Atorvastatin and Thrombogenicity of the Carotid Atherosclerotic Plaque: the ATROCAP Study

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Keywords
Carotid arteries, atherosclerosis, thrombosis, drugs

Summary
Statins appear to have beneficial effects on fibrous cap stabilisation but their effects on plaque thrombogenicity have not been reported. To evaluate the thrombogenicity of human carotid plaques before and after atorvastatin treatment, 59 patients with bilateral carotid stenosis eligible for two-step carotid endoarterectomy (CEA) were randomly assigned to atorvastatin, 20 mg/day, or placebo. Histological and immunohistochemical analyses, Tissue Factor (TF), Tissue Factor Pathway Inhibitor (TFPI) antigens (Ag) and TF activity were determined in endoarterectomy specimens obtained at baseline and after treatment. Mean TFAg and TFPIAg levels from plaques removed at the first CEA were 55 ± 56 and 32 ± 26 pg/mg. After placebo, TFAg and TFPIAg content was higher in the second than the first CEA. Plaques removed at the second CEA from atorvastatin-treated patients had a lower macrophage content than plaques at the first CEA. TFAg and TFPIAg levels, and TF activity in plaques after atorvastatin treatment were lower (respectively 29, 18% and 56%) than after placebo. These findings indicate that atorvastatin reduce the inflammatory/thrombotic phenotype of carotid plaque, suggesting that these drugs may indeed have a beneficial effect on cerebrovascular events.

Introduction
Clinical trials have shown that statins reduce mortality and acute cardiovascular events in patients with high or average cholesterol levels (1). These effects are observed much sooner than was originally predicted – in most cases within two years of randomisation and in one study as early as six months (2). Statins also reduced the risk of acute coronary events out of proportion to their effects on angiographically assessed lumen stenosis (3). This additional beneficial effect results from fibrous cap “stabilisation”, which makes the atheromatous plaque less liable to rupture and thrombosis (4). Statins reduce macrophage accumulation within the vessel wall and endothelial cell activation in experimental atherosclerosis (5). In vitro, these drugs reduce matrix metalloproteinase secretion and tissue factor (TF) expression in human macrophages (6, 7). Cerivastatin was recently shown to reduce macrophage accumulation and TF expression in the aortic wall of Watanabe rabbits (8).

The in vivo effect of cardiovascular drugs is assessed on the basis of clinical benefits (reduction of events) or invasive instrumental (angiography) and non-invasive (B mode) techniques. However, ultrasound gives little information on plaque composition and the biochemical and/or pathological plaque structure can be studied ex vivo only one time, i.e. at the moment of plaque removal. Bilateral carotid atherosclerotic disease provides a unique human model for investigating two symmetrical plaques with similar severity, to test therapeutic interventions in the same patient and at different intervals.

To assess the effect of atorvastatin on plaque stability and thrombogenicity, we designed a prospective study to evaluate plaque ulceration, thrombosis, inflammation, and intraplaque thrombogenic factors (TF and the Tissue Factor Pathway Inhibitor, TFPI) in patients with bilateral carotid stenosis undergoing two-step carotid endoarterectomy (CEA), before (one side) and after 4-5 months (the contralateral side) of treatment with atorvastatin.

Methods
Study Design
A randomised, double-blind, placebo controlled, six-centre study was planned to evaluate thrombogenicity in bilateral carotid plaques before and after atorvastatin plus standardised antiplatelet treatment. Patients with bilateral carotid stenosis eligible for two-step CEA scheduled within 4-6 months were randomly assigned to atorvastatin, 20 mg/day, or placebo. Randomisation was stratified for each surgery centre. All patients received standard antiplatelet therapy (aspirin 100 mg/day) throughout the trial. Patients with aspirin intolerance were given ticlopidine, 400 mg/day.

Diagnosis
Carotid stenosis were considered eligible for surgery according to the European Carotid Trials’ Collaborative Group (9). All patients were examined for coronary and peripheral vascular disease and for risk factors such as hypertension, smoking, diabetes or hyperlipidaemia. Neurological, cardiac and peripheral examination, including ECG and ankle/arm pressure index, and blood analyses were done. Complete history and instrumental data were recorded for all patients at baseline and after follow-up.

Patients were ineligible for the study if they had total and LDL-cholesterol >6.2 and >4.9 mmol/L, respectively, and fasting triglycerides >2.8 mmol/L, or were taking lipid-lowering therapy (resins, statins or fibrates); if they had had a disabling major stroke or had life expectancy less than 24 months, hypothyroidism defined as TSH >8 mIU/L, severe renal (creatinine >3.0 mg/dL) or hepatic failure, or required anticoagulants, insulin or oral antidiabetic therapy.

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Ethics

A Steering Committee was responsible for the study and for all data reports. The study protocol was approved by the regulatory agency of the Italian Ministry of Health and by the Ethical Committees of all centres participating in the trial. Average lipid levels were chosen to justify the “placebo” arm. Written informed consent was obtained from all patients.

Treatment

Out of 105 patients referred for bilateral CEA, 59 met the eligibility criteria. Ineligible patients were on antidiabetic (61%), anticoagulant (7%) or lipid-lowering therapy (7%); 13% were hyperlipidaemic, 9% needed urgent surgery. Of the 59 patients admitted, 18 did not complete the trial, on account of cerebrovascular (2) and coronary events (1); 15 refused to participate further.

Standard CEA was done in 70% and 73% of the first and second operations; evasion endoarterectomy was done in the remaining 30% and 27%. In all surgical procedures 5000 units of heparin were injected intravenously before carotid cross-clamping.

The day after the first CEA, patients were randomly assigned to receive either 20 mg atorvastatin or placebo before the evening meal. Compliance was assessed by counting the number of tablets returned at the end of the study. All patients received antiplatelet therapy with aspirin 100 mg/day (ticlopidine only if aspirin was contraindicated). Test treatment and antiplatelet therapy were continued until after the second CEA. All patients received dietary counselling and were strongly advised to stop smoking.

Tissue Processing

Endoarterectomy specimens were immediately placed on crushed ice and sent to the Central Laboratory where macroscopic evaluation and sampling were done blindly by the same operator. Each specimen was longitudinally divided into multiple samples to provide tissue for morphological and immunohistochemical analysis and for biochemistry. For each lesion at least three adjacent segments were obtained for histopathological, immunohistochemical and biochemical studies.

Histological and Immunohistochemical Analysis

Samples were fixed in 10% buffered formalin and embedded in paraffin. Multiple serial sections were cut at 5 μm thickness and stained with hematoxylin/eosin and with Movat pentachrome stain (10). Further sections were used for immunohistochemical identification of TF (human Tissue Factor 1, Histo-line Lab.) and for the characterisation of inflammatory cells [UCHL-1 (CD20RO, Dako) for T-lymphocytes and PGM-1 (CD68, Dako) for macrophages]. Immunostaining was done as previously described (11, 12). Normal spleen sections were used as positive controls for macrophages, lymphocytes, and endothelial cells. The primary antibody was replaced with normal swine serum or with an unrelated primary antibody as negative control. Positive immunoreactions were scored blindly and semiquantitatively (absent, minimal, moderate and abundant) for cellular and extracellular immunostaining. Quantitative analysis of TF immunoreactivity was done with a computer-assisted colour image analysis system (Image DB 5.1). Slides were scanned with a 1.6 or 2.5 and 16 lens. The two low magnification lenses were used to scan the entire plaque sections and the higher magnification lens to scan multiple adjacent areas of each plaque in order to obtain multiple values that were then averaged. A colour threshold mask for immunostaining was defined to detect brown colour by sampling, and the same threshold was applied to all samples.

Endoarterectomy Specimens for TF and TFPI Determinations

Each carotid specimen, kept in liquid nitrogen, was divided into two halves and TF activity was measured in one and TF Ag and TFPI Ag in the other. Tissue for TF and TFPI antigens was processed as described by Caplice et al. (13); for TF activity it was homogenized in 1 ml of lysis buffer (15 mmol/L octyl-b-D-glycopyranoside, 25 mmol/L Hepes-saline (14). Samples were then sonicated at 20 kHz for three 30 s cycles (Sonicator Ultrasonic Liquid Processor, Heat System Incorporated, Farningdale, N.Y.) and centrifuged at 10,000 rpm for 6 min at 4°C. Samples for TF activity were immediately processed; samples for antigen testing were stored at –20°C.

TF Activity

TF activity was determined as procoagulant activity by a one-stage plasma recalcification assay (15). Clotting times were quantified by a standard curve obtained from serial dilutions of a standard human thromboplastin (Thrombomat, Behring) preparation.

Preincubation of samples with a monoclonal antibody against human TF (cat # 4509, American Diagnostica Inc., Greenwich, Conn) was used to confirm the specificity of the assay. TF activity was inhibited in all the plaques, but the extent of inhibition ranged from 30% to 99%, which suggested to us that in some plaques shortening of clotting times was not completely attributable to TF. Thus TF Ag and TFPI Ag were compared with TF activity only in those plaques where the prolongation of clotting times due to TF antibody was more than 70%. Data are expressed as Units of TF activity per milligram of protein as determined by the Bradford method (16).

TF and TFPI Antigens

TF Ag and TFPI Ag in endoarterectomy homogenates were measured using commercial sandwich ELISA kits (Imubind TF cat # 845, and Imubind Total TFPI cat # 849). Values are expressed as pg of TF and TFPI per milligram of tissue.

Miscellanea

Blood samples for all tests were drawn by clean venipuncture without stasis with a 20-gauge needle from the antecubital vein between 8 and 9:30 AM. For haemostasis, blood was collected into 3.8% trisodium citrate (9:1, vol/vol) and immediately centrifuged at 1250 g for 15 min at room temperature. The supernatant plasma was snap-frozen and stored in small aliquots at –80°C. For lipid measurements, serum was obtained from non anticoagulated blood and stored at –80°C. Fibrinogen levels were determined according to Claus (17). Prothrombin fragment F1+2 was determined by a commercial specific immunoassay (Enzignost F1+2 micro, Dade, Behring). Total and high-density-lipoprotein (HDL) cholesterol and triglycerides (TG) were determined by enzymatic methods (18). HDL was obtained by selective precipitation with dextran-MgCl2 (19). Low-density-lipoprotein (LDL) cholesterol was calculated by Friedewald’s formula (20).

Statistical Methods

All data were blindly submitted to the database manager. Parametric methods were used for continuous variables after ascertaining the normality of the distribution. For these variables within-patients changes were assessed by the signed ranks test; for differences in these changes between treatments Student’s t test was used with the appropriate correction for heterogeneity of the variances, when necessary. The chi-squared test was used for between-patients comparisons of the categorical variables, and within-patient changes were assessed by symmetry tests (McNemar or Bowker). Spearman’s correlation coefficients were computed and linear regressions of dependent continuous variables on the independent categorical variables with more than two classes were fitted and tested by analysis of variance.

Statistical analysis was done only for patients with a complete data set: this is why the number of patients varies from one analysis to another. Data are expressed as mean ± SD.

Results

Patients’ main characteristics are reported in Table 1. The Table includes all 59 patients who underwent a first CEA: values were similar.
for the 41 patients who completed the procedure (first and second CEA) and were included in the analysis. Basal echo-Doppler showed that the mean luminal narrowing (± SD) was 76 ± 9% on the right and 77 ± 10% on the left carotid. No differences in structure and surface characteristics were observed between the two symmetrical series of plaques by B mode echography.

Aspirin was used by 45 patients (76%), ticlopidine in the remaining 14 (24%). Of the 41 patients included in the analysis only 8 were given ticlopidine (3 in the placebo and 5 in the atorvastatin group).

Placebo and atorvastatin treatments (interval between first and second CEA) lasted 132 (± 55) and 133 (± 63) days, respectively. After atorvastatin treatment total and LDL cholesterol and TG showed the expected reductions (total cholesterol 24%; LDL-cholesterol 34%; TG 20%), and HDL-cholesterol was unaffected (Table 2). No significant differences were noted in plasma fibrinogen or in the prothrombin fragment 1+2 between placebo and atorvastatin treated patients (data not shown).

Characteristics of Atherosclerotic Plaques

Plaques removed at the first CEA were atherofibrotic with multiple non-contiguous pultaceous nuclei. TF was identified extracellularly in the core and cap. Figs. 1A and C represent microphotographs of TF immunostaining performed in plaques removed at first CEA. Mean (± SD) TF Ag levels in homogenates of plaques removed at the first CEA were 55 ± 56 pg/mg protein (median 39). TFPI Ag levels were

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>PLACEBO (n = 30)</th>
<th>ATORVASTATIN (n = 29)</th>
</tr>
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<tbody>
<tr>
<td><strong>Number (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20 (67)</td>
<td>19 (66)</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>12 (40)</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Family history of CVD</td>
<td>4 (13)</td>
<td>7 (24)</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>23 (77)</td>
<td>19 (66)</td>
</tr>
<tr>
<td>Smoking</td>
<td>7 (23)</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Ex smoker</td>
<td>12 (40)</td>
<td>16 (55)</td>
</tr>
<tr>
<td>History of TIA</td>
<td>12 (40)</td>
<td>10 (34)</td>
</tr>
<tr>
<td>Stroke</td>
<td>1 (3)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Angina pectoris</td>
<td>10 (33)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>AMI</td>
<td>9 (30)</td>
<td>8 (28)</td>
</tr>
<tr>
<td>Claudicatio</td>
<td>11 (37)</td>
<td>9 (31)</td>
</tr>
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<table>
<thead>
<tr>
<th><strong>Mean ± SD:</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>69.9 ± 7.6</td>
<td>71.3 ± 6.0</td>
</tr>
<tr>
<td>Ankle/arm pressure index: right</td>
<td>86 ± 22</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>left</td>
<td>83 ± 1</td>
<td>87 ± 21</td>
</tr>
</tbody>
</table>

CAD: Coronary artery disease; CVD: Cerebrovascular disease; TIA: Transient ischaemic attack; AMI: Acute myocardial infarction;

Table 2  Lipid values by treatment (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Tot. Cholesterol mmol/L</th>
<th>LDL Cholesterol mmol/L</th>
<th>HDL Cholesterol mmol/L</th>
<th>Triglycerides mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo (n=22)</td>
<td>Atorva (n=24)</td>
<td>Placebo (n=22)</td>
<td>Atorva (n=24)</td>
</tr>
<tr>
<td>Before treatment</td>
<td>5.1±0.8</td>
<td>5.1±1.0</td>
<td>3.5±0.9</td>
<td>3.5±0.8</td>
</tr>
<tr>
<td>After treatment</td>
<td>5.5±1.4</td>
<td>3.9±1.0</td>
<td>3.7±1.2</td>
<td>2.3±0.9</td>
</tr>
<tr>
<td>Diff.</td>
<td>0.4</td>
<td>-1.2</td>
<td>0.2</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

* p= 0.028 (before vs after treatment)

*** p<0.001 (before vs after treatment)
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32 ± 26 pg/mg protein (median 25). TF Ag and TFPI Ag were significantly correlated (p < 0.0001). TF activity was 85.13 ± 98.77 U/mg protein.

Plaques removed from right and left carotids were comparable for their content of inflammatory cells and macrophages and for TF Ag and TFPI Ag content (mean ± SD: right plaques respectively 54.1 ± 63 and 30 ± 27; left plaques 56.4 ± 50.5 and 34 ± 25).

**Effect of Atorvastatin on Atherosclerotic Plaques**

In the placebo group there was no difference in the content of macrophages and inflammatory cells in plaques removed at the first and second CEA (Fig. 2). TF Ag and TFPI Ag content of plaque homogenates from the second CEA was higher than the first (both 45%, p < 0.05 and p = 0.01). The total TF activity in the second CEA was lower than the first (-23%) (Table 3). A direct correlation was found between TF Ag and TF activity (p < 0.05).

Plaques from atorvastatin-treated patients tended to have a lower percentage of inflammatory cells and lower macrophage content (p = 0.059 and p = 0.072; Fig. 2). TF immunoreactivity in the second CEA was less than in the first (Fig. 1 B and D). Computer-assisted image analysis on plaques of ten randomly selected patients assigned to placebo or atorvastatin showed a 35% lower TF positive areas in plaques of atorvastatin treated patients (Fig. 1 E). Accordingly, plaques removed from atorvastatin-treated patients at the second CEA had lower levels of TF Ag and TFPI Ag. Moreover, TF activity was 56% lower than in placebo-treated patients (Table 3).

**Discussion**

The ATROCAP study shows that, in the symmetrical bilateral carotid stenosis model, plaques removed after atorvastatin treatment had lower thrombogenic and inflammatory activities than those of placebo-treated patients. This model provides information for the first time on the direct effect of statins on the TF Ag and TFPI Ag content of human atherosclerotic plaque.

In the placebo group, TF Ag and TFPI Ag were higher in plaque specimens removed at the second CEA than in those at the first although baseline echodoppler indicated a similar percentage of stenosis, and histopathology showed similar characteristics in the two symmetrical plaques. Thus, measurement of TF Ag and TFPI Ag may detect subtle changes in plaque composition occurring in the relatively short time between the two operations. No information is available on how the atherosclerotic plaque develops in vivo in human carotid atheroma within the limited time of this study. The changes seen therefore presumably reflect a limited spontaneous progression of the carotid atheroma, or may even be consequent to surgical stress.

Several studies have identified TF in the intima of human atherosclerotic plaques and have suggested that it is an important determinant of thrombogenicity after plaque rupture (21, 22). Interestingly, in the ATROCAP study, increases in total TF were accompanied by concurrent increases in TFPI, which provides physiological inhibition of TF-initiated coagulation (23, 24). In agreement with Caplice et al., who found measurable levels of TFPI influencing the thrombotic potential of the carotid plaque, we found a direct correlation between TF Ag and TFPI Ag (13).

Atorvastatin treatment results also in a reduction of TF activity determined by one stage clotting assay, which may be explained only in part by the lower levels of TF Ag. It is worth mentioning that TF activity is the result not only of TF Ag levels but also of TFPI. Moreover, it has been recently reported that plaques, in addition to TFPI, possess also TFPI-2, produced probably by macrophages and T cells, which may contribute to TF activity too (25).

### Table 3 TF and TFPI antigens (pg/mg ± SD) and TF activity (U/mg ± SD) from atherosclerotic plaques

<table>
<thead>
<tr>
<th>Patients no.</th>
<th>PLACEBO</th>
<th>ATORVASTATIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st CEA</td>
<td>49±31</td>
<td>63±59</td>
</tr>
<tr>
<td>2nd CEA</td>
<td>72±35</td>
<td>51±24*</td>
</tr>
<tr>
<td>Diff.</td>
<td>22±42*</td>
<td>-12±62*</td>
</tr>
<tr>
<td>TFPI:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st CEA</td>
<td>31±19</td>
<td>46±36</td>
</tr>
<tr>
<td>2nd CEA</td>
<td>45±22</td>
<td>37±32</td>
</tr>
<tr>
<td>Diff.</td>
<td>14±30**</td>
<td>-9±51</td>
</tr>
</tbody>
</table>

** ** p = 0.010 and * p = 0.029 (1st vs 2nd CEA)

^ p = 0.032 (vs placebo )

# p = 0.049 (vs placebo )

* p = 0.031 (vs placebo)

$ p = 0.085 (1st vs 2nd CEA)$
TF has been reported to co-localise with macrophages within the atherosclerotic plaque (8). Smooth muscle cells may also contribute to the TF content of the plaque. In atorvastatin-treated patients the lower levels of TF immunoreactivity were paralleled by a lower TF Ag content in plaque homogenates (30% lower than placebo, p <0.05 and 20% from the first CEA, n.s.). Interestingly these data are in accordance with those published by Crisby et al. who showed that pravastatin reduces metalloproteinase and inflammation in human carotid plaques (26). Changes in plaque TF content resulted in lower TF activity, although other mechanisms, not yet identified, may concur in the overall plaque thrombogenicity. Information on the expression of TF in relation to TFPI is limited and does not explain the upregulation of both proteins. Plasma levels of TF and TFPI were elevated in patients with unstable angina, suggesting that the two proteins may be regulated by the same stimuli (27). Serum and growth factors can increase both TF and TFPI biosynthesis in smooth muscle cells (28, 29). In this study TF Ag and TFPI Ag levels in the atorvastatin and placebo groups and the lower macrophage infiltration suggest a stabilising effect of atorvastatin on plaque thrombogenicity (30, 31).

![Fig. 1](image1.jpg)

Fig. 1 Representative photomicrographs of human carotid endarterectomy specimens, showing immunoreactivity for TF (brown staining, arrows). Low magnification (18x, A and B) and high magnification (120x, C and D) view of plaques before (A and C) and after (B and D) atorvastatin treatment. The larger magnifications are mirror images of the smaller ones. E. Colour-image analysis of TF positive areas in first and second CEs of placebo (n = 5) and atorvastatin (n = 5) treated patients. Bars indicate means ± SD. * p <0.01

![Fig. 2](image2.jpg)

Fig. 2 Number (%) of plaques from the placebo and atorvastatin groups, showing presence (moderate + abundant) of macrophages and inflammation as assessed by semiquantitative analysis (see Methods)
accumulation of cholesteryl esters in macrophages exposed to oxidized LDL (33, 34). Statins inhibit the growth of lymphocytes and other blood mononuclear cells through multiple pathways unrelated to cholesterol metabolism (35), an effect whose therapeutic relevance is currently under investigation.

Statins have been reported to inhibit TF expression through mechanisms independent of cholesterol biosynthesis (7). In particular, lipophilic statins, i.e. fluvastatin, simvastatin and atorvastatin, share the capacity to inhibit TF protein and mRNA, at least in part through impairment of NFkB translocation due to upregulation of IkBo (7). These effects are reverted by geranylglycerol, but not farnesol, which indicates that intermediates of the isoprenoid pathway are involved in these effects. Similarly, fluvastatin reduces TF expression and NFkB in vivo after its administration to cholesterol fed rabbits (36).

In our study in patients with cerebrovascular disease, with normal average plasma cholesterol levels, atorvastatin reduced both plasma cholesterol and triglycerides. Thus, the drug’s beneficial effects on carotid atheroma stabilisation may derive either from cholesterol-lowering or from inhibition of the biosynthesis of intermediates of isoprenoid metabolism, important for cell functions. This trial, however, was not designed to answer this important but controversial question. Rather, our data provide the first demonstration in humans that statins do affect plaque composition, particularly their TF/TFPI content. A major strength of this trial is that the overall amount of TF measured in each lesion corresponds to its plaque content, since adventitial tissue was absent.

In conclusion, these findings indicate that atorvastatin reduces TF in human carotid plaques, which may result in a reduced thrombotic response to rupture and, in line with the clinical findings, suggest a beneficial effect on cerebrovascular events (37). The pooled data indicates that the drug reduces stroke risk and suggests that these effects are consistent across all major subgroups, including those on prophylactic aspirin (38, 39). Interestingly, a meta-analysis of non-statin lipid-lowering therapies failed to detect any significant effect on stroke (40). The reduced plaque thrombogenicity exerted by statins might result in carotid plaque stabilisation and this may be of relevance to explain the lower vascular event rates observed.

The ATROCAP Study Group

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