Diabetic nephropathy : from histological findings to clinical features

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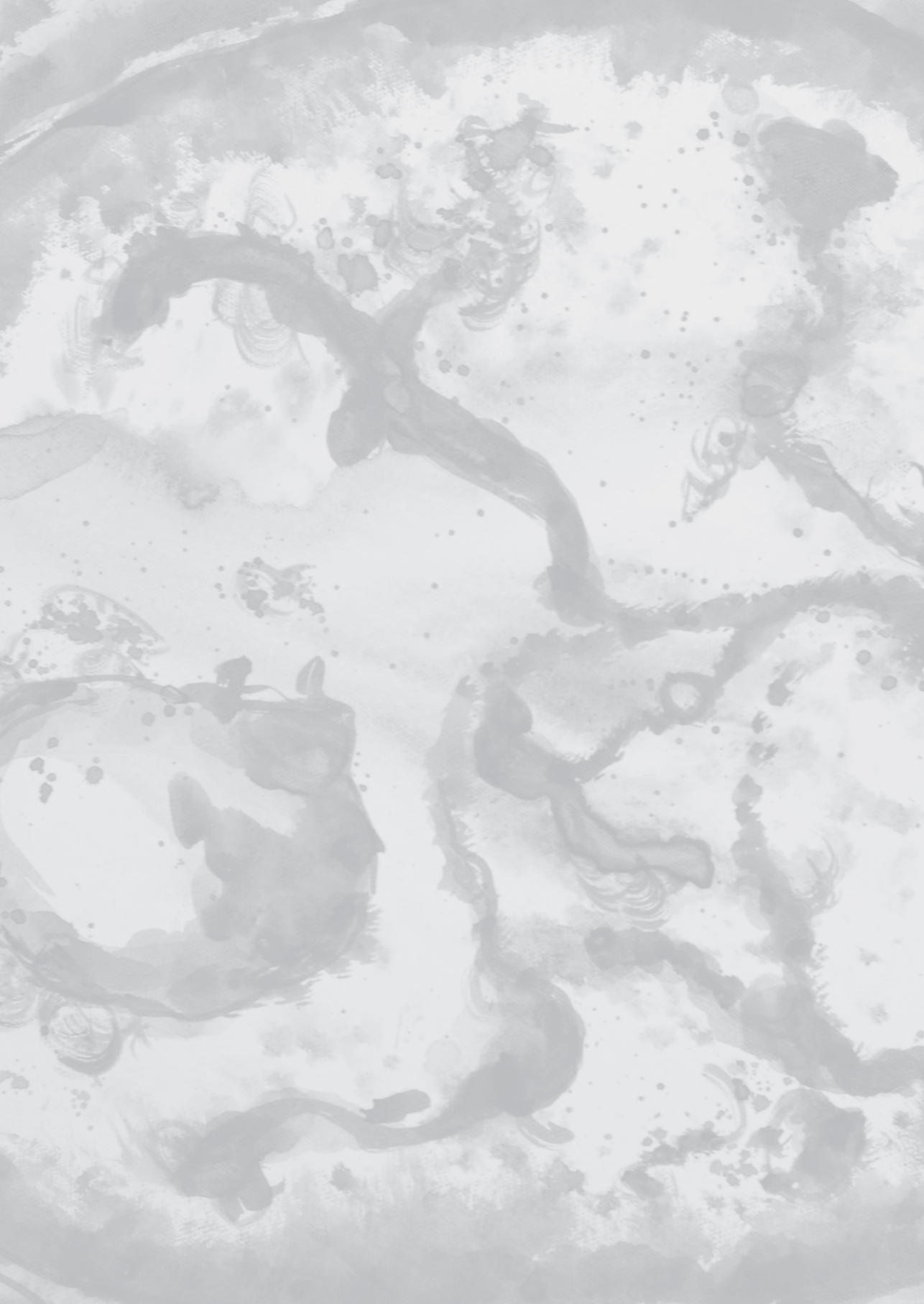

Histologically proven diabetic nephropathy is associated with a leucine repeat of the *CNDP1* gene

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ABSTRACT

The 5-5 homozygous *CNDP1* genotype is associated with a reduced risk for diabetic nephropathy (DN) in patients with type 2 diabetes, based on studies relying on clinically diagnosed DN. The present study investigates whether this association can be confirmed in diabetic patients with histologically proven DN.

Renal autopsy tissue specimens of 204 diabetic patients from Leiden University Medical Center were used to determine the glomerular lesions according to the pathological classification of DN. The *CNDP1* genotype was determined from DNA isolated from spleen and/or liver paraffin-embedded material of these cases.

136 samples were included in the study. 63% of the renal tissue specimens had glomerular lesions conform the pathological DN classification. The frequency of 5-5 leucine repeats *CNDP1* was significantly different between patients with and without histologically proven DN (p=0.031). Furthermore, the 5-5 leucine repeats of *CNPD1* gene was associated with the presence of nodular sclerosis (p=0.013).

This study shows that there is an association between the *CNDP1* gene and DN in histologically proven DN, which confirms the findings of previous studies based on patients with a clinical diagnosis of DN. The *CNDP1* gene may play a role in the development of nodular sclerosis. The direct link between the *CNDP1* genotype and the development of DN remains to be elucidated. However, our results indicate that the *CNDP1* genotype could serve as a genetic biomarker to identify diabetic patients with high risk profiles of DN.

INTRODUCTION

Several studies have shown a reduced susceptibility for the development of diabetic nephropathy (DN) in patients with type 2 diabetes of Caucasian origin and homozygosity for the five leucine repeats of the carnosinase (*CNDP1)* gene compared to diabetic patients with six to eight leucine repeats of the *CNDP1* gene [1-3]. In these studies, DN was diagnosed on the basis of clinical findings; none of these studies performed renal biopsies to confirm the diagnosis of DN by histology. The presence of more than five leucine repeats of *CNDP1* has been shown to be associated with higher levels and activity of serum carnosinase [3, 4]. Serum carnosinase degrades carnosine and other histidine-containing dipeptides. Carnosine has multiple beneficial properties: it is a reactive oxygen scavenger [5], a natural angiotensin converting enzyme (ACE) inhibitor [6], it degrades advanced glycation end products (AGEs) [7], it reduces the synthesis of extracellular matrix components, and it reduces transforming growth factor (TGF)-β in renal cells [1]. These factors have in common that they are disturbed in DN. Therefore, Freedman *et al.* [2] suggested that carnosine and the *CNDP1* gene play a role in the susceptibility to DN in type 2 diabetes.

Glomerular lesions attributable to DN can be classified into four groups according to the pathological classification proposed by Tervaert *et al.* [8]. The exact pathogenesis of these diabetic glomerular lesions remains incompletely understood, especially the development of nodular sclerosis [9], which is scored as Class III in the pathological classification. It has been reported that nodular sclerosis may have a different pathogenesis from more widespread mesangial expansion [9]. However, little is known about the development and variable structures of these nodules [10, 11]. Schwartz *et al.* [11] questioned why some patients with DN develop nodules whereas others do not, especially given that their clinical manifestations are undistinguishable. It might be that a genetic determinant influences the development of nodular sclerosis. Makino *et al.* [12] showed that AGEs, which can be degraded by carnosine, play a role in the development of nodules through impairment of assembly of matrix proteins.

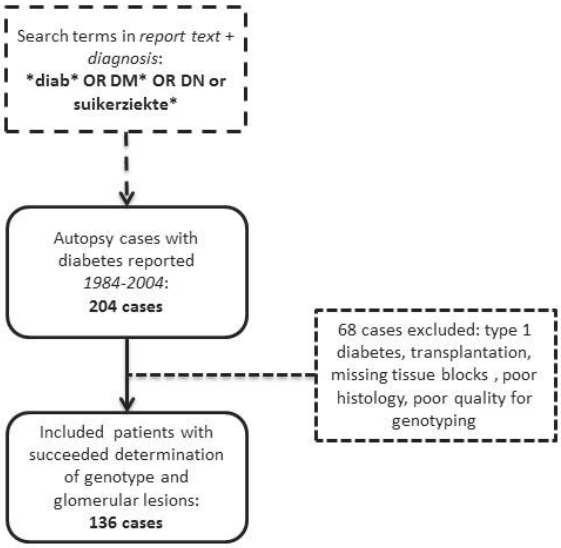
The aim of the current study is to investigate the association between *CNDP1* polymorphism and DN in type 2 diabetes patients with histologically proven DN. In addition, we investigate whether there is an association between *CNDP1* polymorphism and the occurrence of nodular sclerosis.

METHODS

Study design and methods

Patients with diabetes were included retrospectively after a search in the database of the pathology archives from autopsies performed at Leiden University Medical Center between 1984 and 2004. The primary inclusion criteria were the presence of type 2 diabetes in patients who were older than 18 years at the time of autopsy and who had not had a renal transplantation. All samples were handled according to the code of conduct of responsible use. Cases from who the tissue blocks were missing or with poor renal histology due to autolysis were excluded from the study (Figure 1). We collected the paraffin-embedded renal tissue blocks for histological evaluation of the glomerular lesions. Paraffin-embedded tissue blocks from spleen and/or liver were collected for DNA isolation to perform *CNDP1* genotyping of the included patients.

Figure 1. Flowchart of search strategy



The search terms were entered in the database of the pathology archives. The inclusion of patients was based on two subsequent searches to increase the power of the study population

Renal tissue evaluation

Renal tissue was cut at 1- and 3-µm thickness and stained with haematoxylin and eosin, periodic acid–Schiff, and silver stain (3-µm, 3- µm and 1-µm thick sections respectively). Glomerular lesions were scored in renal tissue specimens from autopsy cases containing 100 or more glomeruli by two investigators who were blinded with respect to the genetic profile and clinical data of the patients. The glomerular changes were classified

according to the pathological classification of DN [8]. First, renal tissue specimens were observed by light microscopy. When no light microscopic changes were observed electron microscopic evaluation was performed to measure the glomerular basement membrane (GBM) thickness. Class I, based on electron microscopic measurements, is characterized by thickening of the GBM (GBM width >395nm in females and >430 nm in males). since reprocessing of paraffin tissue for electron microscopy causes artefactual GBM thinning, a correction adding 34 % was used, as described by Nasr *et al*. [13] who showed that this correction is needed to correct for the GBM width in paraffin embedded-material of DN. Taking this calculation into account, the cut off levels described in the histopathogical classification of Tervaert *et al.* [8] could then be used in our study. The remaining classes were evaluated by light microscopy; Class II is characterized by mesangial expansion, subdivided into Class IIa with mild mesangial expansion in >25% of the observed mesangium) and Class IIb with severe mesangial expansion (severe mesangial expansion in >25% of the observed mesangium; Class III is characterized by nodular sclerosis and Class IV by more than 50% global glomerulosclerosis.

CNDP1 genotyping

To determine the CNDP1 genotype, DNA was extracted from paraffin-embedded spleen and/or liver tissue with the automated TPS DNA extraction system (Siemens). A standard PCR protocol was performed with a 5'FAM-labelled forward primer (GC-GGGGAGGGTGAGGAGAAC) and a standard reverse primer (CCCTTCCAGGCTGCGTCC), as described elsewhere [1]. The denaturating, annealing and extension temperatures were 95°C, 60°C, and 72°C, respectively. Fragment analysis was performed on the ABI-3130 analyzer (Perkin Elmer) to determine the number of leucine repeats on each allele. Genemapper® Software was used to determine the CNDP1 polymorphisms and the results were independently analyzed by two investigators.

Statistical analysis

IBM SPSS Statistics, version 20.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses. The 5-5 homozygous *CNDP1* genotype was compared with six or more leucine repeats (5-6, 5-7, 6-6, 6-7, 7-7) of *CNDP1* genotype [3] in patients with and without histologically proven DN. Statistical differences between groups were analyzed using chi-square tests. Difference with a *p*-value less than 0.05 was considered statistically significant.

RESULTS

In this study 136 autopsy cases with type 2 diabetes were included. The baseline characteristics of the included patients are described in Table 1. The mean age was 71 years and 53% of the patients were male. The duration of diabetes was known from 67 patients and the mean duration was 11 years.

Table 1. Baseline characteristics of the included patients

Baseline characteristics (N=136)	
Gender (% male)	53
Age (years)	71 ± 10.6
Diabetes duration (N=67)	11 ± 9
5-5 leucine repeats *CNDP1* gene (%)	38

Data are expressed as mean ± standard deviation, except where indicated otherwise. Diabetes duration expressed in years

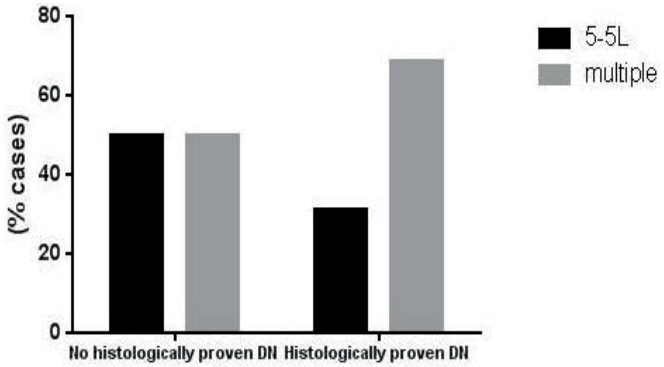
The histological evaluation of the glomeruli revealed that 63% (86/136) of the cases had histological changes according to the pathological classification of DN. In 50 renal tissue specimens no glomerular lesions were observed by light microscopy or electron microscopy and were designated as Class 0. Electron microscopy revealed Class I DN in 18 cases, characterized by thickening of the GBM. Light microscopic evaluation revealed that 26 samples had a class II DN, characterized by mesangial expansion; 16 cases had mild mesangial expansion, designated as Class IIa DN and 10 cases had severe mesangial expansion, designated as Class IIb DN. 36 samples had a class III DN, characterized by nodular sclerosis. The remaining 6 cases had a class IV DN, characterized by more than 50% of glomeruli with global sclerosis. Table 2 presents the distribution of histopathological glomerular lesions of DN between the 5-5 homozygous *CNDP1* and the *CNDP1* genotype with multiple leucine repeats. The frequency of 5-5 leucine repeats in the *CNDP1* gene was significantly lower in patients with histologically proven DN compared to patients without histologically proven DN (p=0.031) (Figure 2). However, no significant difference in the frequency of 5-5 leucine repeats of *CNDP1* was found between the different DN classes (p=0.148).

Table 2. *CNDP1* genotype distribution in histopathological lesions of DN

CNDP1 genotype distribution in histopathological lesions (N=136)			
	5-5 leucine repeats	Multiple leucine repeats	Total
Class 0	25 (50%)	25 (50%)	50 (100%)
Class I	8 (44.4%)	10 (66.6%)	18 (100%)
Class IIa	7 (43.8%)	9 (56.2%)	16 (100%)
Class IIb	3 (30%)	7 (70%)	10 (100%)
Class III	6 (16.7%)	30 (83.3%)	36 (100%)
Class IV	3 (50%)	3 (50%)	6 (100%)
Total	52 (38.2%)	84 (61.8%)	136 (100%)

Data show number of patients and percentages of *CNDP1* distribution per DN class

Figure 2. Distribution of *CNDP1* gene (5-5 leucine repeats and multiple leucine repeats) between histologically proven DN (p=0.031)



We also investigated whether *CNDP1* genotype was associated with the presence of nodular sclerosis (class III DN). Cases with nodular sclerosis (36 cases) were compared to all other cases with histologically proven DN, i.e. Class I, IIa, IIb and IV DN (50 cases), resulting in a significant difference in the presences of nodular sclerosis between cases with 5-5 homozygous *CNDP1* gene and multiple leucine repeats of the *CNDP1* gene (p=0.013). This indicates that cases with homozygosity for the 5-5 leucine repeat of the *CNDP1* gene are less susceptible to develop nodular sclerosis.

DISCUSSION

This study shows that homozygosity for the 5-5 leucine repeat of the *CNDP1* gene is associated with less susceptibility in developing DN in patients with histologically proven DN. This study, relying on histological findings, supports the results of previous studies

in which this genetic association with DN was found in patients with a clinical diagnosis of DN. Since we recently showed that not all cases with histologically proven DN have clinical manifestations [14], it is useful to determine if this genetic association can be found in histological proven DN. We also found an association between the *CNDP1* gene and the occurrence of nodular sclerosis, indicating that this gene may be involved in the development of nodular sclerosis in DN.

The direct effect of the *CNDP1* gene on the pathogenesis of DN remains to be elucidated. It has been suggested that homozygous 5-5 leucine repeats of the *CNDP1* gene result in a lower amount of serum carnosinase in patients, and thereby higher amounts of carnosine [3, 4]. Carnosine has several properties which are affected by diabetes, such as scavenging of reactive oxygen species [15-17], degradation of advanced glycation end-products (AGE) [18], inhibition of mesangial cell proliferation [19], inhibition in both podocytes and mesangial cells of TGF-β–mediated transcription of extracellular matrix proteins [20]. The ability of carnosine to moderate in the protein glycation and the inhibition of AGE formation together with powerful antiglycative and antioxidative effects [21] could indicate that high amounts of carnosine may protect the kidneys against oxidative damage, which plays a central role in the pathogenesis of DN [22].

Regarding nodular sclerosis, it is reported that AGEs also play a role in their development [23]. However, little is known about the development of these nodules and it is suggested that the development of these nodules results from another pathway than mesangial expansion [9, 11]. Although in this study nodular sclerosis occurred in a substantial amount of patients with homozygous 5-5 leucine repeats of *CNDP1* gene, the significant difference between patients with homozygous 5-5 leucine *CNDP1* and patients with multiple leucine repeats *CNDP1* suggests that the *CNDP1* gene may be involved in the development of nodules in diabetic nephropathy. Therefore, this genetic risk factor may help to solve the question of Schwartz *et al.* [11] why certain patients develop nodular sclerosis even when they are clinically undistinguishable from patients with mesangial expansion. More studies are needed to investigate the relationship between the *CNDP1* gene and nodular sclerosis. These additional studies might give insight in the pathogenesis of nodular sclerosis, on the differences in structure and the amount of nodules in patients with different clinical and/or other histological parameters, and could clarify if the *CNDP1* genotype is involved in the development of nodular sclerosis.

In literature, genetic associations in complex diseases such as DN have been plagued by inconsistencies [24]. The lack of reproducibility can often be ascribed to small sample sizes and false positive results [25]. The association with *CNDP1* gene and DN

was first reported in a large cohort that was not treated for the disease, and repeatedly confirmed in other cohorts of patients with type 2 diabetes of Caucasian and Asian ethnicities [2, 3]. The results were also supported by a meta-analysis in Caucasians with type 2 diabetes [26], indicating that the association with *CNDP1* and DN is reproducible.

In this autopsy study we were able to determine histological glomerular lesions attributable to DN in a large amount of glomeruli (>100 per case), although the study had some limitations that merit discussion. First, paraffin-embedded material was used to determine the *CNDP1* polymorphism and glomerular damage. Therefore, some cases needed to be excluded as the material was not sufficient for evaluation due to poor quality. Second, we focused only on the glomerular changes of DN but we did not investigate whether other histological parameters such as interstitial and/or vascular lesions are associated with the *CNDP1* genotype.

In conclusion, this study reports an association between histologically proven DN and the *CNDP1* gene, thereby confirming the results of previous genetic association studies of *CNDP1* and DN which were based on the clinical diagnosis of DN. Large-scale epidemiological studies underscore the need for more extensive characterization of kidney disease in individuals with diabetes to determine a more specific phenotype profile for DN [27]. Additionally, it might be that genetic biomarkers are beneficial to identify individuals risk profiles for DN before it becomes clinically apparent [27]. The direct link between *CNDP1* gene and DN, and nodular sclerosis still needs to be assessed, but our results indicate that the *CNDP1* gene may serve as a potential genetic biomarker to determine diabetic patients with high risk profiles to develop DN. Finally, intervention studies with carnosine supplementation could reveal whether *CNDP1* is of therapeutic value in patients with DN.

DISCLOSURE

All the authors declared no competing interests.

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