ESC European Society of Cardiology

European Heart Journal (2018) **39**, 952–959
doi:10.1093/eurheartj/ehx657

Prognostic impacts of Rho-kinase activity in circulating leucocytes in patients with vasospastic angina

Taro Nihei, Jun Takahashi, Kiyotaka Hao, Yoku Kikuchi, Yuji Odaka, Ryuji Tsuburaya, Kensuke Nishimiya, Yasuharu Matsumoto, Kenta Ito, Satoshi Miyata, Yasuhiko Sakata, and Hiroaki Shimokawa*

Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

Received 3 April 2017; revised 25 June 2017; editorial decision 24 October 2017; accepted 24 October 2017; online publish-ahead-of-print 20 November 2017

See page 960 for the editorial comment on this article (doi: 10.1093/eurheartj/ehx757)

Aims Rho-kinase activity in circulating leucocytes is a useful biomarker for diagnosis and disease activity assessment of vasospastic angina (VSA). The present study aimed to examine the long-term prognostic impact of Rho-kinase activity in circulating leucocytes in VSA patients.

Methods and results We prospectively enrolled 174 consecutive patients with VSA and 50 non-VSA patients, in whom we measured Rho-kinase activity in circulating leucocytes, and they were followed for a median of 16 months. The primary endpoint was cardiac events including cardiac death, non-fatal myocardial infarction, and hospitalization for unstable angina. During the follow-up period, cardiac events occurred in 10 VSA patients (5.7%) but in none of the non-VSA patients. When we divided VSA patients into two groups by a median value of their Rho-kinase activity, the Kaplan–Meier survival analysis showed a significantly worse prognosis in VSA patients with high Rho-kinase activity compared with those with low activity or non-VSA patients (log-rank; $P < 0.05$, respectively). Receiver-operating characteristic curve analysis showed that Rho-kinase activity value of 1.24 was the best cut-off level to predict cardiac events in VSA patients, and multivariable analysis showed that a value above the cut-off point had the largest hazard ratio to predict poor outcome in VSA patients [hazard ratio (95% confidence interval) 11.19 (1.41–88.95); $P = 0.022$]. Importantly, combination of the Japanese Coronary Spasm Association risk score and Rho-kinase activity significantly improved the prognostic impact in VSA patients as compared with either alone.

Conclusion Rho-kinase activity in circulating leucocytes is useful for prognostic stratification of VSA patients.

Keywords Vasospastic angina • Rho-kinase • Prognosis • Biomarkers • Risk factors

Introduction

Vasospastic angina (VSA) is an important functional cardiac disorder characterized by transient myocardial ischaemia due to epicardial coronary artery spasm.[1] Although it has been considered that morbidity of VSA is lower in the Caucasians than in the Asians, recent studies demonstrated that coronary spasm was noted more frequently in the Caucasians with chest pain and unobstructive coronary arteries than ever thought.[2] Accordingly, this cardiovascular functional disorder draws renewed attention.[3–5] Although VSA patients with optimal medical treatment with calcium channel blockers (CCBs) generally have a favoured prognosis,[6] some patients exhibit resistance to the conventional therapy and develop acute myocardial infarction or fatal arrhythmias.[7–9] Thus, it is important for physicians to accurately perform risk stratification in each VSA patient. A number of prognostic factors of VSA have been proposed.[8,10] We have recently developed a useful risk score by the Japanese Coronary Spasm Association (JCSA).[11] However, there is

* Corresponding author. Tel: +81 22 7177151, Fax: +81 22 7177156, Email: shimo@cardio.med.tohoku.ac.jp

no reliable prognostic biomarker available for a long-term risk stratification of VSA patients.

We have previously demonstrated that Rho-kinase plays a central role in the pathogenesis of coronary artery spasm[12] and that Rho-kinase activity in circulating leucocytes is a useful biomarker for the diagnosis and disease activity assessment of VSA patients.[13,14] However, the long-term prognostic impact of Rho-kinase activity in circulating leucocytes in VSA patients remains to be examined.

In the present study, we thus examined whether Rho-kinase activity in circulating leucocytes is a useful biomarker for VSA patients, and if so, whether it could provide additional benefits in the risk stratification when combined with the risk score by the JCSA.[11]

Methods

The present study was conducted following the ethics principles in the Declaration of Helsinki and approved by the Ethics Committees of Tohoku University (No. 2011-417). All patients provided written informed consent before study entry.

Study subjects and spasm provocation test

From December 2011 to May 2014, a total of 768 patients underwent diagnostic cardiac catheterization for evaluation of chest pain and/or ECG abnormalities at the Tohoku University Hospital. Of those, 233 patients, who had symptoms suspected of VSA (e.g. nitrate-responsive rest angina and/or marked diurnal variation in symptom onset and exercise tolerance), underwent acetylcholine (ACh) provocation test for coronary artery spasm. Acetylcholine provocation test and the measurement of Rho-kinase activity in circulating leucocytes were performed as previously reported.[13] The positive provocation test was defined based on the Guidelines by the Japanese Circulation Society,[6] as the development of >90% stenosis accompanied by chest pain and/or ischaemic ECG changes. Microvascular spasm was defined as the presence of myocardial lactate production despite the absence of angiographically demonstrable epicardial spasm during ACh provocation test associated with the reproduction of the patients' symptoms and ischaemic ST-segment changes.[15] Finally, in the present study, we examined 174 VSA patients and 50 non-VSA patients who had neither angiographically demonstrable epicardial spasm nor microvascular spasm with myocardial lactate production during ACh provocation test (see Supplementary material online, *Figure S1*). We have recently developed the JCSA risk score to provide the comprehensive risk assessment and prognostic stratification for VSA patients. The risk score was calculated based on the sum of weighted predictors present in individual patient.[11]

Endpoint and follow-up

The primary endpoint was cardiac events, including cardiac death, non-fatal myocardial infarction, and hospitalization for unstable angina.[11] Clinical long-term follow-up was performed using a questionnaire that was sent to patients and primary physicians in addition to the information available on the medical records.

Statistical analysis

Continuous variables are presented as means ± standard deviations (SD) or medians and interquartile ranges (IQR), and categorical variables as numerals and percentages. Group comparisons for continuous variables were performed by the Kruskal–Wallis test for multiple groups, and the Mann–Whitney *U* test for two groups. The χ^2 test was used for categorical variables. Survival rate from cardiac events was analysed by the Kaplan–Meier method and comparison between groups was performed using the log-rank tests. Receiver-operating characteristic (ROC) curve analysis was performed to determine the best cut-off value of Rho-kinase activity to predict the occurrence of cardiac events in VSA patients. The prognostic value of each variable was tested by univariable and multivariable logistic regression analysis. The model performance of the fitted models was compared by the likelihood ratio test. A *P*-value <0.05 was considered to be statistically significant. Refer to Supplementary material online for further information.

Results

Clinical characteristics of study subjects

In the present study, 174 VSA patients and 50 non-VSA patients were finally analysed (see Supplementary material online, *Figure S1*). Baseline clinical characteristics of the two groups are summarized in*Table 1* and Supplementary material online, *Table S1*. There was no significant difference in demographic background between the two groups, whereas Rho-kinase activity in circulating leucocytes was significantly higher in the VSA compared with the non-VSA group (VSA 1.20 ± 0.33 vs. non-VSA 1.07 ± 0.22, $P = 0.003$) (*Figure 1*). Mean value of Rho-kinase activity in the VSA and the non-VSA groups was distributed on the two sides of the cut-off value 1.18 for the diagnosis of VSA as determined in our previous study.[13] In both groups, no significant correlation was noted between Rho-kinase activity and systemic inflammatory profiles, such as high-sensitivity C reactive protein (hsCRP) and white blood cell (WBC) count (see Supplementary material online, *Figure S2A and B*). Rho-kinase activity of VSA patients was significantly associated with anginal frequency as we previously reported (see Supplementary material online, *Figure S3*).[13] When we divided 174 VSA patients into two groups by a median value of baseline Rho-kinase activity at diagnosis (p-MBS/t-MBS = 1.20), high Rho-kinase activity (p-MBS/t-MBS ≥ 1.20, $n = 87$) and low Rho-kinase activity groups (p-MBS/t-MBS <1.20, $n = 87$), there was no difference in baseline clinical characteristics, including age, gender, or prevalence of coronary risk factors among the three groups (*Table 1*). While all VSA patients were treated with CCBs, nitrates including nicorandil were also used in one-third of them. Between the two groups of VSA patients divided by Rho-kinase activity, medications were almost similar except for statin use (see Supplementary material online, *Table S1*).

Rho-kinase activity in circulating leucocytes and clinical outcomes

During the median follow-up period of 16 months (IQR 9, 25 months), we found the occurrence of 10 cardiac events in the VSA group (5.7%), including cardiovascular death ($n = 1$) and hospitalization for unstable angina ($n = 9$), but none in the non-VSA group (see Supplementary material online, *Table S2*). Vasospastic angina patients tended to develop cardiac events more frequently compared with non-VSA patients (log-rank, $P = 0.09$) (*Figure 2A*). When dividing VSA patients into two groups by the median value of their Rho-kinase activity of 1.20, the high Rho-kinase activity group had a significantly worse prognosis compared with the low activity group or

Table 1 Baseline clinical characteristics of study subjects

	Non-VSA ($n = 50$)	VSA			*P*-value	*P*-value for trend
		Overall ($n = 174$)	Rho-kinase activity <1.20 ($n = 87$)	Rho-kinase activity ≥1.20 ($n = 87$)		
Rho-kinase activity, p-MBS/t-MBS	1.07 ± 0.22	1.20 ± 0.33	0.99 ± 0.14	1.41 ± 0.34	0.003	<0.001
Age (years)	67 (51, 73)	64 (54, 72)	63 (54, 72)	64 (54, 72)	0.48	0.74
Male/female	29/21	116/58	55/32	61/26	0.26	0.34
Medical history, *n* (%)						
Hypertension	28 (56)	99 (57)	45 (52)	54 (62)	0.91	0.39
Dyslipidaemia	13 (26)	80 (46)	40 (46)	40 (46)	<0.05	0.04
Diabetes mellitus	13 (26)	43 (25)	21 (24)	22 (25)	0.85	0.97
Current smoker	3 (6)	36 (21)	15 (17)	21 (24)	<0.05	0.03
Previous myocardial infarction	1 (2)	11 (6)	4 (5)	7 (8)	0.21	0.08
Prior PCI	3 (6)	16 (9)	8 (9)	8 (9)	0.35	0.78
Out-of-hospital cardiac arrest	4 (8)	7 (4)	3 (3)	4 (5)	0.21	0.45
LVEF (%)	72 (65, 77)	71 (66, 77)	70 (64, 77)	71 (66, 77)	0.61	0.82
JCSA risk score	NA	3 (2, 4)	3 (2, 4)	4 (2, 5)	NA	0.50

Results are expressed as mean ± SD or median (IQR). *P*-value was presented for non-VSA vs. total VSA patients. *P* for trend was presented for non-VSA vs. VSA patients with Rho-kinase activity <1.20 vs. VSA patients with Rho-kinase activity ≥1.20.
IQR, interquartile range; JCSA, Japanese Coronary Spasm Association; LVEF, left ventricular ejection fraction; NA, not applicable; PCI, percutaneous coronary intervention; p-MBS, phosphorylated myosin-binding subunit; SD, standard deviation; t-MBS, total myosin-binding subunit; VSA, vasospastic angina.

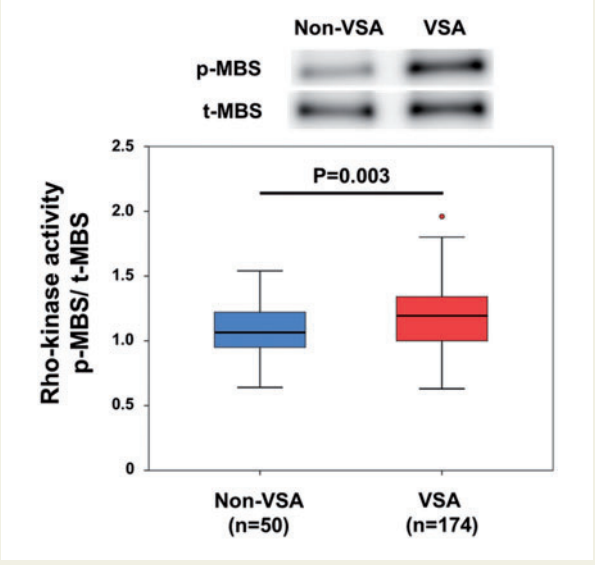


Figure 1 Rho-kinase activity in the circulating leucocytes of VSA and non-VSA patients. Rho-kinase activity was expressed as the ratio of phosphorylated myosin-binding subunit (MBS) to total MBS. p-MBS, phosphorylated myosin-binding subunit; t-MBS, total myosin-binding subunit; VSA, vasospastic angina.

non-VSA group (log-rank; $P < 0.05$, respectively) (*Figure 2B*). In contrast, median values of hsCRP or WBC count of those VSA patients failed to estimate their long-term prognosis (see Supplementary material online, *Figure S4*).

Rho-kinase activity in circulating leucocytes as a predictive biomarker of vasospastic angina patients

The linear regression analysis showed that the level of Rho-kinase activity of VSA patients was not correlated with other established prognostic factors, including history of out-of-hospital cardiac arrest (OHCA), smoking, angina at rest alone, significant organic coronary stenosis, multivessel spasm, ST elevation, or beta-blocker use (see Supplementary material online, *Table S3*).[11] As shown in *Figure 3*, the ROC curve analysis showed that Rho-kinase activity in circulating leucocytes had a moderate accuracy for predicting the incidence of cardiac events in VSA patients with an area under the curve (AUC) of 0.74 [95% confidence interval (95% CI) 0.65–0.83], and a phosphorylated MBS ratio of 1.24 was identified as the best cut-off level. With regard to the value, the sensitivity and specificity for predicting cardiac events were 90% and 64%, respectively, while the negative predictive value was 99%. Univariable Cox proportional hazard analysis showed that significant organic stenosis, use of beta-blockers and Rho-kinase activity in circulating leucocytes ≥1.24 were significantly correlated with the occurrence of cardiac events in VSA patients (*Table 2*). Furthermore, in the multivariable model, Rho-kinase activity in circulating leucocytes ≥1.24 was the most highly correlated with future occurrence of cardiac events in VSA patients (hazard ratio 11.19, 95% CI 1.41–88.95, $P = 0.022$) (*Table 2*).

Combination of the JCSA risk score and Rho-kinase activity in circulating leucocytes

The JCSA risk score assembles multiple prognostic factors and estimates future adverse cardiac events in VSA patients.[11] When we

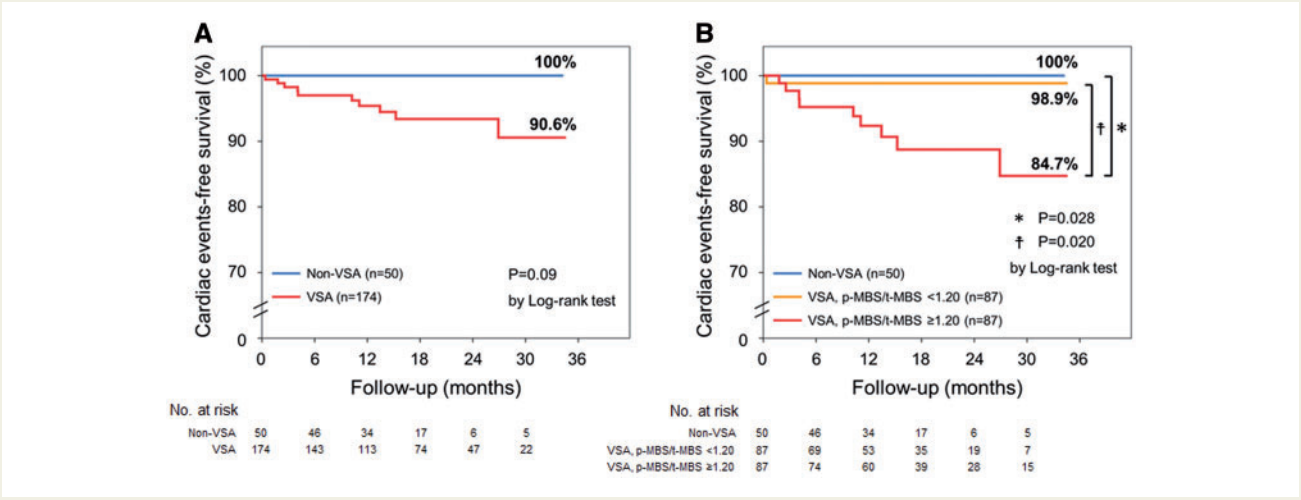


Figure 2 The Kaplan–Meier curve for cardiac events of VSA patients and non-VSA patients. (*A*) Overall VSA patients vs. non-VSA patients. (*B*) VSA patients with higher Rho-kinase activity vs. VSA patients with lower Rho-kinase activity vs. non-VSA patients. VSA, vasospastic angina.

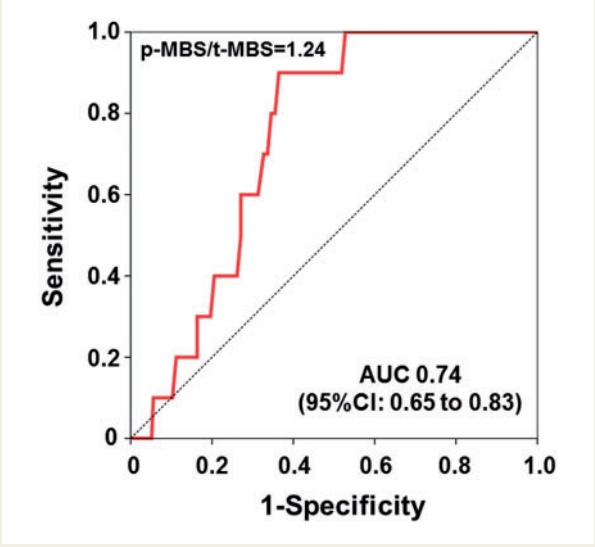


Figure 3 Receiver operating curve analysis for predicting the future development of cardiac events of vasospastic angina patients.

divided VSA patients into two groups by the median value of their JCSA risk score (3 points), whose baseline characteristics are shown in Supplementary material online, *Table S4*, no significant difference in the occurrence of cardiac events was noted between the two groups (*Figure 4A*). However, as we subdivided each group according to Rho-kinase activity level in circulating leucocytes, the Kaplan–Meier survival analysis showed that VSA patients with high JCSA risk score (above 3 points) and a Rho-kinase activity level ≥1.24 had a significantly worse outcome (log-rank test; $P = 0.001$) (*Figure 4B*). To evaluate the effect of the combination of the JCSA risk score and Rho-kinase activity level, we used the logistic regression models of the individual factors and the saturated model with the both factors. In the individual models, the JCSA risk score above the median value (Model 0) was not significant ($P = 0.148$), whereas Rho-kinase activity above the cut-off value (Model 1) was significant ($P = 0.016$). In the saturated model (Model 2), only Rho-kinase activity above the cut-off value was significant ($P = 0.015$) (*Table 3*). We compared the saturated model with the individual models by the likelihood ratio test and found that the saturated model provided significantly better performance ($P = 0.00135$) than the individual model with the JCSA risk score alone (*Table 4*). These results indicate that combination of the JCSA risk score and Rho-kinase activity in circulating leucocytes could substantially improve the risk stratification of VSA patients as compared with either alone.

Acetylcholine-induced angiographical spasm pattern has been shown to be associated with the prognosis of VSA patients.[10,16] Thus, we further examined the prognostic impact of Rho-kinase activity by focal ($n = 16$) and diffuse ($n = 145$) spasm patterns (see Supplementary material online, *Table S5*). Although Rho-kinase activity in circulating leucocytes was significantly higher in the focal spasm group compared with the diffuse spasm group (1.29 ± 0.28 vs. 1.19 ± 0.35, $P = 0.03$), long-term prognosis was comparable between the two groups (see Supplementary material online, *Figure S5A and B*). However, when we subdivided each spasm pattern group according to the cut-off level of Rho-kinase activity of 1.24, VSA patients with high Rho-kinase activity levels had a significantly worse outcome irrespective of spasm pattern (log-rank test; $P = 0.042$) (see Supplementary material online, *Figure S5C*). These results suggest that Rho-kinase activity has more significant prognostic impact in VSA patients than angiographical spasm patterns.

Discussion

The major findings of the present study were as follows: (i) cardiac events occurred more frequently in VSA patients with high Rho-kinase activity in circulating leucocytes compared with those with

Table 2 Correlated factors for cardiac events in VSA patients

Variables	Univariable analysis			Multivariable analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age	1.07	1.00–1.14	0.070	1.09	1.00–1.19	0.040
Male	0.73	0.20–2.57	0.618			
Hypertension	0.69	0.20–2.37	0.549			
Dyslipidaemia	1.10	0.32–3.80	0.882			
Diabetes mellitus	1.26	0.32–4.86	0.742			
Current smoking	0.47	0.06–3.70	0.471			
Previous myocardial infarction	1.46	0.18–11.57	0.722			
Angina at rest alone	0.85	0.24–3.04	0.805			
ST-segment elevation during angina attack	1.89	0.24–14.92	0.547			
Significant organic stenosis	5.30	1.52–18.50	0.009			
Multivessel spasm	3.12	0.81–12.08	0.099			
High Rho-kinase activity (p-MBS/t-MBS ≥1.24)	10.36	1.31–81.92	0.027	11.19	1.41–88.95	0.022
Calcium channel blockers	0.80	0.10–6.34	0.833			
Long-acting nitrate	1.31	0.37–4.66	0.675			
Antiplatelet	3.19	0.67–15.06	0.144			
Beta-blocker	5.27	1.49–18.69	0.010	5.92	1.63–21.49	0.007

CI, confidence interval; HR, hazard ratio; p-MBS, phosphorylated myosin-binding subunit; t-MBS, total myosin-binding subunit; VSA, vasospastic angina.

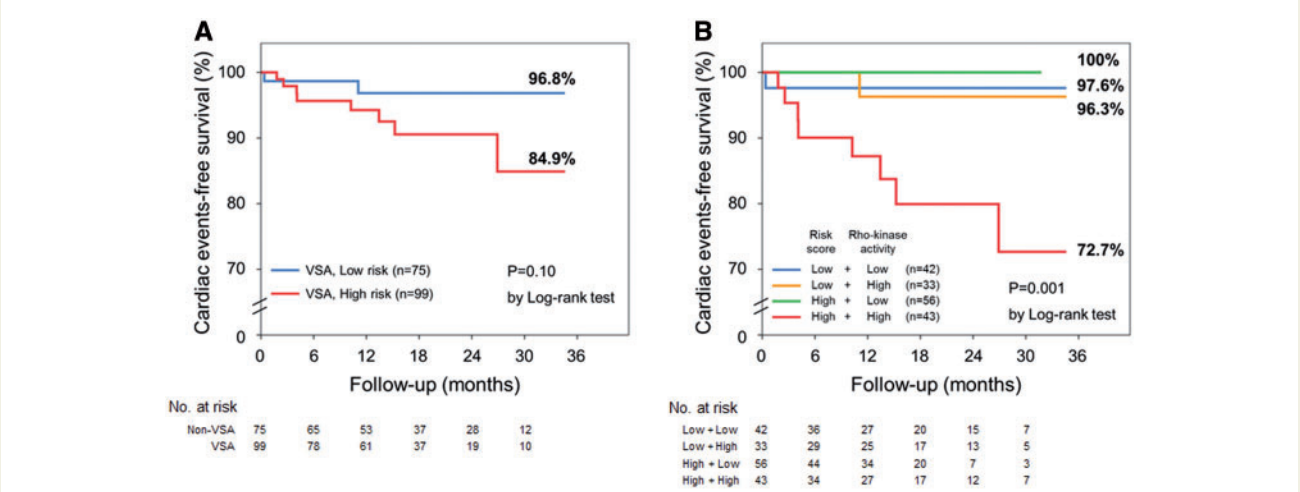


Figure 4 Synergistic effect obtained by a combination of the Japanese Coronary Spasm Association (JCSA) risk score and Rho-kinase activity. (*A*) Vasospastic angina (VSA) patients with higher JCSA risk score tended to have a worse prognosis compared with those with lower JCSA risk score. (*B*) VSA patients with higher JCSA risk score and Rho-kinase activity above the cut-off level 1.24 had a significantly worse prognosis as compared with other three groups.

low Rho-kinase activity or non-VSA patients, (ii) Rho-kinase activity value of 1.24 was identified as the best cut-off level for predicting the incidence of cardiac events in VSA patients, (iii) multivariable analysis for cardiac events showed that Rho-kinase activity in circulating leucocytes ≥1.24 was the significant and independent prognostic factor of VSA patients, and (iv) combination of the JCSA risk score and Rho-kinase activity substantially improved risk stratification of VSA patients as compared with either alone.

Rho-kinase activity in circulating leucocytes as a prognostic biomarker of vasospastic angina

We previously demonstrated that Rho-kinase activity in circulating leucocytes was significantly enhanced in VSA patients, and optimal medications including CCB could decrease the enhanced Rho-kinase activity along with improvement of symptoms.[13] Additionally, Rho-kinase activity in VSA patients showed a distinct circadian variation

Table 3 Multiple logistic regression analysis of variables associated with cardiac events

	Regression coefficient	*P*-value
Model 0		
(Intercept)	-3.597	0
JCSA risk score above the median value	1.166	0.148
Model 1		
(Intercept)	-4.575	0
Rho-kinase activity above the cut-off value (1.24)	2.567	0.016
Model 2		
(Intercept)	-5.444	0
JCSA risk score above the median value	1.234	0.133
Rho-kinase activity above the cut-off value (1.24)	2.603	0.015

JCSA, Japanese Coronary Spasm Association.

Table 4 Synergistic predictive value of combining the JCSA risk score and Rho-kinase activity for long-term incidence of cardiac events of VSA patients

	Residual DF	Residual deviance	DF	Deviance	*P*-value
Model 0	172	74.03			
Model 2	171	63.753	1	10.276	0.00135
Model 1	172	66.452			
Model 2	171	63.753	1	2.6988	0.10040

DF, degree of freedom; JCSA, Japanese Coronary Spasm Association; VSA, vasospastic angina.

with a peak at early morning, associated with alterations in coronary basal tone and vasomotor reactivity.[14] Thus, Rho-kinase activity in circulating leucocytes is useful for diagnosis and disease activity assessment of VSA. In clinical practice of cardiovascular diseases, we obtain considerable benefits from biomarkers that could reflect disease activity of the moment and predict future adverse events, such as B-type natriuretic peptide (BNP) in patients with heart failure and high-sensitivity cardiac troponin T or I in those with acute coronary syndrome (ACS).[17,18] In the present study, we tested our hypothesis that Rho-kinase activity in circulating leucocytes is also useful for risk stratification of VSA patients. Indeed, we found that VSA patients with high Rho-kinase activity (above a median value of this cohort) developed cardiac events more frequently than those with low Rho-kinase activity, although there was no difference in medical treatments including CCBs and long-acting nitrates between the two groups. Importantly, as the linear regression analysis demonstrated, the level of Rho-kinase activity in VSA patients was independent of any other established prognostic factors of VSA, such as history of OHCA, smoking, angina at rest alone, significant organic coronary stenosis, multivessel spasm, ST elevation, and beta-blocker use. Additionally, the significant correlation between Rho-kinase activity ≥1.24 and the incidence of cardiac events was noted in a multivariable model including established predictors of VSA listed above as covariates. These results indicate that Rho-kinase activity in circulating leucocytes is a reliable prognostic biomarker in VSA patients. It has been recently demonstrated that enhanced Rho-kinase activity in circulating leucocytes was correlated with the poor outcome in patients with various cardiovascular disturbances other than VSA, such as cardiovascular risk factors,[19] ACS,[20] and congestive heart failure (CHF).[21] However, systemic Rho-kinase activity could be enhanced non-specifically in a number of thrombotic, inflammatory, or malignant disorders. Thus, in the clinical settings of ACS and CHF, the prognostic implication of Rho-kinase activity might be overestimated by those potential confounding factors. On the other hand, as evidenced by the observation that fasudil, a selective Rho-kinase inhibitor, could suppress coronary hyperconstricting responses in VSA patients, enhanced Rho-kinase activation underlies the pathogenesis of VSA.[12] Thus, we consider that Rho-kinase activity in circulating leucocytes could be a useful prognostic biomarker for VSA patients, although the precise mechanisms for the correlation between enhanced Rho-kinase activity and increased future risk in VSA patients remain to be examined in future studies.

In clinical practice, we need to be aware of the 2 key values of Rho-kinase activity in circulating leucocytes, 1.18 and 1.24, which are the best cut-off levels for the diagnosis[13] and the prediction for poor prognosis of VSA, respectively. As BNP is widely used in cardiovascular practice for screening of HF patients with cut-off level of 100 pg/mL and for identification of those at high risk of developing HF with cut-off level of BNP 40 pg/mL, we could apply the 2 key values of Rho-kinase activity in daily practice of VSA patients. As we have previously demonstrated that 3 months of optimal medical treatment including CCB was significantly decreased the Rho-kinase activity of VSA patients along with symptomatic improvement,[13] repeated measurements of Rho-kinase activity after administration of CCBs could provide useful information about the prognosis of VSA patients.

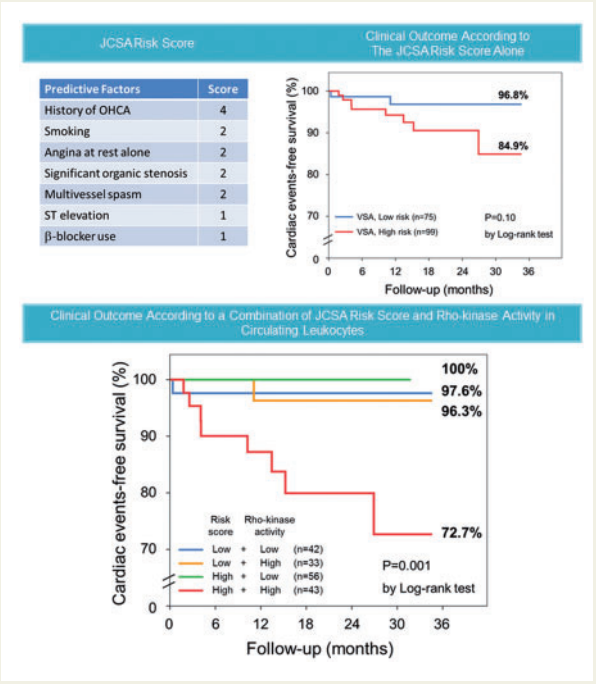
Synergistic effect of the combination of the JCSA risk score and Rho-kinase activity

In the JCSA study, we have previously identified novel prognostic factors of VSA, such as history of OHCA and specific angiographic findings during the spasm provocation tests.[8,10] Subsequently, we developed the JCSA risk score to assemble those multiple prognostic factors and predict the incidence of future adverse cardiac events in VSA patients.[11] As the prediction rate of the JCSA score is ~90%, the score appears to have an acceptable reliability for clinical use. However, in the present study, we found no significant difference in the incidence of future cardiac events between the two groups of VSA patients when simply divided by their JCSA risk score above or below the median value (3.0 points). However, when the same subjects were further divided into four subgroups by combining the JCSA risk score and the Rho-kinase activity in circulating leucocytes, VSA patients with both high JCSA risk score and high Rho-kinase activity showed a worst prognosis compared with other three

subgroups (*Figure 4B*). Furthermore, the likelihood ratio test demonstrated that combination of the JCSA risk score and Rho-kinase activity had an incremental value in predicting cardiac events compared with the JCSA risk score alone. These results suggest that the addition of Rho-kinase activity to clinical risk assessment with the JCSA risk score enables us to identify VSA patients at high risk with a high degree of accuracy. In particular, VSA patients of the intermediate risk stratum (JCSA risk score, 3–5 points), in which approximately half of the subjects of the present study were included, were found to have a wide range of occurrence of MACE from 4.2% (JCSA risk score, 3 points) to 12.3% (JCSA risk score, 5 points).[11] Thus, we consider that VSA patients assigned to the intermediate stratum by the JCSA risk score would obtain great benefit by measurement of Rho-kinase activity in circulating leucocytes. Importantly, the disease activity of VSA often fluctuates during the course of the disease and some patients become intractable even with the conventional treatment. Thus, Rho-kinase activity in circulating leucocytes of VSA patients could provide additional information about disease activity that is not provided by the JCSA risk score.

Study limitations

Several limitations should be mentioned for the present study. First, a small number of events during the follow-up period reduced the statistical power of the present study and might have led to data overfitting. However, we confirmed that high level of Rho-kinase activity was an independent predictor of cardiac events even after adjustment with other prognostic risk factors. Second, we used the composite primary endpoint in the present study. The majority of the cardiac events were hospitalization for unstable angina. Repeated angina is a subjective symptom, and the decision of admission was left to an individual physician. However, in the present study, unstable angina was defined as recurrence or worsening of chest pain or discomfort associated with objective ischaemic ECG changes. Furthermore, the proportion of hospitalization for unstable angina to overall cardiac events in the present study (~80%) was comparable with the previous studies.[8,16] Third, the present study eventually did not include patients with a severe significant organic stenosis of 90% or greater on coronary angiography. If those patients have rest angina and/or marked diurnal variation in symptom onset and exercise tolerance, aggressive functional assessment of coronary artery would be desirable to identify the precise mechanism responsible for angina.[4,5] The prognostic impacts of Rho-kinase activity in circulating leucocytes in VSA patients with the comorbidity of severe significant organic stenosis remains to be elucidated. Fourth, although coronary microvascular angina may be caused by various pathophysiologic mechanisms, we only excluded the patients with ACh-induced microvascular spasm. Thus, patients with microvascular angina with negative response to ACh provocation test might have been included in the non-VSA group. Fifth, management of medical treatment during the follow-up period was individualized at the discretion of each attending physician on the basis of symptoms. Finally, we had no data regarding the changes in symptom or quality of life (e.g. Seattle Angina Questionnaire) and Rho-kinase activity in circulating leucocytes during the follow-up period. This issue remains to be examined in future studies.



Take home figure Synergistic effect obtained by a combination of the JCSA risk score and Rho-kinase activity.

Conclusions

The present study demonstrates that a high level of Rho-kinase activity in circulating leucocytes is an independent and useful prognostic biomarker for VSA patients. The assessment of Rho-kinase activity in combination with the JCSA risk score could substantially improve the risk stratification of VSA patients.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

The authors thank Akemi Saito, Hiromi Yamashita, Teru Hiroi, Takeshi Kato, and Zhulanqiqige Do.e for technical assistance in the present study.

Funding

This study was supported in part by the grants-in-aid from the Ministry of Health, Labor, and Welfare and those from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Conflict of interest: H.S. has received lecture fees from Bayer Yakuhin, Ltd (Osaka, Japan) and Daiichi Sankyo Co., Ltd (Tokyo, Japan).

References

1. Maseri A, Beltrame JF, Shimokawa H. Role of coronary vasoconstriction in ischemic heart disease and search for novel therapeutic targets. *Circ J* 2009;**73**: 394–403.
2. Ong P, Athanasiadis A, Borgulya G, Vokshi I, Bastiaenen R, Kubik S, Hill S, Schaufele T, Mahrholdt H, Kaski JC, Sechtem U. Clinical usefulness, angiographic

characteristics and safety evaluation of intracoronary acetylcholine provocation testing among 921 consecutive caucasian patients with unobstructed coronary arteries. *Circulation* 2014;**129**:1723–1730.
3. Beltrame JF, Crea F, Kaski JC, Ogawa H, Ong P, Sechtem U, Shimokawa H, Bairy Merz CN. Coronary vasomotion disorders international study group (COVADIS). International standardization of diagnostic criteria for vasospastic angina. *Eur Heart J* 2017;**38**:2565–2568.
4. Montalescot G, Sechtem U, Achenbach S, Andreotti F, Arden C, Budaj A, Bugiardini R, Crea F, Cuisset T, Di Mario C, Ferreira JR, Gersh BJ, Gitt AK, Hulot JS, Marx N, Opie LH, Pfisterer M, Prescott E, Ruschitzka F, Sabate M, Senior R, Taggart DP, van der WEE, Vrints CJ, Zamorano JL, Achenbach S, Baumgartner H, Bax JJ, Bueno H, Dean V, Deaton C, Erol C, Fagard R, Ferrari R, Hasdai D, Hoes AW, Kirchhof P, Knuuti J, Kolh P, Lancellotti P, Linhart A, Nihoyannopoulos P, Piepoli MF, Ponikowski P, Sirnes PA, Tamargo JL, Tendera M, Torbicki A, Wijns W, Windecker S, Valgimigli M, Claeys MJ, Donner-Banzhoff N, Frank H, Funck-Brentano C, Gaemperli O, Gonzalez-Juanatey JR, Hamilos M, Husted S, James SK, Kervinen K, Kristensen SD, Maggioni AP, Pries AR, Romeo F, Ryden L, Simoons ML, Steg PG, Timmis A, Yildirir A. 2013 ESC guidelines on the management of stable coronary artery disease: the task force on the management of stable coronary artery disease of the European Society of Cardiology. *Eur Heart J* 2013;**34**:2949–3003.
5. Roffi M, Patrono C, Collet JP, Mueller C, Valgimigli M, Andreotti F, Bax JJ, Borger MA, Brotons C, Chew DP, Gencer B, Hasenfuss G, Kjeldsen K, Lancellotti P, Landmesser U, Mehilli J, Mukherjee D, Storey RF, Windecker S, Baumgartner H, Gaemperli O, Achenbach S, Agewall S, Badimon L, Baigent C, Bueno H, Bugiardini R, Carerj S, Casselman F, Cuisset T, Erol C, Fitzsimons D, Halle M, Hamm C, Hildick-Smith D, Huber K, Iliodromitis E, James S, Lewis BS, Lip GY, Piepoli MF, Richter D, Rosemann T, Sechtem U, Steg PG, Vrints C, Luis Zamorano J. 2015 ESC guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: task force for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *Eur Heart J* 2016;**37**:267–315.
6. JCS Joint Working Group. Guidelines for diagnosis and treatment of patients with vasospastic angina (Coronary Spastic Angina) (JCS2013). *Circ J* 2014;**78**: 2779–2801.
7. Agewall S, Beltrame JF, Reynolds HR, Niessner A, Rosano G, Caforio AL, De Caterina R, Zimarino M, Roffi M, Kjeldsen K, Atar D, Kaski JC, Sechtem U, Tornvall P; on behalf of the WG on Cardiovascular Pharmacotherapy. ESC working group position paper on myocardial infarction with non-obstructive coronary arteries. *Eur Heart J* 2017;**38**:143–153.
8. Takagi Y, Yasuda S, Tsunoda R, Ogata Y, Seki A, Sumiyoshi T, Matsui M, Goto T, Tanabe Y, Sueda S, Sato T, Ogawa S, Kubo N, Momomura S, Ogawa H, Shimokawa H; Japanese Coronary Spasm Association. Clinical characteristics and long-term prognosis of vasospastic angina patients who survived out-of-hospital cardiac arrest: multicenter registry study of the Japanese Coronary Spasm Association. *Circ Arrhythm Electrophysiol* 2011;**4**:295–302.
9. Rodriguez-Manero M, Oloriz T, Le Polain de Waroux JB, Burri H, Kreidieh B, de Asmundis C, Arias MA, Arbelo E, Diaz Fernandez B, Fernandez-Armenta J, Basterra N, Izquierdo MT, Diaz-Infante E, Ballesteros G, Carrillo Lopez A, Garcia-Bolao I, Benezet-Mazuecos J, Exposito-Garcia V, Larraitz G, Martinez-Sande JL, Garcia-Seara J, Gonzalez-Juanatey JR, Peinado R. Long-term prognosis of patients with life-threatening ventricular arrhythmias induced by coronary artery spasm. *Europace* 2017;doi: 10.1093/europace/eux052.
10. Takagi Y, Yasuda S, Takahashi J, Tsunoda R, Ogata Y, Seki A, Sumiyoshi T, Matsui M, Goto T, Tanabe Y, Sueda S, Sato T, Ogawa S, Kubo N, Momomura S, Ogawa H, Shimokawa H; Japanese Coronary Spasm Association. Clinical implications of provocation tests for coronary artery spasm: safety, arrhythmic complications, and prognostic impact: multicentre registry study of the Japanese Coronary Spasm Association. *Eur Heart J* 2013;**34**:258–267.
11. Takagi Y, Takahashi J, Yasuda S, Miyata S, Tsunoda R, Ogata Y, Seki A, Sumiyoshi T, Matsui M, Goto T, Tanabe Y, Sueda S, Sato T, Ogawa S, Kubo N, Momomura S, Ogawa H, Shimokawa H; Japanese Coronary Spasm Association. Prognostic stratification of patients with vasospastic angina: a comprehensive clinical risk score developed by the Japanese Coronary Spasm Association. *J Am Coll Cardiol* 2013;**62**:1144–1153.
12. Shimokawa H, Sunamura S, Satoh K. RhoA/Rho-kinase in the cardiovascular system. *Circ Res* 2016;**118**:352–366.
13. Kikuchi Y, Yasuda S, Aizawa K, Tsuburaya R, Ito Y, Takeda M, Nakayama M, Ito K, Takahashi J, Shimokawa H. Enhanced Rho-kinase activity in circulating neutrophils of patients with vasospastic angina: a possible biomarker for diagnosis and disease activity assessment. *J Am Coll Cardiol* 2011;**58**:1231–1237.
14. Nihei T, Takahashi J, Tsuburaya R, Ito Y, Shiroto T, Hao K, Takagi Y, Matsumoto Y, Nakayama M, Miyata S, Sakata Y, Ito K, Shimokawa H. Circadian variation of Rho-kinase activity in circulating leukocytes of patients with vasospastic angina. *Circ J* 2014;**78**:1183–1190.
15. Odaka Y, Takahashi J, Tsuburaya R, Nishimiya K, Hao K, Matsumoto Y, Ito K, Sakata Y, Miyata S, Manita D, Hirowatari Y, Shimokawa H. Plasma concentration of serotonin is a novel biomarker for coronary microvascular dysfunction in patients with suspected angina and unobstructive coronary arteries. *Eur Heart J* 2017;**38**:489–496.
16. Sato K, Kaikita K, Nakayama N, Horio E, Yoshimura H, Ono T, Ohba K, Tsujita K, Kojima S, Tayama S, Hokimoto S, Matsui K, Sugiyama S, Yamabe H, Ogawa H. Coronary vasomotor response to intracoronary acetylcholine injection, clinical features, and long-term prognosis in 873 consecutive patients with coronary spasm: analysis of a single-center study over 20 years. *J Am Heart Assoc* 2013;**2**:e000227.
17. Fonarow GC, Peacock WF, Phillips CO, Givertz MM, Lopatin M; ADHERE Scientific Advisory Committee and Investigators. Admission B-type natriuretic peptide levels and in-hospital mortality in acute decompensated heart failure. *J Am Coll Cardiol* 2007;**49**:1943–1950.
18. Omland T, Pfeffer MA, Solomon SD, de Lemos JA, Røsjø H, Šaltytė Benth J, Maggioni A, Domanski MJ, Rouleau JL, Sabatine MS, Braunwald E; PEACE Investigators. Prognostic value of cardiac troponin I measured with a highly sensitive assay in patients with stable coronary artery disease. *J Am Coll Cardiol* 2013; **61**:1240–1249.
19. Kajikawa M, Noma K, Maruhashi T, Mikami S, Iwamoto Y, Iwamoto A, Matsumoto T, Hidaka T, Kihara Y, Chayama K, Nakashima A, Goto C, Liao JK, Higashi Y. Rho-associated kinase activity is a predictor of cardiovascular outcomes. *Hypertension* 2014;**63**:856–864.
20. Dong M, Liao JK, Yan B, Li R, Zhang M, Yu CM. A combination of increased Rho kinase activity and N-terminal pro-B-type natriuretic peptide predicts worse cardiovascular outcome in patients with acute coronary syndrome. *Int J Cardiol* 2013;**167**:2813–2819.
21. Dong M, Liao JK, Fang F, Lee AP, Yan BP, Liu M, Yu CM. Increased Rho kinase activity in congestive heart failure. *Eur J Heart Fail* 2012;**14**:965–973.