Diabetic nephropathy : pathology, genetics and carnosine metabolism

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Association between *CNDP1* genotype and diabetic nephropathy is sex-specific

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Abstract

Objective, The 5-5 homozygous *CNDP1* (carnosinase) genotype is associated with a reduced risk for diabetic nephropathy (DN). We investigated whether this association is sex-specific and independent of susceptibility for type 2 diabetes.

Research Design and Methods, Three separate groups of 114, 90, and 66 patients with type 2 diabetes and DN were included in this study, and compared with 93 patients with type 2 diabetes for >15 years without DN, and 472 population controls. The diabetes control group was used to determine an association in the three patient groups separately, and the population control to estimate the genotype risk (odds ratio [confidence interval]) for the population in a pooled analysis. The population controls were also compared with 562 patients with type 2 diabetes without DN to determine whether the association was independent of type 2 diabetes. The *CNDP1* genotype was determined by fragment analysis after PCR amplification.

Results, The frequency of the 5-5 homozygous genotype was 28%, 36%, 41% in the three DN patient groups, and 43% and 42% in the diabetic and population controls, respectively. The 5-5 homozygous genotype occurred significantly less frequently in women in all three patient groups compared with diabetic controls. The genotype risk for the population was estimated to be 0.5 [0.30–0.68] in women and 1.2 [0.77–1.69] in men. The 562 patients with type 2 diabetes without DN did not differ from the general population (P=0.23).

Conclusion, This study shows that the association between the *CNDP1* gene and diabetic nephropathy is sex-specific and independent of susceptibility for type 2 diabetes.

Introduction

Only 20– 40% of patients with type 1 or type 2 diabetes will develop diabetic nephropathy (DN), and if no signs of DN are present in the first 15 years after diagnosis of diabetes, the chance of ever developing DN is small (1). Furthermore, sibling studies show a strong familial component for development of DN (2;3), and certain ethnic groups seem to be at a greater risk of developing DN (2;4). These findings suggest that there is a genetic susceptibility component for DN.

Many genes are thought to be involved in DN (reviewed in (5)). One of the genes associated with DN in both patients with type 1 and type 2 diabetes is the *CNDP1* gene, which encodes serum carnosinase (6). This was confirmed in European Americans with end stage DN due to type 2 diabetes (7), but the association between DN and the *CNDP1* gene could not be confirmed in patients with DN due to type 1 diabetes (8;9) nor in African Americans (7).

Patients with type 2 diabetes of Caucasian origin with homozygosity for 5 leucine repeats in exon 2 demonstrated a reduced susceptibility for developing DN compared with individuals with 6–8 repeats (6;7;10). With increasing numbers of leucine repeats, the secretion of serum carnosinase has been shown to increase (11) and to lead to higher serum carnosinase activity (4;6). Serum carnosinase degrades carnosines and other histidine-containing dipeptides. Carnosines and related dipeptides are known for their reactive oxygen scavenging effects (12), to degrade advanced glycation end products (13), and reduce the TGF-β-induced synthesis of extracellular matrix components (6).

Some genes involved in DN have been shown to have sex-specific effects (14;15). For example, the RANTES receptor gene (*CCR5)* is only associated with DN in men (14), and two single nucleotide polymorphisms in the podocyte slit diaphragm gene (*ACTN4*) were only associated with DN in women (15).

Therefore, we investigated whether the association between the *CNDP1* gene and DN due to type 2 diabetes is sex-specific. Furthermore, we studied whether the association between DN and the *CNDP1* gene is independent of the susceptibility for type 2 diabetes itself.

Study Design and Methods

The institutional Medical Ethics committees of the participating hospitals approved of the studies described below.

CASE GROUPS

For the first case group, female and male Caucasian diabetic patients with DN from the case-control study of Janssen *et al.* were re-assessed separately. DN was defined as diabetes with retinopathy with either macroalbuminuria or who were on dialysis (because of DN). The details of the recruitment of this cohort are described elsewhere (6). In the present analysis, only 114 DN patients with type 2 diabetes were included, and this group will be referred to as DN group 1.

For the second case group, DN patients were selected from the ZODIAC (Zwolle Outpatient Diabetes Project Integrating Available Care) study (16). DN was defined as having either an eGFR < 60 mL/min/1.73 m^2 (17) in combination with an albumin excretion above 30 mg/L (18), or macroalbuminuria (above 300 mg/L) in combination with retinopathy (19). ZODIAC was a cross-sectional, single-centre study, investigating only patients with type 2 diabetes, selected from a population of > 95% Caucasian origin. Patients were recruited from 61 general practitioners from 1998–2000. In this study, 90 DN patients were identified and these will be referred to as DN grou

For the third group, DN patients were selected out of a total of 875 patients from the NECOSAD (Netherlands Cooperative Study on the Adequacy of Dialysis) study (20). NECOSAD is a multicenter, prospective follow-up study of patients with ESRD who were included at the start of dialysis, between 1997 and 2005. For the present analysis, only patients with type 2 diabetes of Caucasian origin and ESRD due to DN were selected; 66 DN patients were included and are referred to as DN group 3.

CONTROL GROUPS

The first control group is a diabetic group, consisting of Caucasian patients with type 2 diabetes without microalbuminuria for at least 15 years, in the absence of ACE inhibitor treatment (6;16). This control group is referred to as diabetic non-nephropathy controls.

The second control group is a population control group selected from the SUNSET (Surinamese in the Netherlands, study for ethnicity and health) study, a population-based, cross-sectional survey (21). In brief, between 2001 and 2003, a random sample of non-institutionalized adults aged 35–60 years was selected. In the present study, only

the 472 white Dutch participants, of whom the genotypic distribution was described in a previous report (4), are used and referred to as population controls.

To investigate whether the association between DN and the *CNDP1* gene is not due to susceptibility for type 2 diabetes, we additionally studied 562 patients with type 2 diabetes without DN (defined as any of the criteria above) selected from the ZODIAC study participants, referred to as the type 2 diabetes population, to compare with the general population.

GENOTYPING

Genotyping was performed as described previously (4). In brief, after PCR amplification, fragment analysis was performed on the ABI-3130 analyzer to determine the number of leucine repeats in each allele. The success rate was, on average, 95% and no errors were detected. Genotyping was performed partially in Leiden and in Mannheim. Some of the samples were measured in both institutes and there was a 100% concordance.

STATISTICAL ANALYSIS

The baseline characteristics of the groups are presented as means and standard deviations or percentages. Continuous variables were tested using the student *t*-test and numeric variables using chi-square. All groups were tested for Hardy-Weinberg equilibrium (HWE), using a chi-square test.

First, the frequency of the 5-5 homozygous genotype in the respective DN groups was compared with the 93 diabetic non-nephropathy controls stratified by sex, to investigate the relevance of the genotype to disease etiology.

Second, the genotype risk for the population was estimated through comparison of the DN groups with population controls. Odds ratios with confidence intervals were calculated. A pooled analysis was performed to determine the total effect for females and males separately, combining the three case groups when compared with sex-matched population controls. The fixed-effects model (inverse variance method) was used when heterogeneity was $P > 0.1$ (chi square) and the random-effects model when heterogeneity was $P < 0.1$.

Finally, to assess whether the susceptibility for DN is independent of susceptibility for type 2 diabetes, we compared the type 2 diabetes population with population controls. The statistical analyses were all performed using SPSS 16.0 and R version 2.9.0.

Results

The baseline characteristics of the three DN groups are described in Table 1 and comparisons are made with diabetic non-nephropathy controls. Baseline characteristics did not differ between diabetic non-nephropathy controls and the type 2 diabetes population, except for diabetes duration (data not shown). There was no significant difference in 5-5 homozygous genotype frequency between the diabetic non-nephropathy controls and the population controls consisting of either all patients ($P = 0.8$), all women ($P = 0.07$), or all men ($P = 0.13$).

All cohorts were in HWE (online appendix).

Table 1. Baseline characteristics

	Diabetic nephropathy groups			T2D* > 15 years without diabetic nephropathy
	1	2	3	
Total	**n = 114**	**n = 90**	**n = 66**	**n = 93**
Age (years)	64.0 ± 11.07	73.4 ± 8.40†‡	66.2 ± 8.97	65.8 ± 11.30
Sex (n (%) male)	64 (56.1)	30 (33.3) †‡	35 (53.0)	47 (50.5)
Diabetes duration (years)	14.3 ± 8.38†	9.7 ± 9.51†‡	15.2 ± 10.99†	22.2 ± 6.78
HbA1c (%)	7.5 ± 1.71	7.5 ± 1.2	-	7.3 ± 1.51
Women				
Age (years)	64.2 ± 11.64	72.2 ± 9.09†‡	67.2 ± 7.57	65.1 ± 12.07
Diabetes duration (years)	14.8 ± 9.24*	9.2 ± 7.77†‡	15.9 ± 11.83†	22.9 ± 6.50
HbA1c (%)	8.0 ± 2.08	7.4 ± 1.12	-	7.5 ± 1.65
Men				
Age (years)	63.7 ± 10.68	75.9 ± 6.22†‡	65.3 ± 10.07	66.4 ± 10.60
Diabetes duration (years)	13.9 ± 7.81†	10.7 ± 12.4†	14.7 ± 10.39†	21.6 ± 7.05
HbA1c (%)	7.2 ± 1.32	7.6 ± 1.38	-	7.6 ± 1.38

*T2D = type 2 diabetes, † $P < 0.05$ compared to T2D > 15 years without diabetic nephropathy, ‡ $P < 0.05$ compared to either DN group 1 or 3. No significant differences were seen between group 1 and 3 or between women and men in each of the DN groups

Relevance of the 5-5 homozygous genotype to disease etiology

Overall, the 5-5 homozygous genotype frequency of the DN groups did not differ from the diabetic non-nephropathy controls or between the DN groups ($P = 0.2$). Women in all three DN groups had a significantly lower frequency of the 5-5 homozygous genotype compared with female diabetic non-nephropathy controls (Table 2), also after Bonferroni adjustment for multiple testing. In contrast, men in all three DN case groups had a higher frequency of the 5-5 homozygous genotype compared with male diabetic non-nephropathy controls.

Table 2. The relation between 5-5 homozygous genotype and DN

	Diabetic nephropathy groups			T2D*>15 years without DN
	1	2	3	
Total	**n = 114**	**n = 90**	**n = 66**	**n = 93**
Frequency 5-5 (%)	28.1	35.6	40.9	43.0
P †	0.03	0.30	0.80	
Women	**n = 50**	**n = 60**	**n = 31**	**n = 46**
Frequency 5-5 (%)	24.0	28.3	25.8	58.7
P †	0.001	0.002	0.006	
Men	**n = 64**	**n = 30**	**n = 35**	**n = 47**
Frequency 5-5 (%)	31.2	50.0	53.4	27.7
P †	0.62	0.05	0.02	

*T2D = type 2 diabetes, † $P < 0.05$ compared to T2D > 15 years without diabetic nephropathy

Genotype risk for the population

No heterogeneity was detected in women ($P = 0.64$), but heterogeneity was detected in men ($P = 0.09$). The three DN groups were pooled for women and men separately, resulting in a genotype risk of 0.5 [0.30–0.68] in women and 1.2 [0.77–1.69] in men (Figure 1).

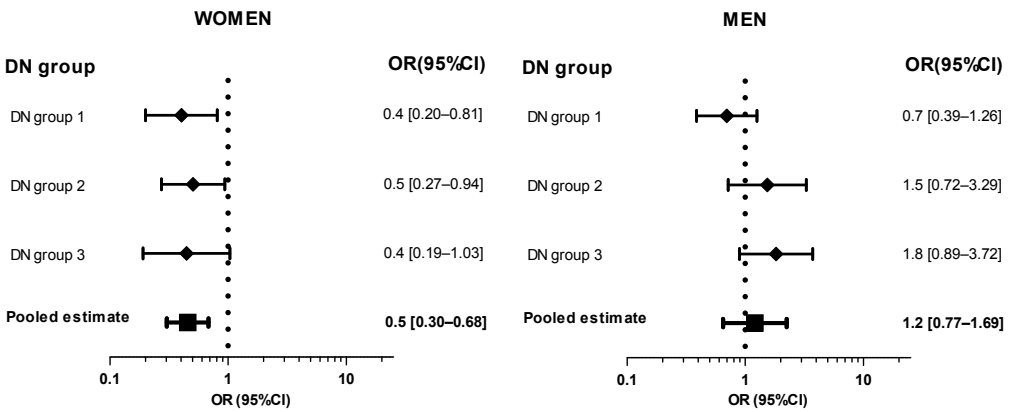

Figure 1. The relationship between the 5-5 homozygous *CNDP1* genotype and diabetic nephropathy in women (upper panel) and men (lower panel) in the three independent diabetic nephropathy groups, and a pooled analysis (total) compared with population controls.

Specificity of 5-5 homozygous genotype for DN, not type 2 diabetes

The 5-5 homozygous genotype frequency of both the 562 type 2 diabetes population and the 472 population controls are shown in Table 3, showing similar frequency of the 5-5 homozygous genotype.

Table 3. Comparison between type 2 diabetes population and population controls

	Type 2 diabetes population	**Population controls**	
Total	**n = 562**	**n = 472**	***P***
Frequency 5-5 (%)	38.1	41.7	0.23
Women	**n = 319**	**n = 239**	
Frequency 5-5 (%)	39.5	43.9	0.23
Men	**n = 243**	**n =233**	
Frequency 5-5 (%)	36.7	39.5	0.58

Discussion

Our results show a sex-specific effect of the *CNDP1* genotype in relation to DN, suggesting that the 5-5 homozygous genotype is only protective in women. The frequency of the 5-5 homozygous genotype was determined in three independent DN groups. These groups were compared with two control groups; patients with type 2 diabetes and a low risk of ever developing DN and a sample from the general population. Compared with the diabetic control group, the 5-5 homozygous genotype frequency was significantly lower in women with DN in all three cohorts, but not in men. The population control group serves to estimate the genotype risk for the population, showing that women with the 5-5 homozygous genotype have a 2-fold reduced risk of ever developing DN. Furthermore, this study shows similar frequencies of the 5-5 homozygous genotype in a large type 2 diabetes population and the general population, underlining that the association with DN is independent of a genetic susceptibility for type 2 diabetes.

The 5-5 homozygous genotype leads to lower carnosinase activity compared with the other genotypes (4;6), leaving more carnosine free to protect the kidney from oxidative stress. Since men have higher carnosine levels in their muscle tissue and women have slightly higher serum carnosinase levels (22), differences in carnosinase activity due to the different *CNDP1* polymorphisms may have a stronger impact in women. Carnosine content in the muscles of female mice was shown to increase after testosterone administration, the increase was 268% (23). This might be because carnosine synthetase,

the enzyme that synthesizes carnosine, is upregulated by testosterone. It is possible that this phenomenon plays a role in DN, because both carnosine synthetase (preliminary results) and androgen receptors are expressed in human kidney (24).

Another explanation for the sex-specific effect found in this study is that the association between the *CNDP1* gene and DN is lost in men due to selective survival by cardiovascular disease. As carnosine has shown to be protective against oxidative stress and hemodynamic damage (6;12;13), this might also explain its role in cardiovascular death in DN patients. Men with DN due to type 2 diabetes have a higher risk for cardiovascular disease than women (25). Therefore this might be more prominent in men. Further support for this theory comes from the ZODIAC study. Men with a diabetes duration < 10 years and the 5-5 homozygous genotype have a significantly lower mortality risk due to cardiovascular disease than patients with more than 10 leucine repeats in the *CNDP1* genotype (data not shown). We found no difference in cardiovascular death between the different genotypes in women.

The relatively older age of DN group 2 might influence the number of subjects with an eGFR < 60 ml/min/1.73m^2. Therefore, a sensitivity analysis was performed, adopting increasingly stringent definitions of DN. The results of this analysis support the conclusion that the 5-5 homozygous genotype is protective in women (online appendix).

The statistical power to detect a similar association as was seen in women in men ranged from 97- 100% within the three DN groups. Insufficient statistical power therefore does not explain the sex-specific effect found in this study.

Limitations of this study are that ethnic origin is not defined by ethnic markers in these Caucasian populations and that sample sizes are relatively small. We performed a sensitivity analysis to exclude population stratification and a permutation analysis to rule out that our results are due to random fluctuation. These analyses support that population stratification or chance are unlikely to explain the sex-specific effect found in this study (online appendix).

Another limitation is that the three DN groups are compared to the same control group.

In conclusion, this study shows a sex-specific effect of the association between the *CNDP1* gene and diabetic nephropathy in three independent patient groups with diabetic nephropathy due to type 2 diabetes, with women being protected by the 5-5 homozygous genotype.

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Disclosure

None of the authors have conflicts of interest to disclose.

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