Assessment of the Genetic Effects of Polymorphisms in the Osteoprotegerin Gene, TNFRSF11B, on Serum Osteoprotegerin Levels and Carotid Plaque Vulnerability

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Background and Purpose—Osteoprotegerin (OPG) is a secretory glycoprotein which belongs to the tumor necrosis factor receptor family. Various mechanisms have been suggested by which calcification might alter atherosclerotic plaque stability, but the significance of this intimal calcification is controversial. High concentrations of OPG have been associated with the presence of vascular and cardiovascular diseases. This study was designed to assess the association between gene polymorphisms of the OPG gene (TNFRSF11B), the serum OPG level, and plaque stability in patients with carotid atherosclerosis.

Methods—We studied 177 patients with internal carotid artery stenosis who underwent carotid endarterectomy and also 303 controls. Carotid endarterectomy samples removed from patients were assessed by immunohistochemistry. Concentrations of OPG were measured and gene polymorphisms were examined by polymerase chain reaction and restriction enzyme analysis and were compared, initially between patients with carotid atherosclerosis and controls, and subsequently between stable and unstable carotid plaques.

Results—We found that the GG genotype of the T245G polymorphism, the CC genotype of the T950C polymorphism, and the CC genotype of the G1181C polymorphism were significantly higher in patients with carotid plaque than in controls (21.5% versus 10.9%, P<0.01; 15.8% versus 7.6%, P<0.01; and 20.3% versus 10.9%, P<0.01, respectively) and that these polymorphisms were associated with high serum OPG levels (4.02 [3.07] versus 2.94 [1.81] pmol/L; P<0.01), which were significantly higher in patients with unstable atherosclerotic plaques (5.86 [4.02] versus 3.53 [1.87] pmol/L; P<0.01).

Conclusions—The TNFRSF11B gene polymorphisms studied are associated with high serum OPG levels and might be potential markers for plaque instability. (Stroke. 2011;42:3022-3028.)

Key Words: carotid artery □ carotid atherosclerosis □ stable and unstable plaques □ osteoprotegerin □ gene polymorphisms

Atherosclerosis is a disease of the elastic and large muscular arteries in which atheroma is the characteristic lesion. The lesions of atherosclerosis enlarge the arterial intima with variable amounts and types of lipids, connective tissues, inflammatory cells, and a variety of extracellular components including matrix proteins, enzymes, and calcium deposits.1 In the past, calcification of normal tissue has been recognized as a common component of atherosclerotic lesions.2 Some evidence suggests that calcification of atherosclerotic arteries is an organized, regulated process, rather than being a passive phenomenon of aging.3,4 Several factors related to bone and mineral formation have been demonstrated within atherosclerotic plaques; these include osteocalcin, osteopontin, osteonectin, osteoprotegerin (OPG), and bone morphogenetic proteins.5,6 The presence of these factors indicates the potential of arterial wall cells to promote
OPG is a soluble member of the tumor necrosis factor receptor superfamily that regulates osteoclastogenesis. OPG has been positively associated with the presence and severity of cardiovascular events: elevated serum OPG concentrations have been found to correlate with the severity of peripheral artery disease and of heart failure, with symptomatic carotid stenosis, unstable angina, vulnerable carotid plaques, and acute myocardial infarction compared with controls with stable atherosclerosis.

The human OPG gene (8q24; TNFRSF11B) consists of 5 exons and 4 introns and is transcribed into 4 transcripts (2.4 kb, 3.0 kb, 4.2 kb, and 6.5 kb lengths). The gene encoding for OPG is affected by common, functionally important genetic polymorphisms that have been associated with osteoporosis among the most important are the T245G (rs2073618) located in exon I, located in the promoter region, the T950C (rs2073617) located in the 5’ untranslated region, and the G1181C (rs2073618) located in exon I. No study has examined the functional relevance of these 3 OPG polymorphisms on circulating OPG levels and vulnerability of carotid plaques. In the present study, we hypothesized that both circulating OPG levels and functional polymorphisms in OPG family genes were associated with altered risk of plaque instability. To test this hypothesis, the aim of our study was to investigate the role of OPG gene polymorphisms on circulating serum OPG levels and the risk of vulnerable carotid plaques.

Methods

Study Population
We studied 177 consecutive patients with internal carotid artery stenosis (ICAS; median [interquartile range] age, 72 years [8]) who underwent carotid endarterectomy and 303 controls, matched for age and sex (median [interquartile range] age, 72 years [7]). The carotid endarterectomies were performed according to established criteria. Additional information is available in the online supplements.

Serum OPG Measurement
Serum OPG was quantified in duplicate by a high-sensitivity ELISA method, using commercial kits according to the supplier’s instructions (R&Dsystems). Additional information is available in the online supplements.

Statistical Analysis
Demographic and clinical data of the 2 groups were compared by Mann-Whitney test and $\chi^2$ test, respectively, for quantitative and qualitative variables. Mann-Whitney $U$ test was chosen because data were not normally distributed as demonstrated by Shapiro-Wilk test.

Figure 1. Light microscopy images illustrating carotid endarterectomy specimens of stable and unstable plaques. L=lumen; F=fibrous cap; H=intraplaque hemorrhage; N=necrotic core. Magnifications as specified on top of the figures.
(P=0.23 current, and P=0.94 former). In contrast, hypercholesterolemia (P<0.01), hypertension (P<0.01), diabetes (P<0.01), coronary artery disease (P<0.01), history of ischemic stroke (P<0.01), and peripheral arterial occlusive disease (P<0.01) were significantly more frequent in patients than in controls.

The genotype frequencies were in line with those for white subjects, and respected the Hardy-Weinberg equilibrium (P≥0.05).

The estimated OPG pairwise haplotype frequencies among the 3 polymorphisms and their linkage disequilibrium values in cases and controls. The pairwise linkage disequilibrium were highly significant in the patient population (P<0.001), and not among controls. Additional information is available in the online supplements (Supplemental Tables I and II).

Table 2 shows the genotype distribution of the T245C, T950C, and G1181C gene polymorphisms. Of 177 patients with carotid plaque, genotype distribution of the T245G gene polymorphism was 38 GG, 97 TG, and 42 TT, which was significantly different to that observed in the 303 controls (33 GG, 153 TG, and 117 TT). The frequency of the GG genotype in patients (21.5%) was significantly higher than in the control subjects (10.9%; P<0.01). Similarly, the genotype distribution of the T950C gene polymorphism was 28 CC, 95 TC, and 54 TT in atherosclerotic patients, which was significantly different to that observed in controls (23 CC, 142 TC, and 138 TT), and the frequency of the CC genotype in patients (15.8%) was significantly higher than in controls (7.6%; P<0.01). In addition, the genotype distribution of the G1181C polymorphism was 36 CC, 75 GC, and 66 GG in patients, which was significantly different to that observed in the control subjects (33 CC, 122 GC, and 148 GG). The frequency of the CC genotype in patients (20.3%) was significantly higher than in controls (10.9%; P<0.01; Table 2).

Interestingly, the median serum OPG level, measured in 169 patients and 295 controls, was 4.02 (3.07) pmol/L in ICAS, which was significantly higher than the median level in control subjects (2.94 (1.81) pmol/L; P<0.01; Table 2).

According to the best genetic model,22 which was the dominant one for SNPs T245G and T950C and the recessive one for SNPs G1181C, after adjusting for relevant confounding variables (age, sex, hypertension, hypercholesterolemia, diabetes, coronary artery disease, peripheral artery occlusive disease, and smoking) we found that the GG, CC, and CC genotypes of the T245G, T950C, and G1181C gene polymorphisms were independently associated with carotid plaque (OR, 1.87 [1.18–2.96], OR, 2.65 [1.35–5.17] and OR, 2.79 [1.27–6.13], respectively; Table 2).

The next step of our study was to assess whether certain genotypes were associated with different OPG serum levels; we found that patients carrying the GG genotype of the T245G gene polymorphism showed a median protein concentration of 8.20 (2.80) pmol/L that was statistically higher than in controls (4.47 [2.47] pmol/L; P<0.01; Table 3). Similarly, the CC genotype of the T950C gene polymorphism and the CC variant genotype of the G1181C gene showed, in ICAS, median serum OPG levels of 8.52 (1.76) pmol/L and 8.15 (2.92) pmol/L, respectively; these were significantly higher than those in control subjects (5.02 [2.31] pmol/L; P<0.01; and 4.46 [1.87] pmol/L; P<0.01, respectively; Table 3).

Subsequently, we divided the 177 patients with ICAS into unstable plaque (USP; n=79) and stable plaque (SP; n=98) groups. In the 79 patients with USP, the genotype distribution
of the T245G gene polymorphism was 31 GG, 34 TG, and 14 TT, which was significantly different to that observed in the 98 patients with SP (7 GG, 63 TG, and 28 TT; Table 4). The frequency of the GG genotype in USP (39.2%) was significantly higher than in SP (7.1%; \(P < 0.01\)). Likewise, frequency of the CC genotype of the T950C polymorphism in USP was more than 10 times higher than in SP (31.6% versus 3.1%; \(P < 0.01\)). The patients with SP showed a CC genotype of the T1181C gene variant in 11.2%, whereas the same genotype was more common in patients with USP (31.6%; \(P < 0.01\); Table 4). After adjustment for the same variables, all the 3 SNPs were demonstrated to be associated independently with the unstable plaque. In this case, the best genetic model was the recessive for SNPs T245G and T950C and the dominant for SNPs G1181C. This means that the shown ORs are applicable to patients having both G and C alleles respectively for SNPs T245G and T950C and at least 1 C allele for SNPs G1181C. This analysis showed that GG, CC, and CC genotypes homozygous of T245G, T950C, and G1181C gene variants are independent risk factors for unstable plaque; in particular, patients carrying the GG and CC, and CC genotypes have a risk >5, >10, and 4 times higher, respectively, of developing unstable carotid plaque (OR, 5.27 [95% CI, 1.97–14.09]; \(P < 0.01\); OR, 10.27 [95% CI, 2.66–39.65]; \(P < 0.01\); and OR, 4.80 [95% CI, 2.19–10.54]; \(P < 0.01\), respectively; Table 4).

Finally, we have found that the median serum OPG level, measured in 74 USP, was 5.86 (4.02) pmol/L and was significantly higher than the median level measured in 95 SP patients (3.53 [1.87] pmol/L; \(P < 0.01\); Table 4). In particular, patients with USP carrying the GG genotype of the T245G gene polymorphism, the CC genotype of the T950C gene variant, and the CC genotype of the G1181C gene polymorphism showed, respectively, median protein concentration of 8.62 (1.50) pmol/L, 8.82 (1.10) pmol/L, and 8.24 (1.94) pmol/L, that was statistically higher than in patients with SP (5.06 [3.08] pmol/L; \(P < 0.01\); 4.93 [2.56] pmol/L; \(P < 0.01\); and 4.59 [1.94] pmol/L; \(P < 0.01\), respectively; Table 5).

Table 3. Association Between Median Serum OPG Levels and Genotype Distribution in Patients With Carotid Plaque and Control Subjects

<table>
<thead>
<tr>
<th>OPG Genotypes</th>
<th>Carotid Plaque</th>
<th>Controls</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>169</td>
<td>295</td>
<td></td>
</tr>
<tr>
<td>SNPs of T245G (rs 3134069)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>8.20 (2.80)*</td>
<td>4.47 (2.47)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>TG</td>
<td>4.02 (2.01)</td>
<td>3.26 (1.43)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>2.02 (1.90)</td>
<td>1.92 (1.12)</td>
<td></td>
</tr>
<tr>
<td>SNPs of T950C (rs 2073617)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8.52 (1.76)</td>
<td>5.02 (2.31)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>TC</td>
<td>4.12 (2.42)</td>
<td>3.52 (1.02)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>2.32 (2.37)</td>
<td>1.96 (1.00)</td>
<td></td>
</tr>
<tr>
<td>SNPs of G1181C (rs 2073618)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8.15 (2.92)</td>
<td>4.46 (1.87)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>GC</td>
<td>4.73 (2.12)</td>
<td>3.37 (1.17)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>2.52 (1.89)</td>
<td>1.98 (1.22)</td>
<td></td>
</tr>
</tbody>
</table>

OPG indicates osteoprotegerin; SNP, single nucleotide polymorphisms.
*Median (interquartile range).
†Kruskall-Wallis test.

Table 4. Genotype Distribution and Median Serum OPG Levels in Patients with Unstable and Stable Carotid Plaques

<table>
<thead>
<tr>
<th>OPG Genotypes</th>
<th>USP</th>
<th>SP</th>
<th>(P)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>79</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNPs of T245G (rs 3134069)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG, no (%)</td>
<td>31 (39.2)</td>
<td>7 (7.1)</td>
<td>&lt;0.01*</td>
<td>5.27 (1.97–14.09)†</td>
</tr>
<tr>
<td>TG, no (%)</td>
<td>34 (43.1)</td>
<td>63 (64.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT, no (%)</td>
<td>14 (17.7)</td>
<td>28 (28.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNPs of T950C (rs 2073617)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC, no (%)</td>
<td>25 (31.6)</td>
<td>3 (3.1)</td>
<td>&lt;0.01*</td>
<td>10.27 (2.66–39.65)†</td>
</tr>
<tr>
<td>TC, no (%)</td>
<td>38 (48.1)</td>
<td>57 (58.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT, no (%)</td>
<td>16 (20.3)</td>
<td>38 (38.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNPs of G1181C (rs 2073618)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC, no (%)</td>
<td>25 (31.6)</td>
<td>11 (11.2)</td>
<td>&lt;0.01*</td>
<td>4.80 (2.19–10.54)†</td>
</tr>
<tr>
<td>GC, no (%)</td>
<td>39 (49.4)</td>
<td>36 (36.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG, no (%)</td>
<td>15 (19.0)</td>
<td>51 (52.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPG levels, pmol/L</td>
<td>5.86 (4.02)‡</td>
<td>3.53 (1.87)‡</td>
<td>&lt;0.01§</td>
<td></td>
</tr>
</tbody>
</table>

OPG indicates Osteoprotegerin; USP, unstable plaques; SP, stable plaques; SNP, single-nucleotide polymorphism; OR, odds ratio; CI, confidence interval; CAD, coronary artery disease; PAOD, peripheral arterial occlusive disease.
*\(\chi^2\) test for categorical values.
†OR adjusted for age, sex, hypertension, hypercholesterolemia, diabetes, CAD, PAOD, smoking.
‡Median (interquartile range).
§Mann-Whitney U test.
Table 5. The Association Between Median Serum OPG Levels and Genotype Distribution in Patients With Unstable and Stable Plaques

<table>
<thead>
<tr>
<th>OPG Genotypes</th>
<th>USP</th>
<th>SP</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>74</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>SNPs of T245G (rs 3134069)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>8.62 (1.50)*</td>
<td>5.06 (3.08)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>TG</td>
<td>5.86 (2.01)</td>
<td>4.00 (1.25)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>2.13 (0.73)</td>
<td>2.03 (1.42)</td>
<td></td>
</tr>
<tr>
<td>SNPs of T950C (rs 2073617)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8.82 (1.10)</td>
<td>4.93 (2.56)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>TC</td>
<td>5.86 (1.65)</td>
<td>3.99 (1.65)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>4.85 (2.61)</td>
<td>3.24 (2.13)</td>
<td></td>
</tr>
<tr>
<td>SNPs of G1181C (rs 2073618)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>8.24 (1.94)</td>
<td>4.59 (1.94)</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>GC</td>
<td>5.86 (2.53)</td>
<td>4.02 (2.17)</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>5.89 (5.24)</td>
<td>3.58 (1.12)</td>
<td></td>
</tr>
</tbody>
</table>

OPG indicates Osteoprotegerin; USP, unstable plaques; SP, stable plaques; SNP, single-nucleotide polymorphism.
*Median (interquartile range). †Kruskal-Wallis test.

Discussion

The current study is the first report showing that both elevated serum OPG levels and variant genotypes of the OPG gene were significantly associated with increased risk of carotid atherosclerosis. More importantly, genetic effects on unstable carotid plaques were more evident among subjects who had higher serum OPG concentration, and also differences in OPG concentrations between atherosclerotic plaque cases and controls were observed among subjects carrying the GG, CC, and CC variant genotypes of the T245G, T950C, and G1181C gene polymorphisms of the OPG gene.

In particular, there were several main findings. First, the frequencies of the GG genotype of the T245G gene polymorphism, the CC genotype of the T950C polymorphism, and the CC genotype of the G1181C polymorphism were significantly and independently associated with ICAS (Table 2). Second, median serum OPG levels were significantly higher in patients than in controls (4.02 [3.07] versus 2.94 [1.81] pmol/L; P<0.01; Table 2), respectively, that the GG, CC, and CC genotypes of T245G, T950C, and G1181C gene variants, respectively, are associated with elevated serum OPG levels in patients with ICAS and are independent risk factors for plaque instability. The results of this study extend previous evidence suggesting an association between genetic variants, elevated circulating OPG levels, and vascular disease. Increasing clinical evidence indicates that OPG, Receptor activator of nuclear factor κ-β ligand (RANKL), and the cytokine network they are part of play an important role in cardiovascular disease. Clinically stable symptomatic coronary heart disease, acute coronary heart disease, coronary heart disease complicated by heart failure, and symptomatic carotid atherosclerosis are associated with increased serum levels and/or expression of OPG, whereas young survivors of uncomplicated myocardial infarction had normal serum levels of OPG collected more than a year after the acute event. Recently, Lief et al observed a positive association between circulating OPG and incident cardiovascular disease and mortality in the largest community-based sample so far, with a rather adverse cardiovascular disease risk-factor profile. Serum levels of OPG have also been shown to predict atherosclerotic plaque growth, incident cardiovascular disease, and cardiovascular mortality in prospective studies in the general population and among postmenopausal women. In addition, elevated serum OPG concentrations have been found to correlate with severity of peripheral artery disease.

Atherosclerosis is a chronic inflammatory condition and previous clinical studies have shown a correlation in elevated OPG levels in patients with atherosclerosis and increased OPG levels with the severity of the disease. The high concentrations of OPG could be responsible for a number of changes within the atherosclerotic plaque that would promote plaque instability. Within bone, OPG has been shown to modulate the release of matrix-degrading enzymes, such as cathepsins, and therefore it may also have an important influence on plaque stability.

OPG is also a receptor for TNF-related apoptosis-inducing ligand, a potent activator of apoptosis.

To gain more insight into the role of OPG in atherosclerotic development, subsequent studies have focused on its functional differences.
relationship with endothelial dysfunction, an important early physiological event in atherosclerosis. The association between elevated serum OPG levels and impaired endothelial function, measured as a decreased flow-mediated dilatation of the brachial artery, was first demonstrated in a cross-sectional survey of type 2 diabetic patients.35 This was followed by prospective studies in newly diagnosed type 1 and type 2 diabetic patients that showed a significant correlation between the decrease in serum OPG levels and improvements in flow-mediated dilatation of the brachial artery endothelial function.36 Similar findings were recently reported for peripheral artery disease patients by Golledge et al, which confirmed the correlation between increased serum OPG levels and decreased flow-mediated dilatation of the brachial artery endothelial function.37

This study has potential limitations. It was a case-control study; therefore a recruitment and survival bias cannot be excluded. Our data were obtained from a cohort of European descent that includes subjects with other cardiovascular diseases, and so comorbidity might represent a confounding factor; also, the generalizability of our findings to other age groups or ethnicities is unclear. The size of the studied population is relatively small and our findings need to be confirmed in larger samples, and should also be tested in groups of different ethnic origins.

Conclusions
In conclusion, variant genotypes of the OPG gene and high serum OPG as an independent risk factor may contribute to the pathogenesis of carotid atherosclerosis. Clearly, the vascular role of OPG is multifaceted and depends on the interplay with its ligands, RANKL and TNF-related apoptosis-inducing ligand, and a bidirectional modulation involving osteogenic, inflammatory, and apoptotic responses. Although the mechanisms linking OPG and vascular disease require additional study, the associations between OPG and carotid plaque demonstrated in this study support additional investigation into the effects of OPG on plaque stability and the possible role of OPG as a biomarker to identify patients with, or at risk of, cerebrovascular events. Targeting OPG may provide a novel therapeutic strategy against carotid plaque rupture.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIALS AND METHODS

Study Population

We studied 177 consecutive patients with internal carotid artery stenosis (ICAS) [median (IQ range) age = 72 years (8)] who underwent carotid endarterectomy, recruited among subjects consecutively admitted to the Department of Vascular Surgery of the A. Gemelli University Hospital of Rome and to the Department of Vascular Surgery of the St M. Goretti Hospital, Latina (Italy), and 303 controls, matched for age and gender [median (IQ range) age = 72 years (7)], without clinical or radiological evidence of cerebrovascular disease, among subjects consecutively admitted to the Department of Internal Medicine of the A. Gemelli University Hospital of Rome, from 1 January 2009 to 15 December 2010. The controls had no relationship with the cases. All subjects were white, from central and southern Italy, and belonged to independent pedigrees. All participants underwent a basic vascular evaluation, including a clinical vascular examination, a thorough color-coded echo flow imaging of the accessible arterial tree, and an ECG at rest. The patients underwent additional neurological evaluation and cerebral CT to assess symptoms and/or cerebral infarction related to ICA stenosis. For each study patient, clinical data and history regarding risk factors such as age, diabetes mellitus, hypertension, hypercholesterolemia and smoking were obtained. Diabetes mellitus was determined by the presence of an existing diagnosis, fasting blood glucose > 126 mg/dL, glycohemoglobin A1c > 5.8%, or by use of antidiabetic medication or insulin. Hypertension was defined as a systolic blood pressure > 140 mmHg and a diastolic blood pressure > 90 mmHg or by use of antihypertensive medications. Hypercholesterolemia was determined by a serum cholesterol value of > 220 mg/dL or by use of cholesterol-lowering medications. Patients were classified as nonsmokers if they had never smoked or if they had stopped smoking ≥ 1 year before the study. All other patients were classified as smokers. The preoperative evaluation included ultrasound assessment of plaque density by color-coded echo flow imaging confirmed by angiography. Patients with malignant neoplasms, severe renal or liver disease, serous membrane
chamber fluid, severe oedema, hypothyroidism, osteoporosis were also excluded from the study. In addition, no patient was taking any drugs, such as oestrogen supplements, thyroxine, glucocorticoid, immunosuppressive, biphosphonate, and warfarin. Approval for this study was provided by the ethics committees of the A. Gemelli University Hospital of Rome and St M. Goretti Hospital, Latina (Italy). Informed consent was obtained from participating patients.

**Histological assays**

After carotid endarterectomy, the specimens were briefly rinsed in normal saline solution and then immersed in buffered 10% formalin fixative and subsequently in a decalcifying solution (formic acid). The plaques were partly decalciﬁed in order to be sectioned. Each specimen was sectioned transversely (perpendicular to the lumen) into 4 mm blocks, starting from the specimen base and then progressing distally until the whole specimen (including the bifurcation) was cut. Each block was processed in paraffin, cut into 4 µm sections and then the proximal end of one slice per block was stained in sequence with hematoxylin and eosin 1% (H&E). The sections were examined for the presence of atheroma, a necrotic core, hemorrhage, fibrosis, calcification and thrombosis. The minimum magnification was used to evaluate the relative necrotic core, whereas higher magnifications were to evaluate the presence of hemorrhage and calcification. The morphological study focused on the level of the largest plaque area, which frequently corresponded to the level of maximal stenosis. The necrotic core was usually located in the deeper regions of the plaque and consisted of cholesterol clefts and amorphous material without any viable cells or admixed collagen. Calcifications manifested as dark blue, sharply demarcated regions devoid of cells in the H&E stains. Intraplaque hemorrhage appeared as debris containing degenerated red blood cells as well as macrophage engulfment of hemosiderin and giant cell development.

According to Stary’s classification¹, stable plaques were considered those having a thick fibrous or fibrocalcific component, lined by endothelium, covering a possible central foamy or necrotic core (types IV, Vb and Vc). Advance atherosclerotic carotid plaques were defined as
unstable, if there were the presence of a thin fibrous cap or fissured cap covering the foamy or necrotic core (type IV, in which the lesion surface contains proteoglycans and macrophage foam cells and only isolated smooth muscle cells and minimal collagen, and type Va), and the presence of overt, hemorrhagic, ulcerated or thrombotic plaques (type VI).

**Serum OPG Measurement**

White blood cell count, serum creatinine, fasting cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein and homocysteine protein levels were measured. Blood samples were collected from all subjects after an overnight fast. Serum was separated by centrifugation of blood samples, stored at −80 °C until assayed and were treated the same way. Monoclonal mouse anti-human OPG antibody was used as a capture antibody and a biotinylated polyclonal goat anti-human OPG antibody was used for detection. The intra- and interassay coefficients of variation were 3.6% and 10.6%, respectively. The sensitivity, defined as the mean ± 3 SD of the 0 standard, was calculated to be 0.15 pmol/ml.

**Statistical analyses**

Median values and interquartile ranges (IQR) were used to report quantitative variables, while qualitative ones were described through absolute and relative frequencies. Demographic and clinical data of the two groups (affected by carotid plaque - cases - and not - controls -) were compared by Mann-Whitney test and χ²-test respectively for quantitative and qualitative variables. Mann-Whitney test was chosen because data were not normally distributed as demonstrated by Shapiro-Wilk test. A χ²-test of Hardy-Weinberg Equilibrium (HWE) for the tested SNPs was performed among controls. The haplotype analysis was conducted for the three tested SNPs by using EH software² and cocaphase³. Genotype frequencies between cases and controls were compared by χ²-test, while serum OPG levels were compared by Mann-Whitney test; Kruskal-Wallis test was employed to
evaluate differences between the same groups stratified by genotypes. Crude Odd Ratios (OR) with 95% Confidence Intervals (95%CI) were calculated to estimate the association between genotypes and the presence of carotid plaques. In order to evaluate the best genetic model the following approach was chosen. First of all OR₁ and OR₂ were computed for the three SNPs:

- **OPG T245G**, TG versus TT (OR₁); GG versus TT (OR₂)
- **OPG T950C**, TC versus TT (OR₁); CC versus TT (OR₂)
- **OPG G1181C**, GC versus GG (OR₁); CC versus GG (OR₂)

According to the following criteria, the best genetic model was identified for each SNPs⁴:

- **Recessive model**: if OR₂ ≠ 1 and OR₁ = 1
- **Dominant model**: if OR₂ = OR₁ ≠ 1,

A multivariate analysis was carried out in order to investigate the role of each three SNPs using the best genetic model and a backward stepwise approach; adjusted ORs with 95%CI were reported in the Tables.

All the procedures which were described were performed also for assessing the differences between unstable and stable plaques.

The analysis was performed using SPSS software version 12.0 per Windows. Statistical significance was set at p ≤ 0.05.
Reference


**SUPPLEMENTARY TABLES**

Supplementary Table 1. Pairwise haplotype frequency in the 177 patients with carotid plaque and 303 controls

<table>
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<th>Estimated haplotype frequency</th>
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<td></td>
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</tr>
<tr>
<td></td>
<td>1 – 1†</td>
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<td></td>
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<td>Cases</td>
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<td>Controls</td>
<td>0.09</td>
<td>0.26</td>
<td>0.21</td>
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</table>

*P-value of χ²-test (cases versus controls) < 0.001
† 1= 245(G)/950(C)/1181(C)
‡ 2= (T)245/(T)950/(G)1181
**Supplementary Table 2.** Estimated OPG haplotype frequencies in 177 patients with carotid plaque and 303 controls.

<table>
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<th>1–2–2</th>
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<td>T245G – T950C</td>
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<tr>
<td>T950C*</td>
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<tr>
<td>Cases</td>
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<tr>
<td>Controls</td>
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<td>0.17</td>
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</table>

* P-value of $\chi^2$-test: cases versus controls <0.001; † 1 = 245(G)/950(C)/1181(C)  
‡ 2 = (T)245/(T)950/(G)1181

The pairwise linkage disequilibria were highly significant in the patient population ($P < 0.001$), and not among controls. Additionally, the pairwise haplotype distribution differed between the two compared groups ($P$-value of $\chi^2$-test < 0.001). The frequency of 1-1 diplotypes was always higher in cases than in controls, while those 1-2 and 2-1 always higher in controls than in cases. When the estimation of haplotype frequencies was extended to all three SNPs results showed a marker difference in the haplotype frequencies among patients and controls ($P <0.001$).