Preprocedural Level of Soluble CD40L Is Predictive of Enhanced Inflammatory Response and Restenosis After Coronary Angioplasty

Francesco Cipollone, MD; Claudio Ferri, MD; Giovambattista Desideri, MD; Leonardo Paloscia, MD; Guido Materazzo, MD; Marco Mascellanti, MD; Maria Fazia, PhD; Annalisa Iezzi, MD; Chiara Cuccurullo, MD; Barbara Pini, MD; Marco Bucci, MD; Anna Santucci, MD; Franco Cuccurullo, MD; Andrea Mezzetti, MD

Background—Inflammation plays a pathogenic role in the development of restenosis after percutaneous transluminal coronary angioplasty (PTCA). CD40–CD40L interaction is involved in the pathogenesis of atherosclerosis; however, its role in the pathophysiology of restenosis is still unclear. We tested the hypothesis that soluble CD40L (sCD40L) may be involved in the process of restenosis and that it exerts its effect by triggering a complex group of inflammatory reactions on endothelial and mononuclear cells.

Methods and Results—We studied 70 patients who underwent PTCA and who had repeated angiograms at 6-month follow-up. Plasma sCD40L was measured before and 1, 5, 15, and 180 days after PTCA, whereas plasma soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, E-selectin, and monocyte chemoattractant protein (MCP)-1 were measured before and 24 hours after PTCA. Furthermore, the release of adhesion molecules and MCP-1 and the ability to repair an injury in endothelial cells, as well as the generation of O2⁻ in monocytes, were analyzed in vitro after stimulation with serum from patients or healthy control subjects. Restenosis occurred in 18 patients (26%). Restenotic patients had preprocedural sCD40L significantly higher than patients with favorable outcomes (2.13 ± 0.3 versus 0.87 ± 0.12 ng/mL, P < 0.0001). Elevated sCD40L at baseline was significantly correlated with adhesion molecules and MCP-1 generation after PTCA and with lumen loss at 6-month follow-up. Furthermore, high sCD40L was directly associated in vitro with adhesion molecules and MCP-1 generation and impaired migration in endothelial cells and with enhanced O2⁻ generation in monocytes.

Conclusions—We conclude that increased sCD40L is associated with late restenosis after PTCA. This may provide an important biochemical link between restenosis and aspirin-insensitive platelet activation. These results provide a rationale for studies with new antiplatelet treatments in patients who underwent PTCA. (Circulation. 2003;108:2776-2782.)

Key Words: angioplasty • restenosis • endothelium • inflammation • platelets

Restenosis after percutaneous transluminal coronary angioplasty (PTCA) is a major clinical problem, and its pathophysiology has not yet been elucidated. In vitro enhanced cytokine synthesis by peripheral blood monocytes before PTCA has been found to predict late lumen loss, suggesting that preprocedural activation of inflammatory cells may play a role in the modulation of vessel wall response to the injury induced by PTCA. This possibility is also supported by the study by Buffon et al showing that increased baseline level of C-reactive protein (CRP) is associated with restenosis after PTCA, but the relation of CRP to restenosis is still controversial because other groups have not found an association. However, the specific mediator(s) involved in the transduction of the hyperresponsiveness of the inflammatory system toward the proliferative and matrix-remodeling response in the injured vessel wall responsible for restenosis after PTCA is still unclear.

CD40 ligand (CD40L, also known as CD154), a transmembrane protein, was originally identified on CD4+ T cells, but it was also recently found on activated platelets. Both membrane-bound and soluble (s) forms of this ligand may interact with CD40, which is constitutively expressed on...
vascular cells, resulting in various inflammatory responses. Recently, the presence of T cells expressing CD40L within atherosclerotic lesions in humans has been reported, as well as the ability of gene disruption or anti-CD40L antibody treatment to limit the evolution and the instability of atherosclerotic plaque. Interestingly, increased sCD40L has been observed in unstable angina and hypercholesterolemia, and it was recently reported that circulating sCD40L has strong independent prognostic value among apparently healthy individuals.  

Thus, it is now generally accepted that the CD40L–CD40 interaction is an initial event in atherothrombosis, leading in turn to the activation of several proinflammatory mediators. In fact, CD40L may promote the expression of matrix metalloproteinases and induce procoagulant activity in vascular cells, and the absence of CD40L affects the stability of arterial thrombi and delays arterial occlusion in vivo. Moreover, ligation of CD40 on endothelial cells (ECs) inhibits cell migration after injury and upregulates monocyte chemotactrant protein (MCP-1), E-selectin, vascular cell adhesion molecule (VCAM)-1, and intercellular adhesion molecule (ICAM)-1, all molecules that have been associated with restenosis after PTCA.  

Thus, it is tempting to hypothesize that the CD40L–CD40 interaction may also contribute to the development of restenosis after PTCA. However, to the best of our knowledge, no studies have demonstrated whether preprocedural sCD40L may influence the inflammatory reaction after vessel injury, which is ultimately responsible for luminal narrowing after PTCA in humans.  

Thus, in the present study, we set out to investigate the possible role of sCD40L in restenosis after PTCA. In addition, we tested the hypothesis that sCD40L exerts its detrimental effect by triggering a complex group of inflammatory reactions both in vascular ECs and in circulating monocytes/macrophages.

### Methods

#### Patients

The study population consisted of 70 of 136 consecutive patients (46 men, 24 women; age 62±5 years) who underwent PTCA on a single nonocclusive coronary stenosis (Table 1). Stenting was applied according to the surgeon’s judgment. In particular, all the patients received 100 mg/d aspirin (plus ticlopidine during stenting) for the entire study period. In addition, 10 nonhospitalized healthy volunteers (5 men, 5 women; age 56±4 years) were studied as a control group for the in vitro experiments. The study was approved by local ethics review committees. Informed written consent was obtained from each patient.

#### Angioplasty Procedure and Follow-Up Evaluation

Balloon angioplasty and quantitative coronary angiography were performed according to standard techniques as described previously. Patients were readmitted for follow-up coronary angiography 6 months after angioplasty.

#### Definitions

Restenosis was defined as recurrent lumen diameter stenosis >50% at follow-up angiography. The continuous variable luminal loss was defined according to the following equation: relative loss = [(postintervention minimal lumen diameter–follow-up minimal lumen diameter)/vessel size]×100%. The vessel size is the value of the reference diameter function at the minimal position of the obstruction.

#### Enzyme Immunoassays

Serum and plasma samples were collected before and 1, 5, 15, and 180 days after PTCA. For serum sampling, blood was collected from each patient and drawn into pyrogen-free blood collection tubes without additives. Tubes were immediately immersed in melting ice and allowed to clot for 1 hour before centrifugation at 1000 g for 10 minutes. Multiple aliquots of serum and plasma were stored at −80°C until analysis. Samples were frozen and thawed only once. Specific immunoassays for sCD40L, sICAM-1, sVCAM-1, and sE-selectin (Bender Medsystems), MCP-1 (BioSource), and high-sensitive CRP (Immulyte hs-CRP, Diagnostic Product Corporation) were used in triplicate as described previously. At our laboratory, the intra-assay and interassay coefficients of variation were <6%.

#### sCD40L-Dependent EC Activation

Human vascular ECs were harvested from fresh human umbilical cord veins and cultured as described previously. For processing the serum, 40 µL of serum from each patient or control subject immediately after thawing was added to EC culture at the start of the culture period. In some experiments, synthetic CD40L (10 µg/mL, Alexis) and anti-human sCD40L blocking monoclonal antibody (10 µg/mL, Calbiochem) were also added to the cell culture. After 3 and

### Table 1. Baseline Characteristics of Study Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>No Restenosis</th>
<th>Restenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62±5</td>
<td>62±4.5</td>
</tr>
<tr>
<td>Male/female</td>
<td>34/18 (65/35)</td>
<td>12/6 (67/33)</td>
</tr>
<tr>
<td>Stable angina/unstable angina</td>
<td>16/36 (31/69)</td>
<td>6/12 (33/67)</td>
</tr>
<tr>
<td>Stenting</td>
<td>27 (52)</td>
<td>9 (50)</td>
</tr>
</tbody>
</table>

Patients with  
Previous myocardial infarction 0 0  
Previous PTCA or CABG 0 0  
Family history of IHD 20 (38) 7 (39)  
Hypertension 32 (62) 11 (61)  
Diabetes mellitus 6 (11) 2 (11)  
Cigarette smoking 15 (29) 5 (28)  
Hypercholesterolemia 29 (56) 10 (55)  

Treatment with  
Calcium channel blockers 22 (42) 8 (44)  
Nitrates 36 (69) 12 (67)  
Unfractionated sodium heparin 52 (100) 18 (100)  
Aspirin 52 (100) 18 (100)  
Aspirin + ticlopidine 27 (52) 9 (50)  
Clopidogrel 0 0  
GP IIb/IIIa antagonists 0 0  

Culprit vessel  
RCA 18 (35) 6 (33)  
LAD 20 (38) 7 (39)  
LCx 14 (27) 5 (28)  

Type B2-C lesions (AHA/ACC) 18 (34) 4 (33)  

Values are given as mean±SD or n (%). IHD indicates ischemic heart disease; RCA, right coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; and AHA/ACC, American Heart Association/American College of Cardiology.
18 hours in culture, the generation of sICAM-1, sVCAM-1, sE-selectin, and MCP-1 was measured.

**EC Migration**

Human vascular ECs were isolated and cultured as described previously. In some experiments, synthetic sCD40L, anti-human blocking monoclonal antibody for CD40L, or control mouse IgG1 (50 μg/mL) was added to cell culture.

**Statistical Analysis**

For clinical data, variables were compared by use of the χ² test. An ANOVA for repeated measures followed by pairwise comparisons (Bonferroni t test) was performed in the 2 groups to test the changes in sCD40L measured over time. Differences in biochemical variables between groups at each collection time were analyzed by Student’s unpaired t test. Correlations were assessed by linear regression analysis. Each variable that proved to be statistically significant in the univariate analysis was then assessed by multiple regression after adjustment for potential confounders to establish whether it was an independent risk factor for late lumen loss. Statistical significance was indicated by a value of P<0.05. All calculations were performed using the computer program SPSS 10.0.7.

**Results**

**Clinical Course**

No patient developed sudden death or infarction during the study. Restenosis occurred in 18 of 70 patients (26%).

### TABLE 2. Time Course of Inflammatory Markers Measured in Plasma Before and After PTCA

<table>
<thead>
<tr>
<th>Marker, ng/mL</th>
<th>Before PTCA</th>
<th>After PTCA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Day</td>
<td>5 Days</td>
</tr>
<tr>
<td>sCD40L</td>
<td>2.13±0.3 vs 0.87±0.12</td>
<td>2.72±0.22 vs 1.47±0.18</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>365±32 vs 115±23</td>
<td>520±42 vs 329±39</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>1102±82 vs 576±44</td>
<td>1879±105 vs 961±82</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>41±3 vs 23±3</td>
<td>56±7 vs 42±4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.55±0.06 vs 0.31±0.03</td>
<td>0.97±0.1 vs 0.54±0.09</td>
</tr>
</tbody>
</table>

**Plasma Level of sCD40L**

Patients who developed restenosis showed higher levels of sCD40L (ng/mL) compared with nonrestenotic patients in the samples collected before PTCA (2.13±0.3 versus 0.87±0.12, P<0.0001; Figure 1). In all patients, sCD40L was significantly increased when measured 24 hours after PTCA (Figure 2). Nevertheless, enhanced sCD40L after PTCA persisted as statistically significant throughout the study in the restenotic patients, whereas they normalized 5 days after the procedure in the nonrestenotic patients (Figure 2). Thus, sCD40L was significantly higher (P<0.0001) in the restenotic patients at days 1, 5, 15, and 180 after PTCA (Figure 2 and Table 2). In the samples collected before PTCA, serum CRP >3 mg/L was observed in 17% (9 of 52) of nonrestenotic patients and in 78% (14 of 18) of restenotic patients. Notably, in patients with restenosis, CRP showed a strong correlation with basal sCD40L (R²=0.711, P<0.0001) and other soluble inflammatory markers.

**In Vivo Biosynthesis of Adhesion Molecules and MCP-1**

Restenotic patients showed higher (P<0.0001) levels of sICAM-1, sVCAM-1, sE-selectin, and MCP-1 than nonrestenotic patients in the plasma samples collected before PTCA (Table 2). Nevertheless, endothelial perturbation after PTCA persisted as statistically significant throughout the study in the restenotic patients, whereas they normalized 5 days after the procedure in the nonrestenotic patients (Table 2). Thus, sICAM-1, sVCAM-1, sE-selectin, and MCP-1 were significantly higher (P<0.0001) in the restenotic patients during
days 1 (Figure 3), 5, 15, and 180 after PTCA. Accordingly, a positive correlation ($P<0.0001$) was found between sCD40L and sICAM-1, sVCAM-1, sE-selectin, and MCP-1 at each collection time, with the best correlation observed in the samples collected before the procedure ($R^2=0.767$, $R^2=0.594$, $R^2=0.636$, and $R^2=0.628$ for sICAM-1, sVCAM-1, sE-selectin, and MCP-1, respectively).

**Effect of sCD40L on Endothelial Activation In Vitro**

We next studied whether the higher grade of endothelial activation observed in restenotic patients after PTCA was a direct consequence of enhanced preprocedural sCD40L. ECs generated considerable levels of sICAM-1 and MCP-1 when cultured with serum collected from patients before PTCA, significantly higher ($P<0.0001$) in the subset of patients with restenosis (581±87 versus 265±32 and 21.7±3 versus 11.4±2 ng/mL, respectively; Figure 4). In contrast, no detectable generation was measured in cells cultured with serum from the healthy donors (Figure 4). The stronger stimulatory effect of sCD40L-rich serum from restenotic patients was replicated by exogenous sCD40L, whereas both were blocked by a blocking monoclonal antibody for CD40L (Figure 4), thus confirming the causal role of sCD40L in sICAM-1 and MCP-1 generation in ECs.

**Effect of sCD40L on Endothelial Migration In Vitro**

Migration (% of control, Figure 5) was significantly ($P<0.001$) abrogated in ECs incubated for 3 hours or 18 hours with serum from the subset of patients with restenosis and high sCD40L levels ($-37±4\%$ and $-53±5\%$, respectively). In contrast, no significant inhibition was measured in ECs cultured with serum from the nonrestenotic patients with low sCD40L levels ($-4±1\%$ and $-7±1\%$, respectively). Importantly, the stronger inhibitory effect of serum from

![Figure 3. Plasma sICAM-1 (a), sVCAM-1 (b), sE-selectin (c), and MCP-1 (d) measured 24 hours after PTCA. Bars and vertical lines represent mean±SD values.](image)

![Figure 4. Effect of serum from patients who underwent PTCA on spontaneous generation of sICAM-1 and MCP-1 in endothelial cells. Bars and vertical lines represent mean±SD values.](image)

![Figure 5. Effect of serum from patients who underwent PTCA on endothelial cell migration. Note inhibitory effect of blocking monoclonal antibody for CD40L. Bars and vertical lines represent mean±SD values.](image)
restenotic patients was replicated by exogenous sCD40L (−42±5% and −57±6%, respectively; P<0.001), whereas it was completely reversed by neutralizing antibody against CD40L (+3±1% and −4±1%, respectively).

**Effect of sCD40L on Monocyte Inflammatory Status In Vitro**

To examine the relation between sCD40L and monocyte activity in patients who had undergone PTCA, healthy monocytes were evaluated for spontaneous and PMA-stimulated O$_2^-$ generation after culturing with either serum from the 70 patients subjected to PTCA with broad-spectrum sCD40L levels (range, 0.34 to 2.7 ng/mL) or serum from 10 healthy control subjects (≤0.4 ng/mL). Monocytes generated considerable levels of O$_2^-$ when cultured with serum collected from patients, higher in the subset of patients with restenosis (9.1±2.1 versus 4.7±1.2 nmol · h$^{-1}$ · 10$^6$ cells, P<0.0001; Figure 6). In contrast, no detectable O$_2^-$ generation was observed in monocytes cultured with serum from healthy donors (Figure 6). The stronger stimulatory effect of serum from restenotic patients was replicated by exogenous sCD40L (4.7±1.2 versus 8.1±1.7 nmol · h$^{-1}$ · 10$^6$ cells, P<0.0001; Figure 6), whereas it was blocked by the blocking antibody for CD40L (9.1±2.1 versus 5.2±1.3 nmol · h$^{-1}$ · 10$^6$ cells, P<0.0001; Figure 6), thus confirming the causal role of sCD40L in enhanced monocyte O$_2^-$ generation. Similar results were also observed in monocytes stimulated with PMA (11.8±2.2 versus 7.4±2.3 nmol · h$^{-1}$ · 10$^6$ cells, P<0.0001; Figure 6). Accordingly, we found a positive correlation (P<0.0001) between circulating sCD40L measured before PTCA and either spontaneous or stimulated monocyte O$_2^-$ generation in vitro (R=0.482 and R=0.593, respectively).

**Variables Predictive for Late Lumen Loss**

To investigate whether sCD40L may contribute to luminal renarrowing after PTCA, we assessed the association between plasma sCD40L and the degree of luminal renarrowing at 6 months after PTCA. Luminal renarrowing showed a positive correlation (P<0.0001) with sCD40L measured both before (R$^2=0.509$, Figure 7) and 1 (R$^2=0.434$), 5 (R$^2=0.412$) and 180 (R$^2=0.473$) days after PTCA. Notably, only 4 of the patients with preprocedural sCD40L values in the lowest quartile but all but 1 (85.7%) of the patients with sCD40L values in the highest quartile developed restenosis (Figure 7). Finally, multiple regression analysis showed that preprocedural sCD40L was an independent predictor (β=0.623, P<0.0001) of vascular renarrowing at 6 months after PTCA.

**Discussion**

To the best of our knowledge, this study is the first to (1) provide evidence in humans of increased sCD40L levels both early and as long as 6 months after PTCA in patients developing restenosis and (2) identify preprocedural sCD40L as an independent predictor of late lumen loss after PTCA.

In this study, patients who developed restenosis showed higher levels of sCD40L before the procedure than nonrestenotic patients. In addition, the increase in sCD40L after PTCA was significantly more manifest and prolonged in restenotic patients. Thus, the differences between the 2 groups became evident before PTCA and persisted as statistically significant during the whole period of examination.

The striking effect of preprocedural sCD40L in increasing late lumen renarrowing after angioplasty raises the question of the origin of this mediator. Platelets are major contributors to enhanced sCD40L in patients with acute coronary syndrome and hypercholesterolemia, and studies on the cellular distribution of CD40L indicate that >95% of the circulating sCD40L exists in platelets. Notably, we recently described, in patients with unstable angina, enhanced levels of thromboxane A$_2$ and isoprostanes, mediators that may induce platelet activation despite chronic aspirin therapy. Furthermore, PTCA is known to disrupt the endothelium, resulting in the exposure of thrombogenic surfaces that
support the adhesion, activation, and aggregation of platelets.\textsuperscript{10}\textsuperscript{10} Thus, the platelet-rich thrombi may be a source of localized high concentrations of proinflammatory CD40L both on the surface and in the immediate environment as they shed sCD40L.\textsuperscript{10}

Activated platelets have been found to stimulate adhesion molecule and MCP-1 production in ECs through enhanced sCD40L secretion and direct platelet–endothelium contact mediated by CD40L expression on the platelet surface.\textsuperscript{4} Such a mechanism was recently found to be operative in patients with unstable angina,\textsuperscript{4} and it is conceivable that such a platelet–endothelium interaction may also contribute to the enhanced expression of adhesion molecules observed in patients who have undergone PTCA.

Importantly, we found no association between aspirin use and sCD40L. In fact, although all patients received aspirin throughout the study, only the patients who developed restenosis showed high levels of sCD40L before PTCA. This observation is in agreement with the recent evidence\textsuperscript{9} showing that aspirin administration did not attenuate the increased risk of future cardiovascular events associated with high sCD40L levels. Thus, although aspirin is used extensively in patients who underwent PTCA and may inhibit some platelet functions, more potent platelet inhibitors (eg, glycoprotein Ib/IIa)\textsuperscript{10} may be necessary to inhibit the enhanced release of sCD40L from these cells.

Alternatively, we cannot exclude the possibility that vascular smooth muscle cells, macrophages, and lymphocytes may be involved, at least in part, in sCD40L generation in the setting of PTCA. Thus, a contribution to enhanced preprocedural sCD40L in restenotic patients could be from inflammatory cells persistently activated in a subset of patients with acute coronary syndromes despite aspirin therapy.\textsuperscript{12}

Thus, our data demonstrate an association between enhanced sCD40L at baseline and vessel hyperresponsiveness to injury, ultimately responsible for luminal narrowing after PTCA. In this light, enhanced preprocedural sCD40L in restenotic patients is not simply a marker of higher immune activation but is also actively involved in pathogenetic processes in these patients. Indeed, whatever the cellular source(s), sCD40L may both indirectly and directly have important pathophysiologic consequences in patients who undergo PTCA, as reflected by the statistically significant relationship between sCD40L and late lumen loss. Intimal hyperplasia and constrictive arterial remodeling are 2 important pathogenic events in the development of restenosis after PTCA, and enhanced CD40L–CD40 interaction may be involved in both these processes through the complex biological effects that it exerts on a number of cell types. First, sCD40L may exercise profound influences on endothelial function. In fact, we demonstrate in this study that sCD40L stimulates the endothelial expression of adhesion molecules critically involved in restenosis;\textsuperscript{14} may activate ECs to release MCP-1, a major signal for the inflammatory cell accumulation after vessel injury and a strong predictor of restenosis when measured after PTCA;\textsuperscript{12} and finally exerts a profound inhibitory effect on endothelial migration, a process considered critical for the reendothelialization of the injured vessel.

In addition to its effects on ECs, sCD40L may exercise profound influences on monocytes as well. In fact, in the present study, we found that the raised circulating sCD40L levels in patients who developed restenosis after PTCA had enhancing effects on spontaneous reactive oxygen species generation in monocytes. Thus, if this enhancement also occurs in vivo, it may be directly involved in the response to vascular injury, because the generation of reactive oxygen species has profound and wide-ranging effects that can dramatically increase vascular toxicity and initiate a cascade of detrimental molecular and cellular responses.

The marked rise in sCD40L after mechanically induced plaque rupture by PTCA might suggest that raised sCD40L levels are merely a secondary phenomenon. However, the observation that PTCA may induce further elevation of sCD40L does not exclude a causal role for sCD40L in this setting. Furthermore, the active role of sCD40L in modulating vascular reactivity has been also demonstrated in this study by the use of sCD40L-neutralizing antibody. Thus, our data seem to suggest that preprocedural sCD40L, in addition to inducing direct inhibition of EC migration, is also involved in the control of endothelium- and monocyte-mediated inflammatory reaction after PTCA, which is ultimately responsible for late luminal narrowing. Thus, systemically detectable inflammatory status may possibly precede the onset of an acute vascular injury, and enhanced sCD40L may, at least in the subgroup of hyperresponsive patients, contribute to the complex phenomena of intimal hyperplasia and constrictive remodeling leading to restenosis.

The critical role of sCD40L in restenosis is also demonstrated in this study by the significant correlation with CRP. However, because in a recent larger study, Dibra et al\textsuperscript{20} recently showed that baseline CRP did not predict restenosis in 1152 patients undergoing stenting, it should be noted that inflammatory marker elevation as a predictor of restenosis still remains controversial.

In conclusion, our results are consistent with the hypothesis that upregulation of sCD40L in a subset of patients undergoing PTCA results in the activation of ECs and in the recruitment of monocytes and tissue macrophages to the injured arterial wall, possibly contributing to restenosis after PTCA. Further understanding of the mechanism(s) by which sCD40L is produced and acts might provide insight into therapies to limit the progression of restenosis after balloon angioplasty.

**Acknowledgment**

This study was supported in part by a grant to the Center of Excellence on Aging of the University of Chieti.

**References**


Preprocedural Level of Soluble CD40L Is Predictive of Enhanced Inflammatory Response and Restenosis After Coronary Angioplasty

Francesco Cipollone, Claudio Ferri, Giovambattista Desideri, Leonardo Paloscia, Guido Materazzo, Marco Mascellanti, Maria Fazia, Annalisa Iezzi, Chiara Cuccurullo, Barbara Pini, Marco Bucci, Anna Santucci, Franco Cuccurullo and Andrea Mezzetti

_Circulation._ 2003;108:2776-2782; originally published online November 17, 2003; doi: 10.1161/01.CIR.0000103700.05109.0D

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/22/2776

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/