Association of Osteoprotegerin With Aortic Stiffness in Patients With Symptomatic Peripheral Artery Disease and in Healthy Subjects

Maksim Zagura1,2, Martin Serg2,3, Priit Kampus1–3, Mihkel Zilmer1,2, Kersti Zilmer1, Jaan Eha2,3, Eve Unt4–6, Jüri Lieberg7,8 and Jaak Kals1,2,7

BACKGROUND
Arterial stiffening is an independent predictor for cardiovascular mortality. Preliminary studies have shown that arterial calcification may have an impact on increased vascular stiffness. However, there are limited data about the role of calcification inhibitor osteoprotegerin (OPG) as an independent predictor for arterial stiffness in patients with peripheral arterial disease (PAD) and in healthy subjects. The aim of this study was to evaluate the association between OPG and arterial stiffness parameters in patients with PAD and in healthy subjects.

METHODS
We studied 69 men with PAD (age 63 ± 7 years) and 68 healthy subjects (age 54 ± 8 years). Serum OPG and oxidized low-density lipoprotein (oxLDL) were measured using the enzyme-linked immunosorbent assay method. Radial and aortic pulse wave velocity (aPWV) and augmentation index (AIx) were determined by applanation tonometry.

RESULTS
The OPG (5.4 ± 1.7 vs. 4.4 ± 1.1 pmol/l; P < 0.001) and aPWV (10.1 ± 2.5 vs. 7.6 ± 1.6 m/s; P < 0.001) were different for the patients and for the controls. There was a linear relationship between OPG and aPWV in patients with PAD (R = 0.37; P = 0.003) and in healthy individuals (R = 0.40; P = 0.001). In multiple regression models after adjustment for potential confounders, OPG was independently associated with aPWV in the patients (R2 = 0.47; P < 0.0001) and in the controls (R2 = 0.44; P < 0.0001). The AIx or radial PWV was not correlated with OPG for either group.

CONCLUSION
The independent association between OPG and aPWV in patients with PAD and in controls suggests that the calcification inhibitor OPG may influence aortic stiffening in atherosclerosis and in clinically healthy subjects.

Keywords: arterial stiffness; blood pressure; hypertension; osteoprotegerin; peripheral arterial disease; pulse wave velocity


Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor superfamily, which has been considered as a possible link between bone metabolism and vascular disease.1 Animal models indicate that OPG has disparate effects within the bones and the arteries. Deficiency of OPG resulted in severe osteoporosis and medial calcification of the aorta and the renal artery in mice.2 Although various animal studies support the protective role of OPG in the arterial system,3 clinical studies on patients have shown a positive association between serum OPG levels and cardiovascular disease. Elevated serum OPG levels have been associated with coronary artery disease, peripheral arterial disease (PAD), diabetic complications, heart failure, abdominal aortic aneurysm, and cardiovascular mortality.4,5

Aortic stiffness has an independent predictive value for all-cause and cardiovascular mortalities in high-risk patients6,7 and in general population.8 Aortic stiffness as measured by aortic pulse wave velocity (aPWV) is the gold standard measure of arterial stiffness as well as yielded prognostic values beyond and above traditional risk factors.9

Previous data support the association between vascular calcification and serum OPG levels.10,11 In newly diagnosed diabetic patients, serum OPG was associated with brachial-ankle PWV and inflammatory markers.12 Circulating OPG level is also associated with abdominal aortic calcification in patients with PAD.13 Elevated serum OPG concentrations have been found to correlate with the severity of PAD.14 Moreover, brachial artery flow-mediated dilatation correlated negatively with serum OPG level in patients with PAD, suggesting that OPG may be a useful marker of endothelial function.15 However, no
studies have assessed possible association between serum OPG and aortic stiffness in patients with PAD in comparison with gender-matched healthy individuals. The goal of this study was to evaluate the association between serum OPG level and aortic stiffness in patients with PAD and in healthy subjects.

**METHODS**

**Study population.** In this cross-sectional study the patient group consisted of 69 PAD patients with stages II, III, or IV of chronic ischemia as defined by Fontaine: stage II = intermittent claudication, stage III = leg pain at rest, and stage IV = tissue loss due to ischemic ulcer or gangrene. A convenient sample of patients according to the inclusion criteria was recruited from the Department of Vascular Surgery, University of Tartu, Estonia. They were all male with angiographically proven PAD, i.e. with occlusion of the arteries of the lower extremities. Their ankle–brachial pressure index (ABPI) was <0.90 (range 0.14–0.89). Patients with any concomitant acute or chronic inflammatory disease, myocardial infarction, coronary revascularization, or cerebrovascular events during the previous 6 months, earlier revascularization procedures at the lower limb, upper limb occlusive arterial disease, cardiac arrhythmias or valve pathologies, diabetes mellitus, malignancies, and renal failure (estimated glomerular filtration rate (eGFR) <60 ml/min/1.73 m²) were excluded from the study. In total, 21 (30.4%) patients with hypertension and 7 (10.1%) patients with coronary artery disease as the comorbidity were included in the study. A convenience sample of clinically healthy men was enrolled as controls. The control group of men (n = 68) was recruited by the family physician and by the doctor of sports medicine and rehabilitation. The exclusion criteria for the control group were the following (based on clinical examination, ECG, and blood tests): any acute or chronic inflammatory disease, coronary artery disease, cardiac arrhythmias or valve pathologies, cerebral or peripheral atherosclerotic disease, diabetes mellitus, malignancies, renal failure (eGFR <60 ml/min/1.73 m²), and regular use of any medication. This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee of the University of Tartu. Written informed consent was obtained from each participant.

**Study protocol.** The subjects were studied and plasma samples were collected between 0800 and 1000 hours after an overnight fast and abstinence from any medications, tobacco, alcohol, and tea or coffee. After 15 min of rest in a supine position blood pressure and ABPI were measured, pulse wave analysis was performed and carotid–femoral and carotid–radial PWVs were determined in all subjects. All measurements were made in duplicate and mean values were used in the subsequent analysis. Thereafter, venous blood samples were drawn from the antecubital fossa. The patients’ height and weight were recorded and body mass index was calculated.

**Laboratory methods.** Serum OPG was measured by an enzyme-linked immunosorbent assay using a commercially available kit (Human Osteoprotegerin ELISA; Biovendor, Heidelberg, Germany). The intra- and interassay precision coefficients of variation for OPG were 3.5 and 5.8%, respectively. The blood samples were centrifuged and the sera for OPG were divided into aliquots and stored at −70°C until analysis. All determination procedures were performed in accordance with the manufacturer’s recommendations.

C-reactive protein was determined by a highly sensitive, latex particle–enhanced immunoturbidimetric assay (Roche Diagnostics, Mannheim, Germany); the measurement range was 0.1–20 mg/l. The levels of the oxidized low-density lipoprotein (oxLDL) were measured using an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). The plasma level of soluble intercellular adhesion molecule-1 was measured by an enzyme-linked immunosorbent assay using a commercially available kit (Human soluble intercellular adhesion molecule-1 Immunoassay; R&D Systems, Minneapolis, MN).

Plasma glucose, total cholesterol, LDL-cholesterol, high-density lipoprotein-cholesterol and triglyceride levels, as well as serum creatinine concentrations were determined by standard laboratory methods using certified assays in the local clinical laboratory.

**Assessment of arterial stiffness.** Peripheral blood pressure was measured in both arms when the subject was seated for 10 min; a validated oscillometric technique was used (OMRON M4-I; Omron Healthcare Europe, Hoofddorp, the Netherlands). Mean arterial pressure was obtained by integration of the radial pressure waveform using the Sphygmocor software (SCOR Px, 7.0; AtCor Medical, Sydney, Australia).

Arterial stiffness was assessed by PWV and pulse wave analysis using a Sphygmocor device. Radial artery waveforms were recorded with a high fidelity micromanometer (SPT-301B; Millar Instruments, Houston, Texas) from the wrist of the dominant arm. Using a transfer function, the corresponding ascending aortic waveforms were then generated, from which central hemodynamics, augmentation index (Alx) and the travel time of the reflected wave were calculated. The Alx was corrected for a heart rate of 75 beats per minute (Alx@75). Heart rate was also determined from the aortic waveform. Carotid–femoral and carotid–radial PWVs were measured as described earlier. All measurements were made in duplicate and mean values used in the subsequent analysis.

The ABI was measured using the Bidirectional Doppler MD 6 (D.E. Hokanson, Bellevue, WA). The lower result of the two measurements was included in statistical analysis for the control group.

**Assessment of GFR.** eGFR was evaluated using the Modification of Diet in Renal Disease formula, equation MDRD 1 (ref. 18).

**Analysis of data.** Data were analyzed with the software R (version 2.8.1 for Windows; The R Foundation for Statistical Computing, Vienna, Austria). All variables were tested for normality using the Kolmogorov–Smirnov and the Lilliefors tests. Normally distributed continuous data are expressed as means ± s.d. Non-normally distributed data are presented as medians with
the interquartile range. Dichotomous variables are presented as prevalence in number and percentage. Comparisons of the patients and the healthy individuals were assessed using two-tailed Student’s t-test (for the means) and the Mann–Whitney U-test (for the medians). The strength of the association between the variables was calculated using linear regression analysis.

Multiple linear regression analysis was used to examine whether simple associations were changed after adjustment for potential confounders. The variables entered into the model were drawn from simple correlation analysis, and from relevant published observations. For multiple regression model building, forward and backward stepwise variable selection procedures were applied. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**Characteristics of the study population**

The baseline characteristics of 69 patients and 68 healthy subjects are presented in Table 1. The groups did not differ with respect to high-density lipoprotein-cholesterol, eGFR, or carotid–radial PWV. Our data revealed that OPG level ($5.4 \pm 1.7$ vs. $4.4 \pm 1.1$ (pmol/l); $P < 0.001$) and aPWV ($10.1 \pm 2.5$ vs. $7.6 \pm 1.6$ (m/s); $P < 0.001$) was different for the patients and for the healthy individuals. There occurred a significant difference in median ABPI and the high-sensitivity C-reactive protein between the groups ($0.5$ vs. $1.1$ mg/l; $P < 0.001$; $4.0$ vs. $0.9$ mg/l, $P < 0.001$).

### Table 1 | Baseline characteristics of the study groups (mean ± s.d.)

<table>
<thead>
<tr>
<th>Variable</th>
<th>PAD patients (n = 69)</th>
<th>Controls (n = 68)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63.2 ± 7.1</td>
<td>53.9 ± 7.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.5 ± 3.9</td>
<td>26.9 ± 3.4</td>
<td>0.03</td>
</tr>
<tr>
<td>Height (m)</td>
<td>174.4 ± 6.1</td>
<td>179.2 ± 7.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Peripheral SBP (mm Hg)</td>
<td>148 ± 20</td>
<td>128 ± 15</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Peripheral DBP (mm Hg)</td>
<td>82 ± 10</td>
<td>77 ± 9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Central SBP (mm Hg)</td>
<td>136 ± 19</td>
<td>118 ± 16</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Central DBP (mm Hg)</td>
<td>83 ± 11</td>
<td>79 ± 10</td>
<td>0.02</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>104 ± 14</td>
<td>95 ± 12</td>
<td>0.3</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>67.2 ± 12.1</td>
<td>58.2 ± 10.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ankle-brachial pressure index</td>
<td>0.5 (0.2–0.6)</td>
<td>1.1 (1.1–1.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.9 ± 1.3</td>
<td>5.2 ± 1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>4.1 ± 1.1</td>
<td>3.6 ± 0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.7 ± 0.7</td>
<td>1.1 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.6 ± 1.0</td>
<td>5.4 ± 0.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>101.8 ± 26.1</td>
<td>98.1 ± 18.0</td>
<td>0.33</td>
</tr>
<tr>
<td>hsCRP (mg/l)</td>
<td>4.0 (1.4–7.9)</td>
<td>0.9 (0.5–1.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>sICAM (ng/ml)</td>
<td>278.6 ± 73.1</td>
<td>183.5 ± 42.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>OPG (pmol/l)</td>
<td>5.4 ± 1.7</td>
<td>4.4 ± 1.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>oxLDL (U/l)</td>
<td>72.5 ± 27.0</td>
<td>56.0 ± 22.4</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Augmentation index (%)</td>
<td>33.1 ± 14.6</td>
<td>21.7 ± 10.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>A1x@75 (%)</td>
<td>28.1 ± 8.1</td>
<td>13.6 ± 11.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PWVcar-fem (m/s)</td>
<td>10.1 ± 2.5</td>
<td>7.6 ± 1.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>PWVcar-rad (m/s)</td>
<td>8.9 ± 1.5</td>
<td>8.9 ± 1.2</td>
<td>0.70</td>
</tr>
<tr>
<td>Current smoking, n (%)</td>
<td>69 (100)</td>
<td>2 (2.9)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The values are represented as means (±s.d.), medians (interquartile range), or prevalence (%).

ABPI, ankle–brachial pressure index; ACE, angiotensin-converting enzyme; A1x@75, augmentation index, corrected for a heart rate of 75 beats per minute; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; MAP, mean arterial pressure; OPG, osteoprotegerin; oxLDL, oxidized low-density lipoprotein; PAD, peripheral arterial disease; PWVcar-fem, carotid–femoral pulse wave velocity; PWVcar-rad, carotid–radial pulse wave velocity; SBP, systolic blood pressure; sICAM, soluble intercellular adhesion molecule; Tr, travel time of the reflected wave.

### Table 2 | Multiple regression model for patients and for healthy men with aortic PWV as the dependent variable.

<table>
<thead>
<tr>
<th></th>
<th>Regression coefficient</th>
<th>s.e.</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG (pmol/l)</td>
<td>0.61</td>
<td>0.20</td>
<td>0.005</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>0.06</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>ABPI</td>
<td>−2.10</td>
<td>0.98</td>
<td>0.04</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>−0.02</td>
<td>0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.24</td>
</tr>
<tr>
<td>Antihypertensive treatment</td>
<td>0.72</td>
<td>0.62</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.10</td>
<td>0.03</td>
<td>&gt;0.001</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>0.05</td>
<td>0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>OPG (pmol/l)</td>
<td>0.33</td>
<td>0.15</td>
<td>0.04</td>
</tr>
</tbody>
</table>

ABPI, ankle–brachial pressure index; MAP, mean arterial pressure; OPG, osteoprotegerin; eGFR, estimated glomerular filtration rate. $R^2 = 0.47$, $P < 0.001$; $n = 69$  $\mu R^2 = 0.44$, $P < 0.001$; $n = 68$.
Osteoprotegerin and Arterial Stiffness

Figure 2: Scatterplot of the biochemical parameters for 69 patients with peripheral arterial disease. Linear correlation was observed between osteoprotegerin (OPG) level and oxidized low-density lipoprotein (oxLDL) ($R = 0.34; P = 0.01$).

Table 3: Multiple regression model for patients and for healthy men with OPG as the dependent variable

<table>
<thead>
<tr>
<th>Regression coefficient</th>
<th>s.e.</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>0.37</td>
<td>0.12</td>
</tr>
<tr>
<td>oxLDL (U/l)</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.93</td>
<td>0.60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>ABPI</td>
<td>0.88</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aPWV (m/s)</td>
<td>0.26</td>
<td>0.11</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>−0.96</td>
<td>0.56</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>oxLDL (U/l)</td>
<td>−0.01</td>
<td>0.008</td>
</tr>
</tbody>
</table>

ABPI, ankle-brachial pressure index; aPWV, aortic pulse wave velocity; oxLDL, oxidized low-density lipoprotein; HDL, high-density lipoprotein.

DISCUSSION

In this study, serum OPG levels were significantly associated with aPWV in the patients with PAD as well as in the healthy subjects. These associations remained significant after adjusting for blood pressure levels, concomitant medications or presence of cardiovascular risk factors. To the best of our knowledge, this is the first study which investigated possible association between serum OPG concentrations and aortic stiffness in a cohort of patients with the established diagnosis of PAD in comparison with clinically healthy individuals. In addition, we demonstrated the existence of relationship of serum OPG with the direct indexes of oxidative stress (oxLDL) for the patient group. However, the causal role of OPG in arterial stiffening and atherosclerosis cannot be explained by the findings of this cross-sectional study.

Human studies have revealed strong association between OPG level and a broad range of cardiovascular diseases. It was hypothesized that OPG concentration may represent a protective response to atherosclerosis aimed at limiting vascular damage.19 In this study, the patients with atherosclerosis had higher values of OPG and increased aortic stiffness compared with the healthy subjects. After adjustment for potential confounders, aPWV remained independently correlated with OPG level in both groups. This finding suggests that OPG might be associated with progression of aortic stiffness in atherosclerosis as well as in healthy condition. We did not observe any correlation between OPG and carotid–radial PWV either in the patients or in the controls. One possible explanation for this is that PAD mainly potentiates stiffness of aorta and the arteries of the lower rather than of the upper extremities.20

Profound oxidative stress is a major contributor to atherosclerosis and cardiovascular dysfunction. In our previous study, we demonstrated association between the indexes of arterial elasticity and urinary F$_2$-IsoPs in patients with PAD.21 In this study, serum oxLDL level was higher in the patients with atherosclerosis, as well as correlated with OPG in the patient group. The relationship between OPG and oxLDL could imply that high-grade oxidative stress may influence the effect of OPG on vascular stiffness in the advanced stages of atherosclerosis. Previous investigations demonstrate that high-grade oxidative stress can
alter arterial wall elasticity. Increased production of reactive species modulates the activity of matrix metalloproteinases, which are associated with destruction of elastic laminae and replacement of elastin fibres by collagen. In addition, high-grade oxidative stress promotes vascular smooth muscle cell hypertrophy. On the other hand, vascular smooth muscle cell are involved in production of OPG, suggesting a mechanistic link between elevated OPG and high-grade oxidative stress.

Several studies have reported significant relationships of serum OPG with endothelium-dependent vasodilatation and arterial stiffness in diabetics. Furthermore, OPG is related to endothelial function in patients with PAD. In this study, we did not assess endothelial function. However, in our previous studies we have demonstrated that arterial stiffness is inversely associated with endothelial function in patients with PAD. These findings could suggest that endothelial dysfunction might be one potential mechanism underlying the relationship between serum OPG level and aortic stiffness.

In agreement with other authors, our finding that OPG is significantly associated with aortic stiffness suggests that measurement of OPG and aPWV may be of importance in assessment of vascular risk. Although previous studies have reported correlation between OPG concentration and severity of PAD, this study did not detect association between OPG and ABPI. However, we demonstrated that ABPI predicted independently aPWV in the patients, which suggests that arterial stiffness is related to severity grade of PAD. Our study has several limitations. First, we studied only male subjects considering that PAD patients are predominantly men and trying to avoid the potential confounding effects of estrogen. Therefore, the extrapolation of our findings to women and younger subjects may be limited. Second, the effect of concomitant medications on hemodynamic parameters and on the concentration of biomarkers remains unclear because we could not withdraw chronically ill patients from their treatment for a long period of time. A potential bias can also be associated with the long-term effects of smoking. Third, as this study is cross-sectional, it is not possible to infer that the associations between OPG and arterial stiffness are causal. Finally, we acknowledge that the data in our study were collected from a relatively small sample. Therefore, larger studies are required to confirm or to refute our findings.

In conclusion, we have demonstrated that OPG concentration is independently associated with aortic stiffness in patients with PAD and in healthy subjects. Our findings also suggest that the association between OPG and aortic stiffness could be influenced by high-grade oxidative stress. This study supports the role of calcification inhibitor OPG in the process of aortic stiffening in atherosclerosis and in healthy individuals. Further studies are needed to clarify precise role of OPG in human vasculature.

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