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## Increased Rho Kinase Activity in a Taiwanese Population With Metabolic Syndrome

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### Abstract

**Objectives**—We sought to determine whether Rho kinase (ROCK) activity is increased in a Taiwanese population with metabolic syndrome (MetS).

**Background**—Recent studies suggest that ROCK may be involved in the pathogenesis of MetS, but clinical studies linking ROCK with MetS are lacking.

**Methods**—We studied 40 Taiwanese subjects (60% men, mean age  $55.5 \pm 5.6$  years) who were diagnosed with MetS with National Cholesterol Educational Program Adult Treatment Panel III criteria and 40 age- and gender-matched control subjects. Subject demographics were recorded, and blood samples were obtained.

**Results**—Compared with control subjects, ROCK activity, as determined by phosphorylation of myosin binding subunit (MBS) in leukocytes, was greater in MetS subjects (mean phospho-MBS/MBS ratio 0.46 vs. 0.35,  $p = 0.002$ ). A cutoff value for ROCK activity of 0.39 predicted the presence of MetS with specificity and sensitivity rates of 70%. Plasma high-sensitivity C-reactive protein was greater (5.5 mg/l, 95% confidence interval [CI] 3.1 to 7.2 mg/l vs. 2.8 mg/l, 95% CI 1.1 to 3.9 mg/l,  $p = 0.01$ ) and adiponectin was lower (4.9  $\mu\text{g/ml}$ , 95% CI 3.2 to 6.1  $\mu\text{g/ml}$  vs. 5.9  $\mu\text{g/ml}$ , 95% CI 4.2 to 7.5  $\mu\text{g/ml}$ ,  $p = 0.01$ ) in MetS subjects compared with control subjects, but plasma levels of interleukin-6 and tumor necrosis factor- $\alpha$  were not different ( $p > 0.05$  for both). Body mass index, waist circumference, fasting glucose, high-sensitivity C-reactive protein, and triglyceride levels were associated with increased levels of ROCK activity. The risk of increased ROCK activity increased with the number of MetS components ( $p$  for trend  $< 0.001$ ).

**Conclusions**—Rho kinase activity is increased in Taiwanese subjects with MetS and is associated with each component of MetS and markers of inflammation. These findings suggest that ROCK activity may be a novel serological marker of MetS.

Metabolic syndrome (MetS) is a cluster of atherosclerotic cardiovascular disease risk factors and is closely associated with insulin resistance and obesity (1,2). The precise diagnosis of MetS, however, is still somewhat controversial, and no serological markers are available for its detection. Nevertheless, according to the criteria of National Cholesterol Educational Program Adult Treatment Panel (ATP)-III (3), the prevalence of MetS in U.S. adults who are older than 20 years of age is approximately 24% (4). Metabolic syndrome is a growing problem

worldwide, particularly in the Asian population (5). The World Health Organization Expert Consultation recommended a lower cut-off point for observed risk in Asian populations than that for non-Asian populations, varying from 22 to 25 kg/m<sup>2</sup> (6). Some studies on migrants from Asian countries to Western countries also indicated that Asian populations might be more susceptible to the Western diet and lifestyle than white ones in developing the MetS (7,8). Patients with MetS have twice the incidence of developing new-onset diabetes compared with those without MetS (9). Metabolic syndrome, therefore, is associated with increasing risk of developing diabetes and cardiovascular disease.

Several inflammatory mediators and adipocytokines, including tumor necrosis factor (TNF)-alpha (10), interleukin (IL)-6 (11), high-sensitivity C-reactive protein (hs-CRP) (12,13), and adiponectin (14) are associated with the development of insulin resistance and MetS (15,16). Despite these associations, the pathogenesis of MetS remains unknown. We recently reported that Rho kinase (ROCK) mediates the expression of plasminogen activator inhibitor-1 under hyperglycemic conditions (17). Furthermore, ROCK is upregulated under inflammatory conditions (18) and may be involved in adipocyte differentiation (19). Thus, growing evidence suggest that ROCK may contribute to the pathogenesis of MetS. However, clinical studies are lacking, which definitively link ROCK activity with MetS.

Rho kinase is a serine/threonine kinase that mediates the downstream signaling of the small guanosine triphosphate-binding protein, Rho, on the actin cytoskeleton (20). ROCK consists of 2 isoforms, ROCK1 and ROCK2. In mostly animal models, the inhibition of ROCK ameliorates many cardiovascular conditions, including hypertension (21), atherosclerosis (22), myocardial fibrosis (23), and stroke (24). Furthermore, ROCK also could regulate insulin signaling and glucose metabolism through direct phosphorylation of the insulin receptor substrate (IRS)-1 (25). Thus, it is likely that ROCK plays an important role in the pathogenesis of MetS and diabetes. However, evidence is lacking showing that ROCK activity is increased in human subjects with MetS. In the current study, we measured ROCK activity in Taiwanese population with MetS and determined whether ROCK activity is an independent marker of MetS and whether it correlates with other components and risk markers of MetS.

## Materials and Methods

### Study subjects

We prospectively enrolled 40 consecutive Taiwanese patients (60% men, ages 35 to 62 years), who were diagnosed with MetS with ATP-III criteria, from an outpatient clinic at the National Cheng Kung University Medical Center from 2005 to 2006. Patients who had a history of percutaneous coronary intervention within 6 months or coronary artery bypass surgery within 1 year were excluded. Other exclusion criteria included patients who had heart failure, cardiomyopathy, or valvular heart diseases. Because statins could affect ROCK activity through modulation of Rho (26), patients taking statins before enrollment also were excluded. A volunteer control group consisting of age- and gender-matched healthy subjects (n = 40, 60% men), with a mean age of 53.8 ± 6.6 years, also was enrolled. These control subjects showed no clinical or electrocardiographic evidence of ischemia or cerebrovascular disease. All subjects provided their informed consent. The study protocol was approved by the Human Research Subjects review committee of the National Cheng Kung University Medical Center.

### Definition of MetS

We used ATP-III criteria for MetS (3), which require 3 or more of the following components: 1) obesity as defined by waist circumference >102 cm (40 inches) in men and >88 cm (35 inches) in women; 2) a low level of high-density lipoprotein cholesterol (HDL-C), defined as HDL-C <40 mg/dl in men and <50 mg/dl in women; 3) high triglycerides (TG), defined as TG

≥150 mg/dl; 4) high level of glucose, defined as fasting glucose ≥110 mg/dl; and 5) increased blood pressure, defined as blood pressure ≥130/85 mm Hg.

### Biochemical analysis

Venous blood samples were drawn from each subject after ≥8 h or overnight fasting. The samples were stored at -80°C until biochemical assay by blinded investigators. Triglycerides were determined enzymatically. High-density lipoprotein cholesterol was measured by the phosphotungstate method. Glucose was enzymatically determined by the hexokinase method. Tumor necrosis factor- $\alpha$  was measured by enzyme immunoassay with a minimum detectable level of 6 pg/ml (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). High-sensitivity IL-6 was also measured by enzyme immunoassay (Dainippon Pharmaceutical Co., Ltd.). High-sensitivity CRP was measured by latex nephelometry (BNII, Dade Behring Co., Ltd., Milton Keynes, Buckinghamshire, United Kingdom). Adiponectin concentrations were determined by enzymelinked immunosorbent assay (Otsuka Pharmaceutical Co., Ltd., Rockville, Maryland). Interassay coefficients of variation of each variable were as follows: TNF- $\alpha$ , <9.6%; IL-6, <7.5%; hs-CRP, <4.0%; adiponectin, <8.6%.

### Leukocyte isolation

Twenty milliliters of blood sample was mixed with Hanks' balanced salt solution (HBSS) in a 50-ml citrate-containing tube. Ten milliliters of Histopaque (Histopaque-1077, Sigma Chemical Co., St. Louis, Missouri) were layered with 25 ml of diluted blood in 2 tubes and centrifuged at room temperature for 30 min at 350 g. The supernatant containing the leukocytes was aspirated, mixed with HBSS and diluted with 2% dextran (1:1 ratio), and kept rest at room temperature for 30 min. The top layer is then aspirated, mixed with HBSS, and centrifuged at room temperature for 5 min at 350 g. The supernatant was discarded, and the pellet containing the leukocytes was resuspended in 3 ml of cold phosphate-buffered saline. After swirling the tubes for 30 s, HBSS was added to stop the lysis. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 5 ml of M199.

After determining cell yield and viability by using the trypan blue exclusion test (usually 4 to  $80 \times 10^6$  viable cells with a viability of more than 95%), we diluted the suspension with HBSS to achieve  $5 \times 10^6$  cells/ml. Then, 400  $\mu$ l of the leukocyte suspension was transferred to 4 sterile 1.5-ml tubes. We added 100  $\mu$ l of fixative solution (50% trichloroacetic acid [Sigma]), 50 mmol/l dichlorodiphenyltrichloroethane (Sigma), and protease inhibitors (Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany) to each tube. After vortexing and centrifuging the samples at 4°C for 5 min at 11,000 g, we removed the supernatant, added HBSS, and centrifuged the samples again at 4°C for 1 min at 11,000 g. Finally, we removed the supernatant with a micropipette. The leukocyte pellets were stored at -80°C until use.

### Assay for ROCK expression and activity

After centrifugation, pellets were dissolved in 10  $\mu$ l of 1 mol/l Tris and mixed with 100  $\mu$ l of extraction buffer (8 mol/l urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol, 0.02% BPB dye). Equal amounts of cell extracts were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, Massachusetts). NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analyses from several membranes. The membranes were incubated with rabbit anti-phospho-specific Thr<sup>853</sup>-myosin binding subunit (MBS) polyclonal antibody (Biosource-Invitrogen, Carlsbad, California) (17), rabbit anti-MBS polyclonal antibody (Covance, Princeton, New Jersey), anti-ROCK2 monoclonal antibody, anti-ROCK1 monoclonal antibody (BD Biosciences, San Jose, California), or antiactin monoclonal antibody (Sigma). Bands were visualized with the use of the ECL detection kit (Amersham Biosciences, a division of GE Healthcare, Piscataway, New

Jersey). Rho kinase activity was expressed as the ratio of phosphorylation levels of myosin binding subunit (pMBS) in each sample per pMBS in each positive control divided by MBS in each sample per MBS in each positive control.

### Statistical analysis

All statistical analyses were conducted with the SPSS statistical package for Windows version 13.0 (SPSS Inc., Chicago, Illinois). The frequencies between MetS and the controls were compared with chi-square analysis. Independent-samples *t* test was used for comparison of mean values of parametric continuous variables (expressed as mean  $\pm$  SD) between MetS and control groups. Because of asymmetric distribution, values for TNF-alpha, IL-6, hs-CRP, and adiponectin were analyzed by nonparametric methods and shown as the geometric means with 95% confidence intervals for those means. For nonparametric analysis, we used Mann-Whitney *U* test to evaluate the difference of levels between groups. Receiver-operating characteristic analysis was performed to determine the best cutoff value of ROCK activity to differentiate MetS and the control subjects. Because smoking status was found to strongly influence ROCK activity (27) and ROCK activity itself was not normally distributed, we used partial correlation coefficients adjusted for smoking status to compute the association between inflammation-related markers and levels of ROCK activity. The odds ratios by number of the MetS components for elevated ROCK activity were also calculated. All reported *p* values were 2-sided, and a *p* value of  $<0.05$  was considered statistically significant.

### Results

The prevalence of cardiovascular risk factors, including hypertension, fasting glucose levels, lipid profiles, smoking, body mass index (BMI), and waist circumference, are shown for control and MetS subjects (Table 1). Compared with the control group, the average age, gender, medications for hypertension and glucose control, and percentage of smokers in the MetS group were similar. Blood pressure, fasting glucose, low-density lipoprotein cholesterol, TG levels, BMI, and waist circumference were all greater among MetS subjects, and HDL-C levels were lower among MetS subjects.

The protein levels of ROCK1 and ROCK2 were similar in control and MetS subjects (Figs. 1A and 1B). However, compared with control subjects, MetS subjects had greater ROCK activities, as measured by pMBS/MBS (Figs. 1C and 1D). Among the inflammatory markers measured, plasma hs-CRP levels were greater in MetS subjects compared with those of control subjects, but not IL-6 or TNF-alpha (Table 2). Adiponectin levels were lower in MetS subjects. The cutoff value for ROCK activity that predicted the presence of MetS was  $>0.39$  by receiver-operating characteristic analysis (area under curve =  $0.76 \pm 0.07$ , range 0.63 to 0.89,  $p = 0.001$ ) (Fig. 2). The sensitivity and specificity rates for this ratio in detecting MetS in our cohort were 70% and 70%, respectively.

To determine whether ROCK activity is a novel risk marker for MetS and whether it correlates with components of MetS, we performed an association analysis to determine the correlation coefficient of ROCK activity with components of MetS (Table 3). Among the associated factors, BMI, waist circumference, fasting glucose, hs-CRP, and TG levels were all positively associated with increased levels of ROCK activity. In contrast, adiponectin and HDL-C levels were negatively associated with ROCK activity. After adjusting for age and smoking, those subjects with a greater number of MetS components ( $\geq 3$ ) had higher odds ratios for elevated ROCK activity when compared with those with  $<3$  components. These findings suggest that elevated ROCK activity requires the presence of several risk components that are associated with MetS (Table 4).

## Discussion

We have shown that ROCK activity is greater among Taiwanese subjects with MetS. Increased levels of ROCK activity (pMBS/MBS ratio  $>0.39$ ), independently predict the diagnosis of MetS. In addition, higher ROCK activity was associated with several components of MetS, such as greater BMI and waist circumference, and correlated with greater hs-CRP and lower adiponectin levels. These findings indicate that ROCK activity is a novel risk marker for MetS and suggest that ROCK may be involved in the development and progression of MetS.

Although the precise mechanism by which ROCK activity is increased among MetS subjects is not known, several possible mechanisms could explain the observed findings. For example, increased ROCK activity is associated with hypertension and phosphorylation of IRS-1 by ROCK, which prevents insulin signaling and glucose transporter-4 translocation, could lead to altered glucose metabolism (19,26). Interestingly, inhibition of ROCK ameliorates insulin sensitivity in obese, but not lean, rats suggesting that ROCK activity is increased by obesity (28). Another possibility that links elevated ROCK activity with MetS is inflammation. Several inflammatory molecules and adipocytokines, including TNF-alpha, IL-6, and hs-CRP, have been shown to be associated with insulin resistance and MetS (10-14). However, in the present cohort, higher hs-CRP, but not TNF-alpha or IL-6, was associated with greater ROCK activity. Interestingly, some studies showed that hs-CRP, but not TNF-alpha or IL-6, is associated with MetS (14,29,30). Nevertheless, the lack of association between ROCK and TNF-alpha or IL-6 might be due to both the wide range of distribution of TNF-alpha and IL-6 in our cohort and the small number of subjects in the present study.

Rho kinase activity was greater among MetS subjects, which may correlate with insulin resistance. In our study, most of our patients were obese with greater BMI and waist circumference values, 2 contributors to MetS and insulin resistance. Insulin resistance acts as bridge between obesity and each component of MetS via different mechanisms, including altering lipid metabolism (19,25) and adipokine secretions (14). Indeed, weight loss can result in clinical meaningful improvements in multiple metabolic parameters and blood pressure (31). Obesity itself can precipitate an inflammatory response and lead to free radical generation (32), thus further augmenting ROCK signaling. Indeed, obesity is one of the major components of MetS. Interestingly, ROCKs are involved in adipocyte differentiation (19), which also may affect the pathogenesis of MetS. As mentioned previously, increased ROCK activity could lead to the phosphorylation of IRS-1 and subsequently impaired the glycogen synthase or glucose transportation in peripheral tissues, thereby increasing insulin resistance.

In our current study, despite increase in leukocyte ROCK activity in MetS subjects, ROCK1 and ROCK2 expression were similar in control and MetS subjects. These findings suggest that the activation of ROCK rather than induction of ROCK expression is the underlying mechanism that is associated with components of the metabolic syndrome. Indeed, agents or conditions that increase ROCK activity generally do not affect ROCK expression because the regulation of ROCKs occurs through their association with activated Rho guanosine triphosphatase in response to G-proteincoupled receptors or growth factors. Thus, conditions associated with components of the metabolic syndrome, such as hypertension, smoking, and dyslipidemia, are more likely to lead to the activation of the Rho/ROCK pathway rather than induction of ROCK expression. It remains to be determined how components of MetS lead to the activation of ROCK.

### Study limitations

Some limitations of this study should be noted. First, the present case-control study did not allow for inferring causality, which should be considered hypothesis generating and requires further confirmation by prospective studies and, if possible, therapeutic clinical trials. Second,

all subjects in the present study were Taiwanese. Thus, the present results may not extrapolate to other ethnic groups. Nevertheless, our finding showing that ROCK activity is an important serological marker of MetS may help clarify the diagnosis of MetS in future patient populations.

## Conclusions

Our results show that ROCK activity is elevated among MetS human subjects and associated with some inflammatory markers. It remains to be determined whether increased ROCK activity correlates with increase cardiovascular event rates in MetS and whether reduction of ROCK activity is associated with therapeutic benefits in subjects with MetS.

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## Abbreviations and Acronyms

BMI, body mass index  
HBSS, Hanks' balanced salt solution  
HDL-C, high-density lipoprotein cholesterol  
hs-CRP, high-sensitivity C-reactive protein  
IL, interleukin  
IRS, insulin receptor substrate  
MBS, myosin binding subunit  
MetS, metabolic syndrome  
ROCK, Rho kinase  
TG, triglycerides  
TNF, tumor necrosis factor

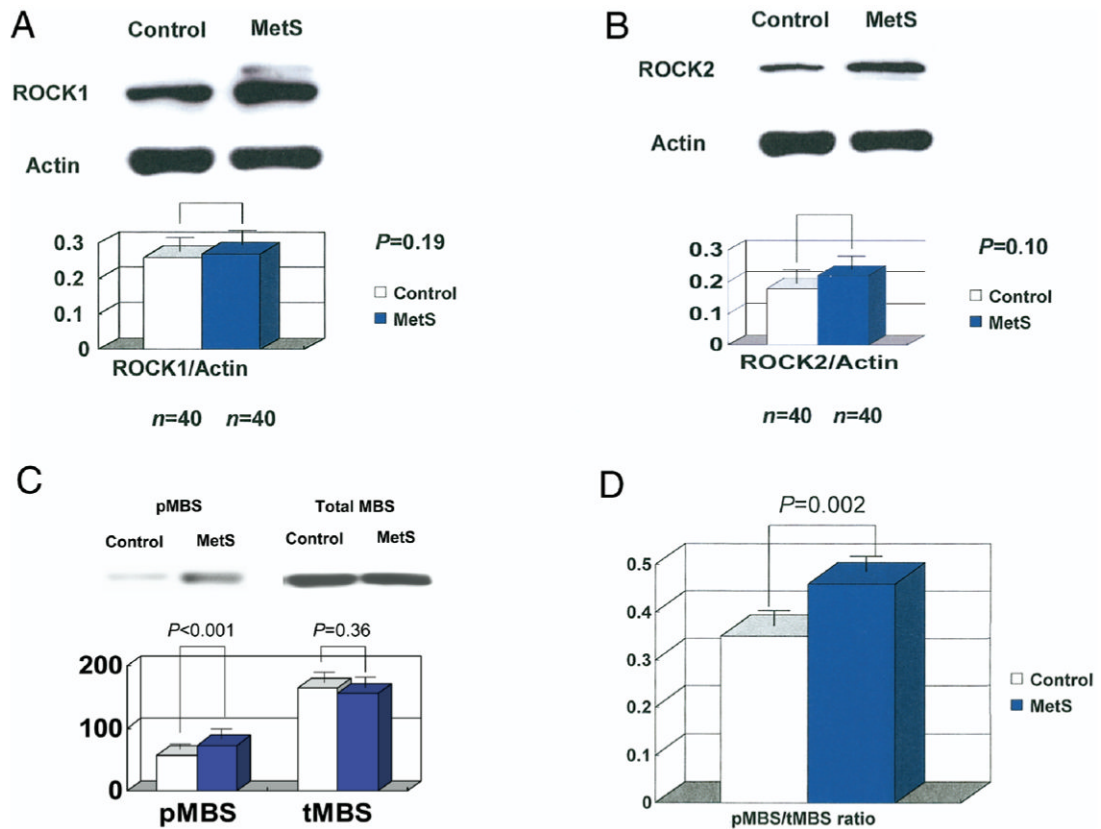
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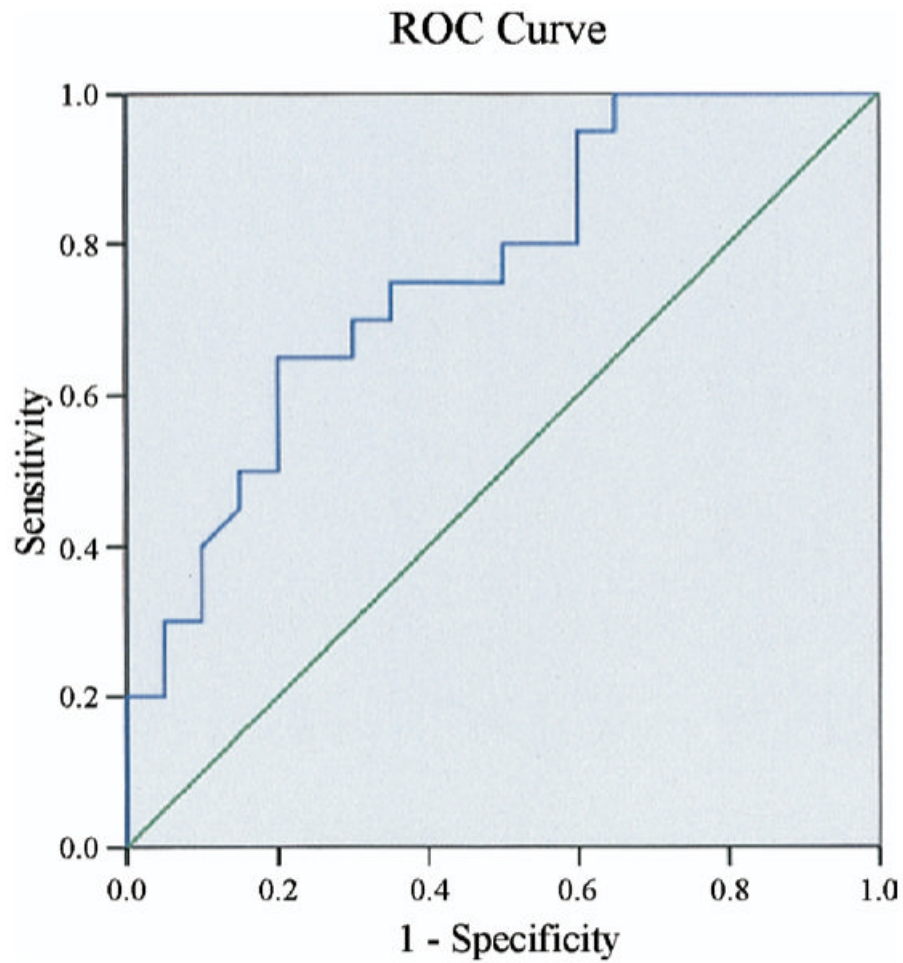
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**Figure 1. ROCK Activity Is Greater Among Asian Subjects With MetS**

(A) Baseline protein expression of Rho kinase (ROCK)1 by immunoblotting method; (B) baseline protein expression of ROCK2 by immunoblotting method; (C) phosphorylation levels of MBS (pMBS) and total MBS (tMBS) were determined by immunoblotting methods; (D) ROCK activity, expressed as pMBS/MBS, between metabolic syndrome (MetS) patients and controls. Three repeated experiments were performed in duplicate.



**Figure 2. ROC Analysis to Determine the Cutoff Value to Differentiate MetS**

The best cutoff value for Rho kinase activity to differentiate the presence of metabolic syndrome (MetS) was 0.39; both sensitivity and specificity rates were 70%. The area under the curve =  $0.76 \pm 0.07$ ,  $p = 0.001$ . ROC = receiver-operating characteristic.

**Table 1**  
**Clinical Manifestations of Metabolic Syndrome and Control Subjects**

|                          | Controls (n = 40) | MetS (n = 40) | p Value |
|--------------------------|-------------------|---------------|---------|
| Age (yrs)                | 59.6 ± 6.5        | 60.1 ± 6.7    | 0.813   |
| Gender (male)            | 24 (60%)          | 24 (60%)      | 0.815   |
| Hypertension             | 8 (20%)           | 18 (45%)      | 0.004   |
| BMI (kg/m <sup>2</sup> ) | 24.1 ± 2.2        | 33.4 ± 1.5    | <0.001  |
| Fasting glucose (mg/dl)  | 93.5 ± 5.9        | 120.8 ± 5.9   | <0.001  |
| LDL-C (mg/dl)            | 122 ± 8           | 131 ± 11      | 0.01    |
| TG (mg/dl)               | 147 ± 18          | 159 ± 22      | 0.02    |
| HDL-C (mg/dl)            | 49 ± 6            | 34 ± 5        | 0.002   |
| Waist circumference (cm) | 84.3 ± 7.2        | 104.3 ± 8.9   | <0.001  |

Values are expressed as mean ± SD or n (%).

BMI = body mass index; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; MetS = metabolic syndrome; TG = triglycerides.

**Table 2**  
**Baseline Inflammatory Markers of Metabolic Syndrome Subjects and Control Subjects**

|                           | Control (n = 40) | MetS (n = 40)   | p Value |
|---------------------------|------------------|-----------------|---------|
| hs-CRP (mg/l)             | 2.8 (1.1-3.9)    | 5.5 (3.1-7.2)   | 0.01    |
| IL-6 (pg/ml)              | 1.7 (0.9-1.9)    | 1.8 (1.1-2.1)   | 0.77    |
| TNF- $\alpha$ (pg/ml)     | 12.0 (10.1-14.1) | 12.8 (9.9-15.2) | 0.91    |
| Adiponectin ( $\mu$ g/ml) | 5.9 (4.2-7.5)    | 4.9 (3.2-6.1)   | 0.01    |

Values are expressed as geometric means with corresponding 95% confidence intervals (in parentheses).

hs-CRP = high-sensitivity C-reactive protein; IL = interleukin; TNF = tumor necrosis factor.

**Table 3**  
**Partial Correlation Coefficient Adjusted for Smoking Status Between Clinical and Biochemical Parameters With Rho Kinase Activity Among Metabolic Syndrome Subjects**

| Covariants          | Coefficient | p Value (2-tail) |
|---------------------|-------------|------------------|
| BMI                 | 0.523       | <0.001           |
| Fasting glucose     | 0.410       | 0.009            |
| Waist circumference | 0.412       | 0.009            |
| Adiponectin         | -0.412      | 0.009            |
| hs-CRP              | 0.359       | 0.023            |
| HDL-C               | -0.352      | 0.026            |
| TG                  | 0.356       | 0.024            |
| Age                 | 0.157       | 0.333            |

Abbreviations as in Tables 1 and 2.

**Table 4**  
**Adjusted Odds Ratios of Greater ROCK Activity With the Number of MetS Components**

| Number of MetS Components | Odds Ratio (95% CI) for Greater ROCK Activity (phospho-MBS/MBS >0.39) <sup>*</sup> |
|---------------------------|--|
| 0                         | 1.0  |
| 1                         | 1.15 (0.94-1.46)   |
| 2                         | 1.29 (0.99-1.93)   |
| 3                         | 1.89 (1.34-2.66) <sup>‡</sup>  |
| ≥4                        | 2.70 (1.98-4.18) <sup>‡</sup>  |
| Trend                     | <0.001   |

CI = confidence interval; MBS = myosin binding subunit; ROCK = Rho kinase.

<sup>\*</sup> Risks are compared with subjects without any risk factor component of metabolic syndrome (MetS) and adjusted for age and smoking status.

<sup>‡</sup>  $p < 0.01$ ;

<sup>‡</sup>  $p < 0.001$ .