49 (7.13 to 7.85)	7.56 (7.21 to 7.91)				
Week 6	7.63 (7.28 to 7.98)	7.41 (7.07 to 7.75)				
Week 12	7.54 (7.20 to 7.87)	7.51 (7.19 to 7.83)				
At 6 weeks	0.14 (-0.48 to 0.75)	-0.15 (-0.74 to 0.44)	0.29 (-0.41 to 0.98)			
At 12 weeks	0.05 (-0.55 to 0.64)	-0.05 (-0.63 to 0.52)	0.10 (-0.59 to 0.78)	0.99	0.67	0.71

Table 2: Continued

SAA (ng/ml)						
Baseline	7.26 (6.95 to 7.58)	7.22 (6.92 to 7.53)				
Week 6	7.29 (6.96 to7.61)	7.07 (6.76 to 7.38)				
Week 12	7.14 (6.83 to 7.46)	7.20 (6.90 to 7.50)				
At 6 weeks	0.02 (-0.53 to 0.57)	-0.15 (-0.68 to 0.37)	0.18 (-0.45 to 0.81)			
At 12 weeks	-0.12 (-0.66 to 0.42)	-0.02 (-0.54 to 0.50)	-0.10 (-0.72 to 0.51)	0.88	0.60	0.67
MPO (ng/ml)						
Baseline	2.90 (2.61 to 3.20)	2.80 (2.51 to 3.09)				
Week 6	2.89 (2.61 to 3.17)	2.67 (2.40 to 2.94)				
Week 12	2.88 (2.61 to 3.16)	2.72 (2.46 to 2.98)				
At 6 weeks	-0.02 (-0.52 to 0.48)	-0.13 (-0.61 to 0.35)	0.11 (-0.45 to 0.68)			
At 12 weeks	-0.02 (-0.51 to 0.47)	-0.08 (-0.56 to 0.39)	0.06 (-0.50 to 0.63)	0.87	0.15	0.92

Data are log-transformed and presented as mean (95% confidence interval) or mean difference (95% confidence interval for difference).

CML, N^{ε}-(Carboxymethyl)lysine; CEL, N^{ε}-(Carboxyethyl)lysine; MG-H1, 5-hydro-5-methylimidazolone; s-ICAM, serum inter-cellular adhesion molecule-1; s-VCAM, serum vascular cell adhesion molecule-1; Hs-CRP, high sensitive C-reactive protein; SAA, serum amyloid A; MPO, myeloperoxidase.

DISCUSSION

We found that 12-week treatment with benfotiamine in patients with type 2 diabetes did not result in a decrease of plasma AGEs, urinary excretion of AGEs, plasma biomarkers of endothelial dysfunction or plasma biomarkers of chronic low-grade inflammation.

Benfotiamine is converted to thiamine pyrophosphate, a co-factor of transketolase. The activation of transketolase plays a crucial role in oxidative and non-oxidative pentosephosphate pathways that inhibit vascular complications of diabetes [3, 10]. In our trial, although we found that benfotiamine resulted in a significant improvement in thiamine status (a significant increase in thiamine levels and transketolase activity), there was no significant difference between the benfotiamine group and the placebo group regarding urinary albumin excretion, urinary excretion of tubular damage markers, or blood pressure [6].

Studies in diabetic animals have shown that benfotiamine inhibited protein kinase C, formation of AGEs and oxidative stress, indicating a protective effect against hyperglycemia-induced endothelial dysfunction and inflammation [2, 4].

To the best of our knowledge, our study is the first randomised controlled trial that has investigated the effect of benfotiamine on AGEs formation and markers of low-grade inflammation in humans. Results from previous studies on thiamine and benfotiamine on endothelial function in humans are conflicting. An earlier cross-sectional analysis in 74 patients with type 1 and type 2 diabetes found an inverse association of thiamine status with sVCAM-1 [11], suggesting an effect of thiamine status on markers of endothelial dysfunction. However, in a study in patients with type 2 diabetes [5], the same group found no effect of thiamine supplementation on sVCAM-1, We also did not find an effect of benfotiamine on markers of endothelial dysfunction, including sVCAM-1. Nevertheless, in a study in 13 patients with type 2 diabetes [12], beneficial effects of benfotiamine (1050mg/day) on the endothelial damage and oxidative stress that occurs after consumption of an AGE-rich meal were suggested to be already present after three days of treatment. While on the basis of this experiment longer periods of treatment with benfotiamine (12 weeks in our study) might be anticipated to be sufficient to cause an effect on formation of AGEs

and endothelial damage, the effects of benfotiamine could also be more directly meal-related rather than affecting steady-state concentrations.

Compared to previous studies in animals [2] and humans [5, 13], a daily dose of 900 mg is considered as a high dose. Due to its pharmacokinetics, benfotiamine is absorbed considerably better than thiamine. However, it is important to realize that intestinal and renal tubular transport of thiamine is tightly regulated to maintain homeostasis. Accordingly, expression of the transporters is up-regulated in presence of deficiency states and down-regulated in response to an excess supply of these nutrients, which may account for a rapid excretion of thiamine in the urine after consumption of a high dose of benfotiamine [14, 15]. Additionally, studies in experimental animals have shown that chronic kidney disease results in down-regulation of expression of key transporters and receptors for thiamine and folate, which can further limit the bioavailability of these micronutrients [16]. In our study, although higher blood thiamine levels and transketolase activity was achieved in the benfotiamine group, thiamine levels in renal cells might still be deficient even in case of high blood thiamine. Our objectives were studied in a relevant study population, including patients with type 2 diabetes and UAE in the high-normal and microalbuminuric range treated with ACE-Is and/or ARBs. An important limitation of this study is that AGEs and biomarkers of endothelial dysfunction and inflammation were measured in urine and blood. Intracellular concentrations of these markers in target organs (e.g. glomeruli and tubuli) may give additional information on effects of benfotiamine on vascular complications. Therefore, assessment of intracellular AGEs in future studies would be necessary.

In conclusion, 12-week benfotiamine treatment did neither significantly affect plasma or urine AGEs nor plasma biomarkers of endothelial dysfunction or chronic low-grade inflammation in patients with type 2 diabetes and UAE in the high-normal and microalbuminuric range. Because small short-term effects can not be excluded and potential effects that take long to ensue can not be studied in a 12-week study, larger or longer term studies are necessary to elucidate whether benfotiamine reduces formation of AGEs or diminishes the risk of hyperglycemia-induced vascular complications.

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Chapter 8

Discussion and summary

Early detection of patients at risk for diabetic nephropathy is possible by various means, but still, disease progression occurs despite an array of different treatments. Therefore, new and earlier markers for risk stratification and alternative therapeutic strategies are needed. This can offer a possibility to start early with effective treatment in high-risk patients, and thereby could prevent renal function loss and the deterioration towards end-stage renal disease (ESRD) in patients with diabetes. In this thesis, two novel approaches for risk stratification in diabetes were evaluated, namely risk assessment by genetic factors, and risk assessment by urinary proteomics. Additionally, it was investigated whether treatment with benfotiamine, addressing thiamine-related pathways as a novel therapeutic target, could be a beneficial intervention for diabetic nephropathy in patients with type 2 diabetes.

Risk stratification: genetics

For genetic risk stratification, we used a candidate gene approach. Accordingly, the investigated genes were selected based on previous studies suggesting relevance of the genotype in known pathways of progressive renal damage and its complications in diabetes. The first investigated gene is the carnosinase gene CNDP1. This gene is of interest because prior cross sectional studies in patients with type 1 and type 2 diabetes suggested that CNDP1 could be a susceptibility locus of diabetic nephropathy [1, 2]. Homozygosity for five copies of a trinucleotide repeat encoding leucine (5L-5L) in CNDP1 was more common in patients without nephropathy, while other genetic variants of CNDP1 (e.g., variants with more leucine repeats: 5L-6L, 6L-6L, 6L-7L, 5L-7L or 7L-7L) were more common in patients with diabetic nephropathy. These findings are consistent with a protective role of the 5L-5L genotype against diabetic nephropathy [1-4]. However, a recent cross sectional study in patients with type 2 diabetes found the association between 5L-5L genotype and a reduced prevalence of diabetic nephropathy to be limited to women [4]. Findings from cross-sectional studies could be subject to selection bias that might results from a possible interaction of the 5L-5L genotype with diabetic nephropathy and/or sex for prediction of mortality. If survival disadvantage in a subgroup exists, it may induce false associations in cross-sectional studies [5]. In **chapter 2**, we presented a prospective study in Caucasian European cohort of 2086 patients with type 1 diabetes (916 cases with diabetic nephropathy

and 1170 controls without diabetic nephropathy). We aimed to investigate whether the 5L-5L homozygous genotype of CNDP1 was prospectively associated with progression to ESRD or mortality in patients with diabetes, and whether any of these associations were sex-specific. Leucine repeats were assessed with a fluorescent DNA analysis system. The median follow-up period was 8.8 years. When looking at short-term follow-up, we found that the risk of progression from diabetic nephropathy to ESRD (defined as need to start chronic dialysis or kidney transplantation) was not significantly different in patients with 5L-5L genotype compared to other genotypes. However, beyond 8 years of follow-up, patients with 5L-5L genotype had a higher risk of developing ESRD compared to patients with other genotypes. No interaction was found between CNDP1 and sex regarding progression to ESRD. Regarding the risk of mortality, there was no difference between 5L-5L genotype and other genotypes. We concluded that the homozygous genotype 5L-5L of CNDP1 was not associated with mortality, but confers increased risk of progression from nephropathy to ESRD in patients with type 1 diabetes after long-term follow-up.

In **chapter 3**, we investigated the same polymorphism in 871 patients with type 2 diabetes with a follow-up of 9.5 years. In this study, we used individual slopes of renal function, expressed as estimated glomerular filtration rate using Modification of Diet in Renal Disease equation (eGFR-MDRD) [6]. We found no significant difference between patients with 5L-5L genotype compared to other genotypes regarding progression of renal function loss. CNDP1 polymorphism was not associated with all-cause mortality. However, women, not men, with 5L-5L genotype had a higher risk of cardiovascular mortality compared to women with other genotypes. We concluded that in patients with type 2 diabetes, CNDP1 predicts cardiovascular mortality in a sex-specific manner, with a higher risk in women with 5L-5L genotype compared to women with other genotypes.

The mechanism by which CNDP1 may reduce risk for diabetic nephropathy as was observed in earlier cross-sectional studies could be through protective effects of lower carnosinase activity in patients with 5L-5L genotype. The number of leucine repeats in CNDP1 has been found to influence serum carnosinase activity, with the lowest activity measured in individuals with homozygosity for five leucine repeats (5L-5L genotype) [1]. Carnosine, which is degraded by carnosinase, is a naturally

occurring dipeptide with protective actions, such as scavenging of free oxygen radicals and inhibition of formation of advanced glycation endproducts (AGEs) [7, 8]. It was therefore hypothesized that subjects with 5L-5L genotype would have higher concentrations of serum carnosine, responsible for the suggested protective role of 5L-5L genotype against diabetic nephropathy. The different impact of CNDP1 polymorphism in women compared to men was suggested to underlie the sex-specific association of CNDP1 and diabetic nephropathy. Since men have higher carnosine levels in muscles and women have higher serum carnosinase levels, differences in carnosinase activity due to the different CNDP1 polymorphism may have a stronger impact in women [9]. However, the suggested effect of 5L-5L in diabetic nephropathy is still a subject of debate. Although genetic variability in CNDP1 has been shown to influence glucose metabolism, animal experiments and other human studies failed to confirm an association between CNDP1 and diabetic nephropathy [8, 10, 11]. If the finding in chapter 3 on increased cardiovascular mortality in women with 5L-5L compared with other genotypes is replicated, I can speculate that survival disadvantage in women with 5L-5L and nephropathy may have resulted in a lower number of women with 5L-5L and nephropathy than the number of women with other genotypes and nephropathy included in cross-sectional studies. This may have induced false associations in previous cross-sectional studies.

The discrepancy between our findings and findings from previous cross-sectional studies may also be explained by other factors. First, it is important to realize that we investigated clinical endpoints, i.e., ESRD and mortality, while cross sectional studies only looked at an association with diabetic nephropathy. In late stages of diabetic renal disease, many other risk factors besides genetic predisposition can overrule the role of a single polymorphism, especially when the suggested role is relatively small and supposed to modulate early pathways of vascular damage. Second, other genetic risk alleles for diabetic nephropathy in the same region of chromosome 18, or even other chromosomes, may play an important role in predicting risk of diabetic nephropathy. Indications for this suggestion have indeed been found in other studies [10, 12]. Third, the associations from cross-sectional studies have been proposed to be the consequence of a primary effect of carnosine on glucose metabolism [1, 7]. In animal experimental studies, carnosine improved serum insulin secretion and other

functional indicators of glucose metabolism in mice [8]. Taking into consideration that diabetic renal disease is a multi-factorial phenomenon that results from activation of multiple cascaded of pathways, the role of genetic variation in CNDP1 could be more pronounced in the early phase of diabetic renal disease, possibly even before albuminuria is established. At this moment, there are no prospective studies on the role of CNDP1 polymorphism in development of new-onset albuminuria and diabetic nephropathy. Such studies are necessary to evaluate the clinical relevance of the CNDP1 polymorphism in diabetic nephropathy.

The second investigated gene in this thesis is CCR2. In **chapter 4**, we prospectively investigated whether CCR2 V64I polymophism, together with ACE I/D, predict the development of new-onset microalbuminuria in patients with type 2 diabetes. These genes are of interest because they have been implicated in renin-angiotensin-aldosterone system (RAAS) and the inflammatory pathways in several populations [13, 14]. The ACE I/D polymorphism in the angiotensin-converting enzyme (ACE) gene has been widely investigated as a potential risk factor for diabetic nephropathy [15, 16]. Activation of RAAS and the resulting renal hemodynamic changes modify the growth and activity of glomerular cells, inducing the expression of chemokines with direct pro-fibrotic and pro-inflammatory responses [17, 18]. Monocyte chemoattractant protein-1 (MCP-1), activated by its C-C chemokine receptor (CCR2), is an essential chemokine that is implicated in chronic monocyte-mediated inflammation and endothelial damage [19]. Recent animal experimental and clinical studies have suggested CCR2 and its gene to play a role in the inflammatory pathways in vascular diseases [20, 21]. Furthermore, CCR2 V64I polymorphism has been shown to be associated with coronary artery disease [13, 14]. Therefore, we hypothesized that the CCR2 V64I polymorphism could play a role in the development of diabetic nephropathy. ACE I/D and CCR2 V64I were genotyped by agarose gel electrophoresis and sequences analysis respectively in 1128 patients from the BErgamo NEphrologic Diabetic Complications Trial (BENEDICT) – a prospective randomized trial evaluating ACE inhibition (ACE-I) effect on new-onset microalbuminuria (albuminuria 20-200 µg/min) in hypertensive type 2 diabetes patients without albuminuria (<20 µg/min) at inclusion. Median follow-up was 42.3 months. We found that ACE I/D was significantly associated with new-onset microalbuminuria, while CCR2 V64I was not

significantly associated with new-onset microalbuminuria. The risk of development of microalbuminuria was higher in patients with combined mutant genotypes from both polymorphisms, i.e. DD from ACE I/D and VI/II from CCR2, compared to carriers of neither of these genotypes, i.e. ID/DD and VV. Thus, we were able to confirm earlier findings on the role of ACE I/D polymorphism as a genetic risk factor for diabetic nephropathy and concluded that CCR2 V64I polymorphism may have additional value besides ACE I/D polymorphism on prediction of renal outcome in patients with diabetes. We did not find a significant interaction between ACE-I treatment and ACE I/D or CCR2 V64I.

In the RENAAL study, the unfavorable renal prognosis conferred by the D allele of ACE I/D polymorphism was opposed by treatment with Losartan in patients with type 2 diabetes [22], In contrast to these findings, we found that treatment with ACE-I was homogeneously renoprotective in all patients, regardless of their ACE I/D genotype. This could be explained by difference in stage of nephropathy between the population of the RENAAL study and the investigated population in chapter 4. All patients in our study were normoalbuminuric at the beginning of the trial, whereas patients in the RENAAL study had advanced diabetic renal disease with macroalbuminuria. Therefore, additional benefit of RAAS-blockade in patients with D allele of ACE I/D seems to be prominent only in later stages of diabetic nephropathy.

Risk stratification: urine proteomics

In the last decade, analysis of urinary proteins using capillary electrophoresis coupled with mass spectrometry (CE-MS) has helped to investigate important biomarkers for diabetic nephropathy [23, 24]. Using such data, a model including 65 urinary biomarkers for diabetic nephropathy has been developed in patients with type 1 diabetes [25]. This model was able to distinguish patients with diabetic nephropathy with 97% sensitivity and specificity (AUC = 0.994). However, this model has not been externally validated in patients with type 2 diabetes. Validation is required before implementation of this model in clinical practice [26]. In **chapter 5**, we evaluated the validity of the previously established proteomic biomarkers with respect to the diagnostic potential in 148 Caucasian patients with type 2 diabetes. Cases were patients with diabetic nephropathy, defined as urinary albumin excretion >300

mg/24h, diabetic retinopathy and at least 5 years duration of diabetes. Controls were patients with diabetes without nephropathy, defined as at least 5 years duration of diabetes without nephropathy (no albuminuria). In this blinded case-control study, proteome analysis was performed using high-resolution capillary electrophoresis coupled with mass spectrometry (CE-MS). Evaluation of data was performed using the previously developed model for diabetic nephropathy. We found this model to have 93.8% sensitivity and 91.4% specificity in the selected cohort of patients with type 2 diabetes, with a combined AUC of 0.95. We concluded that urinary proteome analysis by CE-MS is able to distinguish patients with diabetic nephropathy from normoalbuminuric diabetic patients.

This study did not include patients with microalbuminuria and did not have a longitudinal part, so the diagnostic value of proteome analysis in detection early stages of diabetic nephropathy was not investigated. These limitations have been covered in recent studies on the assessment of urinary collagen fragments in detection of early stages of diabetic nephropathy. Indeed, shortly after our study, a study by Good et al. [27] reported a model based on 273 urinary biomarkers, the so-called CKD273 classifier. This model was shown to have high sensitivity and specificity in detection of chronic kidney disease, irrespective of the underlying pathology. Recently, the CKD273 classifier was prospectively investigated and could identify patients with diabetes who will develop diabetic nephropathy 3-5 years before the development of microalbuminuria [28]. In this study, there was a decrease in collagen fragments before albumin excretion started to increase. It was speculated that the decrease in collagen fragments observed early in the course of diabetic nephropathy could be the related to the increase in extracellular matrix deposition associated with hyperglycemia-induced vascular damage [29]. Therefore, analysis of urinary proteomics seems to be a promising method in assessment of patients at risk for diabetic nephropathy at an early stage.

Benfotiamine; a beneficial treatment for diabetic nephropathy?

In animal experimental studies, benfotiamine and thiamine have been suggested as agents that can prevent occurrence and deterioration of diabetic complications [30, 31]. By activation of thiamine pyrophosphate dependent enzymes, in particular

transketolase (TK), thiamine and benfotiamine are supposed to be responsible for countering the adverse effect of hyperglycemia: activation of hexosamine and protein kinase C (PKC) pathways, mitochondrial dysfunction and formation of AGEs [30, 32]. These pathways are implicated in glucose-mediated vascular damage that underlies diabetic complications, including diabetic nephropathy [33]. Therefore, in **chapter 6**, we investigated the effect of benfotiamine on urinary excretion of albumin (UAE) and the tubular damage markers; kidney injury molecule-1 (KIM-1) and α-1 microglobulin, in patients with diabetic nephropathy. Eighty-six patients with type 2 diabetes and UAE equivalent to 15-300 mg/24h, despite angiotensin-converting enzyme inhibitors (ACE-Is) or angiotensin-receptor blockers (ARBs), were randomly assigned to 12-week benfotiamine (900mg/day) or placebo. Despite a significant improvement in thiamine status and TK activity in the benfotiamine-group, we found that 12-week treatment with benfotiamine did not result in a decrease in urinary excretion of albumin, KIM-1 or α-1 microglobulin compared to placebo. Regarding other clinical parameters, benfotiamine resulted in a small increase in plasma creatinine compared to placebo. We concluded that in patients with type 2 diabetes and nephropathy, high-dose benfotiamine treatment for 12 weeks as add-on to ACE-Is or ARBs did not reverse albuminuria or tubular damage.

As suggested by animal studies, thiamine and benfotiamine are supposed to antagonize detrimental effects of hyperglycemia on tissues, including the formation of AGEs, endothelial damage, and low-grade inflammation [30, 34]. Given the fact that we did not find an effect of benfotiamine on a more distant outcome parameter, such as albuminuria, we wondered whether benfotiamine had effect on parameters that are supposed to be more directly affected by benfotiamine. Therefore, in **chapter 7** we investigated the effect of benfotiamine on markers of these pathways in patients with type 2 diabetes who participated in our randomized controlled trial on benfotiamine in diabetic nephropathy. AGEs (Nε-(carboxymethyl)lysine [CML], Nε-(Carboxyethyl)lysine [CEL], and 5-hydro-5-methylimidazolone [MG-H1]) were measured in plasma and urine at baseline and after 6 and 12 weeks. Plasma markers of endothelial dysfunction (soluble vascular cell adhesion molecule-1 [sVCAM-1], soluble intercellular adhesion molecule-1 [sICAM-1], soluble E-selectin) and markers of low-grade inflammation (high-sensitivity C-reactive protein [hs-

CRP], serum amyloid-A [SAA], myeloperoxidase [MPO]) were also measured at baseline and after 6 and 12 weeks. Compared to placebo, benfotiamine did not result in significant reductions in plasma or urinary AGEs, plasma markers of endothelial dysfunction, or low-grade inflammation. We concluded that 12-week treatment with benfotiamine did not affect intermediate pathways that could finally lead to hyperglycemia-induced vascular complications, including albuminuria. Our findings differ from an earlier pilot study in 40 patients with type 2 diabetes in which 12 weeks of 300mg/day of thiamine resulted in a significant decrease in UAE of 17.7mg/24h [35]. In that study, baseline UAE was 44 [33-121] mg/24h in the thiamine and 51 [32-122]mg/24h in the placebo group, which is almost two times lower than in our study, despite 100% of ACE-I/ARB treatment in our study versus <50% in the pilot study. Thus, thiamine and benfotiamine might prove to have protective effects in earlier diabetic nephropathy stages or in patients without any other form of renoprotective treatment. This is in line with an animal study in which development of albuminuria after induction of diabetes was inhibited by thiamine and benfotiamine [31].

One possible explanation for the lack of effect of benfotiamine could be the short duration of treatment in our trial. For example, in two large intervention studies, it took years of lowering HbA1c before difference in UAE was found between strict metabolic control and standard therapy [36, 37]. Similar delay was found in patients after pancreas transplantation, in whom it took at least five years of normalization of glucose concentrations by pancreas transplantation before glomerular lesions disappeared and albuminuria decreased [38]. The fact, however, that we did not find an effect of benfotiamine on intermediate pathways , including formation of AGEs, endothelial damage, and inflammation, makes it less likely that benfotiamine would have effects in studies of longer duration.

Strengths and limitations

The strengths of the studies on genetic risk stratification in chapter 2, 3 and 4 are their prospective design, the long duration of follow-up, and the well-defined clinically relevant endpoints. The studies on CNDP1 and CCR2 V64I are the first and the largest prospective studies on these polymorphisms in patients with diabetes. Only one other

prospective study was performed on CNDP1 in patients with type 1 diabetes and found, in line with our findings, no significant association between CNDP1 polymorphism and progression to ESRD [11]. In the studies on CNDP1, besides progression to ESRD, we investigated the risk of mortality as a relevant prognostic endpoint. Only in female patients with type 2 diabetes, CNDP1 predicted cardiovascular mortality with increased risk in those with 5L-5L genotype compared to other genotypes. This finding could clarify the sex-specific correlation found in a previous cross-sectional study. Selection bias in cross-sectional studies remains a major pitfall of this kind of studies. This is because cardiovascular mortality can be considered as a competing risk for the development of diabetic nephropathy and ESRD in patients with nephropathy. Another strength of the prospective studies on novel genes is that we tested the proportional hazard (PH) assumption before we applied Cox regression analyses. The PH assumption must be tested for each variable before variables can be included in a Cox regression model. If not, models with time-dependent covariates should be used to estimate hazard ratios [39]. For our studies, we used the tests for non-zero slope of scaled Schoenfeld residuals for functions of time [40]. In these analyses, CNDP1 polymorphism turned out to have a time-dependent association with progression of ESRD in patients with type 1 diabetes. One possible explanation for this pattern of association is gene-environment interaction which may alter over time. Factors such as glycemic control have indeed been shown to play a role in secretion of carnosinase and its activity [41].

The studies in chapter 2, 3 and 4 also have limitations. First, data on changes in clinical parameters (HbA_{1c}, blood pressure, cholesterol level, and cardiovascular status) and use of medication during follow-up were not always available. These factors have influence on the risk of albuminuria, ESRD and mortality. Therefore adjustment for these confounders was not always possible. Second, no yearly measurements on true GFR were performed during follow-up in studies on CNDP1 in chapter 3. As an alternative, we used the estimated GFR as calculated by the MDRD-formula in order to calculate the individual slopes of renal function in patients with type 2 diabetes. Thus, some caution is warranted when interpreting the results on slopes of eGFR-MDRD, taking the limitations of the MDRD formula as an estimate of true GFR into consideration and the fact that slopes of eGFR-MDRD might slightly underestimate

renal function decline in patients with progressive renal disease [42, 43]. Thirdly, follow-up data on yearly urinary albumin excretion were not available in many of the included patients. Therefore, the question whether CNDP1 is associated with development or progression of albuminuria remained unanswered.

The strength of the clinical trial on benfotiamine lies in its design. We performed a randomized double-blind placebo-controlled clinical trial in patients with type 2 diabetes. A power calculation was made to detect a relevant decrease in the primary outcome parameter (urinary albumin excretion). Patients were included after a screening period, in which albuminuria between 15-300 mg/24h was documented in at least 2 different measurements. Changes in antihypertensive treatment, especially ACE-I and/or ARB were not allowed in the screening period and during the study period, and patients were strictly controlled during the study and all changes in co-medications were monitored. In addition to measuring parameters of renal damage in 24-hour urine (albuminuria, tubular damage markers and AGE's), we measured AGEs and parameters of endothelial dysfunction and inflammation in plasma. This enabled us to investigate possible effects of benfotiamine not restricted to the kidneys. Despite the many strengths of our benfotiamine trial, it still has limitations. First, we included patients with albuminuria ranging from 15 to 300 mg/24h under treatment with ACE-I and/or ARB, corresponding with high-normal and microalbuminuric range. Therefore, some patients (< 10%) did not have microalbuminuria at baseline, and without knowledge of the degree of albuminuria they would have had if they had not received ACE-I and/or ARB, an unknown part of these patients could in reality be normoalbuminuric, although on a whole this would probably be a very small portion. This could have resulted in lack of effect of benfotiamine in this subgroup, and thereby affecting to a small extent the general outcome. Since benfotiamine is supposed to influence intermediate pathways between hyperglycemia and microvascular complication, namely thiamine-induced pathways, we assumed the effect of benfotiamine to be more pronounced in patients with early diabetic nephropathy rather in those with advanced nephropathy. Subgroup analyses according to urinary albumin excretion (lower range and upper range) did not reveal a different response to benfotiamine regarding outcome measures.

Second, the trial was powered to detect a relevant decrease in urinary albumin excretion as primary study parameter. Finding no significant differences in secondary study parameters (AGEs, markers of endothelial dysfunction and inflammation) does not exclude effects of benfotiamine treatment on these parameters. However, looking at the measured differences between benfotiamine and placebo in our data, it seems that only small non-relevant differences could be present, if they would be present at all.

Third, power calculation was based on one side-testing to detect a relevant reduction (50% reduction) in urinary albumin excretion, since decrease, and not increase in urinary albumin excretion was hypothesized in our study. Thus, this study was not powered to detect a change but an improvement only in urinary albumin excretion in patients with benfotiamine compared with placebo. A design with 2-sided testing would have been preferable, however when interpreting the confidence intervals of the changes in the several outcome parameters, this probably would not have let to different conclusions.

SUMMARY AND FUTURE PERSPECTIVES

In patients with type 1 diabetes, CNDP1 polymorphism predicts progression to ESRD, but not mortality. In patients with type 2 diabetes, CNDP1 polymorphism did not predict progression of decline of renal function. Cardiovascular mortality was increased in female patients with 5L-5L compared to other genotypes. These results seem to contrast with results of earlier cross-sectional studies. More prospective studies are required to finally be able to discern whether CNDP1 indeed plays a role as a susceptibility factor of diabetic nephropathy and progression of diabetic nephropathy to ESRD. Replication of the finding on increased risk of cardiovascular mortality in women with 5L-5L genotype, preferably in prospective studies, is needed to better understand the role of CNDP1 and serum carnosinase in diabetic complications. Another important question is the role of carnosine as a potential agent for pharmacological intervention, aimed at modifying glucose metabolism and possibly preventing diabetic vascular complication. Randomized controlled trial are needed to elucidate these questions.

In this these I also confirmed the role ACE I/D polymorphism as a risk factor for development of new-onset albuminuria in patients with type 2 diabetes and concluded that CCR2 V64I polymorphism might have additional value besides ACE I/D polymorphism on predicting renal outcome. Larger prospective studies on CCR2 V64I polymorphism are needed to investigate its possible role in genetic risk assessment of diabetic nephropathy.

Analysis of urine proteomics by CE-MS is a helpful diagnostic tool for the detection of diabetic nephropathy. Other studies have shown that urinary proteomics enables noninvasive assessment of diabetic nephropathy risk at an early stage. In a recent study, treatment with ARB (Irbesartan) in patients with microalbuminuria has resulted in improvement in urine peptide identified by CE-MS analysis of urine proteomics [44]. Therefore, it is tempting to speculate that using urine proteomics, renoprotective treatment could be early initiated in those at risk to develop diabetic nephropathy in order to delay or prevent development of microalbuminuria. This subject should be further investigated in upcoming trials. Before urine proteomics becomes a part of screening in patients with diabetes, further research will be necessary to investigate prognostic value above albuminuria and cost-effectiveness of proteome analysis by CE-MS in diabetic renal disease, ESRD and (cardiovascular) mortality.

Based on our randomized controlled trial, our main conclusion is that a 12-week treatment with benfotiamine did not represent a beneficial intervention in patients with diabetic nephropathy. At this moment, based on the current evidence, there are no arguments to recommend the use of benfotiamine as a treatment for patients with diabetic nephropathy. Further research is necessary to elucidate whether longer-term treatment with benfotiamine or initiation of treatment in earlier stages before diabetic nephropathy is already established, could be a beneficial intervention in patients with diabetes.

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Chapter 9

Nederlandse samenvatting

(summary in Dutch)

Dit proefschrift heeft drie doelen: (1) bestuderen van twee recent beschreven genen als risicofactoren voor het ontwikkelen van diabetische nefropathie, (2) onderzoeken of analyse van eiwitten in de urine (urine proteomics) geschikt is om diabetische nefropathie in een vroeg stadium te detecteren, en (3) onderzoeken of benfotiamine (een vetoplosbaar thiaminederivaat) een zinvolle behandeling is bij patiënten met diabetische nefropathie.

Risicostratificatie: genetica

De onderzochte genen zijn geselecteerd op basis van eerdere studies waarin een relevante associatie werd gevonden tussen de betreffende genen en cascades van nierschade in patiënten met diabetes. Hoofdstukken 2 en 3 gaan over het carnosinase-1 gen (CNDP1) op chromosoom 18. Enkele cross-sectionele studies in patiënten met type 2 diabetes suggereerden dat het aantal leucines in het CNDP1 gen is geassocieerd met diabetische nefropathie. Uit die studie is gebleken dat patiënten die homozygoot waren voor het laagste aantal leucines (5L-5L) in CNDP1 een lager risico op diabetische nefropathie hebben dan patiënten met een groter aantal leucines (5L-6L, 6L-6L, 5L-7L, 6L-7L, 7L-7L) in het CNDP1 gen. Dit verband wordt toegeschreven aan de rol van CNDP1 in het metabolisme van carnosine. Het genotype 5L-5L zou leiden tot een lagere activiteit van het enzym carnonisase in het serum, waardoor er meer carnosine beschikbaar is. Carnosine is een dipeptide met beschermende eigenschappen door het remmen van vrije radicalen en “advanced glycation endproducts (AGEs)”. Echter, selectiebias in cross-sectionele studies kan niet uitgesloten worden. Immers, prospectieve studies over CNDP1 ontbraken en enkele cross-sectionele studies hebben de hierboven beschreven associatie niet kunnen bevestigen. Tevens liet een recent onderzoek zien dat de associatie tussen CNDP1 en diabetische nefropathie is beperkt tot vrouwen met type 2 diabetes, waarbij gedacht werd aan een interactie tussen CNDP1 en sekse. Om deze redenen hebben wij CNDP1 in prospectieve studies onderzocht. In **hoofdstuk 2** wordt de associatie tussen CNDP1 en het ontwikkelen van eindstadium nierfalen of mortaliteit bij 2086 patiënten met type 1 diabetes bestudeerd. De mediane follow-up duur was 8.8 jaar. Het risico op eindstadium nierfalen na een lange follow-up was groter in patiënten met 5L-5L vergeleken met patiënten met andere genotypen. Er was geen

verschil in mortaliteit tussen de subgroepen en er was geen interactie tussen het gen en de sekse. In **hoofdstuk 3** wordt CNDP1 bestudeerd in 871 patiënten met type 2 diabetes. De mediane follow-up duur was 9.5 jaar. Genetische variatie in CNDP1 was niet geassocieerd met de achteruitgang van nierfunctie of mortaliteit. Wel was het risico op cardiovasculaire mortaliteit in vrouwen met het 5L-5L genotype verhoogd in vergelijking met vrouwen met andere genotypen. Dit verband ontbrak bij mannen, passend bij een geslachtsafhankelijke relatie tussen CNDP1 en cardiovasculaire mortaliteit bij patiënten met type 2 diabetes. Mogelijk heeft de hogere cardiovasculaire mortaliteit bij vrouwen met 5L-5L gezorgd voor een selectiebias in de cross-sectionele studies.
In **hoofdstuk 4** wordt een "single nucleotide polymorphism" (SNP) in het CCR2 gen (CCR2 V64I) samen met het bekende SNP in het ACE gen (ACE I/D) besproken. Het SNP CCR2 V64I bleek geassocieerd te zijn met vasculaire schade en cardiovasculaire ziekten maar is niet eerder onderzocht bij patiënten met diabetes. Het DD genotype van het ACE I/D SNP is reeds beschreven als risicofactor voor progressie van nierziekten bij patiënten met diabetes. Onze prospectieve analyse bij een cohort van 1128 patiënten met type 2 diabetes laat zien dat ACE I/D is geassocieerd met hoge incidentie van microalbuminurie, terwijl er geen significante associatie werd gevonden tussen CCR2 V64I en de incidentie van microalbuminurie. Een gecombineerde analyse van beide SNPs liet zien dat patiënten met beide gemuteerde varianten (DD in ACE en VI/II in CCR2) een licht verhoogd risico hebben op het ontwikkelen van microalbuminurie vergeleken met patiënten met geen van deze varianten. Echter, het verschil tussen de groepen was niet significant. Mogelijk heeft het CCR2 V64I een toevoegende voorspellende waarde boven het ACE I/D bij patiënten met diabetische nefropathie.

Risicostratificatie: Urine-proteomics

Analyse van eiwitten en collageenfragmenten in de urine door middel van "capillary electrophoresis coupled with mass spectrometry (CE-MS)" heeft in de afgelopen jaren geleid tot het ontdekken van nieuwe experimentele urinemarkers van diabetische nefropathie. Zo werd een model ontwikkeld met 65 biomarkers die een hoge sensitiviteit en specificiteit hebben als het gaat om het voorspellen van nierschade bij patiënten met type 1 diabetes. In **hoofdstuk 5** werd dit model geëvalueerd in 148

patiënten met type 2 diabetes om de diagnostische waarde van het 65-biomarker model te valideren. In deze geblindeerde case-control studie werd een analyse van eiwitten en collageenfragmenten in de urine (urine-proteomics) verricht en werd het 65-biomarker model toegepast. Het model bleek een hoge sensitiviteit en specificiteit te hebben met betrekking tot het voorspellen van diabetische nefropathie bij patiënten met type 2 diabetes (AUC 0.95). Onze studie was de basis voor andere recente studies waarin een uitgebreid model werd ontwikkeld met 273 biomarkers. Dit model bleek een betere diagnostische en voorspellende waarde te hebben voor diabetische nefropathie in een vroeg stadium, namelijk voordat er albuminurie ontstaat. Mogelijk heeft deze ontwikkeling consequenties voor de praktijk. Zo zou een behandeling eerder gestart kunnen worden bij patiënten met een hoog risico op diabetische nefropathie. Gerandomiseerde trials moeten deze vraag beantwoorden.

Benfotiamine; een zinvolle behandeling bij patiënten met diabetische nefropathie?
In **hoofdstukken 6 en 7** wordt de behandeling met benfotiamine bij patiënten met diabetische nefropathie besproken. Experimenten in proefdieren lieten zien dat benfotiamine en thiamine de nierschade en andere vasculaire complicaties van diabetes kunnen tegengaan of beperken. De activatie van het enzym transketolase door thiaminederivaten leidt tot een remming van de cascades die betrokken zijn bij de door hyperglycemie geïnduceerde vasculaire schade; namelijk activatie van hexosamine en protein kinase C (PKC), mitochondriale dysfunctie en formatie van AGEs. In **hoofdstuk 6** wordt het effect van benfotiamine onderzocht bij 86 patiënten met type 2 diabetes en microalbuminurie in een gerandomiseerd dubbelblind placebo-gecontroleerd onderzoek. Alle geïncludeerde patiënten waren reeds ingesteld op een ACE-remmer en/of een angiotensine-receptor blokker (ARB). Na 12 weken bleek de behandeling met benfotiamine geen verlagend effect te hebben op albuminurie of urinemakers van tubulointerstitiële nierschade. Omdat benfotiamine zou ingrijpen op de cascades die betrokken zijn bij de vasculaire schade hebben wij in **hoofdstuk 7** een analyse uitgevoerd naar het effect van benfotiamine op de parameters van de hierboven genoemde cascades, zoals AGEs (Nε-(carboxymethyl) lysine [CML], Nε-(Carboxyethyl)lysine [CEL] en 5-hydro-5-methylimidazolone [MG-H1]) in plasma en urine, markers van endotheliale dysfunctie (soluble vascular

cell adhesion molecule-1 [sVCAM-1], soluble intercellular adhesion molecule-1 [sICAM-1], soluble E-selectin) en inflammatie (high-sensitivity C-reactive protein [hs-CRP], serum amyloid-A [SAA], myeloperoxidase [MPO]). Ook hierop werd geen effect van benfotiamine waargenomen ten opzichte van placebo na 12 weken van de behandeling.
De uitkomst van onze trial komt niet overeen met de bevindingen van de experimentele studies en een pilot-studie naar het effect van thiamine op albuminurie. Het stadium van de nefropathie ten tijde van het starten van de behandeling, de duur van de behandeling en het gelijktijdig gebruik van ACE-remmers en/of ARBs zijn factoren die mogelijk ten grondslag liggen aan de discrepantie tussen onze bevindingen en de eerdere bevindingen waarbij er een gunstig effect werd gezien van thiamine en benfotiamine op diabetische nefropathie. Bij een pilot-studie naar het effect van thaimine bij patiënten met type 2 diabetes uit Pakistan had minder dan 50% van de patiënten een ACE-remmer en/of een ARB. Dit kan van grote invloed zijn op de uitkomsten.

CONCLUSIE

Dit proefschrift laat zien dat het carnosinase-1 gen (CNDP1) voorspellend is voor het risico op eindstadium nierfalen bij patiënten met type 1 diabetes. In tegenstelling tot de bevindingen van cross-sectionele studies bij patiënten met type 2 diabetes, blijkt de homozygote variant voor het laagste aantal leucines (5L-5L) in het CNDP1 gen niet te beschermen tegen achteruitgang van nierfunctie bij patiënten met type 2 diabetes. Vrouwen met 5L-5L lijken een verhoogd risico op cardiovasculaire mortaliteit te hebben vergeleken met vrouwen met andere varianten. Deze bevinding kan van invloed zijn geweest op de resultaten van cross-sectionele studies. Replicatie van deze bevindingen in andere prospectieve studies is noodzakelijk om een beter inzicht te krijgen in de rol van carnosine en het carnosinase-1 gen (CNDP1) bij patiënten met diabetes.
In dit proefschrift heb ik de rol van ACE I/D SNP als genetische risicofactor voor het ontwikkelen van albuminurie bij patiënten met type 2 diabetes kunnen aantonen. Een recent beschreven SNP in het CCR2 (CCR2 V64I) is echter niet voorspellend

gebleken voor het risico op het ontstaan van albuminurie. Grotere prospectieve studies zijn nodig om de potentiële rol van CCR2 V64I in de inflammatoire cascade en als risicofactor voor nefropathie bij patiënten met diabetes in kaart te brengen.
Analyse van eiwitten en collageenfragmenten in de urine (urine-proteomics) is een bruikbare diagnostische methode voor diabetische nefropathie in vroeg stadium. Hierdoor is het in de toekomst wellicht mogelijk om patiënten met diabetes en een verhoogd risico op nefropathie tijdig te behandelen en regelmatig te controleren. Meer onderzoek is echter nodig om te kijken of het routinematige gebruik van urine-proteomics bij patiënten met diabetes een zinvolle en kosteffectieve benadering is.
De laatste conclusie van mijn proefschrift luidt dat er geen bewijs is voor het gebruik van benfotiamine bij patiënten met type 2 diabetes, omdat het niet leidt tot vermindering van albuminurie of andere markers van nierschade.

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This thesis is published within the Diabetes Centre of the Isala Clinics in Zwolle.

Previous dissertations at our Diabetes Centre:

- **Van Hateren K.J.J.** (2013). *Diabetes care in old age*. Promotores: Prof. dr. H.J.G. Bilo, Prof. dr. K. van der Meer. Copromotores: Dr. N. Kleestra, Dr. S.T. Houweling.
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- **Goddijn P.P.M.** (1997*). Improving metabolic control in NIDDM patients referred for insulin therapy*. Promotor: Prof. dr. B. Meyboom-de Jong. Copromotor: Dr. H.J.G. Bilo.

CURRICULUM VITAE

Alaa Alkhalaf is geboren op 12 augustus 1982 en opgegroeid in Damascus, Syrië. In 1999 begon hij met de studie Geneeskunde aan de Universiteit van Damascus. In 2005 rondde hij de opleiding tot arts af. In 2006 behaalde hij tevens het Amerikaanse artsendiploma. In 2007 emigreerde hij naar Nederland waar hij begon met een promotieonderzoek op de afdeling Nefrologie van het Universitair Medisch Centrum Groningen en het Diabetes Kenniscentrum van de Isala Klinieken te Zwolle. Naast dit traject deed hij gedurende zes maanden co-schappen in de Isala Klinieken om zo in 2009 het Nederlandse artsenexamen aan de Rijksuniversiteit Groningen te behalen. Vervolgens is hij in 2010 begonnen met de opleiding tot maag- darm- leverarts in de Isala Klinieken te Zwolle. In 2016 verwacht hij deze specialisatie te voltooien.