Online Supplement

Rho-associated Kinases Activity Is an Independent Predictor of Cardiovascular Events in Acute Coronary Syndrome

Brief title: Rho kinase and acute coronary syndrome

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Supplemental Methods

Rho-associated kinese (ROCK) activity and Cardiovascular Events during the Follow-up Period

ROCK activity and serum concentrations of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, blood urea nitrogen, creatinine, glucose, and electrolytes were measured at the following time points: within 2 hours of acute coronary syndrome (ACS) onset and at 3 and 24 hours after, at 7 days and 14 days after and 6 months after ACS onset. Measurement of the serum creatine kinase levels was performed at admission and at every 3 hours after reperfusion until a peak level. Echocardiographic parameters were evaluated at 7 days after ACS onset.

We obtained information on potential outcomes or adverse events from medical records and/or telephone survey to their primary care doctors. We first assessed the associations of ROCK activity with first major cardiovascular events (death from cardiovascular causes, acute myocardial infarction, ischemic stroke and coronary revascularization) and then assessed the associations with death from cardiovascular causes, acute myocardial infarction, ischemic stroke, coronary revascularization, and hospitalization for heart failure. Coronary revascularization was defined as unplanned percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery, resulting from myocardial ischemia after PCI in ACS. Indications of revascularization and hospitalization for heart failure were determined by cardiology specialists who were blind to the protocol of this study.

Measurement of ROCK Activity

ROCK activity was assayed in peripheral blood leukocytes as the amount of phospho-Thr853 in the myosin-binding subunit of myosin light chain phosphatase (MLCPh), because myosin-binding subunit on MLCPh is one of the downstream targets of ROCK. Blood was collected at room temperature in heparinized tubes (20 U/mL). After adding an equal volume of 2% dextran, each sample was kept at room temperature for 30 minutes. The supernatant was spun at 1450 rpm for 10 minutes. Red blood cells in the resulting cell pellet were lysed with the addition of water and spun at 1450 rpm for 10 minutes after the addition of Hank's balanced salt solution (Hyclone, Logan, UT, USA). The resulting leukocyte pellet was resuspended in medium 199 (Sigma Chemical Co., Saint Louis, Missouri, USA) and the number of cells was counted using a

hematocytometer. Cells were fixed in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane. After centrifugation, the cell pellets were stored at -80°C for Western blot analysis. Cells pellets were dissolved in 10 μL of 1 mol/L Tris base and then mixed with 100 μL of extraction buffer (8 mol/L urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol). Equal amounts of cell extracts were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analyses from several membranes. After serum starvation for 20 hours, confluent cells were stimulated with 10 μmol/L lysophosphatidic acid for 10 minutes and then subsequently fixed and harvested in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane. Following centrifugation at 1450 rpm for 10 minutes at 4°C, precipitates were dissolved in 10 μL of 1 mol/L Tris base and mixed with 100 μL of extraction buffer. An equal volume of positive control cell lysate was used for each gel. Membranes were incubated with rabbit anti-phospho-specific Thr853-myosin-binding subunit polyclonal antibody (Biosource Invitrogen, Carlsbad, California, USA), rabbit anti-myosin-binding subunit polyclonal antibody (Covance Laboratories, Evansville, Indiana, USA), or antiactin monoclonal antibody (Sigma). Bands were visualized using the ECL system (Amersham-Pharmacia Co., London, UK). Images were captured using Adobe Photoshop, and the band intensities were quantified using National Institutes of Health Image 1.61. Rho-associated kinase activity was expressed as the ratio of phospho myosin-binding subunit in each sample to phospho myosin-binding subunit in each positive control divided by total myosin-binding subunit in each sample per total myosin-binding subunit in each positive control.

Supplemental Tables

Table S1. Baseline Clinical Characteristics of ACS Patients on the Basis of Maximum Rho-associated Kinase Activity

Variable	Low group (n=37)	High group (n=36)	P value
Age, yr	67±11	66±11	0.63
Gender, men/women	29/8	31/5	0.38
Body mass index, kg/m^2	24.0±3.7	23.7±2.3	0.71
Systolic blood pressure, mmHg	145±25	143±32	0.85
Diastolic blood pressure, mmHg	79±16	81±20	0.64
Heart rate, bpm	78±20	78±19	0.89
Medical history, n (%)			
Hypertension	26 (70.3)	29 (80.6)	0.30
Dyslipidemia	29 (78.4)	23 (63.9)	0.17
Diabetes mellitus	12 (32.4)	17 (47.2)	0.19
Smokers	28 (75.7)	29 (80.6)	0.61
Previous coronary artery disease	1 (2.7)	1 (2.8)	0.98
Previous stroke	3 (8.1)	4 (11.1)	0.66
Laboratory determinations			
Total cholesterol, mmol/L	5.38±1.16	5.35±0.93	0.44
Triglycerides, mmol/L	1.68±0.87	1.47±0.84	0.28
High-density lipoprotein cholesterol, mmol/L	1.33±0.40	1.32±0.32	0.88
Low-density lipoprotein cholesterol, mmol/L	3.44±0.96	3.49±0.83	0.78
Glucose, mmol/L	8.60±2.94	9.94±3.94	0.12
Maximum creatine kinase, IU/L	1661±1848	3035±2842	0.02
Brain natriuretic peptide, pg/mL	191±464	142±285	0.61
Medications, n (%)			
Antiplatelets	6 (16.2)	3 (8.33)	0.30
Calcium-channel blockers	16 (43.2)	13 (36.1)	0.53
Renin angiotensin system inhibitors	16 (43.2)	13 (36.1)	0.53
Statins	10 (27.0)	3 (8.3)	0.03
Medically treated diabetes			

Any	7 (18.9)	10 (27.8)	0.37
Insulin-dependent	1 (2.7)	1 (2.8)	0.98
Culprit vessel (LAD/LCx/RCA), (%)	37.8/24.3/37.8	39.7/19.2/41.1	0.31
Implantation of stent, n (%)			
Any type	32 (86.4)	34 (94.4)	0.24
Drug-eluting	5 (13.5)	6 (16.7)	0.70
Echocardiographic parameters			
Left atrial diameter, mm	36.3±4.8	37.7±5.8	0.27
Left ventricular end-diastolic dimension, mm	45.9±5.0	47.5±5.4	0.19
Left ventricular ejection fraction, %	54.0±9.6	51.8±7.8	0.29

All results are presented as means±SD.

Low group indicates maximum Rho-associated kinase activity of <1.14, and high indicates maximum Rho-associated kinase activity ≥1.14.

ACS indicates acute coronary syndrome; bpm, beats per minute; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery.

Table S2. Cause of Death

Cause of death	Low group (n=37)	High group (n=36)
Sudden death, n	1	0
Acute myocardial infarction, n	0	1
Ischemic stroke, n	1	0
Heart failure, n	0	1
Cancer, n	1	0

Low group indicates maximum Rho-associated kinase activity of <1.14, and high group indicates ≥1.14.

Table S3. Baseline Clinical Characteristics of the ACS Patients on the Basis of Peak Creatine Kinase Level

Variable	Low group	High group	P value
Age, yr	68±11	64±11	0.15
Gender, men/women	24/10	31/3	0.02
Body mass index, kg/m^2	23.7±3.4	24.1±2.9	0.65
Systolic blood pressure, mmHg	142±25	144±32	0.75
Diastolic blood pressure, mmHg	78±18	81±18	0.57
Heart rate, bpm	79±20	79±18	0.95
Medical history, n (%)			
Hypertension	28 (82.4)	24 (70.6)	0.25
Dyslipidemia	24 (70.6)	26 (76.5)	0.17
Diabetes mellitus	11 (32.4)	17 (50.0)	0.13
Smokers	23 (67.7)	29 (85.3)	0.08
Previous coronary artery disease	1 (2.9)	0 (0)	0.23
Previous stroke	2 (5.9)	3 (8.8)	0.64
Laboratory determinations			
Total cholesterol, mmol/L	5.22±1.11	5.48±1.03	0.32
Triglycerides, mmol/L	1.58±0.84	1.60±0.90	0.93
High-density lipoprotein cholesterol, mmol/L	1.32±0.34	1.32±0.39	0.98
Low-density lipoprotein cholesterol, mmol/L	3.34±0.96	3.57±0.88	0.29
Glucose, mmol/L	8.88±3.39	9.99±3.77	0.22
Maximum creatine kinase, IU/L	602±443	4013±2447	<0.001
Brain natriuretic peptide, pg/mL	212±487	132±280	0.43
Medications, n (%)			
Antiplatelets	6 (17.7)	2 (5.8)	0.12
Calcium-channel blockers	18 (52.9)	11 (32.4)	0.08
Renin angiotensin system inhibitors	16 (47.1)	13 (38.2)	0.46
Statins	7 (20.6)	6 (17.7)	0.75
Medically treated diabetes			
Any	6 (17.7)	10 (29.4)	0.25
Insulin-dependent	1 (2.9)	1 (2.9)	-
Culprit vessel (LAD/LCx/RCA), (%)	38.2/14.7/47.1	41.2/23.5/35.3	0.34

Implantation of stent, n (%)			
Any type	29 (85.3)	32 (94.1)	0.22
Drug-eluting	6 (17.7)	4 (14.7)	0.49

All results are presented as means±SD.

Low group indicates peak creatine kinase level of <1474 and high indicates peak creatine kinase level of ≥1474.

ACS indicates acute coronary syndrome; bpm, beats per minute; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery.

Table S4. Association Between Maximum Rho-associated Kinase Activity and Events during Follow-up

Variable	Unadjusted HR (95% CI) P value	Adjusted* HR (95% CI) P value	Adjusted† HR (95% CI) P value	Adjusted‡ HR (95% CI) P value	Adjusted§ HR (95% CI) P value
First major	3.39 (1.61-7.80)	4.05 (1.87-9.50)	3.94 (1.79-9.44)	3.52 (1.46-8.98)	4.56 (1.98-11.34)
cardiovascular event	0.001	<0.001	<0.001	0.005	<0.001
Death from	1.05 (0.13-8.76)	1.10 (0.13-9.36)	1.00 (0.12-8.83)	3.47 (0.25-89.29)	1.39 (0.14-16.76)
cardiovascular disease	0.96	0.92	0.99	0.36	0.77
Acute myocardial	0.35 (0.02-2.70)	0.30 (0.01-2.36)	0.28 (0.01-2.25)	0.40 (0.02-3.56)	0.20 (0.01-1.64)
infarction	0.32	0.26	0.24	0.43	0.14
Ischemic stroke	2.23 (0.21-48.00)	2.49 (0.23-55.15)	2.95 (0.23-82.55)	1.99 (0.05-109.3)	1.80 (0.12-56.02)
	0.50	0.45	0.41	0.69	0.68
Coronary	3.03 (1.42-7.03)	3.61 (1.65-8.56)	3.39 (1.53-8.16)	2.90 (1.19-7.43)	3.79 (1.63-9.49)
revascularization	0.004	0.001	0.002	0.02	0.002
Hospitalization for heart	3.22 (0.41-65.07)	3.96 (0.50-80.78)	2.06 (0.20-46.39)	1.35 (0.10-39.38)	1.55 (0.17-34.15)
failure	0.27	0.20	0.55	0.83	0.71
Death from any cause	0.70 (0.09-4.21)	0.73 (0.09-4.47)	0.63 (0.08-3.97)	0.22 (0.01-2.23)	0.68 (0.08-5.29)
	0.69	0.72	0.61	0.21	0.70

First major cardiovascular events include cardiovascular death, acute myocardial infarction, ischemic stroke, and coronary revascularization.

HR indicates hazard ratio; CI, confidence interval; NA, not applicable.

Hazard ratios are for the high maximum Rho-associated kinase activity group using the low maximum Rho-associated kinase activity group as a reference.

*Adjusted for age and gender.

†Adjusted for age; gender, presence of hypertension, dyslipidemia, diabetes, and smoking.

‡Adjusted for age; gender, presence of hypertension, dyslipidemia, diabetes, smoking, and maximum creatine kinase value.

§Adjusted for age; gender, presence of hypertension, dyslipidemia, diabetes, smoking, and concomitant treatment with statins.

Table S5. Baseline Clinical Characteristics of ACS Patients on the Basis of Rho-associated Kinase Activity Evaluated at 6 Months after ACS Onset

Variable	Low group (n=34)	High group (n=33)	P value
Age, yr	65±10	66±12	0.72
Gender, men/women	27/7	27/6	0.80
Body mass index, kg/m^2	23.9±3.4	24.4±2.4	0.60
Systolic blood pressure, mmHg	127±22	122±19	0.36
Diastolic blood pressure, mmHg	72±14	74±13	0.63
Heart rate, bpm	69±11	69±8	0.82
Medical history, n (%)			
Hypertension	27 (79.4)	23 (69.7)	0.36
Dyslipidemia	24 (70.6)	25 (75.8)	0.63
Diabetes mellitus	16 (47.1)	10 (30.3)	0.16
Smokers	28 (82.4)	24 (72.7)	0.34
Laboratory determinations			
Total cholesterol, mmol/L	4.60±0.70	4.78±0.70	0.34
Triglycerides, mmol/L	1.81±0.72	1.68±0.84	0.55
High-density lipoprotein cholesterol, mmol/L	1.32±0.31	1.37±0.28	0.38
Low-density lipoprotein cholesterol, mmol/L	2.77±0.59	2.95±0.62	0.23
HbA1c, %	6.4±1.0	6.2±0.8	0.48
Brain natriuretic peptide, pg/mL	89±213	74±111	0.73
Medications, n (%)			
Antiplatelets	34 (100)	34 (100)	NA
Calcium-channel blockers	15 (44.1)	12 (36.4)	0.51
Renin angiotensin system inhibitors	27 (79.4)	24 (72.7)	0.52
Statins	19 (55.9)	21 (63.6)	0.52
Medically treated diabetes			
Any	8 (23.5)	4 (12.1)	0.22
Insulin-dependent	3 (8.8)	1 (3.0)	0.91
Culprit vessel (LAD/LCx/RCA), (%)	41.2/20.6/38.2	36.4/21.2/42.4	0.65
Implantation of stent, n (%)			
Any type	30 (88.2)	30 (90.9)	0.72

Drug-eluting	5 (14.7)	5 (15.2)	0.96

All results are presented as means±SD.

Low group indicates maximum Rho-associated kinase activity of ≤0.70, and high indicates maximum Rho-associated kinase activity >0.70.

ACS indicates acute coronary syndrome; bpm, beats per minute; NA, not applicable; LAD, left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery.

Supplemental Figures

Figure S1

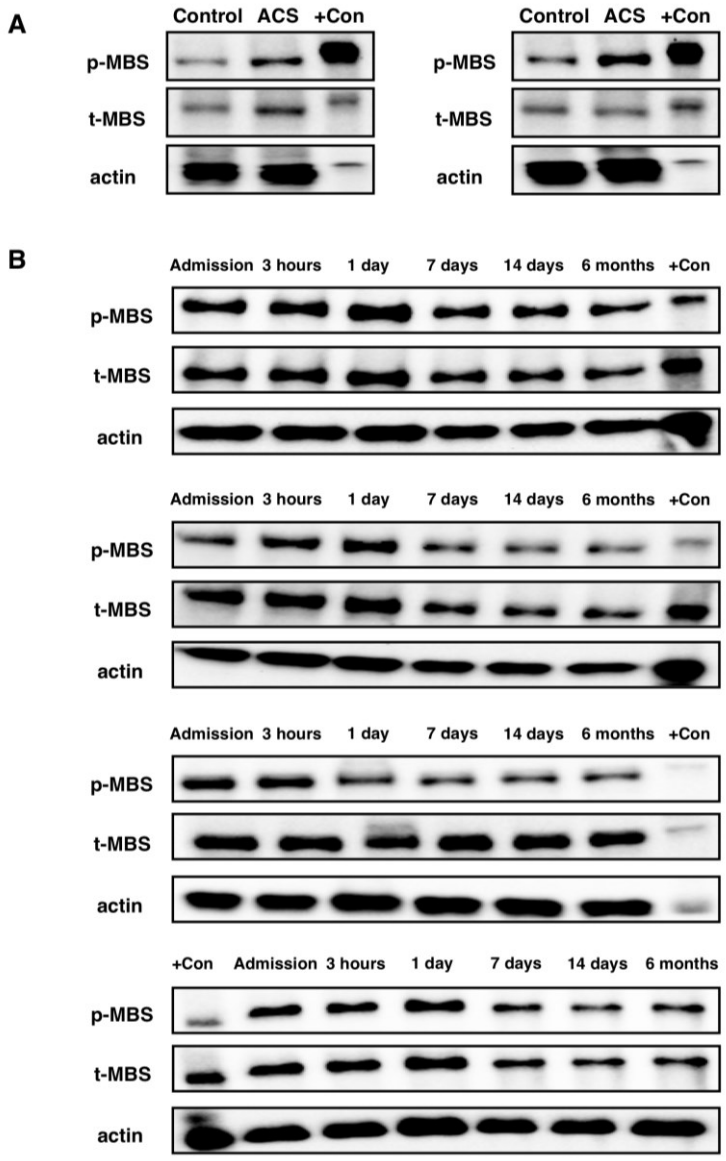

Figure S1 A. Western blot analysis for phospho myosin-binding subunit (p-MBS), total myosin-binding subunit (t-MBS), and actin in peripheral blood leukocytes in 2 control patients and 2 patients with acute coronary syndrome (ACS). **B**. Western blot analysis for phospho myosin-binding subunit (p-MBS), total myosin-binding subunit (t-MBS), and actin in peripheral blood leukocytes in peripheral blood leukocytes within 2 hours of acute coronary syndrome (ACS) onset (admission) and at 3 hours, 1 day, 7 days, 14 days, and 6 months after ACS onset in 4 patients with ACS.

Figure S2

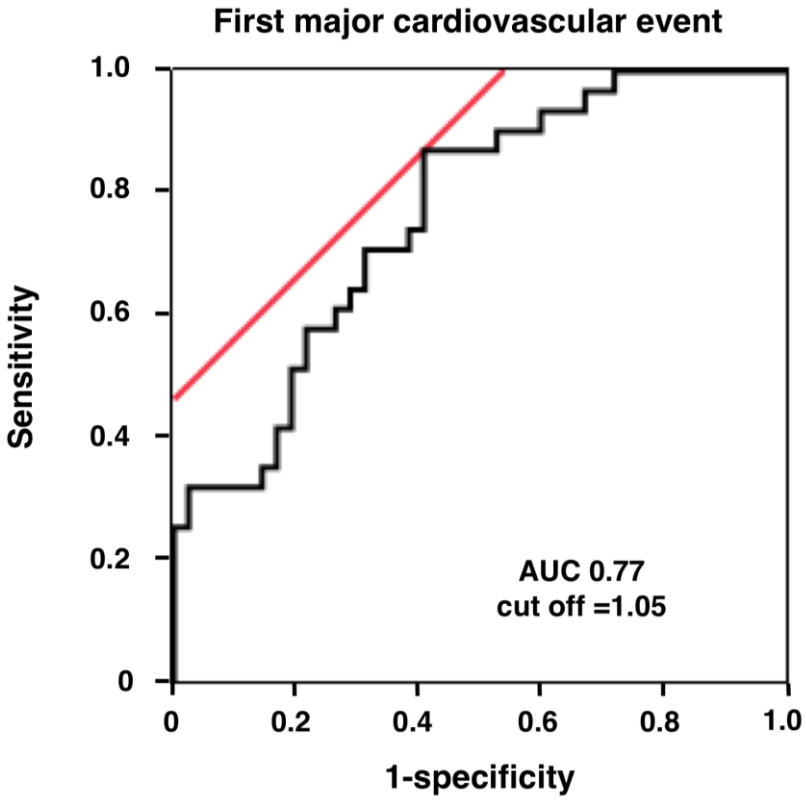

Figure S2. Receiver operating characteristic curves of maximum Rho-associated kinase activity in acute coronary syndrome patients for predicting first major cardiovascular events. AUC indicates area under curve.

Figure S3

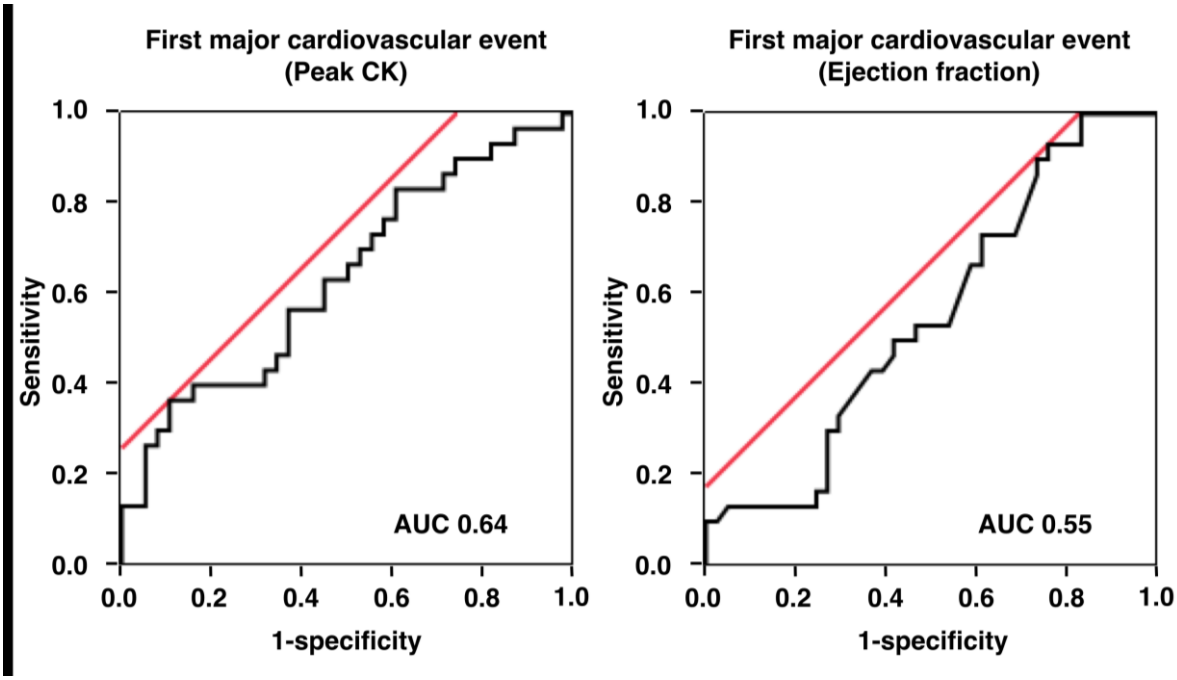

Figure S3. Receiver operating characteristic curves of maximum creatine kinase (CK) and ejection fraction in acute coronary syndrome patients for predicting first major cardiovascular events. AUC indicates area under curve.

Figure S4

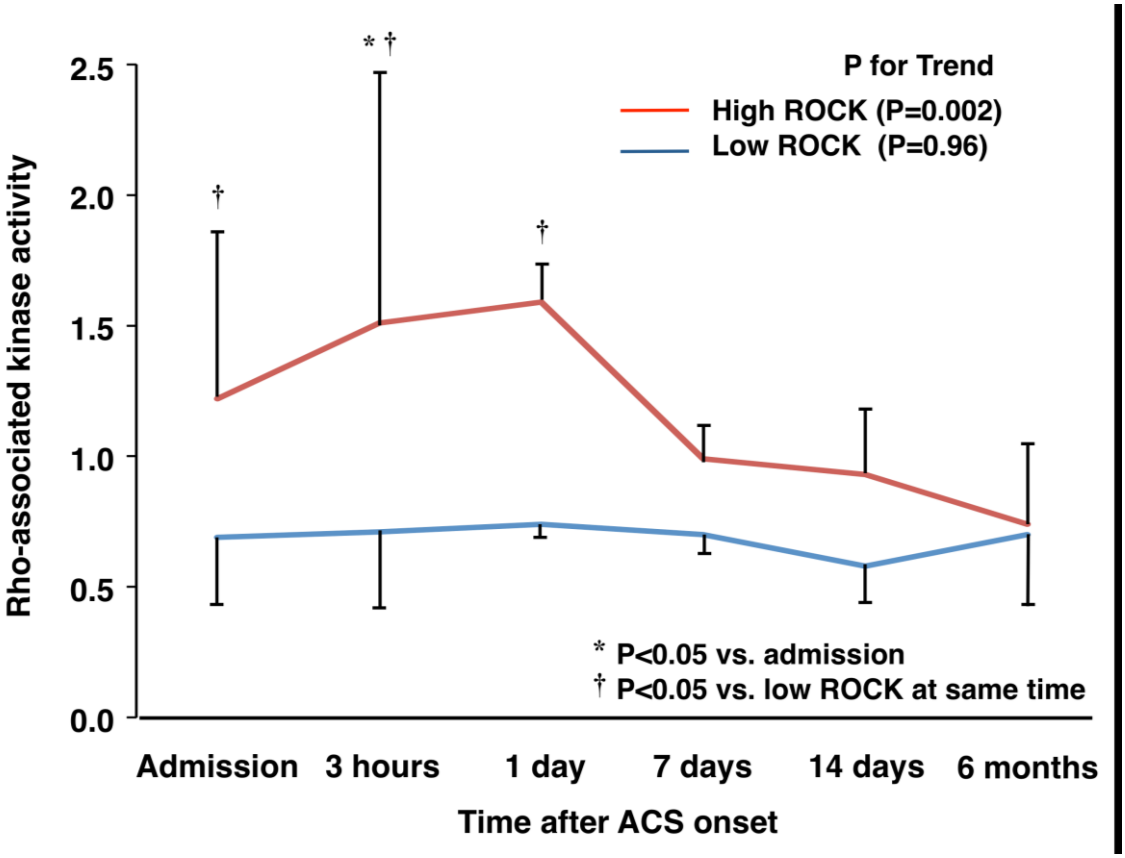

Figure S4. Time-course graphs of the actual Rho-associated kinase (ROCK) activity values in the low and high groups.

Figure S5

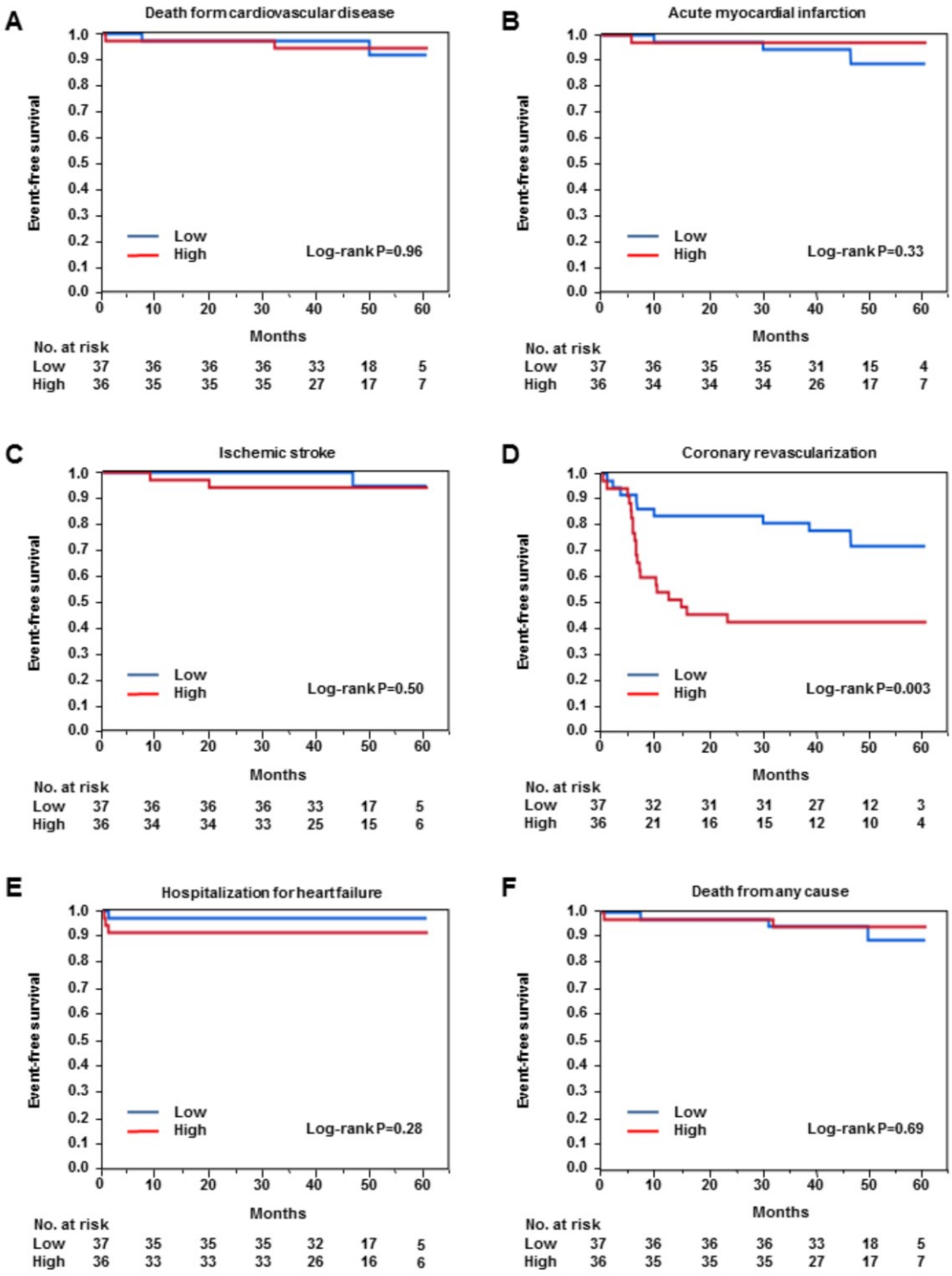
A
Death form cardiovascular disease
Event-free survival
Low
High
Log-rank P=0.96
Months
No. at risk
Low 37 36 36 36 33 18 5
High 36 35 35 35 27 17 7
B
Acute myocardial infarction
Event-free survival
Low
High
Log-rank P=0.33
Months
No. at risk
Low 37 36 35 35 31 15 4
High 36 34 34 34 26 17 7
C
Ischemic stroke
Event-free survival
Low
High
Log-rank P=0.50
Months
No. at risk
Low 37 36 36 36 33 17 5
High 36 34 34 33 25 15 6
D
Coronary revascularization
Event-free survival
Low
High
Log-rank P=0.003
Months
No. at risk
Low 37 32 31 31 27 12 3
High 36 21 16 15 12 10 4
E
Hospitalization for heart failure
Event-free survival
Low
High
Log-rank P=0.28
Months
No. at risk
Low 37 35 35 35 32 17 5
High 36 33 33 33 26 16 6
F
Death from any cause
Event-free survival
Low
High
Log-rank P=0.69
Months
No. at risk
Low 37 36 36 36 33 18 5
High 36 35 35 35 27 17 7

Figure S5. Kaplan-Meier curves of cumulative event-free survival of death from cardiovascular causes (panel A), acute myocardial infraction (panel B), ischemic stroke (panel C), coronary revascularization (panel D), hospitalization for heart failure (panel E), and death from any cause (panel F), according to the median value of maximum Rho-associated kinase activity. Low indicates maximum Rho-associated kinase activity <1.14, and high indicates maximum Rho-associated kinase activity ≥1.14.

Figure S6

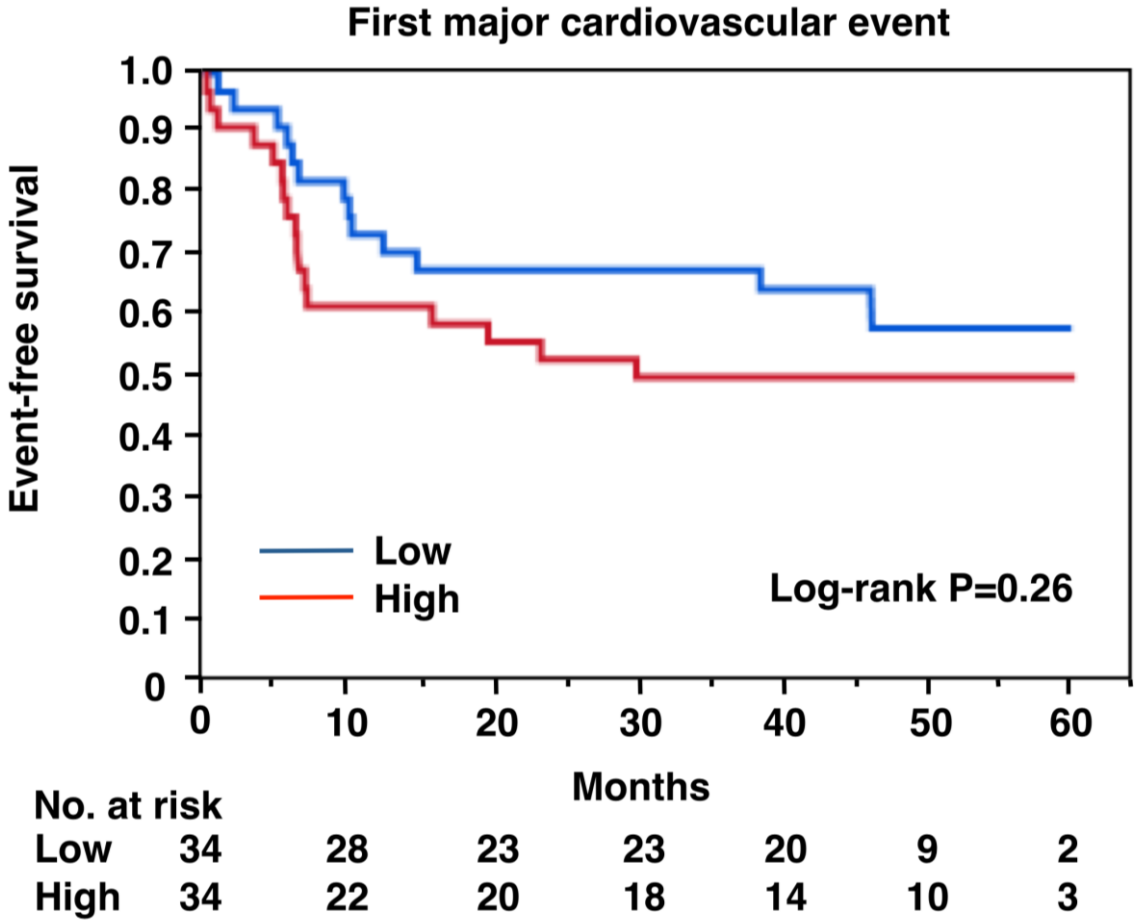

Figure S6. Kaplan-Meier curves of cumulative event-free survival of major cardiovascular events (death from cardiovascular causes, myocardial infarction, ischemic stroke, and coronary revascularization), according to the median value of the peak level of serum creatine kinase. Low indicates median value of the peak serum creatine kinase level of <1474, and high indicates median value of the peak serum creatine kinase level of ≥1474.

Figure S7

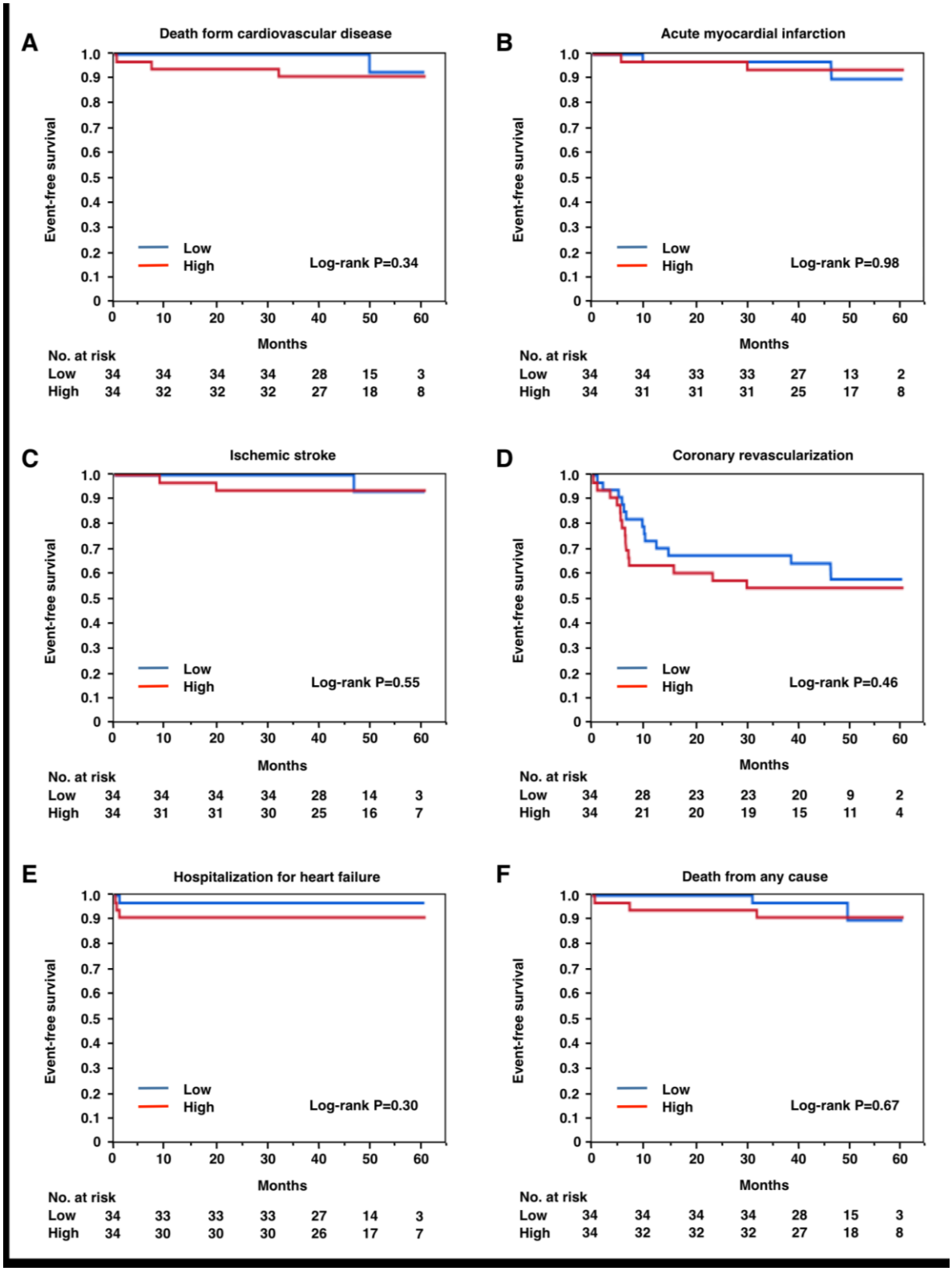
A
Death form cardiovascular disease
Event-free survival
Low
High
Log-rank P=0.34
Months
No. at risk
Low 34 34 34 34 28 15 3
High 34 32 32 32 27 18 8
B
Acute myocardial infarction
Log-rank P=0.98
No. at risk
Low 34 34 33 33 27 13 2
High 34 31 31 31 25 17 8
C
Ischemic stroke
Log-rank P=0.55
No. at risk
Low 34 34 34 34 28 14 3
High 34 31 31 30 25 16 7
D
Coronary revascularization
Log-rank P=0.46
No. at risk
Low 34 28 23 23 20 9 2
High 34 21 20 19 15 11 4
E
Hospitalization for heart failure
Log-rank P=0.30
No. at risk
Low 34 33 33 33 27 14 3
High 34 30 30 30 26 17 7
F
Death from any cause
Log-rank P=0.67
No. at risk
Low 34 34 34 34 28 15 3
High 34 32 32 32 27 18 8

Figure S7. Kaplan-Meier curves of cumulative event-free survival of death from cardiovascular causes (panel A), acute myocardial infraction (panel B), ischemic stroke (panel C), coronary revascularization (panel D), hospitalization for heart failure (panel E), and death from any cause (panel F), according to the median value of the peak level of serum creatine kinase. Low indicates median value of the peak serum creatine kinase level of <1474, and high indicates median value of the peak serum creatine kinase level of ≥1474.

Figure S8

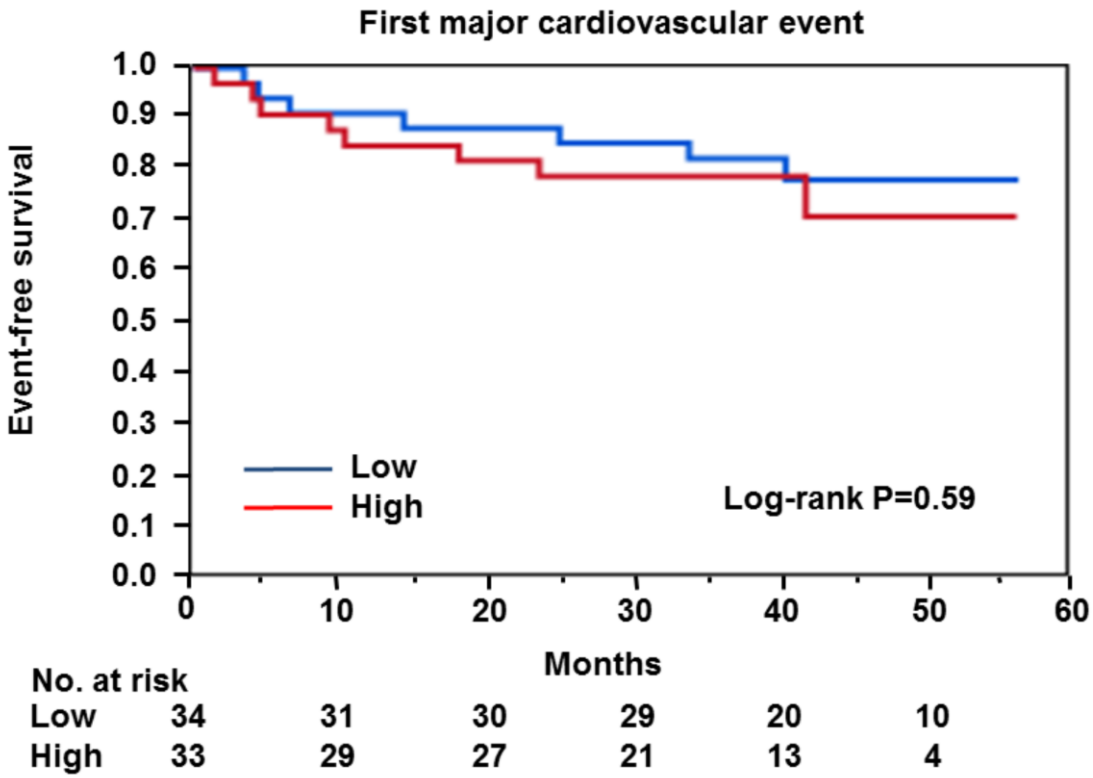

Figure S8. Kaplan-Meier curves of cumulative event-free survival of major cardiovascular events (death from cardiovascular causes, myocardial infarction, ischemic stroke, and coronary revascularization), according to the median value of Rho-associated kinase activity evaluated at 6 months after acute myocardial infarction onset. Low indicates maximum Rho-associated kinase activity <0.70, and high indicates maximum Rho-associated kinase activity ≥0.70

Figure S9

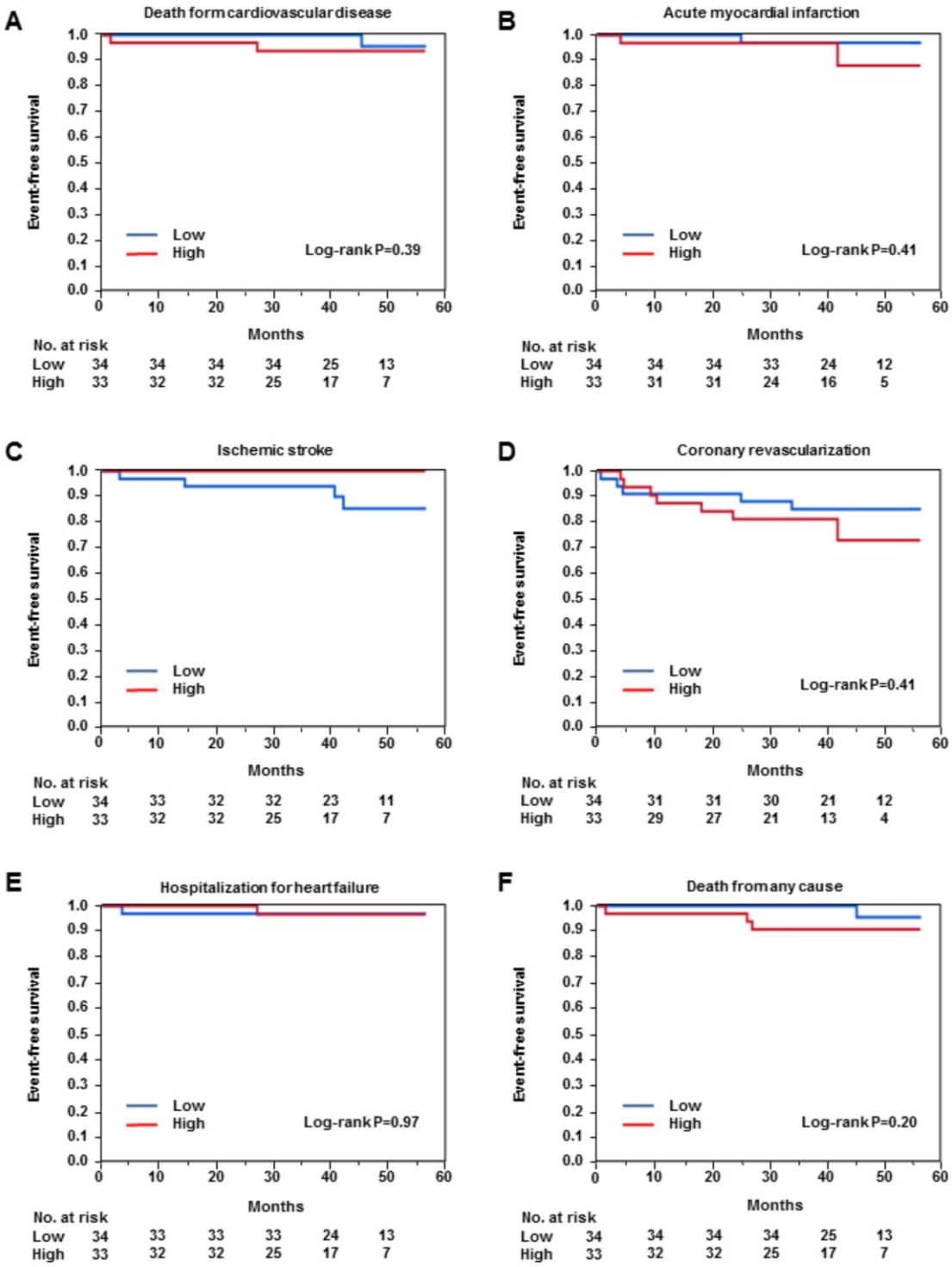
A
Death form cardiovascular disease
Event-free survival
Low
High
Log-rank P=0.39
Months
No. at risk
Low 34 34 34 34 25 13
High 33 32 32 25 17 7
B
Acute myocardial infarction
Event-free survival
Low
High
Log-rank P=0.41
Months
No. at risk
Low 34 34 34 33 24 12
High 33 31 31 24 16 5
C
Ischemic stroke
Event-free survival
Low
High
Months
No. at risk
Low 34 33 32 32 23 11
High 33 32 32 25 17 7
D
Coronary revascularization
Event-free survival
Low
High
Log-rank P=0.41
Months
No. at risk
Low 34 31 31 30 21 12
High 33 29 27 21 13 4
E
Hospitalization for heart failure
Event-free survival
Low
High
Log-rank P=0.97
Months
No. at risk
Low 34 33 33 33 24 13
High 33 32 32 25 17 7
F
Death from any cause
Event-free survival
Low
High
Log-rank P=0.20
Months
No. at risk
Low 34 34 34 34 25 13
High 33 32 32 25 17 7

Figure S9. Kaplan-Meier curves of cumulative event-free survival of death from cardiovascular causes (panel A), acute myocardial infraction (panel B), ischemic stroke (panel C), coronary revascularization (panel D), hospitalization for heart failure (panel E), and death from any cause (panel F), according to the median value of Rho-associated kinase activity evaluated at 6 months after acute myocardial infarction onset. Low indicates maximum Rho-associated kinase activity <0.70, and high indicates maximum Rho-associated kinase activity ≥0.70.