Bone-Related Circulating Proteins as Early Predictors of Coronary Atherosclerosis in Asymptomatic Patients with Known Coronary Artery Disease

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Abstract

Methods. 126 subjects with previously documented asymptomatic coronary artery disease (CAD) were enrolled in the study. CAD was determined with contrast multispiral CT-angiography. Osteopontin (OPN) and osteoprotegerin (OPG) were measured with ELISA. Concentration of high sensitive C-reactive protein (hs-CRP) was measured by nephelometric technique.

Results. Analysis of the results showed that in a patient's cohort, the mean of circulating OPN value was 43.55 ng/mL (95% CI = 31.5 – 57.0 ng/mL). The mean value of circulating OPG was 3849.51 pg/mL (95% CI = 3282.23 - 4413.79 pg/mL). The independent predictors of coronary atherosclerosis determined with Agatston score index and %AS > 50% were OPN, OPG, T2DM, TC, and hs-CRP. The cutoff point for the OPG (AUC = 0.977), OPN (AUC = 0.851) and hs-CRP (AUC = 0.897) concentrations were 3229.8 pg / mL; 48.5 ng / mL and 5.6 mg / L respectively.

In conclusions, we predisposed that circulating OPG in patients with asymptomatic atherosclerosis can be regarded as the best early marker of coronary artery calcification when compared with OPN, and hs-CRP.

Keywords: osteopontin; osteoprotegerin; high sensitive C-reactive protein; asymptomatic coronary artery disease; atherosclerosis; artery calcification
Introduction

Atherosclerosis remains the leading cause of cardiovascular events, including in Western populations [4]. Bone-related proteins and hs-CRP are considered as markers of low-intensity inflammation that modulate atherosclerosis evolution [27]. Osteopontin (OPN) is a secreted molecule belonged to non-collagenous matrix phosphorylated sialoprotein that is highly expressed by several types cells, such as macrophages, endothelial cells, smooth muscle cells and epithelial cells, and interacted with wide types of integrins [12, 29]. OPN as multifunctional protein plays a key role in the pathology of chronic inflammatory diseases including atherosclerosis. Biological effect of OPN is realized thereby regulating of several macrophage functions, such as migration, survival, and tissue accumulation [29, 33]. Results obtained in animal model using ultrastructural analysis through transmission electron microscopy have been suggested that OPN was dramatically over-expressed by vasculature in the presence of oxidized low-density lipoproteins [1]. This mechanism is important to induce atherosclerosis in subintima and vasculature [14]. Moreover, specific histochemical techniques revealed that OPN is detected in vasculature near calcium phosphate precipitates that indicated pivotal role of OPN in regulation of vascular calcification [1]. Finally, OPN has recently emerged as key factors in both vascular remodeling and development of atherosclerosis [6-8].

Osteoprotegerin (OPG) is a member of tumor necrosis factors superfamily, belonging to the class of inhibitors of osteoclastogenesis [19]. OPG is expressed in vivo by osteoblasts, endothelial cells, smooth muscle cells of the arteries and veins of copper and is a specific receptor for the ligand receptor activation of nuclear transcription factor kappa beta and tumor necrosis factor-related apoptosis-inducing ligand [8]. Major inducers of synthesis of the latter are proinflammatory cytokines, such as interleukin (IL)-2, IL-6, monocites chemoattractant protein-1, is produced mainly by mononuclear phagocytes [32]. OPG is often seen as an indicator of proinflammatory activation [21], atherosclerosis and metabolic comorbidities [30]. There is evidence of direct involvement of OPG in the regulation of calcium deposition in the vessel wall [25]. Universal biological potential of OPG, designed to control the intensity of the processes of ossification, is often used as a predictor of early atherosclerotic lesions of arteries [10, 18]. OPG is likely to have an acceptable predictive value with respect to the onset of death in long-term observation of patients with coronary heart disease, type 2 diabetes (T2DM), ischemic stroke, hypertension, and in patients with chronic renal failure [13, 15, 20, 28]. However, currently,
OPN and OPG are considered as markers of vascular calcification [16, 22, 26], but their association with compositional parameters of potential vulnerable plaques in asymptomatic coronary artery disease (CAD) patients with T2DM is not still established.

The aim of the study was to evaluate the interrelation between bone-related circulating protein osteopontin (OPN), osteoprotegerin (OPG), and high sensitive C-reactive protein with coronary atherosclerosis and calcification in asymptomatic CAD patients.

Methods

Population of the study was structured retrospectively after determination of CAD by contrast-enhanced spiral computer tomography-angiography in one hundred twenty six asymptomatic subjects. All subjects gave their written informed consent to participation in the study before enrollment. General characteristics of study patients are presented in Table 1.

Echocardiography examination. Echocardiography in B-mode was performed accordingly to Recommendation of American Society of Echocardiography on scanner ACUSON (Siemens, Germany) using a transducer with a frequency of 2.5-5 MHz. End-diastolic and end-systolic LV volumes were obtained using a two-dimensional reference sector according to Simpson’s method, and LV ejection fraction (LVEF) was calculated accordingly conventional methods [9].

Contrast-enhanced spiral computer tomography angiography. Coronary vessel-wall, and plaque geometrical, and compositional parameters included percentage of area stenosis (%AS) were measured on contrast-enhanced spiral computer tomography (CT) angiography. Contrast-enhanced spiral CT was performed on a “Somatom Volum Zoom” scanner (Siemens, Erlangen, Germany) with 2 rows of detectors during end-expiratory breath-hold. After noncontrast localization image acquisition, injection of nonionic contrast “Omnipak” (Amersham Health, Ireland) was used to determine the optimal coronary arterial image. Images were reconstructed in 0.6-mm axial slices. Coronary artery calcification was quantified by calculating the Agatston’ score index and calcification mass measurement [3]. We determined calcified atherosclerotic plaque, high-density noncalcified plaque (HD-NCP), and low-density noncalcified plaque (LD-NCP). Calcified atherosclerotic plaques (CAP) were classified with attenuation values 150 HU (Hounsfield units) or greater, as HD-NCP with 30 to 149 HU and as LD-NCP with −100 to +30 HU [2, 24].
**Blood Sampling.** All samples were collected in cooling vacutaner and after that it was immediately centrifugated (4°C for 6,000 × 15 min). After centrifugation serum was coded and stored at -70° until used. Osteopontin and osteoprotegerin levels were measured by ELISA technique. For both biomarkers examination Human Quantikine ELISA Kits (R&G, United Kingdom) were used. All determinations were done by duplicate. The mean intra-assay coefficients of variation were <10% for all cases. High-sensitive C-RP (hs-C-RP) level was measured by nephelometric technique and obtained with “AU640 Analyzer” (Olympus Diagnostic Systems Group, Japan). Concentrations of total cholesterol (TC) and high density lipoprotein (HDL) cholesterol were determined by a Dimension Clinical Chemistry System (Dade Behring Inc, Newark, NJ). Low density lipoprotein (LDL) cholesterol was calculated by using the formula of Friedewald W.T., Levy R.I., Fredrickson D.S. (1972).

**Statistical Analysis.** All statistical analyses were performed in SPSS for Windows v. 20.0 (SPSS Inc., Chicago, II, USA). All values were given as mean and 95% CI or median and percentiles. An independent group t-test was used for comparisons for all interval parameters meeting the criteria of normality and homogeneity of variance. For interval parameters not meeting these criteria, the non-parametric Mann-Whitney test was used to make comparisons between groups. Comparisons of categorical variables between groups were performed using the Chi² test, and the Fisher exact test. OPN, OPG, and hs-CRP concentrations were normally distributed (using by Kolmogorov-Smirnov test) and data were no positively skewed. However OPN, OPG, and hs-CRP concentrations were no transformed. The potential factors that may be associated with atherosclerosis extent were identified first with the univariate analysis (ANOVA), then the independent predictors of coronary arteries calcification were searched with the multivariate one-step backward logistic regression analysis, initially including variables for which p value < 0.1 was achieved from the univariate analysis. Predictors of coronary calcification were classified by the k-nearest neighbor algorithm. Receiver operating characteristic (ROC) curves were configured to establish cutoff points of OPN, OPG, and hs-CRP levels that optimally predicted coronary atherosclerosis. A calculated difference of P<0.05 was considered significant.

**Results**

Analysis of obtained results have showed no significant differences between the both cohorts of patients for demographics (age, sex), conventional risk factor
(premature CAD in family anamnesis, smoking, arterial hypertension), biochemical (creatinin, TC) and some hemodynamic parameters (mean systolic BP, heart rate and LV EF). Fasting glucose and HbA1c in patients with were expectantly higher when compared with non-T2DM individuals. The analysis of MSCT figures suggested that as numerous of coronary arteries with plaques determined, as well as both noncalcified and calcified atherosclerotic plaques were considerable higher in T2DM subjects than in non-T2DM patients. Finally, the severity of coronary atherosclerosis measured by Agatston score index was significantly higher in T2DM patients when compared with non-T2DM subjects. All patients were treated according to current clinical guidelines with diet, lifestyle modification and drug therapy that included ACE inhibitors / ARBs, aspirin or other antiagregants, statins, and metformin when needed.

We found that in a patients cohort the mean of circulating OPN and OPG values were 43.55 ng/mL (95% CI=31.5–57.0 ng/mL) and 3849.51 pg/mL (95% CI=3282.23-4413.79 pg/mL). Circulating OPN (52.63 ng/mL [95% confidence interval (CI) =47.11-58.15 ng/mL] and 36.54 ng/mL; 95% CI=31.77–41.31 ng/mL; P<0.0001) and OPG (6073.4 pg/mL [95% confidence interval (CI)=5493.5-6653.3 pg/mL] and 2814.6 pg/mL [95% CI=2411.5–3217.7 pg/mL]; P<0.0001) levels in T2DM subjects were significantly higher when compared with non-T2DM patients. OPN plasma levels were directly related to CAP (r = 0.674, P < 0.001), Agatston’ score index (r = 0.628, P < 0.001), T2DM (r = 0.495, P < 0.001), TC (r = 0.544, P < 0.001), hs-CRP (r = 0.657, P < 0.001), %AS > 50% (r = 0.582, P < 0.001), and inversely to LVEF (r = -0.743, P < 0.001). We observed also that OPG plasma levels were positively considerable related to CAP (r = 0.452, P < 0.001), Agatston’ score index (r = 0.917, P < 0.001), T2DM (r = 0.768, P < 0.001), TC (r = 0.512, P < 0.001), hsC-RP (r = 0.620, P < 0.001), %AS > 50% (r = 0.791, P < 0.001), HD-CAP (r=0.30, P < 0.001), OPN (r = 0.674, P < 0.001), and inversely to LVEF (r = -0.313, P < 0.001). At the same time, the significant association between the levels of circulating OPN and OPG with creatinin plasma level, fasting glucose, HbA1c, mean systolic BP, premature CAD in family anamnesis, and medications for the entire cohort of the patients were not determined. Thus, both biomarkers (OPN and OPG) are correlated well with parameters that determined severity of coronary atherosclerosis, such as Agatston’ score index and %AS > 50%.

The independent predictors of coronary atherosclerosis determined with Agatston’ score index and %AS > 50% were OPN, OPG, T2DM, TC, and hs-CRP (Table 2). Figure 1 is presented results a classified model assigning the predictor of coronary calcification measured by Agatston’ score index using the $k$-nearest neighbor algorithm. In this model ($k=3$) we have suggested that three biomarkers (OPN,
Bone-related circulating proteins as predictors of coronary atherosclerosis

OPG, and hs-CRP) might be considerable predictors for calcium deposition located in coronary arteries as a marker of atherosclerosis. Predictive value of OPG, OPN, and hs-CRP concentrations with respect to the severity of coronary atherosclerosis in patients were performed using ROC-analysis, the results of which are presented in Fig. 2. The findings suggest that the estimated AUC (areas under curve) for OPN, OPG, and hs-CRP were 0.851; 0.977; and 0.897 respectively that reflects this predicted power model as minimum as good for all occasional. However, the cutoff point for the OPG concentrations that had the best prognostic potential on coronary atherosclerosis was 3229.8 pg/mL. The cutoff points for the OPN and hs-CRP concentrations were 48.5 ng/mL and 5.6 mg/L respectively. Thus, these data suggest that for asymptomatic CAD patients circulating elevation of OPG can be considered as the best predictor of atherosclerotic coronary damage an artery calcification.

Discussion

Proteins in the serum of CAD patients predominantly reflected a positive acute phase, inflammatory response and alterations in lipid metabolism, transport, peroxidation and accumulation. Recent studies have been suggested that markers of stromal stem cells with osteogenic potential presumably, such as osteopontin, osteonectin and osteoprotegrin, and also hs-CRP can play an important role in atherogenesis [34]. There were surprisingly few indicators of growth factor activation or extracellular matrix remodeling in the serum of CAD patients except for elevated OPN [8]. These data revealed that many symptomatic patients without significant CAD could be identified by a targeted multiplex serum protein test without cardiac catheterization thereby eliminating exposure to ionizing radiation and decreasing the economic burden of angiographic testing for these patients [31]. Results of presented study are suggested that circulating OPN, OPG and hs-CRP correlate significantly with some key biomarkers of atherosclerosis and with atherosclerosis and calcinosis of coronary arteries. Unfortunately, results of The Cardiovascular Risk in Young Finns Study have been shown that OPN was not associated with carotid intima-media thickness, carotid elasticity, brachial endothelial function or the presence of a carotid plaque in either sex in asymptomatic young adults without symptoms of cardiovascular disease [23]. On the other hand, OPG and hs-CRP are considered as determinants of progression of coronary artery calcification in several patient’ populations [5, 10, 17].We have
compared predicted values of OPN, OPG, and hs-CRP and have suggested that OPG concentration above 3229.8 pg/mL might have the best predicted value toward coronary artery calcification in population of asymptomatic subjects with known CAD.

**In conclusion**, we found that circulating OPG in patients with asymptomatic atherosclerosis can be regarded as the best early marker of coronary artery calcification when compared with OPN, and hs-CRP.

**Limitations of the study**
This study has some limitations. We believed that a greater cohort would be desirable to improve the power of the study. We also relied on clinical data to rule out infection and other inflammatory diseases before sampling, but we cannot exclude that some patients had unrecognized conditions responsible for the elevated OPN, OPG and hs-CRP levels observed. We suppose that these limitations might not have a significant influence to study data interpretation.

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**Ethical declaration.** The study was approved by local ethics committee of State medical university, Zaporozhye, Ukraine. The conduct of the study was in keeping with the declaration of Helsinki.

**Declaration of conflicting interests.** None

**References**

Bone-related circulating proteins as predictors of coronary atherosclerosis

5. D. Silva, A. Pais de Lacerda, High-sensitivity C-reactive protein as a biomarker of risk in coronary artery disease. Rev Port Cardiol. 31(11), (2012), 733-745.


Bone-related circulating proteins as predictors of coronary atherosclerosis

167


Table 1. General Characteristics of Study Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T2DM (n=46)</th>
<th>None-T2DM (n=80)</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td>59.10±2.80</td>
<td>56.6±5.4</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>42 (58.3%)</td>
<td>32 (59.3%)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>30 (65.2%)</td>
<td>54 (67.5%)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>21 (45.6%)</td>
<td>35 (43.8%)</td>
</tr>
<tr>
<td>Premature CAD in family anamnesis, n (%)</td>
<td>4 (8.7%)</td>
<td>8 (10.0%)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>10 (21.7%)</td>
<td>16 (20.0%)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>8.4 (95% CI=7.1-9.5)</td>
<td>4.7 (95% CI=4.0-5.3); P&lt;0.001</td>
</tr>
<tr>
<td>Creatinin, μmol/L</td>
<td>90.30±5.11</td>
<td>84.32±6.20</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>6.90 (95% CI=5.9-9.1)</td>
<td>4.56 (95% CI=3.7-5.5); P&lt;0.001</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>5.39 (95% CI = 4.8 - 6.0)</td>
<td>4.79 (95% CI = 4.13 - 5.55)</td>
</tr>
<tr>
<td>Mean systolic BP, mm Hg</td>
<td>133.80±6.2</td>
<td>130.10±5.94</td>
</tr>
<tr>
<td>Heart rate, beat per min</td>
<td>70.80±3.16</td>
<td>71.10±2.45</td>
</tr>
<tr>
<td>LV EF, %</td>
<td>42.20±0.44</td>
<td>42.92±0.37</td>
</tr>
<tr>
<td>HD-NCP, n (%)</td>
<td>26 (56.5%)</td>
<td>29 (36.3%); P&lt;0.001</td>
</tr>
<tr>
<td>LD-NCP, n (%)</td>
<td>29 (63.0%)</td>
<td>20 (25.0%); P&lt;0.001</td>
</tr>
<tr>
<td>CAP, n (%)</td>
<td>38 (82.6%)</td>
<td>25 (31.3%); P&lt;0.001</td>
</tr>
<tr>
<td>Agatston' score index</td>
<td>672 (95% CI=403-830)</td>
<td>325 (95% CI=216-398); P&lt;0.001</td>
</tr>
<tr>
<td>Numerous of coronary arteries with plaques determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 vessel</td>
<td>27 (58.7%)</td>
<td>19 (23.8%); P&lt;0.001</td>
</tr>
<tr>
<td>2 vessels</td>
<td>25 (54.3%)</td>
<td>17 (21.3%); P&lt;0.001</td>
</tr>
<tr>
<td>3 vessels and more</td>
<td>20 (43.5%)</td>
<td>18 (22.5%); P&lt;0.001</td>
</tr>
<tr>
<td>ACEI/ARBs, n (%)</td>
<td>46 (100%)</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Aspirin, n (%)</td>
<td>46 (100%)</td>
<td>34 (42.5%)</td>
</tr>
<tr>
<td>Other antiagregants, n (%)</td>
<td>0</td>
<td>6 (7.5%)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>39 (84.8%)</td>
<td>55 (68.8%)</td>
</tr>
<tr>
<td>Metformin, n (%)</td>
<td>41 (89.1%)</td>
<td>0</td>
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</table>

Table 2. The independent predictors of coronary atherosclerosis. The multivariate one-step backward logistic regression analysis.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Odds Ratios</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN</td>
<td>1.80</td>
<td>1.18-2.80</td>
<td>0.004</td>
</tr>
<tr>
<td>OPG</td>
<td>2.12</td>
<td>1.60-4.10</td>
<td>0.008</td>
</tr>
<tr>
<td>T2DM</td>
<td>1.14</td>
<td>1.04-1.28</td>
<td>0.001</td>
</tr>
<tr>
<td>TC</td>
<td>1.22</td>
<td>1.10-1.42</td>
<td>0.001</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>1.26</td>
<td>1.04-1.55</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Note: OPN – osteopontin, OPG – osteoprotegerin, T2DM – type two diabetes mellitus, TC – total cholesterol, hs-CRP – high sensitive C-reactive protein

Figure 1. Graphs show results a classified model assigning the predictor of coronary calcification measured by Agatston’ score index using the k-nearest neighbor algorithm.

Predictor Space

Built Model: 3 selected predictors, K = 3

Note: OPN – osteopontin, OPG – osteoprotegerin, hs-CRP – high-sensitive C-reactive protein
**Figure 2.** Receive operation curve analysis: predict value of OPG, OPN, and hs-CRP concentration with respect to severity of coronary atherosclerosis.

Diagonal segments are produced by ties.
### Area Under the Curve

<table>
<thead>
<tr>
<th>Test Result Variables</th>
<th>Area</th>
<th>Cutoff points</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPN</td>
<td>0.851</td>
<td>48.5 ng / mL</td>
</tr>
<tr>
<td>OPG</td>
<td>0.977</td>
<td>3229.8 pg / mL</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.897</td>
<td>5.6 mg / L</td>
</tr>
</tbody>
</table>

Note: OPN – osteopontin, OPG – osteoprotegerin, hs-CRP – high-sensitive C-reactive protein

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