Serum osteoprotegerin in young survivors of myocardial infarction

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Summary

Osteoprotegerin (OPG) is a member of the tumour necrosis factor superfamily and is involved in the regulation of bone metabolism and vascular calcification. Increased serum OPG levels have been reported in patients with stable angina pectoris and survivors of myocardial infarction with heart failure. The purpose of the present study was to determine serum OPG levels in young survivors of acute myocardial infarction (MI), and the relationship between OPG, homocysteine, sCD40L and coagulation factors in blood. Fifty-eight patients with verified MI, 40–60 years of age, were recruited 1–4 years after the acute event into an age- and sex-matched case control study with controls recruited from the general population. Serum OPG levels were similar in cases (2.41 ng/ml, 2.11–2.77 ng/ml) (mean, 95% CI) and controls (2.43 ng/ml, 2.11–2.79 ng/ml) (p= 0.92). Significant correlation between OPG and homocysteine was found in patients (r=0.30, p=0.02) and controls (r=0.35, p=0.007). A significant negative correlation was found between OPG and sCD40L in patients (r=-0.51, p<0.001), but not in controls (r=0.001, p=0.96). No associations were found between serum OPG and markers of coagulation activation. The present study shows that serum OPG level was not increased in young survivors of uncomplicated myocardial infarction. Serum OPG levels were not associated with thrombin generation assessed by thrombin-antithrombin complexes (TAT), but a positive association between serum OPG and homocysteine was found.

Keywords

Osteoprotegerin, homocysteine, coagulation, thrombin generation, myocardial infarction

Introduction

Osteoprotegerin (OPG), a member of the tumour necrosis factor superfamily, has been proposed to represent a link between osteoporosis and vascular calcification (1, 2). This glycoprotein is expressed at high concentrations by a variety of tissues and cell types including main components of the cardiovascular system (3–8) such as arterial smooth muscle cells, endothelial cells and platelets (8–10). OPG is a soluble decoy receptor for receptor activator nuclear factor kappa B ligand (RANKL) (11) and TNF-related apoptosis-inducing ligand (TRAIL) (11, 12). By binding to RANKL and TRAIL, OPG inhibits ligation of these mediators to their cognate receptors and subsequent activation of specific proinflammatory and proapoptotic signalling pathways. RANKL activates its cognate receptor RANK which seems to be an important regulator of dendritic cells, T-cells and osteoclasts (11, 13, 14). Both OPG and RANKL are expressed in non-diseased aortas and in atherosclerotic lesions in humans (6, 15). In vitro, OPG prolongs endothelial cell survival (8) by inhibiting TRAIL and thereby preventing apoptosis (9). Recently, it was reported that OPG is colocalized in endothelial cells with von Willebrand factor (vWF) and remains associated with vWF after secretion from endothelial cells (16). Subsequently, it was hypothesized that OPG may play an important role in thrombosis (16). In humans, increased serum OPG levels have been reported in patients with stable coronary artery disease, and the OPG-level increased with the severity of the disease (17, 18). Furthermore, prospective studies showed that OPG was an independent risk factor for progression of atherosclerosis in carotid arteries and incident cardiovascular disease in the general population (19), and for mortality and cardiovascular events in patients with heart failure after acute myocardial infarction (20).

Inflammation and thrombosis are key elements in the pathogenesis of acute myocardial infarction (21), and a positive correlation between serum OPG and markers of inflammation has been reported (19). The objective of this study was to investigate the relationship between OPG, homocysteine and coagulation.
activation in young survivors of myocardial infarction and in age- and sex-matched controls.

Materials and methods

Patients and study design
Medical records of 120 consecutive patients between 40 and 60 years of age with the diagnosis of acute myocardial infarction (diagnosis code 410, ICD-9) were obtained from the archives at the University Hospital Northern Norway, Tromsø, Norway. The diagnosis acute myocardial infarction was verified by diagnostic criteria such as chest pain, pathological electrocardiogram and increase in serum concentration of cardiac markers in 111 patients. In the remaining 9 subjects the diagnosis could not be verified. The inclusion criteria were: suffering of acute myocardial infarction 1–4 years before the present investigation without any later major coronary events (i.e. hospitalization for angina pectoris, reinfarction, coronary angioplasty, ACB surgery), both genders, age between 40 and 60 years at diagnosis. The exclusion criteria were: regular use of drugs interfering with the coagulation system (i.e. warfarin and heparins), increased bleeding risk (i.e. bleeding disorder or recent trauma or surgery), chronic inflammatory diseases, diabetes mellitus, heart failure following the acute event (LVEF<35%), renal or liver disease, cancer, hypothyreoidism, malignant hypertension, abuse of alcohol or drugs, major psychiatric diseases. Among the 111 patients, 83 patients filled the above-mentioned criteria. These 83 patients were invited to undergo a clinical examination that included a complete medical history, physical examination, and laboratory tests. Fifty-eight patients responded to the invitation and were included in the present study.

In 1994–95, 21,826 subjects, 81.3% of the men aged 20–61 years old and the women aged 20–56 years old living in the municipality of Tromsø, participated in a health survey (The Tromsø Heart Study). All subjects completed a questionnaire about previous diseases, use of drugs, diet and smoking habits. Their height, weight, blood pressure, and non-fasting serum lipid and homocysteine concentrations were measured. From this general population 4 age- and sex- matched apparently healthy (no previous or present disease) persons were randomly selected for each of the 58 cases. One control for each case was invited to undergo the same clinical examination and blood tests as the cases. The regional board of research ethics approved the study protocol, and written informed consent was given by every person included in the study.

Blood sampling
Blood was drawn from an antecubital vein on the right arm (after 12 hours overnight fasting) using a 19-gauge needle in a vacutainer system with minimal stasis. Serum was prepared by clotting whole blood in a glass tube at room temperature for one hour, followed by centrifugation at 2,000 x g for 15 min. Aliquots of 1 ml were transferred into sterile cryovials (Greiner Labor-teknik, Germany), flushed with nitrogen, and stored at −70°C until further analysis. Blood for plasma preparation was collected into vacutainers (Becton Dickinson, Meylan Cedex, France) containing 0.129 M sodium citrate (1 vol anticoagulant and 9 vol whole blood) as anticoagulant. Plasma was prepared by centrifugation at 2,000 x g for 15 min at 22°C, transferred into sterile cryovials in aliquots of 1 ml, flushed with nitrogen and stored at −70°C until further analysis.

OPG, sCD40L, serum lipids, apolipoproteins and homocysteine
The concentration of total OPG (R&D Systems, Abingdon, UK) was analyzed 1 to 4 years after the acute event by an ELISA assay according to the manufacturer’s instructions. The analyses were performed on coded samples. The intra- and interassay coefficients of variation (CV) in our laboratory were 3.2% and 6.8%, respectively. All samples were analyzed in duplicate. Soluble CD40L was analyzed by an ELISA assay (Bender MedSystems) in citrated plasma. Serum lipids were analyzed on a Hitachi 737 Automatic Analyzer (Boehringer Mannheim, Germany) with reagents from the manufacturer. Total cholesterol was measured with an enzymatic colorimetric method (CHOD-PAP), and high density lipoproteins (HDL) cholesterol was assayed by the same procedure after precipitation of lower density lipoproteins with heparin and manganese chloride as described by Burstein et al. (22). Triglyceride concentration was determined with an enzymatic colorimetric test (GPO-PAP). Low density lipoprotein (LDL) cholesterol was calculated by the formula of Friedewald et al. (23): LDL cholesterol = Total cholesterol – HDL cholesterol – 0.47x serum triglycerides. Apolipoproteins A-I (Apo A-I) and B-100 (Apo-B) were measured immunochemically by rate nephelometry, using the Array Protein System from Beckman Instruments Inc. (Brea, CA, USA). Homocysteine was measured by a competitive immunoassay on an IMMULITE 2000 Analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). The lipid and homocysteine analysis was performed at the Department of Clinical Chemistry, University Hospital Northern Norway.

Coagulation analysis
Blood for reference plasma was obtained from 48 healthy (25 males and 23 females) blood donors at the Blood Bank, University Hospital Northern Norway and employees at the University of Tromsø, 22–50 years of age. Plasma samples from the donors were pooled and frozen at −70°C in aliquots of 0.5 ml until further use. The total inhibitory capacity of TFPI (TFPI Ac) was measured by a chromogenic substrate assay based on the ability of a test sample containing TFPI to inhibit TF/factor VIIa catalytic activity in the presence of factor Xa with minor modifications (24). The TFPI activity in the reference plasma was defined as 1 U/ml (intra-assay coefficient of variation (CV) 3.2%, inter-assay CV 4.6%). The carrier-free TFPI antigen (TFPI Ag) was determined by use of a solid-phase two-site enzyme immunoassay (intraassay CV 7.76%, interassay 12.16%) (25). FVIIc activity was measured by a one stage clotting assay using rabbit brain thromboplastin (IL Test™ PT-Fibrinogen HIS, Instrumentation Laboratory SpA, Milan, Italy) and native FVII-deficient plasma (IL Test™, Instrumentation Laboratory) on an automatic analyzer (ACL 3000, Instrumentation Laboratory) (intra-assay CV 3.8%, inter-assay 5.8%). FVIIa was measured by a commercial one stage clotting assay (Staclot VII-rtF, Diagnostica Stago, Asnieres-sur-Seine, France) using a mutant thromboplastin, which does not activate factor VII during the measurement (26) by an automatic analyzer (ACL Futura, Instrumentation Laboratory) (intra-assay CV 4.2%, inter-assay CV 5.7%). Fibrinogen
was measured by a one stage clotting assay (CTS-Fibrinogen, Dade Behring Marburg GmbH, Marburg, Germany) initiated by relative large amounts of thrombin and determined as photometric increase in absorbance on an automatic analyzer (Cobas Faro II, Roche Diagnostics, Basel, Switzerland) (intra-assay CV 2.9%, inter-assay CV 3.6%). Plasma concentration of thrombin-antithrombin complexes was measured with a commercial assay (Enzygnost TAT micro, Dade Behring Marburg GmbH, Marburg, Germany) according to the protocol given by the manufacturer (intra-assay CV 5.4%, inter-assay CV 9.3%).

Statistics
Baseline characteristics were compared by paired t-test between patients and matched controls for continuous data and by the McNemar test for categorical data. OPG, TFPI activity, sCD40L, homocysteine and TAT were logarithmically transformed because of their skewed distribution. Accordingly, the geometric mean is reported. The linear association between two continuous variables was tested by Pearson’s correlation coefficient. Linear trends were tested in a general linear model. Two-sided values of <0.05 were considered statistically significant. The statistical calculations were performed with the SPSS statistical package for Windows version 11.0 (SPSS Inc, Chicago, IL, USA). Confidence intervals for proportions were calculated by the Epi Info software package (Epi Info, version 6.04d, 2001).

Results
Clinical characteristics, blood pressures and serum lipids are shown in Table 1. BMI and triglyceride levels were significantly higher, and HDL cholesterol was significantly lower in the patients. Creatinine clearance was 96.7 ml/min in the patients and 89.9 ml/min in the controls, an difference of borderline significance (Table 1). The medication used in each group is shown in Table 2. Among MI patients, usage of aspirin, \( \beta \)-blockers, statins and ACE inhibitors did not significantly influence the OPG levels.

The serum OPG levels were similar in the two groups, 2.41 ng/ml (95% CI; 2.11–2.77) in the patients and 2.43 ng/ml (95% CI; 2.11–2.79) in the controls (p = 0.92) (Fig. 1). When analyzing the data for men and women separately, no differences in the OPG level were found between patients and controls within each sex (data not shown). The OPG serum level was 7.5% higher in women compared to men, both in patients and controls, but these sex differences were not significant (data not shown). Thirty-two patients had suffered a ST-segment elevation infarction (STEMI) and 26 a non-ST-segment elevation infarction (NSTEMI). Serum OPG was similar in the two groups [2.42 ng/ml (95% CI; 2.02–2.90 ng/ml) vs. 2.41 ng/ml (95% CI; 1.94–3.00 ng/ml)].

Table 1: Baseline characteristics in young survivors of myocardial infarction and healthy age- and sex-matched controls.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=58)</th>
<th>Controls (n=58)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>53.3 (51.9–54.7)</td>
<td>53.4 (52.1–54.8)</td>
<td>0.24</td>
</tr>
<tr>
<td>Male/female</td>
<td>47/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>40 (28–35)</td>
<td>22 (13–35)</td>
<td>0.052</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>28.0 (27.1–29.0)</td>
<td>26.2 (25.5–26.9)</td>
<td>0.003</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>138 (133–144)</td>
<td>140 (136–144)</td>
<td>0.64</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>82 (79–86)</td>
<td>85 (82–88)</td>
<td>0.24</td>
</tr>
<tr>
<td>Total cholesterol (mM)</td>
<td>6.01 (5.73–6.29)</td>
<td>6.15 (5.88–6.41)</td>
<td>0.51</td>
</tr>
<tr>
<td>LDL cholesterol (mM)</td>
<td>3.94 (3.68–4.19)</td>
<td>4.15 (3.91–4.40)</td>
<td>0.24</td>
</tr>
<tr>
<td>HDL cholesterol (mM)</td>
<td>1.25 (1.19–1.30)</td>
<td>1.40 (1.31–1.48)</td>
<td>0.006</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>1.58 (1.29–1.79)</td>
<td>1.14 (1.01–1.29)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>10.0 (9.3–10.9)</td>
<td>9.8 (9.1–10.5)</td>
<td>0.566</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>97 (91–103)</td>
<td>90 (85–95)</td>
<td>0.058</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>60 (56–63)</td>
<td></td>
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</tbody>
</table>

\(^{1}\)Geometric mean, \(^{2}\)n=56, \(^{3}\)n=57, calculated with the Cockcroft & Galt formula, \(^{4}\)Left ventricular ejection fraction after the acute event, assessed either by echocardiography (n=23), Multiple Gated Acquisition scan (MUGA) (n=13) or ventriculography (n=22).

Table 2: Regular use of drugs (%) in young survivors of myocardial infarction and in age- and sex-matched healthy controls.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Patients (n=58)</th>
<th>Controls (n=58)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA(^{1})</td>
<td>83 (48)</td>
<td>3 (2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>( \beta )-blockers</td>
<td>59 (34)</td>
<td>3 (2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACE-inhibitors(^{2})</td>
<td>14 (8)</td>
<td>2 (1)</td>
<td>0.039</td>
</tr>
<tr>
<td>Diuretics</td>
<td>5 (5)</td>
<td>0 (0)</td>
<td>0.250</td>
</tr>
<tr>
<td>Statins</td>
<td>50 (29)</td>
<td>5 (5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^{1}\)Acetylsalicylic acid \(^{2}\)Angiotensin converting enzyme inhibitors.

Figure 1: Box plot of serum OPG levels measured 1 to 4 years after the acute event in young survivors of myocardial infarction and age- and sex-matched controls. The OPG serum levels were similar in the two groups, 2.41 ng/ml (95% CI; 2.11–2.77) in the patients and 2.43 ng/ml (95% CI; 2.11–2.79) in the controls (p = 0.92). The top of the box represent the 75th percentile, the bar the median, the bottom of the box the 25th percentile and the whiskers the largest and smallest values, respectively, which is an outlier.
Twenty-two patients (37.9%) had a coronary angiography at the acute event. The OPG concentration was 1.91 ng/ml (95% CI; 0.87–4.23 ng/ml) in patients with no lesions (n=2), 2.42 ng/ml (95% CI; 1.58–3.70 ng/ml) in patients with 1-vessel disease (n=7), 2.35 ng/ml (95% CI; 1.54–3.59 ng/ml) in patients with 2-vessel disease (n=7) and 2.65 ng/ml (95% CI; 1.68–4.20 ng/ml) in patients with more than 2-vessel disease (n=6), (p for trend=0.90). The sCD40L levels were 0.26 ng/ml (95% CI; 0.21–0.32 ng/ml) in patients and 0.26 ng/ml (95% CI; 0.22–0.31 ng/ml) in controls (p=0.94).

No significant associations between OPG and factor VIIa, factor VIIc, fibrinogen, TFPI; TFPIa; TAT, lipids, apoa, apoB and apoE were found either in MI patients or in controls. A significant negative correlation between OPG and sCD40L was found in patients and between OPG and creatinine clearance in controls (Table 3). In both patients and controls, a significant positive correlation between OPG and age and between OPG and homocysteine was found (Table 3). Trend analysis for changes in OPG across tertiles of homocysteine was performed, and a significant trend for increase in OPG with increasing homocysteine concentration was found both in univariate analysis (p=0.008) and after adjustment for age and sex (p=0.012) (Fig. 2).

Discussion

In our case-control study no difference in serum concentrations of OPG was found between young survivors of myocardial infarction and age- and sex-matched controls. However, confounding factors may be a problem for interpretation of the results. The age- and sex-matched design, with cases and controls from the same population, strengthens the results despite the relatively low number of participants. A difference in serum OPG of 0.6 ng/ml between cases and controls is considered to be clinical relevant (20). The actual difference of 0.0 ng/ml gave an estimated statistical power of 98% to detect equivalence when the margin of equivalence is from −0.6 ng/ml to 0.6 ng/ml. Furthermore, the medication used in the patients did not significantly influence the OPG level, and no significant correlation was found between OPG and recognised risk factors for MI which differed between groups (BMI, HDL cholesterol, triglycerides).

Substantial evidence for impact of OPG on osteoclastic function and inhibition of calcification exist from several experimental, animal and human, studies (27–30). Furthermore, enhanced expression of RANKL in T-cells is reported to be an important feature of unstable atherosclerotic disease (31), indicating that the OPG/RANKL/RANK system may also be involved in atherogenesis and plaque destabilization. Generally, vascular calcification increases with advancing age. The participants in our study were relatively young, and it may be speculated that plaque morphology in this younger age group differs from elderly patients where presence of more calcification would be expected.

In previous studies, higher serum OPG levels have been reported in patients with stable angina pectoris (17, 18) and in patients with ST elevation acute myocardial infarction followed for 4 weeks after the acute event (32). In patients with heart failure after myocardial infarction, the OPG concentration decreased markedly one month after the event, and remained at the same level during 2 years follow-up (20). However, the OPG level remained significantly higher in the patients than in 15 controls (20). Elevated OPG levels in chronic heart failure has been reported with no differences between ischemic and idiopathic cardiomyopathy (33). Proinflammatory cytokines such as interleukin-1β, TNF-α, angiotensin II and growth factors such as platelet derived growth factor induce OPG expression in human vascular smooth muscle cells (7). The inflammatory response to acute MI and heart failure may therefore induce expression of OPG and represent an underlying mechanism for increased serum OPG under these conditions. The findings in the present study may indicate that the presence of heart failure is a stronger determinant for serum OPG levels than the ischemic event in patients with uncomplicated myocardial infarction. In our study, serum OPG was measured 1–4 years after the acute event, and the participants were relatively young with limited comorbidity. Furthermore, the myocardial infarction was uncomplicated without subsequent hospitalization for angina pectoris, reinfarction, co-

### Table 3: Association between OPG, cardiovascular risk factors and coagulation factors. (Pearson correlation coefficients).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Patients</th>
<th>Controls</th>
<th>p-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CD40L (ng/ml)</td>
<td>-0.31 &lt;0.001</td>
<td>0.01 0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>0.33 0.011</td>
<td>0.36 0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homocysteine (µM)</td>
<td>0.30 0.024</td>
<td>0.35 0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine clearance (ml/m)</td>
<td>-0.06 0.65</td>
<td>-0.34 0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VIIa (mU/ml)</td>
<td>0.10 0.48</td>
<td>-0.02 0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor VIIc (%)</td>
<td>0.13 0.34</td>
<td>-0.02 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>-0.04 0.76</td>
<td>-0.01 0.94</td>
<td></td>
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<tr>
<td>TFPI αa (U/mlim)</td>
<td>0.20 0.13</td>
<td>0.06 0.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFPI ag (ng/ml)</td>
<td>0.03 0.82</td>
<td>-0.02 0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (µg/l)</td>
<td>-0.14 0.32</td>
<td>0.10 0.47</td>
<td></td>
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</tbody>
</table>

TFPI; tissue factor pathway inhibitor; TAT; thrombin-antithrombin complex.

![Figure 2: Line graph showing changes in serum OPG concentration (mean ± SEM) across tertiles of homocysteine (tertile; 1< 9 µM), tercile 2; 9–11 µM, tercile 3; >11 µM. (n=114, values for homocysteine missing for 2 persons). Adjusted for age and sex.](image-url)
vascular angioplasty, ACB surgery or heart failure in the time period between the acute event and the inclusion in this study. Lack of increase in serum OPG levels in young survivors of myocardial infarction may in part be explained by these potentially proinflammatory factors.

In our study a significant positive correlation between OPG and homocysteine was found in both groups. Hyperhomocysteinemia is known to cause endothelial dysfunction and injury, followed by platelet activation and thrombus formation (34). Previously, an association between homocysteine and vWF has been reported (35). Homocysteine is known to promote the release of vWF from vascular endothelium and thereby playing a prothrombotic role (34). Our finding may suggest that homocysteine promotes release of OPG from the vascular endothelium, but the role of OPG in hemostasis is uncertain. Recently, it was reported that OPG and vWF were colococalized in endothelial cells and associated in human plasma (16), and thereby suggested that OPG may influence platelet activation and thrombus formation (16).

Activation of the coagulation system is an important factor in the pathogenesis of the acute coronary syndrome (36). In this case-control study, we found no association between OPG and the coagulation factors, including thrombin generation measured years after the acute event. However, a significant negative correlation between sCD40L and OPG was found in patients (r = -0.51, p < 0.001), but not in controls. Soluble CD40L may be involved in platelet activation and thrombus formation (37), and elevation of sCD40L has been reported in coronary disease, particularly in patients with acute coronary syndrome where it also predicts adverse events (38, 39). Both RANK and OPG are upregulated in B-lymphocytes and dendritic cells by CD40 ligation (5). It has been proposed that OPG might be involved in primary hemostasis due to its binding to vWF (16). The associations between OPG and homocysteine, and between OPG and sCD40L, nourish the hypothesis that OPG may be involved in the regulation of platelet activation. The main questions whether OPG is a risk factor for atherosclerosis, vascular calcification, promoting a procoagulatory endothelial state, or represent a counterregulatory protective mechanism is unsettled, and further studies are warranted.

References