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Urinary Amino-Terminal Pro–C-Type Natriuretic Peptide: A Novel Marker of Chronic Kidney Disease in Diabetes

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BACKGROUND: Chronic renal inflammation and fibrosis are common sequelae in diabetes mellitus (DM) and are major causes of premature mortality. Although upregulation of *NPPC* expression occurs in response to renal inflammation in experimental animals, nothing is known of the molecular forms of C-type natriuretic peptide (CNP) products in urine of people with DM or links with renal function.

METHODS: ProCNP products in urine were characterized with HPLC and a range of antisera directed to specific epitopes of amino-terminal proCNP (NTproCNP). The 5-kDa intact peptide was quantified in spot urine samples from healthy adults and 202 participants with DM selected to provide a broad range of renal function.

RESULTS: The predominant products of proCNP in urine were consistent with the 2-kDa fragment (proCNP 3–20) and a smaller peak of intact (5-kDa) fragment (proCNP 1–50, NTproCNP). No peaks consistent with bioactive forms (proCNP 82–103, 50–103) were identified. The urine NTproCNP to creatinine ratio (NCR) was more reproducible than the albumin to creatinine ratio (ACR) and strongly associated with the presence of chronic kidney disease. In models predicting independence, among 10 variables associated with renal function in DM, including plasma NTproCNP, only 3 (sex, ACR, and plasma creatinine) contributed to NCR.

CONCLUSIONS: Characterization of the products of proCNP in urine confirmed the presence of NTproCNP. In spot random urine from study participants with DM, NCR is inversely associated with estimated glomerular filtration rate. In contrast to ACR, NCR reflects nonvascular factors that likely include renal inflammation and fibrosis.

The past 2 decades have seen an explosive global increase in diabetes. There are now an estimated >400 million people with diabetes in the 20–80 years age range, with a worldwide prevalence of 9% *(1)*. A significant proportion of these people are likely to develop chronic kidney disease [CKD,[3] defined by estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2], which markedly increases morbidity and mortality of cardiovascular disorders *(2)* and is a leading cause of end-stage renal failure. Both diabetes-specific kidney disease (DKD), defined by the presence of albuminuria, and CKD due to disorders other than classical DKD commonly coexist in diabetes patients *(3)*. Early detection of these disorders is challenging; when used for detection of DKD, albuminuria is both insensitive and nonspecific, whereas subnormal eGFR is a relatively late sign and usually portends irreversible renal disease *(4, 5)*. Because of these deficiencies, many groups are pursuing novel approaches to improving early detection of renal injury, including composite panels of established risk factors *(6)* and use of specific peptides and other gene products of renal origin *(7)*, but none of these has met the criteria required for detecting early renal injury in the clinic. Current clinical practice is to screen people with diabetes for albuminuria, although 20%–63% of people with diabetes and overt CKD have an albumin to creatinine ratio (ACR) within the normal range *(8)*. Low clinical sensitivity and specificity of ACR for detecting CKD are not surprising in light of the diverse mechanisms—over and above micro-

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[3] Nonstandard abbreviations: CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; DKD, diabetic kidney disease; ACR, urine albumin/creatinine ratio; CNP, C-type natriuretic peptide; proCNP, pro C-type natriuretic peptide; NTproCNP, amino-terminal pro C-type natriuretic peptide; NCR, urine NTproCNP/creatinine ratio; *NPPC*, C-type natriuretic peptide gene; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; SE-HPLC, size-exclusion HPLC; HbA1c, glycated hemoglobin A1c; FE, fractional excretion.

vascular and glomerular disease—that eventually lead to the decline in renal function *(9)*. Clearly, improved early detection of renal injury, enabling timely preventative strategies inhibiting or delaying progression to CKD *(10, 11)*, is an urgent unmet need. In this context, products of renal *NPPC* expression are logical candidates. C-type natriuretic peptide (CNP) is widely produced in the renal macro- and microvasculature, including the glomerulus and podocytes, renal tubules, and extracellular matrix *(12, 13)*, where the peptide likely subserves antiinflammatory, antifibrotic, and vasodilator functions that are renoprotective *(14)*. After unilateral ureteric ligation in rodents, renal *NPPC* expression is upregulated in response to tubular-interstitial fibrosis *(15)* and is increased in both renal cortex and medulla in streptozotocin-induced diabetes *(16)*. Together, these findings suggest that upregulation of CNP expression within renal tissues is an early (adaptive) response to local injury, which potentially could be captured by measuring products of renal proCNP excreted in urine. Although there are many reports of concentrations of CNP products in urine *(17, 18)*, to our knowledge none have provided details validating the methods used to characterize, identify, and quantify the specific urinary molecular species measured in these studies.

After our discovery of the bioinactive 5-kDa peptide product of proCNP, amino-terminal proCNP (NTproCNP) in the circulation *(19)*, we sought its presence in urine. In preliminary studies using RIA and HPLC, the intact peptide could not be identified in urine from healthy adults but was found in urine from neonates, in which plasma concentrations of NTproCNP were greatly increased *(20)*. This finding, together with observations that plasma concentrations of NTproCNP are strongly correlated with serum creatinine within the normal reference range *(21)*, suggests that NTproCNP, whether filtered from plasma or synthetized within the kidney, is normally degraded to smaller fragments during urine formation and excretion unless plasma concentrations are greatly increased and renal function is normal. However, these postulates do not exclude the possibility that intact NTproCNP may be excreted in urine if renal proCNP production is strongly stimulated, for example, in the course of developing DKD. Supporting this, preliminary studies identified large amounts of the intact peptide in urine collected from a diabetic participant with moderate CKD. Clearly, a more detailed study of urine NTproCNP, and the factors affecting its concentration, is required together with study of its excretion in participants with diabetes exhibiting a broad range of renal function.

Here we describe a method that specifically detects NTproCNP in urine and evaluate the assay's potential to predict renal function in people with diabetes. Because urinary NTproCNP has not been studied previously in diabetes, we explored possible associations of urine NTproCNP with albuminuria, renal glomerular function, plasma NTproCNP, and a range of clinical complications in a cross-sectional study of 202 participants. Noting that microvascular disease—in contrast to interstitial tubular inflammation and fibrosis—strongly associates with albuminuria *(3, 22)*, we hypothesized that factors affecting urine NTproCNP would differ from those contributing to albuminuria.

Materials and Methods

STUDY PROCEDURES

The study was approved by the Southern Health and Disability Ethics Committee and conducted in accordance with the Declaration of Helsinki. All study participants gave written informed consent.

PARTICIPANTS

Details of the study protocols relating to healthy control participants and 202 participants with diabetes [101 with type 1 diabetes mellitus (T1DM), 101 with type 2 diabetes mellitus (T2DM)] are provided in the online Data Supplement (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue10). With a stratified approach, participants were recruited to ensure that a broad range of renal function and albuminuria were represented in both T1DM and T2DM participants.

LABORATORY ANALYSES

Plasma NTproCNP was measured with the J39 antiserum, as previously described *(23)*. Three additional antisera recognizing different epitopes across proCNP were used to provide additional characterization of proCNP fragments in urine identified with size-exclusion HPLC (SE-HPLC, TSK-GEL G2000SW, 7.5 × 600 mm, Toyo Soda), as detailed in the online Data Supplement. The following antisera—J39 *(23)*, sheep 45, sheep 42, and sheep 43 *(24)*—were raised against proCNP (1–15), proCNP (10–21), proCNP (26–42), and proCNP (38–50), respectively. These antisera all showed <0.01% cross-reactivity to urodilatin, NTproANP, or NTproBNP. eGFR was calculated by the CKD-EPI creatinine equation *(25)*. The urine ACR and urine NTproCNP to creatinine ratio (NCR) were derived following measurement of urine creatinine in each sample. The fractional excretion (FE) of NTproCNP was calculated according to the formula FE = pNTproCNP/uNTproCNP × uCRN/pCRN, in which p was the plasma and u the urine concentration of analyte.

DETECTION OF proCNP PEPTIDES IN URINE

Owing to the low concentrations of NTproCNP present in urine, a concentrating step was required together with an extraction protocol that removed urine matrix com-

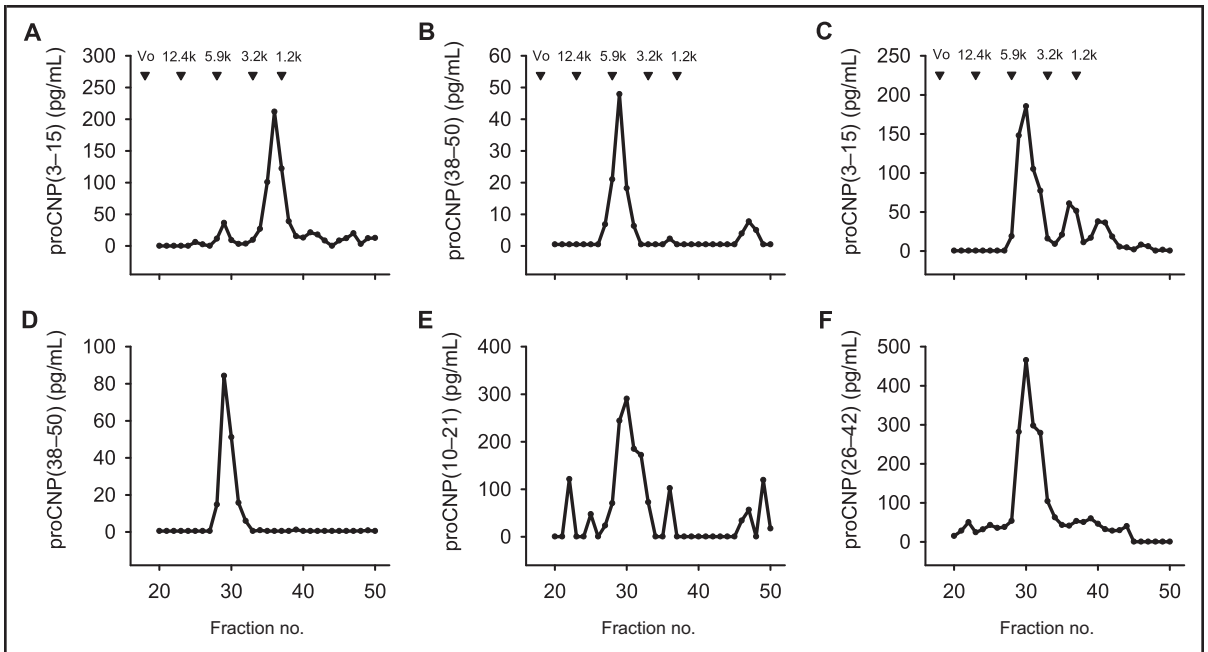

Fig. 1. SE-HPLC profile of urine from a healthy participant (A, B) and a diabetic participant with renal injury (C–F).

Panels A and C show profiles of samples assayed using J39 RIA (proCNP1–15); panels B and D, sheep 43 RIA (proCNP38–50); panel E, sheep 45 RIA (proCNP10–21); panel F, sheep 42 (proCNP26–42). Column void volume (V_0) and molecular weight markers are indicated by triangles in panel A.

ponents known to interfere in immunoassays. Because routine C18 SepPac extraction did not effectively remove urine matrix components, the following extraction protocol was derived, which allowed for a 10-fold concentration of the extract. Three milliliters of urine sample was passed through an Oasis HLB column (3 cc, 60 mg, Waters) preconditioned with 2 mL of methanol and 2 mL of distilled water. After sample addition, each column was washed with 2 mL of 5% acetic acid (v/v) followed by a second wash of 5% methanol in 0.5 M NH_4OH (v/v). The cartridges were dried under suction for 10 s, and the analytes were eluted with 2 mL of 30% isopropanol in 0.1% trifluoroacetic acid into test tubes containing 10 μL of 0.1% Triton X-100. The sample eluents were dried under an air stream at 37 °C before being resuspended in 300 μL of assay buffer (0.1% BSA, 0.01% NaN_3, 0.1% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.4) and stored at −20 °C until assayed. Recovery of synthetic proCNP (1–50)-supplemented (500 pg/mL, GenScript, Piscataway) urine was 73% (72%–77%). SE-HPLC analysis of the supplemented urine revealed a single 5-kDa peak. Recovery of synthetic CNP22-supplemented (22 pg/mL, Bachem) urine was 93% (90%–119%). SE-HPLC analysis of the supplemented urine revealed a single 2-kDa peak.

STATISTICS

Differences between subgroups (T1, T2 DM), CKD present/absent (defined by eGFR <60 ml/min/1.73 m^2) were determined using Mann-Whitney U test or Fisher's exact test as appropriate. Univariate associations between natriuretic peptides and risk factors were assessed with Spearman correlation coefficients. Independence was assessed by multivariable regression analysis with backward stepwise regression in a model comprising 10 separate variables found significant in univariate analyses. All model assumptions were assessed graphically and plasma NTproCNP, NCR, ACR, and creatinine were $\log_{10}$-transformed to satisfy parametric assumptions. All tests were 2-sided, and statistical significance was assumed when $P < 0.05$.

Results

URINE proCNP PEPTIDE PROFILES IN HEALTHY AND DIABETIC PARTICIPANTS

Fig. 1A shows the J39 RIA SE-HPLC profile in urine from a healthy adult participant. The major peak of immunoreactivity was observed in fraction 37 (approximately 2 kDa), whereas a minor peak was present in fraction 29 (approximately 5 kDa, proCNP(1–50)).

With an RIA (sheep 43) that detected the C-terminal end of NTproCNP [proCNP (38–50)], a single peak of immunoreactivity was detected in fraction 29 (Fig. 1B). No peaks were detected with the sheep 42 and sheep 45 RIAs (data not shown). These results showed that the predominant immunoreactive product of proCNP in urine was the smaller N-terminal metabolite proCNP 3–20 (2kDa) or a closely related form (see Fig. 1 in the online Data Supplement). To determine the pattern of urine excretion products in a larger sample of normal participants, further study was performed in 10 healthy adult controls. Demographic and laboratory data for these healthy volunteers, 30–47 years of age, are shown in Table 1 in the online Data Supplement. Renal function was normal, including ACR. As found previously, the 2-kDa peptide was predominant, with median concentrations being approximately 10-fold higher than for the larger (5-kDa) form. However, in a participant with DKD, a different profile of immunoreactive products was found. Fig. 1, C–F, shows the SE-HPLC profile for urine from a study participant with diabetes and eGFR 22 mL/min/1.73 m^2. The major peak of immunoreactivity was observed in fraction 30 for the J39 RIA (Fig. 1C) and fraction 29 for the sheep 43 RIA (Fig. 1D). A minor peak of immunoreactivity was observed at fraction 36–37 with the J39 RIA (Fig. 1C). Sheep 42 (Fig. 1F) and sheep 45 RIAs both detected the major 5-kDa peak at fraction 30, but only sheep 45 [proCNP (10–21)] showed weak cross-reactivity with the 2-kDa peak at fraction 36 (Fig. 1E). Further analysis using HPLC/RIA in 17 participants with diabetes showed that whereas the peak 2-kDa form was predominant in these patients, in patients with impaired renal function the ratio of peak 2 kDa to peak 5 kDa was closer to unity, supporting that the degradation to smaller products may be reduced as renal function deteriorates. Therefore, all subsequent analyses, both in healthy volunteers and those with diabetes, used the extraction method described above and an antiserum (sheep 43) specific to the C-terminus of proCNP (38–50), referred to here as intact NTproCNP in the context of measurements in urine. Overall, there was excellent agreement between the values from this inhouse assay and values calculated from fractions 29–30 in the HPLC-RIA ($r = 0.96$) with the sheep 43 antibody in 27 urine samples obtained from diabetic and healthy control study participants. Values of NCR in healthy participants did not differ from those found in diabetic participants with eGFR >60 mL/min/1.73 m^2. The lower limit of detection of the sheep 43 assay was 2 pg/mL (0.2 pg/mL for urine samples after 10-fold concentrating step). Intra- and interassay coefficients of variation for the sheep 43 urine assay were 5.8% and 7.4%, respectively, at 220 pg/mL. Addition of EDTA to the urine sample to inhibit any potential metalloproteases did not significantly affect NCR measurements ($P = 0.7$).

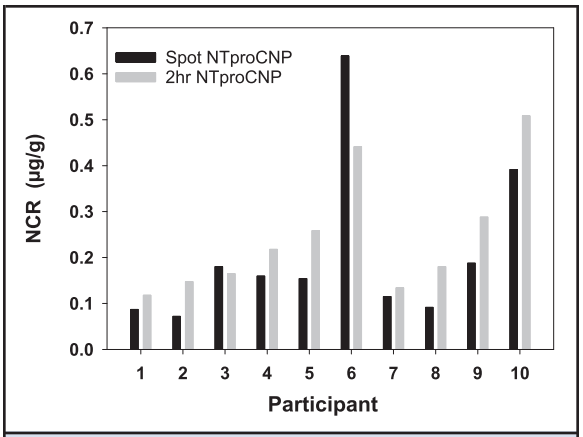

Fig. 2. NTproCNP/creatinine ratio (NCR) from healthy adult spot urine versus complete void 2 h later.

As shown in Fig. 2, NCR did not differ significantly in spot urine samples from those collected 2 h later ($P = 0.2$). In these healthy participants, the FE of NTproCNP was 0.08% (0.05%–0.09%).

CLINICAL AND LABORATORY FINDINGS IN PARTICIPANTS WITH DIABETES

Demographic, clinical, and laboratory data for 202 participants (101 T1DM, 101 T2DM, 17% Maori/Pacifica) are shown in Table 1. For all participants (50% women), the median age was 55 years and the duration of diabetes was 15 years. Overall, 53% reportedly had hypertension, 63% were receiving hypotensive therapy, and 33% were receiving treatment with angiotensin inhibitors or blockers. As shown in Table 1, in which indices in T1DM and T2DM are compared, significant differences in complications of diabetes and related treatments likely reflect the effect of age and diabetes phenotype. Median serum creatinine was 1.0 (0.9–1.2) mg/dL; eGFR, 72 (57–86) mL/min/1.73 m^2; NCR, 0.59 (0.39–0.91) µg/g; and ACR, 1.9 (0.8–17) mg/g. Median ACR and NCR values were significantly higher in women ($P <$ 0.001 for both). Whereas ACR was significantly higher in T2DM, neither plasma NTproCNP nor NCR differed between the 2 groups. The FE of NTproCNP [median, 0.07% (0.04–0.11)] was similar in T1DM and T2DM. FE was inversely associated with eGFR ($r = -0.26$) and positively associated with NCR ($r = 0.88$). FE was significantly higher in patients with CKD (eGFR <60 mL/min) than in patients with normal function [median (interquartile range), 0.09% (0.05%–0.13%) v 0.07% (0.04%–0.10%), respectively, $P = 0.011$].

Table 1. Demographic and laboratory values for participants with diabetes.[a]

	All participants (n = 202)	T1DM (*n* = 101)	T2DM (*n* = 101)	*P*[b]
Age, years	55 (41–66)	46 (30–64)	58 (51–68)	**<0.001**
Sex, % women	50	55	44	0.16
Family history of diabetes, %	70	62	78	**0.020**
Ethnicity, % European	76	88	64	**<0.001**
Duration, years	15 (10–24)	19 (13–30)	12 (6–20)	**<0.001**
Heart disease[c], %	19	9	30	**<0.001**
Retinopathy, %	31	40	23	**0.015**
Hypotensive drugs, %	63	47	80	**<0.001**
Angiotensin inhibitors or blockers, %	33	28	38	0.095
Insulin, %	88	98	77	**<0.001**
Statins, %	55	39	71	**<0.001**
Systolic BP[d], mmHg	135 (123–151)	131 (118–148)	139 (126–153)	**0.006**
Diastolic BP, mmHg	80 (73–86)	79 (71–84)	83 (75–89)	**0.003**
Body mass index, kg/m^2	31 (26–35)	27 (24–31)	34 (30–38)	**<0.001**
HbA1c, mmol/mol	67 (57–84)	66 (56–80)	69 (58–90)	0.13
Total cholesterol/HDL ratio	3.6 (2.9–4.6)	3.2 (2.8–4.1)	4.2 (3.4–5.0)	**<0.001**
Triglycerides, mg/dL	151 (106–213)	106 (80–151)	195 (148–270)	**<0.001**
Urate, mg/dL	5.0 (4.0–6.4)	4.4 (3.5–5.0)	5.7 (4.9–7.1)	**<0.001**
Cystatin C, mg/L	1.0 (0.8–1.2)	0.9 (0.7–1.0)	1.0 (0.8–1.4)	**<0.001**
Serum creatinine, mg/dL	1.03 (0.93–1.21)	1.02 (0.91–1.15)	1.04 (0.94–1.27)	0.19
eGFR, mL/min/1.73 m^2	72 (56–86)	76 (58–88)	70 (52–82)	**0.02**
Plasma NTproCNP, pg/mL	83 (67–111)	82 (67–108)	85 (69–121)	0.45
NCR, μg/g	0.59 (0.39–0.91)	0.56 (0.40–0.86)	0.60 (0.39–0.95)	0.51
ACR, mg/g	1.9 (0.8–17)	1.2 (0.6–4.4)	5.1 (1.1–43)	**<0.001**

[a] Values are medians (interquartile range).
[b] T1DM vs T2DM. Bold values, $P < 0.05$.
[c] Self-reported myocardial infarction, angina, heart failure.
[d] BP, blood pressure.

REPRODUCIBILITY OF ACR AND NCR

In 188 of the 202 participants, ACR and NCR were repeated within 1 month (median interval, 11 days). The coefficient of variation of NCR (36%) was significantly lower than that of ACR (50%, $P = 0.03$).

ASSOCIATIONS OF CLINICAL VARIABLES WITH ACR AND NCR

As shown in Table 2, across all participants there were no significant effects of age or duration of diabetes on either ACR or NCR. However, both body mass index and systolic blood pressure were positively associated with ACR but not with NCR. Positive associations of glycated hemoglobin A1c (HbA1c), urate, total cholesterol/HDL, and triglycerides with ACR were identified, whereas none of these was significantly associated with NCR. By contrast, ACR and NCR are negatively associated with eGFR (Fig. 3). When stratified by sex, all these associations were broadly similar in strength and direction. Significant (positive) associations of plasma NTproCNP and cystatin C with NCR and ACR were also observed.

In assessing independent predictors of NCR (Table 3, model 3), backward stepwise multivariable regression analysis of 10 variables known to be associated with renal function in diabetes (eGFR, diabetes type, ACR, HbA1c, hypertension, plasma NTproCNP, age, sex, and family history of diabetes or heart disease), only sex ($P < 0.001$), ACR ($P = 0.010$), and plasma creatinine ($P = 0.011$) were significant. When eGFR was the dependent variable (Table 3, model 1), age ($P < 0.001$), sex ($P < 0.001$), plasma creatinine ($P < 0.001$), NCR ($P = 0.011$), HbA1c ($P = 0.007$), and family history of heart disease ($P = 0.039$) were independent predictors. Similar findings applied when serum creatinine was the dependent variable, except that plasma NTproCNP ($P < 0.001$) was also an independent predictor. When ACR was the

Table 2. Associations of clinical variables with urine NCR and urine ACR.

	All participants (n = 202)		Women (n = 101)		Men (n = 101)	
	NCR	ACR	NCR	ACR	NCR	ACR
Age	0.12	0.04	0.11	0.03	0.16	0.07
Diabetes duration	0.09	0.04	0.16	−0.05	0.05	0.11
Hypertension	0.12	**0.31**[a]	**0.22**[b]	**0.22**[b]	0.04	**0.40**[a]
Body mass index	−0.07	**0.26**[a]	0.08	**0.23**[b]	−0.06	**0.30**[a]
Systolic BP[c]	0.05	**0.42**[a]	0.15	**0.40**[a]	0.07	**0.36**[a]
Diastolic BP	**−0.16**[b]	**0.24**[a]	−0.05	0.16	−0.14	**0.24**[b]
Retinopathy	0.05	**0.17**[b]	0.08	0.17	0.00	0.18
Ang I/B treatment	0.08	**0.19**[b]	0.14	0.10	0.07	**0.21**[b]
HbA1c	−0.08	**0.26**[a]	−0.12	**0.25**[b]	−0.02	**0.23**[b]
Plasma NTproCNP	**0.15**[b]	**0.35**[a]	**0.22**[b]	0.14	**0.28**[a]	**0.44**[a]
eGFR	**−0.31**[a]	**−0.27**[a]	**−0.28**[a]	**−0.21**[b]	**−0.32**[a]	**−0.35**[a]
Cystatin C	**0.23**[a]	**0.43**[a]	**0.34**[a]	**0.32**[a]	**0.20**[b]	**0.52**[a]
Urate	−0.01	**0.33**[a]	0.06	**0.20**[b]	0.10	**0.40**[a]
Cholesterol/HDL	−0.07	**0.25**[a]	−0.06	**0.24**[b]	0.03	0.18
Triglyceride	−0.04	**0.23**[a]	−0.04	**0.32**[a]	0.08	0.11

[a] $P < 0.001$.
[b] $P < 0.05$.
[c] BP, blood pressure; Ang I/B, angiotensin inhibitors/blockers.

dependent variable (Table 3, model 4), HbA1c (P = 0.001), NCR (P = 0.031), hypertension (P = 0.019), diabetes type (P = 0.027), and creatinine (P = 0.048) were all independent predictors of ACR.

PREDICTION OF CKD

For values of eGFR <60 mL/min/1.73 m^2, using cutoffs for ACR >30 and NCR >0.215, positive predictive values were 64% and 58% and negative predictive values were 76% and 74%, respectively. The area under the ROC curves for ACR or NCR to detect eGFR <60 mL/min/1.73 m^2 were 0.68 (0.60–0.76) and 0.68 (0.61–0.76), respectively.

Discussion

There is strong evidence supporting the renoprotective actions of CNP in counteracting tubular and interstitial inflammation and fibrosis *(14, 26)*. Not only is renal *NPPC* upregulated in animal models of injury-provoked fibrosis but equally importantly, paracrine actions of renal CNP acting via NPR2 and cGMP reduce mesangial cell proliferation and matrix production *(27)* and inhibit renal fibrosis, possibly by inhibiting tumor necrosis factor α and other cell cytokines *(28, 29)*. Because renal fibrosis appears to be a final common path driving renal impairment in diabetes mellitus *(30, 31)*, knowledge of the factors regulating renal CNP, its metabolism, and excretion of degraded products in urine are key to possible applications in the clinic. To this end, we used HPLC-RIA to characterize and identify components of proCNP in urine and focused on one of these (5-kDa NTproCNP) to evaluate the factors affecting its excretion in participants with diabetes.

Little is known of the renal handling of CNP products in any species, including renal clearance (and possible reabsorption), local production of proCNP, absorption into plasma, or degradation within renal tissues and resulting excretion (if any) of proCNP products in urine. Bioactive CNP22 (and possibly CNP53) circulate at low concentrations in healthy individuals and are unlikely to contribute to urine concentrations in view of the strong production of neprilysin and the clearance receptor NPR-3 in renal tissues *(26, 32)*. In contrast, the 5-kDa intact peptide NTproCNP is readily measurable in plasma and is strongly correlated in both sexes with serum creatinine concentrations within the normal range *(21)*. Although likely to be cleared at the glomerulus, the percentage of NTproCNP subject to reabsorption back into plasma—or degraded into smaller fragments within renal tissues—is unknown. Intrarenal processing of proCNP to bioactive and amino-terminal products is another putative source of CNP forms found in urine, par-

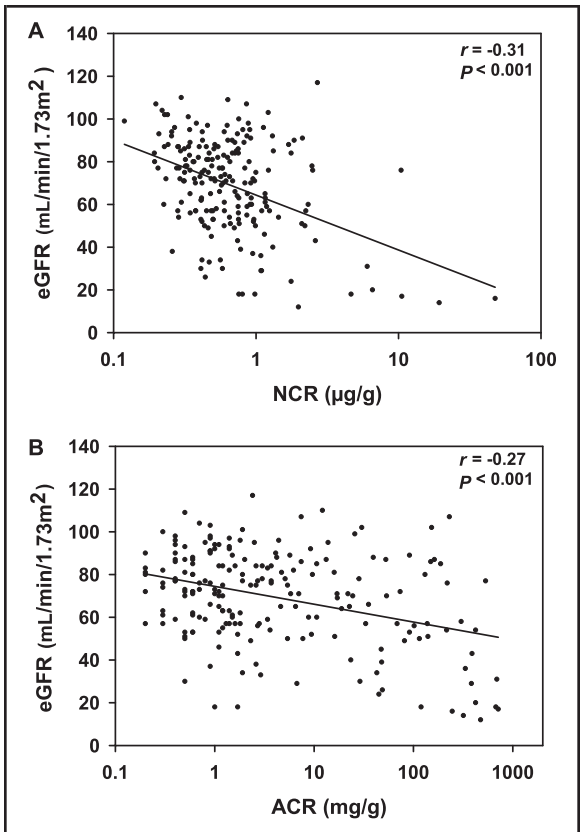

Fig. 3. Association of eGFR with urine NCR (A) and urine ACR (B).

ticularly in light of the upregulation of renal *NPPC* expression after renal injury, as shown in experimental animals. Again, both bioactive and bioinactive forms are presumably subject to both absorption back into plasma, local clearance and hydrolysis (specific to bioactive forms), and/or degradation to lower molecular weight species by renal protease activity before excretion in urine. Except for a report by Mattingly *(33)*, which reported absorbance, not immunoreactivity, we lack proof that any of these molecular forms are present in urine in any species, which seriously questions the reliability of quantitative estimates of urine CNP products reported in human studies *(34)*. Here we characterized the molecular forms of amino-terminal products of proCNP in urine from normal participants and to quantify these forms with an inhouse assay across a wide range of participants with T1DM and T2DM. Using a series of appropriate antisera targeting separate epitopes within the 5-kDa peptide, we show that the predominant form in normal participants is a small molecular weight (2-kDa) peptide. The intact form, NTproCNP, is present at 10-fold lower concentrations, showing that the contribution, if any, from plasma is trivial. Such high rates of degradation of the intact peptide align with previous reports showing the absence of intact NTproBNP from urine even in participants with heart failure, in whom plasma concentrations are greatly increased *(35)*, and are likely to apply to other products of proCNP such as proCNP 51–103 and proCNP 51–82. Consistent with this, we found no evidence of an immunoreactive peak corresponding to either CNP22 or CNP53 when using the Phoenix antiserum in the urine extract used in the HPLC-RIA study (Fig. 1), in which a conspicuous 5-kDa peak was identified. Our decision to focus on the measurement of the 5-kDa peptide was made on the basis of preliminary data in participants with chronic renal impairment, in whom proportionately larger amounts of the intact (compared to later-eluting smaller fragments) were found, raising the possibility that further processing of the 5-kDa form could be compromised as renal function declined. Because the fully developed in-house assay provided results comparable to values calculated from area under the curve analyses of the appropriate immunoreactive peak (HPLC-RIA) on the same urine sample, this assay was used in all subsequent analyses of urine collected from healthy volunteers and study participants. Findings of good reproducibility of random spot NCR and 2-h collections in healthy participant and reasonably consistent reproducibility in test-retesting in diabetes—superior to that of ACR—further attest to the stability of the analyte during collection and storage.

An underlying assumption that urine NTproCNP (indexed to creatinine excretion) reflects increased renal CNP production as function declines receives indirect support from the associative studies undertaken in participants with T1DM and T2DM. First, the data confirm significant associations of NCR with the presence of CKD, similar to those of the currently used marker ACR. Second, simultaneous measures of plasma and urine NTproCNP along with the measurement of renal function, not previously reported in any species, show that FE is very low and inversely associated with GFR. The fact that the apparent FE is significantly higher in those with CKD points to de novo renal production of proCNP as renal function declines. Importantly, the quite different relationships of NCR and ACR with a range of recognized risk factors linked with DKD are relevant in this context. The current test of renal damage, ACR, likely reflects loss of glomerular integrity and endothelial dysfunction *(3)* resulting from chronic hyperglycemia. Consistent with this are our findings of significant (previously identified) associations *(36)* of ACR with self-reported hypertension, body mass index, systolic blood pressure, HbA1C, urate, and lipids in both sexes. Notably, none of these factors were associated with NCR. Factors regulating renal CNP production have not been studied previously in humans with diabetes. The fact that only sex, ACR, and serum creatinine independently associate with

Table 3. Multivariable linear regression analyses.[a]

Model 1					
Dependent variable eGFR	*B*	*SE(B)*	*β*	*t*	*Sig*
(Constant)	344	5.8		59	**0.000**
Age	−0.58	0.02	−0.42	−25	**0.000**
Sex	16.0	0.8	0.36	20	**0.000**
Diabetes type	1.42	0.75	0.03	1.9	0.061
HbA1c	−0.05	0.02	0.05	−2.7	**0.007**
NCR	3.1	1.2	0.05	2.6	**0.011**
Family history of heart disease	−1.5	0.7	−0.03	−3.0	**0.039**
Plasma creatinine	−132	2.8	−0.89	−46	**0.000**
Model 2					
Dependent variable creatinine	*B*	*SE(B)*	*β*	*t*	*Sig*
(Constant)	1.289	0.053		24	**0.000**
Age	0.001	0.000	0.11	2.7	**0.008**
Sex	0.049	0.013	0.16	3.8	**0.000**
HbA1c	0.001	0.000	0.10	2.4	**0.016**
NCR	0.056	0.020	0.13	2.8	**0.005**
Plasma NTproCNP	0.453	0.029	0.71	15	**0.000**
Model 3					
Dependent variable NCR	*B*	*SE(B)*	*β*	*t*	*Sig*
(Constant)	−1.97	0.36		−5.4	**0.000**
Sex	−0.27	0.044	−0.38	−6.2	**0.000**
HbA1c	−0.002	0.001	−0.10	−1.7	0.093
ACR	0.068	0.026	0.18	2.6	**0.010**
Plasma NTproCNP	0.28	0.15	0.19	1.8	0.069
Plasma creatinine	0.64	0.25	0.27	2.6	**0.011**
Model 4					
Dependent variable ACR	*B*	*SE(B)*	*β*	*t*	*Sig*
(Constant)	−3.8	1.0		−3.9	**0.000**
Diabetes type (1/2)	0.26	0.12	0.14	2.2	**0.027**
HbA1c	0.010	0.003	0.20	3.5	**0.001**
NCR	0.38	0.17	0.14	2.2	**0.031**
Plasma NTproCNP	0.75	0.40	0.19	1.9	0.066
Hypertension	0.28	0.12	0.15	2.4	**0.019**
Plasma creatinine	1.25	0.63	0.20	2.0	**0.048**

[a] Plasma NTproCNP, urine NCR, urine ACR, and creatinine were $\log_{10}$ transformed to satisfy parametric assumptions. The coefficient of multiple determination (R^2) for each of the 4 models was 0.95, 0.70, 0.34, and 0.35. Bold values, $P < 0.05$.

NCR, contributing <34% to the measurement, opens up the possibility that factors other than microvascular disease are important. Relevant in this context are findings from experimental animals *(14, 26–28, 37, 38)* linking renal CNP production with renal inflammation and fibrosis markers, which should be measured in future studies.

Our study has several limitations. The identity of the molecular form(s) of the immunoreactive components contributing to the 5-kDa peak requires mass

spectrometry because minor deletions at the C or N terminus are likely to be measured in HPLC-RIA analyses. Although we have not established that NCR directly reflects intrarenal production of proCNP, evidence that NCR and the FE of NTproCNP are inversely associated with eGFR strongly supports that the kidney itself, not plasma NTproCNP, makes the major contribution to NCR. Because only a single spot measurement was made for each participant, future studies of sequential changes, examining the possibility that step up in NCR may predict progressive deterioration and/or those most at risk of rapid decline in renal function *(5)*, are now clearly warranted.

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