Plasma proadrenins and prediction of cause-specific mortality in a middle-aged cohort during long-term follow-up

Ayesha Fawad¹, Andreas Bergmann², Janin Schulte², Zahra A. Butt³, Peter M Nilsson¹, Louise Bennet¹, Marju Orho-Melander¹, and Olle Melander¹

¹Lund University, Department of Clinical Sciences, Malmo, Sweden
²Sphingotec GmbH, Hennigsdorf, Germany
³University of Southern Denmark, Faculty of Health Sciences, Odense, Denmark.

Corresponding author: Ayesha Fawad, Department of Clinical Sciences, Malmo, CRC, Jan Waldenstroems gata 35, building 91, level 12, Skane University Hospital, SE 214 28 Malmo, Sweden, (e-mail: ayesha.fawad@med.lu.se).
Disclosures
This study is supported by research grants from the Knut and Alice Wallenberg Foundation, Göran Gustafsson Foundation, the Swedish Heart- and Lung Foundation, the Swedish Research Council, the Novo Nordisk Foundation, Region Skåne, Skåne University Hospital and the Swedish Foundation for Strategic Research (IRC) to OM. The content of the manuscript is solely the responsibility of the authors. JS is employed by Sphingotec GmbH, a company having patent rights in the pro-neurotensin assay and commercializing it. AB is CEO of Sphingotec GmbH and holds shares in this company. The other authors declare that they have neither disclosures nor financial or non-financial competing interests.
Abstract
Purpose: To examine the prediction of Pro-NT on total and cause-specific mortality in a middle-aged cohort.

Methods: In the population-based middle-aged cohort (n=4632, mean age 57 years) of MDC study, Pro-NT was assessed and total as well as cause-specific mortality was studied. Main cause of death was based on the International Classification of Diseases (ICD).

Results: During a mean follow-up of 20±3 years, 950 men and 956 women died. There was significantly increased mortality risk in subjects belonging to the highest quartile (Q) of Pro-NT (Q4, Pro-NT ≥149 pmol/L) compared with Q 1-3 (Pro-NT <149 pmol/L), hazard ratio (HR), 95% confidence interval (CI) of 1.29 (1.17-1.42; P<0.001). Data was adjusted for sex and age. No significant interaction was observed between Pro-NT and gender on mortality risk. Individuals within Q4 vs. Q 1-3 had a HR of 1.41 (95% CI 1.18 -1.68; P<0.001) for death due to cardiovascular disease (n=595/4632); 2.53 (95% CI 1.37- 4.67; P=0.003), due to digestive tract disease (n= 42/4632), 1.62 (95% CI 1.04 - 2.52; P=0.032) due to mental and behavioral disease (n= 90 /4632); and 1.91(95% CI 1.15 - 3.19; P=0.013) due to unspecific causes (n= 64/4632). There was no significant relationship between Pro-NT and deaths due to cancer, infections, neurological or other causes. Adjustment for cardiovascular risk factors only marginally changed these results.

Conclusions: The relationship between Pro-NT and total mortality risk was mainly driven by cardiovascular mortality, but high Pro-NT also predicts death from digestive, mental and behavioral disease and deaths attributed to unspecific causes.

Keywords: Cardiovascular diseases, digestive tract disease, mortality, obesity, proneurotensin
Introduction
The obesity pandemic crisis and its associated risks for cardiometabolic diseases like type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD) are rising worldwide.[1] Life expectancy is substantially lower in people with cardiometabolic diseases and therefore early identification of subjects at high risk of obesity and its complications is critically important. [2] More than two thirds of obesity-related deaths are due to CVD. [1] This disease burden of developing CVD (due to presence of one or more risk factors such as hypertension, diabetes, hyperlipidemia, or already established but subclinical disease) requires strategies for early detection and management plans.

Neurotensin (NT) is a 13 amino acid peptide, which is released immediately after ingestion of fatty meals, and it facilitates lipid digestion and fat absorption in the small intestines as well as hepatic fat accumulation. [3-5] As the mature hormone NT is unstable and rapidly broken down in the bloodstream in vivo[6], we instead measured Proneurotensin (Pro-NT), which is a stable precursor molecule of NT, produced in equimolar amounts relative to NT from the small intestines [7]. It serves as a robust surrogate marker for the intestinal release of NT, NT produces a wide range of physiological and pharmacological effects by binding to specific NT receptors of which three are well described. [8] In fact also Pro-NT has affinity to and stimulates NT-receptor-1 [8]. Apart from the peripheral endocrine actions of NT, it is produced inside, and is an important neurotransmitter within, the central nervous system [9], although Pro-NT measured in plasma is primarily assumed to stem from the small intestine.[9] Specifically the knockout mice deficient in receptor subtypes NTSR1 [10], NTSR2 [11] and NTSR3[12] have been studied to examine the mechanisms of NT-mediated central and peripheral responses. In addition to NT’s direct effect on cardiac physiology, it acts as a hormone in the gastrointestinal tract where it modulates gastrointestinal motility and pancreaticobiliary secretions. [13] Elevated Pro-NT promotes fat accumulation in the liver, which presents a novel biomarker for prognosis of NAFLD and its complication non-alcoholic steatohepatitis (NASH). [14]

In the central nervous system (CNS), Pro-NT acts as a neuromodulator with dopaminergic system and has endogenous antipsychotic effects. It even interacts with several other systems in the CNS including serotonergic, glutamatergic, cholinergic and GABAergic systems.[15] Even elevated Pro-NT has shown sex-specific associations in, for example, women with cognitive impairment [16]. Pro-NT was found to be a predictor of incident cardiovascular disease (CVD) and even predicted T2D and CVD mortality in population-based studies.[17, 18] [19] Furthermore, associations of circulating Pro-NT with impaired renal function and its relation to all-cause mortality in patients with end-stage kidney disease was recently reported.[20]

Thus, previous research implicates Pro-NT in the pathophysiology of several diseases and its associations with all-cause (ACM) and CVD mortality. [19-21] However, studies supporting associations of Pro-NT with cause-specific mortality (CSM), other than CVD, are still unclear and requires further investigations.

In contrast with previous research evidence suggesting an important role of Pro-NT in obesity and cardiometabolic diseases, the aim of this population-based, cohort study is to assess the association of Pro-NT with CSM. Moreover, given indications that the association between Pro-NT and poor outcomes might be gender specific, [18, 19] we also examined interactions between Pro-NT and gender on mortality risk.


Materials and Methods

Study design and settings

The Malmö Diet and cancer study (MDC) cohort is a population-based prospective cohort from Malmö, Sweden, with the aim to study the epidemiology of cardiometabolic and cancer diseases. In total, 28,449 participants including men (born 1923-1945) and women (born 1923-1950) underwent baseline examinations between 1991 and 1996. About 6103 randomly selected individuals for the study of carotid artery disease and CVD, participated in the cardiovascular Cohort (MDC-CC) between 1991 and 1994. As fasting plasma samples were available for the analysis of Pro-Nt in 4632 participants of MDC-CC, the remaining 1471 participants were excluded from this study due to lack of fasting plasma samples.

Participants, exposures and outcomes

Participants underwent physical examinations and responded to an extensive questionnaire about occupation, previous medical conditions, medications, and dietary and life-style habits. Current smoking was defined by self-reporting of any use within the last year. Diabetes was defined as fasting whole blood glucose level of ≥ 6.1 mmol/L (corresponding to a fasting plasma glucose value of ≥ 7.0 mmol/L or a self-reported physician’s diagnosis of diabetes or use of anti-diabetic medication[22]. Adding HbA1c >6.5% as criterion for diabetes only resulted in one additional case of diabetes. Blood pressure (mmHg) was measured by using an oscillometric device twice after 5 minutes of rest in the supine position, and a mean figure calculated. Height was measured in centimeters to the nearest measured value whereas weight was measured in kilograms without shoes. BMI (kg/m²) was calculated as weight (kg) divided by height (m) squared. All study participants provided written informed consents. The regional ethical committee in (Lund University) Lund, Sweden approved this study (LU 51-90 and Dnr 652/2005).

Blood samples drawn after an overnight fast were immediately stored at ~80 °C. Fasting plasma Pro-Nt levels were centrally quantified in a single lab (ICI immunochemical Intelligence GmbH, Berlin, Germany) with a highly-sensitivity one-step chemiluminometric sandwich immunoassay and coated-tube technique (SphingoTec®, GmbH, Borgsdorf, Germany). The lower limit of detection of Pro-Nt precursor fragment was 1.9 pmol/L. [7] All other analysis including fasting plasma glucose, total cholesterol and triglycerides levels in serum, low-density lipoproteins (LDL), high-density lipoprotein (HDL-cholesterol) and very low-density lipoprotein (VLDL) were measured by standard laboratory methods in a certified laboratory using the Cobas Modular Analyzer Series (Roche, Basel, Switzerland). All analysis were based on the standard and accredited procedures at the Department of Clinical Chemistry, Skane University Hospital.

Mortality endpoints.

The outcomes were ACM and CSM based on the International Classification of Diseases (ICD) codes Ninth or Tenth Revision (ICD-9; ICD-10) recorded as the underlying cause of death. During follow-up, the information of ACM and CSM were retrieved by linking the individual’s 10-digit civil registration number with the Swedish National Cause of Death Register (SNCDR). Mortality ascertaining was according to registered underlying cause of death on the cause of death certificate in accordance with the ICD-9 or ICD-10 codes as follows:

Deaths due to CVD (ICD 9:390-459 or ICD 10: I 00-99), digestive tract diseases (ICD 9:520-579 or ICD 10: K 00-95), mental and behavioral disorders (ICD 9: 290-319 or ICD 10: F 01-99), symptoms and unspecific causes of death (ICD 9:780-789 or ICD 10: R 00-99), respiratory diseases (ICD 9:460-519 or ICD 10: J 00-99), genito-urinary diseases (ICD 9: 580-629 or ICD 10: N 00-99), malignant cancers (ICD
9:140-239 or ICD 10: C 00-97), immunodeficiency and endocrine disorders (ICD 9:240-279 or ICD 10: D 80-89; E00-99), blood disorders (ICD 9:280-289 or ICD 10: D 50-577), diseases in nerve system and ears (ICD 9:320-389 or ICD 10: G 00-99; H 00-99), infectious diseases (ICD 9: 001-139 or ICD 10: A 00-99; B 00-99), dermatological diseases (ICD 9: 680-709 or ICD 10: L 00-99), musculoskeletal diseases (ICD 9:710-739 or ICD 10: M 00-99), injuries and poison incidents (ICD 9:800-999 or ICD 10: U,V,W,X, Y 00-99). Subjects were followed until emigration, death or end of follow-up (December 31, 2018).

Statistics
The distribution of plasma Pro-NT concentration was skewed and therefore logarithmically transformed when analyzed as a continuous variable. Fasting Pro-NT concentrations were divided into population quartiles. Continuous clinical characteristics were compared across quartiles by using ANOVA “linear trend” p-values. Cox proportional hazards models were used to test the relationship of Pro-NT quartile and total mortality in models adjusted for age and sex, as well as in models adjusted for age, sex, BMI, systolic blood pressure, diabetes mellitus, use of anti-hypertensive medication, current smoking status, LDL- and HDL-cholesterol. When adding Hba1c > 6.5% as criterion for diabetes definition, only one additional subject had diabetes (n=419 instead of n=418).[22] Furthermore, risk of ACM and CSM was compared between individuals belonging to Pro-NT Q4 (≥ 149 pmol/L) versus individuals belonging to Pro-NT Q1-3 (<149 pmol/L) and Q4 vs Q1 in similar adjustment models as described above. We tested for interaction between Pro-NT and sex and analyzed data separately for men and women in relation to ACM. No significant multicollinearity was found between the covariates in the multivariate models. Survival and time to event analysis in ACM were visualized by the Kaplan-Meier method.

A 2-sided P-value of less than 0.05 was considered statistically significant. SPSS software version 26.0 (IBM SPSS) was used for all statistical analyses and calculations of cohort data.

RESULTS
The baseline characteristics of the study participants stratified by quartiles of Pro-NT are shown in Table 1. In the crude baseline analysis, Pro-NT in quartiles were significantly related to prevalence of diabetes, smoking, HDL-cholesterol and female gender. Higher quartiles of Pro-NT contained greater proportions of participants with diabetes (P< 0.001) and current smokers (P=0.007). The median (interquartile range) age of patients was 57.7 (51.7 - 63.7) years. Out of 4632 patients (42.5% men, 57.5% women), 1906 (31.3%) died during a follow-up period of 20 ± 3 years.

Pro-NT and all-cause mortality
The cumulative incidence of ACM in quartiles of baseline fasting concentrations of plasma Pro-NT is shown in Table 2. Increased mortality risk with fasting plasma Pro-NT was found in in quartile 4 vs. quartile 1, analysis adjusted for sex and age HR 1.33, 95% CI (1.17-1.42; P<0.001). Mortality risk in quartiles 2 and 3 respectively did not significantly differ from ACM in quartile 1. Moreover, the increased ACM risk observed in quartile 4 vs quartile 1 remained significant in both sexes, and accordingly, no significant interaction was observed between Pro-NT and gender on ACM risk. In line with results of Cox regressions, Figure 1 illustrates the Kaplan-Meier plots for ACM by Pro-NT quartiles during the follow-up. It shows that quartile 4 of Pro-NT deviates from the three lower quartiles.

Subsequently, we compared individuals belonging to quartile 4 with those belonging to quartiles 1-3. Having Pro-NT ≥149 pmol/L increased the relative risk of ACM, HR of 1.29 (1.17-1.43) (P<0.001) in a model adjusted for age and sex, and 1.20 (1.08-1.33) (P=0.001) after additional adjustment for cardiovascular risk factors (Table 3).
**Pro-NT and cause-specific mortality**

People in quartile 4 had increased risk of CSM as compared to people in quartile 1-3 regarding mortality due to CVD, digestive diseases, psychiatry diseases and deaths due to unspecific diseases, observed in both crude and multi-variable adjusted models. Out of total 1906 deaths, significant association of Pro-NT in quartile 4 vs quartile 1-3 and CSM in the cox-regression model were observed, when adjusted for age, sex, BMI, LDL-C, HDL-C, diabetes, smoking, systolic blood pressure and antihypertensive treatment. There were 595 reported deaths (31.2%) due to cardiovascular diseases, HR of 1.41 (1.18 -1.68; P<0.001), 42 reported deaths (2.20%) were due to digestive tract diseases with a HR 2.53 (1.37 - 4.67; P=0.003), 90 reported deaths (4.72%) were due to mental and behavioral diseases with a HR 1.62 (1.04 - 2.52; P=0.032), and 64 reported deaths (3.36%) were due to unspecific causes with a HR of 1.91 (1.15 - 3.19; P=0.013). There was no significant relationship between Pro-NT levels and deaths due to cancer, infections, neurological or other causes as listed in Table 3. In testing Pro-NT as a continuous variable in relation to cause-specific survival in the multivariate Cox regression analysis after adjustment for age, sex, body mass index, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, diabetes, smoking, systolic blood pressure antihypertensive treatment, Pro-NT was a positive predictor of deaths due to CVD and gastrointestinal diseases but the results were not significant for deaths due to mental and behavioral diseases and unspecific diseases as listed in Supplementary table 1. [23]

Calculation of HR by assessing Pro-NT distribution in quartile 4 versus quartile 1 in the same fully adjusted model showed that individuals within quartile 4 vs. those in quartile 1 had a significantly increased risk for all-cause mortality, CVD mortality, gastrointestinal disease mortality and for unspecific disease mortality. No significant relationships were observed between Pro-NT and death due to mental and behavioral diseases as listed in Supplementary table 2. [23]

After adding eGFR (according to MDRD) and HOMA-IR (as marker of insulin resistance) on top of all other covariates the significant associations between Pro-NT and mortality outcomes remained significant, apart from deaths due to mental and behavioral diseases and unspecific diseases, the latter of which were borderline significant, as listed in Supplementary table 3. [23]

The association between Pro-NT and cancer deaths (excluding cancers of unknown primary and hematological cancers) were insignificant with HR=1.12(95% CI 0.93 -1.95), P=0.222 in an age and sex adjusted model, similar to the result of “all cancer mortality” as presented in Table 3.

**DISCUSSION**

To the best of our knowledge, no previous studies have investigated the association of elevated plasma Pro-NT levels and CSM in prospective population-based cohorts. In this population-based cohort study with 20 years of follow-up, elevated plasma Pro-NT predicted ACM as well as CSM in CVD, gastrointestinal, psychiatric diseases, and deaths due to unspecific causes. No specific association was observed with deaths caused by neoplasms, respiratory diseases, endocrine diseases or external causes. Furthermore, sex-stratified analyses showed significant associations between Pro-NT and ACM in both genders. No significant interaction was observed between gender and Pro-NT on ACM. This strongly suggests that the association between Pro-NT and mortality is the same in both men and women. Mature neurotensin hormone is unstable and rapidly breaks down in the circulation, however, wide range of physiological and pharmacological effects of Pro-NT are seen by its affinity to specific NT receptor 1, which is used in this study. [8]
Our result regarding the association of Pro-NT and cardiovascular mortality are in line with previous studies. [19, 21] In contrast to previous study from the MDCS, in which follow-up time was substantially shorter and a gender-specific association with mortality and CVD mortality was observed (but only significant in women), [19], we here find robust associations in both genders during a long-term follow-up.

Observed significance of elevated Pro-NT to predict gastrointestinal disease mortality caused in this study have not been reported previously. However, previous studies have shown that Pro-NT facilitates lipid digestion and fat absorption in the small intestines [4, 5] and a fatty meal is the most potent inducer of NT secretion which results in decreased gastrointestinal motility and gastric acid secretions. [24, 25] In our recent study, Pro-NT contributed to intestinal absorption of lipids into the blood stream resulting in rise of plasma triglycerides. [3] Monten et al observed relationship between elevated postprandial Pro-NT levels and active coeliac disease in a pediatric population suggesting its pro-inflammatory role in the small intestines. [26] Recently, the role of Pro-NT and non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) in obese adults have been investigated, when a strong association between Pro-NT and NAFLD was found. [14] Although, the number of deaths due to gastrointestinal diseases was generally low, but the association remained significant, strengthening our belief in a true relationship between elevated Pro-NT and mortality risk due to gastrointestinal diseases.

The observed association between Pro-NT and diseases due to psychiatric diseases (mental and behavioral disorders) supports the previous study by Charles et al, who reported sex-specific cognitive impairment associated with higher levels of plasma Pro-NT in women.[16] However, our study showed significant associations in all participants in crude and adjusted models, but sex-specific associations were not observed.

Elevated Pro-NT and its receptors have long been recognized and involved in various cancers ranging from tumor growth to metastatic spread, [27] but no overall significant relationship was observed between Pro-NT and cancer mortality in this study. This was despite the proven effects of the neurotensin system on cancer cell proliferation, migration and invasion, as well as their anti-apoptotic effects, resulting in growth stimulation of cancers. [27] It could, however, be that different types of cancer may not share the same possible Pro-NT dependent pathway. A similar lack of association was observed between Pro-NT levels and respiratory, endocrine, infectious, neurological and urogenital diseases.

Strength and Limitations
This is a large prospective cohort study showing comorbidity and mortality in relation to elevated plasma Pro-NT in a middle-aged population. Strength of the study included a long-term follow up of study individuals along with the use of standardized national registry-based diagnose codes and information on potential confounders.

However, this study also has some potential limitations. The study population was dominated by Swedish-born white Europeans, which limits the generalized application of this study to other ethnicities. Furthermore, the mortality is relatively low in this population sample with mean age just below 60 years, giving a lower number of events in some of the major causes of death, especially in non-cardiovascular and non-neoplasm groups. Thus, the power for statistical analysis is low for some of these categories. We also acknowledge lack of data on hepatic function and were thus unable to adjust for this factor. Finally, there is always an uncertainty in the cause of death as judged by the treating physician, so some caution in the interpretation is warranted.

Conclusion
In conclusion, fasting Pro-NT was predictive of all-cause mortality and deaths due to cardiovascular diseases, gastrointestinal diseases, mental and behavioral diseases, and diseases due to unspecific causes. Furthermore, significant associations were observed between elevated levels of plasma Pro-NT and total mortality irrespective of gender. This suggests that Pro-NT may also be a useful biomarker for detecting individuals at higher risk of developing specific diseases and premature deaths and specifically can get benefit from the preventive therapy.

List of abbreviations
- BMI: Body mass Index
- CVD: Cardiovascular Disease
- SBP: Systolic blood pressure
- Pro-NT: Proneurotensin
- SD: Standard deviations
- T2D: Type 2 diabetes
- NAFLD: Non-alcoholic fatty liver disease
- NASH: Non-alcoholic steatohepatitis.
- MDC-CC: Malmö Diet and cancer study-Cardiovascular cohort.
- ICD: International classification of diseases.
- CSM: cause-specific mortality
- ACM: All-cause mortality.
DECLARATIONS:

Ethics approval and consent to participate.
All participants have provided written informed consent to participate.

Consent for publication
The data does not include any individual personal data including individual details, images or videos and hence consent for publication is not applicable in this study.

AUTHORS CONTRIBUTION
AF and OM conceived and conducted data analysis and all authors interpreted the results. AF and ZB drafted the manuscript with inputs from all the authors. All authors have read and approved the final version of submission. There are no conflicts of interest.

Availability of data and materials
All datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.
REFERENCES

1. Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, Lee A, Marczuk L, Mokdad AH, Moradi-Lakeh M, Naghavi M et al: Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *N Engl J Med* 2017, 377(1):13-27.

2. Di Angelantonio E, Kaptoge S, Wormser D, Willeit P, Butterworth AS, Bansal N, O’Keeffe LM, Gao P, Wood AM, Burgess S et al: Association of Cardiometabolic Multimorbidity With Mortality. *Jama* 2015, 314(1):52-60.

3. Fawad A, Fernandez C, Bergmann A, Struck J, Nilsson PM, Bennet L, Orho-Melander M, Melander O: Magnitude of rise in pronerotensin is related to amount of triglyceride appearance in blood after standardized oral intake of both saturated and unsaturated fat. *Lipids in health and disease* 2020, 19(1):191.

4. Gui X, Carraway RE: Enhancement of jejunal absorption of conjugated bile acid by pronerotensin in rats. *Gastroenterology* 2001, 120(1):151-160.

5. Li J, Song J, Zaytseva YY, Liu Y, Rychahou P, Jiang M, Starr ME, Kim JT, Harris JW, Yiannikouris FB et al: An obligatory role for pronerotensin in high-fat-diet-induced obesity. *Nature* 2016, 533(7603):411-415.

6. Aronin N, Carraway RE, Ferris CF, Hammer RA, Leeman SE: The stability and metabolism of intravenously administered pronerotensin in the rat. *Peptides* 1982, 3(4):637-642.

7. Ernst A, Hellmich S, Bergmann A: Pronerotensin 1-117, a stable pronerotensin precursor fragment identified in human circulation. *Peptides* 2006, 27(7):1787-1793.

8. Friry C, Feliciangeli S, Richard F, Kitabgi P, Rovere C: Production of recombinant large pronerotensin/neuromedin N-derived peptides and characterization of their binding and biological activity. *Biochem Biophys Res Commun* 2002, 290(4):1161-1168.

9. Kinkead B, Dobner PR, Egnatashvili V, Murray T, Deltemeyer N, Nemerooff CB: Neurotensin-deficient mice have deficits in prepulse inhibition: restoration by clozapine but not haloperidol, olanzapine, or quetiapine. *J Pharmacol Exp Ther* 2005, 315(1):256-264.

10. Pettibone DJ, Hess JF, Hey PJ, Jacobson MA, Leviten M, Lis EV, Mallorca PJ, Pascarella DM, Snyder MA, Williams JB et al: The Effects of Deleting the Mouse Neurotensin Receptor NTR1 on Central and Peripheral Responses to Neurotensin. *Journal of Pharmacology and Experimental Therapeutics* 2002, 300(1):305-313.

11. Lafrance M, Roussy G, Belleville K, Maeno H, Beaudet N, Wada K, Sarret P: Involvement of NTS2 receptors in stress-induced analgesia. *Neuroscience* 2010, 166(2):639-652.

12. Ruan CS, Yang CR, Li JY, Luo HY, Bobrovskaya L, Zhou XF: Mice with Sort1 deficiency display normal cognition but elevated anxiety-like behavior. *Experimental Neurology* 2016, 281:99-108.

13. Zhao D, Pothoulakis C: Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. *Peptides* 2006, 27(10):2434-2444.

14. Barchetta I, Cimini FA, Leonetti F, Capocci D, Di Cristofano C, Silecchia G, Orho-Melander M, Melander O, Cavallo MG: Increased plasma pronerotensin levels identify NAFLD in adults with and without type 2 diabetes. *The Journal of clinical endocrinology and metabolism* 2018.

15. Antonelli T, Fuxe K, Tomasini MC, Mazzoni E, Agnati LF, Tanganelli S, Ferraro L: Neurotensin receptor mechanisms and its modulation of glutamate transmission in the brain: relevance for neurodegenerative diseases and their treatment. *Progress in neurobiology* 2007, 83(2):92-109.

16. Nicoli CD, Howard VJ, Judd SE, Struck J, Manly JJ, Cushman M: Pro-Neurotensin/Neuromedin N and Risk of Cognitive Impairment in a Prospective Study. *Journal of Alzheimer’s disease* : JAD 2020, 76(4):1403-1412.

17. Januzzi JL Jr., Lyass A, Liu Y, Gaggin H, Trebicka A, Maisel AS, D'Agostino RB, Sr., Wang TJ, Massaro J, Vasan RS: Circulating Pronerotensin Concentrations and Cardiovascular
Disease Events in the Community: The Framingham Heart Study. Arterioscler Thromb Vasc Biol 2016, 36(8):1692-1697.

18. Fawad A, Bergmann A, Struck J, Nilsson PM, Orho-Melander M, Melander O: Proneurotensin Predicts Cardiovascular Disease in an Elderly Population. The Journal of clinical endocrinology and metabolism 2018, 103(5):1940-1947.

19. Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad B, Engstrom G, Kilger U, Nilsson P, Bergmann A et al: Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. Jama 2012, 308(14):1469-1475.

20. Tönjes A, Hoffmann A, Kralisch S, Qureshi AR, Klöting N, Scholz M, Schleinitz D, Bachmann A, Kratzsch J, Nowicki M et al: Pro-neurotensin depends on renal function and is related to all-cause mortality in chronic kidney disease. European journal of endocrinology 2020, 183(3):233-244.

21. Wettersten N, Cushman M, Howard VJ, Hartmann O, Filippatos G, Beri N, Clopton P, Howard G, Safford MM, Judd SE et al: Usefulness of Proneurotensin to Predict Cardiovascular and All-Cause Mortality in a United States Population (from the Reasons for Geographic and Racial Differences in Stroke Study). The American journal of cardiology 2018, 122(1):26-32.

22. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes Care 2018, 41(Suppl 1):S13-S27.

23. Supplementary Table. Plasma concentration of proneurotensin and prediction of cause-specific mortality in a middle-aged cohort during long-term follow-up [https://www.ludc.lu.se/sites/ludc.lu.se/files/2021-08/Supplementary-Tables_Fawad_JCEM_2021.pdf]

24. Spiller RC, Trotman IF, Higgins BE, Ghatei MA, Grimble GK, Lee YC, Bloom SR, Misiewicz JJ, Silk DB: The ileal brake--inhibition of jejunal motility after ileal fat perfusion in man. Gut 1984, 25(4):365-374.

25. Mogard MH, Maxwell V, Sytnik B, Walsh JH: Regulation of gastric acid secretion by neurotensin in man. Evidence against a hormonal role. The Journal of clinical investigation 1987, 80(4):1064-1067.

26. Montén C, Torinsson Naluai Å, Agardh D: Role of proneurotensin as marker of paediatric coeliac disease. Clinical and experimental immunology 2016, 186(3):387-392.

27. Ouyang Q, Zhou J, Yang W, Cui H, Xu M, Yi L: Oncogenic role of neurotensin and neurotensin receptors in various cancers. Clinical and experimental pharmacology & physiology 2017, 44(8):841-846.
Table 1: Clinical characteristics of the study population stratified in quartiles.

|                                | Pro-NT Quartile 1 | Pro-NT Quartile 2 | Pro-NT Quartile 3 | Pro-NT Quartile 4 | P for trend |
|--------------------------------|-------------------|-------------------|-------------------|-------------------|-------------|
| No. of participants            | 1158              | 1158              | 1158              | 1158              |             |
| Men, n (%)                     | 558 (48.2%)       | 497 (42.9%)       | 447 (38.6%)       | 467 (40.3%)       | <0.001      |
| Age, mean (SD), years          | 58.0 ± 6.00       | 57.7 ± 5.98       | 57.6 ± 5.96       | 57.7 ± 5.97       | 0.309       |
| SBP, mean (SD), mmHg           | 142.8 ± 19.7      | 141.6 ± 18.6      | 142.8 ± 19.7      | 142.2 ± 18.8      | 0.430       |
| Antihypertensive therapy, No. (%) | 188 (16.2%) | 197 (17%)         | 180 (15.5%)       | 224 (19.3%)       | 0.112       |
| Diabetes mellitus, No. (%)     | 77 (6.7%)         | 100 (8.7%)        | 100 (8.7%)        | 141 (12.3%)       | <0.001      |
| BMI, mean (SD), kg/m²          | 25.8 ± 3.84       | 25.7 ± 3.75       | 25.9 ± 4.01       | 26.0 ± 4.12       | 0.071       |
| LDL-C mean (SD), mmol/L        | 4.16 ± 1.02       | 4.16 ± 0.96       | 4.21 ± 0.98       | 4.13 ± 0.98       | 0.741       |
| HDL-C mean (SD), mmol/L        | 1.36 ± 0.36       | 1.39 ± 0.38       | 1.39 ± 0.37       | 1.39 ± 0.38       | 0.018       |
| Current smokers, n (%)         | 298 (25.7%)       | 278 (24.0%)       | 290 (25.0%)       | 354 (30.6%)       | 0.007       |

Abbreviations: BMI (body mass index), LDL-C (Low-density lipoprotein cholesterol), HDL-C (High-density lipoprotein cholesterol), SBP (systolic blood pressure).
Table 2: Event Rates and multivariate adjusted Cox Proportional Hazards Models for baseline Pro-NT in relation to All-Cause Mortality *(adjusted for age and sex).*

| All-cause mortality | Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | P value for trend |
|---------------------|-----------|------------|------------|------------|------------------|
| **All participants** |           |            |            |            |                  |
| No./ events No.     | 1158/458  | 1158/442   | 1158/470   | 1158/536   |                  |
| Pro-NT, median (range), pmol/L | 60.0 (3.00 - 75.8) | 89.0 (75.9-105) | 123 (105-149) | 190.4 (149-1150) |                  |
| HR (95% CI)         | REF (1.0) | 0.99 (0.87-1.12) | 1.10 (0.97-1.26) | 1.33 (1.17-1.50) | <0.001 |
| **Women**           |           |            |            |            |                  |
| No./events No.      | 600/194   | 661/213    | 711/252    | 691/291    |                  |
| Pro-NT, median (range), pmol/L | 60.4 (3.00 - 75.8) | 89.1 (75.9-104.6) | 122.6 (104.7-148.5) | 192.0 (148.6-1154.5) |                  |
| HR (95% CI)         | REF (1.0) | 1.03 (0.85-1.25) | 1.11 (0.92-1.34) | 1.42 (1.19-1.70) | <0.001 |
| **Men**             |           |            |            |            |                  |
| No./events No.      | 558/264   | 497/229    | 447/218    | 467/245    |                  |
| Pro-NT, median (range), pmol/L | 59.9 (0.00-75.9) | 89.6 (75.9-123.3) | 123.3 (104.8-188.4) |                  |
| median, pmol/L | 75.8 | 104.5 | 148.3 | 1057.4 |
|----------------|------|-------|-------|--------|
| HR (95% CI)    | REF (1.0) | 0.95 (0.80-1.14) | 1.10 (0.92-1.32) | 1.24 (1.04-1.48) | 0.014 |
Table 3: Event Rates and multivariate Cox Proportional Hazards Models for baseline Pro-NT in relation to Cause-Specific Mortality.

|                      | Age and sex adjusted | Fully adjusted* |
|----------------------|----------------------|-----------------|
|                      | All participants     | Quartile 1-3    | Quartile 4 | P value | All participants, Quartile 1-3 | Quartile 4 | P value |
| All-cause mortality  |                      |                 |           |         |                                 |             |         |
| No./events No.       | 4632/190             | 3474/13         | 1158/53   | 4478/1823 | 3365/1316 | 1113/50 |
| HR (95% CI)          | Ref 1.0              | 1.29            | <0.00     | 1.20      | 0.00  |
|                      | (1.17-1.43)          |                 |           | (1.08-1.33) |         |         |
| Cardiovascular diseases |                    |                 |           |         |                                 |             |         |
| No./events No.       | 4632/595             | 3474/41         | 1158/17   | 4478/562 | 3365/39 | 1113/16 |
| HR (95% CI)          |                      | 1.41            | <0.00     | 1.30      | 0.00  |
|                      | (1.18-1.68)          |                 |           | (1.08-1.56) |         |         |
| Gastrointestinal diseases|                  |                 |           |         |                                 |             |         |
| No./events           | 4632/42              | 3474/24         | 1158/18   | 4478/42 | 3365/24 | 1113/18 |
| No. | HR (95% CI) |
|-----|-------------|
|     | 2.53 (1.37-4.67) | 0.003 |
|     | 2.37 (1.28-4.39) | **0.00** |

**Mental and behavioral diseases**

| No./events | HR (95% CI) | | |
|------------|-------------|---|---|
| No.        | 4632/90  | 3474/61 | 1158/29 |
|            | 1.62 (1.04-2.52) | **0.032** |
|            | 1.56 (0.99-2.34) | **0.05** |

**Unspecific diseases**

| No./events | HR (95% CI) | | |
|------------|-------------|---|---|
| No.        | 4632/64  | 3474/41 | 1158/23 |
|            | 1.91 (1.15-3.19) | **0.013** |
|            | 1.80 (1.07-3.04) | **0.02** |

**Cancer**

| No./events | HR (95% CI) |
|------------|-------------|
| No.        | 4632/683  | 3474/50 | 1158/17 |
|            | 1.10 (0.92-1.30) | **0.293** |
|            | 4478/658  | 3365/49 | 1113/16 |
|            | 1.10 (0.92-1.30) | **0.293** |
| Condition                        | No./events | No.  | HR (95% CI)  | No./events | No.  | HR (95% CI)  | No./events | No.  | HR (95% CI)  | No./events | No.  | HR (95% CI)  | No./events | No.  | HR (95% CI)  |
|---------------------------------|------------|------|--------------|------------|------|--------------|------------|------|--------------|------------|------|--------------|------------|------|--------------|
| **Infectious diseases**         |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No./events                      | 4632/41    | 3474/30 | 1158/11      | 4478/35    | 3365/25      | 1113/10  |  |              |           |      |              |            |      |              |
| HR (95% CI)                     |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No.                             |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| **Endocrine diseases**          |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No./events                      | 4632/48    | 3474/34 | 1158/14      | 4478/45    | 3365/32      | 1113/13  |  |              |           |      |              |            |      |              |
| HR (95% CI)                     |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No.                             |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| **External causes**             |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No./events                      | 4632/58    | 3474/41 | 1158/17      | 4478/52    | 3365/39      | 1113/13  |  |              |           |      |              |            |      |              |
| HR (95% CI)                     |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No.                             |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| **Musculoskeletal diseases**    |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No./events                      | 4632/09    | 3474/6  | 1158/03      | 4478/09    | 3365/06      | 1113/03  |  |              |           |      |              |            |      |              |
| HR (95% CI)                     |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| No.                             |            |      |              |            |      |              |            |      |              |            |      |              |            |      |              |
| Condition                  | HR (95% CI) |    |    |    |    |
|---------------------------|-------------|----|----|----|----|
| Urogenital diseases       | 1.63 (0.41-6.56) |    |    |    |    |
| Respiratory diseases      | 0.91 (0.34-2.43) |    |    |    |    |
| Neurological diseases     | 1.22 (0.82-1.1) |    |    |    |    |
| Hematologica              | 1.11 (0.72-1.72) |    |    |    |    |
| diseases | No./events | HR (95% CI) |
|----------|------------|-------------|
|          | No.        |             |            |
|          | 4632/05    | 0.88 (0.99-7.88) | 0.91 |
|          | 3474/04    |             |            |
|          | 1158/01    |             |            |
|          | 4478/05    |             |            |
|          | 3365/04    |             |            |
|          | 1113/01    |             |            |

Adjusted for age, sex, BMI (body mass index), LDL-C (low-density lipoprotein cholesterol), HDL-C (high-density lipoprotein cholesterol), diabetes, smoking, systolic blood pressure and antihypertensive treatment.
Legends to Figures

FIGURE 1:
Kaplan-Meier plot shows cumulative proportion of all-cause mortality during follow-up in the fourth quartile versus quartiles 1-3 of baseline prononeutensin.
