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

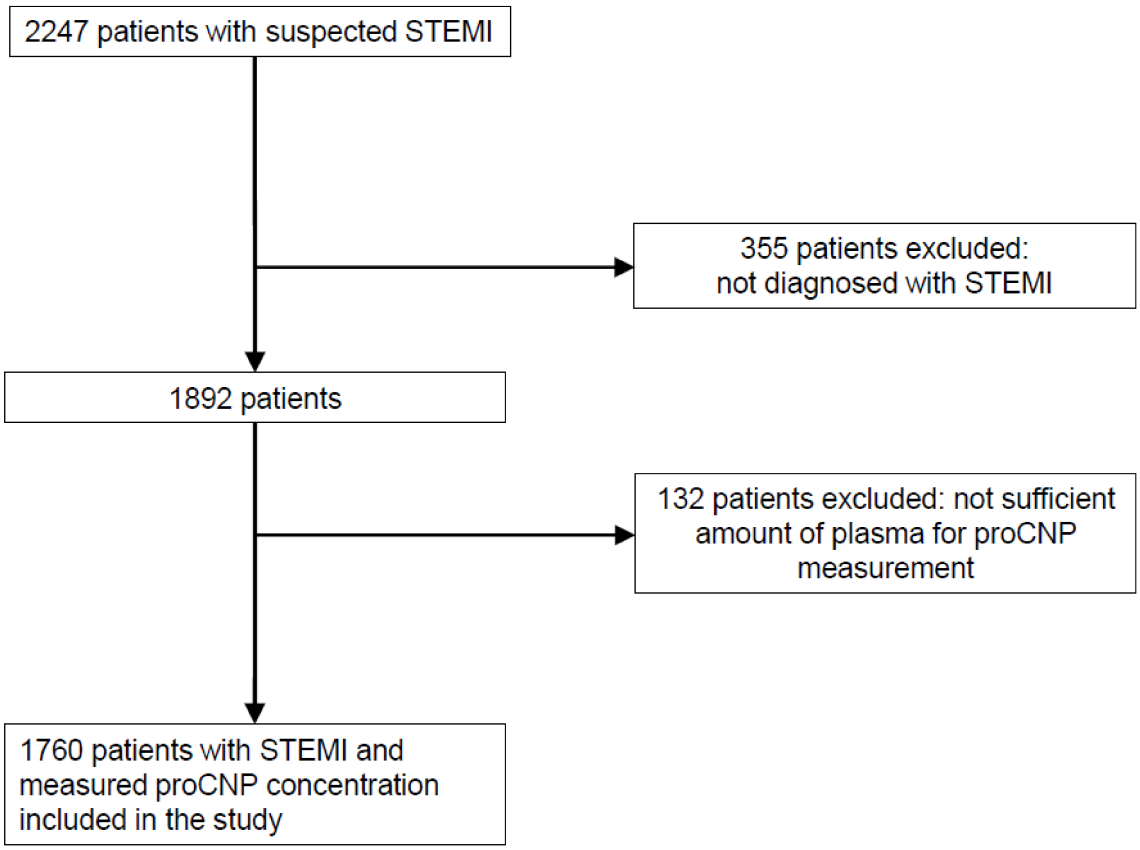
PROCNP MEASUREMENT

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



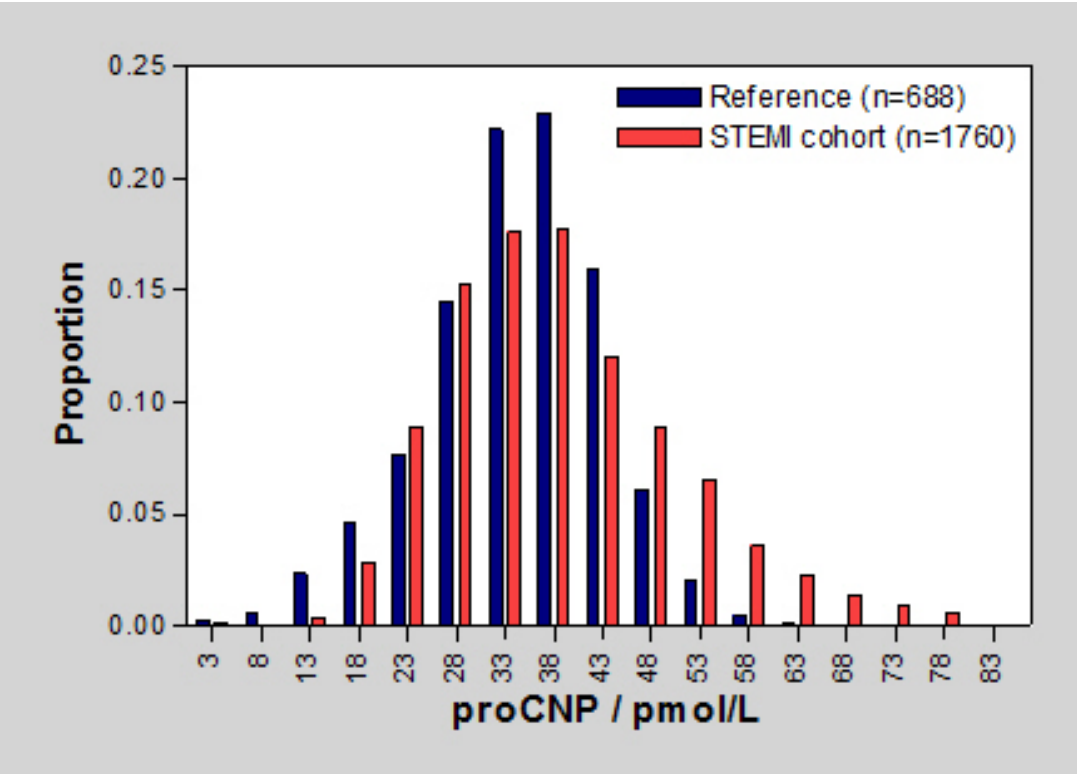
Supplemental Table 1. Reference intervals of proCNP in sex- and age-specific groups.

	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

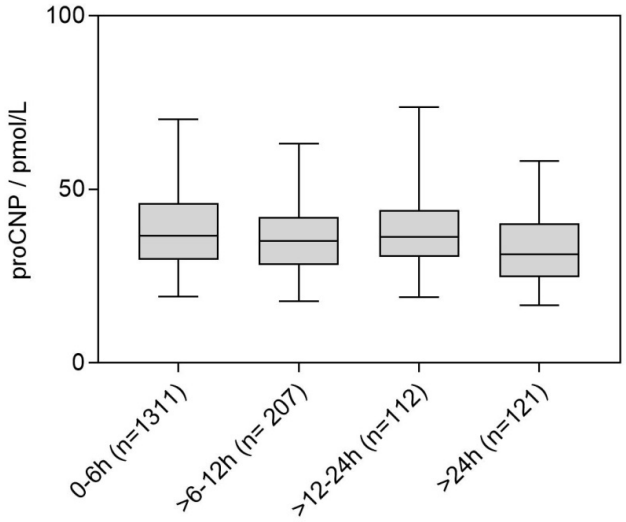
Cardiogenic shock development and cardiac arrest

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.

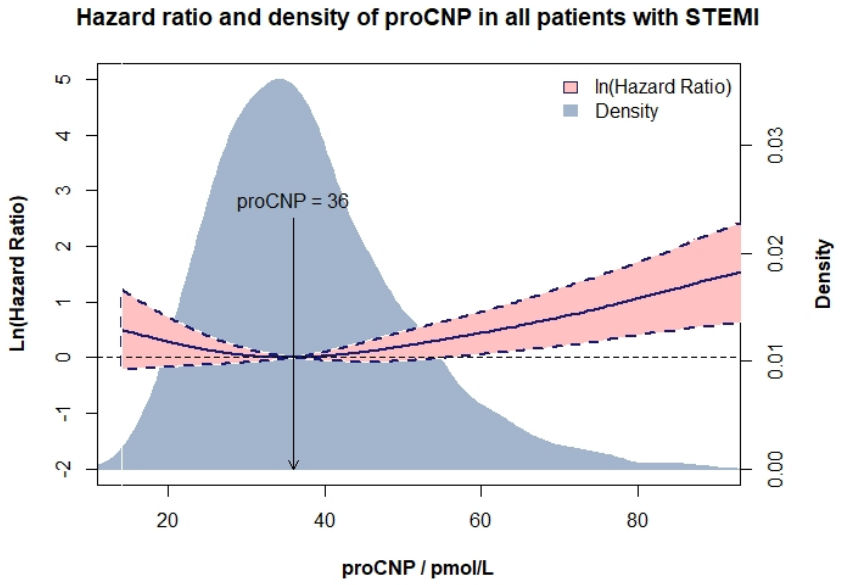
Sensitivity and specificity of increased proCNP and one-year mortality

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.

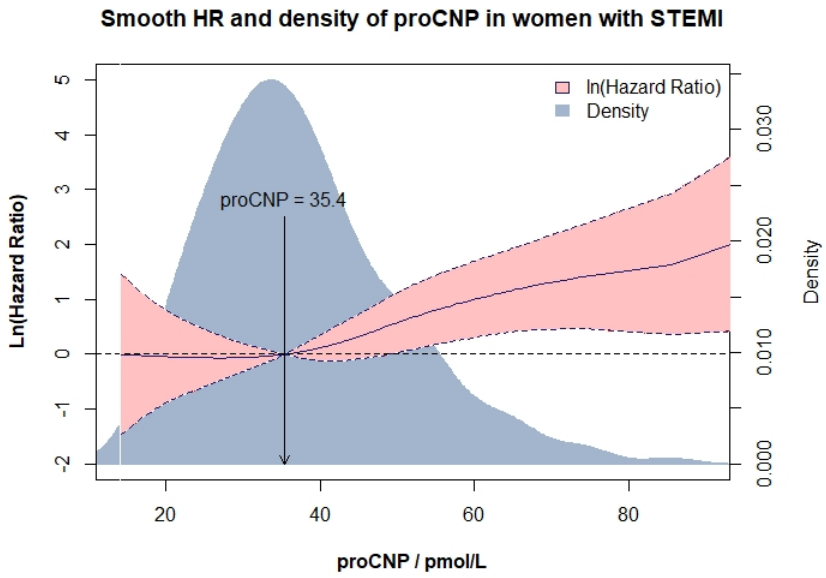
Multivariable Cox regression analyses

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.

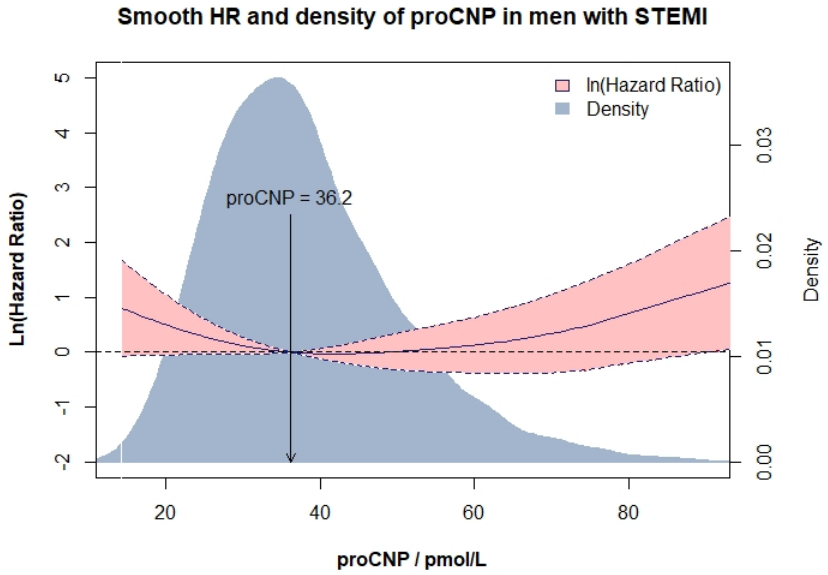
Supplemental Figure 4: Cubic spline and density plots of hazard ratio and proCNP concentrations.


Hazard ratio and density of proCNP in all patients with STEMI
ln(Hazard Ratio)
Density
proCNP = 36
Ln(Hazard Ratio)
Density
proCNP / pmol/L

1


Smooth HR and density of proCNP in women with STEMI
ln(Hazard Ratio)
Density
proCNP = 35.4
Ln(Hazard Ratio)
Density
proCNP / pmol/L

2



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%).

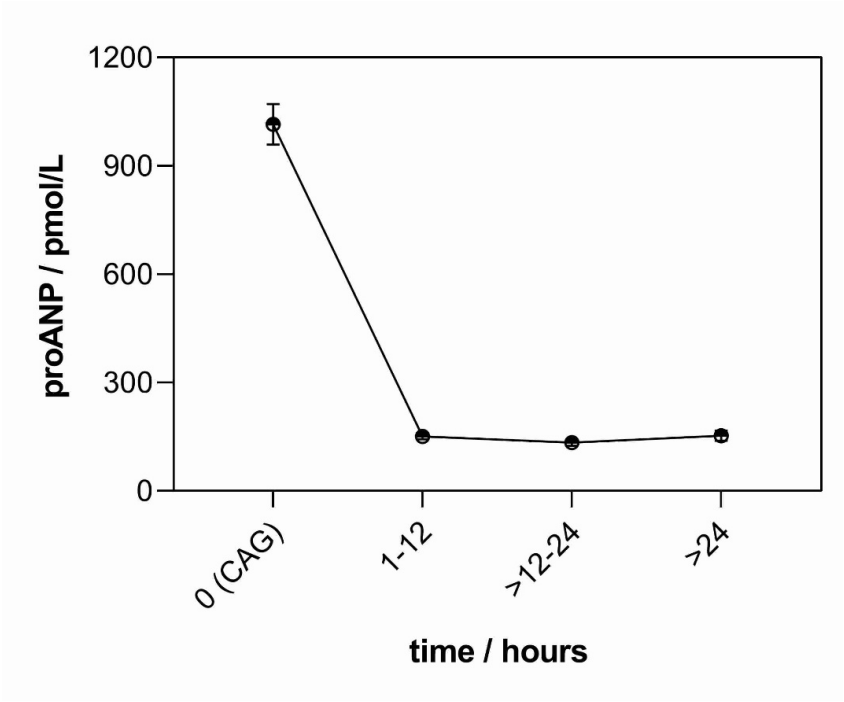
Supplemental Table 3: Number of patients and plasma samples in longitudinal analyses.

	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)

1

2

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.

References

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