Common variants in CNDP1 and CNDP2, and risk of nephropathy in type 2 diabetes.

Ahluwalia, Tarun; Lindholm, Eero; Groop, Leif

Published in:
Diabetologia

DOI:
10.1007/s00125-011-2178-5

2011

Common variants in the *CNDP1* and *CNDP2* genes and risk of nephropathy in type 2 diabetes

T. S. Ahluwalia[1], E. Lindholm[1], L. C. Groop[1]

[1]Department of Clinical Sciences-Diabetes & Endocrinology and Lund University Diabetes Centre, Lund University, University Hospital Skane, Malmö, Sweden.

Corresponding author:

Tarunveer Singh Ahluwalia, PhD

Department of Clinical Sciences- Diabetes and Endocrinology,

Lund University Diabetes Centre,

Entrance 72, Building 91, Plan 12,

Clinical Research Centre (CRC),

University Hospital Skane (UMAS),

20502, Malmo, Sweden.

Email: Tarun.Ahluwalia@med.lu.se

Phone: +46-40-391211

Fax: +46-40-391222

Aims/Hypothesis: Several genome-wide linkage studies have shown association of diabetic nephropathy (DN) with a locus on chromosome 18q harboring two carnosinase genes, *CDNP1* and *CDNP2*. Carnosinase degrades carnosine (β-alanyl-L-histidine) which has been ascribed renal protective effects as a scavenger of reactive oxygen species. We aimed to investigate the putative association of genetic variants in the *CNDP1* and *CNDP2* genes with DN (defined either as micro or macroalbuminuria) and glomerular filtration rate (eGFR) in type 2 diabetic patients from Sweden.

Methods: We genotyped nine single nucleotide polymorphisms (SNPs) and one trinucleotide repeat polymorphism D18S880 (5-7 Leucine repeats) in the *CNDP1* and *CNDP2* genes in a case control set-up including 4,888 unrelated type 2 diabetic individuals (with and without nephropathy) from Sweden (Scania Diabetes Registry).

Results: Two SNPs rs2346061 in *CNDP1* and rs7577 in *CNDP2* were associated with increased risk of DN (rs2346061, p: 5.07 x 10^{-4}; rs7577, p: 0.021) and the latter with eGFR (β: -0.037, p: 0.014), particularly in women. A haplotype including these SNPs (C-C-G) was associated with a 3-fold increased risk of DN (OR: 2.98, 95%CI: 2.43-3.67, p<0.0001).

Conclusions/Interpretation: In conclusion, these data suggest that common variants in the *CNDP1* and *CNDP2* genes play a role in susceptibility to kidney disease in type 2 diabetes.

Keywords: Carnosinase, CNDP, diabetic nephropathy, type 2 diabetic nephropathy, renal function, glomerular filtration rate, genetic polymorphisms, CNDP1, CNDP2, Carnosine, haplotype.

Abbreviations: CNDP: Carnosine dipeptidase, DN: diabetic nephropathy, ESRD: end stage renal disease, eGFR: estimated glomerular filtration rate, SDR: Scania diabetes registry, SNP: single nucleotide polymorphism, ACR: Albumin creatinine ratio, AGEs: advanced glycation end products, UACR: urinary albumin creatinine ratio, LD: Linkage disequilibria. OR: odds ratio, CI: confidence interval

Introduction

Diabetic nephropathy (DN) is one of the most severe complications of type 1 and type 2 diabetes mellitus and the leading cause of end-stage renal disease (ESRD), and renal replacement therapy [1, 2]. DN seems to result from a complex interaction between genetic susceptibility and the diabetic environment characterized by poor glycemic control and hypertension [3-6].

About 35% of type 2 diabetes patients develop DN [6]. Familial clustering has been observed in DN, supporting the presence of a genetic component [4, 7]. Increased urinary albumin excretion is a hallmark of DN and the heritability for albuminuria has been estimated to be about 0.4 [7, 8]. Recent genome wide linkage scans in different ethnic groups have shown association of DN to a locus on chromosome 18q [9-11] which also harbours two carnosine dipeptidase genes, *CDNP1* and *CDNP2*.

The *CNDP1* and *CNDP2* genes lie adjacent on chromosome 18q; *CNDP1* encodes a dipeptidase that hydrolyzes the substrate L-carnosine (*β*-alanyl-L-histidine) specifically, while *CNDP2* encodes a nonspecific dipeptidase [12]. Carnosine has been ascribed a protective role in DN by serving as a scavenger of oxygen radicals which can inhibit formation of advanced glycation end products (AGEs) [13, 14].

Jansen *et al.* also provided the first evidence for an association between a tri-nucleotide repeat variant (D18S880: 5-7 leucine repeats) in the signal peptide of exon 2 of the *CNDP1* gene and DN in type 2 diabetes patients of European and Arab ancestry [15]. This finding was subsequently replicated in type 2 diabetes patients with DN-ESRD of European origin in USA [16]. In a follow-up study in African Americans they identified 2 SNPs in the *CDNP1* and *CNDP2* genes being associated with type 2 diabetes and ESRD [17]. However, several studies have not been able to replicate these findings [18, 19], nor has any association been shown between the CNDP locus and DN in type 1 diabetes [20-22]. The 5L-5L genotype of the D18S880 marker has been reported to be associated with low serum carnosinase (CNDP) concentrations in diabetics [15] making the CNDP genes interesting candidates for studying DN susceptibility. Also, in support of a role for *CDNP1* and *CNDP2* in DN there is differential expression of these genes in kidneys of diabetic animals [23].

Against this background of discrepant results this study was designed to explore whether there is an association between SNPs (including the repeat variant) in the *CNDP* locus and DN in a large well characterized population of patients with type 2 diabetes from Southern Sweden (Scania Diabetes Registry, SDR).

Methods

Study Population: The Scania Diabetes Register (SDR)

All patients were from the Scania Diabetes Registry (SDR) in Southern Sweden (Table 1). At the time of investigation, the registry included 1264 type 1 diabetes and 5123 type 2 diabetes patients with mean disease duration of 14 years (Table 1). The registry contains information on age at onset of diabetes, mode of treatment, time for starting insulin therapy as well as follow-up data on change in BMI, HbA_{1c}, lipids, blood pressure and development of diabetic complications.

Inclusion criteria in the present study: Scandinavian origin, age at onset of diabetes >35 years, diabetes duration of ≥ 10 years, C-peptide ≥ 0.3 nmol/l, and GAD antibody negativity. Diabetes diagnosis was based upon WHO criteria with a fasting plasma glucose (FPG) ≥ 7.0 mmol/l. In total 4,888 type 2 diabetes patients were included in the study. Diabetic patients with other kidney disease (n=35) were excluded

Albumin excretion rate (AER) was measured from timed overnight urine collections or as albumin/creatinine ratio (ACR) in morning spot urine on at least two out of three occasions with 6 month intervals during follow up; albumin concentration in urine was determined by immunonephelometry (Beckman Instruments, CA, USA) until 1998 and thereafter by an immunoturbimetric method (Beckman Coulter, Beckman Instruments, CA, USA).

DN was subdivided into incipient (microalbuminuria) and manifest (macroalbuminuria) DN. Microalbuminuria was defined as a) AER of 20–200 μg/min in at least two timed overnight urine samples or, b) an albumine:creatinine ratio of 2.0–25 g/mol in males and 3.5–35 g/mol in females. Values above the upper limit of the definition for microalbuminuria were indicative of macroalbuminuria. Based upon this definition 880 type 2 diabetes patients were considered to have DN in the SDR (Table 1).

Renal function was estimated from serum creatinine concentrations and expressed as estimated glomerular filtration rate (eGFR). The formula for GFR (Cockroft and Gault) was (mL/min per 1.73 m2) = [(140-age in years) x weight in kg x 1.73]/(plasma creatinine in μmol/L x F x BSA), where F= 0.8 if male and F= 0.85 if female [24].

All patients gave their written informed consent and the local ethics committees approved the study.

Selection of SNPs and haplotyping

Nine SNPs and one microsatellite marker D18S880, in the *CNDP1* and *CNDP2* genes (chromosome 18 position: 70321335 to 70395500 bps) with a minor allele frequency (MAF) > 0.05 in Europeans were selected from the dbSNP (http://www.ncbi.nlm.nih.gov/snp/) and HapMap (http://www.hapmap.org/) data bases. These SNPs capture 50% of variants (36 neighbouring SNPs) with a MAF > 5% at $r^2 \geq 0.8$ from the *CNDP1* gene region (Supplementary table 3; Hapmap 1000 genomes version) [25].

Genotyping

Nine SNPs (7 in *CDNP1* and 2 in *CNDP2*) were genotyped, in 4888 patients from the SDR by an allelic discrimination method on the ABI 7900 platform (TaqMan assay, Applied Biosystems, Foster City, CA); We obtained an average genotyping success rate of 98% and a 99.9% concordance rate, based on 780 duplicate comparisons using Taq Man assays.

Genotyping for the D18S880 microsatellite marker was performed by sequence analysis using an ABI 3130xl capillary sequencer (Applied Biosystems, Darmstadt, Germany) in all the study subjects. The primers used were, AGGCAGCTGTGTGAGGTAAC (forward) and GGGTGAGGAGAACATGCC (reverse) where the forward primer was FAM-labeled at the 5' end (Eurofins MWG Operon, Germany) and a PCR product length of 167bp confirmed the presence of 5 CTG repeat units (five leucine codons). Random samples were sequenced later to confirm a genotyping concordance rate of 99.9%.

Statistical analyses

Data are presented as means ± SD. Non-normally distributed variables (albumin-creatinine ratio (ACR), and eGFR) were logarithmically (natural) transformed for analyses. Variables showing skewed distribution were compared using Mann–Whitney U-test. The risk of developing DN expressed as odds ratio (OR) and 95% confidence intervals (CI) were calculated by logistic regression analyses adjusted for age and sex. Genotype-phenotype correlations were assessed using linear regression analyses adjusted for age, sex, diabetes duration, HbA1c, smoking status, systolic blood pressure and BMI (where appropriate). ANOVA with the Bonferroni test as post hoc test were used to evaluate differences between means.

Deviations from Hardy-Weinberg equilibrium were evaluated with a Pearson's χ^2 goodness-of-fit test. Correction for multiple testing was performed using the QVALUE software package (http://faculty.washington.edu/~jstorey/qvalue/). Haplotypes were reconstructed using the PHASE (2.1v; http://stephenslab.uchicago.edu/software.html) program [26].

All statistical genetic analyses were performed using an additive model (dominant and recessive models were also used) with the Statistical Package for the Social Sciences v.17.0 (SPSS, Chicago, IL), and PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) [27].

Power analysis

The study could detect a genotype RR of 1.15 with a power of at least 70% at $P < 0.05$. To calculate power for SNPs in our study, we used the PS program of Dupont and Plummer (available at http://www.mc.vanderbilt.edu/prevmed/ps/index.htm) [28].

Results

Characteristics of patients

Table 1 compares the clinical characteristics between the 4008 type 2 diabetes patients with normoalbuminuria and the 880 type 2 diabetes patients with DN. While there was no difference in age, the DN patients had longer duration of diabetes than normoalbuminuric type 2 diabetes

patients. The DN patients had lower eGFR, HDL, and cholesterol concentrations but, higher blood pressure, ACR, HbA1c and triglyceride concentrations than normoalbuminuric type 2 diabetes patients. Smoking (51 vs. 36%; p<0.01) and retinopathy (51 vs. 32%; p<0.01) were more common in DN than normoalbuminuric patients.

Association between CNDP SNPs and risk of diabetic nephropathy and eGFR

Of the genotyped SNPs and a microsatellite marker from the *CDNP genes*, rs4891558 (*CNDP2*) was monomorphic (minor allele frequency < 0.05) in this cohort and all the SNPs except one (rs12604675 from *CNDP1*) fulfilled Hardy-Weinberg equilibrium (*P* >0.10). The rs2346061 SNP in the *CNDP1* gene showed a significant association with DN in additive (OR: 1.25, 95%CI: 1.1-1.4, p= 5.07 x 10^{-4}; Table 2 and Figure 2) and alternate models (Table 2) but not with eGFR (Table 3).

The rs7577 SNP in the *CNDP2* gene was nominally associated with DN in additive (OR: 1.15, 95%CI: 1.02-1.3, p=0.021) and recessive models (p=0.016) (Table 2 and Figure 2). The same SNP was also associated with eGFR in all type 2 diabetes subjects with lower eGFR in CC and TC than TT genotype (rs7577) carriers (p=0.014; Table 3) particularly in women. The difference in eGFR between CC and TT/TC genotype carriers (log $eGFR_{women}$: CC: 4.52 ± 0.35, TC: 4.58 ± 0.34, TT: 4.59 ± 0.34, β=0.036, p=0.031; Figure 3) was associated with a higher urinary albumin creatinine ratio (UACR) (log $UACR_{women}$: CC: 2.01 ± 0.55, TC: 1.72 ± 0.54, TT: 1.23 ± 0.63, β=0.11, p=0.0001; Figure 4).

Table 4 shows genotype frequencies for the D18S880 microsatellite marker. As previously reported [17] we identified the 5L, 6L, or 7L leucine repeats, whereas the 4L and 8L repeats were very rare (<0.005%) and not considered for analysis. There were no significant differences in D18S880 genotype frequencies (P=0.17; df=2) between DN and type 2 diabetes patients and this marker was not associated with DN in the SDR cohort (Supplementary table 2).

The other genotyped SNPs (rs7244370, rs7239132, rs12964454, rs12456388, and rs9953129) did not show any significant association with risk of DN or eGFR (Supplementary Table 1 and Supplementary Table 2).

rs7239132 SNP of the *CNDP1* gene was associated with HbA_{1c} levels. The AA genotype carriers had significantly lower HbA_{1c} levels as compared to CA/CC carriers (Table 3).

Haplotype Analysis

The three SNPs having LD values as, D'=0.98 (r^2= 0.75) between rs7577 and rs7244370, D'=0.84 (r^2= 0.24) between rs2346061 and rs7244370, and D'= 0.43 (r^2=0.14) between rs7577and rs2346061, were used for haplotype reconstruction to study the risk associated with different allelic combinations of the common variants. Their frequencies and risk associated with DN have been presented in Table 5.

Haplotype C-C-G (including alleles from rs7577, rs2346061 and rs7244370) was associated with a 3-fold increased risk of DN (Table 5) as well as reduced eGFR (β=-0.039; $p_{adjusted}$=0.011).

Discussion

The key finding of the present study was that the SNP in the 3'UTR of the *CNDP2* gene (rs7577) was associated with increased risk of DN as shown by increased ACR and decreased eGFR, particularly in women. Another SNP in the CNDP1 gene (rs2346061) promoter was associated with DN. However, it did not influence eGFR. A haplotype consisting of these alleles was associated with an increased risk of DN and reduced eGFR.

The SNPs rs2346061 and rs7577are located in the regulatory region of *CNDP1* and *CNDP2* and could thereby modulate carnosinase activity in the same way as reported for another SNP in this region [15]. In previous studies of African American type 2 diabetes patients [17] as well as of European type 1 diabetes patients [22], no association was seen between this SNP and DN. It was recently claimed that the association between DN and *CNDP1* [29], was sex specific and restricted to females. In support of this finding, women with the CC genotype (rs7577) had reduced eGFR as compared to women with the TC/TT genotypes. This finding was further supported by 1.5 times higher albumin-creatinine ratios in women with the CC than in women with TC/TT genotypes. It may not be surprising to find gender specific differences for associations with the CNDP genes as women have been reported to have lower carnosine levels

in muscle than men due higher serum carnosinase levels [30]. Also, in female mice carnosine levels increased > 250% in muscle after testosterone administration [31].

The D18S880 microsatellite marker has been reported to be associated with DN in some [15, 16] but not all [17, 22] studies. Jansen *et al*. reported for the first time that individuals homozygous for the allele with lowest number of leucine repeats (5L) were associated with lower serum carnosinase concentrations, conferring protection from nephropathy [15]. This was further supported by another study showing higher carnosinase concentrations with increasing number of Leucine repeats in COS cells [32]. Since carnosine has been ascribed anti oxidant effects and a potential inhibitor of ACE activity and advanced glycation end products [32], the activity of the enzyme carnosinase may be important in the development of nephropathy.
Our data did not support an association between the leucine repeat and DN in this cohort of Scandinavian patients with type 2 diabetes.

The next question remains whether the promoter SNP (rs2346061) in *CNDP1* really is associated with DN, since there was no effect of the variant on kidney function (eGFR). This SNP was not associated with DN (proteinuria or ESRD) in Caucasian type 1 diabetes patients (11). However, in that study (11) there was a modest association between two other SNPs in the *CNDP1* promoter region (rs12954438, and rs890332) and proteinuria but not ESRD, supporting the view that promoter SNPs in *CNDP1* might influence albuminuria which may not be translated into a progressive deterioration of kidney function. Notably, we excluded patients with ESRD from our study. Also, none of these SNPs are in LD (r^2=0.03; HapMap Version 2 release 24) with rs2346061.
Further support for the view that different SNPs in the *CNDP1* gene influence albuminuria and progression to ESRD comes from a study in African American type 2 diabetes patients which did not find any association between a proxy SNP rs2346061 and ESRD [17].
However, we have no evidence that these are the causal SNPs; functional studies are needed to answer the role of potential functional effects of these SNPs. In lack of such information we also tested whether haplotypes including these two SNPs would confer a stronger effect on DN than the individual SNPs.

The haplotype C-C-G in block 2 (including alleles from rs7577, rs2346061 and rs7244370) was associated with a 3-fold increased risk of DN as well as reduced eGFR. Also another study reported stronger association between haplotypes in the *CNDP2* gene region and ESRD than between individual alleles and ESRD [17].

There are pros and cons of our study. While the well characterized patient groups were large enough to have a power of 75-80% to detect an association between the two key SNPSrs7577 and rs2346061 and DN, they were still underpowered for low frequency SNPs as well as for haplotypes.

In conclusion, we provide evidence for an association between a common SNP rs2346061 in the *CNDP1* gene and DN. As this SNP was not associated with kidney function it is possible that it merely increases risk for albuminuria than for progression of kidney disease as other studies also have failed to demonstrate an association of this SNP with ESRD. The SNP rs7577 in the *CNDP2* gene confers increased risk of nephropathy by altering the kidney function, particularly in women. A 3-allelic haplotype in the regulatory region of the CNDP genes was associated with a 3-fold increased risk of DN and reduced eGFR suggesting that other modifying SNPs are needed to increase risk of progression towards kidney dysfunction.

Acknowledgements:

TA is supported by a grant from the Bo and Kerstin Hjelt Diabetes Foundation (http://www.hjeltfoundations.org/). The study was further supported by grants from the Swedish Research Council, including a Linné grant (2008-6589) and a Strategic Research Grant (Exodiab; 2009-1039), the Heart-Lung Foundation, the Swedish Diabetes Research Foundation, the Knut and Alice Wallenberg Foundation, the Novo Nordisk Foundation, as well as European Community 7th Framework Programme grants (ENGAGE; Health-2007-201413 and an Innovative Medicines Initiative grant (SUMMIT; 2009-115006)). Special thanks to Malin Svensson for her help in genotyping D18S880 polymorphism.

Duality of Interest

The authors declare that there is no duality of interest associated with this manuscript.

References

[1] US Renal Data System, USRDS 2010 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bathedsa, MD, 2010.

[2] ERA-EDTA Registry: ERA-EDTA Registry Annual Report 2008. Academic Medical Center, Department of Medical Informatics, Amsterdam, Netherlands, 2010.

[3] Schmidt S, Ritz E (1997) Genetic determinants of diabetic renal disease and their impact on therapeutic interventions. Kidney Int Suppl 63: S27-31

[4] Conway BR, Maxwell AP (2009) Genetics of diabetic nephropathy: are there clues to the understanding of common kidney diseases? Nephron Clin Pract 112: c213-221

[5] Sallinen R, Kaunisto MA, Forsblom C, et al. Association of the SLC22A1, SLC22A2, and SLC22A3 genes encoding organic cation transporters with diabetic nephropathy and hypertension. Ann Med 42: 296-304

[6] Forbes JM, Fukami K, Cooper ME (2007) Diabetic nephropathy: where hemodynamics meets metabolism. Exp Clin Endocrinol Diabetes 115: 69-84

[7] Freedman BI, Bostrom M, Daeihagh P, Bowden DW (2007) Genetic factors in diabetic nephropathy. Clin J Am Soc Nephrol 2: 1306-1316

[8] Forsblom CM, Kanninen T, Lehtovirta M, Saloranta C, Groop LC (1999) Heritability of albumin excretion rate in families of patients with Type II diabetes. Diabetologia 42: 1359-1366

[9] Iyengar SK, Abboud HE, Goddard KA, et al. (2007) Genome-wide scans for diabetic nephropathy and albuminuria in multiethnic populations: the family investigation of nephropathy and diabetes (FIND). Diabetes 56: 1577-1585

[10] Vardarli I, Baier LJ, Hanson RL, et al. (2002) Gene for susceptibility to diabetic nephropathy in type 2 diabetes maps to 18q22.3-23. Kidney Int 62: 2176-2183

[11] Bowden DW, Colicigno CJ, Langefeld CD, et al. (2004) A genome scan for diabetic nephropathy in African Americans. Kidney Int 66: 1517-1526

[12] Teufel M, Saudek V, Ledig JP, et al. (2003) Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J Biol Chem 278: 6521-6531

[13] Hipkiss AR, Chana H (1998) Carnosine protects proteins against methylglyoxal-mediated modifications. Biochem Biophys Res Commun 248: 28-32

[14] Hipkiss AR, Preston JE, Himsworth DT, et al. (1998) Pluripotent protective effects of carnosine, a naturally occurring dipeptide. Ann N Y Acad Sci 854: 37-53

[15] Janssen B, Hohenadel D, Brinkkoetter P, et al. (2005) Carnosine as a protective factor in diabetic nephropathy: association with a leucine repeat of the carnosinase gene CNDP1. Diabetes 54: 2320-2327

[16] Freedman BI, Hicks PJ, Sale MM, et al. (2007) A leucine repeat in the carnosinase gene CNDP1 is associated with diabetic end-stage renal disease in European Americans. Nephrol Dial Transplant 22: 1131-1135

[17] McDonough CW, Hicks PJ, Lu L, Langefeld CD, Freedman BI, Bowden DW. (2009) The influence of carnosinase gene polymorphisms on diabetic nephropathy risk in African-Americans. Hum Genet 126: 265-275

[18] Kilis-Pstrusinska K, Zwolinska D, Grzeszczak W. (2010) Is carnosinase 1 gene (CNDP1) polymorphism associated with chronic kidney disease progression in children and young adults? results of a family-based study. Arch Med Res 41: 356-362

[19] Kim S, Abboud HE, Pahl MV, et al. (2010) Examination of association with candidate genes for diabetic nephropathy in a Mexican American population. Clin J Am Soc Nephrol 5: 1072-1078

[20] Alkhalaf A, Bakker SJ, Bilo HJ, et al. (2010) 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 mellitus. Diabetologia

[21] Craig DW, Millis MP, DiStefano JK (2009) Genome-wide SNP genotyping study using pooled DNA to identify candidate markers mediating susceptibility to end-stage renal disease attributed to Type 1 diabetes. Diabet Med 26: 1090-1098

[22] Wanic K, Placha G, Dunn J, Smiles A, Warram JH, Krolewski AS (2008) Exclusion of polymorphisms in carnosinase genes (CNDP1 and CNDP2) as a cause of diabetic nephropathy in type 1 diabetes: results of large case-control and follow-up studies. Diabetes 57: 2547-2551

[23] Hu Y, Kaisaki PJ, Argoud K, et al. (2009) Functional annotations of diabetes nephropathy susceptibility loci through analysis of genome-wide renal gene expression in rat models of diabetes mellitus. BMC Med Genomics 2: 41

[24] Farbom P, Wahlstrand B, Almgren P, et al. (2008) Interaction between renal function and microalbuminuria for cardiovascular risk in hypertension: the nordic diltiazem study. Hypertension 52: 115-122

[25] Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI (2008) SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics 24: 2938-2939

[26] Stephens M, Donnelly P (2003) A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am J Hum Genet 73: 1162-1169

[27] Purcell S, Neale B, Todd-Brown K, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81: 559-575

[28] Dupont WD, Plummer WD, Jr. (1998) Power and sample size calculations for studies involving linear regression. Control Clin Trials 19: 589-601

[29] Mooyaart AL, Zutinic A, Bakker SJ, et al. (2010) Association between CNDP1 genotype and diabetic nephropathy is sex specific. Diabetes 59: 1555-1559

[30] Bando K, Shimotsuji T, Toyoshima H, Hayashi C, Miyai K (1984) Fluorometric assay of human serum carnosinase activity in normal children, adults and patients with myopathy. Ann Clin Biochem 21 (Pt 6): 510-514

[31] Penafiel R, Ruzafa C, Monserrat F, Cremades A (2004) Gender-related differences in carnosine, anserine and lysine content of murine skeletal muscle. Amino Acids 26: 53-58

[32] Riedl E, Koeppel H, Brinkkoetter P, et al. (2007) A CTG polymorphism in the CNDP1 gene determines the secretion of serum carnosinase in Cos-7 transfected cells. Diabetes 56: 2410-2413

FIGURES:

Figure 1. Linkage disequilibrium (LD) analysis of nine SNPs in *CNDP2* and *CNDP1*, measured by D'. Dark squares signify high LD values. SNP1, rs4891558; SNP 2, rs7577; SNP 3, rs2346061; SNP 4, rs7244370; SNP 5, rs7239132; SNP 6, rs12604675; SNP 7, rs12964454; SNP 8, rs12456388; SNP 9, rs9953129.

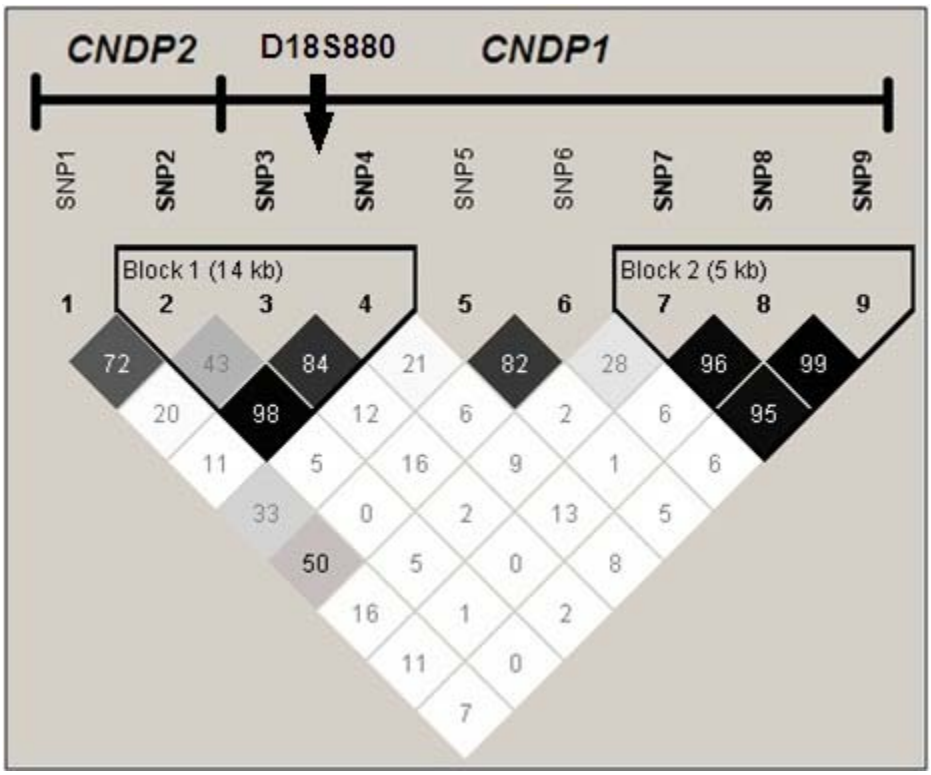


Figure 2. Logistic regression analysis depicting the odds ratios associated with the *CNDP1* and *CNDP2* SNPs. SNP 2, rs7577; SNP 3, rs2346061;D18S880 polymorphism; SNP 4, rs7244370; SNP 5, rs7239132; SNP 7, rs12964454; SNP 8, rs12456388; SNP 9, rs9953129. *P<0.05, **P<0.001. Age, gender, BMI, systolic blood pressure, smoking, duration of diabetes, and HbA1c are included in this model for nephropathy. Error bars depict 95% confidence intervals.

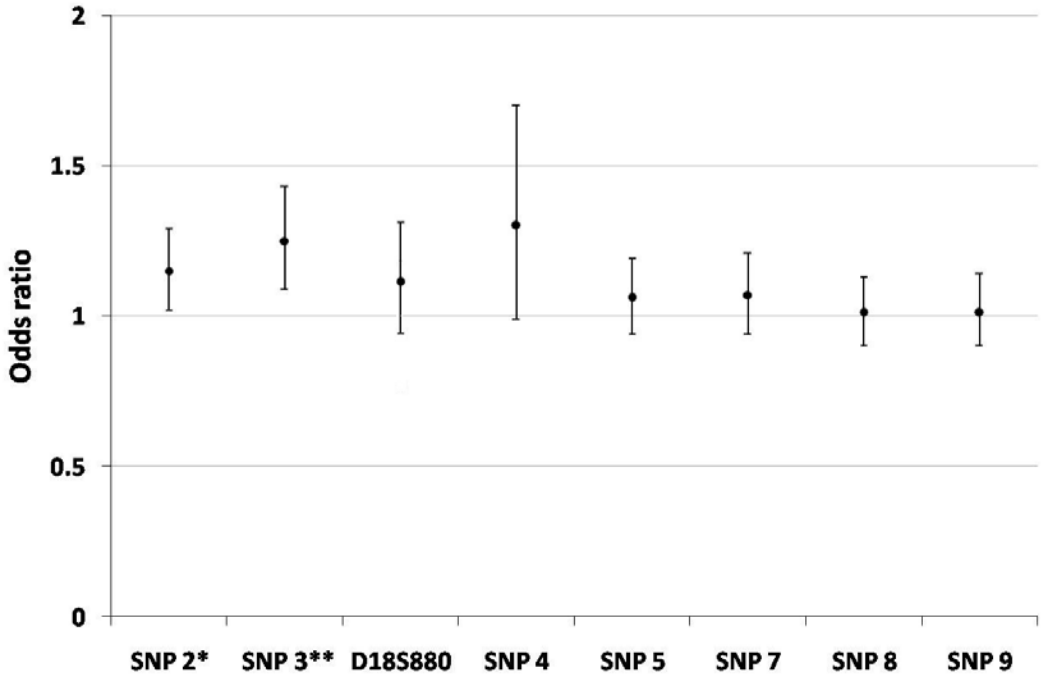


Figure 3. Genotype-based association of *CNDP2* with eGFR. Estimated GFR (ml/min/1.73m^2) was log transformed and expressed as mean ± SD. P value shows the comparison among rs7577 genotype by analysis of variance with Bonferroni test as *post hoc* test. NS; non significant.

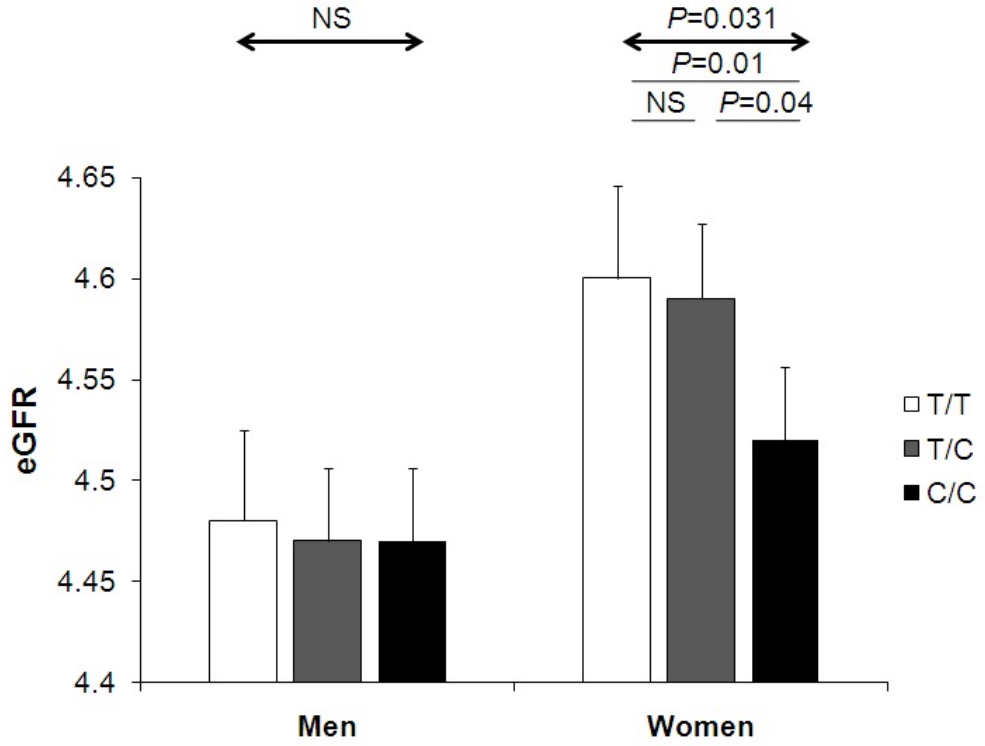
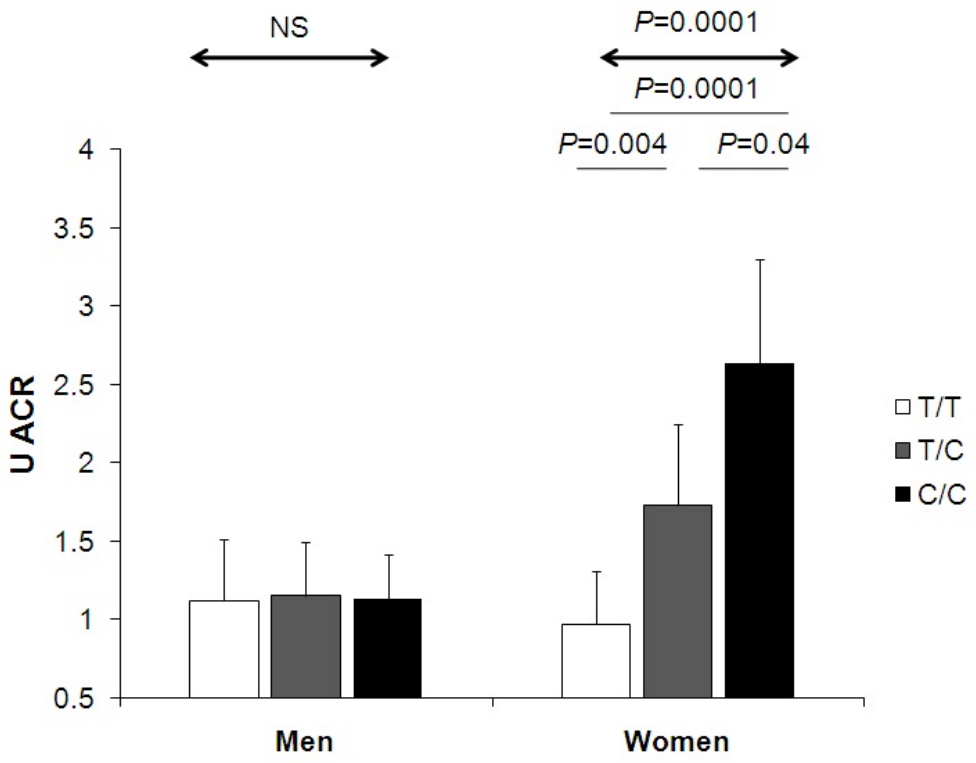


Figure 4. The association between rs7577 genotype and urinary albumin excretion. Urine albumin-creatinine ratio (UACR). UACR values are log transformed. *P*-value shows the comparison among rs7577 genotypes by analysis of variance with Bonferroni test as *post hoc* test. NS: not significant.


NS
P=0.0001
P=0.0001
P=0.004
P=0.04
U ACR
4
3.5
3
2.5
2
1.5
1
0.5
Men
Women
T/T
T/C
C/C

Results

Table 1: Clinical characteristics of DN patients (type 2 diabetes with nephropathy) and type 2 diabetes (without nephropathy) in the Scania diabetes registry (SDR).

	Type 2 diabetes (N=4008)	DN (N=880)
Males (%)	55.4	66.4
Age (years)	54.1 ± 14.3	53.7 ± 11.5
Duration of T2D (years)	13.2 ± 4.4	17.3 ± 6.5*
BMI (Kg/m^2)	28.7 ± 5.	30.1 ± 5.8
SBP (mmHg)	140.9 ± 25.2	147.7 ± 24.3**
DBP (mmHg)	79.4 ±13.5	81.2 ± 12.3
HbA_{1c} (%)	6.8 ± 1.8	7.2 ± 1.6**
S. Creatinine (µmol/L)	83.5 ± 23.0	92.7 ± 26.2**
eGFR (ml/min/1.73m^2)	103.0 ± 34.4	94.2 ± 33.2**
Ln ACR (mg/mmol)	0.26 ± 1.4	2.06 ± 1.5***
HDL (mmol/L)	1.2 ± 0.3	1.1 ± 0.2***
Cholesterol (mmol/L)	5.1 ± 1.0	5.0 ± 0.8*
Triglycerides (mmol/L)	2.1 ± 1.4	2.64 ± 1.9*
Smokers (%)	36.1	51.0**
Retinopathy (%)	32.1	51.4**

Values are Mean ± standard deviation; *P<0.05, **P<0.01 and ***P<0.001 vs. T2D; SBP and DBP: Systolic and diastolic blood pressure; eGFR: estimated glomerular filration rate (baseline); Ln ACR: logarithmic albumin creatinine ratio; Type 2 diabetics (normoalbuminuria); DN: (diabetic nephropathy)

Table 2. Risk of DN predicted by various genotypic models for rs2346061 and rs7577 in the study subjects.

Marker	**Genotypic Association**					
	Dominant		**Additive**		**Recessive**	
	P value	**OR (95%CI)**	**P value**	**OR (95%CI)**	**P value**	**OR (95%CI)**
rs2346061	**2.7E-4**	1.62 (1.24-2.1)	**5.07E-4**	1.25 (1.09-1.43)	**0.0038**	0.8 (0.69-0.93)
rs7577	0.43	1.10 (0.86-1.41)	**0.021**	1.15 (1.02-1.29)	**0.016**	0.83 (0.71-0.96)

Results are shown in all subjects: 880 DN cases, and 4,008 type 2 diabetes (normoalbuminuria) controls;
P values <0.05 are shown in bold; P values for the models have been adjusted for confounding factors including age, gender, BMI, systolic blood pressure, smoking, duration of diabetes, and HbA1c

Table 3: Effects of *CNDP* SNPs on kidney function (eGFR) and HbA_{1c} in the study population.

		rs7577*			Additive model		
	N	TT	TC	CC	β	SE	*P*
eGFR (ml/min/1.73m^2)	4002	101.7 ± 35.5	99.6 ± 34.1	97.3 ± 34.0	-0.037	0.008	0.014
		rs2346061					
	4002	AA	AC	CC	β	SE	*P*
		101.2 ± 35.1	98.7 ± 34.3	100.6 ± 33.0	-0.024	0.009	0.09
HbA_{1c} (%)	3756	**rs7239132***					
		CC	CA	AA	β	SE	*P*
		6.92 ± 1.7	6.90 ± 1.7	6.77 ± 1.6	-0.025	0.008	0.024

Data are Mean± SD, *P<0.05, β and SE from linear regression analysis adjusted for age, sex, BMI, duration of diabetes and denote the effect size of each effect-allele (additive model) on eGFR. eGFR: estimated glomerular filtration rate.

Table 4. The genotype frequencies for D18S880 repeat variant in Scania Diabetes Register. DN= type 2 diabetes with nephropathy (cases); type 2 diabetes without nephropathy (controls); 5L, XL: denotes 5, and X (= 6, or 7) leucine repeats. χ^2 (df=2): 3.59 (p=0.17 for genotype frequencies).

Groups	**Genotype frequencies**		
	5L-5L	**5L-XL**	**XL-XL**
DN (n=820)	0.356	0.401	0.243
Type 2 diabetes (n=4725)	0.387	0.398	0.214

Table 5: Haplotype frequencies of type 2 diabetes patients (with and without nephropathy) and risk associated with DN. The haplotype includes three SNPs (rs7577 (T>C)- rs2346061 (A>C)- rs7244370 (G>T)).

Haplotypes	**Type 2 diabetes (normo)**		**DN**		**OR (95%CI)**	**$P_{unadjusted}$**	**$P_{adjusted}$**
	Frequency	2n=4188	Frequency	2n=1070			
T-A-G	0.46	1922	0.48	522	1.08 (0.95-1.24)	0.21	0.45
C-A-G*	0.20	825	0.16	175	0.78 (0.65-0.93)	0.0065	0.019
C-C-G**	0.061	253	0.16	175	2.98 (2.43-3.67)	<0.0001	<0.0001
T-C-G**	0.16	674	0.093	100	0.52 (0.42-0.65)	<0.0001	<0.0001
T-A-T	0.065	270	0.089	99	1.45 (1.14-1.84)	0.022	0.06

*p<0.05; **p<0.001; N: Number of alleles. $P_{adjusted}$: after multiple testing corrections