Plasma Level of Platelet-Derived Microparticles Is Associated with Coronary Heart Disease Risk Score in Healthy Men

Tetsuya Ueba¹, Shosaku Nomura², Norihito Inami³, Tomofumi Nishikawa¹, Motohiro Kajiwara¹, Ryoichi Iwata¹, and Kohsuke Yamashita¹

¹Department of Neurosurgery, Kishiwada City Hospital, Osaka, Japan
²Department of Hematology, Kishiwada City Hospital, Osaka, Japan
³2nd Department of Internal Medicine, Kansai Medical University, Osaka, Japan

Aim: The aim of this study was to clarify the relationship between platelet-derived microparticles (PDMPs) and the Framingham 10-yr coronary heart disease (CHD) risk score.

Methods: A cross-sectional study of healthy volunteers free of medication, and cardiovascular or cerebrovascular disease was conducted. The subjects were 190 Japanese men (median age 41). An ELISA kit and monoclonal antibodies against CD42b and CD42a (glycoprotein Ib and IX) were used.

Results: PDMPs are correlated with platelet count, high sensitivity C-reactive protein (hsCRP), and diastolic blood pressure by multivariate analysis (R²=0.316, p<0.001). Quartile range of PDMPs is significantly associated with the 10-yr CHD risk score after adjusting for age, platelet count, hsCRP, and hypertension (p=0.033) and for age, platelet count, hsCRP, and presence of metabolic syndrome (MS) (p=0.020). In individuals with a predicted 10-yr risk for CHD ≥8% (corresponding with the highest quartile), compared to those with a predicted 10-yr risk <8%, the odds ratio (OR), adjusted for age, platelet count, hsCRP, and hypertension, was 3.3 (1.2–8.9) and adjusted for age, platelet count, hsCRP, and MS, was 4.5 (1.6–11.8). The age-, platelet count-, hsCRP- and hypertension-adjusted OR for a 10-yr CHD risk score ≥8% was 0.8 (0.5–1.3) for hsCRP and 3.9 (1.6–9.4) for hypertension. The age-, platelet count-, hsCRP- and MS-adjusted OR for a 10-yr CHD risk score ≥8% was 0.7 (0.4–1.2) for hsCRP and 7.9 (2.6–24.5) for MS.

Conclusion: Elevated PDMPs are associated with the 10-yr CHD risk score in healthy men.


Key words: Atherothrombosis, Platelet-derived microparticle, Framingham coronary heart disease risk score, High sensitivity CRP

Introduction

Platelet activation by various agonists or shear stress results in the shedding of submicroscopic membrane vesicles, platelet-derived microparticles (PDMPs)¹-⁴. These are defined as vesicles measuring less than 1.5 μm in diameter that are enriched in procoagulant platelet proteins⁵ and express several platelet receptors, such as CD42b (glycoprotein Ib) and CD42a (glycoprotein IX) and various platelet-derived ligands, i.e. P-selectin and CD40 ligand⁶,⁷. Progressive atherosclerosis induces the elevation of PDMPs via shear-induced platelet activation⁸,⁹. As PDMP levels are also increased in patients with type 2 diabetes mellitus and acute coronary syndrome¹⁰-¹⁶, PDMPs may play a role in the pathogenesis of arterial thrombosis and atherosclerosis¹⁷,¹⁸.

Visceral adiposity, hypertension, insulin resistance, high triglyceride-, and low high-density lipoprotein (HDL) cholesterol levels characterize atherosclerotic metabolic abnormalities, so-called metabolic syndrome (MS)¹⁹-²², which is strongly associated with atherothrombotic events, such as cardiovascular²⁰,²¹ and cerebrovascular disease²². Remnant lipopro-
teins (Rem-L) and high sensitivity C-reactive protein (hsCRP) were also reported to be associated with MS and to promote atherosclerosis. We also reported that PDMPs were associated with positive metabolic syndrome (MS) criteria in healthy volunteers without signs, symptoms, or a history of cardio- or cerebrovascular disease and any medication.

Recently, an adverse prognosis associated with MS in coronary artery disease was reported. A diagnosis of MS might add little in clinical practice, although these are many reports that MS was associated with atherothrombotic events seen in patients with cardio- and cerebrovascular disease.

Clarifying the relationship between PDMPs and the 10-yr coronary heart disease (CHD) risk score in healthy individuals is of importance. We measured the plasma level of PDMP by enzyme-linked sorbent assays (ELISA) using monoclonal antibodies against CD42b (GPIb) and CD42a (GPIX) and analyzed the association of the PDMP level with the 10-yr CHD risk score.

**Subjects and Methods**

**Subjects**

The Institutional Review Board of Kishiwada City Hospital approved our study protocol; prior written informed consent was obtained from all participants. We recruited 190 volunteers without signs, symptoms, or a history of cardio- or cerebrovascular disease; none took anti-hypertensive, anti-hyperlipidemic, anti-diabetic, or steroid or nonsteroid anti-inflammatory drugs on a daily basis. We excluded individuals who took these medications daily because they can affect the PDMP level.

**Measurement of Traditional Cardiovascular Risk Factors, Lipoproteins, and Other Biochemical Parameters**

Anthropometric data (height and weight), blood pressure, and current cigarette smoking were recorded. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

We defined MS according to guidelines promulgated by Adult Treatment Panel III (ATPⅢ), which proposed 5 criteria for MS; however, as the body mass of Japanese tends to be lower than in Caucasians, we found it inappropriate to use the ATPⅢ criteria for abdominal obesity. Instead, as proposed by the Japanese Society for Obesity, we adopted a waist circumference of >85 cm for men and >90 cm for women. MS was defined as the fulfillment of ≥3 of the following 5 criteria: (1) waist circumference >85 cm (men) and >90 cm (women), (2) blood pressure >130/85 mmHg, (3) fasting glycemia ≥110 mg/dL, (4) HDL <40 mg/dL (men) and <50 mg/dL (women), (5) triglyceride (TG) ≥150 mg/dL.

The Framingham 10-yr CHD risk score was determined based on total and high-density lipoprotein cholesterol, systolic and diastolic blood pressure, age and gender, the presence or absence of diabetes mellitus, and current smoking habits.

Fasting blood samples were drawn from a peripheral vein with a vacuum system using a 21-gauge needle. We used kits (Kyowa Medex, Tokyo, Japan) to determine directly the total, high-density and low-density cholesterol (TCho, HDL, LDL, Rem-L) levels.

**Measurement of PDMPs and hsCRP**

For PDMP assay, blood samples were collected with a 21-gauge needle from a peripheral vein in vacutainers containing EDTA-ACD (NIPRO Co. Ltd., Japan) to minimize platelet activation. The samples were handled as described in the manufacturer’s protocol. Briefly, the samples were gently mixed by inverting the tube once or twice, stored at room temperature (RT) for 2–3 hr, and centrifuged at 8,000 g for 5 min at RT. Storage of the samples at RT for 2–3 hr did not affect the PDMP level. Immediately after centrifugation, 200 μL of upper-layer supernatants from 2-ml samples were collected to avoid contamination of the platelets and the samples were stored at −40°C until analysis. The PDMP level was measured in duplicate and the mean values were recorded. For the measurement of PDMPs we used an ELISA kit (JIMRO Co. Ltd., Japan) and monoclonal antibodies against glycoprotein CD42b and CD42a (glycoprotein Ib and IX). Uncoated vacutainers were used for hsCRP assay; these were stored at −40°C until analysis. Immunoradiometric assay for hsCRP was performed as previously described.

**Statistical Analysis**

Variables are expressed as the mean (standard deviation, SD) when they were normally distributed and as the median and minimum-maximum when they were not. The correlation of plasma PDMPs and other response variables was assessed by univariate analysis. Multivariate analysis was used to identify independent predictors of elevated PDMP levels. All variables that were significant (p<0.1) by univariate analysis were entered in the equation. Variables with a high Pearson product-moment correlation coefficient (r) value (>0.8) were excluded from the equation as
potential confounding factors. The association between the quartile range of PDMPs and the 10-yr CHD risk score was assessed by analysis of covariance (ANCOVA). We performed logistic regression analysis to determine whether the PDMP level is associated with a 8%≤10-yr CHD risk score (corresponding with the upper quartile range of the 10-yr CHD risk score). We logarithmically transformed and calculated all variables not in normal distribution for greater symmetry of the distribution. Differences of \( p < 0.05 \) were considered significant. Analyses were performed with the SPSS 14.0.7 program.

**Results**

**Characteristics of the Study Population**

As shown in Table 1, 190 men were included in our cross-sectional study. The median age was 41 years old. Forty out of 190 were positive for MS. The median of the estimated 10-yr CHD risk score was 4.0%. The mean and median values for BMI, waist circumference, systolic and diastolic blood pressure, TC, HDL, LDL, TG, and fasting glucose were within normal limits; 35.3% of the men were smokers. The levels of Rem-L and hsCRP are shown in Table 1. The 10-yr CHD risk score were determined as described in Materials and Methods.

**Level and Distribution of Plasma PDMPs in Healthy Men**

The PDMP value, a response variable, did not exhibit a normal distribution. The empirical cumulative distribution of the logarithmically transformed values in our study sample of 190 men was smooth and symmetrical, indicating that the PDMP values in this population approximated a log-normal distribution. The levels in men were 8.5 IU/mL (Table 1).

**Correlation between PDMPs and Lipoproteins, an Inflammation Marker, and Traditional Cardiovascular Risk Factors**

Univariate analysis showed that BMI, waist circumference, systolic- and diastolic- blood pressure, platelet count, total cholesterol, uric acid, triglycerides, Rem-L, hsCRP, and current smoking were significantly correlated (Table 2). High density cholesterol was inversely correlated. We logarithmically transformed and calculated all variables not in normal distribution. Before multivariate analyses, we performed Pearson’s product-moment correlation analyses among all relevant variables. In cases where the correlation coefficients \( r \) exceeded 0.8 among these variables, we selected those that presented with higher \( \beta \) regression coefficient by univariate analysis. We also included variables with \( p \) values <0.1 in a univariate model. In the multivariate model we calculated the waist circumference, systolic- and diastolic- blood pressure, platelet count, total- and high density- cholesterol, uric acid, fast glucose, Rem-L, hsCRP, and current smoking. As shown in Table 2, diastolic blood pressure, platelet count, and hsCRP were significant factors (\( R^2 = 0.316, \quad p < 0.001 \)).

**Distributions between Each Variable and PDMP Levels**

As shown in Table 2, platelet count and hsCRP were significant factors predicting plasma PDMP levels. The distributions of platelet counts and PDMP levels and of hsCRP levels and PDMP levels are shown in Fig. 1 because the distribution is as critical as the value in the clinical setting. The unadjusted distribution of platelet counts and PDMP levels is shown (\( R^2 = 0.121 \)) (Fig. 1A), as well as the unadjusted distribution of hsCRP and PDMP levels (\( R^2 = 0.077 \)) (Fig. 1B).

The unadjusted distribution of Framingham 10-yr CHD risk scores and PDMP levels is shown in Fig. 2A and the analysis after adjustment for age, platelet count, hsCRP, and MS is shown in Fig. 2B (\( p = 0.015 \)).

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**Table 1. Characteristics of the 190 Study Subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>190</td>
</tr>
<tr>
<td>Age (years)</td>
<td>41.0 (30–71)</td>
</tr>
<tr>
<td>BMI</td>
<td>23.9 (3.0)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>84.6 (8.0)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>125.6 (15.1)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>75.7 (12.0)</td>
</tr>
<tr>
<td>Platelet count (×10^11/μL)</td>
<td>22.9 (4.1)</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>204.8 (35.3)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>59.5 (15.3)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>122.0 (31.3)</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>6.5 (1.2)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>137.0 (37–1,502)</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>94.0 (62–229)</td>
</tr>
<tr>
<td>Rem-L (mg/dL)</td>
<td>5.8 (1.4–61.4)</td>
</tr>
<tr>
<td>hsC-reactive protein (mg/dL)</td>
<td>0.032 (0.020–0.809)</td>
</tr>
<tr>
<td>PDMP (IU/mL)</td>
<td>8.5 (3.0–39.6)</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>40/190 (26.7%)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>67/190 (35.3%)</td>
</tr>
<tr>
<td>CHD risk score (%/10-yr)</td>
<td>4.0 (2–31)</td>
</tr>
</tbody>
</table>

Values in normal distribution are shown as the mean (SD), and values in non-normal distribution as the median (minimum-maximum).
The quartile range of the PDMP level was determined as Q1 (3.0–6.4 IU/mL), Q2 (6.5–8.4 IU/mL), Q3 (8.5–10.7 IU/mL), and Q4 (10.8–39.6 IU/mL), respectively. The number of subjects and the distribution ranges of the 10-yr CHD risk score are shown in Table 3. ANCOVA of the 10-yr CHD risk score in the quartile range of the PDMP level, unadjusted, was significantly associated ($p<0.020$), adjusted for age and platelet count was significantly associated ($p<0.001$), adjusted for age, platelet count, and hsCRP was significantly associated ($p<0.004$), adjusted for age, platelet count, hsCRP, and hypertension was significantly associated ($p<0.033$), and adjusted for age, platelet count, hsCRP, and MS was significantly associated ($p<0.020$) (Table 3). Because the CHD risk scores were significantly high in the presence of MS and the PDMP level was associated with the presence of MS, we used MS, as a confounding factor, in the model. These findings indicate that elevated PDMP levels are associated with the Framingham 10-yr CHD risk score in healthy men.

Association of the Elevated PDMP Level with the 8%<10-yr CHD Risk Score

Next, we investigated whether the PDMP level is associated with the 8%<10-yr CHD risk score. More than 8% of the 10-yr CHD risk score corresponds with the upper quartile range of the 10-yr CHD risk score. The cutoff value of PDMP for CHD risk ≥8%

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Table 2. Predictors of Circulating PDMP Levels by Multivariate Regression Analysis

<table>
<thead>
<tr>
<th>Predictor</th>
<th>β</th>
<th>p</th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.027</td>
<td>0.710*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI</td>
<td>0.208</td>
<td>0.004</td>
<td>0.015</td>
<td>0.840</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>0.190</td>
<td>0.009</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.282</td>
<td>&lt;0.001</td>
<td>-0.053</td>
<td>0.606</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.373</td>
<td>&lt;0.001</td>
<td>0.296</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelet count</td>
<td>0.348</td>
<td>&lt;0.001</td>
<td>0.275</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.144</td>
<td>0.048</td>
<td>0.029</td>
<td>0.713</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.200</td>
<td>0.006</td>
<td>-0.050</td>
<td>0.533</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.142</td>
<td>0.051</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uric acid</td>
<td>0.167</td>
<td>0.022</td>
<td>0.062</td>
<td>0.383</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.198</td>
<td>0.006*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fast glucose</td>
<td>0.139</td>
<td>0.056*</td>
<td>0.082</td>
<td>0.207*</td>
</tr>
<tr>
<td>Rem-L</td>
<td>0.277</td>
<td>&lt;0.001*</td>
<td>0.060</td>
<td>0.504*</td>
</tr>
<tr>
<td>hsC-reactive protein</td>
<td>0.278</td>
<td>&lt;0.001*</td>
<td>0.197</td>
<td>0.003*</td>
</tr>
<tr>
<td>Current smoking</td>
<td>0.160</td>
<td>0.027</td>
<td>0.098</td>
<td>0.132</td>
</tr>
</tbody>
</table>

In multivariate analysis, diastolic blood pressure, platelet count, and hsCRP were significant factors ($R^2=0.316$, $p<0.001$). *means that the variables were not in normal distribution and logarithmically transformed variables were used in the equation.

Fig. 1. As shown in Table 2, platelet count and hsCRP were significant factors predicting plasma PDMP levels. The distributions between each variable are shown since the distribution is as critical as the value in the clinical setting. A) Unadjusted distribution of platelet counts and PDMP levels ($R^2=0.121$). B) Unadjusted distribution of hsCRP levels and PDMP levels ($R^2=0.077$).
was determined as 8.5 IU/mL by the receiver operating characteristic (ROC) curve. The sensitivity and specificity of the elevated PDMP level were 67.9% and 54.5%. The cutoff value of hsCRP for CHD risk was not determined by the ROC curve because the area under curve in the ROC curve of hsCRP for CHD risk was 0.560 (95% CI: 0.474–0.646, p = 0.192), thus, we handled hsCRP as a continuous variable. Compared to subjects with a predicted risk <8% unadjusted and adjusted for age was 4.2 (2.0–8.7) and 7.6 (2.6–21.6), respectively, we used MS, as a confounding factor, in the model. The OR adjusted for age, platelet count, hsCRP, and presence of MS was 4.3 (1.6–11.8) (Table 4). The age-, platelet count-, hsCRP- and hypertension-adjusted OR for a 10-yr CHD risk score ≥8% was 0.8 (0.5–1.3) for hsCRP and 3.9 (1.6–9.4) for hypertension. The age-, platelet count-, hsCRP- and MS-adjusted OR for a 10-yr CHD risk score ≥8% was 0.7 (0.4–1.2) for hsCRP and 7.9 (2.6–24.5) for MS. These findings suggest that the elevated PDMP level could be independently associated with coronary heart disease risk.

Discussion

Our cross-sectional study of 190 healthy men...
clearly evidenced a positive association between the quartile range of the PDMP level and the 10-yr CHD risk score. The elevated PDMP level was associated with the 8% ≥ 10-yr CHD risk score. Indeed, the hsCRP level was indicative of the risk for MS and the 10-yr CHD risk in our study (data not shown) and the PDMP level was significantly associated with MS (data not shown) and with the hsCRP level (Table 2), suggesting that the hsCRP level and the presence of MS might be confounding factors of the PDMP level; however, the PDMP level, measured by ELISA using antibodies against CD42b (GPIb) and CD42a (GPIX), is associated with the 10-yr CHD risk score in healthy volunteers even after adjustment for age, platelet count, hsCRP, and MS, indicating that PDMPs act not only as a marker of disease activity but also as important functional modules in the exacerbation of lesion formation 37-39, i.e. potent procoagulant, proinflammatory, and proatherogenic factors.

PDMPs reportedly play a pivotal role in the acute occlusion of atherosclerotic small arteries or arterioles where pathological levels of fluid shear stress can be found 1, 40-42. Clinically, elevated levels of plasma PDMP are associated with thrombotic disorders 12, 18, 27, 30. PDMPs play an important role in atherothrombotic events; however, their role in the early phase of atherosclerosis in apparently healthy individuals remains to be clarified. This is the first report of the association of the PDMP level and the Framingham 10-yr CHD risk score. Our findings suggest that PDMPs may have a wide range of biological actions, including procoagulant, proinflammatory, and proatherogenic activities 7, 37 in healthy individuals.

Accumulating evidence indicates that PDMPs enhance the expression of cell adhesion molecules, recruit monocytes on the vascular wall, and induce the proliferation of smooth muscle cells 33, 44, thereby exerting pro-atherogenic activity 7, 20, 37, 45. The major factor leading to cellular cross-talk may be the ability of PDMPs to convey biological effectors. Mause et al. 38 showed that PDMPs detected by P-selectin and CD42b (GPIb) played a pivotal role in the delivery of a proinflammatory C-C chemokine, termed regulated upon activation, normal T-cell expressed and secreted (RANTES), by their adhesion to the endothelial surface. They concluded that PDMP rolling facilitates the delivery of RANTES to the inflamed endothelium, favoring monocyte adhesion and plaque infiltration. Activated or apoptotic endothelium also facilitates the release of endothelial cell microparticles (EMPs) 46-48; thus, cross-talk among various cellular microparticles exacerbates atherosclerosis.

Although the Framingham 10-yr CHD risk scores were lower in our study, the PDMP level was associated with the CHD risk score in our healthy volunteers. The PDMP level measured by ELISA in our study may reflect preclinical atherosclerosis, a forerunner of atherosclerosis.

In conclusion, we showed that there is an association between the PDMP level and the Framingham 10-yr CHD risk score. Our data suggest that PDMPs may not only be a marker of disease activity but also act as important functional modules, i.e. as potent procoagulant, proinflammatory, and proatherogenic factors, leading to the occurrence of atherothrombotic events. Cross-sectional or longitudinal studies in large study populations are planned, in which we will measure PDMPs with antibodies against CD42b (GPIb) and CD42a (GPIX) to avoid type II error.

**Limitations of this Study**

Because of the cross-sectional nature of our study we were unable to establish causal relationships. The risk score used in this study is the Framingham 10-yr risk score for Americans and the risk for Japanese is

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**Table 4. Association of PDMP8.5 with the Framingham 10-yr Coronary Heart Disease Risk Score**

<table>
<thead>
<tr>
<th>8% ≥ 10-yr CHD Risk Score</th>
<th>OR</th>
<th>95%CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>2.5</td>
<td>1.3 to 4.9</td>
<td>0.006</td>
</tr>
<tr>
<td>Adjusted for age and platelet</td>
<td>4.0</td>
<td>1.6 to 10.2</td>
<td>0.003</td>
</tr>
<tr>
<td>Adjusted for age, platelet, and hsCRP</td>
<td>4.2</td>
<td>1.6 to 10.8</td>
<td>0.003</td>
</tr>
<tr>
<td>Adjusted for age, platelet, hsCRP, and HT</td>
<td>3.3</td>
<td>1.3 to 8.9</td>
<td>0.016</td>
</tr>
<tr>
<td>Adjusted for age, platelet, hsCRP, and MS</td>
<td>4.3</td>
<td>1.6 to 11.8</td>
<td>0.005</td>
</tr>
</tbody>
</table>

We used logistic regression analysis to determine the odds ratios (OR) for a high Framingham 10-yr coronary heart disease (CHD) risk score status. The cutoff value of PDMP for the 10-yr CHD risk score ≥ 8% (corresponding with the highest quartile) was determined as 8.5 IU/mL (PDMP8.5) by the receiver operating characteristic curve. The sensitivity and specificity of the elevated PDMP level were 67.9% and 54.5%. OR for elevated PDMP in individuals with a predicted Framingham 10-year risk for CHD ≥ 8% compared with those with a risk for < 8%, in each model is shown. HT and MS mean presence of hypertension and metabolic syndrome, respectively.
much lower. Indeed the risk score evaluated by the NIPPON Data for the Japanese over 40 years old is much lower. One hundred four volunteers out of the entire population were evaluated. Twenty-three of 104 men showed a risk of 0.5–1.0% and 6 of 104 men showed the risk of 1.0–2.0%. There was correlation between the PDMP level and the risk score evaluated by the NIPPON Data but it was not significant (data not shown). This could be because of the skewness of the distribution of the risk score evaluated by the NIPPON Data and/or the small number of subjects. A longitudinal study for the Japanese elderly should be planned. In our study we selected, the ELISA method using antibodies against CD42b (GPIb) and CD42a (GPIX) to obtain a large number of data. We should interpret these data carefully because the PDMP level measured by the ELISA method using antibodies against CD42b (GPIb) and CD42a (GPIX) did not represent all types of PDMPs. An ELISA using another antibodies, such as P-selectin et al., should be developed.

Conflict of Interest
There is no conflict of interest in this study.

Acknowledgement
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