The relationship between serum levels of vascular calcification inhibitors and carotid plaque vulnerability

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Objective: **Osteopontin (OPN) and osteoprotegerin (OPG) are well-known vascular calcification inhibitors, which have been recently demonstrated to correlate with inflammation and cardiovascular events incidence. The aim of this cross-sectional study is to survey whether OPN and OPG are involved in carotid plaque vulnerability. For this reason, we assessed serum OPN and OPG levels in patients with carotid stenosis, and we explored their relationship with carotid plaque echogenicity and subsequent cerebrovascular ischemic events.**

Methods: **A total of 164 Whites were selected from a large cohort of 297 subjects to participate. In particular, 114 patients (61 men, 53 women), aged 55 to 80, had recently-diagnosed ICA stenosis higher than 50%. A group of 50 age-, sex-, and body mass index (BMI)-matched healthy individuals served as healthy controls. Patients with renal failure, hypothyroidism, osteoporosis, and lipid-lowering therapy were excluded. Images of both carotids were obtained from all participants using a high-resolution color duplex ultrasound and the gray-scale median (GSM) score was calculated. Brain computed tomography (CT), and magnetic resonance imaging (MRI) scans when CT was questionable, were performed on all patients with carotid stenosis. Clinical parameters, lipid and glycemic indexes, hsCRP, fibrinogen, white blood cells (WBC) count, OPN, and OPG were measured. Independent *t* test, one-way ANOVA, Pearson correlation, and multiple regression analysis were used for statistical analysis.**

Results: **Among patients with carotid stenosis, 60 had history of ipsilateral stroke or TIA and positive CT or MRI findings (group A), while 54 had no neurological symptoms and negative CT and MRI scan (group B). Overall, patients with carotid stenosis showed worse lipid profile and increased waist circumference, blood pressure, hsCRP, fibrinogen, WBC count, OPN, and OPG levels compared with healthy subjects (group C) ($P < .05$). Statistical analysis revealed that group A had significantly lower levels of GSM than group B (57.41 ± 38.19 vs 76.32 ± 36.72; $P = .008$) and higher levels of hsCRP, OPN, and OPG than groups B and C ($P < .05$). Concerning the latter, biochemical markers group B showed only elevated OPG levels compared with group C ($P = .038$). Notably, GSM was considerably associated with serum OPN and OPG and waist circumference in patients with carotid atherosclerosis in univariate ($r = -0.333$; $P = .032$, $r = -0.575$; $P < .001$, $r = -0.590$; $P = .006$, respectively) and multiple regression analysis ($R^2 = 0.445$; $P = .006$).**

Conclusions: **The present study demonstrated elevated serum OPN and OPG levels in patients with carotid stenosis and documented an independent association between these biochemical markers, GSM and carotid-induced symptomatology. Therefore bone-matrix proteins combined with GSM could be potential markers for vulnerable carotid plaques. (J Vasc Surg 2008;47:55-62.)**

Stroke is one of the most common causes of mortality and disability in the Western world. It is well established that carotid atherosclerosis highly predisposes to cerebral ischemic events, and mounting evidence correlates carotid plaque texture with its stability.[1,2] Regarding imaging techniques to assess carotid narrowing and carotid plaque composition, high-resolution ultrasound B-mode has become a safe, noninvasive, and repeatable method. Most recently, the gray-scale median (GSM) score, a quantified index of carotid plaque echogenicity, has emerged as a secondary assessment methodology of carotid plaque vulnerability.[3] Previous researchers have linked histopathological and ultrasonographic findings.[4] In particular, they have documented that echolucent plaque comprises of greater lipid content and hemorrhage than echogenic plaque that mostly contains calcified and fibrous tissue, and it is associated with higher rate of cerebrovascular events.

Ectopic calcification constitutes a prominent feature of several cardiovascular diseases, which is tightly regulated by promoters and inhibitors.[5] Although calcium deposition in carotid plaque augments its stability, coronary artery calcification is a more complex procedure.[6] Quantitative assessment of coronary calcification by electron-beam computed tomography strongly predicts acute coronary syndromes (ACS).[7] On the other hand, intravascular ultrasound has demonstrated that localized plaques from patients with stable angina pectoris are more extensively calcified than

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those from patients with ACS.[8] These findings implicate that coronary artery tree calcification is a risk factor for ACS, while coronary lesions calcification stabilizes atherosclerotic plaque and prevents ACS.

Osteopontin (OPN), a well-known calcification inhibitor, is expressed by many cell-types (osteoblasts, macrophages, etc) in response to biological stressors.[9] Via signaling functions, OPN inhibits mineral deposition and regulates mineralized tissue turnover.[10] Unlike bone, the role of OPN in the arterial vasculature is poorly characterized. Although OPN is expressed by all aortic cells, including activated macrophages of the atheroma, its involvement in atherosclerosis progression is still speculative.[11]

Osteoprotegerin (OPG), a member of the tumor necrosis factor-receptor family, inhibits osteoclastogenesis and the osteoclastic bone resorption.[12] In arterial vasculature, smooth muscle cells and endothelial cells produce OPG, but its precise role in vascular pathophysiology remains obscure.[13] In nonatherosclerotic artery, OPG functions as an angio-protective factor by enhancing immune defence, preventing endothelial cells apoptosis and inhibiting vascular calcification.[10] The inhibition of receptor activator of nuclear factor-kB ligand (RANKL) and tumor necrosis factor (TNF) -related apoptosis-inducing ligand (TRAIL) mediate predominantly the above functions.[10] Contrary to the aforementioned protective role, OPG has been positively associated with the presence and severity of coronary artery disease,[14] and the increased risk of cardiovascular diseases in general population.[15] Most recent evidence suggests that OPG counter-regulate the atherosclerosis-induced mineralization of multipotential vascular cells and thus inhibits atherosclerotic. Further studies are required.

Thus, the primary endpoints of the present study were first to address the question of whether serum OPG and OPN are associated with carotid atherosclerosis and subsequent cerebrovascular ischemic events and second, to test the hypothesis that bone-regulators correlate with carotid plaque echogenicity and thereby could be used as early markers of vulnerable carotid plaques.

METHODS

Study design and subjects. The present, cross-sectional, study was conducted from May 2005 to April 2007. It constitutes a part of an ongoing, prospective project, which aims to identify patients with undiagnosed carotid stenosis and provide optimal therapies. Therefore, the following preliminary data represent the baseline characteristics of our project population. The target population was: (1) patients with recently-diagnosed stenosis, more than 50%, of at least one internal carotid artery (ICA) and (2) healthy subjects. After obtaining a written informed consent, all participants underwent a diagnostic high-resolution color duplex ultrasound of both carotids.

Patients with carotid stenosis were selected from a large cohort of patients recently admitted to our hospitals due to ischemic stroke, transient-ischemic attacks (TIAs), or amaurosis fugax. Candidates for carotid artery examination were also subjects with newly diagnosed peripheral arterial disease, or patients attending our hospital out-patient departments of diabetes, obesity, and hypertension with one or more classical cardiovascular risk factors. In parallel, we looked for healthy volunteers among subjects who had visited our internal medicine department for reasons irrelevant to chronic diseases. All healthy subjects were free from any chronic metabolic or cardiovascular disease. None of them was receiving any long-term medication or was suffering from an acute infection. Exclusion criteria were life-threatening diseases, hypothyroidism, osteoporosis, coronary artery disease, or overt cardiac-origin symptoms, atrial fibrillation, and renal impairment (creatinine levels $>$ 2.0 mg/dl). Trying to limit drug confounders, we did not recruit patients receiving systemic glucocorticoids, immunosuppressants, bisphosphonates, lipid lowering therapy, angiotensin II receptor blockers, estrogen therapy, and warfarin which may affect OPN and OPG levels. Carotid intimal thickening was considered as an exclusion reason for healthy individuals.

In patients with carotid stenosis, the diagnosis of acute ischemic stroke or TIA was based on the comparative evaluation of medical history, neurological examination, and brain computed tomography (CT) scan. The presence of subcortical or cortical ischemic lesions in the territories of carotid arteries was indicative of positive CT scan. When CT findings were questionable, brain magnetic resonance imaging (MRI) examination was additionally performed, just to limit the possibility of misclassifying patients with silent infarcts. After initial evaluation, eligible subjects were categorized into group A, symptomatic patients with carotid stenosis, history of ipsilateral stroke or TIA and positive CT and/or MRI findings; group B, asymptomatic patients with carotid stenosis, no focal neurological symptoms and negative CT and MRI; and group C, healthy individuals. Patients with silent infarcts were assigned to symptomatic group. In symptomatic patients, clinical examination and all imaging tests were performed during their hospitalization.

Diabetes mellitus was considered to be present if a patient fulfilled any of the criteria of diabetes diagnosis (American Diabetes Association). Peripheral arterial disease, smoking habits, and parental medical history were recorded using structured questionnaire.

A physical examination was performed and anthropometrical parameters like body weight, height, waist and hip circumferences, and waist-to-hip ratio (WHR) were obtained from all participants. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the height in meters (kgr/m^{-2}). Blood pressure was measured twice, after keeping participants at a sitting position for 15 minutes. There was a 5-minute interval between the two measurements and the mean value was estimated.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the local ethical committees.

Ultrasound. An ultrasound scanner (General Electric Logiq700, Riverside, Calif) equipped with a 7.5 MHz probe was used. Carotid artery was investigated bilaterally in a suitable longitudinal or transverse view by the same radiologist who was unaware of clinical and laboratory data. Gray-scale and color Doppler images were used in diagnosis and grading of ICA stenosis according to the recommendations of the Society of Radiologists in Ultrasound.[16] All examinations and measurements of plaques were directly recorded on a personal computer and were performed by a single observer blinded to patients' history. Texture analysis of manually selected plaque areas was performed using ANALYSIS, an integrated software system designed to assist interpretation of medical images and GSM was calculated[17]. Normalization of GSM was performed according to the procedures described by Elatrozy et al.[18] Specifically, the images were normalized manually by linearly adjusting the image so that the GSM value of the blood was 0-5 and the GSM value of the adventitia 180 to 190.

In group A, the GSM value of the culprit plaque associated with the ipsilateral brain infarct was used for the study purposes. In case of more than one unilateral atherosclerotic plaque, we measured GSM of each plaque and then averaged the echodensity of these plaques. In asymptomatic patients with more than one carotid plaque, GSM of each single plaque was estimated and the average value was considered for statistical analysis.

Blood analyses. Blood samples were obtained between 8 AM and 9 AM after an overnight fast. In order to restrict the effects of time since event on biochemical markers, we obtained blood samples from symptomatic group at the same time period: 14 days after admission to the hospital and at least 7 days after discharge from the hospital. Glucose and lipid parameters were determined using standard enzymatic methods (Olympus AU560, Hamburg, Germany). High-sensitivity C-reactive protein (hsCRP) and fibrinogen were measured by nephelometric assay (Dade Behrin, BNII, Marburg, Germany) and the Clauss method, respectively. Plasma OPN and OPG were assayed using quantikine immunoassay ELISA kits (R&D Systems Inc, Minneapolis, Minn, and BioVendor Laboratory Medicine Inc, Modrice, Czech Republic). The intra- and inter-assay coefficients of variance were 2.6% and 5.7% for OPN and 7% and 6.8% for OPG, respectively. Samples were frozen and stored (−80°C) until analysis in the same assay.

Statistical analysis. Results are presented as means ± SD. Comparison between groups was performed by student *t* test and one-way ANOVA for parametric parameters. χ^2 test and Mann-Whitney U test was used for nonparametric differences. For multiple comparisons, significant values were adjusted using the Bonferroni method. Normality of distribution was assessed by the Kolmogorov-Smirnov test. Univariate correlations of continuous variables were examined by Pearson's correlation analysis. Partial correlations were used to adjust for age. Multiple regression analysis was conducted to evaluate the relative contributions made by each variable to GSM. All analyses were carried out by software SPSS (13.0 version, SPSS Inc, Chicago, Ill). The chosen significance level was a two-tailed $P < .05$.

RESULTS

At initial screening, significant carotid atherosclerotic lesions were found in 225 patients. Regarding selection criteria, 114 of them were finally considered eligible for our study. On the other hand, 72 healthy individuals were identified and subscribed to participate in our study. From the latter population a body of 50 age-, sex-, and body-mass index (BMI)-matched healthy volunteers was finally included for statistical analysis. Totally, 164 Caucasian patients (90 men and 74 women) were included in the present study. All carotid ultrasound images were of good quality, and therefore, they were concerned for statistical analysis. The ultrasound criteria of the degree of carotid stenosis have been validated in our hospitals' cohort and conform to international recommendations.[17]

The characteristics of all participants are listed in Table I. Patients with carotid stenosis matched healthy subjects in blood pressure and smoking habits, but they differed in waist circumference and WHR ($P < .05$). We detected increased mean fasting plasma glucose, total cholesterol, and LDL concentrations and lower levels of HDL in patients with carotid stenosis than healthy individuals ($P < .05$). Hyperglycemia observed in the carotid group was attributed to the inclusion of diabetic patients (26.32%) and their moderate glycemic regulation. Moreover, the concentrations of inflammatory markers such as hsCRP, fibrinogen, white blood cells (WBC) count, and vascular calcification inhibitors were considerably increased in patients with carotid stenosis compared with control subjects ($P < .001$). We then examined the influence of diabetes on inflammatory and bone-matrix markers, and we identified higher levels of them in the diabetic subpopulation. However, after the exclusion of diabetic patients from statistical analysis, the differences between nondiabetic subgroup and healthy subjects were modestly decreased, without altering the level of significance (data not shown).

Among patients with carotid stenosis, 60 presented with neurological disorders or history of neurological symptoms and positive brain imaging findings (symptomatic, group A) and 54 had no clinical and imaging evidence of cerebral ischemic attack (asymptomatic, group B). Three patients with carotid stenosis reported transient neurological symptoms (amaurosis fugax or dysarthria), which passed before their admission to the hospital. Those patients after thorough examination appeared without residual neurological derangements and negative brain CT and MRI scans. Their symptoms were attributed to conditions other than acute ischemic attacks and, thus, they were assigned to asymptomatic group.

We did not detect significant differences between symptomatic and asymptomatic patients in clinical parameters, lipid profile, blood pressure, fibrinogen, and WBC count, while we found increased levels of hsCRP ($P =$

Table I. General characteristics of patients with carotid atherosclerosis and healthy subjects

Variables	*Carotid atherosclerosis*	*Healthy control*	P
Number	114	50	
Males /Females	61/53	29/20	.598
Smokers (%)	23 (20.18%)	11 (22%)	.766
Diabetes (%)	30 (26.32)	—	
Antihypertensive regimen (%)	46 (40.35%)	—	
PAD	13 (11.4%)	—	
Age (y)	67.57 ± 6.55	65.39 ± 8.88	.846
BMI (kg/m^2)	27.59 ± 3.48	28.15 ± 3.60	.377
Waist circumference (cm)	96.92 ± 9.01	93.54 ± 12.53	.012
WHR	0.95 ± 0.08	0.92 ± 0.09	.039
Systolic BP (mm Hg)	132.25 ± 16.10	128.67 ± 16.13	.214
Diastolic BP (mm Hg)	78.31 ± 7.94	81.22 ± 9.98	.082
Total cholesterol (mg/dl)	237.74 ± 41.63	215.55 ± 51.62	.014
HDL (mg/dl)	41.92 ± 12.47	47.68 ± 12.39	.007
LDL (mg/dl)	161.37 ± 35.78	140.71 ± 42.91	.014
Triglycerides (mg/dl)	164.32 ± 90.42	142.32 ± 60.34	.141
FPG (mg/dl)	128.28 ± 40.97	87.38 ± 11.82	<.001
hsCRP (mg/L)	3.94 ± 2.48	1.68 ± 0.93	.003
WBC (cells/μL)	7164 ± 1899.46	6495 ± 1549.24	.046
Fibrinogen (mg/dl)	421.19 ± 132.61	319.48 ± 95.95	<.001
Osteopontin (ng/ml)	71.33 ± 45.35	43.57 ± 29.63	.008
Osteoprotegerin (pmol/L)	8.00 ± 3.47	5.74 ± 2.39	.002

PAD, Peripheral artery disease; *BMI,* body mass index; *WHR,* waist hip ratio; *BP,* blood pressure; *FPG,* fasting plasma glucose; *hsCRP,* high sensitivity C-reactive protein; *WBC,* white blood cells.
Data are means ± SD.

.046) and lower GSM score ($P = .008$) in symptomatic than asymptomatic group (Table II). As well, the degree of carotid stenosis did not differ between groups significantly. Using one-way ANOVA analysis, we investigated differences in hsCRP, OPN, and OPG levels between symptomatic, asymptomatic, and healthy individuals. These three groups were age-, sex-, and BMI-matched. At post-hoc analysis, symptomatic patients appeared with significantly higher levels of serum hsCRP, OPN, and OPG than asymptomatic counterparts ($P = .041$, $P = .046$, and $P = .016$, respectively) and healthy subjects ($P = .026$, $P = .004$, and $P = .001$, respectively). In comparison with healthy controls, asymptomatic patients showed non-statistically significant higher levels of hsCRP (2.93 ± 1.95 mg/L vs 1.68 ± 0.93mg/L; $P = .329$) and OPN (62.83 ± 41.81ng/ml vs 43.57 ± 29.63 ng/ml; $P = .069$), but considerably elevated levels of OPG (6.78 ± 2.05pmol/L vs 5.74 ± 2.39pmol/L; $P = .038$) (Fig 1).

Correlations. In the total study population, univariate analysis revealed that OPN was positively correlated with OPG, age, total cholesterol, and LDL and inversely with HDL and GSM ($P < .05$). Notably, the relationship between serum OPN and lipid parameters was lost after adjustment for age (total cholesterol: $P = .120$, LDL: $P = .329$, HDL: $P = .540$). Besides OPN, age, hsCRP, BMI, and GSM value were also related to OPG. Adjustment for age attenuated the relationship between OPG and BMI ($P = .255$). Most importantly, the computerized marker of plaque echogenicity was strongly associated with bone-remodeling proteins, waist circumference, and WHR ($P < .05$) (Table III). No other significant relationships were identified between OPN, OPG, GSM, and the rest of study variables.

Multiple regression analysis was then performed to evaluate the above correlations. We observed that GSM retained an independent association with waist circumference, OPN, and OPG levels ($R^2 = 0.445$; $P = .006$). We also used multilinear regression analysis for the rest of the above associations as well. We found that OPN, GSM, and hsCRP constituted independent predictors of OPG after entering multiple regression model ($R^2 = 0.552$; $P = .011$).

DISCUSSION

In the present cross-sectional study, patients with carotid atherosclerosis presented with worse lipid profile, increased inflammatory markers (hsCRP, fibrinogen, WBC), and elevated serum OPN and OPG levels than healthy individuals. The latter biochemical markers showed statistically significant difference between symptomatic and asymptomatic patients with carotid stenosis, and they were independently associated with carotid plaque echogenicity. These data implicate the contributory role of vascular calcification inhibitors to carotid atherosclerosis and plaque stability. In parallel, the independent association of waist circumference with GSM is a promising result with clinical relevance.

OPN is a noncollagenous adhesive protein that increases considerably across coronary artery disease severity.[19] Previous investigators argued that serum OPN is independently associated with early carotid atherosclerosis.[20] Our study extended those findings by demonstrating a 63.71% incre-

Table II. Comparison of variables between symptomatic and asymptomatic patients with carotid atherosclerosis

Variables	*Symptomatic patients*	*Asymptomatic patients*	P
Number	60	54	
Males/females	37/23	31/23	NS
Smokers (%)	11 (18.33%)	12 (22.2%)	0.956
Diabetes (%)	13 (21.67%)	17 (31.48%)	0.262
Antihypertensive regimen (%)	28 (46.67%)	18 (33.33%)	0.195
PAD (%)	7 (11.67%)	6 (11.11%)	0.872
Age (y)	68.91 ± 5.79	65.93 ± 7.12	.77
BMI (kg/m^2)	27.11 ± 3.42	28.12 ± 3.51	.183
Waist circumference (cm)	97.08 ± 8.72	96.74 ± 9.43	.875
WHR	0.96 ± 0.07	0.94 ± 0.10	.465
Systolic BP (mm Hg)	133.33 ± 17.39	130.98 ± 14.54	.494
Diastolic BP (mm Hg)	78.44 ± 8.94	78.17 ± 6.69	.876
FPG (mg/dl)	120.84 ± 29.56	136.71 ± 49.99	.092
*HbA1c (%)	6.52 ± 1.15	6.8 ± 1.42	.470
Cholesterol (mg/dl)	215.80 ± 53.18	215.29 ± 50.64	.966
HDL (mg/dl)	40.16 ± 10.26	43.68 ± 14.27	.220
LDL (mg/dl)	139.68 ± 42.43	141.73 ± 43.93	.837
Triglycerides (mg/dl)	179.03 ± 113.41	148.84 ± 54.58	.137
hsCRP (mg/L)	4.49 ± 2.09	2.93 ± 1.95	.046
WBC (cells/μL)	6972 ± 1710.38	7388 ± 2099.80	.333
Fibrinogen (mg/dl)	432.92 ± 130.55	410.35 ± 135.27	.465
GSM	57.41 ± 38.19	76.32 ± 36.72	.008
Carotid stenosis (%)	71.5 ± 10.23	62.6 ± 8.68	.124

NS, Nonsignificant; *PAD,* peripheral artery disease; *BMI,* body mass index; *WHR,* waist hip ratio; *BP,* blood pressure; *FPG,* fasting plasma glucose; *hsCRP,* high sensitivity C-reactive protein; *WBC,* white blood cells; *GSM,* gray-scale median.
Data are means ± SD.
*HbA1c: comparison only between diabetic patients.

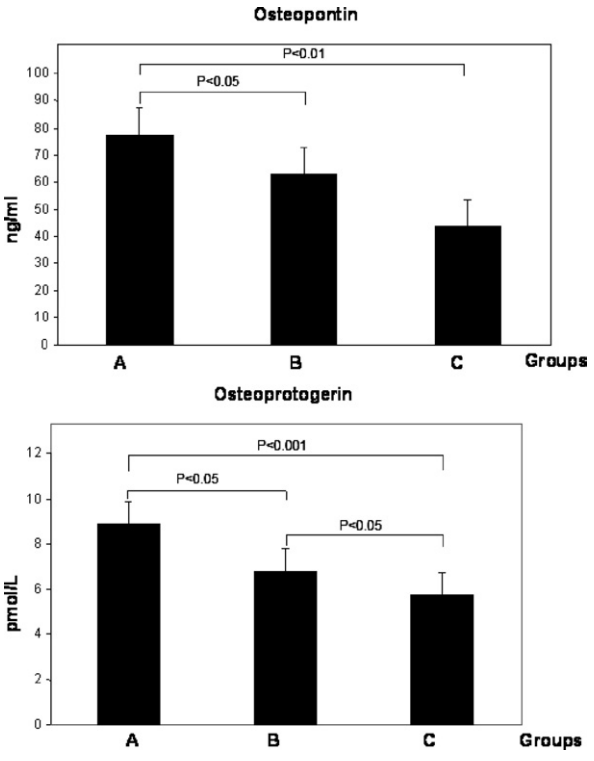

Fig 1. Serum OPN and OPG concentrations in symptomatic, asymptomatic, and healthy control group. **A,** Symptomatic group. **B,** Asymptomatic group. **C,** Healthy individuals.

ment of serum OPN in patients with >50% carotid stenosis. Up to now, the atherogenesis-related mechanisms of OPN were poorly understood. The transgenic overexpression of OPN in high-fat fed C57BL/6 mice resulted in exacerbated atherosclerotic lesions formation,[21] while the simultaneous deficiency of apolipoprotein E and OPN attenuated atherosclerosis only in female mice.[22] OPN functions as a proinflammatory cytokine and augments monocyte/macrophage activation.[23] In addition to this, OPN mediates Th1 immunity and triggers vascular smooth muscle cells proliferation and migration.[24] The above effects are integral to atherosclerosis development and thereby may precipitate atherosclerosis progression.

Most recently Golledge et al observed a fourfold higher OPN expression in carotid specimens from symptomatic patients compared with asymptomatic counterparts.[25] These researchers observed the colocalization of OPN at sites of inflammation. In our investigation, serum OPN levels were significantly increased in symptomatic than asymptomatic patients with carotid stenosis. Moreover, we demonstrated for first time, an inverse correlation between serum OPN levels and GSM value. Carotid echolucent plaques, prone to rupture, have lower content of calcification than echogenic plaques.[4] Our findings collectively support the notion that OPN down- regulates plaque calcification and may promote plaque instability. Aside from direct effects, OPN may facilitate plaque destabilization indirectly. Available data suggest that OPN may induce matrix metalloproteinases release and angiogenesis within the atherosclerotic plaque leading to fibrous cap degrada-

Table III. Correlations of serum osteopontin, osteoprotegerin and GSM value with cardiovascular risk factors

	Osteopontin		*Osteoprotegerin*		*GSM*	
Variables	*r*	P	*R*	P	*r*	P
Osteopontin (ng/ml)	—	—	0.575	<.001	−0.333	.032
Osteoprotegerin (pmol/L)	0.575	<.001	—	—	−0.479	.033
GSM	−0.333	.032	−0.479	.033	—	—
Age (y)	0.289	.009	0.499	<.001	0.159	.491
BMI (kg/m^2)	0.242	.031	0.243	.035	0.336	.156
Waist circumference (cm)	−0.310	.109	−0.092	.641	−0.590	.006
FPG (mg/dl)	0.158	.181	0.252	.035	−0.225	.402
Total cholesterol (mg/dl)	0.360	.002	0.370	.766	−0.231	.408
LDL (mg/dl)	0.298	.011	0.027	.829	−0.150	.593
HDL (mg/dl)	−0.208	.077	−0.110	.370	0.068	.811
Triglycerides (mg/dl)	0.172	.145	0.119	.328	−0.389	.151
hsCRP (mg/L)	0.325	.394	0.311	.045	−0.449	.179

BMI, Body mass index; *FPG,* fasting plasma glucose; *hsCRP,* high sensitivity C-reactive protein; *GSM,* gray-scale median.

tion and hemorrhage, respectively.[26,27,28] Taken all together, OPN seems to assume a key role in atherogenesis and cerebral ischemic events occurrence, but the underlying mechanisms require further research.

The implication of OPG in human cardiovascular diseases has been increasingly recognized.[15] Although the relation of OPG with stroke incidence is under investigation our patients with stroke or TIA appeared with elevated serum OPG levels.[5,29] In agreement with a recent study, we found increased serum OPG levels in patients with carotid stenosis than healthy subjects and strong association between OPG and carotid plaque echogenicity, even after adjustment for age.[30] Furthermore, serum OPG was gradually increased across healthy, asymptomatic and symptomatic subgroups, which reinforces its relationship with carotid disease severity. The latter is subject of controversy, and it may be attributed to different selection criteria.[15,25,30] For instance, we recruited patients with significant carotid stenosis, whereas Vik et al conducted a population health study with small proportion of patients suffering from cardiovascular diseases.[30] They also included control subjects with numerous comorbidities (eg, diabetes, hypertension, etc), which might have attenuated the difference between groups.

OPG has been hypothesized to be a compensatory vasculoprotective response to injurious stimuli of excessive activated inflammatory pathways.[15,29] Up to now, there are conflicting results about the relationship between serum OPG and inflammatory markers.[14,29,31,32] In our study, OPG correlated significantly with hsCRP implicating the interference between OPG and inflammatory atherosclerotic process. hsCRP is a surrogate marker of cardiovascular events and such a relationship is of clinical importance. Whether high serum OPG levels counter-regulate vascular inflammation or constitute an epiphenomenon of inflammatory process remain to be verified.

OPG comprises a crucial inhibitor of vascular calcification. OPG-deficient mice exhibit severe osteoporosis and vascular calcification of the aorta and renal arteries.[33] This phenotype can be prevented by delivery of the recombinant OPG and transgenic overexpression of OPG.[34] Consequently, the elevated OPG levels in our symptomatic patients and their independent relationship with GSM suggested less calcification and a contributory role of OPG to plaque vulnerability. Probably targeting OPG may provide a novel therapeutic strategy against carotid plaque rupture.

It is widely recognized that waist circumference is more closely associated with abdominal visceral adipose tissue accumulation and its consequences than other indexes of adiposity.[35] Centralized body fat predisposes to metabolic disorders (eg, diabetes), cardiovascular diseases, and constitutes the prevailing source of numerous cytokines, which may influence carotid plaque stability.[36] We and others observed greater WHR and waist circumference in patients with carotid stenosis than healthy BMI-matched subjects.[37] We also demonstrated an independent relation between waist girth and carotid plaque echolucency. Future studies combining carotid ultrasound and precise assessments of abdominal obesity (CT, DEXA) will unravel the interaction between fat derivatives (eg, adipocytokines) and carotid plaque stability.

Finally, the expression of both bone-regulators is highly induced by glucose.[38] In agreement with previous researches, higher serum OPN and OPG levels were found in diabetic patients, suggesting that they may be involved in the accelerated atherosclerosis seen in diabetes.[30,39] However, we showed a faint influence of diabetes on the differences between groups, which means a sustained involvement of calcification inhibitors in atherosclerosis independent of diabetes presence.

The principal limitation of our study is the cross-sectional design, which prevents from defining a strong causal relationship. A second limitation is the relative small sample. However, we used numerous selection criteria, and thus, our study sample was adequately homogeneous. Patients were recruited from a cohort of patients with newly-diagnosed cardiovascular diseases and subjects clustering cardiovascular risk factors. Thus, the upregulated levels of bone-remodeling proteins should be interpreted in the framework of generalized atherosclerosis. Perhaps a study

group with early atherosclerotic lesions would confer different results. Finally, we cannot rule out the presence of patients with hidden bone metabolic perturbations in our study, despite the exclusion of patients with diagnosed osteoporosis.

Several points of our study should be considered. In our study, the difference between symptomatic and asymptomatic patients in the degree of carotid stenosis did not reach statistical significance. A plausible explanation derives from the small sample and the inclusion of patients with stenosis >50%. The vast majority of our participants had intermediate degree of stenosis (50%-69%), which might have also attenuated the above difference between groups. In parallel, biochemical markers capability to predict carotid plaque vulnerability and their cut-off points remain to be determined by longitudinal studies. It is well-known that biochemical markers alone are of low predictive power. However, their combination with a valid spectrum of GSM measurements would be valuable mediators of effective risk stratification of patients with carotid stenosis. Finally, we must explain how we succeeded to find a large number of patients with carotid stenosis not receiving lipid-lowering therapy at the entry of the study. First, our patients had recently diagnosed carotid stenosis. Second, a small part of them with dyslipidemia had not complied with their physicians' recommendations. Overall patients with carotid stenosis are usually under-treated and patients with recent stroke/TIA are under investigated for carotid stenosis, as previous authors have referred.[40]

In conclusion, the present study outlined the increased serum OPN and OPG concentrations in patients with carotid atherosclerosis and their independent association with carotid plaque echodensity and neurological symptomatology. GSM and bone-regulators may help to identify potentially unstable plaques that most benefit from intervention. Future prospective studies inquiring the effects of pharmaceutical and surgical interventions on bone-matrix proteins will elucidate their role in long-term cardiovascular outcomes.

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AUTHOR CONTRIBUTIONS

Conception and design: NK
Analysis and interpretation: NK, PK, AK
Data collection: NK, SG, AK
Writing the article: NK, SG, CD
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Final approval of the article: CD, NK
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REFERENCES

1. Kakkos SK, Stevens JM, Nicolaides AN, Kyriacou E, Pattichis CS, Geroulakos G, et al. Texture analysis of ultrasonic images of symptomatic carotid plaques can identify those plaques associated with ipsilateral embolic brain infarction. Eur J Vasc Endovasc Surg 2007;33:422-9.
2. Nighoghossian N, Derex L, Douek P. The vulnerable carotid artery plaque: current imaging methods and new perspectives. Stroke 2005; 36:2764-72.
3. Liapis CD, Kakisis JD, Kostakis AG. Carotid stenosis: factors affecting symptomatology. Stroke 2001;32:2782-6.
4. Grogan JK, Shaalan WE, Cheng H, Gewertz B, Desai T, Schwarze G, et al. B-mode ultrasonographic characterization of carotid atherosclerotic plaques in symptomatic and asymptomatic patients. J Vasc Surg 2005;42:435-41.
5. Johnson RC, Leopold JA, Loscalzo J. Vascular calcification: pathobiological mechanisms and clinical implications. Circ Res 2006;99: 1044-59.
6. Shaalan WE, Cheng H, Gewertz B, McKinsey JF, Schwartz LB, Katz D, et al. Degree of carotid plaque calcification in relation to symptomatic outcome and plaque inflammation. J Vasc Surg 2004;40:262-9.
7. Raggi P, Cooil B, Shaw LJ, Aboulhson J, Takasu J, Budoff M, et al. Progression of coronary calcium on serial electron beam tomographic scanning is greater in patients with future myocardial infarction. Am J Cardiol 2003;92:827-9.
8. Ehara S, Kobayashi Y, Yoshiyama M, Shimada K, Shimada Y, Fukuda D, et al. Spotty calcification typifies the culprit plaque in patients with acute myocardial infarction: an intravascular ultrasound study. Circulation 2004;110:3424-9.
9. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest 2001;107: 1055-61.
10. Abedin M, Tintut Y, Demer LL. Vascular calcification: mechanisms and clinical ramifications. Arterioscler Thromb Vasc Biol 2004;24:1161-70.
11. O'Brien ER, Garvin MR, Stewart DK, Hinohara T, Simpson JB, Schwartz SM, et al. Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. Arterioscler Thromb 1994;14:1648-56.
12. Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler Thromb Vasc Biol 2002;22:549-53.
13. Hofbauer LC, Shui C, Riggs BL, Dunstan CR, Spelsberg TC, O'Brien T, et al. Effects of immunosuppressants on receptor activator of NF-kappaB ligand and osteoprotegerin production by human osteoblastic and coronary artery smooth muscle cells. Biochem Biophys Res Commun 2001;280:334-9.
14. Jono S, Ikari Y, Shioi A, Mori K, Miki T, Hara K, et al. Serum osteoprotegerin levels are associated with the presence and severity of coronary artery disease. Circulation. 2002;106:1192-4.
15. Kiechl S, Schett G, Wenning G, Redlich K, Oberhollenzer M, Mayr A, et al. Osteoprotegerin is a risk factor for progressive atherosclerosis and cardiovascular disease. Circulation. 2004;109:2175-80.
16. Grant EG, Benson CB, Moneta GL, Alexandrov AV, Baker JD, Bluth EI, et al. Carotid artery stenosis: gray-scale and Doppler US diagnosis–Society of Radiologists in Ultrasound Consensus Conference. Radiology 2003;229:340-6.
17. Stoitsis J, Golemati S, Nikita KS. A modular software system to assist interpretation of medical images – application to vascular ultrasound images. IEEE Trans Instrum Meas 2006;55:1944-52.
18. Elatrozy T, Nicolaides A, Tegos T, Zarka A, Griffin M, Sabetai M. The effect of B-mode ultrasonic image standardization of the echodensity of symptomatic and asymptomatic carotid bifurcation plaque. Int Angiol 1998;7:179-6.
19. Ohmori R, Momiyama Y, Taniguchi H, Takahashi R, Kusuhara M, Nakamura H, et al. Plasma osteopontin levels are associated with the

presence and extent of coronary artery disease. Atherosclerosis 2003;170:333-7.
20. Kurata M, Okura T, Watanabe S, Fukuoka T, Higaki J. Osteopontin and carotid atherosclerosis in patients with essential hypertension. Clin Sci (Lond) 2006;111:319-24.
21. Chiba S, Okamoto H, Kon S, Kimura C, Murakami M, Inobe M, et al. Development of atherosclerosis in osteopontin transgenic mice. Heart Vessels 2002;16:111-7.
22. Matsui Y, Rittling SR, Okamoto H, Inobe M, Jia N, Shimizu T, Akino M, et al. Osteopontin deficiency attenuates atherosclerosis in female apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 2003; 23:1029-34.
23. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 2000;287:860-4.
24. Li JJ, Han M, Wen JK, Li AY. Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation. Biochem Biophys Res Commun 2007:356:13-9.
25. Golledge J, McCann M, Mangan S, Lam A, Karan M. Osteoprotegerin and osteopontin are expressed at high concentrations within symptomatic carotid atherosclerosis. Stroke 2004;35:1636-41.
26. Isoda K, Nishikawa K, Kamezawa Y, Yoshida M, Kusuhara M, Moroi M, et al. Osteopontin plays an important role in the development of medial thickening and neointimal formation. Circ Res 2002;91:77-82.
27. Kadoglou NP, Daskalopoulou SS, Perrea D, Liapis CD. Matrix metalloproteinases and diabetic vascular complications. Angiology 2005;56: 173-89.
28. Tureyen K, Vemuganti R, Salamat MS, Dempsey RJ. Increased angiogenesis and angiogenic gene expression in carotid artery plaques from symptomatic stroke patients. Neurosurgery 2006;58:971-77.
29. Browner WS, Lui LY, Cummings SR. Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. J Clin Endocrinol Metab 2001;86:631-7.
30. Vik A, Mathiesen EB, Noto AT, Sveinbjornsson B, Brox J, Hansen JB. Serum osteoprotegerin is inversely associated with carotid plaque echogenicity in humans. Atherosclerosis 2007;191:128-34.
31. Kim SM, Lee J, Ryu OH, Lee KW, Kim HY, Seo JA, et al. Serum osteoprotegerin levels are associated with inflammation and pulse wave velocity. Clin Endocrinol (Oxf) 2005;63:594-8.
32. Anand DV, Lahiri A, Lim E, Hopkins D, Corder R. The relationship between plasma osteoprotegerin levels and coronary artery calcification in uncomplicated type 2 diabetic subjects. J Am Coll Cardiol 2006; 47:1850-7.
33. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, et al. Osteoprotegerin deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 1998;12:1260-8.
34. Min H, Morony S, Sarosi I, Dunstan CR, Capparelli C, Scully S, et al. Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. J Exp Med 2000;192:463-74.
35. Menke A, Muntner P, Wildman RP, Reynolds K, He J. Measures of adiposity and cardiovascular disease risk factors. Obesity (Silver Spring) 2007;15:785-95.
36. Guzik TJ, Mangalat D, Korbut R. Adipocytokines - novel link between inflammation and vascular function? J Physiol Pharmacol 2006;57: 505-28.
37. De Michele M, Panico S, Iannuzzi A, Celentano E, Ciardullo AV, Galasso R, et al. Association of obesity and central fat distribution with carotid artery wall thickening in middle-aged women. Stroke 2002;33: 2923-8.
38. Bidder M, Shao JS, Charlton-Kachigian N, Loewy AP, Semenkovich CF, Towler DA. Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities. J Biol Chem 2002;277: 44485-96.
39. Takemoto M, Yokote K, Nishimura M, Shigematsu T, Hasegawa T, Kon S, et al. Enhanced expression of osteopontin in human diabetic artery and analysis of its functional role in accelerated atherogenesis. Arterioscler Thromb Vasc Biol 2000;20:624-8.
40. Ravipati G, Aronow WS, Ahn C, Channamsetty V, Sekhri V. Incidence of new stroke or new myocardial infarction or death in patients with severe carotid arterial disease treated with and without statins. Am J Cardiol 2006;98:1170-1.

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