Vascular Medicine

Rapid, Direct Effects of Statin Treatment on Arterial Redox State and Nitric Oxide Bioavailability in Human Atherosclerosis via Tetrahydrobiopterin-Mediated Endothelial Nitric Oxide Synthase Coupling

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Background—Treatment with statins improves clinical outcome, but the exact mechanisms of pleiotropic statin effects on vascular function in human atherosclerosis remain unclear. We examined the direct effects of atorvastatin on tetrahydrobiopterin-mediated endothelial nitric oxide (NO) synthase coupling in patients with coronary artery disease.

Methods and Results—We first examined the association of statin treatment with vascular NO bioavailability and arterial superoxide (O₂⁻) in 492 patients undergoing coronary artery bypass graft surgery. Then, 42 statin-naïve patients undergoing elective coronary artery bypass graft surgery were randomized to atorvastatin 40 mg/d or placebo for 3 days before surgery to examine the impact of atorvastatin on endothelial function and O₂⁻ generation in internal mammary arteries. Finally, segments of internal mammary arteries from 26 patients were used in ex vivo experiments to evaluate the statin-dependent mechanisms regulating the vascular redox state. Statin treatment was associated with improved vascular NO bioavailability and reduced O₂⁻ generation in internal mammary arteries. Oral atorvastatin increased vascular tetrahydrobiopterin bioavailability and reduced basal and L-nitro-arginine methyl ester–inhibitable O₂⁻ in internal mammary arteries independently of low-density lipoprotein lowering. In ex vivo experiments, atorvastatin rapidly improved vascular tetrahydrobiopterin bioavailability by upregulating GTP-cyclohydrolase I gene expression and activity, resulting in improved endothelial NO synthase coupling and reduced vascular O₂⁻. These effects were reversed by mevalonate, indicating a direct effect of vascular hydroxymethylglutaryl-coenzyme A reductase inhibition.

Conclusions—This study demonstrates for the first time in humans the direct effects of statin treatment on the vascular wall, supporting the notion that this effect is independent of low-density lipoprotein lowering. Atorvastatin directly improves vascular NO bioavailability and reduces vascular O₂⁻ through tetrahydrobiopterin-mediated endothelial NO synthase coupling. These findings provide new insights into the mechanisms mediating the beneficial vascular effects of statins in humans.

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Key Words: statins ■ tetrahydrobiopterin ■ oxidative stress ■ superoxide ■ nitric oxide synthase

Statins are now considered a fundamental component of the treatment of patients with atherosclerotic vascular disease. Reducing low-density lipoprotein (LDL) cholesterol by statins reduces cardiovascular risk in both primary and secondary prevention and is associated with improvements in other markers of vascular disease risk, including inflammation and endothelial function. In addition to LDL lowering, statins have been demonstrated to exert a number of pleiotropic effects. Reduction of LDL by statin therapy appears to confer a greater reduction of cardiovascular risk than LDL lowering by other modalities, and clinical trials demonstrate that statin treatment improves...
Recent evidence suggested that statin treatment exerts systemic antioxidant effects in patients with atherosclerosis, whereas experimental studies have demonstrated that statins also reduce vascular O$_2^-$ generation in animal and cell culture models. In endothelial cells, statins rapidly increase BH4 bioavailability by upregulating GCH1, encoding GTP cyclohydrolase I (GTPCH I), the rate-limiting enzyme in the BH4 biosynthetic pathway. Recent observations suggest that uncoupled endothelial nitric oxide (NO) synthase (eNOS) is an important mechanism that links impaired endothelial function with increased vascular superoxide production in humans. Reduced levels of the eNOS cofactor tetrahydrobiopterin (BH4) leads to eNOS uncoupling, shifting the enzyme toward the production of NO, and may retard vascular disease progression. Although these observations suggest that eNOS coupling is a potential therapeutic target in vascular disease pathogenesis, it remains uncertain whether eNOS coupling is amenable to therapeutic intervention in humans.

Recent evidence suggested that statin treatment exerts systemic antioxidant effects in patients with atherosclerosis, whereas experimental studies have demonstrated that statins also reduce vascular O$_2^-$ generation in animal and cell culture models. In endothelial cells, statins rapidly increase BH4 bioavailability by upregulating GCH1, encoding GTP cyclohydrolase I (GTPCH I), the rate-limiting enzyme in the BH4 biosynthetic pathway. Recent evidence suggested a direct effect of statins on Rho/Rho kinase pathway in human leukocytes, providing the first strong evidence of a direct pleiotropic effect of statins at the level of cell signaling in humans. The direct effects of statins on human arterial O$_2^-$ generation and their possible impact on eNOS coupling in human blood vessels are unknown.

We sought to investigate the effects and mechanisms of statin treatment on the human vasculature by quantifying the effects of statins on vascular NO bioavailability, vascular O$_2^-$ generation, and BH4-mediated eNOS coupling in patients with coronary artery disease. We first tested for associations between statin treatment and markers of endothelial function in a cohort of coronary artery disease patients scheduled for coronary artery bypass graft surgery (CABG) and then carried out a randomized, placebo-controlled trial of short-term statin treatment. In each study, we evaluated the functional and biochemical effects of statins in samples of blood vessels obtained at the time of CABG. Finally, we probed mechanisms in studies of human vessels exposed to statins ex vivo, thus removing the systemic effects of LDL lowering.

### Methods

#### Study Populations and Clinical Trial Protocol

In study 1, we examined whether treatment with statins was associated with endothelial function and the arterial redox state in patients with coronary artery disease. A total of 886 patients scheduled for elective CABG at the John Radcliffe Hospital in Oxford (n=395) or the Hippokration Hospital in Athens (n=491) were screened prospectively from 2004 to 2010. Of this population, 610 met the inclusion criteria (Oxford, n=285; Athens, n=325) and 492 agreed to participate (Oxford, n=254; Athens, n=238). There were no significant differences in demographic characteristics of patients between the 2 centers. Figure I in the online-only Data Supplement shows a diagram of the recruitment procedure. Exclusion criteria were any inflammatory, infective, liver, or renal disease or malignancy. Patients receiving nonsteroidal antiinflammatory drugs, dietary supplements, or antioxidant vitamins were also excluded. Patients not treated with statins were defined as those who had not received any statin treatment for at least 2 months before recruitment. Blood samples were collected, and endothelial function was evaluated by measuring flow-mediated dilation (FMD) in the brachial artery by ultrasound. At the time of CABG, samples of internal mammary artery (IMA) were collected and used to evaluate arterial O$_2^-$ generation. Endothelial function was also evaluated in saphenous vein segments through ex vivo isometric tension studies in organ baths.

In study 2, we examined whether the associations between statin treatment and vascular redox observed in study 1 were causal. To address that question, a total of 332 patients undergoing elective CABG were screened to identify patients who were not currently taking statins and fulfilled the inclusion criteria (n=66), 42 of whom agreed to participate. Patients were then randomized to receive either atorvastatin 40 mg/d or placebo for 3 days preoperatively in a randomized (using block randomization), double-blind fashion. Flow-mediated dilation was determined at baseline (before treatment initiation) and on the day before CABG. Blood samples were obtained at baseline and on the morning of the operation. During CABG, segments of IMA were collected to evaluate the mechanisms regulating vascular O$_2^-$ and NO bioavailability. Exclusion criteria were any inflammatory, infective, liver, or renal disease; malignancy; and known intolerance to statins. Patients receiving nonsteroidal antiinflammatory drugs, dietary supplements, and antioxidant vitamins and those who received statins during the previous 3 months were also excluded.

In study 3, we examined whether the effect of atorvastatin on vascular redox state in human arteries was independent of the systemic effects of LDL lowering and explored possible alternative mechanisms. We used an ex vivo system of human vessels in which we exposed IMAs directly to atorvastatin in an LDL-free environment. For these experiments, we prospectively screened 54 patients scheduled for elective CABG; 36 fulfilled the inclusion criteria and 26 agreed to participate. Because of the various different experiments, vessels from different patients were used in different experiments as stated. Blood samples from these patients were obtained on the morning before CABG, and segments of IMA were harvested and transferred to the laboratory for the ex vivo experiments. The inclusion criteria were the same as for study 1.

Demographic characteristics of the participants in the 3 studies are presented in Table 1. Risk factors were defined according to the Adult Treatment Panel III criteria for the definition and identification of risk factors as follows. Hypertension was defined as blood pressure $\geq 140/90$ mm Hg on multiple measurements or taking antihypertensive medications. Hypercholesterolemia was defined as a history of hypercholesterolemia for $\geq 3$ months that led to the initiation of lipid-lowering therapy by the primary physician or fasting serum cholesterol value $> 240$ mg/dL. Diabetes mellitus was defined as high fasting blood glucose levels ($> 120$ mg/dL), requiring glucose lowering therapy, or taking antidiabetic treatment. Smoking status was defined as follows: nonsmokers, those who had never smoked; ex-smokers, those who had stopped cigarette smoking for $> 1$ month; and active smokers, those who had smoked any cigarettes during the last month. The study protocol was approved by the local research ethics committees, and each patient gave written informed consent.
Assessment of Endothelial Function
Flow-mediated dilation and endothelium-independent vasodilations of the brachial artery (for studies 1 and 2) were measured by ultrasound (see the online-only Data Supplement).

Vessel Harvesting and Vasomotor Studies
Vasomotor studies in study 1 were performed in saphenous vein samples. Vessel harvesting and the methodology of vasomotor studies are described in the online-only Data Supplement.

Oxidative Fluorescent Microtopography
In situ $O_2^{-}$/H$_{1002}$ production was determined in vessel cryosections from study 3 with oxidative fluorescent dye dihydroethidium, as described in the online-only Data Supplement.

Determination of Vascular Superoxide Production
Vascular $O_2^{-}$ production (in all studies) was measured in fresh segments of intact IMA with lucigenin (5 $\mu$mol/L)-enhanced chemiluminescence (see the online-only Data Supplement).

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction
RNA was extracted from paired IMA rings from the ex vivo study (study 3), and the expression of GCH1 was quantified by quantitative reverse-transcription polymerase chain reaction (see the online-only Data Supplement).

Table 1. Demographic Characteristics and Clinical Details of the Participants

<table>
<thead>
<tr>
<th>Study</th>
<th>Placebo</th>
<th>Atorvastatin</th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (M/F), n</td>
<td>492 (411/81)</td>
<td>21 (20/1)</td>
<td>21 (18/3)*</td>
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<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>65.9±0.41</td>
<td>67.38±1.99</td>
<td>66.14±2.04*</td>
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<tr>
<td>Diabetes mellitus, n</td>
<td>159</td>
<td>9</td>
<td>0</td>
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<tr>
<td>Dyslipidemia, n</td>
<td>311</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Hypertension, n</td>
<td>333</td>
<td>16</td>
<td>18*</td>
</tr>
<tr>
<td>Smokers, n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>185</td>
<td>7</td>
<td>8*</td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>212</td>
<td>10</td>
<td>9*</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
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<td>27.7±0.8</td>
<td>27.5±0.7*</td>
</tr>
<tr>
<td>Serum lipid levels</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>166 (143–195)</td>
<td>205 (157–244)</td>
<td>225 (184–243)</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>...</td>
<td>191 (165–251)</td>
<td>171 (144–204)</td>
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<tr>
<td>Triglycerides, mg/dL</td>
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<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>125 (94–162)</td>
<td>206 (102–264)</td>
<td>132 (94–191)</td>
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<tr>
<td>Posttreatment</td>
<td>...</td>
<td>146 (105–185)</td>
<td>113 (89–166)</td>
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<tr>
<td>HDL, mg/dL</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>39.0 (33.0–43.8)</td>
<td>35.0 (31.0–47.0)</td>
<td>39.0 (35.5–42.8)</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>...</td>
<td>39.0 (32.5–42.0)</td>
<td>37.0 (33.0–45.0)</td>
</tr>
<tr>
<td>Medication, n</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Statins</td>
<td>395</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>ACEi</td>
<td>272</td>
<td>8</td>
<td>11*</td>
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<tr>
<td>ARBs</td>
<td>52</td>
<td>4</td>
<td>6*</td>
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<tr>
<td>CCBs</td>
<td>135</td>
<td>5</td>
<td>5*</td>
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<tr>
<td>$\beta$-blockers</td>
<td>368</td>
<td>13</td>
<td>13*</td>
</tr>
<tr>
<td>Aspirin</td>
<td>390</td>
<td>12</td>
<td>13*</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>154</td>
<td>8</td>
<td>6*</td>
</tr>
<tr>
<td>Diuretics</td>
<td>104</td>
<td>8</td>
<td>10*</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blockers; and CCB, calcium channel blockers. There was no significant difference in the changes of serum lipid levels between the atorvastatin- and placebo-treated groups (as tested by ANOVA for repeated measures with time-by-treatment interaction). Continuous variables are expressed as mean±SEM or median (25th to 75th percentile) as appropriate. *P=NS (P>0.05 vs placebo).

Assessment of Endothelial Function
Flow-mediated dilation and endothelium-independent vasodilations of the brachial artery (for studies 1 and 2) were measured by ultrasound (see the online-only Data Supplement).

Vessel Harvesting and Vasomotor Studies
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RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction
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Measurement of Vascular Rac1 Activation
Rac1 activation was evaluated by an affinity precipitation assay using PAK1-PBD–conjugated glutathione agarose beads (see the online-only Data Supplement).

Ex Vivo Studies (Study 3)
To examine the direct effect of atorvastatin on vascular $O_2^{-}$ generation in human arteries, IMA segments were incubated ex vivo for 6 hours in an organ bath system containing oxygenated (95% $O_2$/5% CO$_2$) Krebs Henseleit buffer at 37°C, as we have previously described. Two rings from the same IMA were incubated with either 0 or 5 $\mu$mol/L atorvastatin for 6 hours. Both basal $O_2^{-}$ and
N-nitro-L-arginine methyl ester (L-NAME)–inhibitable vascular O$_2^-$ were quantified in both rings from each patient at the end of the incubation period. To examine whether the effects of atorvastatin on human IMAs were mediated through direct inhibition of hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase in the vascular wall, we incubated paired IMA segments from 13 patients with/without atorvastatin in the presence or absence of mevalonate (200 μmol/L) for 6 hours. Five of these vessels were used for luminometry experiments and 8 for dihydroethidium staining and fluorescence microtopography. To evaluate the effect of atorvastatin on GTPCH activity, serial IMA rings from 8 patients were incubated for 6 hours (control) in the presence of the GTPCH inhibitor 2,4-diamino-6-hydroxypyrimidine (DAHP; 1 mmol/L), DAHP and atorvastatin (5 mmol/L), or atorvastatin alone. Vascular biopterins were then determined in these vessels to examine whether the effect of atorvastatin on vascular biopterins was due to an increase in GTPCH activity.

Blood Sampling and Serum Lipid Measurements

Fasting venous blood samples were taken after 12 hours of fasting on the morning of the operation (studies 1 and 3) or both at baseline and on the morning of the operation (study 2) (see the online-only Data Supplement for further details).

Determination of Plasma and Vascular Biopterin Levels

Levels of BH4 and total biopterins (BH4 plus dihydriobiopterin [BH2] plus biopterin) in plasma (study 2) or vessel tissue lysates (studies 2 and 3) were determined by high-performance liquid chromatography followed by electrochemical (BH4) and fluorescence (BH2 and biopterin) detection, as we have previously described.24

Statistical Analysis

Continuous variables in all 3 studies were tested for normal distribution by use of Kolmogorov-Smirnov test, and a significance level <0.05 was used to reject the null hypothesis of normal distribution. Nonnormally distributed variables were log transformed for analysis, and they all met the criterion for normality after their first transformation; these variables are presented in a nonlogarithmic format as median (25th to 75th percentile).

Sample size calculations were based on previous data from our laboratory. For study 1, we assumed that 80% of the eligible subjects would be receiving statin treatment. Therefore, we estimated that 420 samples would be able to detect a difference 0.753 relative light units of O$_2^-$ generation with an α of 0.05 and a power of 90% when SD = 2.0 RLU · s$^{-1}$ · mg$^{-1}$, as suggested by previous data from our laboratory in a similar population. For study 3, sample size calculations were performed on the basis of our previous experience on this model,14,25 and we estimated that with 5 pairs of samples (serial rings from the same vessel) we would be able to identify a change of vascular O$_2^-$ by 5.4 RLU · s$^{-1}$ · mg$^{-1}$ and vascular BH4 by 16.6 pmol/g with a power of 90% and an α of 0.05 when the SD for the difference in the response of matched pairs is 2.8 RLU · s$^{-1}$ · mg$^{-1}$ for O$_2^-$ and 8.6 pmol/g for BH4. However, we increased the numbers to 10 in individual experiments, depending on sample availability.

In both studies 1 and 2, continuous variables between 2 independent groups were compared by using unpaired t test, whereas categorical variables were compared by use of the χ2 test as appropriate. For the organ bath experiments, the effect of treatment on vasorelaxations in response to acetylcholine was evaluated by using 2-way ANOVA for repeated measures (examining the effect of acetylcholine concentration–by–statin treatment status [fixed factors] interaction on vasorelaxations) in a full factorial model. Because the sphericity assumption did not hold (Mauchly test of sphericity, P<0.0001), the Greenhouse-Geisser correction was used, which showed a significant difference in vasorelaxations through the course of the study (F = 22.783, P<0.01). The effect of treatment on plasma biomarkers and FMD was compared between the 2 groups in study 2 by use of 2-way ANOVA for repeated measures with time-by-treatment interaction (fixed factors). For the ex vivo experiments in study 3 (in which serial rings from the same vessel were incubated with 0 or 5 μmol/L atorvastatin in the presence or absence of mevalonate or DAHP as stated), we first performed 1-way ANOVA for repeated measures to test whether the type of incubation (fixed factor) would affect superoxide generation or BH4 levels. Compound symmetry was assumed and sphericity was confirmed by the Mauchly test in all ANOVA models in study 3. When a significant overall P value was detected, individual paired comparisons were performed, and P values were corrected with the Bonferroni correction. The assumptions of normality and homogeneity of variance were met in all ANOVA models in the 3 studies.

Correlations between continuous variables were assessed by use of bivariate analysis. The Pearson coefficient was estimated. In study 1, we performed linear regression by using FMD or arterial O$_2^-$ as the dependent variable and the clinical demographic characteristics (age, sex, diabetes mellitus, active smoking dyslipidemia, hypertension, and body mass index) and medication (statins, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, calcium channel blockers, β-blockers, aspirin, clopidogrel, and diuretics) as independent variables that showed an association with the dependent variable in bivariate analysis at the level of 15%. A backward elimination procedure was then used by having P = 0.1 as threshold to remove a variable from the respective model. All statistical tests were performed with SPSS version 18.0. Values of P<0.05 were considered statistically significant.

Table 2. Multivariable Predictive Models of Flow-Mediated Dilation and Arterial Superoxide in Study 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Estimates of β (SE), P</th>
<th>Variable</th>
<th>Regression Estimates of β (SE), P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin treatment</td>
<td>1.3 (0.5), 0.01</td>
<td>Statin treatment</td>
<td>−0.4 (0.8), 0.0001</td>
</tr>
<tr>
<td>ACEi/ARB</td>
<td>2.7 (0.4), 0.001</td>
<td>Diabetes mellitus</td>
<td>0.2 (0.04), 0.002</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>−1.3 (0.5), 0.006</td>
<td>Hypertension</td>
<td>0.1 (0.05), 0.030</td>
</tr>
<tr>
<td>Hypertension</td>
<td>−1.8 (0.5), 0.0001</td>
<td>Active smoking</td>
<td>0.06 (0.03), 0.032</td>
</tr>
<tr>
<td>Active smoking</td>
<td>−1.7 (0.3), 0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FMD indicates flow-mediated dilation in the brachial artery; ACEi, angiotensin-converting enzyme inhibitor; and ARB, angiotensin receptor blockers.
In every analysis, we included only those patients without missing values. Patients with missing values were excluded from the respective analyses.

Results

The demographic characteristics of the study participants are presented in Table 1.

Associations Between Statin Therapy, Endothelial Function, and Vascular Superoxide (Study 1)

In study 1, we first examined whether statin treatment was associated with endothelial function and vascular $O_2^-\cdot$ generation in 492 patients undergoing CABG. In multivariable analysis, we observed that statin treatment was associated with FMD after controlling for risk factors and other medication (Table 2). We observed that those patients receiving statins had significantly greater FMD of the brachial artery in vivo and greater vasorelaxations of saphenous vein in response to acetylcholine ex vivo, findings that suggested improved endothelial NO bioavailability (Figure 1). There was no significant association between statin treatment and either endothelium-independent dilatation of the brachial artery to nitroglycerine or vasorelaxations of saphenous vein to sodium nitroprusside (data not shown). Furthermore, in multivariable analysis, statin treatment was associated with $O_2^-\cdot$ generation in IMA after controlling for risk factors and other medication, with patients receiving statins having significantly lower arterial $O_2^-\cdot$ generation compared with those not receiving statins (Figure 1). A similar effect of statin treatment on vascular $O_2^-\cdot$ was observed in saphenous vein segments obtained from these patients (data not shown).

However, this was an observational, nonrandomized study, and there was large heterogeneity in the type of statin treatment and the dosage administered.

Randomized, Placebo-Controlled Trial of Statin Treatment in Coronary Artery Bypass Graft Patients (Study 2)

Although the results of study 1 revealed associations between the use of statin therapy, vascular NO bioavailability, and

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Figure 1. In study 1 (n=492), patients receiving regular statin treatment, had significantly better flow-mediated dilation (FMD) compared with those not receiving statins (A). In saphenous veins obtained from 256 of these patients during coronary artery bypass graft surgery, ex vivo vasorelaxations in response to acetylcholine (ACh) were significantly greater in subjects receiving statins (n=212) compared with those not receiving statins (n=44; B). In internal mammary artery samples obtained from these patients, vascular $O_2^-\cdot$ was significantly lower in those receiving statin treatment compared with those not receiving statins (C). The statin treatments in this population were simvastatin (63.1%), atorvastatin (26.8%), rosuvastatin (4.3%), pravastatin (3%), lovastatin (1.6%), and fluvastatin (1%). Values expressed as median (25th to 75th percentile; A and C) or mean±SEM (B). *P<0.01 vs no statin treatment (P values were derived from unpaired t test of the log-transformed values for A and C and by 2-way ANOVA for repeated measures for B).

Figure 2. In study 2, there was no significant difference in the change in low-density lipoprotein (LDL) levels between patients who received atorvastatin (n=21) and those who received placebo (n=21; P=0.704; A). However, flow-mediated dilation (FMD) was improved in the atorvastatin-treated group compared with placebo (B). There was no significant difference (P=0.774) in the changes in endothelium-independent dilatation of the brachial artery in response to nitroglycerine (NTG) between the 2 groups (C). P values were derived by using 2-way ANOVA for repeated measures with time-by-treatment interaction on the log-transformed values. Values expressed as median (25th to 75th percentile).

In every analysis, we included only those patients without missing values. Patients with missing values were excluded from the respective analyses.
O$_2^·$ generation in human arteries, these findings did not imply causality, nor could they address statin effects independent of the systemic effects of chronic LDL reduction. Accordingly, we next conducted a double-blind, placebo-controlled clinical trial in which 42 statin-naïve patients were randomized to receive either atorvastatin 40 mg/d or placebo for 3 days before CABG (study 2). This short treatment period was chosen to test the vascular effects of atorvastatin before a significant lowering of LDL was observed. Indeed, after 3 days of treatment, there was no significant reduction of LDL in the atorvastatin-treated group compared with the placebo group (Figure 2). However, atorvastatin induced a significant improvement in FMD compared with placebo, with no change in the response to nitroglycerine (Figure 2). Importantly, there was no correlation between the change in LDL and the respective change in FMD in the study population (r=0.011, P=0.948), further supporting the notion that the effect of atorvastatin on FMD was independent of LDL lowering.

We next examined the effects of atorvastatin treatment on O$_2^·$ production from segments of IMA obtained at CABG. We observed that 3 days of atorvastatin treatment significantly reduced arterial O$_2^·$ compared with placebo (Figure 3). Importantly, vascular O$_2^·$ generation in IMAs from these patients was not correlated with the changes in LDL (r=0.224, P=0.226). To address the possible effects of atorvastatin on eNOS uncoupling, we measured L-NAME–inhibitable O$_2^·$ in these vessels. Treatment with atorvastatin reduced L-NAME–inhibitable O$_2^·$, suggesting an improvement in arterial eNOS coupling (Figure 3). Again, these changes were not correlated with the respective changes in LDL after 3 days of statin treatment (r=−0.269, P=0.150), implying a direct effect of statins on the redox state of human arteries in vivo.

To further investigate the mechanisms underlying the observed effects of atorvastatin on eNOS coupling, we quantified the levels of BH4 in both plasma and vessel tissue obtained at the time of CABG. We observed that the results of study 2 demonstrated that short-term oral treatment with atorvastatin 40 mg/d leads to a significant reduction of O$_2^·$ in human arteries through an
improvement of eNOS coupling, it was still unclear whether this effect was due to a direct impact on the vessel wall or to other systemic effects. Accordingly, we next tested the effects of atorvastatin in an ex vivo study in which paired segments of IMAs were incubated in the presence of either 0 or 5 μmol/L atorvastatin for 6 hours in an LDL-free environment. We observed that atorvastatin rapidly reduced $O_2^-$ generation (Figure 5). In particular, atorvastatin reversed L-NAME–inhibitable vascular $O_2^-$ in these arteries, suggesting that atorvastatin reduces vascular $O_2^-$ by improving enzymatic coupling of eNOS (Figure 5). The effects of atorvastatin on both vascular $O_2^-$ and L-NAME–delta ($O_2^-$) were reversed by the presence of mevalonate (Figure 5). These findings suggested that atorvastatin exerts its striking effect on vascular $O_2^-$ generation in human arteries independently of systemic LDL lowering by inhibiting HMG-CoA reductase directly in the arterial wall. Indeed, incubation with atorvastatin reduced vascular Rac1 activation, an effect that was reversed by mevalonate (Figure 5).

To estimate the exact effects of atorvastatin on endothelium-derived $O_2^-$, we performed dihydroethidium staining in vessels from 8 patients in the ex vivo study (study 3; Figure 6). We observed that atorvastatin reduced endothelium-derived $O_2^-$ and that this effect was reversed by mevalonate (Figure 6). In addition, L-NAME reduced endothelium-derived $O_2^-$ in control vessels and increased endothelium-derived $O_2^-$ in vessels incubated with atorvastatin. This effect of atorvastatin was reversed by mevalonate (Figure 6).

To further explore the mechanisms by which atorvastatin modifies eNOS coupling in human vessels, we quantified the changes in vascular biopterin bioavailability. In keeping with the results from study 2, we observed that after 6 hours of incubation, atorvastatin induced a significant elevation of vascular tissue BH4 and total biopterins (but not the ratio of BH4 to BH2 or BH4 to total biopterins). Furthermore, we observed a significant upregulation of GCH1 gene expression encoding GTPCH I, measured by quantitative reverse-transcription polymerase chain reaction (Figure 7). We also observed that the atorvastatin-induced elevation of vascular biopterins in our ex vivo model was prevented in the presence of the GTPCH I inhibitor DAHP, suggesting that the effect of atorvastatin on vascular biopterins is mediated through GTPCH I activation (Figure 8).

**Discussion**

In this study, we first demonstrate that statin treatment is associated with reduced arterial $O_2^-$ generation and improved endothelial function in patients with atherosclerosis. Moreover, we demonstrate for the first time in humans a direct pleiotropic effect of atorvastatin on the human arterial wall by showing that atorvastatin treatment rapidly reduces vascular $O_2^-$ generation in the human arterial wall and improves endothelial function within 3 days without any significant change in LDL cholesterol. We further demonstrate that these beneficial direct effects of atorvastatin on the human vascular wall, causing direct inhibition of vascular HMG CoA reductase and the mevalonate pathway, could be mediated by improved eNOS coupling through increased BH4 bioavailability. These novel findings provide important evidence indicating a direct effect of statin treatment on arterial eNOS function and redox signaling in human atherosclerosis.

**Statins and Pleiotropic Effects on Endothelial Function**

Inhibition of HMG-CoA reductase by statins has large beneficial impacts on cardiovascular risk. HMG-CoA reductase leads to the synthesis of mevalonate, which is then converted to fernesyl-PP. From this metabolite, the pathway leads either to the synthesis of cholesterol or to the synthesis

Figure 5. In study 3, ex vivo incubation of human mammary arteries with atorvastatin (5 μmol/L) induced a significant reduction of vascular superoxide ($O_2^-$). A) and reversed N-nitro-L-arginine methyl ester (L-NAME)–inhibitable $O_2^-$ (B) in these vessels (n = 10 patients). In a second set of experiments (C, n = 5 patients), the reduction of vascular $O_2^-$ by atorvastatin was reversed in the presence of mevalonate (P = 0.650 vs control after Bonferroni correction). In this experiment (C), L-NAME reduced vascular $O_2^-$ in the absence of atorvastatin and increased vascular $O_2^-$ in vessels preincubated with atorvastatin. Coincubation of these vessels with atorvastatin and mevalonate prevented the effect of atorvastatin on L-NAME–induced changes in vascular $O_2^-$ (P = 0.08 for L-NAME plus atorvastatin plus mevalonate vs L-NAME only). The direct effect of atorvastatin on vascular wall biology was also confirmed by demonstrating a change in vascular Rac1 activation in these vessels (C). – Indicates no atorvastatin or mevalonate; +, atorvastatin 5 μmol/L or mevalonate 200 μmol/L. Values are expressed as median (25th to 75th percentile; A and B) or mean ± SEM (C). One-way repeated measures ANOVA in C revealed a significant effect of treatment in all readouts (P < 0.001). *P < 0.01 for individual comparisons vs control (no atorvastatin, no mevalonate and no L-NAME) after Bonferroni correction; †P < 0.01 for individual comparisons vs L-NAME alone (no atorvastatin, no mevalonate) after Bonferroni correction.
of geranylgeranyl-PP, which results in the activation of geranylgeranylated proteins such as Rho and others (ie, Rac1). These are molecules with a well-characterized role in atherogenesis. However, this information is based largely on animal and cell culture studies, and there is no direct evidence regarding the role of HMG-CoA reductase in human vessels.

Statins have systemic antioxidant effects and improve endothelial function by preventing the oxidation of NO and by upregulating eNOS in animal models. Recently, the first important evidence for a direct pleiotropic effect of statins in humans was shown at the level of cell signaling by studies on the Rho/Rho kinase pathway in human leukocytes after statin treatment. However, most of the mechanistic data implicating a direct pleiotropic effect of statins on the vascular wall are based on cell culture and animal models. Separating the effects of statin therapy exerted by LDL lowering from potential pleiotropic effects is impossible in observational studies or clinical trials because the magnitude of LDL lowering and other effects mediated by HMG CoA reductase inhibition are inextricably linked in individual patients.

An important study by Landmesser at al showed that statin treatment improved endothelial function to a significantly greater extent than ezetimibe despite a similar reduction of LDL. Although this study provided strong evidence for a pleiotropic effect of statins on the human vascular endothelium, the mechanisms mediating vascular pleiotropic effects of statins in humans remain unclear.

In the present study, we observed that statin treatment is a strong independent predictor of endothelial function in patients with advanced coronary atherosclerosis. To further investigate the association between statin treatment and endothelial function, we performed a randomized, double-blind, placebo-controlled clinical trial in which we observed that atorvastatin treatment for just 3 days improved endothelial function in the brachial artery in patients with atherosclerosis before any significant change in, and independently of, LDL cholesterol levels.

Statins and Oxidative Stress
Clinical evidence suggests that treatment with statins is associated with an early reduction of systemic oxidative stress, partly as a result of the reduction of NADPH oxidase activity in the arterial wall. Incubation of human umbilical vein endothelial cells with atorvastatin rapidly increased NO and reduced ONOO\textsuperscript{-} generation. In experimental diabetes mellitus, atorvastatin improved eNOS coupling by increasing...
the bioavailability of the eNOS cofactor BH4. This effect was proposed to be due to upregulation of GTPCH I, the rate-limiting enzyme in BH4 biosynthesis, in the vascular wall. Indeed, Hattori et al first reported that statins rapidly upregulate GCH1 gene expression in human umbilical vein endothelial cells, leading to increased BH4 bioavailability. Despite the previous reports on the impact of statins on redox state of endothelial cells in experimental animal models, it is not known whether these mechanisms regulate eNOS coupling in the human arterial wall. This is an important question not just for understanding the mechanism of action of statins in human atherosclerosis but also for validating the therapeutic potential of these mechanisms as targets for other novel agents. We now demonstrate for the first time in humans that short-term atorvastatin treatment (40 mg/d for 3 days) reduces vascular O2·− generation in human IMAs of patients with atherosclerosis. By using an ex vivo model of human IMAs, we also demonstrate that atorvastatin reduces arterial O2·− generation even in the absence of LDL. This effect was due largely to an improvement in eNOS coupling in these arteries, and it was reversed by mevalonate, as was confirmed by estimating the effects of atorvastatin and mevalonate on endothelium-derived O2·− in these vessels. These findings suggest that direct inhibition of HMG-CoA reductase in the arterial wall is an important mechanism by which statins exert their vascular effects.

To further investigate the underlying mechanisms by which atorvastatin improves eNOS coupling in the human arterial wall, we examined the impact of treatment on vascular BH4 bioavailability. Because BH4 is a critical cofactor of eNOS that is required to maintain eNOS enzymatic uncoupling in the vascular wall, we hypothesized that statin treatment exerts its effects on vascular redox by modifying the BH4-mediated eNOS coupling. Indeed, we observed that short-term atorvastatin treatment had a striking effect on BH4 and total biopterins (tBio), indicating that an effect on BH4 biosynthesis occurs in vivo. This finding was also confirmed in the ex vivo model of human arteries in which short-term incubation of IMAs with atorvastatin also increased vascular BH4 and total biopterins. This is due largely to increased biosynthesis of biopterins in the vascular wall, a conclusion supported not only by the increase of vascular total biopterins (including BH4, BH2, and biopterin) but also by the upregulation of GCH1 gene expression and increase in GTPCH activity in these arteries.

Despite previous studies suggesting that the ratio of BH4 to BH2 or BH4 to total biopterins may be important for eNOS biology in endothelial cells, atorvastatin treatment had no impact on either plasma or vascular ratios of BH4 to BH2 or
BH4 to total biopterins in our clinical study. In the vascular wall of patients with advanced coronary artery disease, these relationships appear to be much more complex. For example, the absolute vascular BH4 was a much stronger determinant of vascular endothelial function or $O_2^-$ generation than the ratio of BH4 to BH2 or BH4 to total biopterins, reflecting the biological complexity in human vessels (which comprise multiple cell types, not just endothelial cells) or technical limitations of biopterin analysis in retrieved clinical material.

In contrast to the effect of atorvastatin on vascular tissue biopterins, we observed that atorvastatin treatment for 3 days was associated with a modest but significant reduction in plasma biopterins. As we have previously documented, there is a discordance between plasma and vascular BH4 levels in human atherosclerosis. Plasma biopterins levels are driven by inflammatory mechanisms, and under conditions of systemic low-grade inflammation, which is observed in patients with atherosclerosis, plasma biopterins are inversely correlated with vascular biopterins. Therefore, the reduction of plasma biopterins after statin treatment is compatible with a systemic antiinflammatory effect of statins. Statin treatment is also known to increase eNOS expression in endothelial cell systemic antiinflammatory effect of statins. Statin treatment was associated with a modest but significant reduction in plasma biopterins, as we have previously documented, there is a discordance between plasma and vascular BH4 levels in human atherosclerosis.

Conclusions

We demonstrate for the first time in humans that statins have a direct impact on the arterial redox state and NO bioavailability by reducing uncoupled eNOS-derived $O_2^-$. This effect was accompanied by an increase in vascular BH4 bioavailability and upregulation of the GCH1 gene encoding GTPCH I. Our findings support the notion that these effects of atorvastatin on the vascular wall are independent of LDL lowering and result directly from inhibition of HMG-CoA reductase. These novel findings provide an additional mechanism by which statins exert their beneficial effects in atherosclerosis and identify eNOS coupling and vascular BH4 availability as rational therapeutic targets in vascular disease.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

Statin treatment reduces cardiovascular risk. Although statins exert their antiatherogenic effects mainly by reducing low-density lipoprotein, experimental studies demonstrated a number of pleiotropic effects directly on the vascular cells. However, mechanistic studies examining the pleiotropic effect of statins on endothelial nitric oxide (NO) bioavailability and the vascular redox state in human arteries are remarkably limited. In the present study, we first demonstrate that in a real-life population of 492 patients undergoing coronary artery bypass graft surgery, the use of statins was a predictor of both improved endothelial function and reduced vascular superoxide (O$_2^-$) in internal mammary arteries (IMAs). Next, in a randomized, clinical trial with 42 patients undergoing coronary artery bypass graft surgery, we demonstrated that 3 days of treatment with atorvastatin 40 mg/d before coronary artery bypass graft surgery improved endothelial function and reduced vascular O$_2^-$ in internal mammary arteries of these patients by improving endothelial NO synthase coupling with increased vascular levels of the endothelial NO synthase cofactor tetrahydrobiopterin. We then performed a number of mechanistic ex vivo experiments in which atorvastatin rapidly upregulated GTP-cyclohydrolase I gene expression in the arterial wall, increased GTP cyclohydrolase I activity, and stimulated the synthesis of vascular biopterins, resulting in an improvement of endothelial NO synthase coupling, a reduction of endothelium-derived vascular O$_2^-$, and an improvement in endothelial function in a low-density lipoprotein-free environment. These effects were due to a direct inhibition of hydroxymethylglutaryl-coenzyme A reductase in the vascular wall. Therefore, our study demonstrates for the first time in humans that high-dose treatment with atorvastatin rapidly modifies the vascular redox state and endothelial function via tetrahydrobiopterin-mediated endothelial NO synthase coupling, providing one of the first direct reports of a pleiotropic effect of statins on the human arterial endothelium.
Rapid, Direct Effects of Statin Treatment on Arterial Redox State and Nitric Oxide Bioavailability in Human Atherosclerosis via Tetrahydrobiopterin-Mediated Endothelial Nitric Oxide Synthase Coupling

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SUPPLEMENTAL MATERIAL

Rapid, Direct Effects of Statin Treatment on Arterial Redox State and Nitric Oxide Bioavailability in Human Atherosclerosis via Tetrahydrobiopterin-Mediated eNOS Coupling

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Supplemental Methods

Patients’ recruitment process

The patients’ recruitment flowchart in the 3 studies is presented below:

**Study 1**

886 patients scheduled for elective CABG screened

610 met the criteria

492 agreed to participate

**Study 2**

332 patients scheduled for elective CABG screened

66 met the criteria

42 agreed to participate

**Study 3**

54 patients scheduled for elective CABG screened

36 met the criteria

26 agreed to participate

**Online figure 1**: Flow chart of patients’ recruitment in the 3 studies.

**Assessment of Pre-operative Endothelial Function**

FMD and endothelium-independent vasodilatations of the brachial artery were measured as previously described, using a linear array transducer, Philips Sonos 5500 and automated offline analysis (Vascular Analyser, Medical Imaging Applications LLC). Briefly, brachial artery
diameter was recorded before, and sixty seconds after a five minutes forearm blood flow occlusion. A further measurement was made three minutes after a sublingual spray of glyceryl trinitrate (GTN, 200 µg). FMD and endothelium-independent dilatation of the brachial artery were defined as the %change in vessel diameter after forearm ischaemia or sublingual GTN, respectively.

**Vessel Harvesting**

Samples of IMA or SV were obtained at the time of CABG as we have described previously.3, 4 Briefly, samples of IMA or SV were transferred into oxygenated (95%O₂ / 5% CO₂) ice-cold Krebs Hensleit buffer. Excess adventitia was removed and the lumen was flushed gently using an insulin syringe to remove blood. Segments of SV were immediately transferred to the lab for the organ bath experiments (Study 1). Some segments of IMA were snap frozen in the operating room. Additional IMA segments of appropriate size were also transferred to the laboratory within 30 minutes, and they were either used immediately for luminometry experiments (Studies 1 and 2) or for the *ex vivo* experiments (Study 3).

**Vasomotor studies**

Endothelium-dependent and endothelium-independent relaxations were assessed in saphenous vein (SV) rings obtained at the time of CABG, using isometric tension studies.5, 6 The rationale for using SV segments to evaluate endothelial function was based on a) the technical advantages of this model system over IMAs b) The lack of
enough IMA tissue for organ bath studies and vascular redox state measurements) c) The use of FMD in the brachial artery as a measure of arterial endothelial function.

Briefly, vascular segments were transferred to the lab in ice-cold Krebs-Hensleit buffer within 30 minutes from harvesting, and were used immediately for the organ bath experiments. Vessels were equilibrated in the organ bath for 60 minutes, to achieve a resting tension of 3g. Contractile responses were tested by exposure to Krebs-Hensleit buffer containing potassium chloride (60mM). Four rings from each vessel were pre-contracted with phenylephrine (3x10^{-6}M), then endothelium-dependent relaxations were quantified using acetylcholine (ACh, 10^{-9}M to 10^{-5}M). Finally, relaxations to the endothelium-independent NO donor sodium nitroprusside (SNP, 10^{-10}M to 10^{-6}M), were evaluated in the presence of the NOS inhibitor L-NAME (100μM), as we have previously described.⁵ ⁶

**Determination of Vascular Superoxide Production**

Vascular O$_2^-$ production was measured in fresh segments of intact IMA using lucigenin (5 μmol/L)-enhanced chemiluminescence, as we have described previously.³ ⁷ Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 minutes in oxygenated (95% O$_2$/5% CO$_2$) Krebs-HEPES buffer (pH 7.4) at 37°C. As lucigenin-enhanced chemiluminescence favors redox cycling of lucigenin at high concentrations (250μM), we used low lucigenin concentration (5μMol/L) as suggested in the past.⁷ The contribution of uncoupled nitric oxide synthase (NOS) to vascular O$_2^-$ production was quantified as the change of O$_2^-$ from baseline, after 20 minutes of
incubation with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L).³

**Oxidative Fluorescent Microtopography**

In situ $O_2^-$ production was determined in vessel cryosections with the oxidative fluorescent dye dihydroethidium (DHE).⁵¹³ Serial rings of mammary arteries from 8 patients were incubated with/without atorvastatin 5μmol/L in the presence and absence of mevalonate (200μmol/L) for 6 hours, and the they were snap frozen in OCT. Cryosections (30μM) were incubated with DHE (2μmol/L for 5 minutes) in Kreps-Hepes buffer, with or without L-NAME (100μmol/L). Fluorescence images of the endothelium (x63, Zeiss LSM 510 META laser scanning confocal microscope) were obtained from each vessel quadrant. In each case, segments of vessel rings (with and without L-NAME) were analyzed in parallel with identical imaging parameters. DHE fluorescence was quantified by automated image analysis using Image-Pro Plus software (Media Cybernetics), while all analyses were performed in a blinded fashion.

**Ribonucleic acid (RNA) isolation and quantitative real time-polymerase chain reaction (qRT-PCR).**

Paired rings of IMA (incubated with 0 or 5 μmol/L atorvastatin for 6 hours in the *ex vivo* study) were initially lysed in Trizol reagent (Tri-Reagent, Sigma, St. Louis, Missouri), followed by RNA purification from the aqueous phase using the RNeasy Micro kit (Qiagen, Stanford, California). Ribonucleic acid was converted into complementary DNA (Superscript II reverse transcriptase, Invitrogen, Carlsbad, California), then subjected to
qPCR using the TaqMan system (Applied Biosystems, Foster City, California; Assay ID GCH _ Hs00609198_m1, Assay ID GAPDH _ Hs02758991_g1) and analyzed on an iCyclerIQ (Biorad, Hercules, California). Relative expression was calculated using the $2^{-ΔΔT}$ method.

**Measurement of Vascular Rac1 Activation**

Rac1 activation was evaluated by an affinity precipitation assay using the PAK1-PBD conjugated glutathione agarose beads according to the manufacturer’s instructions (Millipore, Temecula, USA). Briefly, human IMA samples were homogenized on ice in 500 μL of lysis buffer. Pull-down of GTP-bound Rac1 was performed by incubating tissue lysates (100 μg) with GST fusion protein corresponding to the p21-binding domain of PAK-1 bound to glutathione-agarose for 1 h at 4 °C. The beads were washed three times with lysis buffer, and the protein bound to the beads was eluted with Laemmli buffer and analyzed for the amount of GTP-bound Rac1 by immunoblotting using a Rac1 monoclonal antibody. Rac1 activation was defined by the ratio GTP-Rac1/total Rac1, as appropriate.

**Blood Sampling and Serum Lipid Measurements**

Fasting venous blood samples were taken after 12 hours of fasting, on the morning of the operation (Studies 1 and 3), or both at baseline and at the morning of the operation (Study 2). After centrifugation at 2000 g at 4°C for 15 min, plasma or serum was collected and stored at −80 °C until assayed. Serum total cholesterol, LDL, HDL and
triglycerides were measured by using a chromatographic enzymatic method in a Technicon automatic analyzer RA-1000 (Dade Behring, Marburg, Germany).

Supplemental References