Lack of Observed Association between High Plasma Osteoprotegerin Concentrations and Ischemic Stroke Risk in a Healthy Population

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BACKGROUND: Several studies suggest that osteoprotegerin (OPG) concentrations may be associated with the risk of ischemic stroke, but no large prospective studies have been conducted. We conducted a nested case-control study within a large cohort to elucidate a possible relation.

METHODS: The study was done within a follow-up study including 57,053 men and women. Baseline data included OPG concentrations, lifestyle factors, and medical history. Median length of follow-up was 3.1 years. We assessed the relationship between OPG and stroke risk using conditional logistic regression to adjust for known risk factors (smoking, blood pressure, cholesterol, diabetes, body mass index, alcohol use, polyunsaturated fatty acids, and education).

RESULTS: We identified 254 cases with verified incident acute ischemic stroke and 254 age- and sex-matched controls. Median plasma OPG concentration among cases was 1.84 µg/L (25th–75th percentile 1.45–2.30 µg/L) compared with 1.87 µg/L (1.49–2.27 µg/L) in the control group. The adjusted odds ratio was 0.87 (95% CI 0.46–1.63) comparing participants in the highest quartile of OPG concentrations with those in the lowest quartile.

CONCLUSIONS: These findings provide no support for the hypothesis that plasma OPG concentrations are associated with an increased risk of ischemic stroke. This result could indicate a different pathogenic process in stroke development from that in ischemic heart disease, where OPG is a strong predictor.

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Osteoprotegerin (OPG),7 first described by Simonet et al. (1), serves as a soluble decoy receptor for 2 members of the tumor necrosis factor receptor superfamily, RANKL and TRAIL (receptor activator of nuclear factor-κB ligand and tumor necrosis factor–related apoptosis-inducing ligand). Apart from being an important regulating molecule in bone turnover, OPG also serves as a vascular calcification inhibitor, as indicated in recent studies. OPG production has been demonstrated in many different tissues, including bone, heart, and vasculature (2), and is also present in plasma, but at considerably lower concentrations than in bone and arterial tissue (3, 4). Nevertheless, plasma OPG has been shown to correlate with bone and arterial diseases (5), and recent studies have shown that OPG is a strong, independent predictor of cardiovascular disease (CVD) (6, 7).

Ischemic stroke is associated with the presence of coronary calcifications (8). As plasma OPG is associated with vascular calcifications (9), the molecule may possibly also be involved in the development of ischemic stroke (10–12); however, no large prospective studies have been conducted. We therefore examined the association between plasma OPG concentrations and ischemic stroke risk within a large cohort to elucidate whether this new cardiovascular risk marker is associated with future risk of ischemic stroke.

Materials and Methods

DESIGN AND STUDY POPULATION
We conducted a nested case-control study within the Danish follow-up study Diet, Cancer, and Health (13). People born in Denmark who resided in the area of

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Received May 14, 2008; accepted August 21, 2008. Previously published online at DOI: 10.1373/clinchem.2008.110593

Nonstandard abbreviations: OPG, osteoprotegerin; CVD, cardiovascular disease; ICD, International Classification of Diseases; OR, odds ratio.
Copenhagen or Aarhus and had no previous cancer diagnosis registered in the Danish Cancer Registry were eligible for the study. From December 1993 through May 1997, 80 996 men and 79 729 women ages 50–64 years were invited to participate in the study; 27 177 men (33.6%) and 29 876 women (37.3%) accepted the invitation. All cohort members completed questionnaires on education and lifestyle factors. At baseline, anthropometric features, blood pressure, and plasma cholesterol were measured.

We obtained information on death or emigration using the Civil Registration System that records data of all changes in vital status of the entire Danish population through their civil registry number, a personal identification number given to all Danish citizens at birth or on immigration. We retrieved information on hospitalizations from the Danish National Registry of Patients (14), including date of admission to and discharge from the hospital, surgical procedures, and up to 20 discharge diagnoses, classified according to the Danish version of the International Classification of Diseases, 8th Revision (ICD-8) until 1993 and subsequently according to ICD-10.

To ensure that OPG concentrations and possible confounding factors were not influenced by prevalent CVD at baseline, we excluded 2500 hospitalized participants (4.4%) before enrollment due to CVD [i.e., stroke, transient ischemic attack, ischemic heart disease, or peripheral arteriosclerosis (ICD-8: 410–414, 430–438, 440; ICD-10: G45, I20–25, I60–70)]. Furthermore, we excluded 47 participants (0.08%) with ≥10 items blank on the questionnaires.

CASES AND CONTROLS
We defined potential cases as participants with a discharge diagnosis of stroke or transient ischemic attack (ICD-10: I60–I69.8 and G45). Cases were identified through 1998 for persons living in the Copenhagen area and through 1999 for persons living in the Aarhus area. A single researcher reviewed medical records using a detailed standardized form to verify the diagnosis (15). The review was based on all available information in the medical records including radiology reports, results from laboratory tests, etc. All cases underwent computed tomography or magnetic resonance scanning.

We used the WHO definition of stroke, i.e., an acute disturbance of focal or global cerebral function with symptoms lasting >24 h or leading to death of presumed vascular origin (16). The distinction between ischemic stroke, intracerebral hemorrhage, and subarachnoid hemorrhage was based on computed tomography or magnetic resonance scan, spinal fluid examination, or an autopsy report. All cases of ischemic stroke were subclassified on the basis of the presumed etiology according to the Trial of ORG10172 in Acute Stroke Treatment (TOAST) classification: large-artery atherosclerosis, cardiac embolism, small-vessel occlusion, stroke of other determined etiology, and stroke of undetermined etiology (17). This classification is based on clinical features, i.e., cortical or cerebellar dysfunction and lacunar syndrome, and on data (e.g., location and size of infarct) collected by tests such as brain imaging, cardiac imaging, duplex imaging of extracranial arteries, arteriography, and laboratory assessments for a prothrombotic state. Only cases of ischemic stroke were included in this study, since the number of participants with intracerebral hemorrhage or subarachnoid hemorrhage was too small for further analyses. One control was selected for each case, matched by sex and age (within 5 years) using the risk set sampling technique (18).

BLOOD SAMPLING
Blood samples were drawn from each participant in a nonfasting state. The samples were spun and divided into tubes of 1 mL. All samples were processed and frozen within 2 h at −20 °C. At the end of the day of collection, all samples were stored in liquid nitrogen vapor (maximum −150 °C) serving as a biorepository, where samples were kept unthawed until analysis.

OSTEOPROTEGERIN MEASUREMENT
We measured OPG concentration in EDTA-plasma samples from the biorepository using a commercially available kit (R&D Systems). The assay was a sandwich ELISA using a mouse antihuman OPG as capturing antibody and a biotinylated goat antihuman OPG for detection, done by horseradish peroxidase–conjugated streptavidin. The analytical range was 62.5–4000 pg/mL. The interassay imprecision (CV) was 8% (n = 18), and the intraassay imprecision judged from duplicate measurements was 3%. The method measured total OPG in the sample (both free and bound to RANKL). Addition of RANKL to the incubation wells did not interfere with or alter the OPG measurement.

STATISTICAL ANALYSES
We categorized OPG concentrations into quartiles based on the distribution among cases and estimated risk of ischemic stroke using the lowest quartile as the reference risk group. Conditional logistic regression was used to estimate odds ratio (OR) adjusted for the following possible confounding variables: smoking status (current, former, and never smoker), systolic and diastolic blood pressure at baseline (included as continuous variables), total nonfasting plasma cholesterol at baseline (≤6 mmol/L vs >6 mmol/L), self-reported history of diabetes (yes/no), body mass index (BMI) (included as a continuous variable), alcohol intake...
(≤14 vs >14 units of alcohol per week for women and ≤21 vs >21 units per week for men), intake of n-3 polyunsaturated fatty acids from fish and other sources, and length of school education (7, 8–10, and >10 years). We also examined the association between OPG and risk of ischemic stroke with second-degree fractional polynomial regression to obtain smooth representations of the OR as a continuous function of OPG (19). The median OPG value was used as a reference value in this analysis. Analyses were done separately for different subtypes of ischemic stroke, and 95% CIs were calculated for all ORs. Analyses were performed using Stata Statistical Software (Stata Corp.).

## ETHICS
Diet, Cancer, and Health and the substudy reported here were approved by the regional ethics committees and by the Danish Data Protection Agency.

## Results

### CHARACTERISTICS OF CASES AND CONTROLS
Two hundred sixty-six study participants were hospitalized with a verified diagnosis of acute ischemic stroke, but only 254 cases had provided plasma samples at baseline. We found no substantial differences in baseline characteristics when comparing the 12 excluded cases with the remaining 254 cases. Median length of follow-up was 3.1 years (range 0.0–5.1 years).

Table 1 shows characteristics of the 254 cases and 254 controls. Data on all variables were available for 498 participants (98.0%).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>254</td>
<td>254</td>
</tr>
<tr>
<td>Median age, years (10th–90th percentile)</td>
<td>60.4 (53.7–65.9)</td>
<td>60.5 (54.0–66.1)</td>
</tr>
<tr>
<td>Sex, % M/F</td>
<td>61/39</td>
<td>61/39</td>
</tr>
<tr>
<td>Smoking status, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>57.1</td>
<td>33.9</td>
</tr>
<tr>
<td>Former</td>
<td>21.0</td>
<td>31.9</td>
</tr>
<tr>
<td>Never</td>
<td>21.8</td>
<td>34.3</td>
</tr>
<tr>
<td>Median systolic blood pressure at baseline, mmHg (25th–75th percentile)</td>
<td>156.0 (24.3)</td>
<td>142.7 (23.5)</td>
</tr>
<tr>
<td>Median diastolic blood pressure at baseline, mmHg (25th–75th percentile)</td>
<td>89.7 (11.5)</td>
<td>84.8 (11.5)</td>
</tr>
<tr>
<td>Hyperlipidemia at baseline, %a</td>
<td>57.5</td>
<td>48.0</td>
</tr>
<tr>
<td>Self-reported history of diabetes, %</td>
<td>5.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Median body mass index, kg/m² (25th–75th percentile)</td>
<td>26.5 (4.0)</td>
<td>26.6 (4.1)</td>
</tr>
<tr>
<td>High alcohol intake, %b</td>
<td>27.6</td>
<td>20.9</td>
</tr>
<tr>
<td>Years of school education, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>42.9</td>
<td>39.4</td>
</tr>
<tr>
<td>8–10</td>
<td>42.5</td>
<td>40.6</td>
</tr>
<tr>
<td>&gt;10</td>
<td>14.6</td>
<td>20.1</td>
</tr>
<tr>
<td>Median osteoprotegerin, μg/L (25th–75th percentile)</td>
<td>1.84 (1.45–2.30)</td>
<td>1.87 (1.49–2.27)</td>
</tr>
<tr>
<td>Ischemic stroke subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large-artery atherosclerosis</td>
<td>26 (10.2)</td>
<td></td>
</tr>
<tr>
<td>Cardiac embolism</td>
<td>22 (8.7)</td>
<td></td>
</tr>
<tr>
<td>Small-vessel occlusion</td>
<td>112 (44.0)</td>
<td></td>
</tr>
<tr>
<td>Other determined etiology</td>
<td>2 (0.8)</td>
<td></td>
</tr>
<tr>
<td>Undetermined etiology</td>
<td>92 (36.2)</td>
<td></td>
</tr>
</tbody>
</table>

*a Nonfasting total cholesterol >6 mmol/L.

*b >14/21 units of alcohol per week for women and men, respectively.

## OPG AND RISK OF ISCHEMIC STROKE
Baseline median OPG concentration among cases was 1.84 μg/L (25th–75th percentile 1.45–2.30) compared with 1.87 μg/L (1.49–2.27) in the control group, a non-
significant difference. Cutpoints for the OPG quartiles were 1.45, 1.84, and 2.30 μg/L for cases and 1.49, 1.87, and 2.27 μg/L for controls. A crude OR of 0.88 (95% CI 0.54–1.42) was found when comparing participants in the top quartile with participants in the bottom quartile (Table 2). After adjustment for a wide range of possible confounding factors, the OR remained virtually unchanged at 0.87 (95% CI 0.46–1.63). The second-degree fractional polynomial regression could suggest a possible threshold pattern in the association between OPG concentration and risk of ischemic stroke, which indicated a U-shaped tendency (not shown). Only few cases were present in the extremes of the curve, however, and the curve had relatively wide confidence intervals and can only be used as an indicator for further analysis. Separate analyses of the different subtypes of ischemic stroke did not reveal any significant differences (data not shown).

Discussion

In this study, we show that increased plasma OPG concentrations are not associated with ischemic stroke risk in a large cohort after adjustment for known risk factors. As recent studies have shown that plasma OPG is a strong, independent predictor of CVD, our findings also indicate a different pathogenic process in stroke development from that in cardiovascular disease in general.

OPG, a vascular calcification inhibitor, is upregulated when calcifications evolve. This may be the reason for the presence of increased circulating amounts in patients with vascular calcifications (9). A reason for the nonexisting association between plasma OPG and development of ischemic stroke in this study could be that vascular calcification is not involved in development of stroke per se. It is well-established, however, that carotid atherosclerosis, which often contains calcified areas, highly predisposes to cerebral ischemic events, and evidence is accumulating that correlates carotid plaque texture with plaque stability (20, 21). A new study has found increased serum OPG concentrations in patients with carotid stenosis and documented an independent association between this marker and vulnerable carotid plaques (22). Of note, most of the patients in that study had a recently diagnosed internal carotid artery stenosis, whereas our study differs considerably in population composition, as it was conducted in a larger, unselected cohort from the general population. Other previous studies indicating a correlation between plasma OPG concentrations and acute stroke were also carried out either in a risk population (10, 12) or in a rather small population (11). We find that our follow-up study estimates the stroke risk in a general population better than when investigating risk populations, where a complex risk profile complicates establishment of a straightforward cause–consequence relation. Furthermore, the nested case-control design used here is more methodologically favorable and usually gives more solid data.

Recently, a polymorphism in the gene coding for OPG (OPG 1181C/C genotype) was found to be associated with first-ever intracerebral hemorrhage (OR 6.04), but no correlation was found between this genotype and ischemic stroke (23). OPG concentrations were not measured in that study, and thus it cannot be assessed with relation to our findings.

The finding of plasma OPG as a predictor of CVD could seem counterintuitive, as the molecule inhibits vascular calcifications and high amounts and therefore should be beneficial. Of note, a spline analysis (not shown), in which normally is preferred when handling nonlinear associations, suggested that low OPG concentrations actually increase stroke risk more than high concentrations. This is consistent with the study by Vik et al. (24), who found an inverse correlation between OPG concentrations and carotid plaque echogenicity. The wide confidence intervals in the extremes of the curve, however, do indicate that the shape of the curve should be interpreted cautiously. Speculatively, lack of OPG as a vascular calcification inhibitor could increase the risk of vascular calcification, whereas the considerably increased concentrations are seen late in the pathophysiological process, when the high amounts of OPG may no longer be beneficial. As mentioned, our findings are in contrast to the association described for CVD in general; one could therefore speculate on whether the OPG molecule plays different roles in the 2 settings. Clarification of this must await further investigations.

Limitations of our study exist. OPG concentrations are known to be affected by the length of time

### Table 2. ORs and 95% CIs for ischemic stroke in relation to OPG.

<table>
<thead>
<tr>
<th>OPG quartile</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0.86 (0.52–1.42)</td>
<td>0.89 (0.45–1.73)</td>
</tr>
<tr>
<td>3</td>
<td>0.72 (0.43–1.21)</td>
<td>0.51 (0.25–1.05)</td>
</tr>
<tr>
<td>4</td>
<td>0.88 (0.54–1.42)</td>
<td>0.87 (0.46–1.63)</td>
</tr>
</tbody>
</table>

* Cutpoints for the OPG categories were 1.45, 1.84, and 2.30 μg/L for cases and 1.49, 1.87, and 2.27 μg/L for controls.

* Adjusted for smoking, systolic and diastolic blood pressure, total plasma cholesterol, diabetes, body mass index, alcohol intake, intake of n-3 polyunsaturated fatty acids, and years of school education.
between sample collection and sample processing (25). Our sampling procedure was as rapid as possible, however, and the samples were frozen within a few hours. We therefore do not suspect that any preanalytical effects were introduced in this aspect. As far as the effect of storage on the OPG concentrations measured in EDTA samples is concerned (25), the fact that the samples from cases and controls were treated similarly substantiates that our findings are genuine and not affected by loss of OPG due to freezing. Furthermore, the OPG concentrations in our control group are compatible with others reported and do therefore not indicate such a problem. One should however be aware of these preanalytical issues when interpreting data from studies like ours.

Our study had a prospective design with a complete follow-up through population-based registries followed by a detailed assessment of all potential cases, and the risk of selection and surveillance bias was therefore limited. We were able to include only strokes that led to hospitalization, but given the age profile of our study cohort, it is likely that most patients with clinical symptoms of acute stroke were referred to a hospital for further evaluation. Of note, administrative registers are not necessarily 100% sensitive, nor is a population-based register as the one used in this study. It is unlikely that the sensitivity should be related to the OPG concentrations, however, and the possibility of a bias in this relation therefore seems very limited. In this regard, it is important to emphasize that all the cases in our study were validated through a computed tomography/magnetic resonance imaging scan.

In conclusion, our findings do not support involvement of OPG in the pathogenesis of ischemic stroke in a general population. This result could indicate a different pathogenic process in stroke development from that in CVD, where OPG is a strong predictor. The seeming discrepancy in those 2 settings warrants further investigations to elucidate the exact pathological mechanism connected to the OPG molecule.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors’ Disclosures of Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. No authors declared any potential conflicts of interest.

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Stock Ownership:** None declared.

**Honorary:** None declared.

**Research Funding:** None declared.

**Expert Testimony:** None declared.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

**References**