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Biomarkers

Proenkephalin and Prognosis After Acute Myocardial Infarction

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Objectives The goal of this research was to assess the prognostic value of proenkephalin (PENK) levels in acute myocardial infarction (AMI) by using N-terminal pro–B-type natriuretic peptide and Global Registry of Acute Coronary Events (GRACE) scores as comparators and to identify levels that might be valuable in clinical decision making.

Background PENK is a stable analyte of labile enkephalins. Few biomarkers predict recurrent AMI.

Methods We measured PENK in 1,141 patients (820 male subjects; mean age 66.2 ± 12.8 years) with AMI. Endpoints were major adverse events (composite of death, myocardial infarction [MI], and heart failure [HF] hospitalization) and recurrent MI at 2 years. GRACE scoring was used for comparisons with PENK for the death and/or MI endpoint at 6 months.

Results During follow-up, 139 patients died, and there were 112 HF hospitalizations and 149 recurrent AMIs. PENK levels were highest on admission and were related to estimated glomerular filtration rate, left ventricular wall motion index, sex, blood pressure, and age. Multivariable Cox regression models found that the PENK level was a predictor of major adverse events (hazard ratio [HR]: 1.52 [95% confidence interval (CI): 1.19 to 1.94]), death and/or AMI (HR: 1.76 [95% CI: 1.34 to 2.30]), and death and/or HF (HR: 1.67 [95% CI: 1.24 to 2.25]) (all comparisons $p < 0.001$), as well as recurrent AMI (HR: 1.43 [95% CI: 1.07 to 1.91]; $p < 0.01$). PENK levels were independent predictors of 6-month death and/or MI compared with GRACE scores. PENK-adjusted GRACE scores reclassified patients significantly (overall category-free net reclassification improvement [>0] of 21.9 [95% CI: 4.5 to 39.4]; $p < 0.014$). PENK levels <48.3 pmol/l and >91 pmol/l detected low- and high-risk patients, respectively.

Conclusions PENK levels reflect cardiorenal status post-AMI and are prognostic for death, recurrent AMI, or HF. Cutoff values define low- and high-risk groups and improve risk prediction of GRACE scores. (J Am Coll Cardiol 2014;63:280–9)

Although the endogenous opioid systems (enkephalins, endorphins, and dynorphins) have been well described in analgesia, recent evidence suggests a role in cardiovascular regulation (1). The distribution of preproenkephalin A and proenkephalin (PENK) is widespread, including in the nervous system, adrenal medulla, and immune system, and enkephalins are coreleased from nerve terminals with catecholamines. In the heart, enkephalins are secreted by both myocytes (2) and nonmyocytes (3), and they may have an autocrine/paracrine effect, mainly on delta receptors.

Activation of opioid receptors (OPRs) have a predominantly depressor effect (causing hypotension and bradycardia) via central and peripheral mu and delta receptors, as well as inhibition of norepinephrine release and sympathetic vasoconstriction (4). OPR activation attenuates the beta-adrenergic receptor-mediated positive inotropic effect and the increase in cyclic adenosine monophosphate, but also has an independent, direct, negatively inotropic effect (5). Administration of a delta OPR antagonist in dogs with experimental heart failure (HF) increased blood pressure, cardiac output, and blood flow to kidney, heart, splanchnic

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bed, and skeletal muscle (6). The density of delta and other OPRs, although widely distributed, is highest in the kidney (7).

Conversely, delta receptors have also been implicated in ischemic pre-conditioning, although effects may be dependent on dose and duration of ischemia (8,9). Met-enkephalin is also a ligand for the widely distributed opioid growth (or zeta) receptor, which maintains a tonic inhibitory effect on cell proliferation via cyclin-dependent inhibitory kinase (p16, p21) pathways (10) and can inhibit ventricular deoxyribonucleic acid synthesis (11). Manipulation of PENK levels may affect apoptosis, and PENK associates with histone deacetylase in a transcriptional repression complex that controls pro-apoptosis (12).

Acute stress such as that seen in acute myocardial infarction (AMI) (13) activates a number of neurohormones, including the PENK and vasopressin systems. Although previous studies on met-enkephalin in AMI demonstrated no change over 4 days (14), interpretation may have been hampered by the short half-life of met-enkephalin. Recently, an assay for stable PENK was developed (15), and we have investigated the utility of this marker compared with existing risk stratification techniques (N-terminal pro–B-type natriuretic peptide [NT-proBNP] and risk scores) in AMI. Biomarkers such as NT-proBNP (16) show greatest association with risk of death and HF post-AMI but are less useful for predicting the endpoint of readmission with AMI (recurrent [re-]AMI), justifying a need for improved prediction of this endpoint.

Methods

Study population. We studied 1,141 ST-segment elevation myocardial infarction and non–ST-segment elevation myocardial infarction (NSTEMI) patients admitted to University Hospitals of Leicester NHS Trust between August 2004 and April 2007, predominantly on weekdays (9 AM to 4 PM). NSTEMI patients were recruited when a positive troponin result was observed. This observational cohort study complied with the Declaration of Helsinki and was approved by the local ethics committee; written informed consent was obtained from patients.

AMI was diagnosed if a patient had a cardiac troponin I level above the 99th percentile with at least 1 of the following: chest pain lasting >20 min or diagnostic serial electrocardiographic changes consisting of new pathological Q waves or ST-segment and T-wave changes (17). Patients with known malignancy, renal replacement therapy, or surgery in the previous month were excluded. Estimated glomerular filtration rate (eGFR) was calculated from the simplified Modification of Diet in Renal Disease formula (18). All patients received standard medical treatment and revascularization at the discretion of the attending physician.

Plasma samples. Blood samples (anticoagulated with ethylenediaminetetraacetic acid and aprotinin) were drawn after 15 min of bed rest, immediately after diagnosis, and within 36 h of symptom onset (mean ± SEM 20.75 ± 0.35 h). Plasma was stored at −80°C until assayed in a single batch for blinded determination of plasma PENK and NT-proBNP.

Echocardiography. Transthoracic echocardiography was performed in 895 (78.4%) patients during the index admission, using either a Sonos 5500 or IE 33 instrument (Philips Medical Systems, Reigate, United Kingdom). A 16-segment left ventricular wall motion index score was performed based on the American Society of Echocardiography method (19). In suitable patients, left ventricular ejection fraction was calculated using the biplane method of discs formula. Left ventricular systolic dysfunction (LVSD) was defined as either a left ventricular ejection fraction <40% or a left ventricular wall motion index >1.8.

GRACE scoring. Based on an international observational database of patients with acute coronary syndromes, Global Registry of Acute Coronary Events (GRACE) scores can be calculated on initial presentation to predict in-hospital mortality (20) or for 6-month major adverse cardiac events (MACE), defined as death and/or re–myocardial infarction (MI) (21). We used GRACE scores on discharge for comparison with 6-month death and/or re-AMI.

Biomarker assays. The Centaur cTnI Ultra immunoassay (Siemens Healthcare Diagnostics, Deerfield, Illinois) was used to measure troponin I, which has a coefficient of variation of 10% at 0.03 μg/l with a 99th percentile of 0.04 μg/l. The NT-proBNP assay was based on a noncompetitive assay, as previously published (16). An assay for stable PENK (amino acids 119 to 159 of proenkephalin A) has been previously reported in detail (15) and was modified as follows: in brief, 2 mouse monoclonal anti-PENK antibodies were developed by immunization with PENK peptide (amino acids 119 to 159 of proenkephalin A). One antibody (2 μg) was used to coat polystyrene tubes. The other antibody labeled with methylacridinium ester served as the detector antibody. Standards (PENK peptide; amino acids 119 to 159 of proenkephalin A) and samples (50 μl) were incubated in tubes with the detector antibody (150 μl). After equilibration, the tubes were washed, and bound chemiluminescence was detected with a luminometer (LB952T/16, Berthold Technologies GmbH & Co., Wildbad, Germany). The lower detection limit of the immunoassay was 5.5 pmol/l. Intra-assay and interassay coefficients of variation were 6.4% and 9.5% at 50 pmol/l, and 4.0% and 6.5% at 150 pmol/l, respectively. The mean ±

Abbreviations and Acronyms

AMI = acute myocardial infarction
eGFR = estimated glomerular filtration rate
HF = heart failure
MACE = major adverse cardiac event(s)
LVSD = left ventricular systolic dysfunction
MI = myocardial infarction
NRI = net reclassification improvement
NSTEMI = non–ST-segment elevation myocardial infarction
NT-proBNP = N-terminal pro–B-type natriuretic peptide
OPR = opioid receptor
PENK = proenkephalin
re- = recurrent

SEM normal range was 46.6 ± 0.21 pmol/l, with a median of 45 (range: 9 to 518) pmol/l.

Endpoints. The primary composite endpoint was MACE, including all-cause mortality, HF hospitalization, or re-AMI, which were evaluated within 2 years. Hospitalization for HF was defined as a hospital readmission for which HF was the primary reason requiring treatment with high-dose diuretics, inotropes, or intravenous nitrate. Recurrent AMI was diagnosed using the universal definition (17). Secondary endpoints were composites of death and/or re-AMI and death and/or HF readmission, and re-AMI individually. The endpoint of death and/or re-AMI at 6 months was used in analyses involving the GRACE score because this time point was used in development of the risk score. Endpoints were obtained by reviewing the local hospital databases and the Office of National Statistics Registry and by telephone calls to patients, and these data were verified by reviewing medical records. We achieved 100% follow-up.

Statistical analysis. Statistical analyses were performed using SPSS version 20 (IBM SPSS Statistics, IBM Corporation, Armonk, New York) and Stata version 12.1 (Stata Corp., College Station, Texas). Assuming an event rate of 15% and that the covariates predict up to 30% of the variance of the biomarker, a sample size of 609 patients would be powered (90% at $p < 0.05$) to detect a hazard ratio (HR) of the biomarker of 1.5, using the command stpower cox in Stata 12.1. All biomarker levels were $\log_{10}$ transformed. HRs for these refer to 1 SD increment of the $\log_{10}$ transformed biomarker. GRACE scores were used as the

Table 1 Characteristics of the 1,141 AMI Patients According to PENK Quartiles on Admission

		PENK Quartiles				
	All (N = 1,141)	1 (<39.9 pmol/l; n = 285)	2 (40.0–55.5 pmol/l; n = 286)	3 (55.6–83.2 pmol/l; n = 285)	4 (>83.3 pmol/l; n = 285)	p Value
PENK (pmol/l)	71.1 ± 52.9	30.9 ± 6.1	47.3 ± 4.3	66.9 ± 7.8	139.1 ± 65.6	<0.0005
NT-proBNP (pmol/l)	1,850 ± 2,109	998 ± 1,162	1,268 ± 1,642	1,713 ± 1,822	3,419 ± 2,629	<0.0005
Demographics						
Age (yrs)	66.2 ± 12.8	60.0 ± 10.6	61.8 ± 11.6	67.6 ± 12.4	75.5 ± 10.4	<0.0005
Male	820 (72)	243 (85)	229 (80)	202 (71)	145 (51)	<0.001
STEMI	548 (48)	148 (52)	140 (49)	134 (47)	120 (42)	NS
History						
MI	251 (22.0)	40 (14)	51 (18)	63 (22)	97 (34)	<0.001
Angina pectoris	228 (20)	34 (12)	43 (15)	69 (24)	77 (27)	<0.001
Heart failure	46 (4)	3 (1)	9 (3)	6 (2)	23 (8)	<0.001
Hypertension	592 (52)	116 (41)	140 (49)	151 (53)	185 (65)	<0.001
Diabetes mellitus	262 (23)	51 (18)	66 (23)	63 (22)	86 (30)	<0.007
Killip class >1	419 (40)	60 (26)	78 (29)	119 (43)	167 (61)	<0.001
Glucose (mmol/l)	8.9 ± 4.2	8.5 ± 3.8	8.6 ± 3.6	8.1 ± 3.3	10.3 ± 5.7	<0.0005
Troponin I (μg/l)	13.1 ± 25.8	12.9 ± 25.4	13.1 ± 26.2	14.6 ± 28.5	11.7 ± 22.9	NS
eGFR (ml/min/1.73 m^2)	65.6 ± 20.1	76.7 ± 15.7	73.3 ± 17.4	65.2 ± 16.4	47.4 ± 16.9	<0.0005
Risk markers on discharge						
Echocardiographic LVSD (n = 893)						
LV wall motion index	1.47 ± 0.42	1.41 ± 0.40	1.40 ± 0.39	1.49 ± 0.41	1.58 ± 0.44	<0.0005
LV ejection fraction	42.1 ± 14.5	45.5 ± 12.5	41.7 ± 14.8	42.2 ± 14.3	38.8 ± 15.7	<0.0005
GRACE score	120 ± 33	103 ± 26	108 ± 28	122 ± 30	144 ± 30	<0.0005
Treatment						
Aspirin	957 (84)	255 (90)	243 (85)	228 (80)	228 (80)	<0.002
Beta-blocker	911 (80)	246 (87)	237 (83)	223 (78)	207 (73)	<0.001
ACE inhibitor or ARB	934 (82)	246 (87)	243 (85)	235 (82)	210 (74)	<0.001
Statin	992 (87)	266 (94)	263 (92)	252 (88)	214 (75)	<0.001
Revascularization	306 (27)	77 (27)	87 (30)	77 (27)	65 (27)	NS
Endpoints (2 yrs)						
MACE	323 (28)	36 (13)	51 (18)	81 (28)	155 (54)	<0.001
Death	139 (12)	9 (3)	11 (4)	28 (10)	91 (32)	<0.001
Nonfatal MACE	230 (20)	32 (11)	45 (16)	61 (21)	92 (32)	<0.001
Heart failure	112 (18)	12 (4)	18 (6)	30 (10)	52 (18)	<0.001
Re-AMI	149 (13)	21 (7)	32 (11)	42 (15)	54 (19)	<0.001

Values are mean ± SD or n (%). p values are quoted for the Kruskal-Wallis or chi-square tests for continuous or categorical variables, respectively.

ACE = angiotensin-converting enzyme; AMI = acute myocardial infarction; ARB = angiotensin 2 receptor blocker; eGFR = estimated glomerular filtration rate; GRACE = Global Registry of Acute Coronary Events; LV = left ventricular; LVSD = left ventricular systolic dysfunction; MACE = major adverse cardiac events; MI = myocardial infarction; NT-proBNP = N-terminal pro–B-type natriuretic peptide; PENK = proenkephalin; Re- = recurrent; STEMI = ST-segment elevation myocardial infarction.

Table 2 Univariate General Linear Model Showing Independent Predictors of PENK Levels

	F Statistic	p Value
eGFR	151.195	0.000
Age	16.504	0.000
Wall motion score index	11.399	0.001
Female	11.125	0.001
Diastolic blood pressure	7.048	0.008
History of diabetes	3.545	NS
History of IHD	2.058	NS
Heart rate	.146	NS
History of hypertension	.005	NS
Adjusted R2	0.41	<0.0005

IHD = ischemic heart disease; other abbreviations as in Table 1.

original scores. In comparisons of continuous variables between PENK quartiles, the Kruskal-Wallis test was used because the data were not normally distributed. Chi-square tests were used for categorical variables. Independent predictors of PENK levels were assessed using univariate general linear models. To assess the prognostic value of the biomarkers, a base model was generated using Cox survival analysis, which included variables that were significantly ($p < 0.10$) associated with any of the study endpoints on univariable analysis (age, sex, history of ischemic heart disease, hypertension or diabetes, Killip class, eGFR, echocardiographic evidence of systolic dysfunction, therapies including in-hospital revascularization, and biomarkers [log troponin I and log NT-proBNP]). PENK was added to this base model to evaluate its relative prognostic value, with all variables entered simultaneously. A second comparative Cox model was used to assess the relative prognostic power of these biomarkers and the GRACE score. The additional prognostic value of PENK to the GRACE score was evaluated by using reclassification analysis with calculation of category-free net reclassification improvement (NRI) as described by Pencina et al. (22). We constructed classification trees by using chi-square Automatic Interaction Detection (analysis performed by using SPSS), which chooses at each step the biomarker that has the strongest interaction with the dependent variable.

Results

Patient characteristics. The characteristics of the study population according to PENK quartiles are shown in Table 1. Patients with higher PENK levels were older; were female; had a history of hypertension, ischemic heart disease, diabetes, and HF; and had higher GRACE scores and NT-proBNP levels. They also had more impaired cardiac and renal function. Revascularization frequencies were similar between PENK quartiles.

Correlation analysis. Spearman analysis (r_s) revealed that PENK was significantly correlated with age (0.488), eGFR (−0.583), heart rate (0.108), diastolic blood pressure (−0.196), glucose (0.107), NT-proBNP (0.406), and ejection fraction (−0.173) (all $p < 0.0005$). PENK was not correlated with troponin or peak creatine kinase levels and was weakly correlated with time from symptom onset ($r_s = -0.068$, $p = 0.023$).

A univariate general linear model indicated the following independent predictors of PENK level, in descending order according to variance accounted for in the model (Table 2): eGFR, age, left ventricular wall motion score, female sex,

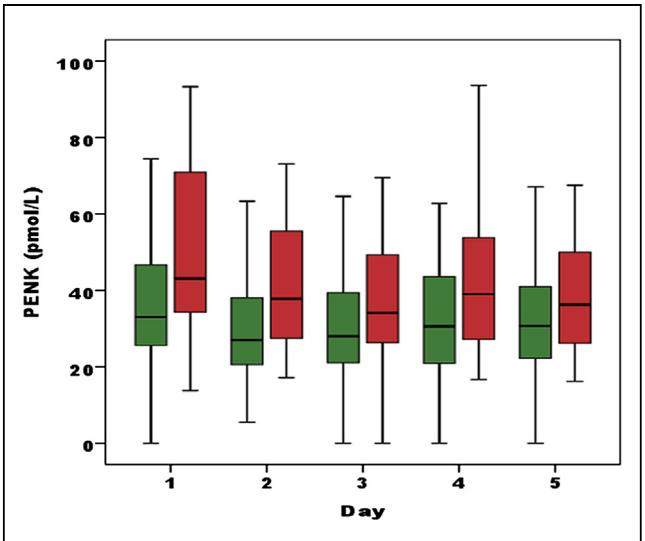

Figure 1 Profile of Plasma PENK During the 5 Days After AMI

Values are given for those with major adverse cardiac events (MACE) **(red)** or without MACE **(green)** at 2 years. **Boxes** represent interquartile ranges, with the median marked. **Whiskers** represent 1.5 times the interquartile range. AMI = acute myocardial infarction; PENK = proenkephalin.

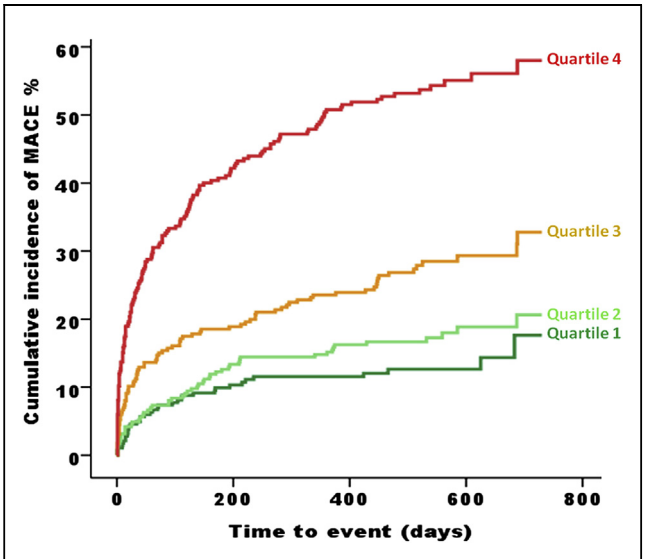

Figure 2 Cumulative Incidence of MACE According to PENK Quartiles

Cumulative incidence plot of MACE according to PENK quartiles. Abbreviations as in Figure 1.

Table 3 Cox Regression Analysis for MACE at 2 Years Post-AMI

	Univariable	p Value	Multivariable Model 1	p Value	Multivariable Model 2	p Value
Age (yrs)	1.05 (1.04–1.06)	0.000	1.04 (1.02–1.06)	0.001	1.03 (1.01–1.05)	0.001
Male	0.63 (0.50–0.79)	0.000	1.08 (0.74–1.58)	NS	1.15 (0.79–1.69)	NS
ST-segment elevation	1.09 (0.88–1.36)	NS	1.91 (1.25–2.91)	0.003	1.73 (1.13–2.65)	0.012
Killip class >1	2.68 (2.12–3.37)	0.000	1.56 (1.07–2.28)	0.022	1.40 (0.95–2.06)	NS
eGFR (ml/min/1.73 m^2)	0.97 (0.96–0.98)	0.000	0.99 (0.98–1.00)	NS	1.00 (0.99–1.01)	NS
Heart rate (beats/min)	1.01 (1.01–1.01)	0.000	0.99 (0.99–1.01)	NS	0.99 (0.99–1.00)	NS
SBP (mm Hg)	0.99 (0.99–1.00)	0.05	0.99 (0.99–1.00)	NS	0.99 (0.99–1.00)	NS
LVSD (echo)	2.24 (1.74–2.88)	0.000	1.44 (1.00–2.07)	0.048	1.50 (1.04–2.16)	0.028
History						
Ischemic heart disease	1.52 (1.22–1.90)	0.000	1.02 (0.69–1.48)	NS	0.97 (0.66–1.42)	NS
Hypertension	1.64 (1.31–2.05)	0.000	0.87 (0.59–1.27)	NS	0.88 (0.60–1.29)	NS
Diabetes	1.55 (1.22–1.96)	0.000	1.36 (0.93–1.99)	NS	1.31 (0.89–1.92)	NS
Treatment						
Revascularization	1.44 (1.14–1.82)	0.002	1.89 (1.25–2.84)	0.002	1.91 (1.27–2.88)	0.002
Aspirin	0.57 (0.44–0.75)	0.000	0.78 (0.53–1.16)	NS	0.70 (0.47–1.05)	NS
Beta-blockers	0.52 (0.41–0.66)	0.000	0.98 (0.65–1.47)	NS	0.89 (0.59–1.35)	NS
ACE inhibitor/ARB	0.55 (0.43–0.71)	0.000	0.63 (0.41–0.98)	0.040	0.70 (0.45–1.09)	NS
Statins	0.40 (0.31–0.52)	0.000	0.78 (0.47–1.28)	NS	0.86 (0.52–1.43)	NS
Diuretics	2.36 (1.89–2.94)	0.000	2.15 (1.48–3.13)	0.000	2.04 (1.41–2.96)	0.000
Biomarkers						
Log troponin (μg/l)	1.11 (0.99–1.26)	NS	1.10 (0.85–1.42)	NS	1.11 (0.92–1.35)	NS
Log NTproBNP (pmol/l)	1.92 (1.64–2.24)	0.000	1.14 (0.94–1.38)	NS	1.04 (0.80–1.34)	NS
Log PENK (pmol/l)	1.98 (1.80–2.19)	0.000			1.52 (1.19–1.94)	0.001
C statistic			0.795 (0.755–0.834)		0.810 (0.772–0.847)	0.031
PENK quartiles						
1	Reference	0.000			Reference	0.000
2	1.35 (0.88–2.07)	NS			1.22 (0.75–1.99)	NS
3	2.28 (1.54–3.38)	0.000			1.66 (1.05–2.64)	0.032
4	5.44 (3.78–7.82)	0.000			2.78 (1.71–4.51)	0.000

Values are hazard ratio (95% confidence interval). Multivariable analysis results are reported for model 1, which included variables and biomarkers (except PENK) that were significant on univariable analysis. Multivariable model 2 used the variables in model 1 with the addition of PENK as a continuous variable. Hazard ratios for PENK entered as quartiles are reported at the bottom of the table.

NT-proBNP = N-terminal pro–B-type natriuretic peptide; SBP = systolic blood pressure; other abbreviations as in Table 1.

and diastolic blood pressure. eGFR accounted for the majority of the variance (27%).

Day curves for PENK. Sequential plasma samples for 5 days were available for 107 patients, 28 of whom had MACE within 2 years. Figure 1 demonstrates the plasma profile along with a general linear model with repeated measures that shows significant changes in PENK over time ($p < 0.001$) and higher levels in those with MACE ($p < 0.01$). In post-hoc testing, PENK levels on day 1 were higher than all other days ($p < 0.001$, Bonferroni-corrected for multiple comparisons). PENK levels on days 2 to 5 were similar. There was no statistically significant interaction of the changes of PENK with time and MACE.

Survival analysis. During a 2-year follow-up, there were 139 deaths, 112 HF hospitalizations, and 149 re-AMIs. Patients with elevated PENK levels (log_{10} transformed and expressed as a continuous variable) had more MACE, deaths, and rehospitalizations with HF or re-AMI (Table 1). Figure 2 illustrates the cumulative incidence of MACE according to PENK quartiles ($p < 0.0005$). Table 3 reports the univariable HRs of various factors that affected the outcome of MACE at 2 years. In multivariable analysis for predicting MACE at 2 years, the base model (multivariable model 1 in Table 3) included the following independent predictors: age, ST-segment elevation on electrocardiography, Killip class >1, LVSD, revascularization, and treatment with angiotensin-converting enzyme inhibitors/angiotensin receptor blockers and diuretics. Addition of PENK to the base model (model 2 in Table 3) retained the following predictors: age, ST-segment elevation on electrocardiography, LVSD, revascularization, treatment with diuretics, and log PENK (HR: 1.52 [95% confidence interval (CI): 1.19 to 1.94]; $p < 0.001$). The C statistic improved significantly from 0.795 to 0.810 ($p = 0.031$) (Table 3). Tests for multicollinearity revealed variance inflation factors of <2.1 for all variables, suggesting that multicollinearity did not affect the analysis.

Entering PENK as quartiles to the MACE multivariable model (Table 3) revealed that the top PENK quartile had an HR of 2.78 (95% CI: 1.71 to 4.51) compared with the lowest quartile ($p < 0.0005$).

Category-free reclassification analysis was used as described by Pencina et al. (22) to calculate the NRI (>0) so that no arbitrary cutoff probabilities are chosen for analysis. The models were well calibrated as indicated by nonsignificant Hosmer-Lemeshow goodness-of-fit tests

Table 4 Cox Regression Analysis for Death and/or MI at 2 Years Post-AMI

	Univariable	p Value	Multivariable Model 1	p Value	Multivariable Model 2	p Value
Age (yrs)	1.05 (1.04–1.06)	0.000	1.04 (1.02–1.07)	0.000	1.04 (1.02–1.06)	0.000
Male	0.66 (0.51–0.85)	0.001	1.08 (0.70–1.65)	NS	1.15 (0.74–1.78)	NS
ST-segment elevation	1.04 (0.81–1.32)	NS	1.47 (0.90–2.38)	NS	1.35 (0.83–2.19)	NS
Killip class >1	2.09 (1.62–2.70)	0.000	1.24 (0.81–1.91)	NS	1.07 (0.69–1.66)	NS
eGFR (ml/min/1.73 m^2)	0.97 (0.96–0.98)	0.000	0.99 (0.98–1.01)	NS	1.00 (0.99–1.02)	NS
Heart rate (beats/min)	1.01 (1.00–1.01)	0.001	1.00 (0.99–1.01)	NS	1.00 (0.99–1.01)	NS
SBP (mm Hg)	0.99 (0.99–1.00)	0.050	1.00 (0.99–1.01)	NS	1.00 (0.99–1.01)	NS
LVSD (echo)	2.14 (1.61–2.83)	0.000	1.28 (0.85–1.91)	NS	1.35 (0.90–2.02)	NS
History						
Ischemic heart disease	1.60 (1.26–2.05)	0.000	1.27 (0.84–1.94)	NS	1.19 (0.78–1.83)	NS
Hypertension	1.55 (1.20–1.99)	0.001	0.88 (0.57–1.34)	NS	0.89 (0.58–1.37)	NS
Diabetes	1.54 (1.18–1.99)	0.001	1.41 (0.93–2.15)	NS	1.34 (0.88–2.05)	NS
Treatment						
Revascularization	1.65 (1.28–2.13)	0.000	2.09 (1.35–3.25)	0.001	2.19 (1.41–3.40)	0.001
Aspirin	0.64 (0.48–0.87)	0.004	0.96 (0.60–1.52)	NS	0.87 (0.54–1.39)	NS
Beta-blockers	0.59 (0.45–0.77)	0.000	1.12 (0.71–1.79)	NS	0.99 (0.62–1.57)	NS
ACE inhibitor/ARB	0.49 (0.37–0.64)	0.000	0.70 (0.43–1.14)	NS	0.80 (0.49–1.31)	NS
Statins	0.38 (0.29–0.51)	0.000	0.65 (0.38–1.13)	NS	0.72 (0.42–1.25)	NS
Diuretics	1.84 (1.43–2.36)	0.000	1.61 (1.05–2.46)	0.028	1.48 (0.97–2.26)	NS
Biomarkers						
Log troponin (μg/l)	1.05 (0.92–1.21)	NS	1.15 (0.92–1.44)	NS	1.11 (0.89–1.38)	NS
Log NTproBNP (pmol/l)	1.82 (1.53–2.16)	0.000	1.02 (0.77–1.35)	NS	0.94 (0.71–1.24)	NS
Log PENK (pmol/l)	1.94 (1.74-2.16)	0.000			1.76 (1.34–2.30)	0.000
C statistic			0.788 (0.748–0.828)		0.802 (0.763–0.840)	0.11
PENK quartiles						
1	Reference				Reference	
2	1.40 (0.86–2.27)	NS			1.31 (0.62–2.74)	NS
3	2.35 (1.50–3.67)	0.000			1.62 (0.80–3.28)	NS
4	5.46 (3.61–8.28)	0.000			2.50 (1.17–5.36)	0.01

Values are hazard ratio (95% confidence interval). Multivariable analysis results are reported for model 1, which included variables and biomarkers (except PENK) that were significant on univariable analysis. Multivariable model 2 used the variables in model 1 with the addition of PENK. Hazard ratios for PENK entered as quartiles are reported at the bottom of the table.

Abbreviations as in Tables 1 and 3.

(while acknowledging the limitations of this test) and visualization of the calibration plots. The NRI in those without the MACE endpoint was 13.0 (95% CI: 4.8 to 21.2; $p = 0.002$), indicating that 13% of those without MACE who were deemed high risk in model 1 had been down-classified after addition of PENK to the model. In those with the endpoint, NRI was 18.1 (95% CI: 5.1 to 31.1; $p = 0.007$), indicating that 18.1% of those with MACE who were deemed low risk in model 1 had been up-classified after addition of PENK to the model. Overall NRI (>0) was 31.1 (95% CI: 15.7 to 46.4; $p < 0.0005$), suggesting that PENK improved the risk stratification of the base model.

In other models for prediction of the secondary composite endpoints of death and/or re-AMI (Table 4) and death and/or HF readmission (Table 5), PENK remained an independent predictor (death/MI HR: 1.76 [95% CI: 1.34 to 2.30]; death/HF HR: 1.67 [95% CI: 1.24 to 2.25]; $p < 0.001$ for both) with corresponding increases in C statistic (to 0.802 [$p = 0.11$] and 0.799 ($p = 0.041$], respectively). The HR of the top quartile of PENK was significantly higher than the lowest reference quartile for both composite endpoints.

Table 6 reports the univariable and multivariable HRs for the endpoint of re-AMI at 2 years. The base model (model 1 in Table 6) included the following significant predictors: age, ST-segment elevation, revascularization, and diuretic treatment. Addition of PENK to this model revealed PENK as a predictor (HR: 1.43 [95% CI: 1.07 to 1.91]; $p < 0.01$) together with ST-segment elevation and revascularization. The C statistic improved from 0.712 to 0.735 ($p = 0.004$) after the addition of PENK.

Comparison with GRACE scores. The widely used GRACE risk score (21) was originally derived for prediction of death and/or MI at 6 months. GRACE scores and the biomarkers NT-proBNP and PENK were predictors of MACE, death and/or MI, and death and/or HF in univariable analysis (Table 7). In multivariable analysis for MACE and death and/or MI at 6 months, GRACE score and PENK remained predictors, whereas NT-proBNP was only retained for the death and/or HF model.

For the 6-month death/MI endpoint, the C statistic only increased from 0.693 (95% CI: 0.647 to 0.739) for GRACE scoring to 0.734 (95% CI: 0.691 to 0.778) with the addition of PENK ($p < 0.0047$). No further improvement in the area under the curve was observed with

Table 5 Cox Regression Analysis for Death and/or Heart Failure at 2 Years Post-AMI

	Univariable	p Value	Multivariable Model 1	p Value	Multivariable Model 2	p Value
Age (yrs)	1.07 (1.06–1.09)	0.000	1.03 (1.00–1.06)	0.032	1.03 (1.00–1.05)	0.050
Male	0.51 (0.39–0.66)	0.000	1.19 (0.76–1.89)	NS	1.27 (0.79–2.01)	NS
ST-segment elevation	0.99 (0.77–1.30)	NS	1.33 (0.79–2.22)	NS	1.21 (0.72–2.03)	NS
Killip class >1	3.78 (2.81–5.08)	0.000	2.02 (1.24–3.30)	0.005	1.75 (1.06–2.87)	0.027
eGFR (ml/min/1.73 m^2)	0.96 (0.95–0.97)	0.000	0.98 (0.97–0.99)	0.003	0.99 (0.98–1.01)	NS
Heart rate (beats/min)	1.01 (1.01–1.02)	0.000	1.00 (0.99–1.00)	NS	1.00 (0.99–1.00)	NS
SBP (mm Hg)	0.99 (0.98–0.99)	0.004	0.99 (0.98–0.99)	0.010	0.99 (0.98–0.99)	0.006
LVSD (echo)	3.07 (2.25–4.18)	0.000	1.62 (1.04–2.54)	0.033	1.73 (1.10–2.71)	0.017
History						
Ischemic heart disease	1.57 (1.21–2.04)	0.001	0.94 (0.60–1.49)	NS	0.88 (0.56–1.40)	NS
Hypertension	1.69 (1.29–2.22)	0.000	0.73 (0.46–1.16)	NS	0.74 (0.47–1.18)	NS
Diabetes	1.57 (1.19–2.09)	0.002	1.33 (0.84–2.10)	NS	1.25 (0.79–1.97)	NS
Treatment						
Revascularization	1.02 (0.76–1.36)	NS	1.28 (0.75–2.19)	NS	1.31 (0.76–2.26)	NS
Aspirin	0.50 (0.37–0.68)	0.000	0.69 (0.43–1.10)	NS	0.61 (0.38–0.99)	0.044
Beta-blockers	0.36 (0.27–0.47)	0.000	0.81 (0.51–1.29)	NS	0.74 (0.46–1.19)	NS
ACE inhibitor/ARB	0.47 (0.36–0.63)	0.000	0.70 (0.42–1.17)	NS	0.79 (0.47–1.32)	NS
Statins	0.28 (0.21–0.38)	0.000	0.66 (0.38–1.16)	NS	0.73 (0.41–1.29)	NS
Diuretics	3.17 (2.43–4.11)	0.000	2.32 (1.50–3.60)	0.000	2.24 (1.44–3.47)	0.000
Biomarkers						
Log troponin (μg/l)	1.15 (0.99–1.33)	0.051	1.13 (0.89–1.43)	NS	1.11 (0.87–1.40)	NS
Log NT-proBNP (pmol/l)	3.20 (2.56–4.00)	0.000	1.49 (1.04–2.15)	0.032	1.40 (0.97–2.01)	NS
Log PENK (pmol/l)	2.35 (2.09–2.64)	0.000			1.67 (1.24–2.25)	0.001
C statistic			0.785 (0.745–0.826)		0.799 (0.760–0.838)	0.041
PENK quartiles						
1	Reference				Reference	
2	1.24 (0.69–2.26)	NS			1.22 (0.45–3.18)	NS
3	2.70 (1.60–4.57)	0.000			1.66 (0.68–3.94)	NS
4	7.98 (4.93–12.92)	0.000			2.51 (1.01–6.30)	0.05

Values are hazard ratio (95% confidence interval). Multivariable analysis results are reported for model 1, which included variables and biomarkers (except PENK) that were significant on univariable analysis. Multivariable model 2 used the variables in model 1 with the addition of PENK. Hazard ratios for PENK entered as quartiles are reported at the bottom of the table.

Abbreviations as in Tables 1 and 3.

the addition of NT-proBNP (0.734, 95% CI: 0.691 to 0.778; p = NS).

Figure 3 shows the reclassification plot as proposed by Steyerberg et al. (23), with probabilities of events by using GRACE scoring alone plotted against probabilities of events by using PENK-adjusted GRACE scores. Probabilities adjusted by using PENK in those with adverse outcomes were up-classified (i.e., above the diagonal in Fig. 2). Hosmer-Lemeshow goodness-of-fit tests indicated good calibration of models. Category-free reclassification analysis demonstrated that the NRI (>0) in those without the endpoint of death and/or MI at 6 months was −1.5 (95% CI: −8.3 to 5.2; p = NS) and in those with the endpoint, it was 23.5 (95% CI: 7.4 to 39.5; p < 0.004), with an overall NRI (>0) of 21.9 (95% CI: 4.5 to 39.4; p < 0.014). These findings suggest that PENK improved the risk stratification from GRACE scoring (predominantly from up-classifying risk in those with death/MI).

For the endpoint of MACE at 6 months, the NRI in those without MACE was 1.0 (95% CI: −5.9 to 7.9; p = NS) and in those with the endpoint, it was 26.0 (95% CI: 11.9 to 40.2; p < 0.000). The overall NRI (>0) was 27.0 (95% CI: 11.3 to 42.8; p < 0.001).

Decision tree analysis. To determine optimal cutoff points for biomarkers, we constructed a decision tree (by using PENK and NT-proBNP levels and GRACE scores) to classify patients into survivors or those with endpoints. For the endpoint of death and/or MI at 6 months, PENK was selected as the optimal initial classifier (Fig. 4), with NT-proBNP used at another node. For those with PENK <48.3 pmol/l, a low-risk group (n = 456 [40% of the cohort]) was defined with 30 deaths and/or MI events at 6 months. At 30 days, this low-risk group had 12 events, including 2 deaths. Patients with PENK >91 pmol/l were at high risk of death and/or MI (18.9% at 30 days, 34.2% at 6 months).

Discussion

In this observational cohort study, we describe the use of a novel PENK assay for risk stratification after AMI, measuring an analyte that is stable in plasma, unlike previous assays of labile enkephalins. Analysis of variance suggests that the major independent factors which influenced PENK levels are renal function (eGFR), age, cardiac function (left ventricular wall motion score index), sex, and diastolic blood

Table 6 Cox Regression Analysis for Re-MI at 2 Years Post-AMI

	Univariable	p Value	Multivariable Model 1	p Value	Multivariable Model 2	p Value
Age (yrs)	1.02 (1.01–1.03)	0.004	1.03 (1.00–1.05)	0.035	1.02 (1.00–1.05)	NS
Male	0.85 (0.60–1.21)	NS				
ST-segment elevation	1.15 (0.83–1.58)	NS	2.04 (1.23–3.39)	0.006	1.96 (1.19–3.25)	0.009
Killip class >1	1.64 (1.18–2.27)	0.003	0.92 (0.57–1.48)	NS	0.83 (0.51–1.36)	NS
eGFR (ml/min/1.73 m^2)	0.99 (0.98–0.99)	0.016	1.00 (0.99–1.02)	NS	1.00 (0.99–1.02)	NS
Heart rate (beats/min)	1.00 (0.99–1.01)	NS				
SBP (mm Hg)	1.00 (0.99–1.01)	NS				
LVSD (echo)	1.60 (1.12–2.29)	0.010	1.48 (0.94–2.31)	NS	1.50 (0.96–2.35)	NS
History						
Ischemic heart disease	1.73 (1.25–2.39)	0.001	1.33 (0.82–2.13)	NS	1.29 (0.80–2.08)	NS
Hypertension	1.56 (1.12–2.18)	0.008	1.17 (0.73–1.85)	NS	1.15 (0.72–1.83)	NS
Diabetes	1.60 (1.13–2.26)	0.008	1.18 (0.73–1.90)	NS	1.18 (0.73–1.90)	NS
Treatment						
Revascularization	1.92 (1.38–2.68)	0.000	2.16 (1.34–3.48)	0.002	2.21 (1.37–3.57)	0.001
Aspirin	0.99 (0.63–1.55)	NS				
Beta-blockers	1.29 (0.82–2.03)	NS				
ACE inhibitor/ARB	0.74 (0.50–1.09)	NS				
Statins	1.03 (0.61–1.76)	NS				
Diuretics	1.51 (1.07–2.13)	0.018	1.67 (1.03–2.71)	0.038	1.59 (0.98–2.59)	NS
Biomarkers						
Log troponin (μg/l)	1.01 (0.84–1.20)	NS	1.09 (0.86–1.38)	NS	1.08 (0.86–1.37)	NS
Log NT-proBNP (pmol/l)	1.25 (1.04–1.52)	0.019	1.07 (0.79–1.45)	NS	1.02 (0.76–1.38)	NS
Log PENK (pmol/l)	1.46 (1.26–1.71)	0.000			1.43 (1.07–1.91)	0.010
C statistic			0.712 (0.667–0.756)		0.735 (0.692–0.778)	0.004
PENK quartiles						
1	Reference				Reference	
2	1.44 (0.83–2.49)	NS			1.47 (0.69–3.14)	NS
3	1.96 (1.16–3.31)	0.01			1.78 (0.85–3.70)	NS
4	3.01 (1.82–4.98)	0.000			2.05 (0.93–4.57)	0.07

Values are hazard ratio (95% confidence interval). Multivariable analysis results are reported for model 1, which included variables and biomarkers (except PENK) that were significant on univariable analysis. Multivariable model 2 used the variables in model 1 with the addition of PENK. Hazard ratios for PENK entered as quartiles are reported at the bottom of the table.

Abbreviations as in Tables 1 and 3.

pressure. Infarct size (troponin and creatine kinase levels) had no influence on PENK levels. PENK may therefore closely integrate the cardiorenal status of an individual, and the observation that levels peaked on presentation with AMI may represent an advantage for early risk assessment. Previous studies such as those from the GRACE investigators (20,21) have emphasized the importance of cardiac and renal function in determining risk after acute coronary syndromes, which may explain the prognostic performance of PENK for adverse events post-AMI. In addition, PENK was associated with cardiovascular outcomes such as death, re-AMI, and HF rehospitalization. Existing biomarkers such as NT-proBNP

Table 7 Cox Regression Analysis for Endpoints at 6 Months (MACE, Death and/or MI, Death and/or HF)

	Univariable	p Value	Multivariable	p Value
MACE				
GRACE score	1.02 (1.02–1.03)	0.000	1.01 (1.00–1.02)	0.000
Log NT-proBNP (pmol/l)	2.01 (1.66–2.42)	0.000	1.23 (0.99–1.52)	NS
Log PENK (pmol/l)	1.95 (1.74–2.19)	0.000	1.56 (1.32–1.83)	0.000
Death and/or MI				
GRACE score	1.02 (1.01–1.02)	0.000	1.01 (1.00–1.01)	0.016
Log NT-proBNP (pmol/l)	1.87 (1.52–2.31)	0.000	1.20 (0.95–1.52)	NS
Log PENK (pmol/l)	1.91 (1.68–2.17)	0.000	1.65 (1.38–1.99)	0.000
Death and/or HF				
GRACE score	1.03 (1.02–1.04)	0.000	1.02 (1.01–1.02)	0.000
Log NT-proBNP (pmol/l)	3.20 (2.45–4.18)	0.000	1.72 (1.26–2.34)	0.001
Log PENK (pmol/l)	2.27 (1.98–2.59)	0.000	1.57 (1.29–1.91)	0.000

Values are hazard ratio (95% confidence interval).

HF = heart failure; other abbreviations as in Tables 1 and 3.

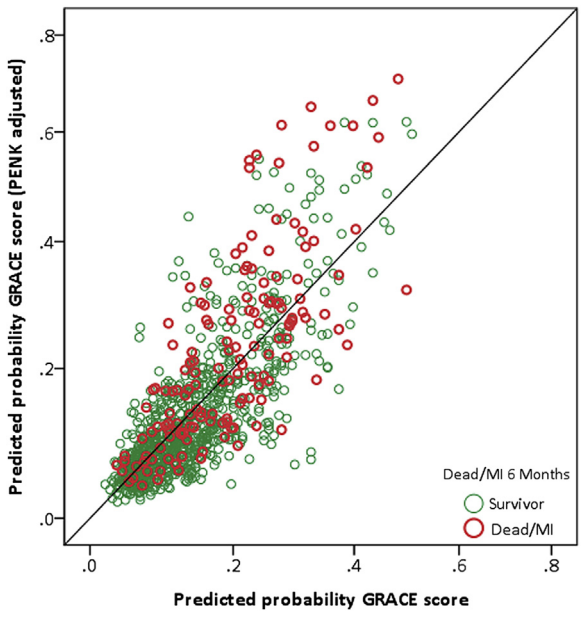

Figure 3 **Up- and Down-Reclassification of the Probabilities of Events in Survivors and Patients With the Endpoint of Death and/or MI at 6 Months**

The Global Registry of Acute Coronary Events (GRACE) score–derived probability of death/myocardial infarction (MI) in subjects with endpoints **(red)** has been up-classified (above the diagonal line of equivalence) after adjustment by using PENK, with a small preponderance (1.5%) of those without the endpoint **(green)** being up-classified.

were mainly predictive of mortality and HF, with poorer performance for re-AMI.

The prognostic performance of PENK was confirmed by using a panel of different tests, including Cox survival

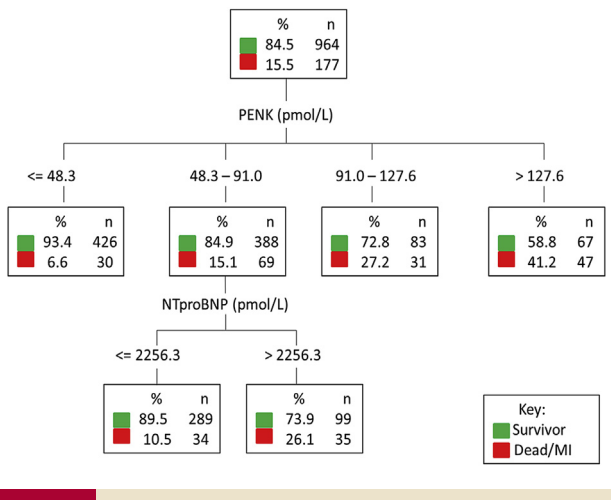

Figure 4 **Classification Tree for Death/MI at 6 Months**

MI = myocardial infarction; NT-proBNP = N-terminal pro–B-type natriuretic peptide; PENK = proenkephalin.

analysis, reclassification analysis, and classification by using decision trees. PENK retained its independent predictive value and demonstrated additional utility to the GRACE score in reclassification analysis, the major effect being on up-classification of those with endpoints. PENK levels were also prognostic for both short- and long-term adverse events up to 2 years. Current guidelines recommend that post-AMI, patients should undergo risk stratification so that appropriate therapy can be instituted (24), as intensification of therapy is especially efficacious in high-risk patients. In addition, PENK levels <48.3 pmol/l may define a low-risk group of patients who potentially could be discharged earlier from the hospital.

This association of PENK with poor outcomes may reflect a direct link between pathophysiological actions of enkephalins and adverse events. The enkephalins act mainly on delta OPRs, with a predominantly depressor effect on the cardiovascular system (5,6) and on tissue perfusion, including the kidneys. OPRs are widely distributed, with high concentrations in the kidney (7). In addition, delta OPRs have been implicated in ischemia pre-conditioning (8,9), although this effect may be dependent on the duration and concentration of ischemia. Enkephalins also act on the zeta or opioid growth receptor, which exerts an antiproliferative or pro-apoptotic effect (10,11). Our studies suggest a correlation between PENK (a surrogate of enkephalin levels) and adverse events, and there may be a causal relationship between enkephalins and these adverse events. Previous research on heroin addicts suggest an up-regulation of platelet alpha$_2$-adrenoceptors (25). An opioid antagonist (naltrexone) suppressed adrenaline-induced platelet aggregation and alpha$_2$-adrenoceptor density. In post-MI patients, it is unknown whether enkephalins affect the adrenaline-induced platelet aggregation in vivo. PENK levels were inversely correlated with renal function, echocardiographic evidence of left ventricular impairment, and blood pressure. These findings are hypothesis generating for investigating the effect of OPR antagonists on MACE and platelet function after reperfusion, when the beneficial effects of enkephalins on reperfusion injury have been accrued.

Study limitations. Our findings are based on a population from a single center, with 2 admitting hospitals, and should be verified in other larger populations. The rate of early revascularization in our NSTEMI population was low and may not reflect a more contemporary invasive approach of revascularization within 72 h of presentation. However, it is unlikely that the relationship of PENK with adverse events would have been confounded by higher early revascularization rates. Another advantage of a registry-like study (as opposed to a clinical trial) is that endpoints may not be affected by investigational therapies. We excluded patients with unstable angina, some of whom may have been regarded as NSTEMI on more contemporary high-sensitivity troponin assays; this action may also have affected the Cox regression analysis models. In addition, we used an in-house NT-proBNP assay; this assay is well established and

correlated well with the Roche NT-proBNP assay (r_s = 0.90). Reclassification analyses have not been fully validated in the published literature, and significance tests need to be confirmed (26). Prospective studies on the clinical effectiveness of using this biomarker for management strategies, whether in low- or high-risk groups, need to be performed.

Conclusions

After AMI, circulating PENK levels reflect cardiorenal status and provide prognostic information, over and above that provided by the GRACE score and the current gold standard biomarkers NT-proBNP and troponin. The ability of PENK to predict re-AMI, in addition to mortality and HF events, may confer clinical utility on this endogenous opioid in risk stratification after AMI.

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