Pro-neurotensin depends on renal function and is related to all-cause mortality in chronic kidney disease

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Abstract

Background: Patients with chronic kidney disease (CKD) have a high risk of premature cardiovascular diseases (CVD) and show increased mortality. Pro-neurotensin (Pro-NT) was associated with metabolic diseases and predicted incident CVD and mortality. However, Pro-NT regulation in CKD and its potential role linking CKD and mortality have not been investigated, so far.

Methods: In a central lab, circulating Pro-NT was quantified in three independent cohorts comprising 4715 participants (cohort 1: patients with CKD; cohort 2: general population study; and cohort 3: non-diabetic population study). Urinary Pro-NT was assessed in part of the patients from cohort 1. In a 4th independent cohort, serum Pro-NT was further related to mortality in patients with advanced CKD. Tissue-specific *Nts* expression was further investigated in two mouse models of diabetic CKD and compared to non-diabetic control mice.

Results: Pro-NT significantly increased with deteriorating renal function ($P < 0.001$). In meta-analysis of cohorts 1–3, Pro-NT was significantly and independently associated with estimated glomerular filtration rate ($P \leq 0.002$). Patients in the middle/high Pro-NT tertiles at baseline had a higher all-cause mortality compared to the low Pro-NT tertile (Hazard ratio: 2.11, $P = 0.046$). Mice with severe diabetic CKD did not show increased *Nts* mRNA expression in different tissues compared to control animals.

Conclusions: Circulating Pro-NT is associated with impaired renal function in independent cohorts comprising 4715 subjects and is related to all-cause mortality in patients with end-stage kidney disease. Our human and rodent data are in accordance with the hypotheses that Pro-NT is eliminated by the kidneys and could potentially contribute to increased mortality observed in patients with CKD.

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Introduction

Cardiometabolic diseases, such as obesity (1), type 2 diabetes (T2D) (2), and chronic kidney disease (CKD) (3) are a major global health burden contributing to an increased morbidity and mortality in affected patients. Among others, a pro-inflammatory milieu (4) and a dysregulation of circulating cytokines might link these interrelated disease states. As an example, several cytokines, including adipocyte fatty acid-binding protein (AFABP) (5, 6, 7), are increased in cardiometabolic diseases and associate with cardiovascular (CV) events and mortality (8).

Recently, neurotensin (NT) was discovered as a peptide predominantly secreted by the intestine (9). Pro-NT 1–117, the stable NT precursor fragment in human blood, is associated with markers of obesity (10), new-onset obesity (11), nonalcoholic fatty liver disease (NAFLD) (12), T2D (13), and gestational diabetes mellitus (14, 15) in several independent cohorts. Importantly, Pro-NT was not only linked to metabolic disease states but also predicted incident CV diseases (13, 16) and mortality (13) in the Malmö Diet and Cancer Study and the Framingham Heart Study. Taking these data into account, NT appears to be a metabolically adverse peptide linking obesity and T2D with CV diseases and mortality.

Importantly, all these cardiometabolic diseases also act as independent risk factors for CKD. However, the regulation of Pro-NT in patients with CKD that show a very high CV and non-CV mortality (17) has not been investigated to date. Furthermore, the association of Pro-NT with markers of renal function has not been determined in the general population comprising diabetic and non-diabetic participants. Moreover, mortality analyses have not been carried out in patients with end-stage kidney disease (ESKD) to investigate whether Pro-NT is also linked to mortality in a CKD cohort with a narrow range of estimated glomerular filtration rate (eGFR).

Therefore, we measured serum levels of Pro-NT in three independent human cohorts comprising 4715 participants. Furthermore, we related Pro-NT levels to mortality in 163 ESKD patients. Moreover, we investigated NT regulation in two mouse models of diabetic kidney disease (DKD) vs two groups of non-diabetic control mice (18).

We hypothesized that Pro-NT as a cardiometabolic risk marker (1) is increased in patients with CKD; (2) is associated with markers of renal dysfunction also in general human cohorts; and (3) is an independent predictor for mortality in ESKD.

Subjects and methods

Human studies

Cohort 1 – cross-sectional (CKD, n = 581)

For this cross-sectional study, 581 patients (men: $n=338$; women: $n=243$) were recruited by the Department of Endocrinology and Nephrology, University of Leipzig, as well as from three different outpatient Nephrology Care Units (Hospital St. Georg, Division of Nephrology, KfH Renal Unit, Leipzig; outpatient Nephrology Care Units, Leipzig). Study design has been described previously (6, 19, 20, 21, 22). Briefly, inclusion criteria were an age >18 years, nonpregnant, and written informed consent. Exclusion criteria were end-stage malignant diseases, acute generalized inflammation, acute infectious disease, and history of drug abuse. Blood samples were taken from all participants after an overnight fast. In all hemodialysis patients, blood was obtained just before hemodialysis started. Based on their eGFR, patients were classified into eGFR categories G1 to G5 according to the Kidney Disease Improving Global Outcomes guidelines (23). Furthermore, a spot urine sample was obtained in subjects with preserved urinary excretion. In a sub-cohort, urinary Pro-NT was quantified in ten age-, sex-, and BMI-matched subjects of each eGFR category. The study was approved by the local ethics committee of the University of Leipzig (Reg. No: 180-13-15072013) and all subjects gave the written informed consent before taking part in the study.

Cohort 2 – cross-sectional (Sorbs, n = 1041)

In a second cohort, 1047 subjects were recruited by the Department of Endocrinology and Nephrology, University of Leipzig, between 2005 and 2007. All subjects are part of a sample from a self-contained population of the Sorbs from Eastern Germany, have not been specifically selected for metabolic or CVD states, and were recruited in medical practices at their hometowns of the upper Lusatia area. Design of the present study has been described previously (5, 24, 25). For the present analyses, 1041 samples were available. After an overnight fast, blood and spot urine samples were taken in the morning. All subjects gave written informed consent before taking part in the study which was approved by the ethics committee of the University of Leipzig (Reg. No. 088-2005).

Cohort 3 – cross-sectional (LIFE-Adult, n = 3093)

In a third cohort, 3093 non-diabetic subjects were included from the baseline examination of the LIFE-Adult

study of the Leipzig Research Centre for Civilization Diseases (LIFE) (26). LIFE-Adult is a large population-based study investigating prevalence, early onset markers, genetic predispositions, and the role of lifestyle factors on major civilization diseases in inhabitants of the city of Leipzig in Germany (26). Recruitment started in August 2011 and ended in November 2014 when having reached the planned sample size of 10 000 participants. Inclusion criteria for this study were a glycated hemoglobin A1c (HbA1c) <6.5% and/or an available oral glucose tolerance test. Exclusion criteria were a pre-existing or novel (e.g. based on the oral glucose tolerance test) diabetes mellitus, medication interfering with glucose homeostasis (e.g. glucocorticoids), manifest thyroid diseases, and active malignant diseases. The study was approved by the responsible institutional ethics board of the Medical Faculty of the University of Leipzig (Reg. No: 263-2009-14122009) and all subjects gave written informed consent before taking part in the study.

Cohort 4 – longitudinal (MIA, n = 163)

For mortality analyses, Pro-NT was investigated in a fourth, prospective, and observational study comprising incident dialysis patients as previously described (27, 28). Briefly, study participants were recruited among consecutive patients initiating dialysis at the Department of Renal Medicine, Karolinska University Hospital, from August 2004 to May 2016. For the present analyses, 163 serum samples at baseline were analyzed. Exclusion criteria were age <18 years, signs of overt clinical infection, and unwillingness to participate. The Ethics Committee of the Karolinska Institutet at the Campus Flemingsberg (EPN) Stockholm, Sweden, approved the study protocol and written informed consent was obtained from each patient.

Study protocols of all four human studies adhered to the statutes of the Declaration of Helsinki.

Animal studies

Animal experiments are described in the Supplementary methods (see section on supplementary materials given at the end of this article).

Assays

In all human cohorts, serum concentrations of Pro-NT were centrally quantified in a single lab by sphingotec (sphingotec GmbH, Hennigsdorf, Germany) from 2018 till 2019 using a chemiluminometric sandwich immunoassay (29). In recent, own validation analyses of this Pro-NT assay, intra-assay and inter-assay coefficients of variation were 3.6% and 4.3%, respectively (14). In cohorts 1–3, high sensitivity interleukin 6 (hsIL-6, R&D Systems) as well as leptin and adiponectin (both Mediagnost, Reutlingen, Germany) were determined using enzyme linked immunosorbent assays according to the manufacturers' instructions. Serum creatinine, fasting glucose (FG), fasting insulin (FI), HbA1c, TG, total, high density lipoprotein (HDL), low density lipoprotein (LDL) cholesterol, and urinary albumin and creatinine were measured in a certified laboratory by standard methods. For cohort 4, all biochemical analyses were performed at the Clinical Chemical Laboratory of Karolinska University Hospital, Stockholm, Sweden (30).

Statistical analysis

SPSS software version 24.0 (IBM) was used for statistical analyses of cohort data 1–3. In cohort 1 (CKD, $n = 581$), continuous parameters were adjusted for age, sex, and BMI and differences of the parameters between the eGFR categories were analyzed by Kruskal–Wallis test followed by post-hoc analysis. For adjustment, non-standardized residuals were calculated which were then taken forward as a new variable in the Kruskal–Wallis test. Categorical parameters were analyzed using the chi-squared-test. Univariate correlations of Pro-NT with anthropometric and clinical parameters were analyzed by non-parametric Spearman's rank correlation method. Afterward, linear regression analysis was conducted to identify independent predictors of Pro-NT. In the multivariate model, parameters that correlated significantly with Pro-NT in univariate analysis were included except for covariates. Before performing multivariate linear regression analyses, all continuous parameters were logarithmically transformed. In a sub-cohort, urinary Pro-NT/creatinine ratio and albumin/creatinine ratio (ACR) were compared using one-way ANOVA with prior logarithmic transformation.

For meta-analysis, MedCalc version 13 (MedCalc Software, Ostend, Belgium) was used. Standardized mean difference of circulating Pro-NT in patients with CKD compared to non-CKD subjects with 95% CIs for the fixed effects model and the random effects model were calculated using the Hedges g statistic. CKD status in all cohorts was defined as a single measurement of eGFR <60 mL/min/1.73 m^2 and/or an ACR ≥30mg/g (23) and all subjects with an eGFR ≥60 mL/min/1.73 m^2 and an ACR <30 mg/g were classified as non-CKD controls. In a second meta-analysis, the weighted summary regression

coefficient was calculated using the Hedges–Olkin method. Therefore, linear regression analysis between Pro-NT and eGFR with adjustment for age, sex, and BMI was performed in each cross-sectional cohort, that is, cohorts 1–3, separately and the respective standardized β was then taken forward as the correlation coefficients.

In cohort 4, statistical analyses were performed using Statistical Software Stata 16.0 (Stata Corporation) and SAS Version 9.4 (SAS Institute Inc., Cary, NC). The cohort was divided into Pro-NT tertiles. Multivariate Fine and Gray competing risk regression analysis (31), which is believed to be one of the most appropriate methods for prognostic studies especially in nephrology (32), was used for analyzing hazard ratios for middle/high Pro-NT tertiles vs low Pro-NT tertile (33) for all-cause mortality. For adjustment, the Framingham CVD risk score was calculated according to age- and sex-stratified tables with specific scores assigned for systolic blood pressure, diabetes mellitus, anti-hypertensive medication, total cholesterol, HDL cholesterol, and smoking status (30). Moreover, the analysis was further adjusted for BMI, history of CV diseases, as well as eGFR as a marker of residual renal function.

A *P*-value of <0.05 was considered as statistically significant in all analyses.

Results

Cohort 1 (CKD, *n* = 581)

Circulating Pro-NT levels increase with deteriorating renal function

Baseline characteristics of cohort 1 (CKD) divided into the eGFR categories G1 to G5 are shown in Table 1. Median (interquartile range) serum Pro-NT levels in the total

Table 1 Baseline characteristics of study population 1 (CKD, *n* = 581), divided into five eGFR categories. Values for median (interquartile range) or total number (percentage) are shown. Continuous parameters were adjusted for age, sex, and BMI and analyzed by Kruskal–Wallis test followed by post-hoc analysis. Categorical parameters were analyzed using the chi-squared-test. *P*-values for overall group differences are depicted. Numbers in superscript indicate $P < 0.05$ as compared to eGFR categories 1, 2, 3, or 4 in *post-hoc* tests.

	eGFR category					*P*
	G1	G2	G3	G4	G5	
n	57	87	133	74	230	
Pro-NT (pmol/L)	129.0 (60.6)	143.8 (77.2)	178.3 (106.7)[1,2]	224.9 (167.3)[1,2,3]	283.6 (184.2)[1,2,3,4]	<0.001
Age (years)	54.2 (18.8)	64.8 (14.6)	72.3 (13.7)[1,2]	72.9 (14.8)[1,2]	66.5 (21.2)[1,3,4]	<0.001
Sex (m/f)	27/30	41/46	89/44	40/34	141/89	0.012
Diabetes (%)	20 (35)	35 (40)	56 (42)	26 (35)	90 (39)	0.840
BMI (kg/m^2)	26.4 (5.9)	28.7 (5.8)	28.3 (5.4)	26.5 (5.4)	26.9 (7.2)[2]	0.001
WHR	0.91 (0.10)	0.92 (0.12)	0.96 (0.09)	0.96 (0.10)	0.97 (0.10)[1,2,3]	<0.001
WHtR	0.56 (0.11)	0.60 (0.13)	0.60 (0.10)	0.60 (0.10)	0.61 (0.13)[1,2,3,4]	<0.001
SBP (mmHg)	129 (20)	130 (30)	135 (25)	140 (22)	130 (28)[4]	0.011
DBP (mmHg)	80 (15)	80 (20)	80 (17)	80 (19)	73 (15)[2,3,4]	<0.001
Creatinine (µmol/L)	63 (18)	82 (24)	138 (44)[1,2]	214 (62)[1,2,3]	687 (371)[1,2,3,4]	<0.001
eGFR (mL/min/1.73 m^2)	98.8 (11.8)	74.1 (12.8)	41.7 (14.2)[1,2]	23.7 (6.0)[1,2,3]	5.9 (4.2)[1,2,3,4]	<0.001
ACR (mg/g)	7.1 (10.0)	11.6 (43.0)	22.5 (62.7)	94.4 (232.7)[1,2,3]	231.5 (461.0)[1,2,3,4]	<0.001
FG (mmol/L)	5.3 (1.7)	5.8 (2.0)	5.8 (2.0)	5.7 (1.7)	4.9 (1.8)[1,2,3,4]	<0.001
FI (pmol/L)	55.6 (52.2)	71.5 (71.8)	76.6 (95.8)	74.0 (56.8)	45.5 (71.6)[3,4]	<0.001
HOMA-IR	2.0 (2.2)	2.5 (3.0)	2.6 (3.8)	2.7 (2.6)	1.4 (2.4)[3,4]	<0.001
HbA1c (%)	5.5 (0.5)	5.7 (0.7)	5.8 (0.6)	5.8 (0.6)	5.4 (0.7)[3,4]	<0.001
HbA1c (mmol/mol)	36.6 (4.9)	38.8 (7.7)	39.9 (6.6)	39.3 (6.8)	35.5 (7.7)[3,4]	<0.001
TG (mmol/L)	1.17 (0.79)	1.52 (0.92)[1]	1.59 (1.07)[1]	1.64 (1.35)[1]	1.57 (1.02)[1]	<0.001
Cholesterol (mmol/L)	5.3 (1.5)	5.5 (1.4)	5.3 (1.7)	6.2 (2.4)	4.6 (1.7)[1,2,3,4]	<0.001
HDL cholesterol (mmol/L)	1.4 (0.6)	1.3 (0.4)	1.3 (0.5)	1.4 (0.7)	1.1 (0.5)[1,2,3,4]	<0.001
LDL cholesterol (mmol/L)	3.3 (1.4)	3.2 (1.4)	3.0 (1.4)	3.2 (1.8)	2.6 (1.4)[1,2,3,4]	<0.001
hsIL-6 (ng/L)	1.5 (1.4)	1.7 (1.2)	2.4 (1.9)[1]	2.9 (3.1)[1,2]	5.0 (5.6)[1,2,3,4]	<0.001
Leptin (µg/L)	11.4 (15.6)	17.6 (25.0)	20.6 (40.5)[1,2]	21.9 (40.1)[1,2]	20.2 (48.1)[1,2]	<0.001
Adiponectin (mg/L)	6.4 (6.4)	6.7 (7.0)	9.1 (9.0)[1,2]	10.9 (12.6)[1,2]	15.6 (14.0)[1,2,3]	<0.001

ACR, albumin/creatinine ratio; CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FG, fasting glucose; FI, fasting insulin; HbA1c, glycated hemoglobin A1c; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; hsIL-6, high sensitivity interleukin 6; LDL, low density lipoprotein; NT, neurotensin; SBP, systolic blood pressure; TG, triglycerides; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

sample were 201.4 (153.1) pmol/L. Pro-NT adjusted for age, sex, and BMI significantly increased with deteriorating renal function (Table 1, $P < 0.001$). Circulating Pro-NT did not depend on sex (female subjects: 190.9 (153.2) pmol/L; male subjects: 206.6 (151.2) pmol/L; $P=0.387$). In contrast, patients with T2D (214.7 (160.3) pmol/L; $n=227$) had significantly higher Pro-NT levels as compared to non-diabetic subjects (185.7 (150.9) pmol/L; $n=354$) ($P=0.012$).

Uni- and multi-variate correlations

In univariate correlation analysis, serum levels of Pro-NT were significantly and positively correlated with waist-to-hip ratio, creatinine, ACR, hsIL-6, and adiponectin (Table 2). In contrast, Pro-NT was negatively and significantly associated with BMI, systolic and diastolic blood pressure, eGFR, FG, FI, homeostasis model assessment of insulin resistance (HOMA-IR), total cholesterol, HDL cholesterol, and LDL cholesterol in univariate analysis (Table 2). To identify independent associations, multiple linear regression analysis was carried out. eGFR was the strongest, negative predictor of Pro-NT serum levels after adjustment for age, sex, as well as for markers of obesity, blood pressure, dyslipidemia, inflammation, and glucose homeostasis (Table 2). Furthermore, LDL cholesterol was independently and negatively correlated with Pro-NT in multivariate analysis (Table 2). In a further linear regression model used for the meta-analysis, eGFR remained a significant and negative predictor of Pro-NT after adjustment for age, sex, and BMI (data not shown).

Urinary Pro-NT

In a sub-cohort, urinary Pro-NT/creatinine ratio was quantified in ten age-, sex-, and BMI-matched subjects of each eGFR category. Mean ± SD. of urinary Pro-NT levels were 4.5 ± 6.1 pmol/L and were around the limit of detection of the assay. After adjustment for urinary creatinine, urinary Pro-NT/creatinine ratio was significantly different between the eGFR categories ($P=0.017$) (Supplementary Fig. 1).

Cohort 2 (Sorbs, *n* = 1041)

Baseline characteristics of cohort 2 are summarized in Table 3. Median circulating Pro-NT was 114.7 (59.1) pmol/L.

Table 2 Univariate correlations with serum Pro-NT in study population 1 (CKD, $n = 581$) and multiple regression analysis between Pro-NT (dependent variable) and eGFR adjusted for age, sex, BMI, DBP, HDL cholesterol, LDL cholesterol, hsIL-6, and adiponectin. Non-normally distributed variables as assessed by Shapiro–Wilk-test were logarithmically transformed prior to multivariate testing. *r*- and *P*-values, as well as standardized β-coefficients, and *P*-values are given.

	Univariate correlations		Multiple regression analysis	
	r	*P*	β	*P*
Age (years)	0.031	0.457	0.030	0.431
Sex	-	-	0.004	0.926
BMI (kg/m^2)	**−0.112**	**0.007***	−0.044	0.272
WHR	**0.098**	**0.021***	-	-
WHtR	0.010	0.804	-	-
SBP (mmHg)	**−0.086**	**0.038***	-	-
DBP (mmHg)	**−0.190**	**<0.001***	−0.040	0.303
Creatinine (µmol/L)	**0.553**	**<0.001***	-	-
eGFR (mL/min/1.73m^2)	**−0.558**	**<0.001***	**−0.510**	**<0.001**†
ACR (mg/g)	**0.371**	**<0.001***	-	-
FG (mmol/L)	**−0.100**	**0.019***	-	-
FI (pmol/L)	**−0.091**	**0.033***	-	-
HOMA-IR	**−0.105**	**0.013***	-	-
HbA1c (%)	−0.070	0.154	-	-
TG (mmol/L)	0.035	0.418	-	-
Cholesterol (mmol/L)	**−0.191**	**<0.001***	-	-
HDL cholesterol (mmol/L)	**−0.113**	**0.008***	0.035	0.456
LDL cholesterol (mmol/L)	**−0.201**	**<0.001***	**−0.111**	**0.006**†
hsIL-6 (ng/L)	**0.276**	**<0.001***	−0.068	0.135
Leptin (µg/L)	0.069	0.112	-	-
Adiponectin (mg/L)	**0.262**	**<0.001***	0.074	0.115

Abbreviations are indicated in Table 1.
*Indicates significant correlation as assessed by Spearman's correlation method; †Indicates significant correlation in multivariate analysis.

Table 3 Baseline characteristics and multivariate regression analysis in study population 2 (Sorbs, *n* = 1041). Baseline characteristics of study population 2 (Sorbs, *n* = 1041) and linear regression analyses with Pro-NT (dependent variable), age, sex, and BMI as covariates, as well as creatinine, eGFR, or ACR, respectively. All parameters were logarithmically transformed prior to regression analysis.

	Baseline characteristics	Multivariate regression analyses		
		Covariates	β	*P*
n	1041			
Pro-NT (pmol/L)	114.7 (59.1)			
Age (years)	48.4 (23.7)			
Sex (m/f)	422/619			
Diabetes (%)	114 (11.0)			
BMI (kg/m^2)	26.4 (6.5)			
Creatinine (µmol/L)	70 (18)	Age, sex, BMI	**0.133**	**0.001***
eGFR (mL/min/1.73 m^2)	97.9 (21.2)	Age, sex, BMI	**−0.171**	**<0.001***
ACR (mg/g)	6.7 (9.0)	Age, sex, BMI	-	n.s.

n.s., Not significant; all other abbreviations are indicated in Table 1. Values for median (interquartile range) or total number (percentage), as well as standardized β-coefficients and *P*-values, are shown.
*Indicates $P < 0.05$ for each separate linear regression analysis.

In linear regression models, eGFR remained a significant and negative predictor of Pro-NT after adjustment for age, sex, and BMI (Table 3). Furthermore, when creatinine instead of eGFR was included in the model, creatinine was significantly and positively related to Pro-NT (Table 3). In contrast, ACR was not an independent predictor of Pro-NT levels in this cohort (Table 3).

Cohort 3 (LIFE-Adult, *n* = 3093)

Baseline characteristics of cohort 3 are summarized in Table 4. Median circulating Pro-NT was 107.5 (56.0) pmol/L. In linear regression models, creatinine was the strongest renal predictor of serum concentrations of Pro-NT after adjustment for age, sex, and BMI (Table 4). Furthermore, eGFR was significantly and negatively correlated with Pro-NT (Table 4). Again, ACR was not an independent predictor of Pro-NT levels in this cohort (Table 4). Abbreviations are indicated in Table 1.

Meta-analyses of the cohorts 1–3 (*n* = 4715)

All three cohorts were included in a first meta-analysis comparing the means of circulating Pro-NT between patients with CKD (total $n = 1063$) as compared to non-CKD patients (total $n = 3652$) (Fig. 1A). The standardized mean difference (95% CI) between patients with CKD as compared to non-CKD patients was 0.402 (0.267–0.537) ($P < 0.001$) as assessed by a random effects model and 0.387 (0.307–0.467) ($P < 0.001$) by a fixed effects model (Fig. 1A). Cochran's Q was 4.233 ($P = 0.121$) and I^2 statistic was 52.75 % (0.00–86.43), respectively.

Table 4 Baseline characteristics and multivariate regression analysis in study population 3 (LIFE-Adult, *n* = 3093). Baseline characteristics of study population 3 (LIFE-Adult, *n* = 3093) and linear regression analyses with Pro-NT (dependent variable), age, sex, and BMI as covariates, as well as creatinine, eGFR, or ACR, respectively. All parameters were logarithmically transformed prior to regression analysis. Values for median (interquartile range) or total number (percentage), as well as standardized β-coefficients and *P*-values, are shown.

	Baseline characteristics	Multivariate regression analyses		
		Covariates	β	*P*
n	3093			
Pro-NT (pmol/L)	107.5 (56.0)			
Age (years)	64.3 (16.7)			
Sex (m/f)	1445/1648			
Diabetes (%)	0 (0)			
BMI (kg/m^2)	26.7 (5.4)			
Creatinine (µmol/L)	79 (20)	Age, sex, BMI	**0.208**	**<0.001***
eGFR (mL/min/1.73 m^2)	78.6 (20.8)	Age, sex, BMI	**−0.204**	**<0.001***
ACR (mg/g)	5.4 (7.2)	Age, sex, BMI	0.030	0.108

*Indicates $P < 0.05$ for each separate linear regression analysis.

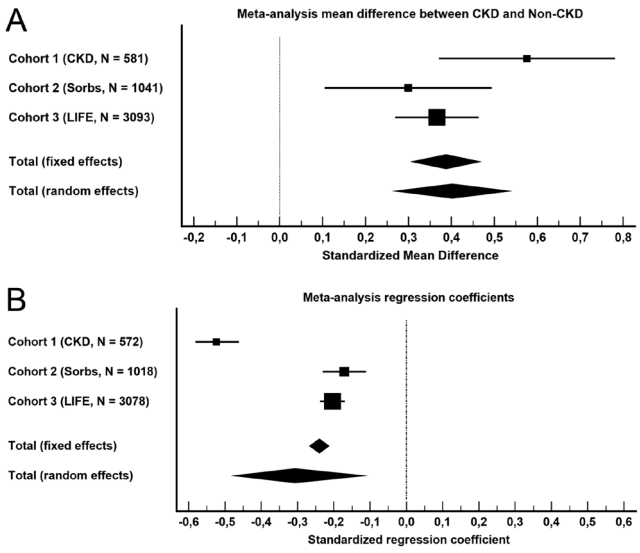

Figure 1

Meta-analyses of serum concentrations of Pro-NT in the three cross-sectional cohorts, that is, cohort 1 (CKD), cohort 2 (Sorbs), and cohort 3 (LIFE-Adult). (A) Standardized mean difference of circulating Pro-NT with 95% CIs in patients with CKD (total $n = 1063$) as compared to non-CKD patients (total $n = 3652$) were analyzed using the Hedges g statistic. CKD status in all cohorts was defined as an estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 and/or an albumin/creatinine ratio (ACR) ≥30 mg/g. Marker size is relative to study weight, respectively. (B) Standardized meta-regression coefficient of the association between Pro-NT and eGFR as assessed by the Hedges–Olkin method. Linear regression analysis between Pro-NT and eGFR with adjustment for age, sex, and BMI was performed in each cross-sectional cohort, that is, cohorts 1–3 separately, and the standardized β was then taken forward in the meta-analysis as the correlation coefficients, respectively. Marker size is relative to study weight, respectively. In both meta-analyses, total effects are presented based on a fixed effects model and a random effects model.

Furthermore, the standardized β coefficients of the association between Pro-NT and eGFR obtained in multivariate regression models from cohorts 1–3 were taken forward in a second meta-analysis. Using these standardized β-coefficients adjusted for age, sex, and BMI, the meta-regression coefficient between Pro-NT and eGFR was investigated. The meta-regression coefficient of the association between Pro-NT and eGFR adjusted for age, sex, and BMI was −0.307 (−0.478 to −0.115) ($P=0.002$) as assessed by a random effects model and −0.240 (−0.267 to −0.213) ($P < 0.001$) by a fixed effects model (Fig. 1B). Cochran's Q was 74.328 ($P < 0.001$) and I^2 statistic was 97.31 % (94.70–98.63), respectively. Heterogeneity was caused by cohort 1 showing a significantly stronger effect than the other cohorts. However, effect was significant and direction consistent in all three cohorts.

Cohort 4 (longitudinal MIA cohort, *n* = 163)

The patients were followed from the inclusion date until renal transplantation ($n=89$), death ($n=45$), completion of 60 months of follow-up, or until 1 January 2018. After stratifying the cohort into baseline Pro-NT tertiles (Table 5), patients in the middle/high Pro-NT tertiles had a higher all-cause mortality as compared to patients in the low Pro-NT tertile (Hazard ratio (95% CI): 2.11 (1.01–4.41), $P=0.046$) after adjustment for baseline BMI, presence of CV disease, the Framingham CV disease risk score, as well as eGFR as a marker of residual renal function (Fig. 2 and Table 6).

Animal experiments

Figure 3 summarizes animal data on the regulation of the *Nts* mRNA expression in mice with severe DKD ($eNOS^{-/-}$;*db/db*) and mild DKD (*db/db*) compared to two non-diabetic control groups ($eNOS^{-/-}$ and *db/+* mice). In mice with DKD, mRNA expression of *Nts* was reduced in BAT compared to non-diabetic control mice (Fig. 3A). Furthermore, mice with severe DKD had reduced *Nts* expression in the hypothalamus compared to control animals (Fig. 3F). Mice with mild DKD, that is, *db/db* mice, had lower mRNA expression of *Nts* in SAT, as well as in the kidney, compared to $eNOS^{-/-}$ mice (Fig. 3C and E). In contrast, no significant differential expression of *Nts* was found in VAT and the liver (Fig. 3B and D).

Discussion

We show that circulating Pro-NT is significantly increased in human CKD depending on the disease severity and Pro-NT is independently associated with impaired renal function in the general population. Furthermore, Pro-NT is a predictor of all-cause mortality in incident dialysis patients independent of traditional risk factors. Importantly, *Nts* mRNA expression in mice with DKD is not significantly increased in adipose tissue depots, liver, kidney, and hypothalamus compared to non-diabetic control mice with preserved renal function.

Table 5 Baseline characteristics of study population 4 (longitudinal MIA, $n = 163$) stratified into two groups according to tertiles (33) of Pro-NT. Values for median (interquartile range) or total number (percentage) and *P*-values are shown.

	Lowest Pro-NT tertile	Middle and highest Pro-NT tertiles	*P*
n	54	109	
Pro-NT (pmol/L)	142 (86.2–174.5)	278 (195.3–413.9)	**0.001***
Age (years)	54.0 (47.1–63.0)	57.5 (44.0–65.5)	0.437
Sex (m/f)	31/23	75/34	0.154
Diabetes (%)	13 (24.5%)	40 (37.0%)	0.107
CVD (%)	20 (37.7%)	42 (38.9%)	0.887
BMI (kg/m^2)	25.3 (21.5–28.4)	24.0 (22.3–27.9)	0.533
eGFR (mL/min/1.73 m^2)	5.3 (4.7–7.4)	5.9 (4.9–7.5)	0.481
IL-6 (ng/L)	6.1 (3.7–9.2)	6.2 (3.0–10.3)	0.996

CVD, Cardiovascular disease; all other abbreviations are indicated in Table 1.
*Indicates $P < 0.05$ for group differences as assessed by Mann–Whitney *U*-test.

Our results indicate that circulating levels of the adverse peptide Pro-NT are increased with deteriorating renal function. Many other metabolic cytokines are also crucially regulated by renal function including leptin (34), adiponectin (35), visfatin (36), progranulin (21, 37), AFABP (6), FGF21 (20), and follistatin-like 3 (22). Importantly, sex-specific associations of Pro-NT with incident diabetes, CV disease, and mortality have been reported (13) and Pro-NT is mechanistically linked to fat mass (11). Thus, all analyses have been adjusted for age, sex, and BMI which did not affect the observed association of Pro-NT with renal function in all analyzed cohorts.

Pro-NT has been linked to incident CV diseases (13, 16) and mortality (13) in the Malmö Diet and Cancer Study and the Framingham Heart Study. Hypothetically, increased Pro-NT could contribute to the substantially elevated risk for CV diseases and mortality in renal dysfunction (38). Therefore, we investigated whether Pro-NT predicts mortality in ESKD patients with a narrow range of eGFR. Since we find that Pro-NT is a significant predictor of all-cause mortality even after adjustment for BMI and residual renal function, as well as traditional CV diseases risk factors, Pro-NT potentially could be a link between CKD/ESKD and mortality. Further and larger studies need to investigate the association of Pro-NT with mortality in earlier CKD stages.

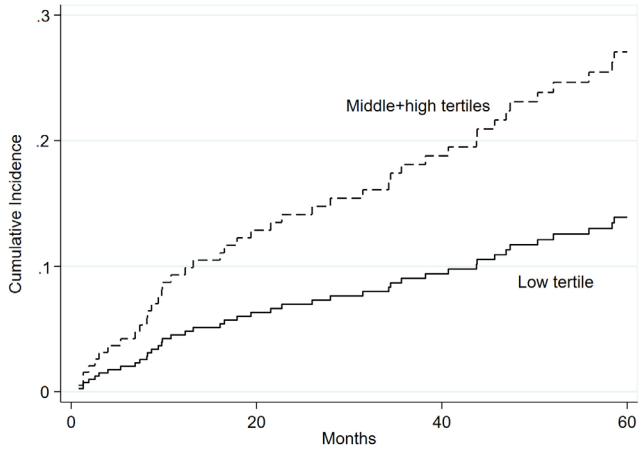

Figure 2
Multivariate Fine and Gray competing risk regression analysis in cohort 4, that is, longitudinal MIA cohort. The cohort was split into Pro-NT tertiles. Cumulative incidence of all-cause mortality over the follow-up period of about 60 months is depicted for the groups middle/high Pro-NT tertiles vs low Pro-NT tertile. We adjusted for history of cardiovascular diseases (CVD), 1-s.d. of body mass index, 1-s.d. of eGFR as a marker of residual renal function, as well as 1-s.d. of Framingham's CVD risk score. Abbreviations as indicated in Fig. 1.

Table 6 Multivariate Fine and Gray competing risk regression analysis for Pro-NT tertiles (middle/high Pro-NT tertiles vs low Pro-NT tertile) and all-cause mortality adjusted for history of cardiovascular diseases (CVD), 1-s.d. of body mass index (BMI), 1-s.d. of estimated glomerular filtration rate (eGFR) as a marker of residual renal function, as well as 1-s.d. of Framingham's CVD risk score. Values are presented as sub-hazard ratios (sHR) with 95% CIs and *P*-values.

	sHR	95% CI	*P*
Pro-NT tertile (low vs middle/high)	2.113	1.013–4.407	**0.046**†
1-s.d. of Framingham CVD score	1.521	1.084–2.135	**0.015**†
1-s.d. of BMI (kg/m^2)	0.707	0.479–1.043	0.080
1-s.d. of eGFR (mL/min/1.73 m^2)	1.20	0.863–1.667	0.277
Presence of CVD	3.272	1.457–7.344	**0.004**†

†Indicates $P < 0.05$ as assessed by competing-risks regression analysis. Abbreviations are indicated in Table 1.

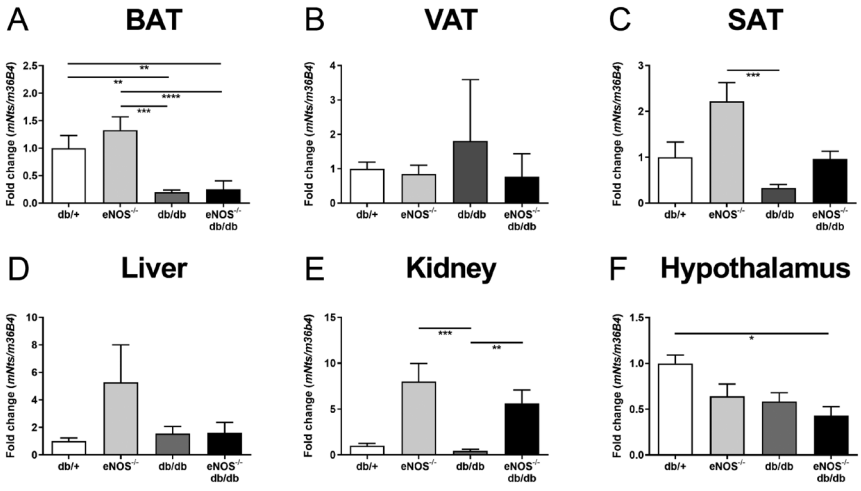

Figure 3

Regulation of mRNA expression of *Nts* in mice with severe chronic kidney disease (CKD) due to diabetic kidney disease, that is, eNOS$^{-/-}$;*db/db* mice (black bars) and mild CKD, that is, *db/db* mice (dark grey bars), as compared to two groups of non-diabetic control mice, that is, eNOS$^{-/-}$ mice (light grey bars) and *db/+* mice (white grey bars). *Nts* mRNA expression in (A) brown adipose tissue (BAT), (B) visceral adipose tissue (VAT), (C) subcutaneous adipose tissue (SAT), (D) liver, (E) kidney, and (F) hypothalamus was determined and normalized to *36B4* expression as described in the Supplementary Methods section. Results are shown as means ± S.E.M. *P*-values were assessed by one-way ANOVA with Tukey's multiple comparisons test with prior logarithmic transformation. $n \geq 3$ per group. *Indicates $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

Different mechanisms for the observed increase of Pro-NT in CKD are possible. Thus, renal Pro-NT retention due to impairment of renal function could increase circulating Pro-NT as seen for other cytokines including leptin (39). Alternatively or in addition, upregulated *Nts* expression in target tissues could also contribute to increased circulating levels of the peptide. Our animal studies indicate that the *Nts* mRNA expression is not significantly upregulated in organs that show a different physiology in the four groups of mice investigated, that is, adipose tissue depots, liver, and kidney. In contrast, *Nts* mRNA expression in the hypothalamus, which is a major organ contributing to systemic metabolic effects of NT (40), is downregulated in more advanced models of murine CKD compared to non-diabetic control mice. Taking our mouse and human data into consideration, increased circulating Pro-NT in renal dysfunction is likely caused by reduced renal elimination rather than increased *Nts* expression. Interestingly, the same regulation pattern has been shown for the adipokine leptin with reduced expression of the leptin-coding *ob*-gene in patients with advanced CKD (39). In accordance with the hypothesis of reduced Pro-NT elimination in CKD, urinary Pro-NT levels are very low and around the limit of detection of the assay in the human CKD study. Despite the low urinary Pro-NT levels, urinary Pro-NT/creatinine ratio was significantly different between the eGFR categories, further supporting the observed regulation of Pro-NT and renal function. Indeed, Shulkes *et al*. showed that plasma NT levels are increased in ten patients with ESKD as compared to ten healthy controls (41). Furthermore, they demonstrated a decreased metabolic clearance rate of NT compared to controls (41). Thus, since both NT and Pro-NT appear to be increased in CKD, studies need to determine whether Pro-NT is causally involved in the pathogenesis of CKD. Based on our findings, adjustment for renal function should always be done when circulating Pro-NT is related to outcome.

With regards to the association of Pro-NT with cardiometabolic diseases and mortality (13, 16), it is notable that Pro-NT is not independently related to an adverse cardiometabolic profile, for example, LDL cholesterol, hsIL-6, and blood pressure, in patients with CKD. This supports data from the Framingham Heart Study showing no association of Pro-NT with LDL cholesterol, age, and sex (16). In contrast, Melander *et al*. demonstrate a weak but significant association between Pro-NT and cardiometabolic risk factors, for example, FI (13). It should be noted that, in patients with advanced CKD/ESKD, the associations between distinct cardiometabolic

risk factors with mortality differ as compared to the general population. As an example, LDL cholesterol is not predictive of vascular outcome (42) and treatment with atorvastatin does not have a statistically significant effect on a CV end point in patients with T2D and ESKD (43). Since it is likely that inflammation and/or protein energy wasting change the risk factor profile in ESKD (44), the predictive effect of NT and Pro-NT needs to be assessed in inflamed vs non-inflamed patients.

Limitations of the present study include its cross-sectional design in cohorts 1–3, preventing us from drawing conclusions on mechanisms by which NT promotes CKD. Moreover, we did not measure Pro-NT in the animal experiment since there is no reliable, commercial ELISA available. In addition, circulating Pro-NT levels are not necessarily associated with the mRNA expression of the *Nts* gene. Consequently, we cannot prove a causal nexus behind our observations. However, phenotyping for all cohorts and the mouse experiments were performed at a high level of standardization by a trained study team. Furthermore, we used the same ELISA system for the quantification of human Pro-NT like all other cohorts (11, 13, 14, 16) limiting potential technical aspects.

In conclusion, circulating Pro-NT is significantly associated with impaired renal function. Furthermore, Pro-NT is independently associated with increased all-cause mortality in ESKD. Animal experiments suggest that renal retention of Pro-NT is likely the cause for increased circulating Pro-NT. Our results are in accordance with the hypotheses that Pro-NT is eliminated by the kidneys and could potentially contribute to increased mortality observed in patients with CKD. Further mechanistic studies need to determine whether Pro-NT is causally involved in the pathogenesis of CKD and its complications.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EJE-20-0087.

Declaration of interest

M Sch receives funding from Pfizer Inc. for a project not related to this research. The other authors have nothing to declare.

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Author contribution statement

A T, A H, M F, and T E wrote the manuscript and researched data. S K, A R Q, N K, M S, D S, A B, J K, M N, S P, K W, C E, R B, J B, M A, and I B researched data, supervised the analyses, and/or reviewed/edited the manuscript. M B, P K, M-Z Z, R C H, P S, and M S contributed to the discussion and reviewed/edited the manuscript. Dr Thomas Ebert is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. A Tönjes, A Hoffmann, M Fasshauer and T Ebert contributed equally to this work.

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