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# Sex-specific Mortality Prediction by Pro-C-type Natriuretic Peptide Measurement in ST-elevation Myocardial Infarction

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**Keywords:** Natriuretic peptides, C-type natriuretic peptide, CNP, ANP, Reference intervals, Myocardial infarction.

**List of abbreviations:** CNP, C-type natriuretic peptide; ACS, acute coronary syndrome; NT-proCNP, amino-terminal proCNP; STEMI, ST-elevation myocardial infarction; proCNP (see Nomenclature); NOBIDA, Nordic Reference Interval Project Biobank and Database; RH, Copenhagen University Hospital, Rigshospitalet; OUH, Odense University Hospital; CAG, coronary angiography; ECG, electrocardiogram; BMI, body mass index; LVEF, left ventricular ejection fraction; hs-CRP, high sensitivity c-reactive protein; sTM, soluble thrombomodulin.

## Abstract

**Objective:** To determine the predictive value of pro-C-type natriuretic peptide (proCNP) measurement in plasma sampled on admission from patients presenting with ST-elevation myocardial infarction (STEMI).

**Design:** Prospective cohort study.

**Setting:** Two University Hospitals in Denmark.

**Participants:** 1760 consecutive patients (470 females and 1290 males) with confirmed STEMI.

**Main Outcomes and Measures:** The main outcome was all-cause mortality at 30 days and one year after presentation and the primary measure was proCNP concentration in plasma at admission in all patients and longitudinal measurements in a consecutive subgroup of 287 patients. A reference population (n = 688) defined cut-off values of increased proCNP concentrations.

**Results:** In all patients, an increased proCNP concentration was associated with a higher all-cause mortality after one year (HR: 1.6 (1.1-2.4), $P_{logrank}$= .009) including an interaction of sex ($P$= .03). In separate sex-stratified analyses, female patients showed increased all-cause mortality ($HR_{one\ year}$: 2.6 (1.5-4.6), $P_{logrank}$ < .001), whereas no differences were found in male patients ($HR_{one\ year}$: 1.1 (.7-1.9), $P_{logrank}$ = .66). After adjusting for potential risk factors, we found increased proCNP concentrations ≥ the median value to be independently associated with increased risk of mortality in female patients within one year (HR per 1 pmol/L increase: 1.04 (1.01-1.06), $P$ = .007).

Moreover, we found indications of sex differences in proCNP concentrations over time (higher proCNP in males (4.4 (-.28 – 9.1) pmol/L, P= .07) and interaction of sex and time (P= .13)), and that hypertension was independently associated with higher proCNP (4.5 (.6-8.4) pmol/L, P= .03).

**Conclusions:** In female but not male patients presenting with STEMI, increased concentrations of proCNP at admission independently indicate a higher risk of all-cause mortality. The findings are remarkably specific for female patients, suggesting a different vascular phenotype beyond traditional measures of coronary artery flow compared to male patients.

## Strengths and limitations of this study

- This is the first study to investigate the prognostic potential of measurement of peptides derived from pro-C-type natriuretic peptide (proCNP) using predefined sex- and age-specific cut-off values based on a reference population.
- As a novel approach, a large cohort of patients are examined during the acute phase of ST-elevation myocardial infarction with plasma sampling at admission and all-cause mortality within one year as main outcome.
- To clarify the temporal pattern of proCNP concentrations, longitudinal measurements during admission in a subgroup of the cohort are used to further examine sex differences and baseline associations.
- The sex-specific analyses are exploratory and the present report should be considered a hypothesis-generating study.
- As all-cause mortality within one year is the only available outcome measure, other clinical end-points and long-term follow-up is not investigated.

## Introduction

C-type natriuretic peptide (CNP) is a paracrine/autocrine peptide expressed in many tissues,[1] including endothelial cells.[2] Experimental studies have shown that endothelial-derived CNP is locally involved in regulation of vascular tone[3,4] and angiogenesis.[5] Also, a recent preclinical investigation has suggested that CNP plays a key protective role in cardiac pathophysiology.[6]

Clinical reports on the general population as well as patients with heart disease have examined circulating proCNP-derived peptides as prognostic markers.[7–9] In patients presenting with acute coronary syndrome (ACS), high concentrations of amino-terminal proCNP (NT-proCNP) in plasma measured 4-6 weeks after the event were reported to be the only natriuretic peptide to independently predict cardiac readmission and death in the patients with unstable angina.[7] Taken together, experimental and epidemiological data suggest CNP to be an important regulator of cardiovascular function and that increased concentrations of proCNP-derived peptides in plasma of patients reflect an unfavorable cardiovascular condition. However, no large study has explored the prognostic value of proCNP measurement in the acute phase of an ST-elevation myocardial infarction (STEMI).[10]

In this study, we measured the precursor of C-type natriuretic peptides (proCNP) in plasma from a large cohort of patients presenting with STEMI. We have previously reported on this method for accurate quantification of “total” proCNP in plasma by a processing-independent radioimmunoassay.[11] In total, 1760 patients with STEMI were included, and proCNP was measured in plasma sampled at admission to determine its

predictive value in 30-day and one-year all-cause mortality. Moreover, we investigated a subgroup of 287 patients with STEMI with longitudinal plasma samples collected during the hospital admission to examine proCNP concentrations over time and further analyze baseline associations between proCNP concentrations and vascular diseases. Finally, we included a large sample of healthy individuals (n = 688) in order to, independently of the patient cohort, establish age- and sex-specific reference intervals for proCNP concentrations in plasma.

*Nomenclature*

ProCNP: In the present article, proCNP refers to a specific amino-acid sequence (human proCNP 11-27) within the prohormone sequence of CNP; the epitope of the antiserum of our radioimmunoassay. In this processing-independent methodology, we utilize this fragment after enzymatic cleavage in vitro as a proxy measure of all proforms released to plasma irrespective of prohormone post-translational processing.

ProCNP-derived peptides: Collective term for any fragment of the prohormone of CNP.

## Methods

REFERENCE POPULATION

For establishment of reference intervals, we used plasma samples from the Nordic Reference Interval Project Biobank and Database (NOBIDA), originally consisting of 3002 subjects.[12] A subgroup of 853 subjects from this population was randomly selected with the aim to represent sex, age, and country of origin equally, as previously described.[13]

## COHORT OF PATIENTS WITH STEMI

Patients with suspected STEMI were consecutively included from two Danish hospitals over a period of one year (2015/2016) (Copenhagen University Hospital, Rigshospitalet (RH), and Odense University Hospital (OUH)). The procedure of inclusion has been described previously.[14] From this cohort of patients with suspected STEMI and triaged for acute coronary angiography (CAG) (based on assessment of symptoms and the individual electrocardiogram (ECG)), we only included patients with confirmed STEMI.[15] All patients underwent CAG (See further details on data collection in the Supplemental Material).

## PATIENT AND PUBLIC INVOLVEMENT

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

## ETHICS

Patients gave written informed consent. When patients were not able to provide this (e.g. comatose cardiac arrest patients), consent was obtained by the patients' next of kin and general practitioners in accordance with national legislation. The study was approved by the local ethics committee (Copenhagen) (Ref. H-2-2014-110).

## BIOCHEMICAL ANALYSES

Plasma proANP and proCNP concentrations were quantified by the previously reported processing-independent assay technology and procedures.[11,16,17] Information of other biochemical analyses can be found in the Supplemental Material.

## ALL-CAUSE MORTALITY

The Danish Civil Registration System was used for all-cause mortality assessment. All Danish citizens are recorded with a unique 10-digit civil registration number, and deaths are registered within 2 weeks. Initial follow-up began on the date of admission and continued until date of death, or October 30th, 2017.

## STATISTICS

### *Reference Population*

We divided the reference population into groups based on sex and age (<50 and ≥50 years) and used the RefVal software[18] to calculate 95% reference intervals based on a non-parametric bootstrapping method.

### *STEMI Cohort*

Based on their respective sex- and age-specific reference interval from the reference population, all STEMI patients were stratified according to a) increased proCNP concentration (higher than the 95% reference interval), b) normal proCNP concentration (within the 95% reference interval), and c) decreased proCNP concentration (lower than the 95% reference interval). Dichotomous variables are presented as numbers (n) and percentages (%). Continuous variables are presented as medians with 25th–75th percentiles. Comparisons between groups were done using independent non-parametric t-tests and Fisher's exact two-sided test. Spearman's correlation analyses were used to assess the relationships between proCNP and other biochemical analytes. Differences in median proCNP concentrations at different time intervals from onset of

symptoms to blood sample were assessed by Kruskal-Wallis tests (this time parameter also reflects total ischemic time; see Supplemental Material). All-cause mortality after 30 days and one year was assessed in patients stratified into normal or increased proCNP concentrations and depicted by Kaplan-Meier plots and then compared with the log-rank test and estimates of hazard ratio including 95% confidence intervals. We performed a statistic test of interaction between sex and groups of proCNP. To test the relation of mortality and proCNP concentrations on a continuous scale, we performed cubic spline plots with the density distribution and the logarithm of hazard ratios. We focused our further mortality analyses on proCNP concentrations ≥ median, where we observed an effect of increasing proCNP concentrations on mortality. Patients were divided into groups of ≥ vs. < median proCNP according to sex- and age-specific median proCNP concentrations in the reference population. Multivariable Cox proportional hazard models including proCNP, age, plasma creatinine, plasma proANP, time from onset of symptoms to blood sample, number of coronary vessels affected and tertiles of peak plasma troponin concentrations were constructed for mortality assessment. Both plasma proANP and creatinine were logarithmically transformed, where peak troponin T and troponin I were combined in one variable of tertiles (represented by values of one to three) as a proxy myocardial infarction size, before being entered into the model. For longitudinal analyses, we constructed four time points/intervals for statistical analyses (1: samples from admission; 2: 1 to 12 hours after admission; 3: >12 to 24 hours after admission; 4: >24 hours after admission). We used linear mixed models of unstructured co-variances to examine changes in concentrations and associations with co-variables over time. Statistical analyses were performed using RefVal software[20] for calculation of

reference intervals, statistical software R version 3.6.1 (R Core Team, Vienna, Austria[19]) for linear mixed models (nlme package), and IBM SPSS Statistics 22 (SPSS Inc., Chicago, Illinois, United States) for other analyses. A *P*-value <.05 was considered statistically significant.

## Results

In the NOBIDA reference population, we measured proCNP concentrations in available plasma from 688 subjects (358 females; 330 males). From these measurements, 95% reference intervals for females vs. males and < vs. ≥ 50 years were calculated (see results in the Supplemental Material).

From the cohort of 2247 patients with suspected STEMI, 1760 patients (460 females and 1290 males) with verified STEMI and available plasma were included in our study (see Supplemental Material for histograms of the distribution of plasma proCNP concentrations in the reference population). When compared to the sex- and age-specific reference intervals, a total of 283 (16.1%) of the patients had an increased proCNP concentration; no difference in sex-specific proportions was observed (*P* = .42).

Table 1 shows the baseline characteristics and follow-up in patient groups defined by normal or increased proCNP concentrations. Five patients (one male and four females) displayed decreased proCNP concentrations and were not included in Table 1. Missing individual information on each variable in each group was between .1% and 4.4%, except for age, sex, mortality, cardiogenic shock, cardiac arrest coma, time from onset of symptoms to blood sample, and TIMI grade flow, where information from all patients was available. Female patients with increased proCNP concentrations

at admission had a higher prevalence of hypertension ($P$ = .003), diabetes mellitus ($P$ = .022), peripheral artery disease ($P$ = .002), and kidney disease ($P$ < .001), whereas male patients had higher prevalence of hypertension ($P$ = .004), stroke ($P$ = .012), and kidney disease ($P$ < .001). Increased proCNP was associated with higher concentrations of proANP ($P$ = .019) and soluble thrombomodulin (sTM) ($P$ = .001) in female patients and with higher concentrations of creatinine ($P$ < .001), syndecan-1 ($P$ = .019), and sTM ($P$ = .003) in male patients. Lastly, we found a higher one-year mortality rate ($P$ = .001) and prevalence of cardiogenic shock development ($P$ = .013) in female patients with increased proCNP concentrations, whereas no differences were found in male patients. For biochemical markers with positive associations to proCNP, we performed Spearman's correlation analyses (see the Supplemental Material for results).

Thirty-days and one-year all-cause mortality plots of normal and increased proCNP concentrations of all patients and stratified by sex are depicted in Figure 1. Different mortality rates were found for all patients at both time points ($HR_{30\ days}$ = 1.6 (1.0-2.6), $P_{logrank}$ = .03 and $HR_{one\text{-}year}$ = 1.6 (1.1-2.4), $P_{logrank}$ = .009). However, we found an interaction of sex and groups of proCNP ($P$ = .03). In sex-specific estimates, only female patients showed different mortality rates ($females_{30\ days}$: HR = 2.8 (1.4-5.6), $P_{logrank}$ = .002, $females_{one\text{-}year}$: HR = 2.6 (1.5-4.6), $P_{logrank}$ < .001, $males_{30\ days}$: HR = 1.1 (0.6-2.1), $P_{logrank}$ = .87, and $males_{one\text{-}year}$: HR = 1.1 (.7-1.9), $P_{logrank}$ = .66). In cubic spline plots, we observed an effect of increasing proCNP as a continuous variable on mortality from median concentrations in both sexes. We therefore focused on this upper range of proCNP concentrations in Cox regression analyses (see Supplemental Figure 3). In a univariate Cox proportional hazard model, we found an elevated hazard ratio (HR) of

all-cause mortality with increases of plasma proCNP for both sexes (results shown in Table 2). When including age and plasma creatinine in a multivariable model (Model 1), proCNP was independently associated with mortality in female but not in male patients (see Table 2): HR (95% CI) for female patients was 1.04 (1.01-1.07) (*P* = .008) for 30-day and 1.03 (1.01-1.06) (*P* = .010) for one-year mortality, whereas HR was 1.00 for both all patients and male patients at both time points (see Table 2). In a model where proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to admission were also added (Model 2), risk estimates of proCNP (pr. one pmol/L increase) were: HR (95% CI) = 1.04 (1.01-1.07), *P* = .016 for 30-day mortality, and HR (95% CI) = 1.04 (1.01-1.06), *P* = .007 for one-year mortality.

To examine proCNP concentrations over time during a STEMI and further test the baseline associations of vascular disease and increased proCNP, we used a set of longitudinal plasma samples from a consecutive subgroup of the cohort consisting of 287 STEMI patients (64 females and 223 males). Results are shown in Figure 2 and Table 3. An overall decrease in proCNP concentration was estimated to be 3.8 pmol/L (~10%) (*P* = .001) from admission to >24 hours. In a multivariate model including sex, age, and chronic diseases (Model 3), kidney disease and hypertension were independently associated with higher concentrations of proCNP (*P* < .001 and *P* = .03, respectively), whereas time and age were not independently associated with changes in proCNP. Also, both Model 2 and 3 implied a positive association of proCNP concentration and male sex, and an interaction of time and sex; however, the statistical uncertainty of these estimates was substantial (*P* = .07 and *P* = .13, respectively). Figure 2 shows proCNP concentrations over time in overall and sex-specific graphs

including graphs of kidney disease and hypertension (see the Supplemental Material for further results of longitudinal measurements).

## Discussion

In this study, we report on a marked sex-specific prognostic information profile for proCNP measurement in patients presenting with a STEMI. A major advantage of our present approach comes from an independent establishment of a sex-specific proCNP reference interval prior to patient measurement. This allowed us to perform clinically meaningful divisions of normal, decreased, or increased proCNP concentrations in plasma specific to sex, rather than testing differences only within the patient cohort as a whole (see the Supplemental Material for discussion of age groups).

In this cohort of consecutive patients with a verified STEMI, we show that 16.1% have increased concentrations of proCNP during the early phase of the myocardial infarction compared with the sex- and age-specific intervals. Interestingly, besides higher prevalence of kidney disease, we found a markedly higher prevalence of cardiovascular disease including hypertension in both sexes; diabetes mellitus and peripheral artery disease for female patients; and stroke for males among patients with increased proCNP. Our longitudinal analyses of a subgroup of the STEMI patients corroborates that the association to both kidney disease and hypertension is consistent even over time and is independent of sex, age, and other cardiovascular diseases. A putative explanation for the linkage of hypertension and increased proCNP concentrations is the upregulation of CNP expression by vascular shear stress.[20,21] The positive association of proCNP and a vascular marker, sTM, for both sexes also

support the relation of circulating proCNP with vascular stress. In contrast, the baseline associations of increased proCNP concentrations and diabetes, peripheral artery disease and stroke, respectively, are not statistically independent in longitudinal analyses. However, given the sex-specific pattern in the baseline findings and the limited number of patients with the respective diseases in longitudinal analyses, the results may be too preliminary to sufficiently conclude on the potential associations to diabetes, peripheral artery disease, and stroke. Also, the statistical uncertainty of the suggested effect of sex and interaction of sex and time in longitudinal analyses calls for a cautious interpretation (see the Supplemental Material for further discussion of longitudinal analyses).

The risks of death within 30 days and one year were higher for female patients with increased proCNP concentrations – but not for male patients. This marked sex-specific association was confirmed when the mortality rate was analyzed by increases of proCNP ≥ median, where proCNP proved to be an independent predictor in a model (Model 1 in Table 2) including two additional variables, age and plasma creatinine, which have previously established associations with NT-proCNP in plasma.[22] To further test the prognostic potential of proCNP, we added plasma proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to admission into a multivariable model (Model 2). In this model, increases of proCNP were still independently associated with the risk of death, and the estimates were of similar size at both time points. Recent data on patients with STEMI found no independent prognostic value on mortality rate for NT-proCNP concentrations.[7] However, that report differed from ours in several aspects including radioimmunoassay

principle and duration of follow-up, and, importantly, plasma was sampled 4-6 weeks after admission in contrast to our real-time STEMI investigation. These differences make a direct comparison difficult.

Our results firmly suggest a sex-specific association with survival, where increased proCNP concentrations indicate a poorer prognosis in female patients. Previously, studies have shown that CNP acts as a more potent vasodilator in female porcine arteries[23] and that the female sex hormone estradiol upregulates the expression of CNP in vascular endothelial cells.[24] Furthermore, recent preclinical reports have convincingly pointed to a pivotal role of CNP and its receptors in the regulation of the microcirculation[4] and in cardiac homeostasis.[25] Taken together, these previous findings suggest that the CNP system is a critical modulator of vascular integrity and function including a more pronounced vasodilatory capacity in females that may be provided by female sex hormones. Clinical evidence shows that females display a sharp rise in the incidence of cardiovascular disease after menopause and that changes in sex hormones by complex mechanisms play a key role in the loss of vasoprotection.[26,27] Notably, postmenopausal females are at higher risk of coronary microvascular dysfunction and HFpEF compared with their male counterparts,[28] and high concentrations of NT-proCNP have previously been independently linked to an adverse outcome in patients with HFpEF.[8] In this perspective, we speculate that our observed sex-specific effect of increased proCNP concentrations in plasma of patients with STEMI partly reflects ongoing vascular dysfunction in females in particular and, hence, represents an independent signal of poor cardiovascular prognosis. However,

there is a need for translational research to elucidate this association between increased proCNP in females with vascular complications and increased mortality rate.

*Limitations*

While the number of patients with STEMI in the cohort is high, the follow-up period is relatively short. We examined only all-cause mortality as outcome in our follow-up multivariable Cox regression analyses, and other clinical endpoints, e.g., cardiac readmission, were not tested. Hence, the number of events limits the number of possible covariates in the multivariable Cox regression analyses (Model 3), and sex-specific estimates of 30-day mortality in this model should be interpreted cautiously. Moreover, this study focused on increased proCNP and the upper range of proCNP concentrations in plasma, whereas associations of decreased proCNP concentrations were not investigated. With regard to measurement of troponins in plasma, two different analytes (TnT and TnI) were measured at the two hospitals of inclusion. Thus, a combined variable of tertiles of peak troponins (discrete values of one to three) was included in the multivariable models with less quantitative information than the measured concentrations. Baseline biochemical analyses were performed on arterial plasma, whereas venous plasma was used from the reference population and in repeated measurements. A previous report has shown slightly lower plasma concentrations of NT-proCNP in arterial compared with venous plasma.[29] Thus, in theory, our approach will underestimate the proportion of STEMI patients with increased proCNP concentration and the initial longitudinal decrease in proCNP concentration from baseline to the second time point (0 to 1-12 hours).

## Conclusions

We show here that increased proCNP concentrations in plasma from patients presenting with STEMI are associated with a higher all-cause mortality rate within one year among female patients with STEMI, whereas male patients display no such pattern. Moreover, we report that an increase of proCNP in the upper range of plasma concentrations in female patients is an independent prognostic marker of mortality at both 30 days and one year. The findings are remarkably specific for female patients, suggesting a different vascular phenotype beyond traditional measures of coronary artery flow compared to male patients.

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## Contributors

Designed the study: PDM, MF, CH, JPG. Designed and conducted the STEMI cohort study: MF, OKLH, LH, JEM, CH. Undertook biochemical measurements: PDM, SRO, PIJ, JPG. Performed the data analysis: PDM, MF, TCRP. Wrote the manuscript draft: PDM. Revised the paper based on intellectual contribution: PDM, MF, OKLH, LH, JEM, PIJ, SRO, TCRP, CH, JPG.

## Funding

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## Competing interests

None to report.

## Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

## Ethics approval

The study was approved by the local ethics committee (Copenhagen) (Ref. H-2-2014-110).

## Data availability statement

Data are available upon reasonable request (email: JPG@dadlnet.dk).

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**Table 1. Sex-specific characteristics, medical history, and biochemical results in groups of proCNP concentrations in the STEMI-cohort.**

| | **All patients (*n* = 1755)** | | | **Females (*n* = 466)** | | | **Males (*n* = 1289)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value |
| **Number, *n* (%)** | 1472 (83.6) | 283 (16.1) | - | 385 (81.9) | 81 (17.2) | - | 1087 (84.2) | 202 (15.6) | - |
| **Age, *years*** | 63 (54-72) | 66 (56-76) | .046 | 67 (56-77) | 72 (60-80) | .16 | 62 (53-70) | 64 (55-74) | .21 |
| **Males, *n* (%)** | 1087 (73.8) | 202 (71.4) | .42 | - | - | - | - | - | - |
| **Height, *cm*** | - | - | - | 165 (160-169) | 164 (160-168) | .13 | 178 (173-182) | 178 (174-183) | .87 |
| **BMI, *kg/m²*** | 26.3 (24.1-29.3) | 26.3 (23.8-30.1) | .96 | 25.4 (20.0-29.1) | 25.7 (22.5-31.2) | .61 | 26.5 (24.5-29.3) | 26.5 (24.2-29.9) | .76 |
| **Smoking, *n* (%)** | 1045 (73.3) | 203 (74.9) | .60 | 250 (68.1) | 54 (70.1) | .79 | 795 (75.1) | 149 (76.8) | .65 |
| **Time from symptom to blood sample, *min*** | 190 (128-376) | 178 (125-292) | .34 | 210 (136-460) | 216 (141-349) | >.99 | 185 (125-356) | 167 (123-271) | .18 |
| **LVEF, %** | 45 (40-55) | 45 (35-55) | .33 | 50 (40-55) | 45 (35-55) | .62 | 45 (40-55) | 45 (36-55) | .36 |
| **Culprit vessel** | | | | | | | | | |
| **None, *n* (%)** | 55 (3.7) | 7 (2.5) | .38 | 21 (5.5) | 5 (6.2) | .79 | 34 (3.1) | 2 (1.0) | .10 |
| **Left main coronary artery, *n* (%)** | 31 (2.1) | 5 (1.8) | >.99 | 15 (3.9) | 2 (2.5) | .75 | 16 (1.5) | 3 (1.5) | >.99 |
| **Left anterior descending coronary artery, *n* (%)** | 623 (42.3) | 119 (42.0) | .95 | 144 (37.4) | 31 (38.3) | .90 | 479 (44.1) | 88 (43.6) | .94 |
| **Right coronary artery, *n* (%)** | 529 (35.9) | 115 (40.6) | .14 | 148 (38.4) | 34 (42.0) | .62 | 381 (35.1) | 81 (40.1) | .18 |
| **Left circumflex, *n* (%)** | 221 (15.0) | 36 (12.7) | .36 | 54 (14.0) | 9 (11.1) | .59 | 167 (15.4) | 27 (13.4) | .52 |
| **Graft, *n* (%)** | 10 (0.7) | 1 (0.4) | >.99 | 1 (0.3) | 0 (0) | >.99 | 9 (0.8) | 1 (0.5) | >.99 |
| **Number of vessels affected** | | | | | | | | | |
| **No-vessel disease, *n* (%)** | 29 (2.0) | 2 (0.7) | .21 | 14 (3.6) | 2 (2.5) | >.99 | 15 (1.4) | 0 (0) | .15 |
| **One-vessel disease, *n* (%)** | 903 (61.5) | 172 (60.7) | .84 | 243 (63.4) | 46 (56.8) | .31 | 660 (60.8) | 126 (62.4) | .70 |
| **Two-vessels disease, *n* (%)** | 333 (22.7) | 61 (21.6) | .76 | 75 (19.6) | 20 (24.7) | .29 | 258 (23.8) | 41 (20.3) | .32 |
| **Three-vessels disease, *n* (%)** | 204 (13.9) | 48 (17.0) | .20 | 51 (13.3) | 13 (16.0) | .48 | 153 (14.1) | 35 (17.3) | .23 |
| **Thrombolysis in Myocardial Infarction (TIMI) grade flow** | | | | | | | | | |
| **0, *n* (%)** | 96 (6.8) | 21 (7.4) | .60 | 28 (7.2) | 8 (9.9) | .49 | 68 (6.3) | 13 (6.4) | .88 |
| **1, *n* (%)** | 6 (0.4) | 1 (0.4) | >.99 | 1 (0.3) | 0 (0) | >.99 | 5 (0.5) | 1 (0.5) | >.99 |
| **2, *n* (%)** | 49 (3.3) | 7 (2.5) | .58 | 17 (4.4) | 2 (2.5) | .55 | 32 (2.9) | 5 (2.5) | >.99 |
| **3, *n* (%)** | 1321 (89.7) | 254 (89.8) | >.99 | 339 (88.1) | 71 (87.7) | .85 | 982 (90.3) | 183 (90.6) | >.99 |
| **Medical history, *n* (%)** | | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Hypertension** | 612 (42.6) | 156 (56.1) | <.001 | 177 (46.9) | 52 (65.8) | .003 | 435 (41.0) | 104 (52.3) | .004 |
| **Diabetes mellitus** | 177 (12.3) | 51 (18.3) | .009 | 47 (12.5) | 18 (22.2) | .022 | 130 (12.2) | 33 (16.5) | .11 |
| **Peripheral artery disease** | 72 (5.0) | 24 (8.5) | .023 | 15 (4.0) | 11 (13.6) | .002 | 57 (5.3) | 13 (6.4) | .50 |
| **Stroke** | 91 (6.3) | 30 (10.6) | .015 | 30 (7.9) | 8 (9.9) | .51 | 61 (5.7) | 22 (10.9) | .012 |
| **Kidney disease** | 41 (2.8) | 40 (14.1) | <.001 | 10 (2.6) | 11 (13.6) | <.001 | 31 (2.9) | 29 (14.4) | <.001 |
| **Ischemic heart disease** | 218 (14.8) | 43 (16.3) | .53 | 44 (11.5) | 10 (12.3) | .85 | 174 (16.0) | 36 (17.9) | .53 |
| **Heart failure** | 38 (2.6) | 13 (4.6) | .079 | 6 (1.6) | 3 (3.7) | .19 | 32 (2.9) | 10 (5.0) | .13 |
| **Biochemical analyses** | | | | | | | | | |
| **Acute Troponin I, *ng/L*** | 220 (53-1576) | 323 (86-887) | .52 | 293 (54-3220) | 813 (176-1814) | .57 | 188 (53-1481) | 211 (58-844) | .99 |
| **Acute Troponin T, *ng/L*** | 1610 (329-3940) | 953 (222-3370) | .018 | 1520 (394-3540) | 571 (166-2753) | .076 | 1640 (287-4058) | 1250 (242-3710) | .24 |
| **Peak Troponin I, *ng/L*** | 20264 (3413-50000) | 19981 (5508-50000) | .91 | 12970 (2734-41856) | 11721 (4014-50000) | .98 | 24507 (3654-50000) | 20575 (5771-47927) | .56 |
| **Peak Troponin T, *ng/L*** | 3110 (1230-6920) | 2430 (880-8060) | .23 | 2520 (995-5930) | 2290 (614-8123) | .55 | 3380 (1400-7603) | 2860 (923-8075) | .23 |
| **Plasma creatinine, *µmol/L*** | 81 (70-96) | 95 (80-126) | <.001 | 69 (59-84) | 83 (63-113) | .072 | 85 (74-98) | 97 (86-131) | <.001 |
| **proANP, *pmol/L*** | 822 (515-1315) | 1005 (558-1742) | .005 | 978 (664-1504) | 1382 (747-2064) | .019 | 758 (488-1241) | 899 (508-1499) | .10 |
| **hs-CRP ≥2 mg/L, *n* (%) *, †** | 474 (65.0) | 111 (63.1) | .66 | 117 (67.2) | 34 (68.0) | >.99 | 357 (64.3) | 77 (61.1) | .54 |
| **ST2, *ng/ml*** | 40.0 (29.1-56.8) | 39.8 (30.4-59.7) | .95 | 35.3 (27.0-55.0) | 34.8 (26.2-58.4) | .92 | 41.5 (30.2-57.5) | 41.5 (32.1-60.0) | .95 |
| **Syndecan-1, *ng/ml*** [b] | 92.3 (64.2-137.0) | 103.5 (69.4-154.0) | .001 | 82.2 (52.5-137.3) | 99.3 (61.8-140.8) | .21 | 94.4 (68.1-137.0) | 113.5 (71.6-155.1) | .019 |
| **Soluble thrombomodulin, *ng/ml*** [b] | 7.35 (5.98-8.91) | 8.49 (6.67-10.86) | <.001 | 6.71 (5.42-8.15) | 8.05 (6.16-10.80) | .001 | 7.57 (6.19-9.18) | 8.53 (6.70-10.87) | .003 |
| **Follow-up, *n* (%)** | | | | | | | | | |
| **Number of deaths within one year** | 114 (7.7%) | 35 (12.4%) | .014 | 37 (9.6%) | 19 (23.5%) | .001 | 77 (7.1%) | 16 (7.9%) | .66 |
| **Cardiogenic shock** | 122 (8.3%) | 34 (12.0%) | .052 | 32 (8.3%) | 15 (18.5%) | .013 | 90 (8.3%) | 19 (9.4%) | .58 |
| **Cardiac arrest coma** | 66 (4.5%) | 19 (6.7%) | .13 | 14 (3.6%) | 3 (3.7%) | >.99 | 52 (4.8%) | 16 (7.9%) | .084 |

Continuous variables are given in median (inter-quartile range).

***** Only patients with a time from onset of symptoms to CAG of <6 hours were included.

† Only patients admitted at RH (313 females and 873 males) were included.

**Table 2. Cox regression analyses of mortality in patients with proCNP ≥ median.**

| | | Univariate | | | Model 1* | | | Model 2 † | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HR | 95% CI | *P* Value | HR | 95% CI | *P* Value | HR | 95% CI | *P* Value |
| **All patients with proCNP ≥ median (*n* = 928)** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.99-1.01 | .89 | 1.00 | 0.99-1.02 | .57 |
| | 1-year mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.99-1.01 | .89 | 1.00 | 0.99-1.02 | .58 |
| **Females with proCNP ≥ median (n=260)** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.05 | 1.02-1.07 | <.001 | 1.04 | 1.01-1.07 | .008 | 1.04 | 1.01-1.07 | .016 |
| | 1-year mortality | 1.04 | 1.02-1.07 | <.001 | 1.03 | 1.01-1.06 | .010 | 1.04 | 1.01-1.06 | .007 |
| **Males with a proCNP ≥ median (*n* = 668)** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.02 | 1.01-1.03 | .001 | 1.00 | 0.98-1.02 | .86 | 1.00 | 0.98-1.02 | .92 |
| | 1-year mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.98-1.01 | .72 | 1.00 | 0.98-1.02 | .86 |

* Multivariable model with age and plasma creatinine included as co-variables.

† Multivariable model with age, plasma creatinine, proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to blood sample included as co-variables.

HR, hazard ratio; CI, confidence interval.

**Table 3: Linear mixed models of longitudinal measurements of proCNP in 287 patients with STEMI.**

| | ***Univariate*** | | ***Model 1*** | | ***Model 2*** | |
|---|---|---|---|---|---|---|
| | Value (95% CI) / pmol/L | *P* Value | Value (95% CI) / pmol/L | *P* Value | Value (95% CI) / pmol/L | *P* Value |
| **Time (per time point)** | -1.3 (-2.1 – -0.5) | .001 | -0.25 (-1.8 – 1.3) | .75 | -0.25 (-1.8 – 1.3) | .76 |
| **Age (per year)** | | | 0.16 (0.02 – 0.30) | .02 | 0.06 (-0.08 – 0.20) | .39 |
| **Sex (effect of male sex)** | | | 4.5 (-0.6 – 9.6) | .09 | 4.4 (-0.28 – 9.1) | .07 |
| **Interaction of time and sex** | | | -1.4 (-3.2 – 0.4) | .13 | -1.4 (-3.2 – 0.36) | .13 |
| **Kidney disease** | | | | | 19.7 (13.3 – 26.1) | <.001 |
| **Hypertension** | | | | | 4.5 (0.6 – 8.4) | .03 |
| **Diabetes** | | | | | -4.0 (-9.7 – 1.7) | .17 |
| **Peripheral artery disease** | | | | | -5.1 (-13.9 – 3.7) | .26 |
| **Stroke** | | | | | 2.7 (-5.3 – 10.7) | .51 |

The table shows the associated estimated changes in proCNP concentration in pmol/L (95%CI) when each variable is entered in a linear mixed model.

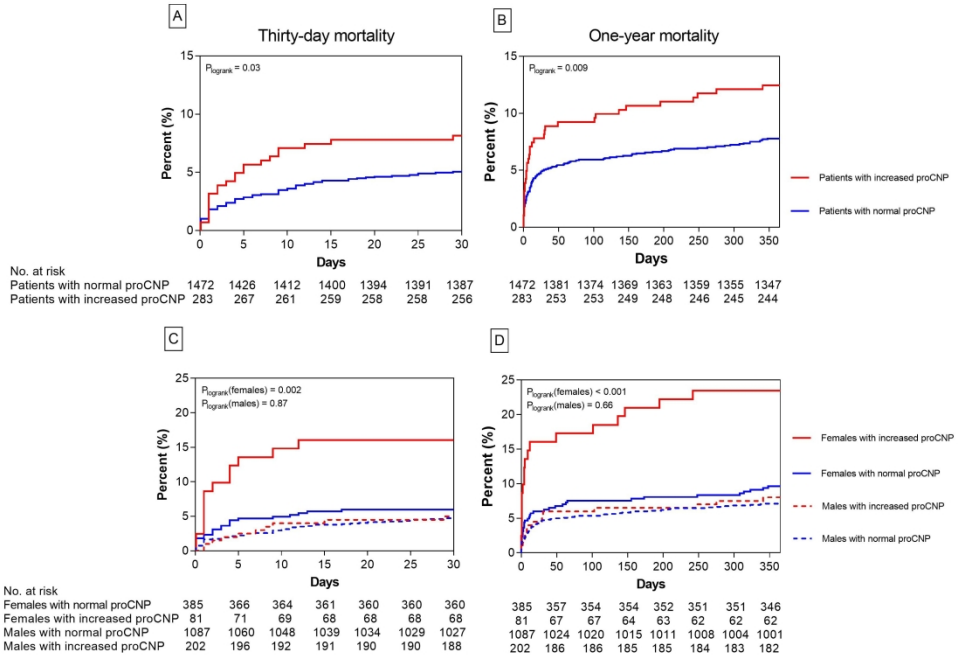
CI, confidence interval.

**Figure 1. Mortality rates for increased and normal proCNP in all patients and stratified by sex.**

Display of all-cause mortality rates for groups of normal vs. increased proCNP concentrations in plasma. Graphs of 30-day and one-year mortality rate for all patients are shown in section A and B, respectively. Sex-specific 30-day and one-year mortality rates are shown in section C and D, respectively. The numbers of patients at risk in subgroups are given beneath each graph.

**Figure 2. Longitudinal concentrations of proCNP in plasma.**

Concentrations are shown as mean (point) and standard error of the mean (error bars). ProCNP concentrations over time for 287 patients with STEMI are shown in section A. In section B, C, and D, these patients are grouped based on sex, hypertension, and kidney disease, respectively.

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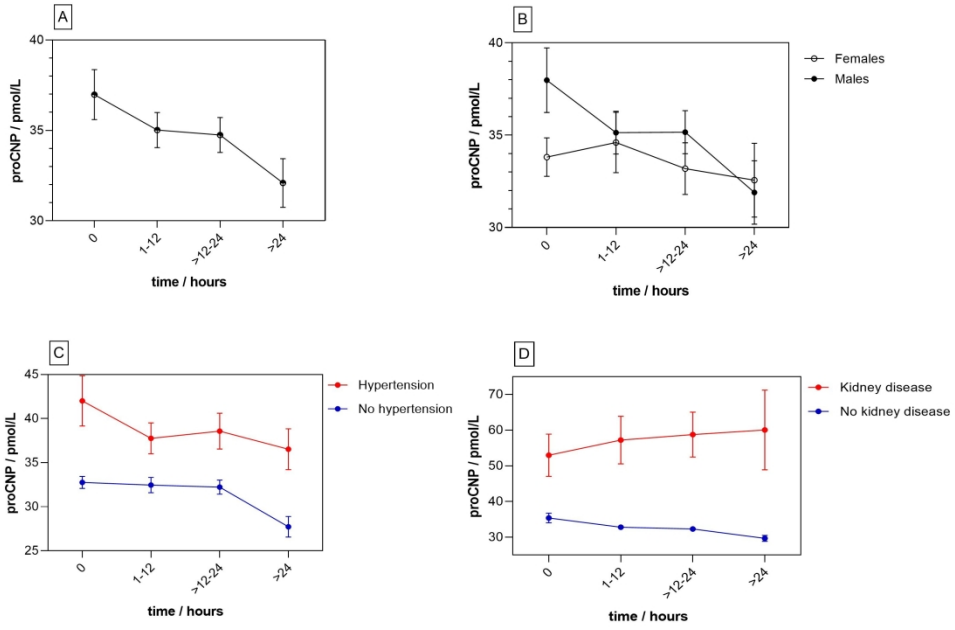


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# Supplemental Material

## METHODS

### COHORT OF PATIENTS WITH STEMI

Information on age, sex, height, body mass index (BMI), smoking status, development of cardiogenic shock, and cardiac arrest coma, time from onset of symptoms to blood sample, time from blood sample to percutaneous coronary intervention balloon angioplasty, number of coronary vessels affected (defined as the number coronary arteries with at least one stenosis of >70% of the lumen diameter (discrete values of zero to three) from the coronary angiography procedure (CAG)), culprit coronary vessel anatomy, Thrombolysis In Myocardial Infarction (TIMI ) grade flow, medical history, left ventricular ejection fraction (LVEF), and routine laboratory measurements was used in the data analyses. Blood samples for baseline biochemical measurements were collected from the femoral or radial sheath on admission immediately before CAG. LVEF was determined by 2D echocardiography performed on admission or within 48 hours of admission. From a consecutive subgroup of the cohort, we collected repeated venous plasma samples during the first days after admission from January to March 2016 at Rigshospitalet (RH) for longitudinal assessment of potential changes in proCNP concentrations. Longitudinal plasma samples were collected at least twice within the first day of admission, and once daily in subsequent days of admission.

### BIOCHEMICAL ANALYSES

Troponin T was measured in patients admitted to RH by Elecsys Troponin T hs assay (Cobas, by Roche, Basel, Switzerland), whereas troponin I was measured in patients admitted to Odense Universitetshospital (OUH) using Architect *STAT* High Sensitive Troponin-I (Abbott, Chicago, Illinois, United States). Both measurements from samples on admission and measured peak values during admission (the latter as a proxy of myocardial infarction size), were used in our statistical analyses. Plasma creatinine was measured using CREP2 assay and high sensitivity C-reactive protein (hs-CRP) using CRPHS assay (Cobas, by Roche, Basel, Switzerland). ST2 was measured by Presage ST2 Assay (Critical Diagnostics, Inc., San Diego, California). We included measurement of soluble thrombomodulin (sTM) and syndecan-1 as markers of endothelial cell and glycocalyx damage, respectively. Assay procedures of sTM and syndecan-1 have been described previously.[1] With regards to measurement of hs-CRP, the results were analyzed as the proportion of patients with a concentration of ≥2mg/L. This cut-off value has been introduced as a definition of chronic inflammation in cardiac disease.[2] Moreover, patients with a time from onset of symptoms to blood sample of ≥6 hours are excluded from hs-CRP analyses because an increase in hs-CRP concentration can be expected due to myocardial damage.[3,4] For measurement of syndecan-1, sTM, and hs-CRP, only patients admitted at RH were analyzed. All biochemical analytes, apart from peak troponins and longitudinal proCNP and proANP, were measured in blood samples collected on admission.

## RESULTS

## PROCNP MEASUREMENT

Coefficients of variation of proCNP measurement in plasma were 13.8% for 20 pmol/L and 13.1% for 40 pmol/L.

## REFERENCE POPULATION AND STEMI COHORT

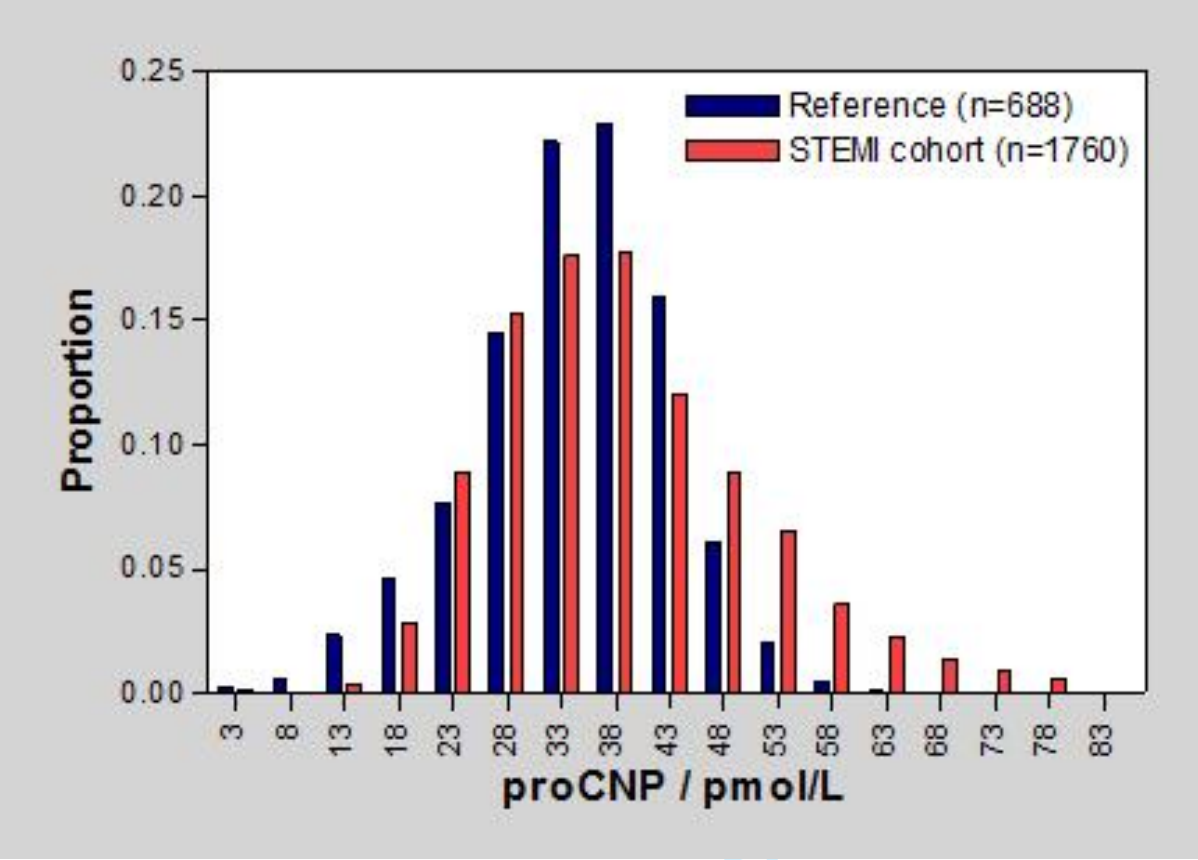
By inspection of histograms of proCNP concentrations, we concluded that there were no outliers among the individuals. Table 1 shows sex- and age-specific 95% reference intervals of proCNP concentrations in the reference population. Plasma concentrations in males were marginally higher compared to those in females ($P$ = 0.015). Women ≥50 years had higher proCNP concentrations compared to women <50 years ($P$ = 0.011), where no difference was observed among men ($P$ = 0.44). The distributions of proCNP concentrations in the reference population and the STEMI cohort are shown in Supplemental Figure 1.

**Supplemental Table 1. Reference intervals of proCNP in sex- and age-specific groups.**

| | Men | | Women | |
|---|---|---|---|---|
| **Age groups (years)** | <50 | ≥50 | <50 | ≥50 |
| **Number of subjects** | 157 | 173 | 179 | 179 |
| **95% reference interval** | 10.2 – 52.2 | 13.9 – 49.6 | 13.6 – 48.9 | 13.4 – 49.4 |
| **Median** | 35.2 | 36.0 | 32.2 | 34.6 |
| **Range** | 4.4 – 55.4 | 11.4 – 52.4 | 8.2 – 55.4 | 4.4 – 60.0 |

Reference intervals, median, and range of proCNP concentrations of subgroups are all given in pmol/L.

**Supplemental Figure 1. Histograms of relative frequencies of plasma proCNP concentrations.**




The reference population is represented by blue bars and the STEMI cohort by red bars. Each bar represents an interval of 5 pmol/L.

Values of >85 pmol/L are not shown.

**Cardiogenic shock development and cardiac arrest**

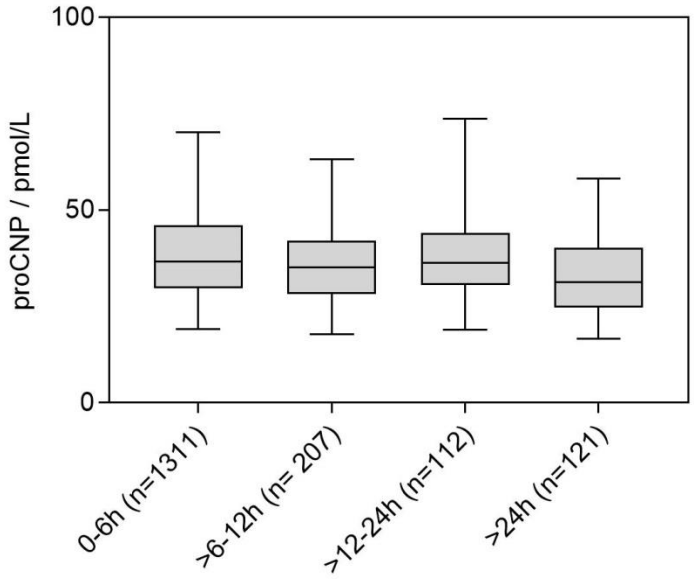
When median proCNP concentrations were tested in patients with cardiogenic shock development vs. no cardiogenic shock development and cardiac arrest coma vs. no

cardiac arrest coma or cardiogenic shock development, no differences were observed (*P* = 0.41 and *P* = 0.28 for females, respectively, and *P* = 0.97 and *P* = 0.46 for males, respectively).

**Time from onset of symptoms to blood sample and correlation of biochemical markers**

In Supplemental Figure 2, median plasma proCNP concentrations are shown in subgroups of different time intervals from onset of symptoms to blood sample. A difference in proCNP was observed across subgroups (*P* < 0.001). However, when patients with a time interval of >24 hours were excluded, no difference was found (*P* = 0.091). Information on time from baseline blood sampling (performed when the CAG procedure was initiated) to percutaneous coronary intervention balloon angioplasty (time of reperfusion) was obtained on 862 patients (227 women, 635 men). Median (interquartile range) values in minutes were: 5 (4-9) and 5 (3-9) (*P* = 0.90) for women with normal and increased proCNP respectively, and 6 (4-10) and 5 (4-10) (*P* = 0.17) for men with normal and increased proCNP respectively. For biochemical markers with positive associations to proCNP, we performed Spearman's correlation analyses, and for proCNP vs. creatinine, proANP, syndecan-1, and sTM, Spearman r were 0.26, 0.082, 0.098, and 0.24, respectively (*P* < 0.001 for all).

**Supplemental Figure 2: Box-plots of proCNP concentrations in groups of time from onset of symptoms to admission.**



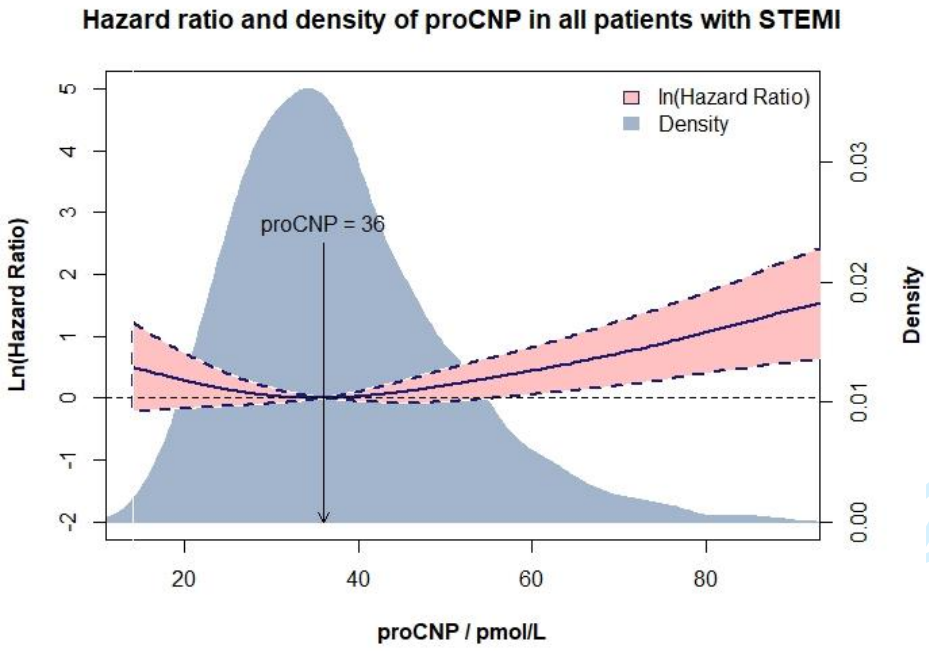

Boxes indicate median and inter-quartile range, and error bars indicate the interval from 2.5 to 97.5 percentile.

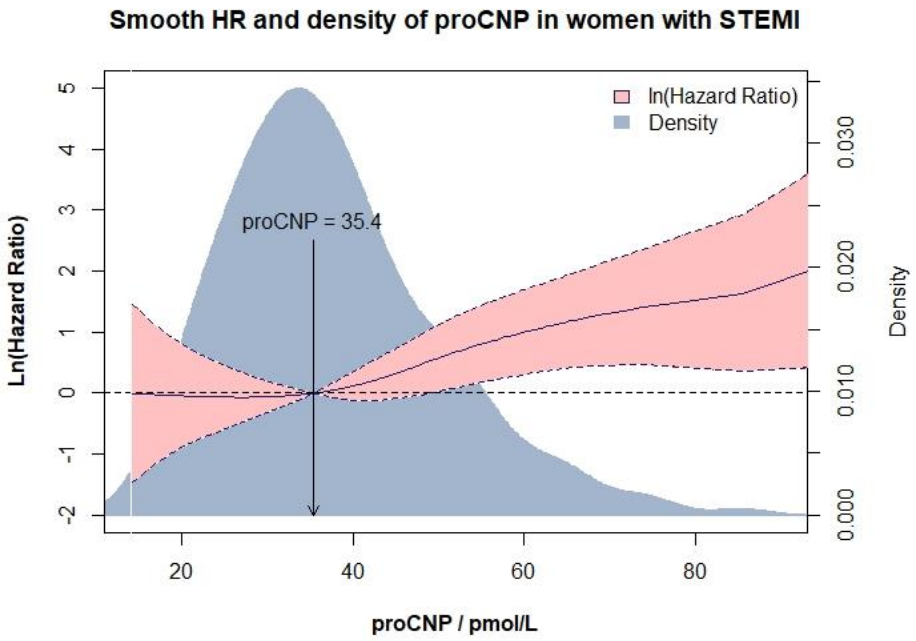
**Multivariable Cox regression analyses**

We examined the effect of proCNP concentrations as a continuous variable on the hazard ratio (HR) of one-year all-cause death in all patients as well as females and males separately by cubic spline plots, using median concentrations as a reference point (shown in Supplemental Figure 3). In females, there was no effect of proCNP below median concentrations, whereas the estimated HR increased from approximately median concentrations to the highest measured concentrations. In males, a trend

towards a U-shaped relation was observed with increasing estimated hazard ratios for decreases of proCNP below median and for increases of proCNP above median. Based on these observations we focused our Cox regression analyses on the upper range of proCNP, where we observed that increasing proCNP was associated with increasing HR in both females and males.

**Supplemental Figure 3: Cubic spline and density plots of hazard ratio and proCNP concentrations.**



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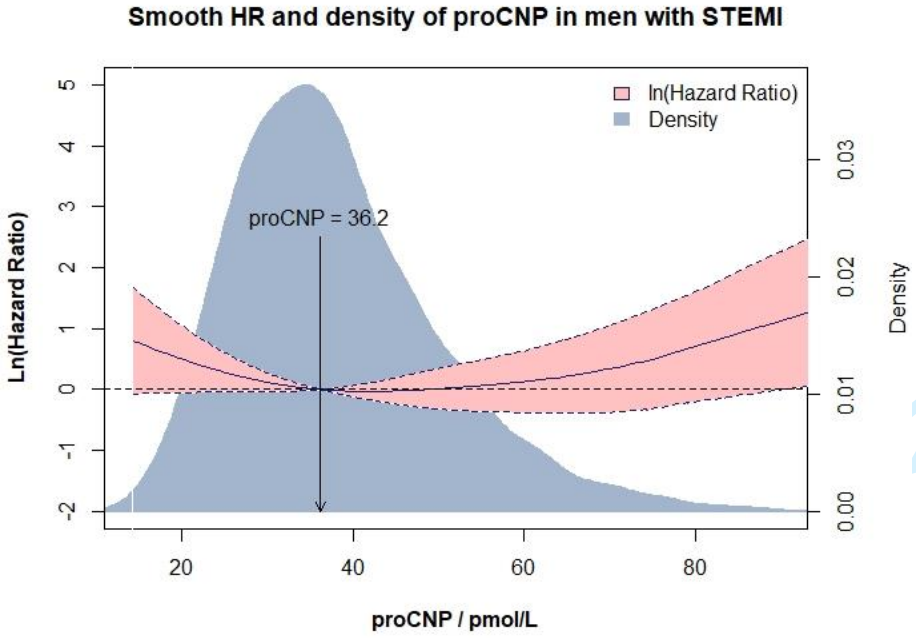


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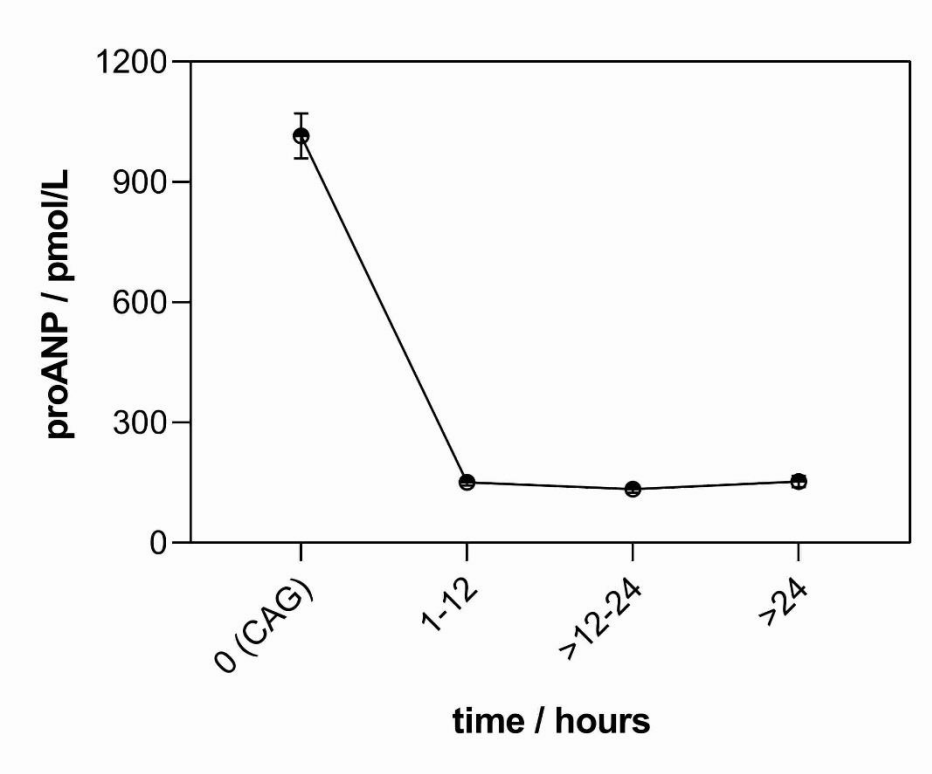
## Longitudinal plasma measurements

In Supplemental Table 2, the number of patients and samples in groups stratified according to diseases are shown. Longitudinal measurements of proANP are shown in Supplemental Figure 4, where the initial decrease from first (0 hours) to second (1-12 hours) timepoint is estimated to be ~850 pmol/L (~-85%).

**Supplemental Table 2: Number of patients and plasma samples in longitudinal analyses.**

| | **Number of patients** (females/males) | **Number of samples** (from females/from males) |
|---|---|---|
| **Overall** | 287 (64/223) | 907 (211/696) |
| **Kidney disease** | 26 (6/20) | 81 (20/61) |
| **Hypertension** | 131 (32/99) | 412 (96/316) |
| **Diabetes** | 34 (8/26) | 107 (20/87) |
| **Stroke** | 15 (5/10) | 46 (16/30) |
| **PAD** | 12 (2/10) | 40 (6/34) |

**Supplemental Figure 4: Longitudinal concentrations of proANP in plasma.**




Concentrations are shown as mean (SEM) for patients with longitudinal plasma samples.

## DISCUSSION

*Assay Principle*

Our proCNP radioimmunoassay is developed in accordance with a processing-independent principle,[5,6] where different fragments of the prohormone of CNP can be accurately quantitated in circulation regardless of post-translational processing of the prohormone. Also, there is no cross-reactivity to the structurally related cardiac natriuretic propeptides.[7]

*Reference Intervals*

We based our calculated 95% reference intervals on two age groups of < and ≥ 50 years. The reason for choosing this division is a previous report showing that NT-proCNP concentrations in plasma in healthy individuals increase from ~50 years of age.[8] We therefore assume that the two age groups represent two different stages of adulthood with regard to circulating NT-proCNP concentrations and, hence, that the two age-specific intervals (for each sex) constitute a meaningful reference for interpretation of measured proCNP concentrations in the STEMI cohort.

*Time from onset of symptoms to blood sampling and balloon angioplasty*

In our baseline and multivariable Cox regressions analyses we have included the time from onset of symptoms as a variable, where we have data from the 99.5% of the included patients. Our results on time from blood sampling to balloon angioplasty (data was obtained from 49.1% of the included patients) show no differences in time between groups of normal and increased proCNP in both sexes, where the median time duration was 5-6 minutes in all groups. Assuming that the total ischemic time of the STEMI patients is equal to the time from onset of symptoms to balloon angioplasty (time of reperfusion), our analyses support that the time onset of symptoms to blood sampling as a variable also reflects the total ischemic time of the patients.

*Correlation Analyses of Biochemical Measurements*

Correlation analyses showed that the association of proCNP with sTM is superior in comparison to both that of syndecan-1 and proANP. A likely explanation may be that both proCNP and sTM are released from endothelial cells, whereas the glycocalyx and the cardiomyocytes are the major sources of syndecan-1 and proANP, respectively. In contrast to the vascular markers, however, no associations with markers of inflammation, hs-CRP, and ST2 were observed. Thus, proCNP concentrations in plasma do not seem to be affected by general inflammation in patients with STEMI.

*Longitudinal Analyses*

Repeated measurements displayed a statistically significant decrease in proCNP concentrations over time in a univariate analysis. For comparison we have included repeated measurements of proANP, shown in Supplemental Figure 4. The dynamic response of proANP differs markedly from proCNP with a steep decrease from 0 to 1-12 hours and a flat curve from 1-12 to >24 hours. These differences highlight the distinct biological roles of CNP vs. ANP.

For proCNP, both multivariate linear mixed models (Model 1 and 2) show that the effect of time was reduced in magnitude and was non-significant, indicating that changes over time are better explained by other variables than time per se. Model 1 found an independent effect of age with higher concentrations of proCNP per year, but Model 2 found that the effect of age disappeared when kidney disease and other vascular diseases were included. Thus, age per se does not seem to explain an increase in proCNP concentration; more likely, chronic diseases (where prevalence increases with age) appear a confounder of the crude effect of age. Although a statistically insignificant

finding, Model 1 indicates an effect of male sex and an interaction of sex and time that are unchanged after inclusion of chronic diseases in Model 2. Consistent with the effect of male sex are previously reported results[8] and the results of the reference population of the present study, where males have slightly higher concentrations of proCNP-derived peptides in the circulation. The possible interaction of sex and time, where males display a relative decrease over time compared with females, has not previously been reported. However, our longitudinal analyses lack the statistical power to sufficiently conclude on a potential interaction of sex and time on proCNP concentrations. In model 2, we find that kidney disease and hypertension are independently associated with higher proCNP concentrations, consistent with baseline associations.

Of the (cardio)vascular diseases with a crude positive association to increased proCNP in baseline results, only hypertension is statistically independent in the multivariate repeated measurement analysis. Unexpectedly, the independent effects of diabetes mellitus and peripheral artery disease seem to be oppositely directed, where the diseases are independently associated with lower proCNP concentrations. However, these results were statistically insignificant, and further studies with more statistical power are needed to determine if such negative independent associations exist.

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2

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

| | Item No | Recommendation | Page No |
|---|---|---|---|
| **Title and abstract** | 1 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract<br>(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found | 1<br>3-4 |
| **Introduction** | | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported | 7-8 |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses | 7-8 |
| **Methods** | | | |
| Study design | 4 | Present key elements of study design early in the paper | 8-12 |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection | 8-11 |
| Participants | 6 | (*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up<br>(*b*) For matched studies, give matching criteria and number of exposed and unexposed | 8-10 |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable | 8-11 + suppl. |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group | 8-10 + suppl. |
| Bias | 9 | Describe any efforts to address potential sources of bias | - |
| Study size | 10 | Explain how the study size was arrived at | 8 |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why | 10-11 |
| Statistical methods | 12 | (*a*) Describe all statistical methods, including those used to control for confounding<br>(*b*) Describe any methods used to examine subgroups and interactions<br>(*c*) Explain how missing data were addressed<br>(*d*) If applicable, explain how loss to follow-up was addressed<br>(*e*) Describe any sensitivity analyses | 10-12 + suppl. |
| **Results** | | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed<br>(b) Give reasons for non-participation at each stage<br>(c) Consider use of a flow diagram | 12-15 + table 1-2 + figure 1 +suppl |
| Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders | 12-15 + table 1-2 + figure 1 |

| | | | |
|---|---|---|---|
| | | (b) Indicate number of participants with missing data for each variable of interest<br>(c) Summarise follow-up time (eg, average and total amount) | +suppl |
| Outcome data | 15* | Report numbers of outcome events or summary measures over time | table 2 + figure 1 |

| | | | |
|---|---|---|---|
| Main results | 16 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included<br><br>(*b*) Report category boundaries when continuous variables were categorized<br>(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period | 12-15 + table 1-2 + figure 1 +suppl |
| Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses | 12-15 + table 1-2 + figure 1-2 +suppl |
| **Discussion** | | | |
| Key results | 18 | Summarise key results with reference to study objectives | 15-19 |
| Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias | 18 |
| Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence | 15-19 |
| Generalisability | 21 | Discuss the generalisability (external validity) of the study results | 15-18 |
| **Other information** | | | |
| Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based | |

*Give information separately for exposed and unexposed groups.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

BMJ Open

# Sex-specific Mortality Prediction by Pro-C-type Natriuretic Peptide Measurement in a Prospective Cohort of Patients with ST-elevation Myocardial Infarction



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# Sex-specific Mortality Prediction by Pro-C-type Natriuretic Peptide Measurement in a Prospective Cohort of Patients with ST-elevation Myocardial Infarction

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**Keywords:** Natriuretic peptides, C-type natriuretic peptide, CNP, ANP, Reference intervals, Myocardial infarction.

**List of abbreviations:** CNP, C-type natriuretic peptide; ACS, acute coronary syndrome; NT-proCNP, amino-terminal proCNP; STEMI, ST-elevation myocardial infarction; proCNP (see Nomenclature); NOBIDA, Nordic Reference Interval Project Biobank and Database; RH, Copenhagen University Hospital, Rigshospitalet; OUH, Odense University Hospital; CAG, coronary angiography; ECG, electrocardiogram; BMI, body mass index; LVEF, left ventricular ejection fraction; hs-CRP, high sensitivity c-reactive protein; sTM, soluble thrombomodulin.

## Abstract

**Objective:** To determine the predictive value of pro-C-type natriuretic peptide (proCNP) measurement in plasma sampled on admission from patients presenting with ST-elevation myocardial infarction (STEMI).

**Design:** Prospective cohort study.

**Setting:** Two University Hospitals in Denmark.

**Participants:** 1760 consecutive patients (470 females and 1290 males) with confirmed STEMI.

**Main Outcomes and Measures:** The main outcome was all-cause mortality at one year after presentation and the primary measure was proCNP concentration in plasma at admission in all patients and longitudinal measurements in a consecutive subgroup of 287 patients. A reference population (n = 688) defined cut-off values of increased proCNP concentrations.

**Results:** In all patients, an increased proCNP concentration was associated with a higher all-cause mortality after one year (HR: 1.6 (1.1-2.4), $P_{logrank}$= .009) including an interaction of sex ($P$= .03). In separate sex-stratified analyses, female patients showed increased all-cause mortality ($HR_{one\ year}$: 2.6 (1.5-4.6), $P_{logrank}$ < .001), whereas no differences were found in male patients ($HR_{one\ year}$: 1.1 (.7-1.9), $P_{logrank}$ = .66). After adjusting for potential risk factors, we found increased proCNP concentrations ≥ the median value to be independently associated with increased risk of mortality in female patients within one year (HR per 1 pmol/L increase: 1.04 (1.01-1.06), $P$ = .007).

Moreover, we found indications of sex differences in proCNP concentrations over time (higher proCNP in males (4.4 (-.28 – 9.1) pmol/L, P= .07) and interaction of sex and time (P= .13)), and that hypertension was independently associated with higher proCNP (4.5 (.6-8.4) pmol/L, P= .03).

**Conclusions:** In female but not male patients presenting with STEMI, high concentrations of proCNP (≥ median) at admission independently indicate a higher risk of all-cause mortality. The findings are remarkably specific for female patients, suggesting a different vascular phenotype beyond traditional measures of coronary artery flow compared to male patients.

## Strengths and limitations of this study

- The is the first study to investigate the prognostic potential of measurement of peptides derived from pro-C-type natriuretic peptide (proCNP) by using predefined sex- and age-specific cut-off values based on a reference population.
- As a novel approach, a large cohort of patients are examined during the acute phase of ST-elevation myocardial infarction with plasma sampling at admission and all-cause mortality within one year as main outcome.
- To clarify the temporal pattern of proCNP concentrations, longitudinal measurements during admission in a subgroup of the cohort are used to further examine sex differences and baseline associations.
- The sex-specific analyses are exploratory and the present report should be considered a hypothesis-generating study.
- As all-cause mortality within one year is the only available outcome measure, other clinical end-points and long-term follow-up is not investigated.

## Introduction

Despite the structural resemblance to the cardiac hormones, atrial and B-type natriuretic peptides (ANP and BNP), C-type natriuretic peptide (CNP) differs functionally from the two other family members.[1] CNP is a paracrine/autocrine peptide expressed in many tissues,[2] including endothelial cells.[3] Experimental studies have shown that endothelial-derived CNP is locally involved in regulation of vascular tone[4,5] and angiogenesis.[6] Also, a recent preclinical investigation has suggested that CNP plays a key protective role in cardiac pathophysiology.[7]

Clinical reports on the general population as well as patients with heart disease have examined circulating proCNP-derived peptides as prognostic markers.[8–10] In patients presenting with acute coronary syndrome (ACS), high concentrations of amino-terminal proCNP (NT-proCNP) in plasma measured 4-6 weeks after the event were reported to be the only natriuretic peptide to independently predict cardiac readmission and death in the patients with unstable angina.[8] Taken together, experimental and epidemiological data suggest CNP to be an important regulator of cardiovascular function and that increased concentrations of proCNP-derived peptides in plasma of patients reflect an unfavorable cardiovascular condition as a compensatory response to cardiovascular disease. However, no previous large study has explored the prognostic value of measurement of proCNP-derived peptides in the acute phase of an ST-elevation myocardial infarction (STEMI).[11]

In this study, we measured concentrations of the precursor of C-type natriuretic peptides (proCNP) in plasma from a large cohort of patients presenting with STEMI,

where baseline blood samples were collected when patients were admitted (before coronary intervention). Previously, we have reported on this method for accurate quantification of "total" proCNP in plasma by a processing-independent radioimmunoassay.[12] We determined proCNP concentration as a predictor of all-cause mortality within one year and examined for potential sex-differences. Moreover, we investigated a large consecutive subgroup of patients with STEMI from the cohort with longitudinal plasma samples collected during the hospital admission to examine proCNP concentrations over time and further analyze baseline associations between proCNP concentrations and vascular diseases. Finally, we included a large sample of healthy individuals in order to, independently of the patient cohort, establish age- and sex-specific reference intervals for proCNP concentrations in plasma.

*Nomenclature*

ProCNP: In the present article, proCNP refers to a specific amino-acid sequence (human proCNP 11-27) within the prohormone sequence of CNP; the epitope of the antiserum of our radioimmunoassay. In this processing-independent methodology, we utilize this fragment after enzymatic cleavage in vitro as a proxy measure of all proforms released to plasma irrespective of prohormone post-translational processing.

ProCNP-derived peptides: Collective term for any fragment of the prohormone of CNP.

## Methods

REFERENCE POPULATION

For establishment of reference intervals, we used plasma samples from the Nordic Reference Interval Project Biobank and Database (NOBIDA), originally consisting of 3002 subjects.[13] A subgroup of 853 subjects from this population was randomly selected with the aim to represent sex, age, and country of origin equally, as previously described.[14]

## COHORT OF PATIENTS WITH STEMI

Patients with suspected STEMI were consecutively included from two Danish hospitals over a period of one year (2015/2016) (Copenhagen University Hospital, Rigshospitalet (RH), and Odense University Hospital (OUH)). The procedure of inclusion has been described previously.[15] From this cohort of patients with suspected STEMI and triaged for acute coronary angiography (CAG) (based on assessment of symptoms and the individual electrocardiogram (ECG)), we only included patients with confirmed STEMI.[16] All patients underwent CAG and baseline blood samples were obtained immediately before CAG was performed (See further details on data collection including blood sampling in the Supplemental Material).

## PATIENT AND PUBLIC INVOLVEMENT

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

## ETHICS

Patients gave written informed consent. When patients were not able to provide this (e.g. comatose cardiac arrest patients), consent was obtained by the patients' next of

kin and general practitioners in accordance with national legislation. The study was approved by the Local Committee on Health Research Ethics (Copenhagen) (Ref. H-2-2014-110).

## BIOCHEMICAL ANALYSES

Plasma proANP and proCNP concentrations were quantified by the previously reported processing-independent assay technology and procedures.[12,17,18] Information of other biochemical analyses can be found in the Supplemental Material.

## ALL-CAUSE MORTALITY

The Danish Civil Registration System was used for all-cause mortality assessment. All Danish citizens are recorded with a unique 10-digit civil registration number, and deaths are registered within 2 weeks. Initial follow-up began on the date of admission and continued until date of death, or October 30th, 2017.

## MAIN OUTCOMES

The primary outcome was one-year all-cause mortality. We tested 30-day all-cause mortality as a secondary outcome. We focused on sex-specific estimates as we found a statistical interaction of sex and proCNP concentrations on mortality.

## STUDY DESIGN

The study design is summarized in Figure 1.

## STATISTICS

### *Reference Population*

We divided the reference population into groups based on sex and age (<50 and ≥50 years) and used the RefVal software[19] to calculate 95% reference intervals based on a non-parametric bootstrapping method. See Supplemental Table 1 for results on reference intervals.

*STEMI Cohort*

Based on their respective sex- and age-specific reference interval from the reference population, all STEMI patients were stratified according to a) increased proCNP concentration (higher than the 95% reference interval), b) normal proCNP concentration (within the 95% reference interval), and c) decreased proCNP concentration (lower than the 95% reference interval). Dichotomous variables are presented as numbers (n) and percentages (%). Continuous variables are presented as medians with $25^{th}$–$75^{th}$ percentiles. Comparisons between groups were done using independent non-parametric t-tests and Fisher's exact two-sided test. Spearman's correlation analyses were used to assess the relationships between proCNP and other biochemical analytes. Differences in median proCNP concentrations at different time intervals from onset of symptoms to blood sample were assessed by Kruskal-Wallis tests (this time parameter also reflects total ischemic time; see Supplemental Material). All-cause mortality after 30 days and one year was assessed in patients stratified into normal or increased proCNP concentrations and depicted by Kaplan-Meier plots and then compared with the log-rank test and estimates of hazard ratio including 95% confidence intervals. We performed a statistic test of interaction between sex and groups of proCNP. To test the relation of mortality and proCNP concentrations on a continuous scale, we performed cubic spline plots with the density distribution and the logarithm of hazard ratios. We focused our

further mortality analyses on proCNP concentrations ≥ median, where we observed an effect of increasing proCNP concentrations on mortality (patients were divided into groups of ≥ vs. < median proCNP according to sex- and age-specific median proCNP concentrations of the reference population shown in the Supplemental Material). Thus, for patients with a baseline proCNP concentration ≥median, multivariable Cox proportional hazard models including proCNP, age, plasma creatinine, plasma proANP, time from onset of symptoms to blood sample, number of coronary vessels affected and tertiles of peak plasma troponin concentrations were constructed for mortality assessment. Both plasma proANP and creatinine were logarithmically transformed, where peak troponin T and troponin I were combined in one variable of tertiles (represented by values of one to three) as a proxy myocardial infarction size, before being entered into the model. For longitudinal analyses, we constructed four time points/intervals for statistical analyses (1: samples from admission; 2: 1 to 12 hours after admission; 3: >12 to 24 hours after admission; 4: >24 hours after admission). We used linear mixed models of unstructured co-variances to examine changes in concentrations and associations with co-variables over time. Statistical analyses were performed using RefVal software[20] for calculation of reference intervals, statistical software R version 3.6.1 (R Core Team, Vienna, Austria[20]) for linear mixed models (nlme package), and IBM SPSS Statistics 22 (SPSS Inc., Chicago, Illinois, United States) for other analyses. A *P*-value <.05 was considered statistically significant.

## Results

*Reference population*

We measured proCNP concentrations in available plasma from 688 subjects (358 females; 330 males). From these measurements, 95% reference intervals for females vs. males and < vs. ≥ 50 years were calculated (see results in the Supplemental Table 1).

*STEMI Cohort*

From the cohort of 2247 patients with suspected STEMI, 1760 patients (460 females and 1290 males) with verified STEMI and available plasma were included in our study (see a flowchart of the inclusion in Supplemental Figure 1 and the relative frequencies of measured proCNP concentrations in Supplemental Figure 2). When compared to the sex- and age-specific reference intervals, a total of 283 (16.1%) of the patients had an increased proCNP concentration; no difference in sex-specific proportions was observed ($P$ = .42).

*Baseline analyses*

Table 1 shows the baseline characteristics and follow-up in patient groups defined by normal or increased proCNP concentrations. Five patients (one male and four females) displayed decreased proCNP concentrations and were not included in Table 1. Missing individual information on each variable in each group was between .1% and 4.4%, except for age, sex, mortality, cardiogenic shock, cardiac arrest coma, time from onset of symptoms to blood sample, and Thrombolysis in Myocardial Infarction (TIMI) grade flow, where information from all patients was available (see Supplemental Table 2 for results on culprit vessel, number of coronary vessels affected and TIMI grade flow). Female patients with increased proCNP concentrations at admission had a higher

prevalence of hypertension ($P$ = .003), diabetes mellitus ($P$ = .022), peripheral artery disease ($P$ = .002), and chronic kidney disease (CKD) ($P$ < .001), whereas male patients had higher prevalence of hypertension ($P$ = .004), stroke ($P$ = .012), and CKD ($P$ < .001). Increased proCNP was associated with higher concentrations of proANP ($P$ = .019) and soluble thrombomodulin (sTM) ($P$ = .001) in female patients and with higher concentrations of creatinine ($P$ < .001), syndecan-1 ($P$ = .019), and sTM ($P$ = .003) in male patients. Lastly, we found a higher one-year mortality rate ($P$ = .001) and prevalence of cardiogenic shock development ($P$ = .013) in female patients with increased proCNP concentrations, whereas no differences were found in male patients. For biochemical markers with positive associations to proCNP, we performed Spearman's correlation analyses (see the Supplemental Material for results). For results on median proCNP concentrations in groups of time from onset of symptoms to blood sample, see Supplemental Material including Supplemental Figure 3).

*Mortality analyses*

Thirty-days and one-year all-cause mortality plots of normal and increased proCNP concentrations of all patients and stratified by sex are depicted in Figure 2. Different mortality rates were found for all patients at both time points ($HR_{30\ days}$ = 1.6 (1.0-2.6), $P_{logrank}$ = .03 and $HR_{one\text{-}year}$ = 1.6 (1.1-2.4), $P_{logrank}$ = .009). However, we found an interaction of sex and groups of proCNP ($P$ = .03). In sex-specific estimates, only female patients showed different mortality rates ($females_{30\ days}$: HR = 2.8 (1.4-5.6), $P_{logrank}$ = .002, $females_{one\text{-}year}$: HR = 2.6 (1.5-4.6), $P_{logrank}$ < .001, $males_{30\ days}$: HR = 1.1 (0.6-2.1), $P_{logrank}$ = .87, and $males_{one\text{-}year}$: HR = 1.1 (.7-1.9), $P_{logrank}$ = .66). In cubic spline plots, we observed an effect of increasing proCNP as a continuous variable on mortality

from median concentrations in both sexes. We therefore focused on this upper range of proCNP concentrations in Cox regression analyses (see Supplemental Figure 4). In a univariate Cox proportional hazard model, we found an elevated hazard ratio (HR) of all-cause mortality with increases of plasma proCNP for both sexes (results shown in Table 2). When including age and plasma creatinine in a multivariable model (Model 1), proCNP was independently associated with mortality in female but not in male patients (see Table 2): HR (95% CI) for female patients was 1.04 (1.01-1.07) (*P* = .008) for 30-day and 1.03 (1.01-1.06) (*P* = .010) for one-year mortality, whereas HR was 1.00 for both all patients and male patients at both time points (see Table 2). In a model where proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to admission were also added (Model 2), risk estimates of proCNP (pr. one pmol/L increase) were: HR (95% CI) = 1.04 (1.01-1.07), *P* = .016 for 30-day mortality, and HR (95% CI) = 1.04 (1.01-1.06), *P* = .007 for one-year mortality.

*Longitudinal analyses*

To examine proCNP concentrations over time during a STEMI and further test the baseline associations of vascular disease and increased proCNP, we used a set of longitudinal plasma samples from a consecutive subgroup of the cohort consisting of 287 STEMI patients (64 females and 223 males). Results are shown in Figure 3 and Table 3. An overall decrease in proCNP concentration was estimated to be 3.8 pmol/L (~10%) (*P* = .001) from admission to >24 hours. In a multivariate model including sex, age, and chronic diseases (Model 3), CKD and hypertension were independently associated with higher concentrations of proCNP (*P* < .001 and *P* = .03, respectively), whereas time and age were not independently associated with changes in proCNP.

Also, both Model 2 and 3 implied a positive association of proCNP concentration and male sex, and an interaction of time and sex; however, the statistical uncertainty of these estimates was substantial ($P$ = .07 and $P$ = .13, respectively). Figure 3 shows proCNP concentrations over time in overall and sex-specific graphs including graphs of CKD and hypertension (see the Supplemental Material including Supplemental Table 3 and Supplemental Figure 5 for further results of longitudinal measurements).

## Discussion

In this study, we report on a marked sex-specific prognostic information profile for proCNP measurement in patients presenting with a STEMI. A major advantage of our present approach comes from an independent establishment of a sex-specific proCNP reference interval prior to patient measurement. This allowed us to perform clinically meaningful divisions of normal, decreased, or increased proCNP concentrations in plasma specific to sex, rather than testing differences only within the patient cohort as a whole (see the Supplemental Material for discussion of age groups).

In this cohort of consecutive patients with a verified STEMI, we show that 16.1% have increased concentrations of proCNP during the early phase of the myocardial infarction compared with the sex- and age-specific intervals. Interestingly, besides higher prevalence of CKD, we found a markedly higher prevalence of cardiovascular disease including hypertension in both sexes; diabetes mellitus and peripheral artery disease for female patients; and stroke for males among patients with increased proCNP. Our longitudinal analyses of a subgroup of the STEMI patients corroborates that the association to both CKD and hypertension is consistent even over time and is

independent of sex, age, and other cardiovascular diseases. A putative explanation for the linkage of hypertension and increased proCNP concentrations is the upregulation of CNP expression by vascular shear stress.[21,22] The positive association of proCNP and a vascular marker, sTM, for both sexes also support the relation of circulating proCNP with vascular stress. In contrast, the baseline associations of increased proCNP concentrations and diabetes, peripheral artery disease and stroke, respectively, are not statistically independent in longitudinal analyses. However, given the sex-specific pattern in the baseline findings and the limited number of patients with the respective diseases in longitudinal analyses, the results may be too preliminary to sufficiently conclude on the potential associations to diabetes, peripheral artery disease, and stroke. Also, the statistical uncertainty of the suggested effect of sex and interaction of sex and time in longitudinal analyses calls for a cautious interpretation (see the Supplemental Material for further discussion of longitudinal analyses).

The risks of death within 30 days and one year were higher for female patients with increased proCNP concentrations – but not for male patients. This marked sex-specific association was confirmed when the mortality rate was analyzed by increases of proCNP ≥ median, where proCNP proved to be an independent predictor in a model (Model 1 in Table 2) including two additional variables, age and plasma creatinine, which have previously established associations with NT-proCNP in plasma.[23] To further test the prognostic potential of proCNP, we added plasma proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to admission into a multivariable model (Model 2). In this model, increases of proCNP were still independently associated with the risk of death, and the estimates were of

similar size at both time points. Recent data on patients with STEMI found no independent prognostic value on mortality rate for NT-proCNP concentrations.[8] However, that report differed from ours in several aspects including radioimmunoassay principle and duration of follow-up, and, importantly, plasma was sampled 4-6 weeks after admission in contrast to our real-time STEMI investigation. These differences make a direct comparison difficult.

Our results firmly suggest a sex-specific association with survival, where increased proCNP concentrations indicate a poorer prognosis in female patients. Previously, studies have shown that CNP acts as a more potent vasodilator in female porcine arteries[24] and that the female sex hormone estradiol upregulates the expression of CNP in vascular endothelial cells.[25] Furthermore, recent preclinical reports have convincingly pointed to a pivotal role of CNP and its receptors in the regulation of the microcirculation[5] and in cardiac homeostasis.[26] Taken together, these previous findings suggest that the CNP system is a critical modulator of vascular integrity and function including a more pronounced vasodilatory capacity in females that may be provided by female sex hormones. Clinical evidence shows that females display a sharp rise in the incidence of cardiovascular disease after menopause and that changes in sex hormones by complex mechanisms play a key role in the loss of vasoprotection.[27,28] Notably, postmenopausal females are at higher risk of coronary microvascular dysfunction and HFpEF compared with their male counterparts,[29] and high concentrations of NT-proCNP have previously been independently linked to an adverse outcome in patients with HFpEF.[9] In this perspective, we speculate that our observed sex-specific effect of increased proCNP concentrations in plasma of patients

with STEMI partly reflects ongoing vascular dysfunction in females in particular and, hence, represents an independent signal of poor cardiovascular prognosis. However, there is a need for translational research to elucidate this association between increased proCNP in females with vascular complications and increased mortality rate. Furthermore, there is a paucity of reports examining a potential racial impact on concentrations of circulating proCNP-derived peptides. As the majority of Danish and Scandinavian population are Caucasian, it remains to be investigated whether our results can be extrapolated to other populations.

*Limitations*

While the number of patients with STEMI in the cohort is high, the follow-up period is relatively short. We examined only all-cause mortality as outcome in our follow-up multivariable Cox regression analyses, and other clinical endpoints, e.g., cardiac readmission, were not tested. Hence, the number of events limits the number of possible covariates in the multivariable Cox regression analyses (Model 3), and sex-specific estimates of 30-day mortality in this model should be interpreted cautiously. Moreover, this study focused on increased proCNP and the upper range of proCNP concentrations in plasma, whereas associations of decreased proCNP concentrations were not investigated. With regard to measurement of troponins in plasma, two different analytes (TnT and TnI) were measured at the two hospitals of inclusion. Thus, a combined variable of tertiles of peak troponins (discrete values of one to three) was included in the multivariable models with less quantitative information than the measured concentrations. Baseline biochemical analyses were performed on arterial plasma, whereas venous plasma was used from the reference population and in

repeated measurements. A previous report has shown slightly lower plasma concentrations of NT-proCNP in arterial compared with venous plasma.[30] Thus, in theory, our approach will underestimate the proportion of STEMI patients with increased proCNP concentration and the initial longitudinal decrease in proCNP concentration from baseline to the second time point (0 to 1-12 hours).

## Conclusions

We show here that increased proCNP concentrations in plasma from patients presenting with STEMI are associated with a higher all-cause mortality rate within one year among female patients with STEMI, whereas male patients display no such pattern. Moreover, we report that an increase of proCNP in the upper range of plasma concentrations (≥median) in female patients is an independent prognostic marker of mortality at both 30 days and one year. The findings are remarkably specific for female patients, suggesting a different vascular phenotype beyond traditional measures of coronary artery flow compared to male patients.

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## Contributors

Designed the study: PDM, MF, CH, JPG. Designed and conducted the STEMI cohort study: MF, OKLH, LH, JEM, CH. Undertook biochemical measurements: PDM, SRO, PIJ, JPG. Performed the data analysis: PDM, MF, TCRP. Wrote the manuscript draft: PDM. Revised the paper based on intellectual contribution: PDM, MF, OKLH, LH, JEM, PIJ, SRO, TCRP, CH, JPG.

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## Competing interests

None to report.

## Patient and public involvement

Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

## Ethics approval

The study was approved by the local ethics committee (Copenhagen) (Ref. H-2-2014-110).

## Data availability statement

Data are available upon reasonable request (email: JPG@dadlnet.dk).

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**Table 1. Sex-specific characteristics, medical history, and biochemical results in groups of proCNP concentrations in the STEMI-cohort.**

| | **All patients (*n* = 1755)** | | | **Females (*n* = 466)** | | | **Males (*n* = 1289)** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value |
| **Number, *n* (%)** | 1472 (83.6) | 283 (16.1) | - | 385 (81.9) | 81 (17.2) | - | 1087 (84.2) | 202 (15.6) | - |
| **Age, *years*** | 63 (54-72) | 66 (56-76) | .046 | 67 (56-77) | 72 (60-80) | .16 | 62 (53-70) | 64 (55-74) | .21 |
| **Males, *n* (%)** | 1087 (73.8) | 202 (71.4) | .42 | - | - | - | - | - | - |
| **Height, *cm*** | - | - | - | 165 (160-169) | 164 (160-168) | .13 | 178 (173-182) | 178 (174-183) | .87 |
| **BMI, *kg/m²*** | 26.3 (24.1-29.3) | 26.3 (23.8-30.1) | .96 | 25.4 (20.0-29.1) | 25.7 (22.5-31.2) | .61 | 26.5 (24.5-29.3) | 26.5 (24.2-29.9) | .76 |
| **Smoking, *n* (%)** | 1045 (73.3) | 203 (74.9) | .60 | 250 (68.1) | 54 (70.1) | .79 | 795 (75.1) | 149 (76.8) | .65 |
| **Time from symptom to blood sample, *min*** | 190 (128-376) | 178 (125-292) | .34 | 210 (136-460) | 216 (141-349) | >.99 | 185 (125-356) | 167 (123-271) | .18 |
| **LVEF, %** | 45 (40-55) | 45 (35-55) | .33 | 50 (40-55) | 45 (35-55) | .62 | 45 (40-55) | 45 (36-55) | .36 |
| **Medical history, *n* (%)** | | | | | | | | | |
| **Hypertension** | 612 (42.6) | 156 (56.1) | <.001 | 177 (46.9) | 52 (65.8) | .003 | 435 (41.0) | 104 (52.3) | .004 |
| **Diabetes mellitus** | 177 (12.3) | 51 (18.3) | .009 | 47 (12.5) | 18 (22.2) | .022 | 130 (12.2) | 33 (16.5) | .11 |
| **Peripheral artery disease** | 72 (5.0) | 24 (8.5) | .023 | 15 (4.0) | 11 (13.6) | .002 | 57 (5.3) | 13 (6.4) | .50 |
| **Stroke** | 91 (6.3) | 30 (10.6) | .015 | 30 (7.9) | 8 (9.9) | .51 | 61 (5.7) | 22 (10.9) | .012 |
| **Chronic kidney disease** | 41 (2.8) | 40 (14.1) | <.001 | 10 (2.6) | 11 (13.6) | <.001 | 31 (2.9) | 29 (14.4) | <.001 |
| **Ischemic heart disease** | 218 (14.8) | 43 (16.3) | .53 | 44 (11.5) | 10 (12.3) | .85 | 174 (16.0) | 36 (17.9) | .53 |
| **Heart failure** | 38 (2.6) | 13 (4.6) | .079 | 6 (1.6) | 3 (3.7) | .19 | 32 (2.9) | 10 (5.0) | .13 |
| **Biochemical analyses** | | | | | | | | | |
| **Acute Troponin I, *ng/L*** | 220 (53-1576) | 323 (86-887) | .52 | 293 (54-3220) | 813 (176-1814) | .57 | 188 (53-1481) | 211 (58-844) | .99 |
| **Acute Troponin T, *ng/L*** | 1610 (329-3940) | 953 (222-3370) | .018 | 1520 (394-3540) | 571 (166-2753) | .076 | 1640 (287-4058) | 1250 (242-3710) | .24 |
| **Peak Troponin I, *ng/L*** | 20264 (3413-50000) | 19981 (5508-50000) | .91 | 12970 (2734-41856) | 11721 (4014-50000) | .98 | 24507 (3654-50000) | 20575 (5771-47927) | .56 |
| **Peak Troponin T, *ng/L*** | 3110 (1230-6920) | 2430 (880-8060) | .23 | 2520 (995-5930) | 2290 (614-8123) | .55 | 3380 (1400-7603) | 2860 (923-8075) | .23 |
| **Plasma creatinine, *µmol/L*** | 81 (70-96) | 95 (80-126) | <.001 | 69 (59-84) | 83 (63-113) | .072 | 85 (74-98) | 97 (86-131) | <.001 |
| **Estimated glomerular filtration rate, *mL/min*** | 83 (67-95) | 70 (45-85) | <.001 | 79 (61-93) | 61 (39-86) | .095 | 85 (69-96) | 72 (49-86) | <.001 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **proANP, *pmol/L*** | 822 (515-1315) | 1005 (558-1742) | .005 | 978 (664-1504) | 1382 (747-2064) | .019 | 758 (488-1241) | 899 (508-1499) | .10 |
| **hs-CRP ≥2 mg/L, *n* (%) *, †** | 474 (65.0) | 111 (63.1) | .66 | 117 (67.2) | 34 (68.0) | >.99 | 357 (64.3) | 77 (61.1) | .54 |
| **ST2, *ng/ml*** | 40.0 (29.1-56.8) | 39.8 (30.4-59.7) | .95 | 35.3 (27.0-55.0) | 34.8 (26.2-58.4) | .92 | 41.5 (30.2-57.5) | 41.5 (32.1-60.0) | .95 |
| **Syndecan-1, *ng/ml*** [b] | 92.3 (64.2-137.0) | 103.5 (69.4-154.0) | .001 | 82.2 (52.5-137.3) | 99.3 (61.8-140.8) | .21 | 94.4 (68.1-137.0) | 113.5 (71.6-155.1) | .019 |
| **Soluble thrombomodulin, *ng/ml*** [b] | 7.35 (5.98-8.91) | 8.49 (6.67-10.86) | <.001 | 6.71 (5.42-8.15) | 8.05 (6.16-10.80) | .001 | 7.57 (6.19-9.18) | 8.53 (6.70-10.87) | .003 |
| **Follow-up, *n* (%)** | | | | | | | | | |
| **Number of deaths within one year** | 114 (7.7%) | 35 (12.4%) | .014 | 37 (9.6%) | 19 (23.5%) | .001 | 77 (7.1%) | 16 (7.9%) | .66 |
| **Cardiogenic shock** | 122 (8.3%) | 34 (12.0%) | .052 | 32 (8.3%) | 15 (18.5%) | .013 | 90 (8.3%) | 19 (9.4%) | .58 |
| **Cardiac arrest coma** | 66 (4.5%) | 19 (6.7%) | .13 | 14 (3.6%) | 3 (3.7%) | >.99 | 52 (4.8%) | 16 (7.9%) | .084 |

Continuous variables are given in median (inter-quartile range).

***** Only patients with a time from onset of symptoms to CAG of <6 hours were included.

† Only patients admitted at RH (313 females and 873 males) were included.

**Table 2. Cox regression analyses of all-cause mortality.**

| | | Univariate | | | Model 1* | | | Model 2 † | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HR | 95% CI | *P* Value | HR | 95% CI | *P* Value | HR | 95% CI | *P* Value |
| **All patients with proCNP ≥ median (*n* = 928) are included** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.99-1.01 | .89 | 1.00 | 0.99-1.02 | .57 |
| | 1-year mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.99-1.01 | .89 | 1.00 | 0.99-1.02 | .58 |
| **Females with proCNP ≥ median (n=260) are included** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.05 | 1.02-1.07 | <.001 | 1.04 | 1.01-1.07 | .008 | 1.04 | 1.01-1.07 | .016 |
| | 1-year mortality | 1.04 | 1.02-1.07 | <.001 | 1.03 | 1.01-1.06 | .010 | 1.04 | 1.01-1.06 | .007 |
| **Males with a proCNP ≥ median (*n* = 668) are included** | | | | | | | | | | |
| **ProCNP (per 1 pmol/L increase)** | 30-day mortality | 1.02 | 1.01-1.03 | .001 | 1.00 | 0.98-1.02 | .86 | 1.00 | 0.98-1.02 | .92 |
| | 1-year mortality | 1.02 | 1.01-1.03 | <.001 | 1.00 | 0.98-1.01 | .72 | 1.00 | 0.98-1.02 | .86 |

All patients with a proCNP ≥ median of the sex- and age-specific reference interval are included in the analyses to test the predictive value of proCNP as a continuous variable (per 1 pmol/L increase).

* Multivariable model with age and plasma creatinine included as co-variables.

† Multivariable model with age, plasma creatinine, proANP, tertiles of peak troponins, number of vessels affected, and time from onset of symptoms to blood sample included as co-variables.

HR, hazard ratio; CI, confidence interval.

**Table 3: Linear mixed models of longitudinal measurements of proCNP in 287 patients with STEMI.**

| | ***Univariate model*** | | ***Multivariate model 1*** | | ***Multivariate model 2*** | |
|---|---|---|---|---|---|---|
| | Value (95% CI) / pmol/L | *P* Value | Value (95% CI) / pmol/L | *P* Value | Value (95% CI) / pmol/L | *P* Value |
| **Time (per time point)** | -1.3 (-2.1 – -0.5) | .001 | -0.25 (-1.8 – 1.3) | .75 | -0.25 (-1.8 – 1.3) | .76 |
| **Age (per year)** | Variables not included | | 0.16 (0.02 – 0.30) | .02 | 0.06 (-0.08 – 0.20) | .39 |
| **Sex (effect of male sex)** | | | 4.5 (-0.6 – 9.6) | .09 | 4.4 (-0.28 – 9.1) | .07 |
| **Interaction of time and sex** | | | -1.4 (-3.2 – 0.4) | .13 | -1.4 (-3.2 – 0.36) | .13 |
| **Chronic kidney disease** | | | Variables not included | | 19.7 (13.3 – 26.1) | <.001 |
| **Hypertension** | | | | | 4.5 (0.6 – 8.4) | .03 |
| **Diabetes** | | | | | -4.0 (-9.7 – 1.7) | .17 |
| **Peripheral artery disease** | | | | | -5.1 (-13.9 – 3.7) | .26 |
| **Stroke** | | | | | 2.7 (-5.3 – 10.7) | .51 |

The table shows the associated estimated changes in proCNP concentration in pmol/L (95%CI) when each variable is entered in a linear mixed model. All variables included in the models are shown in the table.

CI, confidence interval.

**Figure 1. Study design.**

STEMI, ST-elevation myocardial infarction; CAG, coronary angiography; proCNP, pro-C-type natriuretic peptide (see *Nomenclature*).

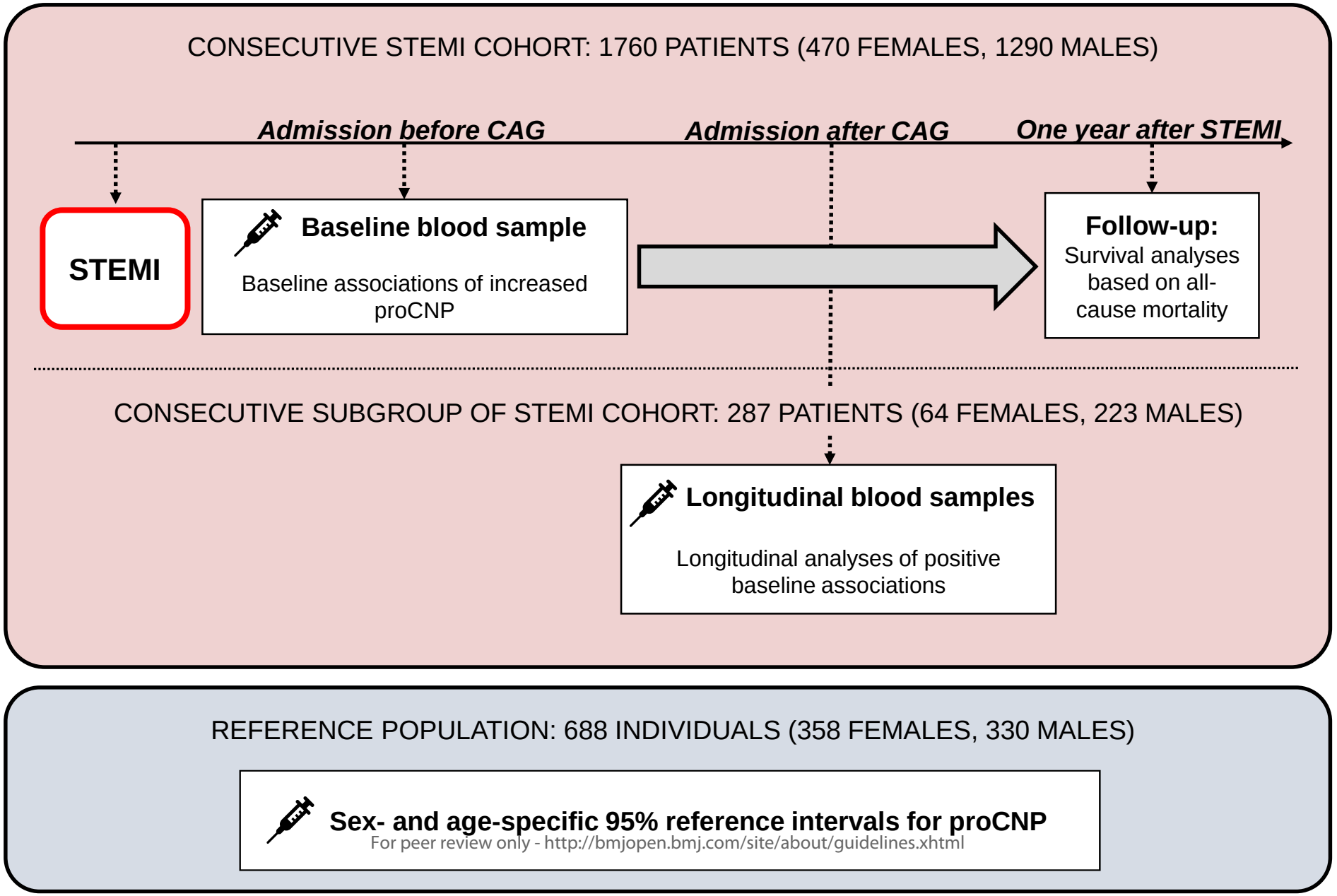
**Figure 2. Mortality rates for increased and normal proCNP in all patients and stratified by sex.**

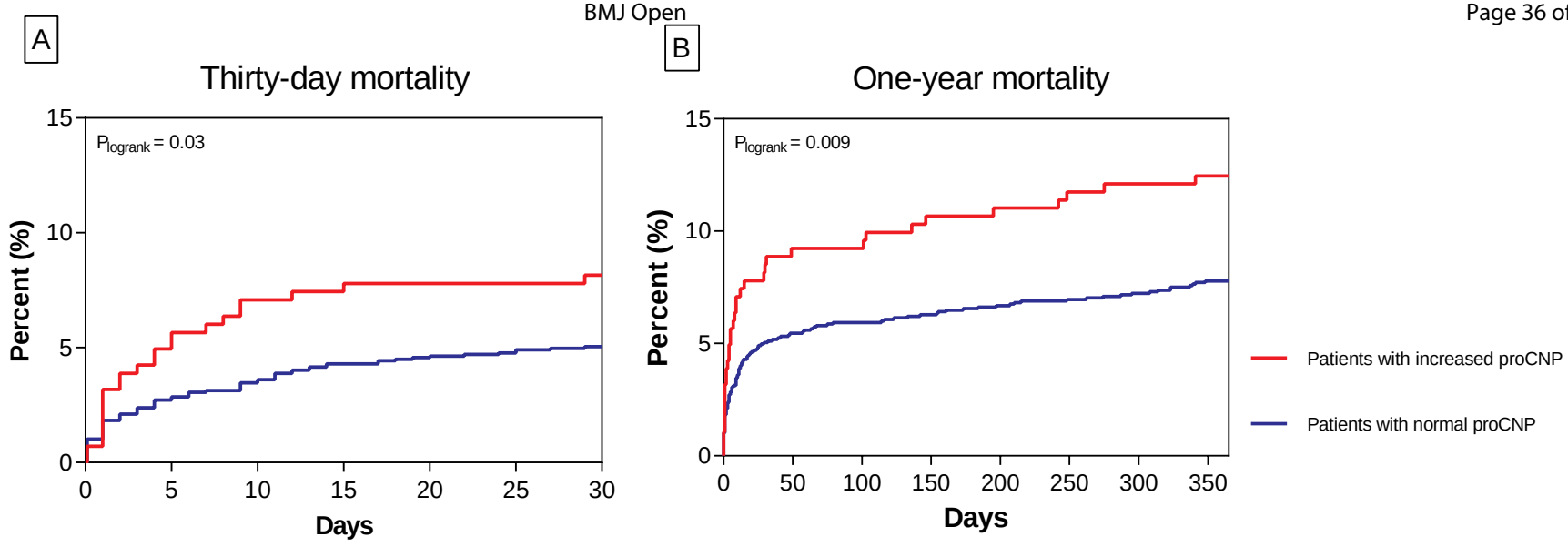
Display of all-cause mortality rates for groups of normal vs. increased proCNP concentrations in plasma. Graphs of 30-day and one-year mortality rate for all patients are shown in section A and B, respectively. Sex-specific 30-day and one-year mortality rates are shown in section C and D, respectively. The numbers of patients at risk in subgroups are given beneath each graph.

**Figure 3. Longitudinal concentrations of proCNP in plasma.**

Concentrations are shown as mean (point) and standard error of the mean (error bars). ProCNP concentrations over time for 287 patients with STEMI are shown in section A. In section B, C, and D, these patients are grouped based on sex, hypertension, and chronic kidney disease, respectively.

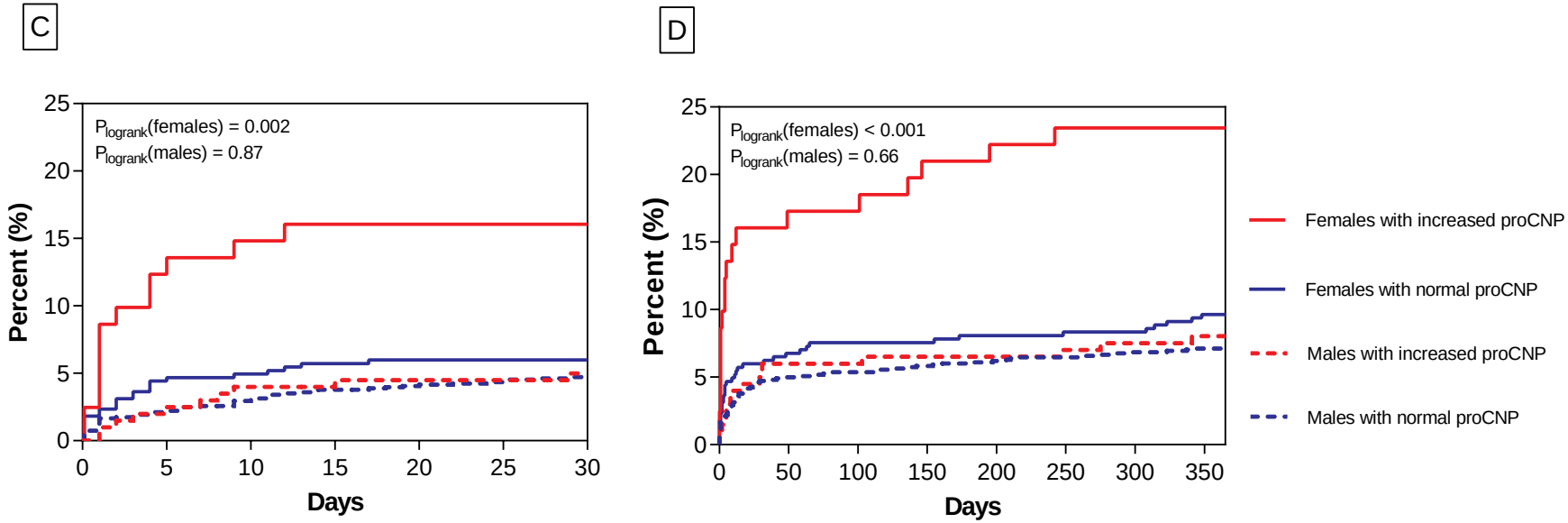
# STUDY DESIGN





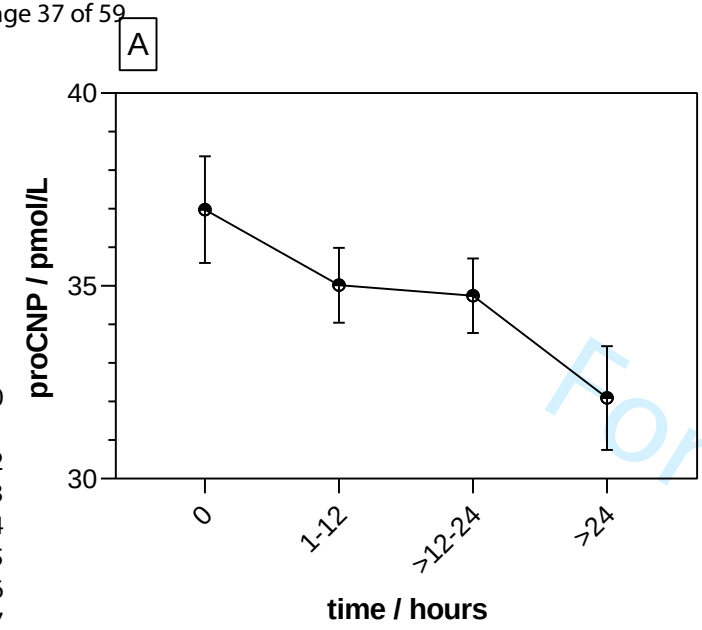

No. at risk

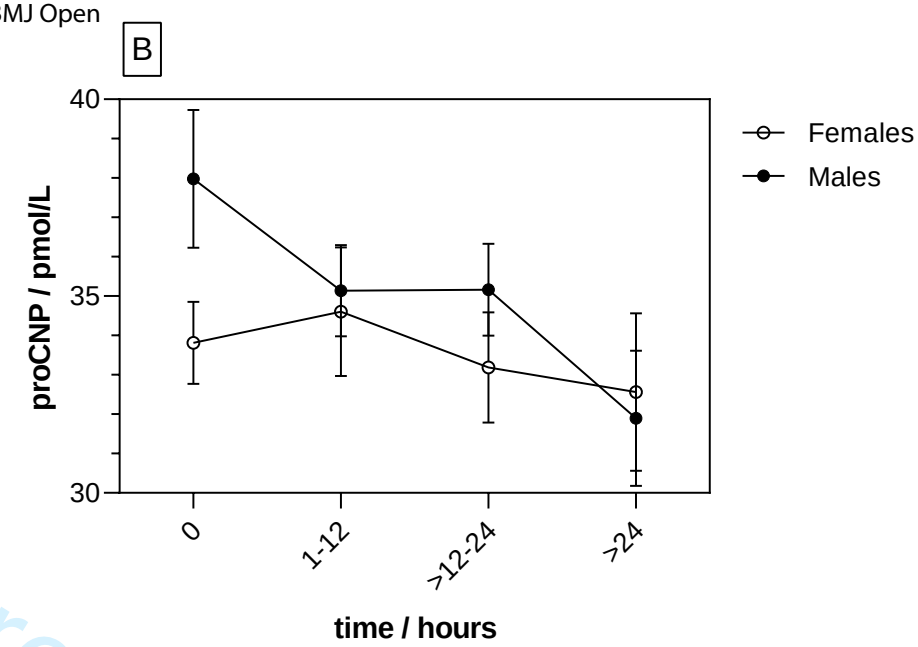
| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patients with normal proCNP | 1472 | 1426 | 1412 | 1400 | 1394 | 1391 | 1387 | 1472 | 1381 | 1374 | 1369 | 1363 | 1359 | 1355 | 1347 |
| Patients with increased proCNP | 283 | 267 | 261 | 259 | 258 | 258 | 256 | 283 | 253 | 253 | 249 | 248 | 246 | 245 | 244 |

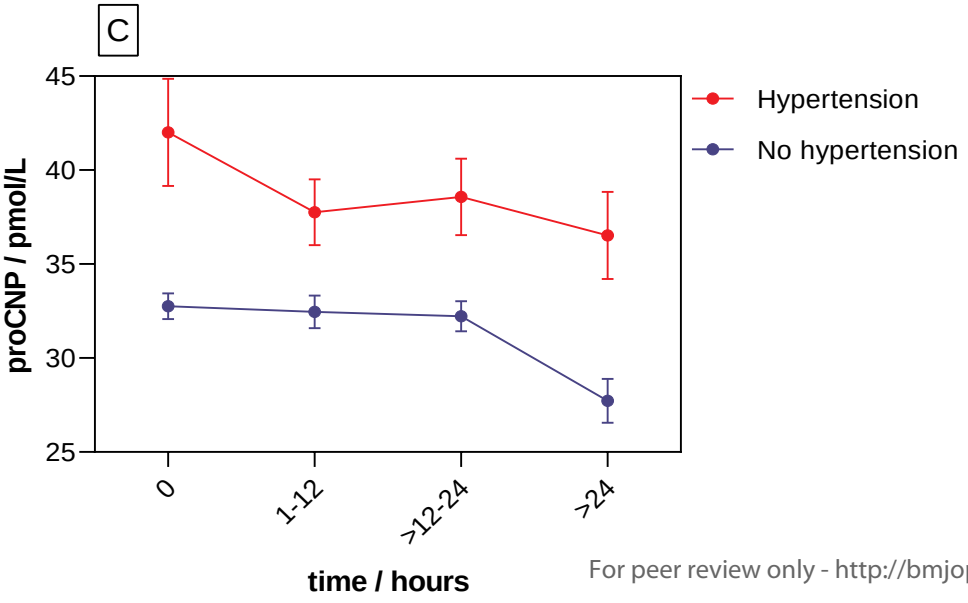


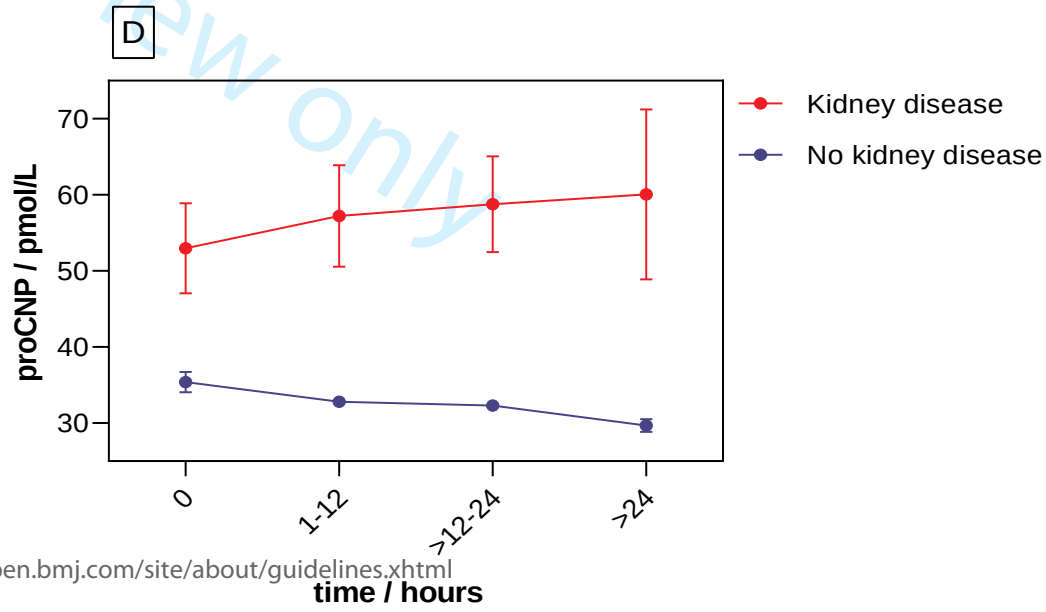

No. at risk

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Females with normal proCNP | 385 | 366 | 364 | 361 | 360 | 360 | 360 | 385 | 357 | 354 | 354 | 352 | 351 | 351 | 346 |
| Females with increased proCNP | 81 | 71 | 69 | 68 | 68 | 68 | 68 | 81 | 67 | 67 | 64 | 63 | 62 | 62 | 62 |
| Males with normal proCNP | 1087 | 1060 | 1048 | 1039 | 1034 | 1029 | 1027 | 1087 | 1024 | 1020 | 1015 | 1011 | 1008 | 1004 | 1001 |
| Males with increased proCNP | 202 | 196 | 192 | 191 | 190 | 190 | 188 | 202 | 186 | 186 | 185 | 185 | 184 | 183 | 182 |



# Supplemental Material

## METHODS

### COHORT OF PATIENTS WITH STEMI

Information on age, sex, height, body mass index (BMI), smoking status, development of cardiogenic shock, and cardiac arrest coma, time from onset of symptoms to blood sample, time from blood sample to percutaneous coronary intervention balloon angioplasty, number of coronary vessels affected (defined as the number coronary arteries with at least one stenosis of >70% of the lumen diameter (discrete values of zero to three) from the coronary angiography procedure (CAG)), culprit coronary vessel anatomy, Thrombolysis In Myocardial Infarction (TIMI ) grade flow, medical history, left ventricular ejection fraction (LVEF), and routine laboratory measurements was used in the data analyses. Blood samples for baseline biochemical measurements were collected from the femoral or radial sheath on admission immediately before CAG was performed. LVEF was determined by 2D echocardiography performed on admission or within 48 hours of admission. From a consecutive subgroup of the cohort, we collected repeated venous plasma samples during the first days after admission from January to March 2016 at Rigshospitalet (RH) for longitudinal assessment of potential changes in proCNP concentrations. Longitudinal plasma samples were collected at least twice within the first day of admission, and once daily in subsequent days of admission. All patients with longitudinal plasma samples are included in the analyses.

### BIOCHEMICAL ANALYSES

Troponin T was measured in patients admitted to RH by Elecsys Troponin T hs assay (Cobas, by Roche, Basel, Switzerland), whereas troponin I was measured in patients admitted to Odense Universitetshospital (OUH) using Architect *STAT* High Sensitive Troponin-I (Abbott, Chicago, Illinois, United States). Both measurements from samples on admission and measured peak values during admission (the latter as a proxy of myocardial infarction size), were used in our statistical analyses. Plasma creatinine was measured using CREP2 assay and high sensitivity C-reactive protein (hs-CRP) using CRPHS assay (Cobas, by Roche, Basel, Switzerland). ST2 was measured by Presage ST2 Assay (Critical Diagnostics, Inc., San Diego, California). We included measurement of soluble thrombomodulin (sTM) and syndecan-1 as markers of endothelial cell and glycocalyx damage, respectively. Assay procedures of sTM and syndecan-1 have been described previously.[1] With regards to measurement of hs-CRP, the results were analyzed as the proportion of patients with a concentration of ≥2mg/L. This cut-off value has been introduced as a definition of chronic inflammation in cardiac disease.[2] Moreover, patients with a time from onset of symptoms to blood sample of ≥6 hours are excluded from hs-CRP analyses because an increase in hs-CRP concentration can be expected due to myocardial damage.[3,4] For measurement of syndecan-1, sTM, and hs-CRP, only patients admitted at RH were analyzed. All biochemical analytes, apart from peak troponins and longitudinal proCNP and proANP, were measured in blood samples collected on admission.

## RESULTS

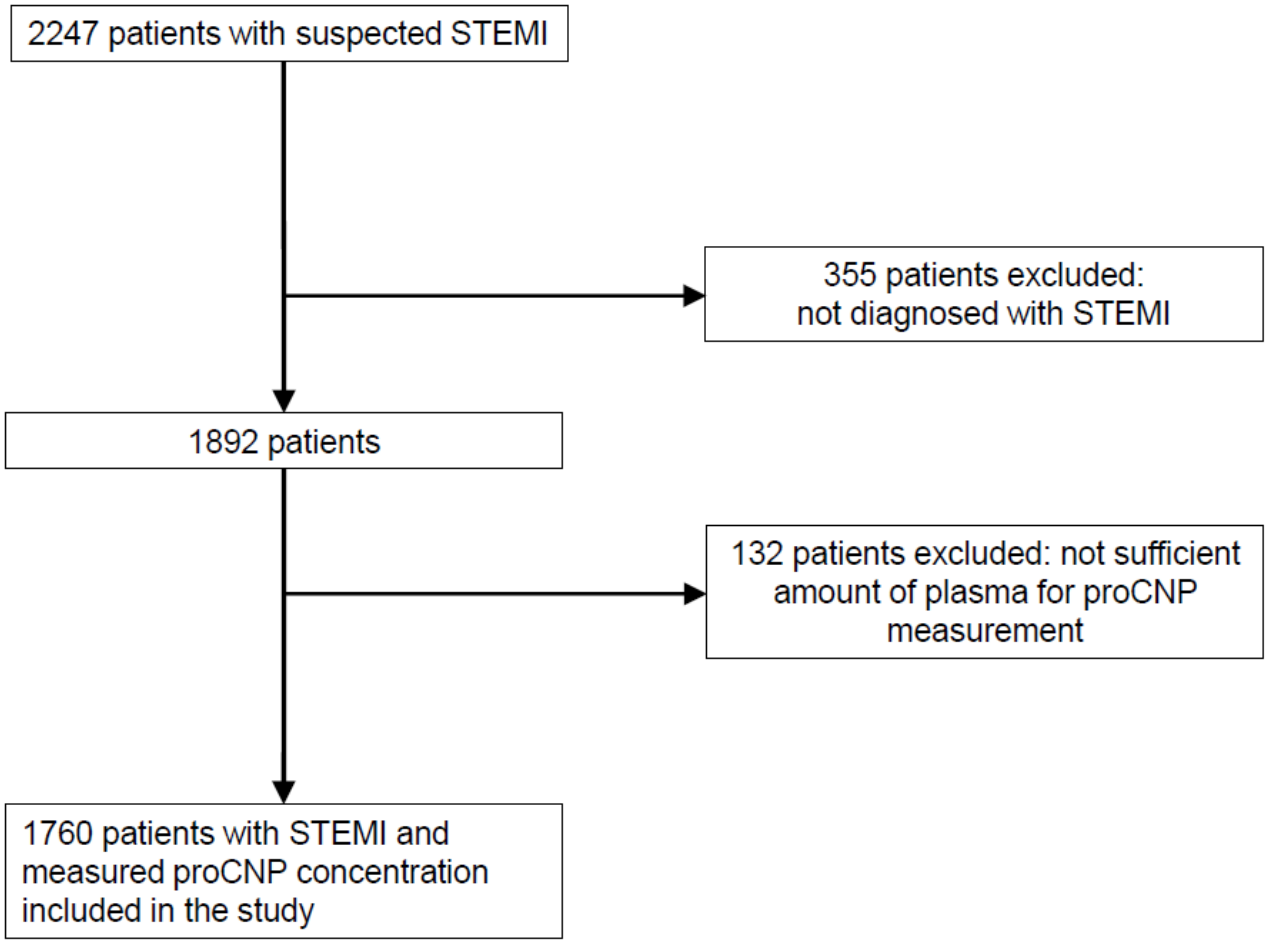
## PROCNP MEASUREMENT

Coefficients of variation of proCNP measurement in plasma were 13.8% for 20 pmol/L and 13.1% for 40 pmol/L.

## REFERENCE POPULATION AND STEMI COHORT

By inspection of histograms of proCNP concentrations, we concluded that there were no outliers among the individuals. Table 1 shows sex- and age-specific 95% reference intervals of proCNP concentrations in the reference population. Plasma concentrations in males were marginally higher compared to those in females ($P$ = 0.015). Women ≥50 years had higher proCNP concentrations compared to women <50 years ($P$ = 0.011), where no difference was observed among men ($P$ = 0.44). A flowchart of the inclusion of patients is shown in Supplemental Figure 1. The distributions of proCNP concentrations in the reference population and the STEMI cohort are shown in Supplemental Figure 2.

**Supplemental Figure 1: Flowchart of the inclusion of patients in study**



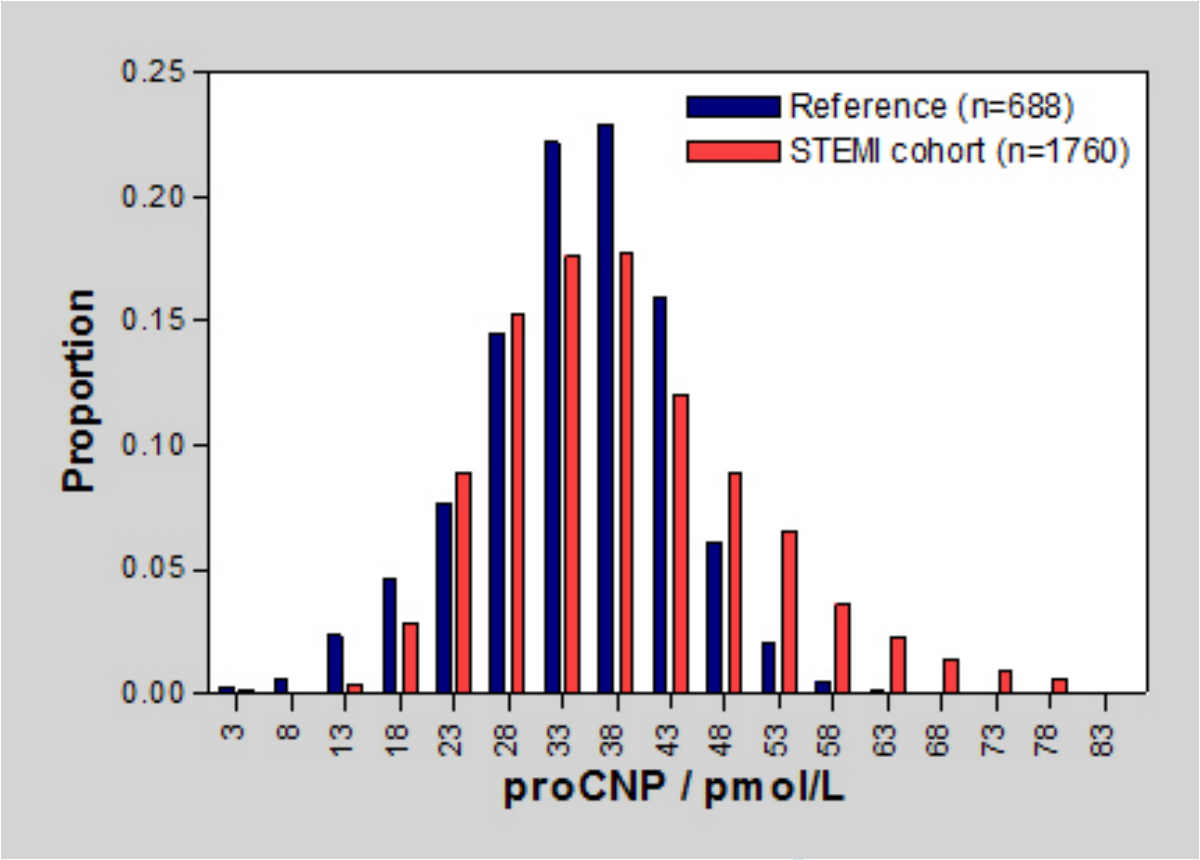

**Supplemental Table 1. Reference intervals of proCNP in sex- and age-specific groups.**

| | Men | | Women | |
|---|---|---|---|---|
| **Age groups (years)** | <50 | ≥50 | <50 | ≥50 |
| **Number of subjects** | 157 | 173 | 179 | 179 |
| **95% reference interval** | 10.2 – 52.2 | 13.9 – 49.6 | 13.6 – 48.9 | 13.4 – 49.4 |
| **Median** | 35.2 | 36.0 | 32.2 | 34.6 |

| | | | | |
|---|---|---|---|---|
| **Range** | 4.4 – 55.4 | 11.4 – 52.4 | 8.2 – 55.4 | 4.4 – 60.0 |

Reference intervals, median, and range of proCNP concentrations of subgroups are all given in pmol/L.

**Supplemental Figure 2. Histograms of relative frequencies of plasma proCNP concentrations.**




The reference population is represented by blue bars and the STEMI cohort by red bars.

Each bar represents an interval of 5 pmol/L.

Values of >85 pmol/L are not shown.

**Information from coronary angiography**

In Supplemental Table 2 data from coronary angiography on culprit vessel, numbers of coronary vessels affected and Thrombolysis in Myocardial Infarction (TIMI) grade flow is shown in all patients and sex-specifically.

**Supplemental Table 2. Culprit vessel, number of coronary vessels affected and Thrombolysis in Myocardial Infarction grade flow.**

| | All patients (*n* = 1755) | | | Females (*n* = 466) | | | Males (*n* = 1289) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value | Normal proCNP | Increased proCNP | *P* Value |
| **Culprit vessel** | | | | | | | | | |
| **None, *n* (%)** | 55 (3.7) | 7 (2.5) | .38 | 21 (5.5) | 5 (6.2) | .79 | 34 (3.1) | 2 (1.0) | .10 |
| **Left main coronary artery, *n* (%)** | 31 (2.1) | 5 (1.8) | >.99 | 15 (3.9) | 2 (2.5) | .75 | 16 (1.5) | 3 (1.5) | >.99 |
| **Left anterior descending coronary artery, *n* (%)** | 623 (42.3) | 119 (42.0) | .95 | 144 (37.4) | 31 (38.3) | .90 | 479 (44.1) | 88 (43.6) | .94 |
| **Right coronary artery, *n* (%)** | 529 (35.9) | 115 (40.6) | .14 | 148 (38.4) | 34 (42.0) | .62 | 381 (35.1) | 81 (40.1) | .18 |
| **Left circumflex, *n* (%)** | 221 (15.0) | 36 (12.7) | .36 | 54 (14.0) | 9 (11.1) | .59 | 167 (15.4) | 27 (13.4) | .52 |
| **Graft, *n* (%)** | 10 (0.7) | 1 (0.4) | >.99 | 1 (0.3) | 0 (0) | >.99 | 9 (0.8) | 1 (0.5) | >.99 |
| **Number of vessels affected** | | | | | | | | | |
| **No-vessel disease, *n* (%)** | 29 (2.0) | 2 (0.7) | .21 | 14 (3.6) | 2 (2.5) | >.99 | 15 (1.4) | 0 (0) | .15 |
| **One-vessel disease, *n* (%)** | 903 (61.5) | 172 (60.7) | .84 | 243 (63.4) | 46 (56.8) | .31 | 660 (60.8) | 126 (62.4) | .70 |
| **Two-vessels disease, *n* (%)** | 333 (22.7) | 61 (21.6) | .76 | 75 (19.6) | 20 (24.7) | .29 | 258 (23.8) | 41 (20.3) | .32 |
| **Three-vessels disease, *n* (%)** | 204 (13.9) | 48 (17.0) | .20 | 51 (13.3) | 13 (16.0) | .48 | 153 (14.1) | 35 (17.3) | .23 |
| **Thrombolysis in Myocardial Infarction (TIMI) grade flow** | | | | | | | | | |
| **0, *n* (%)** | 96 (6.8) | 21 (7.4) | .60 | 28 (7.2) | 8 (9.9) | .49 | 68 (6.3) | 13 (6.4) | .88 |
| **1, *n* (%)** | 6 (0.4) | 1 (0.4) | >.99 | 1 (0.3) | 0 (0) | >.99 | 5 (0.5) | 1 (0.5) | >.99 |
| **2, *n* (%)** | 49 (3.3) | 7 (2.5) | .58 | 17 (4.4) | 2 (2.5) | .55 | 32 (2.9) | 5 (2.5) | >.99 |
| **3, *n* (%)** | 1321 (89.7) | 254 (89.8) | >.99 | 339 (88.1) | 71 (87.7) | .85 | 982 (90.3) | 183 (90.6) | >.99 |

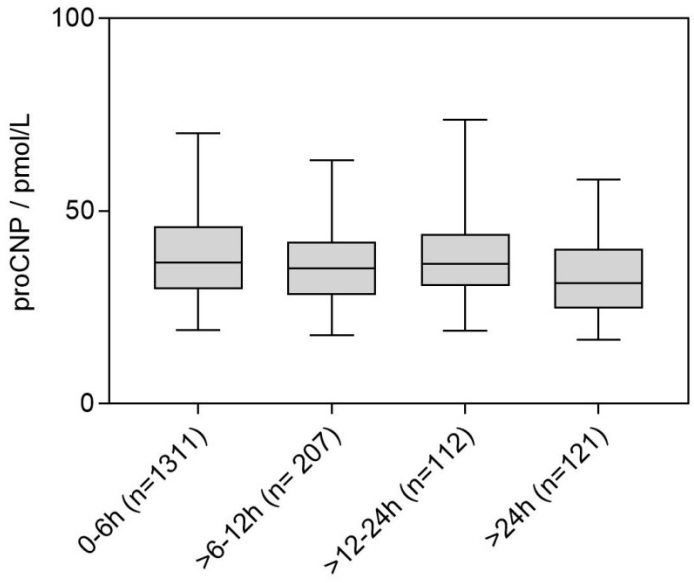
## Cardiogenic shock development and cardiac arrest

When median proCNP concentrations were tested in patients with cardiogenic shock development vs. no cardiogenic shock development and cardiac arrest coma vs. no cardiac arrest coma or cardiogenic shock development, no differences were observed ($P = 0.41$ and $P = 0.28$ for females, respectively, and $P = 0.97$ and $P = 0.46$ for males, respectively).

## Time from onset of symptoms to blood sample and correlation of biochemical markers

In Supplemental Figure 3, median plasma proCNP concentrations are shown in subgroups of different time intervals from onset of symptoms to blood sample. A difference in proCNP was observed across subgroups ($P < 0.001$). However, when patients with a time interval of >24 hours were excluded, no difference was found ($P = 0.091$). Information on time from baseline blood sampling (performed when the CAG procedure was initiated) to percutaneous coronary intervention balloon angioplasty (time of reperfusion) was obtained on 862 patients (227 women, 635 men). Median (interquartile range) values in minutes were: 5 (4-9) and 5 (3-9) ($P = 0.90$) for women with normal and increased proCNP respectively, and 6 (4-10) and 5 (4-10) ($P = 0.17$) for men with normal and increased proCNP respectively. For biochemical markers with positive associations to proCNP, we performed Spearman's correlation analyses, and for proCNP vs. creatinine, proANP, syndecan-1, and sTM, Spearman r were 0.26, 0.082, 0.098, and 0.24, respectively ($P < 0.001$ for all).

**Supplemental Figure 3: Box-plots of proCNP concentrations in groups of time from onset of symptoms to admission.**




Boxes indicate median and inter-quartile range, and error bars indicate the interval from 2.5 to 97.5 percentile.

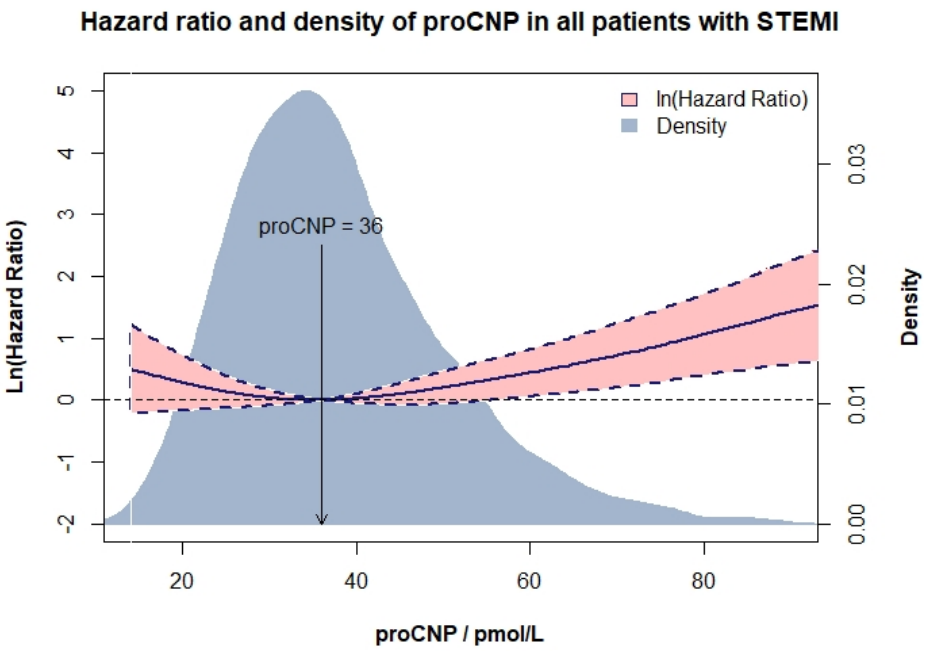
**Sensitivity and specificity of increased proCNP and one-year mortality**

For one-year mortality, the sensitivity (defined as the proportion of patients that died, who had increased proCNP) vs. specificity (defined as the proportion of patients that survived, who had normal proCNP) were 34% vs. 85% for females and 17% vs. 84% for males.

**Multivariable Cox regression analyses**

We examined the effect of proCNP concentrations as a continuous variable on the hazard ratio (HR) of one-year all-cause death in all patients as well as females and males separately by cubic spline plots, using median concentrations as a reference point (shown in Supplemental Figure 4). In females, there was no effect of proCNP below median concentrations, whereas the estimated HR increased from approximately median concentrations to the highest measured concentrations. In males, a trend towards a U-shaped relation was observed with increasing estimated hazard ratios for decreases of proCNP below median and for increases of proCNP above median. Based on these observations we focused our Cox regression analyses on the upper range of proCNP, where we observed that increasing proCNP was associated with increasing HR in both females and males.

**Supplemental Figure 4: Cubic spline and density plots of hazard ratio and proCNP concentrations.**

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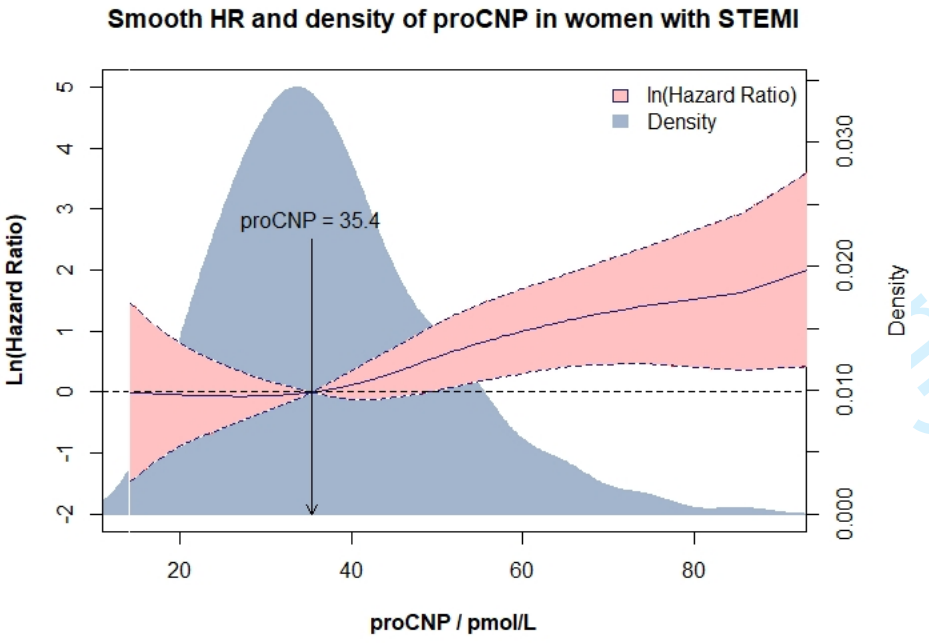


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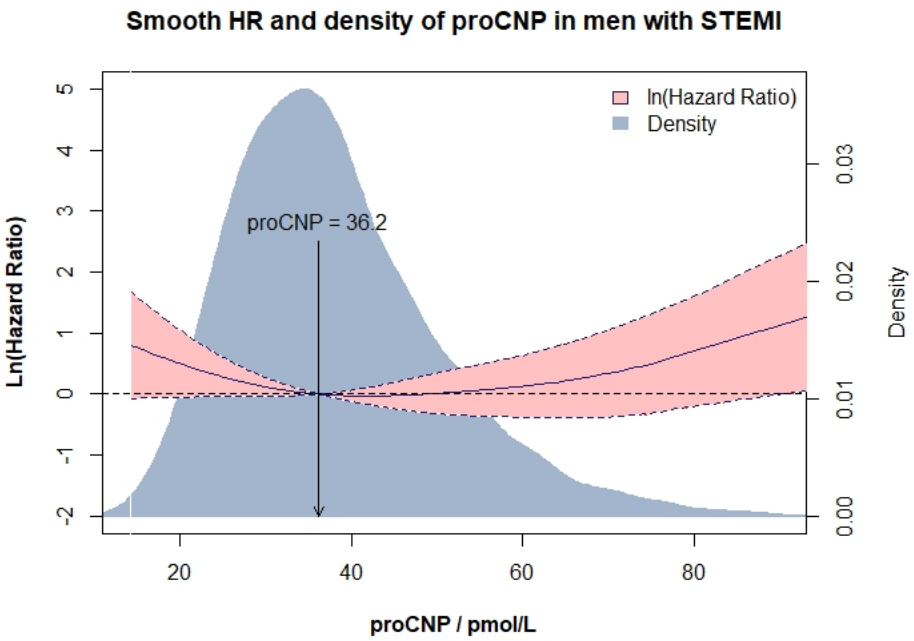


## Longitudinal plasma measurements

In Supplemental Table 3, the number of patients and samples in groups stratified according to diseases are shown. Longitudinal measurements of proANP are shown in Supplemental Figure 5, where the initial decrease from first (0 hours) to second (1-12 hours) timepoint is estimated to be ~850 pmol/L (~-85%).

**Supplemental Table 3: Number of patients and plasma samples in longitudinal analyses.**

| | **Number of patients** (females/males) | **Number of samples** (from females/from males) |
|---|---|---|
| **Overall** | 287 (64/223) | 907 (211/696) |
| **Chronic kidney disease** | 26 (6/20) | 81 (20/61) |
| **Hypertension** | 131 (32/99) | 412 (96/316) |
| **Diabetes** | 34 (8/26) | 107 (20/87) |
| **Stroke** | 15 (5/10) | 46 (16/30) |
| **Peripheral artery disease** | 12 (2/10) | 40 (6/34) |

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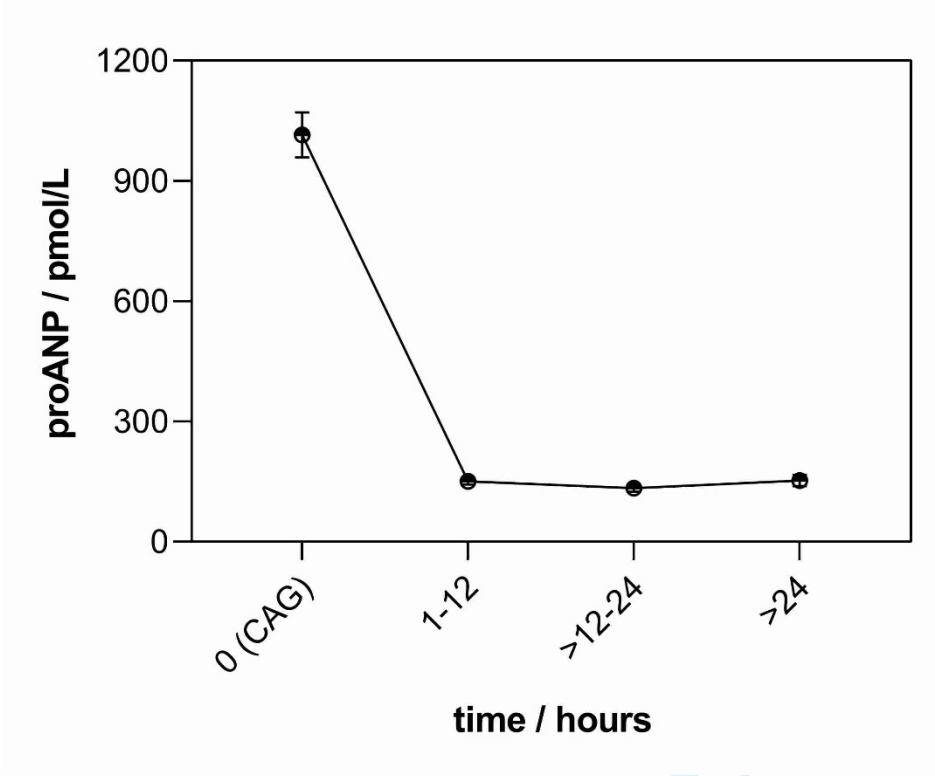
**Supplemental Figure 5: Longitudinal concentrations of proANP in plasma.**




Concentrations are shown as mean (SEM) for patients with longitudinal plasma samples.

## DISCUSSION

*Assay Principle*

Our proCNP radioimmunoassay is developed in accordance with a processing-independent principle,[5,6] where different fragments of the prohormone of CNP can be accurately quantitated in circulation regardless of post-translational processing of the prohormone. Also, there is no cross-reactivity to the structurally related cardiac natriuretic propeptides.[7]

*Reference Intervals*

We based our calculated 95% reference intervals on two age groups of < and ≥ 50 years. The reason for choosing this division is a previous report showing that NT-proCNP concentrations in plasma in healthy individuals increase from ~50 years of age.[8] We therefore assume that the two age groups represent two different stages of adulthood with regard to circulating NT-proCNP concentrations and, hence, that the two age-specific intervals (for each sex) constitute a meaningful reference for interpretation of measured proCNP concentrations in the STEMI cohort.

*Time from onset of symptoms to blood sampling and balloon angioplasty*

In our baseline and multivariable Cox regressions analyses we have included the time from onset of symptoms as a variable, where we have data from the 99.5% of the included patients. Our results on time from blood sampling to balloon angioplasty (data was obtained from 49.1% of the included patients) show no differences in time between groups of normal and increased proCNP in both sexes, where the median time duration was 5-6 minutes in all groups. Assuming that the total ischemic time of the STEMI patients is equal to the time from onset of symptoms to balloon angioplasty (time of reperfusion), our analyses support that the time onset of symptoms to blood sampling as a variable also reflects the total ischemic time of the patients.

*Correlation Analyses of Biochemical Measurements*

Correlation analyses showed that the association of proCNP with sTM is superior in comparison to both that of syndecan-1 and proANP. A likely explanation may be that both

proCNP and sTM are released from endothelial cells, whereas the glycocalyx and the cardiomyocytes are the major sources of syndecan-1 and proANP, respectively. In contrast to the vascular markers, however, no associations with markers of inflammation, hs-CRP, and ST2 were observed. Thus, proCNP concentrations in plasma do not seem to be affected by general inflammation in patients with STEMI.

*Longitudinal Analyses*

Repeated measurements displayed a statistically significant decrease in proCNP concentrations over time in a univariate analysis. For comparison we have included repeated measurements of proANP, shown in Supplemental Figure 5. The dynamic response of proANP differs markedly from proCNP with a steep decrease from 0 to 1-12 hours and a flat curve from 1-12 to >24 hours. These differences highlight the distinct biological roles of CNP vs. ANP.

For proCNP, both multivariate linear mixed models (Model 1 and 2) show that the effect of time was reduced in magnitude and was non-significant, indicating that changes over time are better explained by other variables than time per se. Model 1 found an independent effect of age with higher concentrations of proCNP per year, but Model 2 found that the effect of age disappeared when chronic kidney disease and other vascular diseases were included. Thus, age per se does not seem to explain an increase in proCNP concentration; more likely, chronic diseases (where prevalence increases with age) appear a confounder of the crude effect of age. Although a statistically insignificant finding, Model 1 indicates an effect of male sex and an interaction of sex and time that are unchanged after inclusion of chronic diseases

in Model 2. Consistent with the effect of male sex are previously reported results[8] and the results of the reference population of the present study, where males have slightly higher concentrations of proCNP-derived peptides in the circulation. The possible interaction of sex and time, where males display a relative decrease over time compared with females, has not previously been reported. However, our longitudinal analyses lack the statistical power to sufficiently conclude on a potential interaction of sex and time on proCNP concentrations. In model 2, we find that chronic kidney disease and hypertension are independently associated with higher proCNP concentrations, consistent with baseline associations.

Of the (cardio)vascular diseases with a crude positive association to increased proCNP in baseline results, only hypertension is statistically independent in the multivariate repeated measurement analysis. Unexpectedly, the independent effects of diabetes mellitus and peripheral artery disease seem to be oppositely directed, where the diseases are independently associated with lower proCNP concentrations. However, these results were statistically insignificant, and further studies with more statistical power are needed to determine if such negative independent associations exist.

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2

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

| | Item No | Recommendation | Page No |
|---|---|---|---|
| **Title and abstract** | 1 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract<br>(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found | 1<br>3-4 |
| **Introduction** | | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported | 7-8 |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses | 7-8 |
| **Methods** | | | |
| Study design | 4 | Present key elements of study design early in the paper | 8-12 |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection | 8-11 |
| Participants | 6 | (*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up<br>(*b*) For matched studies, give matching criteria and number of exposed and unexposed | 8-10 |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable | 8-11 + suppl. |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group | 8-10 + suppl. |
| Bias | 9 | Describe any efforts to address potential sources of bias | - |
| Study size | 10 | Explain how the study size was arrived at | 8 |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why | 10-11 |
| Statistical methods | 12 | (*a*) Describe all statistical methods, including those used to control for confounding<br>(*b*) Describe any methods used to examine subgroups and interactions<br>(*c*) Explain how missing data were addressed<br>(*d*) If applicable, explain how loss to follow-up was addressed<br>(*e*) Describe any sensitivity analyses | 10-12 + suppl. |
| **Results** | | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed<br>(b) Give reasons for non-participation at each stage<br>(c) Consider use of a flow diagram | 12-15 + table 1-2 + figure 1 +suppl |
| Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders | 12-15 + table 1-2 + figure 1 |

| | | | |
|---|---|---|---|
| | | (b) Indicate number of participants with missing data for each variable of interest<br>(c) Summarise follow-up time (eg, average and total amount) | +suppl |
| Outcome data | 15* | Report numbers of outcome events or summary measures over time | table 2 + figure 1 |

| | | | |
|---|---|---|---|
| Main results | 16 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included<br><br>(*b*) Report category boundaries when continuous variables were categorized<br>(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period | 12-15 + table 1-2 + figure 1 +suppl |
| Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses | 12-15 + table 1-2 + figure 1-2 +suppl |
| **Discussion** | | | |
| Key results | 18 | Summarise key results with reference to study objectives | 15-19 |
| Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias | 18 |
| Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence | 15-19 |
| Generalisability | 21 | Discuss the generalisability (external validity) of the study results | 15-18 |
| **Other information** | | | |
| Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based | |

*Give information separately for exposed and unexposed groups.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.