High Level of Fasting Plasma Proenkephalin-A Predicts Deterioration of Kidney Function and Incidence of CKD

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ABSTRACT

High levels of proenkephalin-A (pro-ENK) have been associated with decreased eGFR in an acute setting. Here, we examined whether pro-ENK levels predict CKD and decline of renal function in a prospective cohort of 2568 participants without CKD (eGFR > 60 ml/min per 1.73 m²) at baseline. During a mean follow-up of 16.6 years, 31.7% of participants developed CKD. Participants with baseline pro-ENK levels in the highest tertile had significantly greater yearly mean decline of eGFR (Ptrend < 0.001) and rise of cystatin C (Ptrend = 0.01) and creatinine (Ptrend < 0.001) levels. Furthermore, compared with participants in the lowest tertile, participants in the highest tertile of baseline pro-ENK concentration had increased CKD incidence (odds ratio, 1.51; 95% confidence interval, 1.18 to 1.94) when adjusted for multiple factors. Adding pro-ENK to a model of conventional risk factors in net reclassification improvement analysis resulted in reclassification of 14.14% of participants. Genome-wide association analysis in 4150 participants of the same cohort revealed the strongest association of pro-ENK levels with rs1012178 near the PENK gene, where the minor T-allele associated with a 0.057 pmol/L higher pro-ENK level per allele (P = 4.67x10^-21). Furthermore, the T-allele associated with a 19% increased risk of CKD per allele (P = 0.03) and a significant decrease in the instrumental variable estimator for eGFR (P < 0.01) in a Mendelian randomization analysis. In conclusion, circulating plasma pro-ENK level predicts incident CKD and may aid in identifying subjects in need of primary preventive regimens. Additionally, the Mendelian randomization analysis suggests a causal relationship between pro-ENK level and deterioration of kidney function over time.


With an estimated prevalence of 8%–16% worldwide and expected disproportional growth in incidence in developing countries, CKD is becoming a growing public health issue. CKD is usually diagnosed, and the severity stage determined, by eGFR and albuminuria. Etiology of CKD is complex and involvement of hypertension, diabetes, and metabolic syndrome in the pathophysiology have been suggested. Kidney function has an impact on hemodynamic, vascular, inflammatory, and metabolic diseases due to its role in circulation and consequently a decreased kidney function is associated with increased risk of cardiovascular events, hospitalization, and death. Thus, early detection of decreased kidney function is important and therefore screening of certain risk groups, such as individuals with family predisposition as well as of patients with diabetes, hypertension, cardiovascular disease, autoimmune diseases, hereditary

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renal diseases, and persons with structural disease of the renal tract is recommended.\textsuperscript{1–4} The commonly used markers for kidney function are creatinine and cystatin C.\textsuperscript{5–7} However, serum creatinine and eGFR are rather insensitive in identification of individuals at high risk of CKD and therefore novel, more sensitive biomarkers need to be identified.\textsuperscript{8} Although CKD denotes a vascular insult that puts individuals at high risk of ESRD, the disease is not restricted to the kidneys but affects the entire vascular tree with coronary heart disease and stroke as the most common end points. Thus, from a cardio-renal perspective, it is essential to identify individuals at high risk of CKD before the vascular insult has occurred in order to focus primary preventive use of nonpharmacologic and pharmacologic tools on individuals who are likely to benefit the most from such efforts.

In 1975, enkephalins were the first ever discovered endogenous opioids.\textsuperscript{9} They are all encoded by the proenkephalin gene (PENK) on chromosome 8,\textsuperscript{10} and similar to other neuropeptides, biosynthesis of the active enkephalins involves several steps including proteolytic cleavage of the precursor preproenkephalin (pro-ENK), through which four copies of methionine(met)-enkephalin and one copy of each leucine(leu)-enkephalin, and a hexa- and an octa-peptide are processed. The precursor pro-ENK is produced throughout the human body in neuronal as well as non-neuronal cells.\textsuperscript{11,12} Although enkephalins were discovered almost 40 years ago, their physiologic role still remains to be fully understood. In two earlier observational studies enkephalins have been implicated in kidney function.\textsuperscript{13,14} Recently, high plasma concentration of pro-ENK was observed to associate with decreased eGFR and a worse prognosis after acute myocardial infarction.\textsuperscript{13} In another study, among patients with sepsis or a septic shock, elevated pro-ENK concentrations was found to associated with AKI.\textsuperscript{14}

However, no study has investigated if pro-ENK predicts development of CKD in the population.

Therefore, we aimed to test if circulating plasma levels of pro-ENK associate with changes in eGFR, creatinine, and cystatin C levels per year, and predict future CKD in the prospective population-based Malmö Diet and Cancer Study Cardiovascular Cohort (MDCS-CC). Furthermore, to gain insight as to whether any such relationship may be causal, we performed a genome wide association study (GWAS) for pro-ENK levels and used the effect of the strongest GWAS-significant single-nucleotide polymorphism (SNP) as instrumental variable (IV) in a Mendelian randomization (MR) approach for deterioration of kidney function and development of CKD.

**RESULTS**


Baseline characteristics of the study participants are shown in Table 1. High levels of pro-ENK were significantly associated with older age and lower anthropometric characteristics as well as lower systolic BP (SBP), diastolic BP (DBP), and fasting glucose at baseline (Table 2). The baseline mean eGFR was 89.2 ml/min per 1.73 m$^2$ (range 14.09–153.2) and high pro-ENK levels were associated with lower eGFR ($P<0.001$) as well as with higher creatinine and cystatin C levels when adjusted for age and sex. Further adjustment for body mass index (BMI), body fat mass, body lean mass, fasting glucose, cystatin C, and eGFR did not markedly change the results (Supplemental Table 1).

Longitudinal Changes in Kidney Function from Baseline to Follow-Up Re-Examination in Relation to Fasting Plasma Pro-ENK Concentration at Baseline

Subsequently, we examined the relationship between fasting plasma pro-ENK concentration at baseline and change in markers of renal function between baseline and follow-up re-examination in 2908 participants from MDCS-CC. The mean eGFR of the study population was 66.3 ml/min per 1.73 m$^2$ (range 5.2–118.4) at re-examination and on average the annual decline in eGFR was 1.46 ml/min per 1.73 m$^2$. Participants with high pro-ENK at baseline had a greater decline of eGFR compared with participants with low pro-ENK (1.56 versus 1.42 ml/min per 1.73 m$^2$; $P_{\text{trend}}<0.001$) (Table 3). Yet, in subgroup analysis, we did not observe that the small number of participants with the

| Table 1. Clinical characteristics of the MDCS participants at baseline examination (1991–1996) included in analyses for prediction of CKD at follow-up re-examination (2007–2012) |
|---------------------------------------------|-------|-------|-------|
| % cases$^a$                                | All (n=2568) | Men (n=1046) | Women (n=1522) |
| Age at baseline, yr                        | 31.7 | 28.0 | 34.2 |
| BMI, kg/m$^2$                              | 56.44 (5.69) | 56.51 (5.83) | 56.39 (5.60) |
| Waist, cm                                  | 25.40 (3.66) | 25.93 (3.28) | 25.03 (3.86) |
| SBP, mmHg                                  | 82.41 (12.27) | 92.00 (9.37) | 75.83 (9.36) |
| DBP, mmHg                                  | 139.59 (17.85) | 141.26 (17.23) | 138.44 (18.18) |
| Fasting blood glucose, mmol/L              | 86.27 (9.12) | 87.98 (9.23) | 85.10 (8.86) |
| Plasma creatinine C, mg/dl                 | 5.55 (1.08) | 5.72 (1.10) | 5.43 (1.06) |
| Plasma cystatin C, mg/dl                   | 0.76 (0.12) | 0.78 (0.12) | 0.74 (0.12) |
| Plasma creatinine, µmol/L                  | 83.52 (13.46) | 91.26 (13.11) | 78.20 (10.87) |
| eGFR (CKD-EPI 2012)                        | 90.85 (12.28) | 94.57 (12.02) | 88.30 (11.80) |
| pro-ENK, µmol/L                            | 46.34 (14.60) | 43.12 (9.99) | 48.56 (16.71) |
| Antihypertensive treatment, %              | 14.7 | 15.2 | 14.4 |
| Current smoking, %                         | 22.5 | 23.0 | 22.2 |

Data expressed either as mean (SD) for continuous variables or as percentage for categorical variables.

eGFR CKD-EPI 2012 based on Inker et al.$^{41}$

$^a$CKD defined as eGFR (CKD-EPI 2012)<60 ml/min per 1.73 m$^2$. 

$^b$Mean eGFR was calculated based on the most recent re-examination (2007–2012) included in analyses for prediction of CKD at follow-up re-examination (2007–2012).
most rapid decline of eGFR were driving the relationship between eGFR and pro-ENK (ΔeGFR > 3 ml/min per 1.73 m² per year, n = 106; ΔeGFR > 30%, n = 1002; ΔeGFR > 50%, n = 154). In addition, high pro-ENK associated with greater increase of plasma cystatin C and plasma creatinine (P_trend < 0.01 and P_trend < 0.001, respectively) when the analyses were adjusted for age at follow-up, sex, and corresponding baseline measures of renal function (Figure 1, A–C, Table 3).

Table 2. Cross-sectional relationship between tertiles of pro-ENK levels and phenotypic characteristics of the MDCS participants at baseline (1991–1994)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>Fasting Plasma Pro-ENK Concentrationa</th>
<th>P_Trendb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td></td>
<td>Low  (67.16 (0.152)) Medium (67.30 (0.152)) High (67.80 (0.152))</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>2905</td>
<td>0.081 (0.005) 0.819 (0.005) 0.804 (0.005)</td>
<td>0.7</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>2905</td>
<td>0.609 (0.015) 0.569 (0.015) 0.560 (0.015)</td>
<td>0.02</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>2905</td>
<td>0.278 (0.035) 0.199 (0.035) 0.245 (0.035)</td>
<td>0.51</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>2905</td>
<td>0.160 (0.020) 0.195 (0.020) 0.215 (0.020)</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>2905</td>
<td>0.067 (0.043) 0.033 (0.043) 0.225 (0.044)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>2905</td>
<td>0.024 (0.001) 0.023 (0.001) 0.026 (0.001)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR CKD-EPI 2012</td>
<td>2905</td>
<td>0.024 (0.001) 0.023 (0.001) 0.026 (0.001)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antihypertensive treatment, %</td>
<td>2905</td>
<td>0.081 (0.005) 0.081 (0.005) 0.084 (0.005)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data is presented as mean and SEM. n.a., not applicable.

aGeneral specific pro-ENK tertile cut-offs in pmol/L. Males: low: mean 43.74 (19.20–40.90); medium: mean 44.92 (41.00–44.90); high: mean 57.01 (49.50–172.00). Females: low: mean 35.79 (21.40–40.90); medium: mean 45.33 (41.00–44.90); high: mean 59.30 (49.50–518.10).
bGeneral linear model adjusted for age and sex.
cFasting whole blood was converted into plasma value by multiplication with the factor 1.11.

Table 3. Association between tertiles of fasting plasma pro-ENK at baseline examination (1991–1996) and mean changes per year in kidney function and other clinical characteristics during the follow up re-examination (2007–2012) in the MDCS

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>Fasting Plasma Pro-ENK Concentration</th>
<th>P_Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, kg/m²</td>
<td>2905</td>
<td>0.081 (0.005) 0.819 (0.005) 0.804 (0.005)</td>
<td>0.7</td>
</tr>
<tr>
<td>Waist, cm</td>
<td>2905</td>
<td>0.609 (0.015) 0.569 (0.015) 0.560 (0.015)</td>
<td>0.02</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>2905</td>
<td>0.278 (0.035) 0.199 (0.035) 0.245 (0.035)</td>
<td>0.51</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>2905</td>
<td>0.160 (0.020) 0.195 (0.020) 0.215 (0.020)</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>2905</td>
<td>0.067 (0.043) 0.033 (0.043) 0.225 (0.044)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine, µmol/L</td>
<td>2905</td>
<td>0.024 (0.001) 0.023 (0.001) 0.026 (0.001)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>eGFR CKD-EPI 2012</td>
<td>2905</td>
<td>0.024 (0.001) 0.023 (0.001) 0.026 (0.001)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data is presented as mean and SEM. n.a., not applicable.

aGeneral specific pro-ENK tertile cut-offs in pmol/L. Males: low: mean 43.74 (19.20–40.90); medium: mean 48.43 (41.00–44.90); high: mean 57.01 (49.50–172.00). Females: low: mean 35.79 (21.40–40.90); medium: mean 48.43 (41.00–44.90); high: mean 59.30 (49.50–518.10).
bGeneral linear model adjusted for age and sex.
cFasting whole blood was converted into plasma value by multiplication with the factor 1.11.

Prospective Analysis of Association between Fasting Plasma Pro-ENK Concentration at Baseline and New-Onset CKD

During 16.6 (13.3–20.2) years of follow-up, 31.7% of the eligible 2568 participants with baseline eGFR > 60 ml/min per 1.73 m² developed CKD. The majority of our participants were classified into CKD stage 3A (74.8%, n = 608), but also more advanced stages were observed (stage 3B, 22.0%, n = 179; stage 4, 3.0%, n = 24; and stage 5, 0.2%, n = 5). The event rate during the follow-up time was 19.2 per 1000 person-years and CKD occurred significantly more often in women than in men (20.7 versus 17.2 per 1000 person-years; X² = 0.001). We observed a significantly increased incidence of CKD at follow-up re-examination with increasing pro-ENK levels in the age, sex, baseline eGFR, and follow-up time-adjusted logistic regression model (odds ratio [OR], 1.16; P_trend = 0.013). Participants classified in the highest baseline concentration (third tertile) of pro-ENK had a higher incidence of CKD compared with participants having the lowest levels (first tertile) at baseline (OR, 1.34; 95% confidence interval [95% CI], 1.05 to 1.69). Adding further risk factors for kidney function (fasting glucose, SBP, antihypertensive medication, and BMI at baseline) into the model strengthened the association (OR, 1.51; 95% CI, 1.18 to 1.94) and the corresponding OR per increase of one SD pro-ENK concentration was 1.17 (P_trend = 0.01) (Figure 1D). The risk increase for high pro-ENK concentration at baseline was somewhat stronger in women than in men, but the difference was not statistically significant (P_interaction = 0.80). For sensitivity analyses we excluded individuals with diabetes and cardiovascular disease at baseline, which did not change the results in the remaining 2453 individuals (OR, 1.54 for highest pro-ENK concentration at baseline; 95% CI, 1.19 to 1.97).

Risk Discrimination and Net Reclassification Improvement Analyses

To test the incremental value in predicting CKD we added pro-ENK to a model with conventional risk factors (i.e., sex, age, eGFR, BMI, fasting glucose, SBP, and antihypertensive medication at baseline) and follow-up time. The observed area under the curve (AUC) was 0.789 without pro-ENK and adding pro-ENK to a model only marginally improved the
C-statistics with an AUC of 0.791 ($P=0.08$). However, adding pro-ENK to the model led to a significant net reclassification improvement (NRI) of 14.14% of the individuals ($P<0.001$). Model calibration was good for both models i.e., with and without pro-ENK (Hosmer-Lemeshow $P>0.05$ for both).

Age Stratified Analyses of the Relationship between Pro-ENK and Kidney Function

Given the known relationship between age and decline of eGFR, we stratified the participants by tertiles of baseline age and observed that high pro-ENK was significantly associated with decline in eGFR at follow-up in the two youngest tertiles, but not in the oldest tertile (Figure 2). Interestingly, we observed a significant interaction between baseline age and pro-ENK ($P_{interaction}<0.001$) for decline in eGFR. Furthermore, per one SD of log transformed pro-ENK, the individuals in the youngest age tertile had the highest OR of 1.33 for incidence of CKD (95% CI, 1.04 to 1.68), compared with ORs of the middle and oldest age tertiles of 1.17 (95% CI, 0.97 to 1.40) and 1.10 (95% CI, 0.93 to 1.29), respectively. However, adding pro-ENK to the risk model did not significantly increase AUC for any of the age groups. Yet, NRI was 29.85% ($P=0.002$) in youngest tertile, which is almost twice as high compared with individuals in the middle (NRI=14.98; $P=0.04$) and oldest (NRI=17.37%; $P=0.01$) tertiles.

Genome-Wide Analysis for Fasting Plasma Concentration of Pro-ENK and Conditional Analyses

We performed a genome-wide association (GWA) analysis based on 850,658 directly genotyped variants in 4150 participants from MDCS-CC to identify SNPs associated with fasting plasma concentration of pro-ENK. Twenty-four SNPs in the vicinity of the PENK locus (up to 420 kBP up- and 79 kBP downstream) were identified as associated with altered pro-ENK concentration at $P<5\times10^{-8}$. The strongest association was observed with rs1012178 where the minor T-allele with a frequency of 22% was associated with 0.057 pmol/L higher pro-ENK levels per allele ($P=4.7\times10^{-21}$) (Figure 3).

To distinguish which SNPs in the region have independent associations we performed conditional analyses for the variants with the strongest association signals located upstream (rs1012178) and downstream (rs17760660) of PENK and identified the rs1012178 as the strongest...
associated variant (Supplemental Figure 1), and thereby used it in further analyses.

**Association between the rs1012178 Genotype and Incidence of CKD and Change in Kidney Function during the Follow-Up**

We next tested the association between the lead SNP rs1012178 and incidence of CKD. We observed that during the mean follow-up time of 16.6 years, the minor allele of rs1012178 was associated with 19% increased incidence of CKD per allele ($P_{\text{trend}}=0.03$) (Figure 4A). Further, the minor allele carriers had a significantly greater decline of eGFR (−0.083 ml/min per 1.73 m$^2$, $P=0.001$ per year and allele), as well as a greater increase of cystatin C (+0.001 mg/L, $P=0.03$ per year and allele) and creatinine (+0.123 μmol/L, $P=0.002$ per year and allele) (Figure 4, B–D, Table 4). All associations were somewhat stronger among male participants, but the differences between genders were not statistically significant ($P_{\text{interaction}}=0.32$).

**IV Analyses with rs1012178 for Incidence of CKD and Deterioration of Kidney Function by Time**

To investigate if the pro-ENK levels could be causally linked to incidence of CKD and deterioration of kidney function we performed IV-analyses with the lead SNP rs1012178 and observed a nonsignificant trend for increased incidence of CKD (OR, 1.95; 95% CI, 0.97 to 3.92; $P=0.06$) in a multivariate model. The imputed SNP rs2068321, in high LD with rs1012178 ($D^2=1.00$, $r^2=0.951$; in Utah residents with ancestry from northern and western Europe (CEU) using 1000G reference population SNAP version 2.2 Broad Institute) reached the strongest association ($P=4.6\times10^{-21}$) with an associated effect size comparable to that of rs2068321. LD, linkage disequilibrium.
adjusted logistic regression model. Further, the IV-estimator for eGFR significantly decreased ($P<0.01$) and for plasma creatinine increased ($P=0.02$) (Figure 5).

**DISCUSSION**

**Main Findings: Clinical Analyses**

The main finding of our study was that high levels of fasting plasma pro-ENK associated significantly with faster decline of renal function and increased risk of new-onset CKD during a mean follow-up time of 16.6 years. Further, by GWA analysis we identified genetic variation at the PENK locus that associates with higher pro-ENK levels and with significant longitudinal deterioration of kidney function and higher incidence of CKD, pointing at a causal relationship between circulating pro-ENK levels and CKD.

These findings add pro-ENK as a potentially valuable tool in a completely new clinical situation. Whereas elevated pro-ENK levels have previously been shown to have prognostic value for renal outcome in critically ill and acute conditions, i.e., in sepsis and after myocardial infarction, our study indicates that pro-ENK may identify subjects with baseline
eGFR $\geq 60$ ml/min per 1.73 m$^2$ who are at increased risk of future CKD (eGFR $\leq 60$ ml/min per 1.73 m$^2$) and faster decline of GFR over time. Importantly, these associations were found to be independent of all known risk factors for CKD, including baseline eGFR. Furthermore, when adding pro-ENK to a model of conventional risk factors in NRI analysis, 14.14% of the study participants were reclassified into the correct direction. Yet, adding pro-ENK to the risk discrimination model did not significantly improve the AUC, which may at least partially be explained by the fact that the receiver operating characteristic curve is predominantly used for diagnostic and not for prediction purposes.

In our study population we observed a rather high incidence rate of about 30% of new-onset CKD during the follow-up time when defining the incidence of CKD as having an eGFR $< 60$ ml/min per 1.73 m$^2$. The clinical significance of defining CKD at this level of eGFR in the elderly has been questioned, as deterioration of kidney function can be considered as a natural part of ageing, and for instance studies have indicated that the risk for mortality in patients $> 65$ years old does not increase until eGFR is declined to $< 45$ ml/min per 1.73 m$^2$. The age of most of our study participants ranged from around 50–60 years at baseline to around 65–75 years at the end of the follow-up, and their annual decline of GFR during the follow-up was on average 1.46 ml/min per 1.73 m$^2$, which is rather normal for Swedish individuals in their late middle age. To clarify the relationship between age, pro-ENK, and decline in eGFR we stratified the population by tertiles of baseline age and observed a significant interaction between baseline age and pro-ENK for decline in eGFR. Interestingly, high pro-ENK was significantly associated with decline in eGFR in the two youngest tertiles, but not in the oldest tertile. Hence, the observed markedly stronger association between pro-ENK and decline in eGFR among the youngest participants suggests that pro-ENK may serve as a predictive marker for deterioration of kidney function even considering the age-related decline, and thus may have relevance for preventive therapies. Such predictive markers of CKD could provide an important clinical advantage because the awareness of CKD in populations is low in general, and it is often not detected until at later stages when clear symptoms begin to occur and possibilities for preventive approaches are diminished.

Given the strong link between CKD and cardiovascular disease, potential primary preventive regimens that may counterbalance the increased risk seen in subjects with high pro-ENK levels would not be restricted to those used to preserve renal function (e.g., pharmacologic and nonpharmacologic BP reduction) but, at least theoretically, could also include tools used for primary prevention of cardiovascular disease e.g., statin therapy. Thus, although certainly more studies are needed both to test the clinical value of pro-ENK in predicting CKD, and whether high CKD risk indicated by a high level of pro-ENK can be reduced by lifestyle and pharmacologic treatment, our study clearly points in that direction.

![Figure 4](www.jasn.org)
Table 4. Relationship between rs1012178 genotypes and change in phenotypic characteristics over time

| Characteristics | n (%) | CC (Mean ± SD) | CT (Mean ± SD) | TT (Mean ± SD) | P<sub>Trend</sub>  
|----------------|-------|---------------|---------------|---------------|---------------
| BMI, kg/m²      | 3309  | 0.081 (0.004) | 0.089 (0.005) | 0.076 (0.013) | 0.55          |
| Waist, cm       | 3312  | 0.568 (0.010) | 0.561 (0.014) | 0.517 (0.036) | 0.27          |
| SBP, mmHg       | 3310  | 0.272 (0.024) | 0.331 (0.032) | 0.232 (0.082) | 0.46          |
| DBP, mmHg       | 3308  | -0.192 (0.013) | -0.152 (0.018) | -0.201 (0.046) | 0.27          |
| Glucose, mmol/L | 3110  | 0.036 (0.002) | 0.042 (0.002) | 0.029 (0.006) | 0.46          |
| Creatinine, μmol/L | 2978 | -0.041 (0.031) | 0.092 (0.041) | 0.183 (0.105) | 0.002         |
| Cystatin C, mg/L| 2843  | 0.024 (0.000) | 0.042 (0.001) | 0.025 (0.001) | 0.03          |
| eGFR CKD-EPI 2012 | 1.411 (0.019) | 1.507 (0.025) | 1.544 (0.065) | <0.001        |
| Incidence of CKD, n (%) | 3214 | 624 (58.8) | 380 (35.8) | 58 (5.5) | n.a.          |
| Follow-up time, yr<sup>a</sup> | 3316 | 16.65 (1.51) | 16.73 (1.51) | 16.62 (1.44) | n.a.          |

Data is presented as mean (SEM). CC, homozygote major allele carriers; CT, heterozygote allele carriers; TT, homozygote minor allele carriers with pro-ENK level increasing 0.05729 pmol/L per minor allele; n.a. not applicable.

<sup>a</sup>General linear model adjusted for age at follow-up, sex, and value at baseline.

<sup>b</sup>Mean (SD).

Figure 5. IV analysis for rs1012178 genotype and longitudinal kidney function, and risk for incident CKD in the MDCS. (A) IV analysis for rs1012178 genotype for risk of incident CKD in 2308 participants of MDCS-CC. The logistic regression model was adjusted for sex, age, baseline eGFR, antihypertensive medication, BMI at baseline, and follow-up time. IV analysis for rs1012178 genotype for changes in (B) eGFR, (C) creatinine, and (D) cystatin C through rs1012178 genotypes in a linear model adjusted for age, (sex), and baseline levels.

and should stimulate such further research in the area. In fact, our MR analysis, which indicated that genetically elevated pro-ENK is associated with increased risk of incident CKD and faster decline of eGFR over time, suggests a causal relationship between pro-ENK and risk of CKD. Although the results from the MR analysis support our findings, and we adjusted for baseline eGFR, we cannot completely exclude residual confounding. However, the results suggest that treatments that specifically block the effect of enkephalins may have beneficial effects on renal function.

Previous studies have indicated that the retention of met-enkephalin in blood may be involved in the pathogenesis of uremic syndrome. In uremic patients, the plasma concentration of met-enkephalin has been described to be four times higher than in healthy individuals, and directly related to plasma levels of creatinine and urea.<sup>15</sup> Elevated levels of met-enkephalin in renal failure have been suggested to either be due to impaired clearance of met-enkephalin or of the precursor, or because of its increased production.<sup>20</sup> Nevertheless, we cannot rule out the possibility that pro-ENK is accumulated because of decreased clearance in the kidney. A molecular mass of 4386 Da for pro-ENK and no protein binding suggests renal clearance and accumulation in the very low range of GFR. However, in this cohort with a mean eGFR of 89.2 ml/min per 1.73 m² and 99% of the participants having a baseline eGFR of ≥45 ml/min per 1.73 m² it is unlikely that increased pro-ENK level is due to impaired renal clearance.

Met-enkephalin is also known as the opioid growth factor (OGF) which name refers to its involvement in inhibition of cell proliferation.<sup>21,22</sup> Recent studies put forward the potential of met-enkephalin in modulating the cyclin-dependent kinase inhibitory pathway to delay G1/S interface after interacting with the OGF-receptor on the outer nuclear envelope.<sup>21</sup> Indeed, the OGF-OGF-receptor axis has been suggested to play a major role in regulation of cell proliferation of human cancer cells.<sup>23</sup> Similarly, nuclear pro-ENK has been shown to be involved in stress-activated apoptosis in vitro by having a physical association with the transcriptional corepressor histone deacetylase, propounding the nuclear pro-ENK as a component of a transcription repressor complex that contributes to anti-tumor response by proapoptotic actions.<sup>24</sup> If plasma concentration of pro-ENK reflects or affects nuclear levels of pro-ENK is yet unknown. Further investigations are needed to study if and how nuclear pro-ENK and/or an unbalanced OGF-OGF-receptor axis may affect cell proliferation and apoptosis in cells of renal tissues and which long-term consequences such actions would entail.

The kidneys are provided with around 1.2 L of blood per minute from the heart, making renal blood flow almost similar to those of the brain or liver (20%–25% versus 15% versus 20%). However, when considering the much smaller mass of...
kidneys, the renal perfusion rate is by far the highest of all organs.\textsuperscript{23} Despite a lower expression of PENK in kidney cortex compared with expression measured in liver or several parts of the brain,\textsuperscript{26} the higher perfusion rate may suggest that this may result in a higher exposure to circulating pro-ENK for renal tissues. Whether this is plausible remains to be clarified, considering the rather short half-time of <15 minutes for active enkephalins in human plasma.\textsuperscript{27–29}

**Main Findings: Genetic Analyses**

Our complementary genetic analysis gave additional insights indicating that fasting plasma pro-ENK concentration is influenced by a rather narrow genomic area around 8q12.1, the location of PENK gene. In conditional GWA analysis we identified the variant rs1012178 as having the strongest association in this region. Further, our results demonstrate that the minor T-allele of rs1012178, that associates strongly with increased pro-ENK levels, also associates with increased incidence of CKD. Moreover, when we regressed the longitudinal changes in kidney function traits from baseline to follow-up re-examination, the rs1012178 T-allele was observed to significantly associate with a greater decline in eGFR and a greater increase of creatinine and cystatin C by time.

Compared with the strong significant association between plasma concentrations of pro-ENK and CKD, the association with the genetic variant rs1012178 was only found to be borderline significant. This may not be surprising taking into account that one variant alone only explains a minor part of the variation in pro-ENK concentration.

Previous studies investigating the effects of structural differences within the enkephalin precursor gene have indicated that a particular secondary structure of DNA is required to enable a single-binding side for the cAMP-responsive enhancer for specific and effective transcription of PENK.\textsuperscript{30} In this context it is interesting that our lead SNP rs1012178 is located in a sequence that encodes for a noncoding long intergenic noncoding RNA (lincRNA) (LINC00968), and these are known to regulate gene expression of nearby genes.\textsuperscript{31} In addition, rs1012178 is located only a couple of nucleotides downstream of an area of open chromatin (ENSR0000172233). This is intriguing as epigenetic silencing of PENK has been reported in a plethora of cancer forms\textsuperscript{32–39} suggesting hypermethylation of PENK as a biomarker for cancer detection.\textsuperscript{36} It is therefore probable that genetic variation up- and downstream of PENK may affect the DNA structure and methylation of the PENK locus, and therefore its expression.

**Strengths and Limitations**

Our study is the first to investigate association between fasting plasma concentration of pro-ENK and CKD, in a large population based study with a long follow-up time. The prospective design of the study minimized the risk of reverse causation and our observation that fasting plasma concentration of pro-ENK predicts deterioration of kidney function was additionally supported by the MR analyses.

However, our study also suffers from several limitations that need to be recognized. First, the plasma pro-ENK measurement was limited to one assessment at baseline examination, which might not be optimal considering the cross-sectional observation of a positive association between pro-ENK with age. It might appear convincing though, that the lack of sequential measurements may not have influenced the results in a major manner, as we adjusted all analyses for age, and for the time of follow-up when applicable. Second, an important aspect in assessing the risk for CKD is albuminuria\textsuperscript{40} and it would have been favorable to define CKD based on albuminuria and eGFR. Yet, we were not able to take albuminuria into consideration for the definition of CKD, as no urine samples were available at baseline. Therefore, we instead used the most current formula by the CKD Epidemiology Collaboration (CKD-EPI) 2012,\textsuperscript{41} which incorporates both creatinine and cystatin C in the formula to estimate GFR and has shown to perform better than equations based on only one of the markers.\textsuperscript{41} Third, we acknowledge the lack of directly measured GFR as a limitation, although this is hard to accomplish in a large population cohort. Fourth, our findings concerning change in eGFR were based on measurements of creatinine and cystatin at only two time points, and more measurements would have been desirable as also recommended in the current Kidney Disease Improving Global Outcomes 2012 CKD guidelines.\textsuperscript{40} Nevertheless, the rather long duration of the follow-up increases our confidence concerning assurance of progression.\textsuperscript{40} Fifth, the statistically significant difference in change in eGFR between low and high pro-ENK might be less relevant from a clinical perspective. Finally, our results were obtained in a middle-aged cohort from southern Sweden and replications in other cohorts are needed to validate and generalize the findings. However, the genetic variants in PENK, that were strongly associated with pro-ENK levels, were also significantly associated with deterioration of kidney function, which provides further evidence of a role for pro-ENK in kidney function.

**Significance**

This study not only provides evidence that fasting plasma concentration of pro-ENK is a biomarker with potential for clinical implication for future prediction of CKD risk, but also links genetic variation in PENK to deterioration of kidney function over time. Therefore, apart from suggesting that pro-ENK may be of clinical value in terms of identifying normal subjects at high risk of future CKD, our genetic results indicate that pro-ENK may be causally related to CKD development, thus highlighting the possibility that future therapies influencing pro-ENK levels might have protective cardio-renal effects.

**Future**

The results of our study are in line with previous cross-sectional observations and add valuable novel information that pro-ENK can identify subjects in need of pharmacologic and nonpharmacologic primary preventive cardio-renal regimens.
in future. However, our observations give rise to new questions that remain to be addressed in future studies. First of all, confirmation of the clinical findings and genetic associations in additional cohorts are needed. Second, examining the genetic region in the vicinity of PENK in more depth, including functional studies, is required to provide insights for mechanisms and if an underlying biologic causality exists. Last but not least, it remains particularly interesting to examine the association shared between environmental exposures and pro-ENK concentration in relation to CKD, considering that environmental exposures, such as dietary intake,2,42,43 have been speculated to be involved in CKD. Likewise, hypothalamic expression of PENK has been supposed to be effected by environmental exposures, such as dietary fat44,45 and alcohol46 in rodents, and nicotine in vitro.47

In conclusion, our findings provide evidence from a large prospective population-based cohort that circulating pro-ENK is a biomarker with potential for clinical implication to predict risk of CKD. The genome-wide analysis followed by analyzing risk of CKD by genetic variation in PENK suggests that the relationship between pro-ENK and deterioration of kidney function may be causal.

CONCISE METHODS

Subjects
The background population for this study is the population-based Malmö Diet and Cancer Study (MDCS) of which 28,098 healthy men and women born between 1923–1945 and 1923–1950, respectively, participated in the baseline examination between 1991 and 1996. The total participation rate was 40.8%48 and a detailed description of the cohort has been published elsewhere.49 MDCS was approved by the Ethics Committee at Lund University (LU 51–90) and written informed consent was given by all the participants.

For this study we included individuals from 6103 randomly selected participants of the MDCS who underwent additional phenotyping, designed to study epidemiology of carotid artery disease, in the MDCS Cardiovascular Cohort (MDCS-CC) between 1991 and 1994. This random sample was invited to a follow-up re-examination between 2007 and 2012, as described previously.50 MDCS was approved by the Ethics Committee at Lund University (LU 51–90) and written informed consent was given by all the participants.

To achieve normal distribution, we stratified subjects by gender and cystatin C in 2601, 2767, and 2636 individuals, respectively, in all participants for whom measurements were available at both examinations.

Clinical Examination and Assays
All participants underwent a physical examination during baseline examination and height (cm), weight (kg), and waist and hip circumference were measured by nurses. SBP and DBP (in mmHg) were measured in supine position with a mercury column sphygmomanometer after 10 minutes of rests by trained personnel. Questions concerning socio-economic status, lifestyle factors, and medical history were answered by the participants via a self-administered questionnaire.49 Fasted blood samples were drawn and immediately frozen to −80°C and stored in a biologic bank.51 Plasma creatinine (μmol/L) and cystatin C (mg/L) were measured. Creatinine was analyzed with the Jaffe method, and traceable to the International Standardization with isotope dilution mass spectrometry. Cystatin C was measured using a particle-enhanced immunonephelometric assay (N Latex Cystatin; Dade Behring, Inc., Newark, NJ). The values of cystatin C were not standardized because they were analyzed before the introduction of the world calibrator in 2010. The reference value for the method was 0.53–0.95 mg/L. In addition, whole blood glucose (mmol/L) was quantified.

During the follow-up re-examination (2007–2012) the following anthropometric characteristics were measured following similar approaches as at baseline: height, weight, waist and hip circumference, SBP, and DBP. Further, using the same analytical methods as at baseline, the plasma concentrations of glucose (mmol/L), creatinine (μmol/L), and cystatin C (mg/L) were quantified from fasted blood samples.

PENK A 119–159, a surrogate marker for endogenous pro-ENK A precursor, was measured in fasting plasma from 4634 participants at MDCS-CC baseline examination using a chemiluminometric sandwich immunoassay. The assay utilizes a stable solid phase and tracer antibodies detecting the endogenous pro-ENK A precursor fragments.27 Due to lack of available plasma from baseline we were unable to measure pro-ENK in 1460 of the participants. These individuals were observed to be slightly younger, had a marginally higher BMI and plasma creatinine as well as lower SBP, fasting glucose, and hemoglobin A1c at baseline examination, but did not differ in gender, cystatin C, or the frequency of antihypertensive treatment when compared with the participants for which pro-ENK levels could be determined (Supplemental Table 2). To achieve normal distribution, we transformed the positively skewed concentration of fasting plasma pro-ENK with the natural logarithm. Additionally, individuals were stratified to tertiles based on their pro-ENK concentration, defining the first tertile (T1 = lowest pro-ENK concentration) as the reference. Because that women had a significantly higher mean pro-ENK concentration at baseline compared with men (one-way ANOVA P<0.001), sex specific tertiles were used as exposure categories.

Incidence of CKD
CKD was defined as presence of an eGFR of <60 ml/min per 1.73 m² calculated according to the previously reported CKD-EPI 2012 equation41 which considers blood concentration of creatinine as well as cystatin C (Supplemental Table 3). As the MDCS cohort is a homogenous cohort of people of white race, we did not apply correcting factors for race in the equations.

Genotyping, Quality Control, GWA Analyses, and Imputation to 1000 Genomes
We used the GWAS approach to identify genetic markers that significantly associate with pro-ENK levels. Genotyping was performed using Illumina HumanOmnigene BeadChip v. 1 at Broad
Institute, Cambridge, MA. During the quality control procedures (QC) we removed individuals having a call rate of <0.95, an inbreeding coefficient of >3 SD away from the mean, discordance between inferred and reported gender, duplicate samples, unexpected high proportion of identity by descent sharing, first and second degree relatives, or deviating from the common population structure in the MDCS-CC (exceeding 8 σ on first two principal components). SNPs were filtered out if they were monomorphic or had a call rate of <0.95, an extreme deviation from Hardy-Weinberg equilibrium (P<1×10⁻⁶⁰⁷), were missing in either cases or controls (P<1×10⁻⁰⁷ and minor allele frequency >0.01) or plate assignment (P<1×10⁻⁰⁸ and minor allele frequency >0.01).

After QC, 5453 individuals and a total number of 850,658 SNPs were eligible in the dataset. The GWA analysis on pro-ENK concentration was performed in 4150 with measured fasting plasma levels. Additionally, this dataset was imputed up to 38,028,468 genetic variants with the 1000 Genomes Phase 1 version 3 reference panel using SHAPEIT2 for prephasing and IMPUTE54 at the Division of Translational Medicine and Human Genetics, Perelman School of Medicine, University of Pennsylvania, PA. After QC, 21,575,257 imputed variants were left for the analysis.

**Variant Selection for MR and IV Analysis**

We next conducted MR analysis, which uses genetic markers to assess causal relationship between a risk factor (often a biomarker) and disease outcome. In our study the genetic marker from GWAS with strongest association with pro-ENK was used as a proxy for pro-ENK. As genes are randomly allocated at conception, the genetic marker divides the study population into random groups and similar to a randomized clinical trial, participants who inherit the biomarker-raising allele are assigned to a group with higher levels randomly, whereas those who do not inherit this allele are assigned to the group with lower levels randomly. To identify the most suitable SNP variant associated with pro-ENK for the IV analyses we performed conditional analyses. IV analysis was conducted using a two-stage least square regression approach in which we first regressed pro-ENK concentration on the strongest independent SNP in an age- and sex-adjusted linear regression model to obtain the IV estimator. Subsequently, we performed a logistic regression for the binary outcome, presence of CKD at follow-up re-examination (0=eGFR>60 ml/min per 1.73 m²; 1=eGFR≤60 ml/min per 1.73 m²), to calculate the OR for the IV estimator. For linear outcomes (eGFR and plasma concentrations of creatinine and cystatin C) linear regression models were used. Adjustments for covariates are stated in the results section.

**Statistical Analyses**

Association analyses between concentration of fasting plasma pro-ENK at baseline examination (1991–1994) and phenotypic characteristics at baseline, and change in phenotypic characteristics from baseline to follow up re-examination (2007–2012), were performed using linear regression. For the latter, we divided the variable “mean change over time” (value at follow-up minus value at baseline) by follow-up time in years to account for different length in follow up.

Association analyses between fasting plasma pro-ENK concentration at baseline and the risk of CKD at follow-up re-examination were performed using logistic regression adjusting for follow-up time in years, age, sex and baseline level of eGFR, SBP, BMI, fasting glucose, and antihypertensive medication.

SPSS (version 21, IBM, White Plains, NY) was used for the clinical epidemiologic analyses and all analyses were adjusted for sex and age. Additional adjustments for covariates in specific models are reported in the results section. The null hypothesis was rejected if a two-sided P value of <0.05 was observed and the association was considered as statistically significant.

In addition, we tested the model discrimination by calculating C-statistics using “roccomp” command in STATA for risk factor models with and without pro-ENK. Global calibration of the risk models was assessed calculating Hosmer–Lemeshow statistics for models with and without pro-ENK using “estat gof” command in STATA. We further evaluated the ability of pro-ENK to reclassify risk calculating the continuous net-reclassification improvement (NRI), a category-free version of the NRI, using “nri” command in STATA for the package id (integrated discrimination improvement) from http://personalpages.manchester.ac.uk/staff/mark.lunt. The STATA version 13.1 (StataCorp., College Station, TX) was used for all analysis.

The GWA analyses for fasting plasma concentration of pro-ENK were performed using a linear additive genetic model adjusting for age and sex using the PLINK version 1.07. To identify the SNP with strongest evidence for association with pro-ENK levels for further analyses, we performed conditional analyses adding the potential variants to an age and sex adjusted GWA model. GWA analysis of the imputed dataset was computed using SNptest version v2.5. The P values of <5×10⁻⁸ were considered as significant. IV analyses were performed in SPSS (version 21, IBM).

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