Biomarkers

ISSN: 1354-750X (Print) 1366-5804 (Online) Journal homepage: www.tandfonline.com/journals/ibmk20

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To cite this article: Lyanne M. Kieneker, Oliver Hartmann, Andreas Bergmann, Rudolf A. de Boer, Ron T. Gansevoort, Michel M. Joosten, Joachim Struck & Stephan J. L. Bakker (2018) Proenkephalin and risk of developing chronic kidney disease: the Prevention of Renal and Vascular End-stage Disease study, Biomarkers, 23:5, 474-482, DOI: 10.1080/1354750X.2018.1443514

To link to this article: https://doi.org/10.1080/1354750X.2018.1443514

Published online: 08 Mar 2018.

BIOMARKERS
2018, VOL. 23, NO. 5, 474–482
https://doi.org/10.1080/1354750X.2018.1443514

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RESEARCH ARTICLE

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Proenkephalin and risk of developing chronic kidney disease: the Prevention of Renal and Vascular End-stage Disease study

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ABSTRACT

Background: Proenkephalin (pro-ENK) was recently found to be associated with low estimated glomerular filtration rate (eGFR). The association of pro-ENK with urinary albumin excretion (UAE), another marker for chronic kidney disease (CKD), has not been investigated. We examined the association of pro-ENK with eGFR and UAE as markers of CKD.

Methods: We included 4375 subjects of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study. CKD_{eGFR} was defined as development of eGFR <60 ml/min/1.73 m^2 and CKD_{UAE} as albuminuria >30 mg/24 h.

Results: Baseline median pro-ENK was 52.2 (IQR: 44.9–60.5) pmol/L. After a median follow-up of 8.4 (IQR: 7.9–8.9) years, 183 subjects developed CKD_{eGFR} and 371 developed CKD_{UAE}. The association of pro-ENK with CKD_{eGFR} was modified by sex ($P_{interaction} < 0.1$), in such a way that after adjustment, the association only remained significant in men (adjusted hazard ratio per *SD* increase in ^{10}log-transformed pro-ENK, 1.65; 95% CI: 1.15–2.36) and not in women (0.83; 0.58–1.20). No significant association was observed between pro-ENK and CKD_{UAE} risk (0.83; 0.58–1.20).

Conclusions: High pro-ENK is associated with increased risk of CKD_{eGFR} in men, but not in women. No association of pro-ENK with CKD_{UAE} was observed. These results should be interpreted with caution, since residual confounding and potential overfitting of models could have influenced the results.

ARTICLE HISTORY
Received 1 December 2017
Revised 12 February 2018
Accepted 18 February 2018

KEYWORDS
Biomarker; chronic kidney disease; epidemiology

Introduction

Chronic kidney disease (CKD) is a major public health problem, with adverse outcomes of kidney failure, cardiovascular disease and premature death (Levey *et al*. 2009). Early identification of CKD is needed to prevent disease progression, reduce the risk of cardiovascular morbidity and mortality, and improve survival and quality of life.

Recently, pro-enkephalin (pro-ENK) was introduced as a potential new biomarker for identifying normal subjects at high risk of future decline in renal function. Pro-ENK is a stable precursor fragment of unstable enkephalins, which are well-known endogenous opioid peptides, and it has been hypothesized that enkephalins play an important biological function in renal physiology because of dense expression of enkephalin-responsive delta-opioid receptors in the kidney (Denning *et al*. 2008). It has been shown that stimulation of delta-opioid receptors with selective agonists results in a profound diuretic and natriuretic response in conscious rats (Sezen *et al*. 1998), which likely results in a reduction of energy consumption and workload of the kidneys (Kiil 1977). Enkephalins have also been implicated in decreasing energy consumption and protection of the heart (van den Brink *et al*. 2003). Together, an increase in pro-ENK may be a compensatory response to either an initial decline of function of the heart, decline of renal function, or both, protecting organ function from further decline in both the acute and chronic situation (Kieneker *et al*. 2017, Ng *et al*. 2017).

In previous clinical studies, cross-sectional associations were observed for elevated levels of pro-ENK with severity of acute kidney injury (Smith *et al*. 1981, Zoccali *et al*. 1987), or chronic renal failure (Marino *et al*. 2015, Shah *et al*. 2015), but also a prospective association was found between high pro-ENK and increased risk of graft failure among renal transplant recipients (Kieneker *et al*. 2017). Moreover, recently Schulz *et al*. (2017) observed that high concentrations of circulating pro-ENK were associated with renal function decline during follow-up in 2568 participants of the Malmö Diet and Cancer

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Supplemental data for this article can be accessed at here.

study. Interestingly, genome-wide association analysis in 4150 participants of the same cohort revealed genetic variation at the PENK locus that was associated with higher pro-ENK levels and with higher incidence in CKD, suggesting a causal relationship between pro-ENK and CKD defined as an estimated glomerular filtration rate (eGFR) < 60 ml/min/1.73 m^2. However, it is unclear whether pro-ENK is also associated with increased risk of developing increased albuminuria, the other main variable that defines CKD (Levey *et al*. 2011, 2015), which Schulz and colleagues could not take into account. In stable renal transplant recipients, a higher pro-ENK was cross-sectionally associated with urinary albumin excretion (UAE) (Kieneker *et al*. 2017), however, no prospective data are yet available.

Therefore, the aim of the present study was to replicate the association of pro-ENK with low eGFR as a marker of CKD, and to investigate whether pro-ENK is associated with risk of developing high UAE.

Clinical significance

- Early identification of CKD is needed to prevent disease progression, reduce the risk of cardiovascular morbidity and mortality, and improve survival and quality of life.
- In the present study, high concentrations of plasma pro-ENK are associated with an increased risk of developing CKD_{eGFR} in men, but not in women.
- Further investigations are needed to determine whether pro-ENK might aid in early identification of subjects at high risk of future CKD_{eGFR}.

Methods

Study design and population

The Prevention of Renal and Vascular End-stage Disease (PREVEND) study is a prospective investigation of albuminuria, renal and cardiovascular disease in a large cohort drawn from the general population. The details of this study are described elsewhere (Hillege *et al*. 2001). In brief, from 1997 to 1998, all inhabitants of Groningen, the Netherlands aged 28–75 years, were sent a questionnaire and a vial to collect a first morning void urine sample. Pregnant women and subjects with type 1 diabetes mellitus were excluded. Urinary albumin concentration (UAC) was assessed in 40,856 responders. Subjects with a UAC of $\geq$10 mg/L ($n = 7768$) were invited to participate, of whom 6000 were enrolled. In addition, a randomly selected group with a UAC of $<$10 mg/L ($n = 3394$) was invited to participate in the cohort, of whom 2592 were enrolled. These 8592 individuals form the PREVEND cohort and completed an extensive examination in 1997 and 1998 (baseline). The second screening took place from 2001 through 2003 ($N = 6894$), which was the starting point of the present evaluation. Of these 6894, we excluded

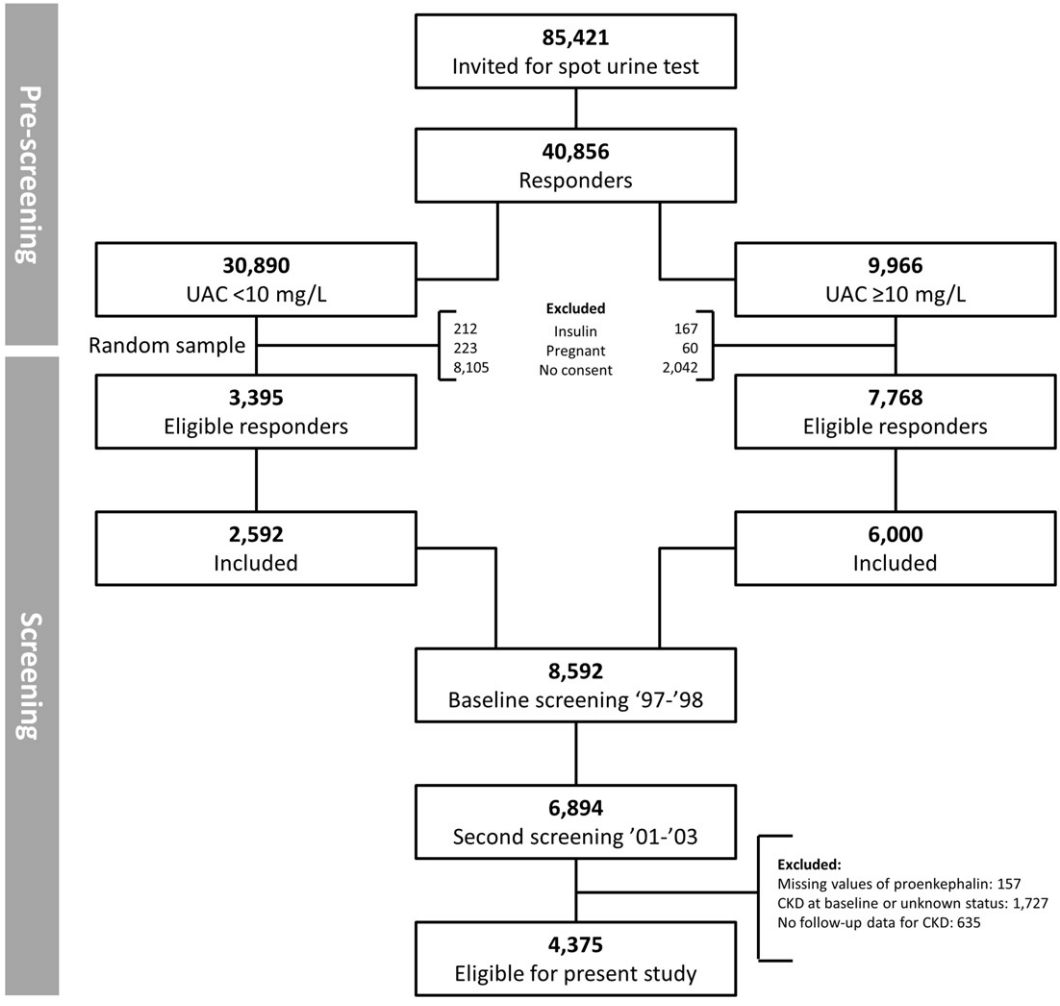

Figure 1. Flow diagram of the participants in the study.

subjects with missing values of pro-ENK ($n = 157$), subjects with CKD at baseline or unknown CKD status ($n = 1727$), or with no follow-up data available for CKD ($n = 635$), leaving 4375 participants for the analyses. The flow diagram of the participants is displayed in Figure 1.

The PREVEND study has been approved by the medical ethics committee of the University Medical Center Groningen and is performed in accordance with Declaration of Helsinki guidelines. Written informed consent was obtained from all participants.

Data collection

The procedures at each examination in the PREVEND study have been described in detail previously (Halbesma *et al*. 2008). In brief, each examination included two visits to an outpatient clinic separated by 3 weeks. Prior to the first visit, all participants completed a self-administered questionnaire regarding demographics, cardiovascular and renal disease history, smoking habits, alcohol consumption and medication use. Information on medication use was combined with information from community pharmacies. During the first visit, participants' height and weight were assessed. During each examination and during each visit, blood pressure was measured on the right arm, every minute for 10 and 8 min, respectively, by an automatic Dinamap XL Model 9300 series device (Johnson-Johnson Medical, Tampa, FL). The mean of the last two recordings from each of the two visits was used. In the last week before the second visit, subjects had to collect two consecutive 24-h specimens after thorough oral and written instruction. The collected urine was stored cold (4 °C) for a maximum of 4 days before the second visit. After handing in the urine collections, the urine specimens were stored at −20 °C. Furthermore, fasting blood samples were provided and stored at −80 °C.

Laboratory procedures

Urine electrolytes were directly analyzed according to standard laboratory procedures. Concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides and glucose were determined as previously described (Verhave *et al*. 2004, Oterdoom *et al*. 2009). Measurement of serum creatinine was performed by an isotope dilution mass spectrometry (IDMS) traceable enzymatic method on a Roche Modular analyzer using reagents and calibrators from Roche (Roche Diagnostics, Mannheim, Germany), with intra- and interassay coefficients of variation of 0.9% and 2.9%, respectively. Serum cystatin C concentrations were measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). Cystatin C was calibrated directly using the standard supplied by the manufacturer (traceable to the International Federation of Clinical Chemistry Working Group for Standardization of Serum Cystatin C) (Grubb *et al*. 2010). The intra- and interassay coefficients of variation were <4.1% and <3.3%, respectively. UAC was measured by nephelometry with a threshold of 2.3 mg/L, and intra- and interassay coefficients of variation of 2.2% and 2.6%, respectively (Dade Behring Diagnostic, Marburg, Germany).

Measurement of proenkephalin (pro-ENK)

An assay for stable pro-ENK (amino acids 119–159 of pro-ENK A) has been previously reported (Ernst *et al*. 2006) and was modified as recently described (Ng *et al*. 2014). In brief, two mouse monoclonal anti-pro-ENK antibodies were developed by immunization with pro-ENK peptide (amino acids 119–159 of pro-ENK A). One antibody (2 μg) was used to coat polystyrene tubes. The other antibody labelled with methyl-acridinium ester served as the detector antibody. Standards (pro-ENK peptide; amino acids 119–159 of pro-ENK A) and samples (50 μl) were incubated in tubes with the detector antibody (150 μl). After equilibration, tubes were washed and bound chemiluminescence was detected with a luminometer LB952T/16 (Berthold, Bad Wildbad, Germany). The lower detection limit of the assay was 5.5 pmol/L. Intra- and interassay coefficients of variation were, 6.4% and 9.5% at 50 pmol/L, and 4.0% and 6.5% at 150 pmol/L, respectively. The assay was provided by Sphingotec GmbH (Hennigsdorf, Germany).

Ascertainment of outcomes

Development of CKD was defined as reaching an eGFR <60 ml/min per 1.73 m^2 (CKD_{eGFR}) or an UAE >30 mg per 24 h (CKD_{UAE}) *de novo* (Koning *et al*. 2015). We estimated GFR with the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology (CKD-EPI) Collaboration equation from 2012, taking into account age, sex and race (Inker *et al*. 2012). UAC was multiplied by urine volume to obtain UAE expressed as mg per 24 h. The two 24-h UAE values of each subject per examination were averaged.

Assessment of covariates

Smoking status was defined as self-reported never smoker, former smoker or current smoker (<6, 6–20 or >20 cigarettes/day). Parental history of CKD was defined as having a first-degree relative who had a renal disease requiring dialysis for >6 weeks. Educational level was defined as low (primary education or intermediate vocational education), middle (higher secondary education) and high (higher vocational education and university).

Statistical analyses

Baseline characteristics are presented according to sex-specific quintiles of pro-ENK. Continuous data are presented as mean with *SD* or as median and interquartile range (IQR) in case of skewed distribution. Categorical data are presented as percentiles. Univariable and multivariable linear regression were performed to determine whether patients' characteristics were associated with pro-ENK concentrations. The Pearson's product–moment correlation coefficient was calculated for the associations of pro-ENK with UAE and eGFR.

To study the prospective association of pro-ENK with risk of developing CKD_{eGFR} and CKD_{UAE} while taking to account the oversampling of subjects with albuminuria, design-based Cox proportional hazards regression analysis was used to

calculate hazard ratios (HRs) and 95% confidence intervals (CIs). Pro-ENK was analyzed as a continuous term per *SD* ^{10}log-transformed increment of pro-ENK and in quintiles. Nonlinearity was tested by using the likelihood ratio test, comparing nested models with linear or linear and cubic spline terms. We first calculated HRs (95% CIs) for the crude model. Second, age- and sex-adjusted HRs (95% CIs) were calculated (multivariable model 1). Additionally, we calculated HRs (95% CIs) which were further adjusted for body mass index (BMI), alcohol consumption, smoking status, and systolic blood pressure, HDL, triglycerides, glucose, and urinary urea, creatinine, and albumin excretion (multivariable model 2); and eGFR (multivariable model 3). We evaluated potential effect modification by sex, age, BMI, and baseline eGFR and UAE in the analyses of risk of CKD by fitting models containing both main effects and their cross-product terms.

In case we observed an association, we evaluated whether pro-ENK adds additional prognostic information for risk prediction on top of baseline eGFR and a multivariable model including baseline eGFR, by testing differences in the Harrell's C-statistics and the −2 log likelihood of prediction models with and without inclusion of pro-ENK in the model.

We performed sensitivity analyses to examine the robustness of the associations between pro-ENK and risk of CKD, by defining CKD_{eGFR} as an eGFR <66 ml/min/1.73 m^2 (instead of <60 ml/min per 1.73 m^2) and CKD_{UAE} as an UAE >25 mg/24 h (instead of >30 mg/24 h) for a more pronounced decline in kidney function in order to reach the primary outcome of CKD.

All *p* values are two-tailed. A *p* value <0.05 was considered statistically significant. All analyses were conducted with the use of the statistical package IBM SPSS (version 22; SPSS Inc., Chicago, IL) and RStudio (version 0.99.893; RStudio, Boston, MA).

Results

Baseline characteristics

Baseline median pro-ENK was 52.2 pmol/L (IQR: 44.9–60.5 pmol/L), with higher values in women (54.4; IQR: 47.0–63.0 pmol/L) than in men (50.2; IQR: 43.2–57.7 pmol/L; $p < 0.001$). Baseline characteristics are shown according to quintiles of pro-ENK (Table 1). Compared to subjects with low pro-ENK at baseline, subjects with high pro-ENK concentrations were more likely to be female, older, to consume less alcohol, to have a lower BMI, lower systolic and diastolic blood pressure, and to have slightly higher HDL cholesterol and lower triglycerides concentrations. Additionally, these same individuals were more likely to have a lower plasma glucose, a lower eGFR, and a lower urinary excretion of albumin, creatinine and urea.

Cross-sectional associations

Baseline pro-ENK was inversely correlated with eGFR ($r = -0.27$, $p < 0.001$; Figure 2) and UAE ($r = -0.09$, $p < 0.001$). Table 2 shows the associations between log-transformed pro-ENK levels and variables of interest due to their association with pro-ENK or because of their involvement in the development of CKD. In crude analyses, female sex, age, and HDL cholesterol were positively associated with pro-ENK, whereas use of alcohol, BMI, systolic blood pressure, triglycerides, glucose, eGFR and urinary excretions of albumin, creatinine and urea were inversely associated with pro-ENK.

When adding all the variables in a multivariable model, significant independent positive associations with pro-ENK were observed for HDL cholesterol, and use of antihypertensive and glucose-lowering drugs, whereas inverse

Table 1. Baseline characteristics of the 4375 participants of the PREVEND study according to quintiles of pro-enkephalin.

	Quintiles of pro-ENK, pmol/L					
	<43.3	43.3–49.3	49.4–55.3	55.4–62.9	>62.9	*p* value for trend[a]
Participants, *N*	875	875	875	875	875	
Women, %	38.7	47.8	51.1	57.9	65.7	<0.001
Age, years	51.1 ± 9.9	51.6 ± 10.9	51.7 ± 11.0	51.2 ± 11.5	52.5 ± 11.9	0.03
Race, whites, %	96.6	96.1	96.0	96.9	94.7	0.13
Parental history of CKD, %	0.9	1.6	1.8	1.3	2.6	0.02
Smoking status, never, %	31.5	28.8	31.2	32.5	32.6	0.82
Alcohol consumption, none, %	20.7	22.1	22.7	23.8	27.1	<0.001
Education, high, %	31.7	35.2	35.2	34.5	31.3	0.13
BMI, kg/m^2	27.5 ± 4.3	26.7 ± 4.1	26.3 ± 4.0	25.6 ± 4.0	25.3 ± 3.8	<0.001
Systolic blood pressure, mm Hg	125 ± 16	124 ± 16	123 ± 16	121 ± 17	122 ± 18	<0.001
Diastolic blood pressure, mm Hg	74 ± 8	73 ± 9	72 ± 9	71 ± 8	72 ± 9	<0.001
Antihypertensive drugs, %	16.3	16.1	13.6	14.8	19.0	0.35
Total cholesterol, mmol/L	5.4 ± 1.0	5.5 ± 1.0	5.4 ± 1.0	5.4 ± 1.1	5.4 ± 1.0	0.20
HDL cholesterol, mmol/L	1.2 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	<0.001
LDL cholesterol, mmol/L	3.6 ± 0.9	3.6 ± 0.9	3.6 ± 0.9	3.5 ± 1.0	3.5 ± 0.9	0.13
Triglycerides, mmol/L	1.17 (0.82–1.69)	1.13 (0.80–1.55)	1.03 (0.77–1.49)	1.05 (0.77–1.44)	1.02 (0.74–1.47)	<0.001
Lipid-lowering drugs, %	6.2	5.9	7.1	9.0	6.7	0.14
Glucose, mmol/L	5.1 ± 1.2	5.0 ± 0.9	4.8 ± 0.9	4.8 ± 0.9	4.8 ± 0.8	<0.001
Glucose-lowering drugs, %	2.3	0.9	1.0	1.7	0.6	0.13
eGFR, ml/min/1.73 m^2	100 ± 13	97 ± 14	95 ± 14	95 ± 14	89 ± 15	<0.001
Urinary albumin excretion, mmol/24 h	8.2 (6.3–11.8)	8.3 (5.9–11.8)	7.6 (5.7–11.0)	7.3 (5.7–10.6)	7.1 (5.4–10.2)	<0.001
Urinary creatinine excretion, mg/24 h	13.6 (11.3–16.0)	12.5 (10.4–15.0)	11.9 (10.2–14.8)	11.5 (9.7–13.8)	10.7 (9.0–13.1)	<0.001
Urinary urea excretion, mmol/24 h	391 (319–474)	368 (290–438)	360 (294–436)	345 (284–412)	324 (263–392)	<0.001

Continuous variables are reported as mean ± *SD* or median (interquartile range) and categorical variables are reported as percentage.
[a]Determined by linear-by-linear association chi-square test (categorical variables) and linear regression (continuous variables).
BMI: body mass index; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; PREVEND: Prevention of Renal and Vascular End-stage Disease.

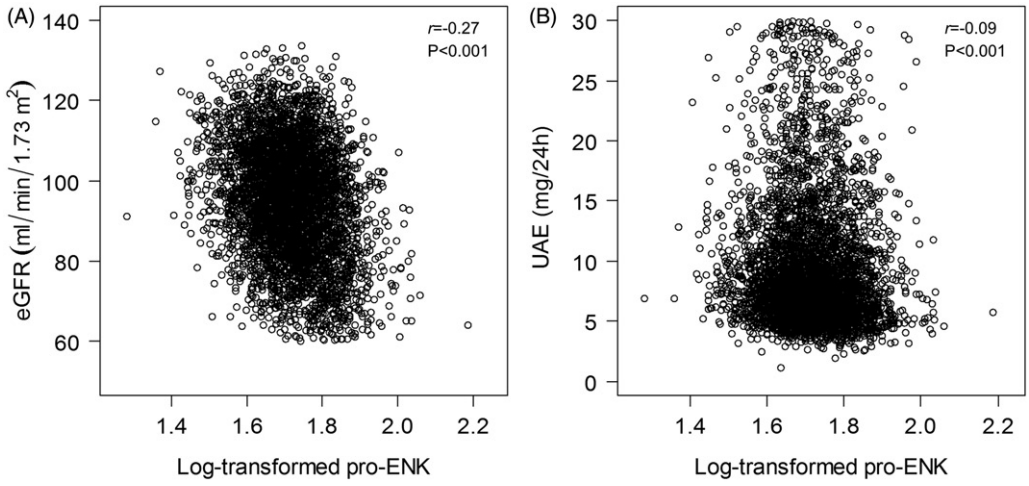

Figure 2. Association of pro-ENK with baseline eGFR (A) and UAE (B) in 4375 participants of the PREVEND study. eGFR: estimated glomerular filtration rate; UAE: urinary albumin excretion; PREVEND: Prevention of Renal and Vascular End-stage Disease; pro-ENK: proenkephalin.

Table 2. Uni- and multivariable linear regression analyses with log-transformed pro-enkephalin as dependent variable in 4375 participants of the PREVEND study.

	Univariable			Multivariable ($R^2 = 0.204$)		
Variables	R^2	Standardized β	p value	Partial R^2	Standardized β	p value
Sex, female vs. male	0.036	0.191	<0.001	0	0.008	0.69
Age, years	0.001	0.041	0.007	0.018	−0.201	<0.001
Caucasian, yes vs. no	<0.001	−0.023	0.13	0	0.007	0.61
Parental history of CKD, yes vs. no	<0.001	−0.004	0.81	0	−0.003	0.85
Smoking, yes vs. no	0.001	0.038	0.11	0	−0.007	0.63
Alcohol, yes vs. no	0.003	−0.057	<0.001	0	−0.022	0.13
Education high, yes vs. no	<0.001	−0.013	0.39	0	−0.018	0.24
BMI, kg/m^2	0.037	−0.193	<0.001	0.019	−0.169	<0.001
Systolic blood pressure, mm Hg	0.006	−0.079	<0.001	0	0.002	0.91
Antihypertensive drugs, yes vs. no	<0.001	0.023	0.16	0.001	0.032	0.04
HDL cholesterol, mmol/L	0.022	0.148	<0.001	0.001	0.036	0.04
LDL cholesterol, mmol/L	<0.001	−0.019	0.18	0	0.008	0.60
Triglycerides, mmol/L	0.009	−0.098	<0.001	0.001	−0.032	0.04
Lipid-lowering drugs, yes vs. no	<0.001	0.018	0.25	0	0.015	0.34
Glucose, mmol/L	0.012	−0.111	<0.001	0.003	−0.056	<0.001
Glucose-lowering drugs, yes vs. no	<0.001	0.024	0.11	0.005	0.072	<0.001
eGFR, ml/min/1.73 m^2	0.072	−0.269	<0.001	0.093	−0.400	<0.001
Urinary albumin excretion, mmol/24 h	0.007	−0.085	<0.001	0	0.004	0.80
Urinary creatinine excretion, mmol/24 h	0.075	−0.275	<0.001	0.015	−0.181	<0.001
Urinary urea excretion, mmol/24 h	0.040	−0.200	<0.001	0.001	−0.035	0.04

BMI: body mass index; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; LDL: low-density lipoprotein; PREVEND: Prevention of Rena land Vascular End-stage Disease; pro-ENK: proenkephalin.

independent associations were observed for age, BMI, triglycerides, glucose, eGFR, and urinary creatinine and urea excretion. When excluding either baseline eGFR or urinary creatinine excretion, female sex was significantly associated with higher levels of pro-ENK (standardized $\beta = 0.048$, $p = 0.03$ and standardized $\beta = 0.021$, $p < 0.01$, respectively).

The multivariable model had an adjusted R^2 of 0.204, with eGFR being the most important contributing determinant (partial $R^2 = 0.093$), suggesting that a large percentage of circulating pro-ENK levels may be explained by eGFR.

Association of pro-ENK with risk of developing CKD

During a median follow-up of 8.4 years (IQR: 7.9–8.9 years), 183 cases of CKD_{eGFR} and 371 cases of CKD_{UAE} occurred. The association of pro-ENK with CKD_{eGFR} risk was modified by sex ($P_{interaction} < 0.1$). The Kaplan–Meier curves for CKD_{eGFR} and CKD_{UAE} survival according to quintiles of pro-ENK and stratified for sex are presented in Figure 3. In the crude model stratified for sex, high pro-ENK was associated with increased risk of CKD_{eGFR} in men (HR: 2.66; 95% CI: 2.13–3.32; Table 3) and in women, although less strong (HR: 1.64; 95% CI: 1.25–2.15). The associations remained similar after adjustment for age, BMI, alcohol consumption, smoking status, systolic blood pressure, HDL cholesterol, triglycerides, glucose, and urinary urea, creatinine, and albumin excretion (HR for men: 2.71; 95% CI: 1.96–3.77 and HR for women: 1.58; 95% CI: 1.16–2.16). However, after further adjustment for baseline eGFR, associations were strongly attenuated in both men and women, resulting in a weakened, but significant association in men (HR: 1.65; 95% CI: 1.15–2.36) and a non-significant association in women (HR: 0.83; 95% CI: 0.58–1.20). When adjusting for baseline eGFR alone, the association of pro-ENK with CKD_{eGFR} risk remained in men (HR: 1.42; 95% CI:

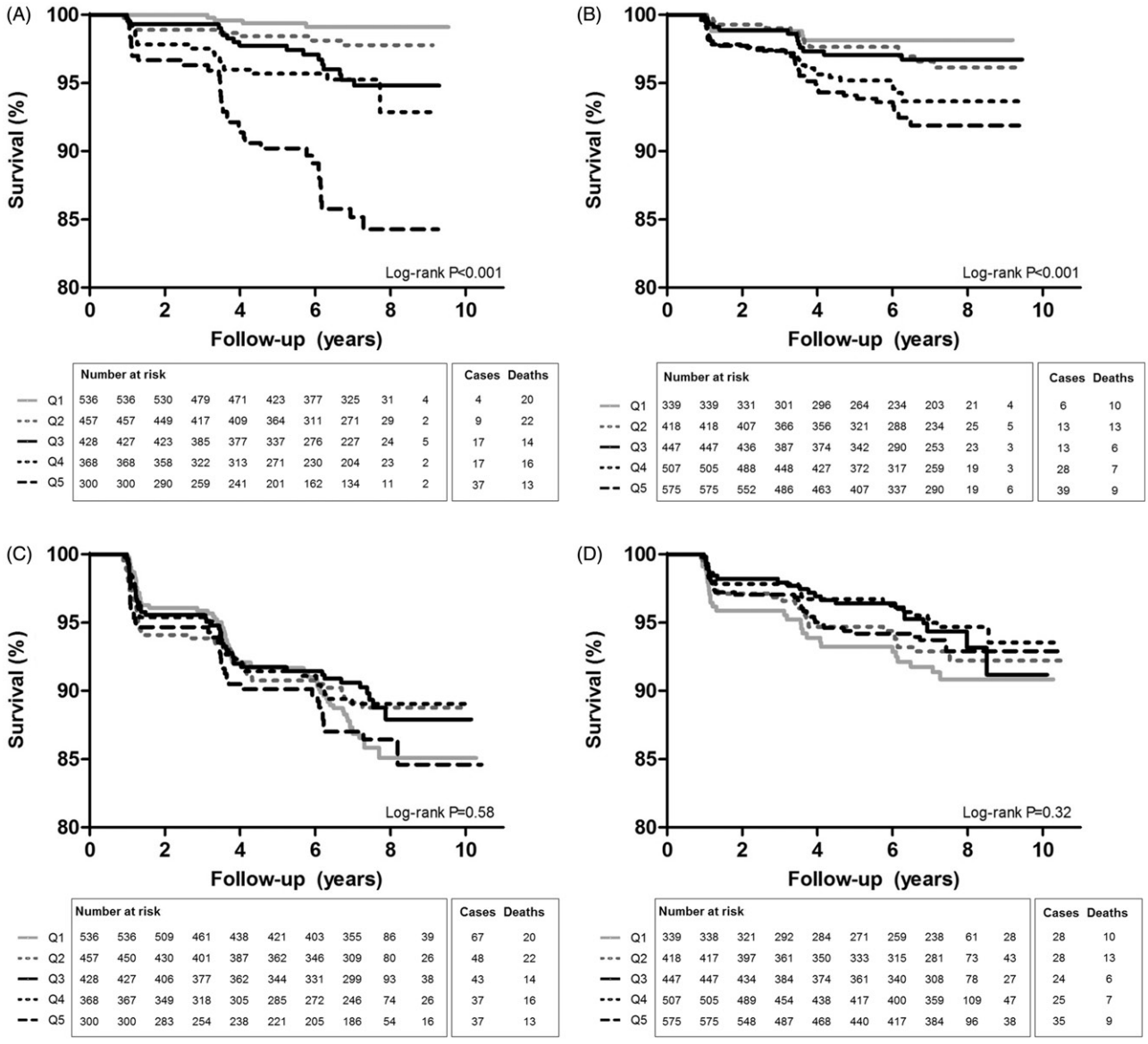

Figure 3. Kaplan–Meier curves for CKD_{eGFR} and CKD_{UAE} survival according to quintiles of pro-ENK. Upper panels represent the Kaplan–Meier survival curves for CKD_{eGFR} for men (A) and women (B), whereas the lower panels represent the Kaplan–Meier survival curves for CKD_{UAE} for men (C) and women (D). CKD_{eGFR}: chronic kidney disease defined as estimated glomerular filtration rate <60 ml/min/1.72 m^2; CKD_{UAE}: chronic kidney disease defined as urinary albumin excretion >30 mg/24 h; pro-ENK: proenkephalin.

1.06–1.89), but not in women (HR: 0.81; 95% CI: 0.61–1.08). We did not find evidence for effect-modification by age and BMI in the association of pro-ENK with risk of CKD_{eGFR} (all $P_{interaction}>0.1$).

No associations were observed for pro-ENK with risk of CKD_{UAE} in the crude (HR: 0.90; 95% CI: 0.76–1.06; Table 3) or multivariable adjusted model (HR: 0.95; 95% CI: 0.77–1.17). Also no evidence for effect-modification by sex, age and BMI was observed (all $P_{interaction} > 0.1$). We did not analyze the combined endpoint of CKD defined as eGFR <60 ml/min/1.73 m^2 and/or UAE >30 mg/24 h, because of the different associations (linear vs. tendency to U-shape).

Results remained essentially similar when we excluded subjects at baseline with an eGFR <66 ml/min/1.73 m^2 (instead of <60 ml/min/1.73 m^2) ($N = 4282$, $n = 120$; HR for men: 1.65; 95% CI: 1.21–2.26 and HR for women: 0.89; 95% CI: 0.67–1.18) and a UAE >25 mg/24 h (instead of >30 mg/24 h) ($N = 4256$, $n = 293$; Q1: HR: 1.71; 95% CI: 1.19–2.46; Q5:1.37; 95% CI: 0.93–2.01) for a more pronounced decline in kidney function during follow-up before reaching the CKD endpoints.

Additional prognostic value of pro-ENK

When analyzing the additional prognostic value of pro-ENK on top of baseline eGFR for the prediction of CKD_{eGFR} in men by comparing Harrell's C-statistics of prediction models with and without pro-ENK, no significant difference was observed (p for comparison = 0.15; Supplementary Table S1). However, when investigating differences in the −2 log likelihood of the model including baseline eGFR with and without inclusion of pro-ENK, the −2 log likelihood significantly improved with pro-ENK included in the model (p for comparison <0.01; Supplementary Table S1).

Table 3. Association between pro-ENK and risk of developing chronic kidney disease (CKD_{eGFR} and CKD_{UAE}) in 4375 participants of the PREVEND study[a].

	Log pro-ENK Per *SD* increment	Quintiles of pro-ENK, pmol/L				
		<43.3	43.3–49.3	49.4–55.3	55.4–62.9	>62.9
CKD_{eGFR} – men						
Person-years	12,981	3410	2916	2675	2253	1727
Cases	84	4	9	17	17	37
Crude model	2.66 (2.13–3.32)	0.10 (0.03–0.33)	0.61 (0.18–2.03)	1.00 (ref)	1.51 (0.56–4.09)	4.96 (2.15–11.45)
Age-adjusted model	2.38 (1.81–3.11)	0.13(0.04–0.46)	0.73 (0.22–2.49)	1.00 (ref)	1.28 (0.45–3.58)	4.55 (1.97–10.49)
Multivariable model 1[b]	2.57 (1.87–3.54)	0.13 (0.04–0.48)	0.63 (0.18–2.27)	1.00 (ref)	1.25 (0.45–3.50)	3.95 (1.64–9.54)
Multivariable model 2[c]	2.71 (1.96–3.77)	0.09 (0.02–0.34)	0.51 (0.13–1.94)	1.00 (ref)	1.28 (0.43–3.84)	3.79 (1.70–8.44)
Multivariable model 3[d]	1.65 (1.15–2.36)	0.17 (0.04–0.76)	0.86 (0.22–3.38)	1.00 (ref)	1.50 (0.42–5.33)	2.00 (0.83–4.78)
CKD_{eGFR} – women						
Person-years	13,947	2134	2610	2745	3068	3390
Cases	99	6	13	13	28	39
Crude model	1.64 (1.25–2.15)	0.67 (0.19–2.31)	0.99 (0.35–2.77)	1.00 (ref)	1.96 (0.81–4.72)	2.42 (1.05–5.58)
Age-adjusted model	1.44 (1.09–1.90)	1.06 (0.30–3.79)	1.13 (0.40–3.22)	1.00 (ref)	2.09 (0.86–5.07)	2.45 (1.05–5.68)
Multivariable model 1[b]	1.56 (1.17–2.09)	0.89 (0.23–3.42)	1.11 (0.39–3.17)	1.00 (ref)	2.04 (0.82–5.09)	2.67 (1.08–6.60)
Multivariable model 2[c]	1.58 (1.16–2.16)	0.61 (0.13–3.02)	1.23 (0.42–3.59)	1.00 (ref)	2.04 (0.79–5.22)	2.53 (0.95–6.76)
Multivariable model 3[d]	0.83 (0.58–1.20)	1.78 (0.43–7.42)	2.56 (0.86–7.61)	1.00 (ref)	1.94 (0.80–4.72)	1.13 (0.44–2.88)
CKD_{UAE}						
Person-years	28,707	5704	5749	5808	5853	5593
Cases	372	95	76	67	62	72
Crude model	0.90 (0.76–1.06)	1.37 (0.86–2.16)	1.08 (0.67–1.76)	1.00 (ref)	0.81 (0.49–1.36)	0.98 (0.60–1.60)
Age- and sex-adjusted model	0.90 (0.76–1.08)	1.44 (0.91–2.29)	1.13 (0.70–1.84)	1.00 (ref)	0.87 (0.52–1.46)	1.04 (0.63–1.70)
Multivariable model 1[b]	0.89 (0.74–1.06)	1.47 (0.92–2.35)	1.12 (0.69–1.82)	1.00 (ref)	0.84 (0.50–1.41)	1.01 (0.62–1.64)
Multivariable model 2[c]	0.93 (0.76–1.13)	1.54 (0.93–2.53)	0.90 (0.55–1.49)	1.00 (ref)	0.96 (0.56–1.65)	1.10 (0.66–1.82)
Multivariable model 3[d]	0.95 (0.77–1.17)	1.18 (0.72–1.93)	0.82 (0.72–1.93)	1.00 (ref)	0.98 (0.58–1.66)	1.35 (0.79–2.28)

CKD_{eGFR} is defined as eGFR <60 ml/min/1.73 m^2 and CKD_{UAE} is defined as urinary albumin excretion >30 mg/24 h.
[a]HRs (95% CIs) were derived from design-based Cox proportional hazard models.
[b]Multivariable model 1 is additionally adjusted for BMI, alcohol consumption, smoking status and systolic blood pressure.
[c]Multivariable model 2 is additionally adjusted for HDL cholesterol, triglycerides, glucose, and urinary urea, creatinine, and albumin excretion.
[d]Multivariable model 3 is additionally adjusted for eGFR.
BMI: body mass index; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein; PREVEND: Prevention of Renal and Vascular End-stage Disease; pro-ENK: proenkephalin; UAE: urinary albumin excretion.

When analyzing the additional prognostic value of pro-ENK on top of the multivariable model including baseline eGFR for the prediction of CKD_{eGFR} in men, we observed significant improvement of both the Harrell's C-statistics as well as the −2 log likelihood when pro-ENK was included in the model ($p = 0.02$ and $p < 0.01$, respectively; Supplementary Table S1).

Discussion

In this prospective population-based cohort study free of CKD at baseline, a higher concentration of pro-ENK was associated with an increased risk of developing CKD_{eGFR} in men, but not in women. No association of pro-ENK with risk of developing CKD_{UAE} was observed.

Despite comparable baseline eGFR and pro-ENK levels in this study compared to the study of Schulz *et al.* (2017), we could only replicate their finding of a decreased eGFR during follow-up with a higher pro-ENK in men, but not in women. In total, they studied 2568 participants without CKD at baseline of the population-based Malmö Diet and Cancer Study Cardiovascular cohort during a mean follow-up of 16.6 years. An increased risk of CKD_{eGFR} was observed for subjects with a higher baseline pro-ENK. A possible explanation for the difference in observed associations could be the difference in baseline age range between the cohorts, since the age most of the study participants of the Malmö Diet and Cancer Study Cardiovascular cohort study ranged between 50–60 years (mean age 56.4 ± 5.7 years) versus 33–75 years (mean age 51.6 ± 11.1 years) in the PREVEND study. Therefore, the incidence rate of CKD_{eGFR} was about 30% in contrast to our incidence rate of 4%. An interaction of age was observed in their study, however, in such a way that high pro-ENK was associated with decline in eGFR in the youngest individuals. No interactions with age were observed in the present study.

When comparing prediction models for CKD_{eGFR} risk in men by means of Harrell's C-statistics for models including baseline eGFR with and without pro-ENK, no significant difference was observed between both models. However, since the Harrell's C-statistics is based on ranks rather than on continuous data, it is very insensitive to detect differences (Harrell 2001, Cook 2007). To avoid injudicious discarding of otherwise promising biomarkers, it is therefore currently recommended to also compare prediction models by means of more sensitive methods, like the −2 log likelihood method (Harrell 2001, Cook 2007). When using the −2 log likelihood method, we observed a significantly improvement for CKD_{eGFR} risk in men of the model including pro-ENK. When comparing the additional prognostic value of pro-ENK on top of the multivariable adjusted model including baseline eGFR for the prediction of CKD_{eGFR} in men, significant improvements of both the Harrell's C-statistics as well as the −2 log likelihood were observed when pro-ENK was included in the model. This indicates that the model including pro-ENK had a modest, but significant, higher predictive capacity for CKD_{eGFR} risk in men on top of other risk factors including baseline eGFR.

This study is the first to examine the association of pro-ENK with risk of CKD defined as UAE >30 mg/24 h. Albuminuria is an important component in the diagnosis and

classification of CKD, because albuminuria is an early predictor of progressive renal function loss and also may indicate a worse renal prognosis (de Jong and Curhan 2006). In the study of Schulz *et al*. (2017), no data on UAE were available, so they could not take UAE into account when excluding subjects with CKD at baseline and investigate the prospective association of pro-ENK with risk of developing CKD based on albuminuria. We did not observe a significant association of pro-ENK with risk of CKD_{UAE} in the present study.

Due to the low molecular weight of pro-ENK (4586.60 g/mol), it is assumed to be freely filtered through the glomerulus and therefore the proposed association of enkephalins with renal disease therefore likely implies impaired clearance or increased production of enkephalins in renal disease. Genome-wide association analysis revealed genetic variation at the PENK locus that was associated with higher pro-ENK levels and with higher incidence in CKD, suggesting a causal relationship between pro-ENK and CKD_{eGFR} (Schulz *et al*. 2017). Besides inflammation, various other physiological functions have been shown to be modulated by enkephalins, including processes of cell growth, differentiation, and apoptosis (Ovadia *et al*. 1996, McTavish *et al*. 2007, Denning *et al*. 2008, Awad *et al*. 2012). In an experimental study in conscious Sprague-Dawley rats, activation of the *delta* opioid system by infusion of a *delta* opioid receptor agonist evoked a profound diuretic and natriuretic response, which suggest significant changes in renal excretory function (Sezen *et al*. 1998). We could therefore speculate that an increment in pro-ENK concentrations could be a reflection of counteracting the decreasing functionality of the kidney by promoting kidney function. Further experimental studies blocking or stimulating the opioid receptors could demonstrate the potential role of pro-ENK in this relationship.

Some limitations of the present study should be noted. First, the association of pro-ENK with risk of CKD_{eGFR} was strongly attenuated by adjustment for baseline eGFR. Arguably, the association observed in men could also be the result of residual confounding with respect to pro-ENK as a marker for renal function in addition to eGFR as an estimation of true renal function. Second, because sex was an effect modifier in the association of pro-ENK with risk of CKD_{eGFR}, we had to stratify the analyses and therefore the number of cases could have become insufficient to either detect an association in women, or overfitting of the model could be an issue. Third, pro-ENK was only assessed at baseline, and therefore we could not take into account changes over time in pro-ENK concentrations. However, when the intra-individual variability of variables is taken into account, this results in general in strengthening of associations (Koenig *et al*. 2003, Danesh *et al*. 2004). Therefore, our use of a single pro-ENK measurement at baseline rather than multiple ones, will likely provide an underestimation of the true effect, whereas we now already found an association. Second, eGFR as measure of kidney function might be a less precise measure for renal function compared to measured GFR, which was not available in the PREVEND study, but which is also not feasible in large scale epidemiological studies with serial follow-up. Finally, we could not use the combined endpoint of CKD defined as eGFR <60 ml/min/1.73 m^2 and/or UAE >30 mg/24 h, because of the different associations (linear vs. tendency to U-shape).

A strength of this study includes the measurement of UAE, so that we could assess the association of pro-ENK with risk of CKD_{UAE}. Another strength of this study is the use of estimated GFR with the combined creatinine cystatin C-based CKD-EPI Collaboration equation from 2012, which includes serum creatinine and cystatin C (Inker *et al*. 2012). Other strengths of this study are the prospective design and the relatively large sample size.

Conclusions

In this prospective cohort study, high concentrations of plasma pro-ENK are associated with an increased risk of developing CKD_{eGFR} in men, but not in women. These results should be interpreted with caution, since residual confounding and potential overfitting of models could have influenced the results. Further investigations are needed to determine the association of pro-ENK with risk of CKD_{eGFR} and to determine whether pro-ENK might aid in early identification of subjects at high risk of future CKD.

Disclosure statement

A.B. has shares in and is CEO of Sphingotec GmbH, the company providing and having patent rights on the pro-ENK assay. O.H. and J.S. are employed by Sphingotec GmbH.

Funding

The Prevention of Renal and Vascular End-Stage Disease (PREVEND) study has been made possible by grants from the Dutch Kidney Foundation. The funders of the work had no role in the conception, execution or analysis of the research and had no role in drafting the manuscript.

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