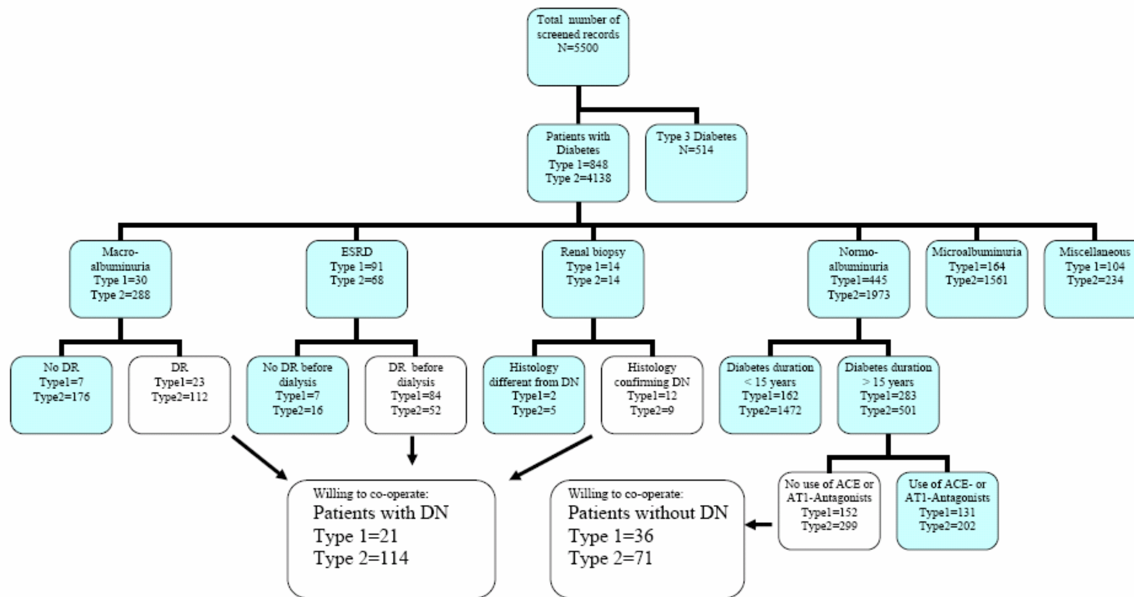
Supplementary material for the manuscript:

# Carnosine as a protective factor in diabetic nephropathy:

# Association with a leucine repeat of the carnosinase gene *CNDP1*.




The flowchart shows the decision making during the screening process implementing the in- and exclusion criteria. It also shows the number of patients excluded for one or another reason. Approximate percentages can be found in the text. Patients who fulfilled the inclusion criteria (i.e. patients in the small white boxes) and did not take part in the study were not willing to cooperate. ESRD= end-stage renal disease; DR= diabetic retinopathy; DN= diabetic nephropathy.

Addendum Methods:

*Selection of patients:*

The number of patients with gestational diabetes is higher in our population than in the general population, because records are not eliminated from the archive after termination of pregnancy and because we screened specialised diabetology centers, where gestational diabetes is overrepresented.

Cases (i.e. patients with DN) fulfilled the following criteria: macroalbuminuria (albumin excretion rate > 300 mg/d) necessarily in combination with diabetic retinopathy, this combination was chosen to exclude cases with proteinuria due to renal disease other than DN. Patients without diabetic retinopathy included had to have a renal biopsy with a histological diagnosis of DN. Exclusion criteria were urinary tract infection or fever at the time of urine investigation and renal disease other than DN. Also, diabetic patients already on dialysis or transplanted had to have diabetic retinopathy at the onset of renal disease or a renal biopsy confirming diabetic nephropathy.

Criteria used for inclusion of controls (i.e. patients without DN) were a diabetes duration of at least 15 years and normoalbuminuria (albumin excretion rate < 30 mg/d) without use of ACE-Inhibitors, AT1-Antagonists or NSAIDs. Patients with microalbuminuria were not included in the study.

Patients and controls were matched by centres.

In a first phase, in order to be able to obtain haplotype information instead of single genotypes, ‘trios’, consisting of the patient and partner and a child of the

couple, were recruited. In these trios, it was possible to determine which alleles on chromosome 18q22.3-q23, were inherited from the same parent, and which alleles were in linkage disequilibrium. The trio-patients were designated ‘group 1’.

Single type 2 and type 1 diabetic patients (designated ‘group 2’ and ‘3’ respectively), were recruited according to the criteria mentioned above in the next phase to be able to perform confirmatory testing of the sequence variant selected in the first phase.

The following data were obtained from each patient: age, gender, retinopathy status, albumin excretion rate, hypertension, onset of diabetes, onset of DN, diabetes duration at inclusion, ethnicity. 15 ml of blood from patients and from two relatives for group 1 was obtained from a cubital vein. All patients and relatives gave written informed consent before data and blood-sample collection. Overall, the high number of patients screened for the study was largely due to the difficulty retrieving patients without diabetic nephropathy with a diabetes duration of more than 15 years.

The medical ethical boards of all participating centres had reviewed the protocols before the start of sample and data collection.

*Indirect immunofluorescence (IIF)*

To detect changes in the extracellular matrix accumulation, podocytes were cultured for 14 days at non-permissive conditions (37°C) in the presence or absence of 20mM L-Carnosine under normo- and hyperglycaemic conditions. 25mM L-Glucose was used as osmotic control. Afterwards, indirect immunofluorescence stainings were performed. To this end, the cells were extensively washed with ice cold PBS and subsequently fixed in methanol. The cells were incubated with a mouse monoclonal antibody, recognizing all fibronectin splice variants (1:500, IgG1, Santa Cruz, Heidelberg, Germany), and an anti-fibronectin antibody, that binds to a TGFβ-dependent side in the ED-A domain of human cellular fibronectin exclusively (1:200, mouse monoclonal IgG1, Cambridge, United Kingdom). To detect additional changes in extra-cellular matrix , we also used antibody against Collagen VI (1:200, rabbit polyclonal, Acris Antibodies, (Hiddenhausen, Germany) . Incubation with the primary antibodies was performed overnight at 4°C. After three washes with ice cold PBS, the appropriate secondary antibody conjugated to FITC (all from Dako, Glostrup, Denmark) was added as recommended by the manufacturer. All dilutions were made in PBS/BSA (1% w/v). All slides were photographed, coded and independently evaluated by three persons.

*ELISA*

Human mesangial cells were cultured for 10 days with or without 20mM L-Carnosine under normo- (5.5mM D-glucose) and hyperglycaemic (25mM D-glucose) conditions. 25mM L-glucose was used as osmotic control. To detect differences in the production of TGFβ1 and TGFβ2 in the culture supernatants, ELISA technique was applied according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

*RNA isolation and real-time PCR on human glomeruli*

Real-time-PCR was used to determine *CNDP1* expression in human glomeruli from normal healthy patients or from patients with DN. Kidneys were obtained from Eurotransplant (n=7). Pathologic criteria for DN include glomerular hypertrophy, diffuse mesangial and focal nodular glomerulosclerosis, arteriolar hyalinosis, and focal and segmental glomerulosclerosis, hyaline drops between Bowman's capsule and epithelial cells, and interstitial fibrosis. Isolation of glomeruli, RNA extraction and real-time PCR were described earlier [17,18]. Glomeruli were isolated using differential sieving technique and RNA was isolated with Trizol® according to the instruction of the manufacturer. Quantitative real-time PCR was used to quantify the gene transcription level for *CNDP1* (Forward primer: TTCAATCCGTCTAGTCCCTCACATG; Reverse primer: TGCAATCCACG GGTGTAGTCC). Each PCR reaction consisted of the following components: 300 pmol primers, 1 µl SYBrgreen®, 0.25 mM dNTPs, 5 mM $MgCl_2$, 0.5 U of Hot Gold Star polymerase, 1 x real-time PCR buffer (Eurogentec). The transcription levels for CNPD1 were determined and related to a panel of three housekeeping genes, i.e., glyceraldehyde-phosphate-dehydrogenase, hypoxanthine phosphoribosyl transferase, and TATA-binding protein [17].

*CNDP1-Antibodies*

According to Teufel et al., two synthetic peptides containing an N-terminal cystein and *CNDP1* residues 256–272 (peptide 256C17E272) and 312–329 (peptide 312Y18K329) were used to raise polyclonal rabbit antibodies (C17E andY18K) at Eurogentech (Seraing, Belgium). Purified antibodies were stored at -20 °C in 50% glycerol at a final concentration of 0.5–1 mg/ml.

*Immunohistochemistry*

Sections from human kidneys (7 from diabetic patients (DM 2) with nephropathy, 3 pre-transplantation biopsies and 2 from patients after undergoing tumour related nephrectomy) were deparaffinised and processed using the microwave antigen retrieval approach in target unmasking fluid buffer (Pharmingen) according to the manufacturer's protocol. The sections were pre-treated with 10% of normal goat serum in phosphate-buffered saline and then incubated with primary antibodies diluted in phosphate-buffered saline/2% normal goat serum o/n at 4 °C. Antibody dilutions were 1:1000 for anti-C17E and 1:500 for anti-Y18K. Specific staining was detected with a commercial kit (ABC; Vector Laboratories) according to the manufacturer's instructions. In control experiments, primary antibodies were pre-adsorbed with the corresponding *CNDP1* peptide or primary antibodies were omitted and replaced by phosphate-buffered saline/2% normal goat serum.

# Statistics for Figure 3

## **Kruskal-Wallis test**

Variables: $5^0$, $5^1$, $5^{10}$, $5^{20}$
(5,5 mM D-glucose +/- l-carnosine)

Groups = 4

df = 3

total observations = 12

T = 10,167
P = 0,0172

Adjusted for ties:

T = 10,202
P = 0,0169

At least one of the sample populations tends to yield larger observations than at least one other sample population.

Variables: $25^0$, $25^1$, $25^{10}$, $25^{20}$
(25,5 mM D-glucose +/- l-carnosine)

T = 8,744
P = 0,0329

Adjusted for ties:

T = 8,836
P = 0,0315

A least one of the sample populations tend to yield larger observations than at least one other sample population.

## **Kruskal-Wallis: all pairwise comparisons (Conover-Inman)**

Critical t (8 df) = 2,306

| Comparison | | Result | |
|---|---|---|---|
| $5^0$ and $5^1$ | (3,167 > 2,191) | significant | P = 0,0103 |
| $5^0$ and $5^{10}$ | (5,833 > 2,191) | significant | P = 0,0003 |
| $5^0$ and $5^{20}$ | (9 > 2,191) | significant | P < 0,0001 |
| $5^1$ and $5^{10}$ | (2,667 > 2,191) | significant | P = 0,023 |
| $5^1$ and $5^{20}$ | (5,833 > 2,191) | significant | P = 0,0003 |
| $5^{10}$ and $5^{20}$ | (3,167 > 2,191) | significant | P = 0,0103 |

| Comparison | | Result | |
|---|---|---|---|
| D0 and D1 | (4,667 > 3,605) | significant | P = 0,0175 |
| D0 and D10 | (2,333 > 3,605) | not significant | P = 0,1739 |
| D0 and D20 | (8,333 > 3,605) | significant | P = 0,0007 |
| D1 and D10 | (2,333 > 3,605) | not significant | P = 0,1739 |
| D1 and D20 | (3,667 > 3,605) | significant | P = 0,047 |
| D10 and D20 | (6 > 3,605) | significant | P = 0,005 |