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Statin Treatment Is Not Associated With Consistent Alterations in Inflammatory Status of Carotid Atherosclerotic Plaques A Retrospective Study in 378 Patients Undergoing Carotid Endarterectomy

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- **Background and Purpose**—Anti-inflammatory qualities are held partially responsible for the reduction of cardiovascular events after statin treatment. We examined the phenotype of carotid atherosclerotic plaques harvested during carotid endarterectomy in relation to the previous use of different statins prescribed in clinical practice.
- *Methods*—Three hundred and seventy-eight patients were included. Atherosclerotic plaques were harvested, immunohistochemically stained and semiquantitively examined for the presence of macrophages (CD68), smooth muscle cells, collagen and fat. Adjacent atherosclerotic plaques were used to study protease activity and interleukin levels. Patients' demographics were recorded and blood samples were stored.
- **Results**—Serum cholesterol, low-density lipoprotein, apolipoprotein B, and C-reactive protein levels were lower in patients treated with statins compared with patients without statin treatment. Atheromatous plaques were less prevalent in patients receiving statins compared with patients without statin therapy (29% versus 42%, P=0.04). An increase of CD68 positive cells was observed in patients receiving statins compared with nonstatin treatment (P=0.05). This effect was specifically related to atorvastatin treatment. In patients treated with atorvastatin, the increased amount of CD68 positive cells were not associated with increased protease activity. In contrast, a dose-dependent decrease in protease activity was shown in the atorvastatin group. Interleukin 6 expression was lower in plaques obtained from patients treated with statins (P=0.04).
- *Conclusions*—Statin use may exert pleiotropic effects on plaque phenotype. However, not the presence of macrophages but activation with subsequent protease and cytokine release may be attenuated by statin use. (*Stroke*. 2006;37:2054-2060.)

Key Words: carotid endarterectomy ■ inflammation ■ pharmacology

Hyperlipidemia is a risk factor for cardiovascular disease and is related to the increase of plaque atheroma.¹ Treatment with 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) clearly results in reduced cardiovascular-related mortality and morbidity.^{2,3}

Next to the lipid-lowering effects, statin treatment has potential pleiotropic effects by modulating various inflammatory responses that are involved in the initiation and progression of atherosclerotic disease.⁴ Statin-induced reductions of serum interleukin (IL) levels, C-reactive protein (CRP) and matrix metalloproteinase (MMP) activity have been described previously.^{3,5–9} However, pleiotropic effects of statin treatment on atherosclerotic plaque characteristics have mainly been described in animal studies. Pravastatin has been investigated in many experimental cardiovascular studies.^{10–13} The modifying effects of atorvastatin or simvastatin on human plaque characteristics are relatively unexplored. A prospective longitudinal study including a nontreated placebo

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group would be the best design to answer our research question. However, considering the beneficial effects of statins in a population experiencing atherosclerotic cardiovascular disease, the compliance of the practitioner and patient would be a major limitation.

In this retrospective study, we analyzed the pleiotropic effects of 3 regularly prescribed statins on plaque characteristics in 378 patients undergoing carotid endarterectomy (CEA).

Methods

Baseline Data

Patients have been included in the longitudinal cohort bio-bank study Athero-Express. The study design of Athero-Express has been published previously.¹⁴

Briefly, all patients receiving operative treatment for carotid artery disease in the participating centers are enrolled. Patients may have been symptomatic or asymptomatic. Operation is indicated based on the recommendations as published by ACST and NASCET.^{15,16} Patients with a terminal malignancy and those who are referred back to a hospital outside The Netherlands immediately after surgery are excluded. The Medical Ethical Committees of the participating hospitals have approved of the study.

From patients included in Athero-Express extensive baseline patient characteristics are available (history of cardiovascular disease, medication, etc).¹⁴ In cases of doubt about medication use or dosage, the patient's general practitioner or pharmacy was consulted. Statin dosages were recorded and divided into 2 categories: low-dose (simvastatin, pravastatin and atorvastatin, 10 and 20 mg) and high-dose (simvastatin and atorvastatin, 40 mg). All analyses have been performed in the same cohort of 378 patients. The total number of 378 plaques were all used for immunohistochemistry and protein isolation was available for 293 patients. IL levels were assessed in 133 randomly selected protein isolates.

Of the 378 patients, blood samples were available of 228 patients. Total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein (LDL) cholesterol, apolipoprotein B and high-sensitive CRP were assessed before surgery. Baseline patient characteristics are presented in Table 1.

Tissue Processing

The atherosclerotic plaque obtained during CEA was immediately processed and dissected in parts of 0.5 cm. The culprit lesion was fixated in formaldehyde 4%, paraffin embedded and used for histological stainings. The segments adjacent to the culprit lesion were immediately frozen in liquid nitrogen and stored at -80° C.

All stained sections were independently scored by 2 observers as described previously.¹⁷ Plaques stainings were categorized as follows:

- 1. Picro Sirius red; collagen staining using polarized light microscopy: (1) no or minor staining= staining along part of the luminal border; (2) moderate or heavy staining=staining along the entire luminal border.
- CD68 positive cells: (1) absent or minor staining=negative or few scattered positive cells; (2) moderate or heavy staining=stained clusters of cells with >10 cells present.
- 3. α-actin positive cells: (1) no or minor staining=discontinuous over the entire circumference with absent staining at parts of the circumference of the arterial wall; (2) positive cells along the entire circumference of the luminal border, with at least minor staining locally with few scattered cells.
- 4. Hematoxylin and elastin stains were used to assess overall morphology. The percentage of atheroma of the total area of the plaque was visually estimated using the picro Sirius red with polarized light and hematoxylin stains. Three groups were defined based on the percentage of atheroma in the plaque: fibrous plaques <10% fat, fibro-atheromatous 10% to 40% or atheromatous >40% fat.
- 5. Human leukocyte antigens (HLA-DR) staining was performed in patients with moderate or heavy CD68 stained plaques and treated with atorvastatin (n=20) or no statin (n=22). Three groups were defined based on the percentage of cells being activated in the plaque <25%, 25% to 75% or >75%.

The CD68 (macrophage) staining was also analyzed quantitatively by computerized analyses using Analysis software. The stained sections were scored quantitatively as a percentage of the plaque area.

	No Statin	All Statins	P Value	Simvastatin	Pravastatin	Atorvastatin	P Value
Included in study	129	249		91	44	98	
Coronary intervention in the past	13.5% (17/126)	25.0% (58/232)	0.01	29.1% (23/79)	27.9% (12/43)	20.2% (19/94)	0.36
Myocardial infarction in the past	17.5% (21/120)	22.9% (50/218)	0.24	21.6% (16/74)	30% (12/40)	18.2% (16/88)	0.32
Peripheral artery intervention ever	17.6% (22/125)	25% (58/232)	0.11	31.6% (25/79)	18.6% (8/43)	20.2% (19/94)	0.14
Hypertension ever	61.2% (71/116)	72.0% (157/218)	0.04	71.2% (52/73)	75% (30/40)	71.1% (64/90)	0.89
Diabetes	13.2% (16/121)	25.0% (54/216)	0.01	26.3% (20/76)	16.7% (6/36)	26.7% (24/90)	0.46
Hypercholesterolemia	27.8% (32/115)	78.1% (171/219)	< 0.001	78.5% (62/79)	82.1% (32/39)	74.2% (66/89)	0.59
Smoker	30.3% (36/119)	25.9% (56/216)	0.46	30.1% (22/73)	15.8% (6/38)	25.6% (23/90)	0.33
BMI>25	60.4% (64/106)	61.7% (116/188)	0.82	60.0% (36/60)	60.5% (23/38)	66.3% (53/80)	0.71
High dose		43.5% (100/230)		44.4% (40/90)	95.5% (42/44)	18.8% (18/96)	< 0.001
Symptoms of Carotid disease							
Asymptomatic	28% (35/125)	23% (54/235)		13.1% (11/84)	21.4% (9/42)	28.4% (27/95)	
TIA or prior stroke	58.4% (73/125)	61.5% (145/235)	0.56	72.6% (61/84)	64.3% (27/42)	53.7% (51/95)	0.10
Amaurosis fugax	13.6% (17/125)	15.3% (36/235)		14.3% (12/84)	14.3% (6/42)	17.9% (17/95)	

TABLE 1. Baseline Patient Characteristics

Numbers and percentages of patients for the displayed categories are presented.

The adjacent segments were used for protein isolation. Protein was isolated by 2 protocols. One part of segment 1 was treated with 1 mL Tripure Isolation Reagent (Boehringer Mannheim) according to the manufacturer's protocol. The other part was dissolved in 1 mL 40 mmol TrisHCl (pH=7.5; 4°C) and centrifuged at max rpm after which the vials were stored at -80° C. Protein concentrations for both isolation techniques were measured according to the manufacture's protocol (Bio-Rad Laboratories).

For a randomly selected subgroup of 133 plaques, MMP-2, MMP-8 and MMP-9 activities were measured in isolated protein using the Biotrak activity assays RPN 2631, RPN 2635 and RPN 2634, respectively (Amersham Biosciences).^{18,19} IL-6 and -8 were measured in 293 protein isolates using a multiplex suspension array system according to the manufacture's protocol (Bio-Rad Laboratories).

Statistical Analyses

Statins were associated with plaque characteristics using a χ^2 and Fisher exact test. Additionally, a binary logistic regression analysis was used in cases of confounding. In the cases of nonparametric distributed continuous variables, comparison of the variables was performed by a Mann–Whitney test and a Kruskal Wallis test. For normal distributed parameters, *t* tests and Anova tests were used. Probability values of <0.05 were considered statistically significant.

Results

Patients who received statin therapy showed a higher prevalence of cardiovascular morbidity and risk factors in the past (Table 1). These risk factors were equally distributed among the 3 major statins described. We examined the relations between all baseline characteristics and the investigated markers for plaque phenotype to rule out confounding of baseline characteristics in the studied correlations between statin use and plaque phenotype. Overall, plaque phenotype (eg, amount of fat, protease activity and IL levels) was neither associated with any of the risk factors nor with clinical history (all P > 0.10). CD68 positive stained cells were more prevalent in plaques obtained from patients with prior coronary intervention in the past and a tendency was noticed for hypertension (P=0.04 and P=0.06).

Baseline characteristics of serological assessments are presented in Table 2. Serum levels of cholesterol, LDL and apolipoprotein B as well as CRP were significantly lower among statin users (Table 2).

Atheromatous ("lipid rich") plaques were less prevalent in patients receiving statins compared with patients without statin therapy (29% versus 42%; P=0.04; Table 3). The

prevalence of atheromatous plaques was not associated with
statin type or dosage. A tendency toward a lower prevalence
of atheromatous plaques was noticed for patients receiving
statins for >1 year (no statin: 41.9% atheromatous plaques,
<1 year statin 32.9% and >1 year statin 27.7%; $P=0.07$).

In contrast to the decreased level of unstable "lipid rich plaques," a significant increase in CD68 positive cells was observed in the statin group (P=0.05; Table 3 and Figure 1). To rule out confounding between statin use and the previously mentioned baseline characteristics that were related to CD68 positive cells (P<0.1; hypertension and prior coronary intervention), a binary logistic regression analysis was performed to identify the independent relations. Statin use was independently related to an increased number of CD68 positive cells in the plaque (P=0.02), whereas prior coronary intervention and high blood pressure were not (P=0.25 and 0.06).

These observations would appear to conflict with previous studies demonstrating that pravastatin treatment is related to lower macrophage content in the atherosclerotic plaque. Therefore, we decided to perform subgroup analyses for the different, frequently prescribed statins. Plaques derived from patients receiving atorvastatin showed significantly more CD68 positive cells than those receiving pravastatin (Table 3; P=0.01). Prolonged statin therapy (>1 year) and high dosage were not related to a decreased prevalence of CD68 positive cells (Figure 1).

The increased amount of CD68 positive cells in patients who received statin treatment was not associated with an increased MMP activity (Table 3). In contrast, patients treated with high doses of statin showed less protease activity compared with patients treated with low doses of statin (Figure 2A). In the nonstatin-treated group MMP-8 and -9 activities were associated with the degree of CD68 positive staining. The relation between CD68 staining and MMP activities was abolished in the atorvastatin-treated group but remained significant in the pravastatin-treated group (Figure 2B). Patients with strong CD68 staining and high dose atorvastatin revealed lower activity levels of MMP-8, and a tendency was shown for MMP-9 compared with patients with low dose and strong CD68 staining (P=0.02 and P=0.06; Figure 3). Statin use did not influence MMP-2 activity (data not shown).

Serum Characteristics							
(mean \pm SD) n=228	No Statin	All Statins	P Value	Pravastatin	Simvastatin	Atorvastatin	P Value
Cholesterol (mmol/l)	5.7±1.3	4.7±0.9	< 0.001	$4.7\!\pm\!0.9$	$4.7\!\pm\!0.9$	4.7±1.0	0.71
Triglicerydes (mmol/l)	$2.0{\pm}0.9$	2.1±1.1	0.58	$2.1\!\pm\!0.9$	2.3±1.0	2.0±1.0	0.19
HDL (mmol/l)	1.2±0.4	1.2±0.4	0.66	1.2±0.3	1.2±0.3	$1.2 {\pm} 0.4$	0.79
LDL (mmol/l)	3.7±1.2	$2.7{\pm}0.7$	< 0.001	$2.9{\pm}0.8$	$2.7{\pm}0.7$	$2.6{\pm}0.8$	0.39
ApoB (g/l)	1.1 ± 0.3	$0.9{\pm}0.2$	< 0.001	$0.9{\pm}0.2$	$0.9{\pm}0.2$	$0.9{\pm}0.2$	0.27
CRP (median/IQR mg/l)	4.8/2.1–12.9	2.8/1.5-6.0	0.002	2.2/1.5-5.1	2.6/1.5-5.4	3.4/1.6-8.9	0.31

Parametrically distributed variables are presented with mean and standard deviation, whereas nonparametric variables are presented with median and interquartile range (IQR). Normal values for: cholesterol <5.0 mmol/l; triglicerydes 0.8–2.2 mmol/l; HDL 0.9–2.0 mmol/l; LDL <3.5 mmol/l; APO B 0.55–1.4 g/l; HSCRP: <10mg/l.

ApoB indicates apolipoprotein B; HDL, high-density lipoprotein; HsCRP, high-sensitive C-reactive protein.

TABLE 2. Serum Characteristics

Plaque Characteristics (%/number)	No Statin	All Statins	<i>P</i> Value	Pravastatin	Simvastatin	Atorvastatin	<i>P</i> Value
Minor collagen	25%/32	24%/59	0.86	12%/5	28%/25	26%/25	0.10
Heavy collagen	75%/97	76%/187		88%/38	72%/64	75%/73	
Minor SMC	34%/44	33%/80	0.72	23%/10	36%/32	33%/32	0.35
Heavy SMC	66%/84	68%/166		77%/33	64%/58	67%/65	
Minor calcifications	48%/62	47%/117	0.97	43%/19	49%/45	46%/45	0.96
Heavy calcifications	52%/67	53%/132		57%/25	51%/46	54%/53	
Minor M ϕ	53%/68	43%/106	0.05	61%/26	44%/40	34%/33	0.01
Heavy M ϕ	47%/59	57%/141		40%/17	56%/50	66%/65	
Fibrous	29%/37	33%/83	0.04	32%/14	31%/28	37%/36	0.59
F-Atheromatous	29%/38	38%/94		43%/19	34%/31	37%/36	
Atheromatous	42%/54	29%/72		25%/11	35%/32	26%/26	
M ϕ quantitative (median/IQR)	0.3/0.01-0.3	0.4/0.1–1.3	0.16	0.2/0.01-1.0	0.4/0.1-1.2	0.6/0.1-1.5	0.04
MMP 8 (median/IQR)	6.3/2.9–12	5.8/2.7-9.8	0.32	3.6/0.0-9.3	6.9/3.7-9.3	6.2/3.1-13.2	0.25
MMP 9 (median/IQR)	1.7/0.8-5.2	2.1/1.0-4.9	0.43	1.4/0.8-3.8	2.6/0.9-5.8	2.1/1.3-4.8	0.39
IL 6 (median/IQR)	11.0/4.2-23	6.9/1.6-48	0.04	4.8/0.4–13.9	6.9/2.2-19.6	7.9/2.1–19.5	0.29
IL 8 (median/IQR)	34.4/0.6–97	42.9/7.3–138	0.29	32.4/0.8–178	43.3/8.8–113	44.1/6.8–152	0.95

 TABLE 3.
 Plaque Characteristics

Plaque stainings are in absolute numbers and percentages. Parametrically distributed variables are presented with mean and standard deviation, whereas nonparametric variables are presented with median and interquartile range (IQR). $M\phi$ indicates CD68 staining; SMC, smooth muscle cell staining.

IL-6 levels were lower in plaques obtained from patients who were on statin treatment compared with plaques derived from patients who did not receive statin treatment (P=0.04; Table 3). No significant differences were observed for IL-8 among patients groups (Table 3).

As an indication of activated CD68 cells we measured plaque HLA-DR expression in patients with high staining for CD68. No difference between the percentage of activated cells was observed between patients treated with atorvastatin and patients without statin treatment (P=0.99).

Besides the observed relations for statin treatment, other often prescribed medications in cardiovascular disease (angiotensin-converting enzyme inhibitors, calcium-antagonists, β blockade) were not associated with plaque phenotype (data not shown).

Discussion

Statin treatment of cardiovascular patients results in improved clinical outcome and reduction of serum LDL and CRP levels.^{3,20,21} Recently, a significant difference in hazard ratio for cardiovascular events was reported for symptomatic patients pretreated with statins at the time of carotid endarterectomy.²² Reduction of serum IL levels has also been described in relation to statin treatment.^{8,23} However, statin-related effects on plaque characteristics have only been described in a limited number of studies that were limited by the number of specimens ana-



Figure 1. Percentage of plaques with minor or heavy staining for CD68 positive cells. Prav indicates pravastatin; simv, simvastatin; ator, atorvastatin; low, low dose; high, high dose; <1 year, statin therapy shorter than 1 year; >1 year, statin therapy longer than 1 year.

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Figure 2. A, Patients treated with high statin dose showed a significant decrease in MMP-8 activity, whereas a tendency is observed for MMP-9. Bars indicate median. B, MMP-8 and -9 levels of atherosclerotic plaques with moderate/heavy (+) staining for CD68 positive cells compared with plaques with no/minor (-) staining and different groups of statin treatment. Bars indicate median.

lyzed.^{10–13,24} In this retrospective study, we analyzed the inflammatory status of atherosclerotic plaques obtained during CEA in relation to statin therapy.

It has been acknowledged that inflammatory mechanisms couple dyslipidemia to atheroma formation.²⁵ Statin treatment results in reduced serum cholesterol levels, impairs inflammatory processes and might stabilize atheromatous plaques.^{12,24,26} The current study confirms the earlier experimental observations that statin treatment results in lower fat content of atherosclerotic plaques. However, in contrast with previous studies, we found a slight but significant increase of CD68 positive cells in plaques obtained from statin-treated patients which was mainly attributable to the inclusion of the atorvastatin-treated patients. This was even more surprising considering the recently published clinical trial demonstrating the beneficial outcome for atorvastatin users.²⁷ Our observation does not imply that the inflammatory response is en-

hanced in atherosclerotic tissue of atorvastatin-treated patients. CD68 is considered a pan macrophage marker and does not provide information about the functional status of macrophages. The question rises whether the CD68 positive cells in patients treated with statins are inactivated. Within the atherosclerotic plaque, statins could suppress the production of cytokines and MMP by CD68 positive cells. This hypothesis is supported by our observations and is also substantiated by earlier fundamental research.^{12,24} We cannot explain the increased number of CD68 positive cells in the atorvastatin group. Chemokine expression is attenuated after atorvastatin treatment and increased adhesion of monocytes is not a likely explanation.²⁸ In an experimental model it has been shown that statin treatment reduced apoptosis in macrophages which could theoretically increase relative macrophage content.²⁹

Heavy CD68 stained plaques correlated with increased protease activity. This association was eliminated in atorvastatin-



Figure 3. MMP-8 and -9 levels in a selected group of patients. Selection was based on CD68 plaque staining and atorvastatin treatment. All patients were treated with atorvastatin, and those who showed moderate or heavy CD68 plaque staining were selected. In this selected group of patients, patients receiving low-dosage atorvastatin were compared with patients with high-dosage atorvastatin. Bars indicate median.

but not in pravastatin-treated patients. Moreover, IL-6 levels were lowered among patients treated with statins. It could be suggested that the pleiotropic effect of statin treatment is more reflected on a macrophage activation than on a macrophage recruitment level. For this reason, we studied HLA-DR expression, as an indication for macrophage activation. However, any conclusive remarks regarding inactive macrophages merit careful consideration when based on immunohistochemistry. HLA-DR expression did not differ significantly between statin users and nonstatin users. This observation makes a dramatic effect of statins on HLA-DR unlikely. However, HLA-DR expression on inflammatory cells on immune activation is not a de novo ("on-off") feature; on activated cells the number of HLA-DR molecules increases drastically. Such a phenomenon may result in increased staining intensity, which we were unable to demonstrate, but which does not allow the tracing of discrete differences in expression (and hence activation).

Besides the above-mentioned possibilities to explain the observed differences in CD68 staining, hypercholesterolemia itself could be an explanation. Significantly more patients in the statin group had a prior history of hypercholesterolemia. However, baseline serum lipid spectra are lower in the statin group compared with the nonstatin group. We also noted significantly lower serum CRP (activated plaque macrophages could produce CRP) levels in patients treated with statins compared with patients with no statin. Strictly hypothetically, the observed increased number of macrophages in the atherosclerotic plaques could be necessary for organizing plaque lipid remains.

What do these observations mean for clinical practice? First, imaging modalities are being developed to visualize the vulnerable plaque and the patient who is at risk for plaque rupture and subsequent cardiovascular symptoms. The present study suggests that in patients receiving statins, the inflammatory status of the atherosclerotic plaque should not be based on the number of inflammatory cells which may not always relate to plaque vulnerability.

Secondly, long-term statin treatment may be necessary to optimally influence plaque phenotype. We report a tendency that the statin associated effects on plaque characteristics are more pronounced in patients receiving statins longer than 1 year (P=0.07). The reported effect of statins on LDL serum levels and incidence of myocardial infarction has also been observed after prolonged treatment.³⁰ Sever et al also reports significant differences in hazard ratio between atorvastatin and placebo occurring at 2-year follow-up.³¹

Limitations

This study is based on retrospective data. No causality can be inferred from the current data.

Besides the modifying effect of therapy duration, we also observed a dose-response effect. We report a dose-response relation between plaque MMP levels and statin dose. This relation was not evident for IL-6 and IL-8. The dosages among the different statins were not equally distributed and could be of influence because the reported effects on inflammation differ among statins.

Our selected population could induce a bias. Higher rates of prior coronary intervention, hypertension, diabetes and previous cardiovascular events were reported in the statintreated group. Patients with these characteristics would be expected to have more advanced and more severe atherosclerosis and quite likely, greater inflammation and other biological derangements so typical of atherosclerosis. However, we excluded confounding and this cannot explain the differences observed among simvastatin, pravastatin and atorvastatin.

Sampling error could be an issue. Adjoining tissue showed less protease and IL-6 activity than would be expected from the amount of macrophages in the section examined by histology. Plaque characteristics may be heterogeneous within a short distance. Therefore, expression of protein or RNA may not always reflect the association with an immunohistochemically assessed phenotype. This limitation merits consideration when a limited number of samples is used. However, the relation we observed in control tissues in this and previous studies between MMP-9 and CD68 stainings, for instance, supports the idea that inflammatory markers are present in both adjacent segments.

Any conclusions regarding inactive macrophages cannot be drawn from the immunochemistry study alone. However, the finding that an increase in macrophage content was not associated with increased activity of MMP-8 and MMP-9 in the atorvastatin group supports the immunohistochemical observations.

Summary

Statin therapy was associated with a decreased prevalence of carotid atheromatous plaques. The number of CD68 positive cells increased slightly in patients receiving atorvastatins. Not the presence of macrophages but activation with subsequent protease and cytokine release seem attenuated by statin use. None.

Disclosures

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