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ORIGINAL ARTICLE

# Detection of carnosinase-1 in urine of healthy individuals and patients with type 2 diabetes: correlation with albuminuria and renal function

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## Abstract

Low serum carnosinase (CN-1) concentrations are associated with low risk for development of diabetic nephropathy (DN) in patients with type 2 diabetes (T2D). Although CN-1 is expressed in the kidney, urinary CN-1 (CNU) excretion and its pathological relevance in patients with T2D have not been investigated to date. The present study therefore assessed the extent of CNU excretion in healthy subjects ($n=243$) and in patients with T2D ($n=361$) enrolled in the DIAbetes and LifEstyle Cohort Twente-1 (DIALECT-1) in relation to functional renal parameters. CNU was detected in a high proportion of healthy individuals, 180 (74%); median CNU excretion was 0.25 mg/24 h [(IQR 0–0.65 mg/24 h]. In patients with T2D the prevalence and extent of CNU increased in parallel with albuminuria ($r=0.59$, $p<0.0001$; median CNU 0.1 vs 0.2 vs 1.5 mg/24 h, $p<0.0001$; prevalence of CNU 61 vs. 81 vs. 97% $p<0.05$ in normo- ($n=241$), micro- ($n=80$) and macroalbuminuria ($n=40$), respectively). Patients with estimated glomerular filtration rate (eGFR) < 30 ml/min/1.73 $m^2$ displayed higher median CNU excretion rates in comparison to patients with preserved eGFR (> 90 ml/min/1.73 $m^2$) (1.36 vs 0.13 mg/24 h, $p<0.05$). Backward stepwise multivariate linear regression analysis revealed albuminuria, eGFR and glycosuria to be independent factors of CNU excretion rates, all together explaining 37% of variation of CNU excretion rates ($R^2=0.37$, $p<0.0001$). These results show for the first time that CN-1 can be detected in urine and warrants prospective studies to assess the relevance of CNU for renal function deterioration in diabetes patients.

**Keywords** Carnosinase-1 · Urine · Diabetic nephropathy · Albuminuria

## Abbreviations

| | |
|---|---|
| DN | Diabetic nephropathy |
| CNU | Urinary carnosinase-1 |
| ESRD | End-stage renal disease |
| CN-1 | Carnosinase-1 |
| eGFR | Estimated glomerular filtration rate |
| HbA1c | Hemoglobin A1c |
| ACEi/ARB | Angiotensin converting enzyme inhibitor/ angiotensin receptor blocker |

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## Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) worldwide and imparts an increased risk for cardiovascular disease and death in patients with type 1 and type 2 diabetes (Cusick et al. 2005).

Diabetic nephropathy develops insidiously, starting with microalbuminuria as an early sign of impaired glomerular filtration. Accompanied by a gradual decline in renal function, patients may progress to macroalbuminuria, overt proteinuria and ultimately end-stage renal disease (ESRD) (Ismail et al. 1999; Campbell et al. 2003). Genetic

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susceptibility to DN has been suggested in numerous studies (Palmer and Freedman 2012). Amongst these, a trinucleotide repeat $(CTG)_n$ polymorphism in the signal sequence of the *CNDP1* gene encoding for the protein carnosinase-1 (CN-1) showed a strong association with DN in patients with T2D (Janssen et al. 2005; Freedman et al. 2007; Mooyaart et al. 2009; Ahluwalia et al. 2011; Yadav et al. 2016). The $(CTG)_n$ polymorphism is functional since it influences the efficacy of CN-1 secretion from the hepatic cells into the circulation. The short allelic variant, i.e., $(CTG)_5$, also referred to as the *Mannheim allele,* was found to be more common in T2D patients without nephropathy and is associated with lower serum CN-1 concentrations and enzymatic activities (Janssen et al. 2005; Riedl et al. 2007). Apart from the $(CTG)_n$ genotype, hyperglycemia also enhances CN-1 secretion and enzymatic activity in vitro and in vivo (Riedl et al. 2010; Peters et al. 2012).

CN-1 is the rate limiting enzyme for hydrolysis of the histidine-containing dipeptide: carnosine (β-alanyl-L-histidine), anserine (β-alanyl-*N*-methyl-histidine) and homocarnosine (*N*-(4-amino-butyryl)-L-histidine). In keeping with a plethora of beneficial properties reported for carnosine, e.g., ROS scavenging (Nagasawa et al. 2001; Aydın et al. 2010), inhibition of AGEs formation (Alhamdani et al. 2007) and anti-glycemic properties (Sauerhöfer et al. 2007; Albrecht et al. 2017), carnosine has widely gained scientific interest as potential food supplement in T2D patients. Based on the genetic association between the *CNDP1* gene and DN and the beneficial effects of carnosine, the hypothesis has been put forward that low CN-1 concentrations/enzymatic activity may result in higher renal carnosine concentrations affording protection against hyperglycemia-mediated renal damage (Boldyrev et al. 2013).

Although in humans, CN-1 is predominantly produced in the liver from where it is secreted into the circulation, a number of studies have suggested that CN-1 is also expressed in the kidney, albeit to a much lower extent (Janssen et al. 2005; Mooyaart et al. 2009; Peters et al. 2015). In the kidney, CN-1 expression was mostly found in distal tubular cells and podocytes of healthy individuals, but seemed to be reallocated to proximal tubuli in diabetic patients with DN (Peters et al. 2015).

Based on the current knowledge that CN-1 is expressed in renal tissue and contains a signal peptide, it is conceivable that CN-1 can be secreted into the tubular lumen and consequently be detected in urine. To test this, we validated our ELISA assay for the detection of CN-1 in urine and subsequently measured CN-1 in 24-h urine collected from healthy subjects (living kidney donors evaluated prior to donation) ($n = 243$) and patients with T2D enrolled in the DIAbetes and LifEstyle Cohort Twente-1 (DIALECT-1) (Gant et al. 2017) ($n = 361$). In the latter group, we also investigated possible correlations between urinary CN-1 and renal parameters including albuminuria and renal function.

## Results

### CN-1 is detectable in urine (CNU)

We have recently reported the detection of CN-1 in serum or plasma using an in-house developed ELISA system (Adelmann et al. 2012). To assess if this ELISA is also suitable for detection of CN-1 in urine, a known concentration of recombinant CN-1 (rCN-1) was spiked into spot urine samples of healthy individuals or into standard phosphate-buffered saline (PBS). By comparing the detected and expected rCN-1 concentration at different dilutions, a spike recovery of nearly 100% was detected for both matrices. Linearity in the dilution curves was observed in a concentration range between 100 and 1000 ng/ml and was similar for urine and PBS (Fig. 1a). Since CN-1 in rodents is not a secreted protein, we further validated the specificity of the CN-1 ELISA by testing serum and urine of human *CNDP1*-overexpressing transgenic BTBR and wild-type mice.

While CN-1 was clearly detected in serum and urine of *CNDP1* transgenic mice, the CN-1 ELISA was completely negative on serum and urine of wild-type mice (Fig. 1b). The presence of CN-1 in urine of CNDP1 transgenic mice was further confirmed by Western blotting (Fig. 1c).

### CNU in healthy subjects

Although spiking of rCN-1 in PBS or urine gave similar results, we observed in some individuals that the measured CN-1 concentrations were significantly higher than expected at high rCN-1 dilutions when using urine as diluent. Indeed the blanks, i.e., urine without spiked CN-1, showed traces of CN-1 in the samples of these individuals. To substantiate these findings we collected 24-h urine of living kidney donors and assessed if, and to what extent, CN-1 is present in urine of healthy individuals. In 180 (74%) out of 243 subjects CN-1 was detected in urine. The median CNU excretion rate was 0.25 mg/24 h (IQR 0–0.65 mg/24 h). No differences were detected between females and males. CN-1 enzymatic activity was not detected in any of the urine samples (data not shown).

### CNU in patients with T2D

CNU was studied in a total of 361 type 2 diabetes patients (T2D) stratified for 24-h albumin excretion into normoalbuminuria ($n = 241$), microalbuminuria ($n = 80$) or macroalbuminuria ($n = 40$).

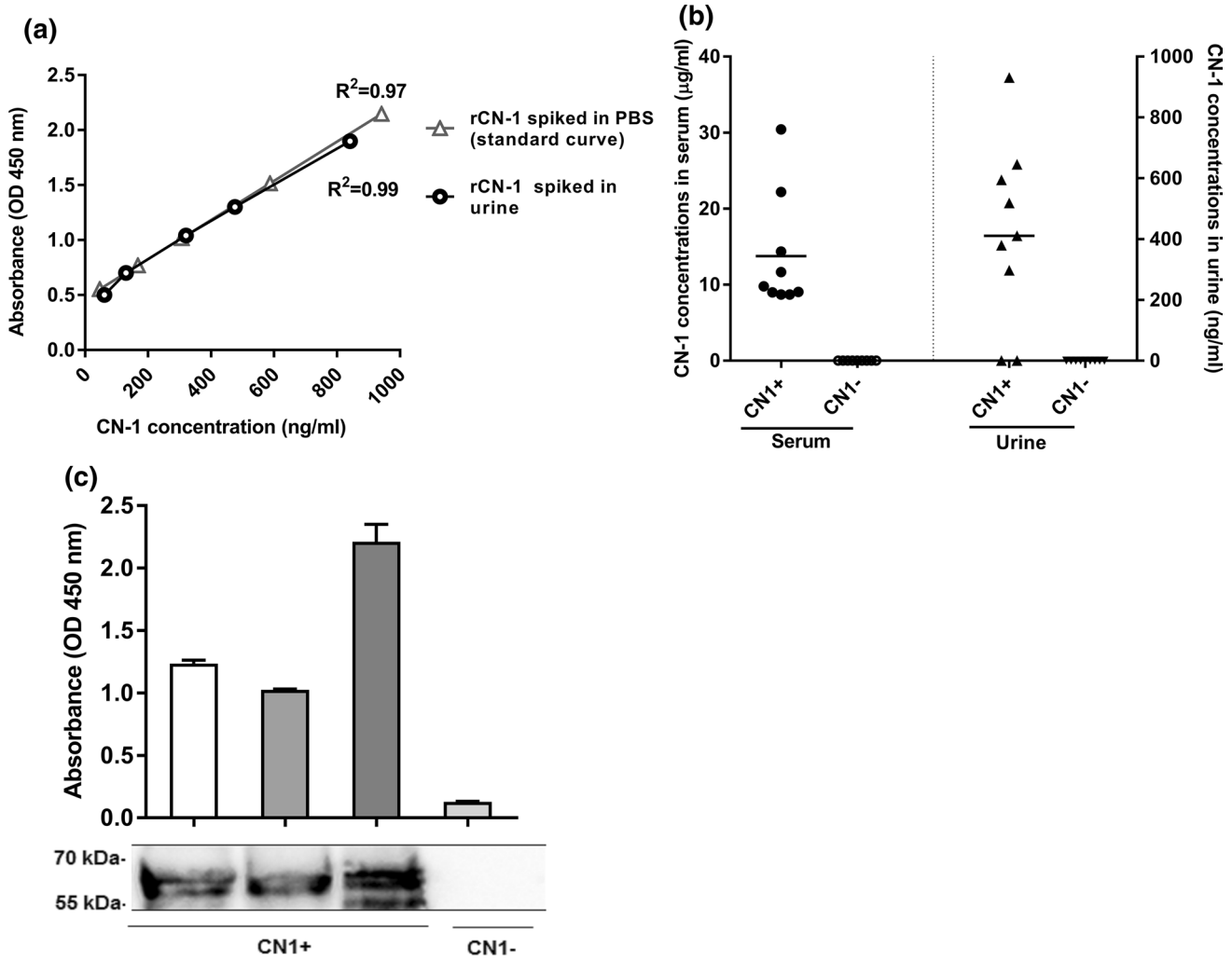


**Fig. 1** **a** rCN-1 was spiked in human urine or PBS. CN-1 was detected by ELISA in serial dilutions using urine or PBS as diluent. Detected CN-1 concentrations of rCN-1 spiked in urine were compared with the expected concentrations to estimate spike recovery %. Both dilution curves were plotted using linear regression. **b** Specificity of the ELISA system: CN-1 concentrations in serum and in urine of mice overexpressing human CN-1 (CN1+) ($n=9$). Note that no human CN-1 was detected in serum or urine of wild-type mice (CN1−) ($n=9$). **c** ELISA and Western blotting of CN-1 in urine obtained from three different transgenic mice overexpressing human CN-1 and one wild-type mouse

The baseline characteristics of patients are shown in Table 1. In the macroalbuminuria group, there were significantly more men. The patients in this group were also older and had a higher systolic blood pressure. In addition, lower eGFR and creatinine clearance rates, and higher serum creatinine concentrations and proportion of patients on ACEi/ARB therapy were found in the macroalbuminuria group compared with the other two groups.

In macroalbuminuric patients, 97% of the urine samples were positive for CN-1, while in the microalbuminuria and normoalbuminuria group, the percentage of CN-1 positive samples was 81 and 64%, respectively ($p=0.016$).

The median CNU excretion rates increased in parallel to the extent of albuminuria: i.e., from 0.1 mg/24 h (IQR 0–0.4 mg/24 h) to 0.2 mg/24 h (IQR 0.08–0.8 mg/24 h) and 1.5 mg/24 h (IQR 0.5–3.8 mg/24 h) in the normoalbuminuria, microalbuminuria and macroalbuminuria groups, respectively ($p<0.0001$) (Fig. 2a). Of note, when comparing CNU excretion rates between patients with T2D and healthy subjects, differences in CNU excretion rates were only significant between healthy individuals and T2D patients with macroalbuminuria ($p<0.0001$) (Fig. 2a).

To have a closer look at the relationship between CNU and kidney function, all patients with T2D were stratified on the basis of estimated glomerular filtration rate (eGFR) in > 90, 60–90, 30–60 and < 30 ml/min/1.73 $m^2$ and defined as normal renal function, mildly, moderately and severely decreased GFR, respectively.

**Table 1** Baseline characteristics of T2D patients related to albuminuria

| Characteristics | T2D | | | *p* value |
|---|---|---|---|---|
| | Normoalbuminuria | Microalbuminuria | Macroalbuminuria | |
| | *n* = 241 | *n* = 80 | *n* = 40 | |
| Demographic | | | | |
| Men, *n* (%) | 118 (49) | 56 (70)[a] | 31 (78)[a,b] | 0.016 |
| Age (years) | 62.8 ± 8.8 | 63.6 ± 9.0 | 67.5 ± 7.9[a] | 0.007 |
| Clinical | | | | |
| Systolic blood pressure (mmHg) | 134.6 ± 15.3 | 139.4 ± 16.8 | 140.3 ± 19.4[a] | 0.002 |
| Diastolic blood pressure (mmHg) | 74.1 ± 9.0 | 75.3 ± 10.3 | 75.2 ± 10.8 | 0.55 |
| Body mass index (kg/m$^2$) | 32.7 ± 6.0 | 33.0 ± 5.7 | 32.8 ± 6.1 | 0.75 |
| Duration of diabetes (years) | 11 [6–18] | 12 [6–18] | 10 [6–19] | 0.65 |
| Retinopathy, *n* (%) | 55 (22) | 26 (33) | 11 (28) | 0.21 |
| HbA1c (%) | 7.3 ± 1.0 | 7.6 ± 1.2 | 7.2 ± 1 | 0.16 |
| Kidney function | | | | |
| Serum creatinine (mg/dl) | 0.9 ± 0.3 | 1.1 ± 0.6[a] | 1.4 ± 0.5[a,b] | < 0.0001 |
| Creatinine clearance rate (ml/min) | 126.1 ± 48.4 | 117.9 ± 59.6 | 82.8 ± 38.7[a,b] | < 0.0001 |
| eGFR (ml/min/1.73 m$^2$) | 81 ± 22 | 75 ± 26 | 54 ± 22[a,b] | < 0.0001 |
| Urine osmolarity (mOsm/kg) | 486.8 ± 210.0 | 494.6 ± 217.9 | 415.0 ± 170.4 | 0.10 |
| Urine volume (ml) | 2024 ± 783 | 2071 ± 775 | 2153 ± 961 | 0.48 |
| Glycosuria excretion (mg/24 h) | 451 [85–5263] | 1137 [105–8393] | 326 [74–4349] | 0.17 |
| Albuminuria excretion (mg/24 h) | 4.4 [1.5–11.1] | 82.4 [59.6–145.8][a] | 508.0 [365.4–900.1][a,b] | < 0.0001 |
| Urinary creatinine (mg/24 h) | 1556 ± 550.3 | 1579 ± 572.4 | 1514 ± 424.3 | 0.82 |
| ACEi/ARB, *n* (%) | 158 (63.5) | 57 (71.4) | 34 (85)[a] | 0.016 |
| CN-1 detection, *n* (%) | 147 (61) | 65 (81) | 39 (97)[a,b] | 0.016 |

Data are shown as mean ± standard error, median [interquartile range] or absolute number (proportion). *p* values were calculated using one-way ANOVA, Kruskal–Wallis or Chi$^2$

*eGFR* estimated glomerular filtration rate, *ACEi* angiotensin converting enzyme inhibitor, *ARB* angiotensin II receptor blocker, *CN-1* carnosinase-1

[a]Compared to patients with normoalbuminuria

[b]Compared to patients with microalbuminuria

When patients with T2D were allocated in the aforementioned groups, patients with severely decreased renal function (< 30 ml/min/1.73 m$^2$) displayed significantly higher CNU excretion rates compared to patients with normal (> 90 ml/min/1.73 m$^2$) and mildly decreased renal function (60–90 ml/min/1.73 m$^2$). This was also true when comparing patients with moderately decreased (30–60 ml/min/1.73 m$^2$) and mildly decreased renal function ($p < 0.05$) (Fig. 2b).

To identify potential associations between CNU and relevant clinical parameters we performed Pearson correlation analysis.

As displayed in Table 2, there was a significant positive correlation between CNU excretion rates and albuminuria ($r = 0.59$, $p < 0,0001$) (Fig. 3, to the left) and between CNU and glycosuria ($r = 0.13$, $p = 0.017$). In contrast, CNU excretion was negatively associated with eGFR ($r = -0.24$, $p < 0.0001$) (Fig. 3, to the right), urine osmolality ($r = -0.17$, $p < 0.0001$) and creatinine clearance rate ($r = -20$, $p = 0.0002$).

To assess the independent influence of these variables on CNU excretion, log (CNU+1) was set as the dependent variable and a backward stepwise multivariate linear regression was performed.

As shown in Table 3, albuminuria, eGFR and glycosuria appeared to be independent determinants of CNU excretion rates, all together explaining 37% of variation in log-transformed (CNU+1) excretion rates ($R^2 = 0.37$; $p < 0.0001$). The variables remaining in the model showed a variance inflation factor (VIF) < 5, which is the recommended threshold to rule out multicollinearity.

## Discussion

There is compelling evidence suggesting that CN-1 is expressed in human kidneys. Because exon1 and 2 of the *CNDP1* gene encode a signal peptide, CN-1 is targeted to the secretory pathway (Teufel et al. 2003). Yet, unlike in serum

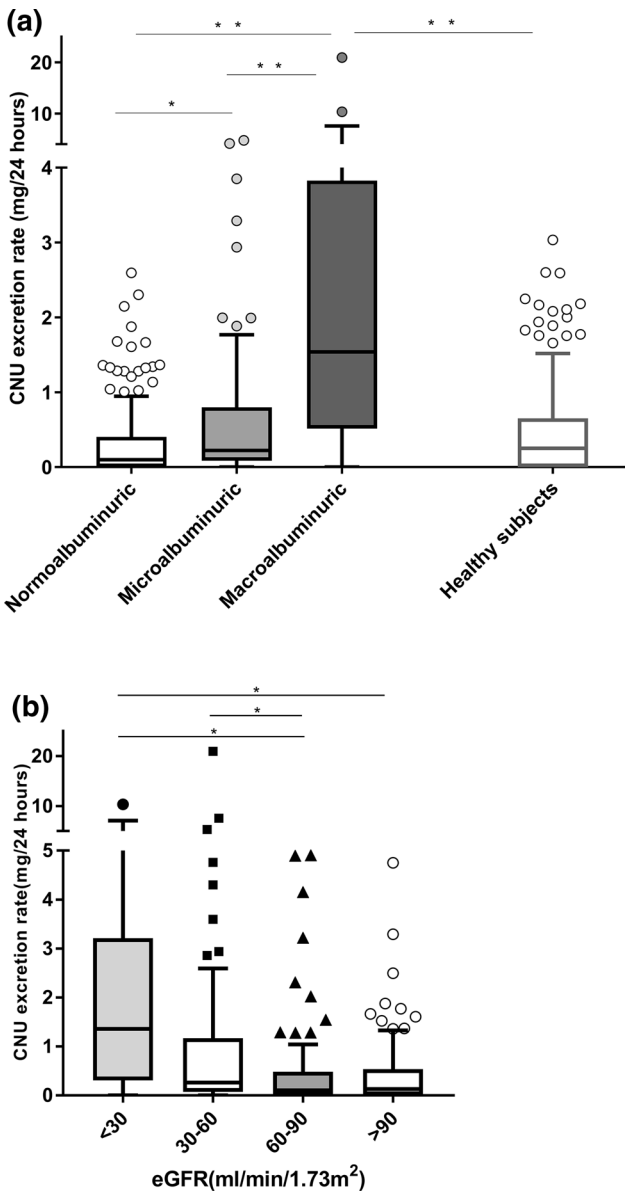


**Fig. 2** Detection of CNU. **a** Distribution of CNU excretion rates in T2D with normoalbuminuria, microalbuminuria, macroalbuminuria and healthy subjects. Boxes and whiskers represent the median and IQR. Outliers are indicated with circles. *$p<0.05$; **$p<0.0001$. **b** Distribution of CNU excretion rates of T2D according to eGFR. T2D patients ($n=361$) were stratified into those with normal renal function ($n=128$), and mildly ($n=140$), moderately ($n=74$) and severely decreased ($n=13$) eGFR. Boxes and whiskers represent the median and IQR. Outliers are indicated by circles. *$p<0.05$

and cerebrospinal fluid (CSF), the presence of CN-1 in urine thus far has not been reported.

The present study demonstrates for the first time that, apart from being detectable in serum, CN-1 is also detectable in urine. We were able to detect CNU in 74% of the healthy subjects and in up to 97% of diabetes patients using an in-house developed ELISA system (Adelmann et al. 2012). Our results also show that high CNU excretion rates in T2D patients were associated with increased albuminuria and low eGFR, the main prognostic predictors of diabetic nephropathy (Berhane et al. 2011). Likewise, glycosuria appeared to be an independent factor influencing CNU excretion rates.

**Table 2** Significant correlations between log (CNU+1) excretion rate and clinical parameters in T2D patients

| Clinical parameter | *R* | *p* value |
|---|---|---|
| Albuminuria (mg/24 h) | 0.59 | < 0.0001 |
| eGFR (ml/min) | − 0.24 | < 0.0001 |
| Log (glycosuria excretion + 1) | 0.13 | 0.017 |
| Urine osmolarity (mOsm/kg) | − 0.17 | 0.0009 |
| Creatinine clearance rate (ml/min) | − 0.20 | 0.0002 |

An intrinsic carnosine metabolism in human kidneys has been reported based on the presence of carnosine synthesizing and degrading enzymes, i.e., carnosine synthase and CN-1, respectively (Peters et al. 2015). Immunehistological staining of human renal biopsies suggested that these enzymes are in different tubular compartments. While carnosine synthesis might be important for protecting renal tissue from oxygen radicals, degradation of carnosine might play a role in maintaining a low pH that is required for the exchange of protons with K+ and Na+ ions in the distal tubules (Peters et al. 2015). It should be underscored, however, that we were unable to detect CN-1 activity in the urine samples of our study (data not shown) and that to our knowledge formal proof for the latter assumption is therefore lacking. Nonetheless, as reported for other enzymes secreted in urine, lack of enzyme activity might be caused by several unfavorable factors intrinsic to urine, including high urea concentrations, long retention in the bladder and low pH (Jung et al. 1982, 1983). Indeed, the reported pH activity curve of CN-1 enzyme shows a maximum between pH 7.5 and 8.5 (Teufel et al. 2003).

The concept that glomerular filtration is restricted by the size and charge barriers of the glomerular basement membrane and the underlying slit diaphragm is widely accepted. It is believed that small negatively charged proteins such as albumin are not completely retained by the glomerular filtration barrier. Most of the filtered albumin does, however, not reach the bladder as it is reabsorbed mainly in the proximal convoluted tubule. In keeping with the molecular weight (~ 65 kDa) and pI of CN-1 monomers, it is conceivable that they may be filtered through the glomerulus with similar sieving characteristics as for albumin. In its homodimer conformation, which is believed to be the major conformation of CN-1 in serum (Pavlin et al. 2016), CN-1 is therefore too large (~ 130 kDa) to pass the filtration barrier.

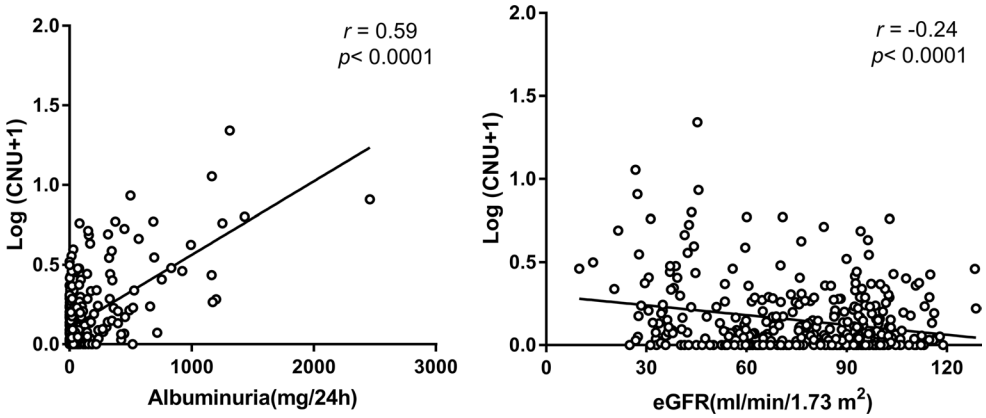


**Fig. 3** Graph on the left reveals a positive correlation between CNU (log (CNU+1)) and albuminuria; graph on the right displays an inverse correlation between CNU (log (CNU+1)) and eGFR

**Table 3** Multivariate linear regression analysis with log-transformed (CNU+1) excretion rate as the dependent variable in T2D patients

| Predictive parameter | Beta value | 95% CI | *p* value |
|---|---|---|---|
| eGFR(ml/min) | −0.001 | −0.002 to −0.00015 | 0.019 |
| Log (glycosuria excretion + 1) | 0.014 | 0.016 to 0.048 | 0.0007 |
| Albuminuria (mg/24 h) | 0.0004 | 0.0004 to 0.0005 | < 0.0001 |

Goodness of fit of the full model: $R^2 = 0.37$, $p < 0.0001$

*CI* confidence interval

Small molecules like organic anions and cations (H+, creatinine, drug metabolites, etc.) can be cleared from the blood via tubular secretion. This process requires specific transporters and mostly occurs in the proximal tubule and to a lesser extent in other segments of the nephron. Because tubular secretion is selective and active CN-1 transporters are not known to be present in the kidney, the presence of CN-1 in urine cannot be explained by tubular secretion.

Hence, the appearance of CN-1 in urine of healthy individuals might be due to local renal production and secretion into the tubular lumen or alternative to filtration of small amounts of CN-1 monomers from the circulation.

CN-1 is detected in the vast majority of T2D patients. Among these patients, CNU excretion rates are significantly higher in patients with macroalbuminuria. Since the glomerular filtration barrier is severely impaired in these patients it can be expected that, like albumin, CN-1 will appear in the urine, thus explaining the association between albuminuria and CNU.

Of note, CNU excretion rates did not differ between healthy subjects and patients with microalbuminuria, possibly because the amount of filtered proteins did not exceed the renal reabsorption capacity in the tubuli of these patients. This observation can also explain why in diabetic patients with proteinuria CN-1 reallocates from the distal to the proximal tubuli, where almost 90% of proteins are reabsorbed (Peters et al. 2015).

Although it is elusive how much of CN-1 is reabsorbed in the tubule, the finding that CNU does not correlate with urinary creatinine in T2D patients without nephropathy (normoalbuminuria and renal function eGFR > 60 ml/min/1.73 $m^2$), in which a greater fraction of the urinary creatinine is derived from glomerular filtration, suggests that CN-1 is probably not freely filtered through a healthy glomerular basement membrane.

An increase in urine albumin excretion rate typically precedes a fall in glomerular filtration rate in patients developing chronic kidney disease. Here, we show that T2D patients with kidney disease, as reflected by poor eGFR, display high CNU excretion rates. Multivariate linear regression analysis suggests that both albuminuria and eGFR can be considered as independent factors associated with CNU excretion rates in T2D patients. It is likely that CNU excretion increase along with renal function deterioration; however, this should be addressed in future prospective studies.

Likewise, glycosuria also appeared as an independent factor positively associated with CNU excretion rates. If CN-1 is locally synthesized in the kidney, it is possible that high glucose levels in the tubular lumen enhance CN-1 synthesis and secretion (Riedl et al. 2010).

Current ongoing studies are addressing if urinary CN-1 excretion could result in decreased serum CN-1 concentrations. If so, our findings of enhanced urinary CN-1 excretion might be considered as positive, since low serum CN-1 concentrations may protect patients with T2D from developing DN. Yet, it should be emphasized that patients with macroalbuminuria already have significant pathologic lesions and thus it remains to be assessed if a decreased carnosine turnover due to low serum CN-1 concentrations can still influence the course of nephropathy in patients with advanced lesions. However, it should be underlined that increased CNU will not necessarily result in low serum CN-1 concentrations, since depletion of serum proteins, e.g., serum albumin, is efficiently replenished by increased protein synthesis in the liver (Prinsen et al. 2003).

In conclusion, our study demonstrates for the first time that CN-1 can be detected in urine of healthy individuals and in patients with T2D. These findings are compatible with previous immune-histological and mRNA data that showed that CN-1 is expressed in renal tissue. The physiological role of CNU is, however, still elusive and warrants further studies to assess if CNU can influence carnosine stores in kidney and if the CNU in healthy individuals is also influenced by the $(CTG)_n$ polymorphism in the CNDP1 gene. We also demonstrate that CNU excretion rates are increased in T2D patients with macroalbuminuria; yet, the potential relevance hereof for renal function deterioration cannot be discerned in this cross-sectional study.

## Materials and methods

### Participants and sampling

*Participants:* A total of 361 T2D patients from the DIALECT-1 cohort were included in this study. DIALECT is a prospective study of T2D patients recruited in the ZGT Hospital, a secondary health-care center in Almelo, The Netherlands (Gant et al. 2017). T2D patients older than 18 years, treated in a secondary healthy-care center and able to understand the informed consent were eligible. Patients with renal replacement therapy were excluded.

The study was approved by the relevant local institutional review boards. (METc-registration numbers METc-Twente NL57219.044.16 and METc-Groningen 1009.68020), and is registered in the Netherlands Trial Register (NTR trial code 5855). The study was performed according to the guidelines of good clinical practice, and all patients signed an informed consent form prior to participation.

The control group consisted of 243 healthy individuals that were regularly checked for living kidney donation in the University Medical Center Groningen. None had a medical history of diabetes, kidney or cardiovascular disease. The institutional board approved the study protocol (METc 2008/186).The study was performed in accordance with the guidelines of good clinical practice and the Declaration of Helsinki.

*Sampling:* For the 24-h urine collection, patients were instructed to dispose of the first morning void urine and thereafter collect all urine in a provided container until the first urine of the next day. In between voids, patients were instructed to store the container in a dark place and preferably in a refrigerator.

Albuminuria, glycosuria and urinary CN-1 excretion rates were calculated by multiplying concentrations with the volume of the 24-h urine collection. Normoalbuminuria was defined as 24-h urinary albumin excretion of < 30 mg/day. Micro- and macroalbuminuria were defined as 24-h urinary albumin excretion of 30–300 mg/day and > 300 mg/day, respectively. Estimated glomerular filtration rate (eGFR) was calculated according to the CKD-EPI formula. Creatinine clearance was calculated according to the formula: GFR = [urine creatinine × urine volume]/serum creatinine. Tests for assessing HbA1c, serum creatinine and cholesterol were performed in venous blood using routine laboratory procedures.

### Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1984 Helsinki Declaration and its later amendments or comparable ethical standards.

### Generation of CN-1 transgenic mice

Generation of human CN-1 transgenic mice was carried out in the context of ongoing studies. CN-1 transgenic mice were used in the current study to demonstrate the specificity of our ELISA. A detailed description on the generation and characterization of human CN-1 (hCN-1) transgenic mice will be addressed in a separate manuscript. Briefly, the cDNA of human CN-1 including the endogenous signal peptide of six leucines was amplified from IMAGE clone acc. no. BX094414 using the primers CN1 forward 5′PCACCATGGATCCCAAACTCGGGA3′ and CN1 reverse 5′PTCAATGGAGCTGGGCCATCT3′. PCR β product was ligated into the Stu1 site of plasmid pTTR1ExV3 which contains the transthyretin promoter and drives hCN1 expression specifically within the liver and brain. After fragment digestion using HindIII, the purified fragment was injected into the pronuclei of the fertilized ovum of BTBR (Black and Tan BRachyuric) ob/wt female mouse (purchased from Jackson Laboratories, Bar Harbor, ME, USA). All animal procedures were approved by the Regierungspräsidium Karlsruhe (AZ 35-9185.81/G-116/14). Genomic DNA was isolated from tail biopsies by making use of the Invisorb spin tissue mini kit according to the manufacturer's instructions. The transgene TTP-hCN1 mutation was genotyped by PCRs as described in Sauerhöfer et al. (2007). Mice were housed at 22 °C in a 12 h light/dark cycle and fed regular chow and water ad libitum. At week 24 of age, blood samples were collected from the orbital plexus under anesthesia and serum was isolated by centrifugation. To obtain morning spot urine samples, animals were placed in metabolic cages at the beginning of the light cycle. Serum and urinary carnosinase concentrations were measured with ELISA as previously described.

## CN-1 concentrations and enzymatic activities in urine

CN-1 concentrations in urine were measured by ELISA as described (Adelmann et al. 2012). In brief, high-absorbent microtiter plates (Greiner, Labortechnik, Frickenhausen, Germany) were coated overnight with 100 µl of goat polyclonal anti-human CN-1 (10 µg/ml) (R&D, Wiesbaden Germany). The plates were extensively washed and incubated with 0.05% of dry milk powder to avoid unspecific binding. Serum samples from CN-1 transgenic mice were tested in a dilution of 1:100 or 1:200. Urine samples were tested undiluted. The plates were placed on a shaker for 1 h and subsequently extensively washed with 1 × PBS/Tween. Thereafter, rabbit polyclonal (ATLAS, Abcam plc, Cambridge, United Kingdom) was added for 1 h followed by extensive washing. Goat anti-rabbit IgG HRP-conjugated antibody was added for 30 min followed by extensive washing. Deep-blue peroxidase (POD) (Roche diagnostics, Mannheim, Germany) was used for color development and the reaction was stopped after 10 min by the addition of 50 µl of 1 M $H_2SO_4$. The plates were directly read at optical density (OD) of 450 nm. A serial dilution of pooled serum with known carnosinase concentration (2 µg/µl) was used as standard. CN-1 protein concentrations were assessed in the linear part of the dilution curve with a lower sensitivity of 31 ng/ml. Since no linear relation was observed between CN-1 concentrations and OD450 below a concentration of 31 ng/ml this was set as threshold of sensitivity. Readings below the threshold were considered as undetectable and were given the value of 0. On each ELISA plate a blank (PBS) was included. The OD450 of the blank was subtracted from the OD450 of the sample before curve fitting. Validation of the CN-1 ELISA for urine samples was in essence performed by making dilution curves of recombinant CN-1- protein spiked into urine samples of healthy individuals or into 1× phosphate buffer. Concentrations below detection limit were considered as CN-1 negative.

CN-1 activities in urine and in CN-1 recombinant protein were measured as described by Peters et al. (2010). Briefly, the reaction was initiated with the addition of carnosine into the samples and stopped with 1% trichloroacetic acid in predetermined time points. Supernatants were taken after centrifugation and placed in a new tube in combination with *o-Phthalaldehyde*. Fluorescence liberated by histidine substrate was read using a Tecan plate reader ($\lambda_{Ex}$: 360 nm; $\lambda_{Em}$: 460 nm).

## Western blotting

Detection of CN-1 by Western blotting was performed to demonstrate the presence of carnosinase in urine of human CN-1 transgenic mice as supportive line of evidence for the specificity of the CN-1 ELISA. Western blotting was used for qualitative but not quantitative assessment of CN-1 in urine of these mice. Since all mice were healthy and protein concentrations in urine were below the detection limit, urine samples were concentrated by the addition of 1 volume of 100% trichloroacetic acid in 4 volumes of sample and centrifuged for 10 min at 12,000 rpm. The pellets were washed twice with 200 µl of cold acetone and let dry for 10 min in 95 °C heat block before dissolving in Laemmli sample buffer containing β-mercaptoethanol. All samples were boiled for 5 min prior to separation on 10% SDS-PAGE for 1 h at 150 V.

SDS-PAGE was run at 150 V for 1 h and proteins were subsequently transferred to a PVDF membrane by means of semidry blotting. After blocking the membrane with Tris-buffered saline (TBS)–Tween 20 containing 5% w/v milk powder for 1 h at room temperature, the membrane was subsequently incubated at 4 °C overnight with rabbit anti-human CNDP1 IgG (ATLAS, Sigma, Munich, Germany; 1:500 diluted in 5% milk powder) as primary antibody, followed by incubation with goat anti-rabbit IgG HRP (1:2000 diluted in 5% milk powder) (Santa Cruz, Heidelberg, Germany) for 1 h at room temperature the next day. In between the incubations and after the last incubation, the membrane was thoroughly washed with TBS–Tween 20 (5% w/v milk powder). Detection of immune-reactive bands was performed by Western Lightning Plus-ECL solution as recommended by the manufacturer (Perkin Elmer, Rodgau, Germany) and visualized by chemiluminescence.

## Statistical analysis

Continuous variables were expressed as mean ± SD or median and interquartile range (IQR). Categorical variables were expressed as numbers and percentages. For comparison of the groups, independent Student's *t* test was applied for continuous variables if normally distributed or Mann–Whitney *U* or Kruskal–Wallis test for data with non-normal distribution. The $Chi^2$ test was used for categorical variables.

Because of skewed distribution and the presence of zero values in some samples, a constant of 1 was added to CNU (CNU+1) and glycosuria (glycosuria excretion + 1) and thereafter logarithmically transformed: log (CNU+1), log (glycosuria excretion + 1). Correlations between log (CNU+1) with other continuous variables were assessed using the Pearson correlation test.

Backward stepwise regression analysis was employed with log-transformed (CNU+1) as the dependent variable to determine the best multivariate model predicting CNU excretion rates. All statistical tests were two sided and a $p$ value < 0.05 was considered statistically significant in all analyses. The analyses were assessed with GraphPad Prism version 7.02 and SAS version 9.3.

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## Compliance with ethical standards

**Statement on the welfare of the animals** All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

**Conflict of interest** The authors declare that they have no conflict of interest.

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