Increased Bioavailability of Nitric Oxide After Lipid-Lowering Therapy in Hypercholesterolemic Patients

A Randomized, Placebo-Controlled, Double-blind Study

Stefan John, MD; Markus Schlaich, MD; Matthias Langenfeld, MD; Horst Weihprecht, MD; Gerd Schmitz, MD; Gottfried Weidinger, MD; Roland E. Schmieder, MD

Background—Impaired endothelium-dependent vasodilation is an early sign of atherosclerosis in hypercholesterolemic patients. We hypothesized that lipid-lowering therapy can improve endothelial function and that this effect is mainly mediated by increased bioavailability of nitric oxide (NO).

Methods and Results—In a randomized, double-blind, placebo-controlled trial, we studied 29 patients (age, 50±12 years) with hypercholesterolemia (LDL cholesterol ≥160 mg/dL) randomly assigned to receive either fluvastatin (40 mg twice daily; 17 patients) or placebo (12 patients). Forearm blood flow was measured by plethysmography before and after 24 weeks of treatment. Endothelium-dependent vasodilation was assessed by intra-arterial infusion of acetylcholine (ACh; 3, 12, 24, and 48 μg/min) and basal NO synthesis rate by intra-arterial infusion of Nω-monomethyl-L-arginine (L-NMMA; 1, 2, and 4 μmol/min). Simultaneous intra-arterial infusion of L-NMMA (4 μmol/min) and ACh (12, 24, and 48 μg/min) was used to test whether any increase in endothelium-dependent vasodilation after lipid-lowering therapy could be blocked by this NO synthase inhibitor. Endothelium-dependent vasodilation improved significantly after 24 weeks of lipid-lowering therapy compared with before therapy (ACh 24 μg/min: 240±34% before versus 347±50% after therapy; P≤0.01) and placebo (changes between after and before therapy with ACh 24 μg/min: 108±39% for fluvastatin versus −26±32% for placebo; P≤0.05). This improvement in endothelium-dependent vasodilation could be blocked by simultaneous administration of L-NMMA (ACh 24 μg/min plus L-NMMA 4 μmol/min: 170±69% before versus 219±47% after treatment; P=NS).

Conclusions—Lipid-lowering therapy with fluvastatin can improve disturbed endothelial function in hypercholesterolemic patients compared with placebo. This improvement is mediated by increased bioavailability of NO. (Circulation. 1998;98:211-216.)

Key Words: atherosclerosis ■ endothelium ■ blood flow ■ hypercholesterolemia ■ nitric oxide ■ vasodilation

The endothelium plays a major role in determining vascular tone through the production and release of different vasoconstrictor and vasodilator substances that control the activity of the underlying smooth muscle layer.1 The most important endothelium-derived vasodilator substance is nitric oxide (NO), which helps to prevent atherosclerosis by maintaining vasodilation and inhibiting platelet aggregation, leukocyte adhesion, and proliferation of smooth muscle cells.2 Impaired endothelial function appears to be an early sign of atherosclerosis, appearing long before the formation of atherosclerotic lesions.3 Recent studies have confirmed that endothelium-dependent vasodilation is impaired in hypercholesterolemic patients4–6 and in patients with other cardiovascular risk factors.5,6 Hypercholesterolemic patients seem to have a defect in the bioavailability of NO that may explain their impaired endothelium-dependent vasodilation, and there is increasing evidence for a central role of the L-arginine/NO pathway in the pathogenesis of atherosclerosis in hypercholesterolemia.8

Hypercholesterolemia is a severe risk factor for atherosclerosis and cardiovascular morbidity and mortality.9 Recent studies have shown that cholesterol-lowering therapy improves endothelium-dependent vasodilation in coronary arteries and that this effect may explain the reduced incidence of adverse coronary events that is known to result from cholesterol-lowering therapy.10,11 However, whether this improvement of endothelium-dependent coronary vasorelaxation is mediated by an increased bioavailability of NO has never been demonstrated, mainly because of the potential risk of an intra-arterial infusion of NO–blocking agents into the coronary circulation in hypercholesterolemic patients. A close relationship between endothelium-dependent vasomotor responses of the
coronary arteries to acetylcholine and flow-mediated vasodilation in the brachial artery has been shown. Stroes et al demonstrated in a small series of hypercholesterolemic patients that lipid-lowering therapy reverses disturbed endothelium-dependent vasorelaxation in peripheral arteries and that endothelial dysfunction returns rapidly when hypercholesterolemia is restored.

The first aim of the present study was to determine whether lipid-lowering therapy can improve endothelial function of the forearm vasculature in hypercholesterolemic patients in a larger placebo-controlled, randomized, double-blind study design. The second objective was to investigate in this readily accessible vascular bed whether the expected improvement in endothelial function is mediated by increased bioavailability of NO.

**Methods**

**Study Population**

Patients were eligible for the study if they were between 18 and 70 years of age, had a history of polygenic hypercholesterolemia, had a serum LDL cholesterol level \( \geq 160 \text{ mg/dL} \) and a serum triglyceride level \( \leq 300 \text{ mg/dL} \), and were not taking cholesterol-lowering medication. Exclusion criteria were as follows: pregnant or lactating women; familial hypercholesterolemia; secondary hyperlipoproteinemia; vascular abnormalities in the forearm vasculature; diabetes mellitus; liver or kidney disease (aspartate aminotransferase and alanine aminotransferase levels \( >120\% \) of upper normal limit; alkaline phosphatase, bilirubin, and serum creatinine \( >150\% \) of upper normal limit); history of myocardial infarction; unstable angina; congestive heart failure categorized as NYHA class III or IV; history or clinical signs of peripheral artery disease; inadequately controlled arterial hypertension (diastolic blood pressure \( \geq 95 \text{ mm Hg} \) or systolic blood pressure \( \geq 160 \text{ mm Hg} \)) or treatment with \( >1 \) blood pressure–lowering agent; therapy with calcium antagonists, ACE inhibitors, or \( \beta \)-blocking agents; use of lipid-lowering medication; and use of steroids, immunosuppressive agents, erythromycin, or nonsteroidal anti-inflammatory drugs.

Twenty-nine patients were enrolled in this randomized, double-blind, placebo-controlled trial. They were randomly assigned by a randomization list in a 2:1 fashion to the treatment (n = 17) or placebo (n = 12) group.

Two patients, 1 in the placebo group and 1 in the fluvastatin group, did not complete the study (withdrawal of consent). Thus, 16 patients in the fluvastatin group and 11 in the placebo group could be statistically analyzed after conclusion of the study. Baseline characteristics of these patients were not different between the treatment and placebo groups (Table 1).

Moreover, 30 healthy subjects, all nonsmokers, with normal total cholesterol (177±30 mg/dL) and LDL cholesterol (92±23 mg/dL) levels, normal blood pressure, and normal fasting blood sugar were examined at baseline and served as a control group to the baseline examination of the hypercholesterolemic patients.

**Study Design**

The study was approved by the ethics committee of the University of Erlangen-Nürnberg. Written informed consent was obtained from patients before they entered the study. In the first phase (weeks 4 to 0), inclusion and exclusion criteria, baseline laboratory values, and baseline vasomotor responses were evaluated. During this time period, all patients received dietary instructions from a registered dietician according to the European Atherosclerosis Society guidelines. The subsequent treatment phase (weeks 0 to 24) lasted 6 months.

**Assessment of Forearm Blood Flow**

Forearm blood flow and responses to different vasoactive drugs were assessed by forearm plethysmography at baseline and again after

<table>
<thead>
<tr>
<th>TABLE 1. Baseline Characteristics and Lipid Profiles of Hypercholesterolemic Patients (Mean±SD, n=29) in the 2 Treatment Groups</th>
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<tr>
<td>Sex, m/f</td>
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<td>Age, y</td>
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<tr>
<td>Height, m</td>
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<tr>
<td>Weight, kg</td>
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<td>Body mass index, kg/m²</td>
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<td>Body surface area, m²</td>
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<td>Blood pressure, mm Hg</td>
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<tr>
<td>Systolic</td>
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<td>Diastolic</td>
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<tr>
<td>Smoker, yes/no</td>
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<tr>
<td>Hypertensives, yes/no</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<td>HDL cholesterol, mg/dL</td>
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<td>LDL cholesterol, mg/dL</td>
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<td>Triglycerides, mg/dL</td>
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<td>Thrombomodulin, mg/dL</td>
</tr>
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<td>Fibrinogen, mg/dL</td>
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<tr>
<td>Apoprotein B, mg/dL</td>
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<tr>
<td>Apoprotein A1, mg/dL</td>
</tr>
</tbody>
</table>

\( P=NS \) for all comparisons.

Treatment. An intra-arterial line was inserted into the brachial artery of the left arm, then subjects rested for 30 minutes before the study was begun. Forearm vascular responsiveness to vasoactive agents was assessed by venous occlusion plethysmography (EC 5R plethysmography, Hokanson). Drugs were given via intra-arterial infusion at the rate of 2 mL/min. The following substances were administered (each dose was infused for 5 minutes): (1) acetylcholine, to assess endothelium-dependent vasodilation, at sequential doses of 3, 12, 24, and \( 48 \text{ mg/} \mu\text{g/min} \); (2) sodium nitroprusside, to test endothelium-independent vascular relaxation, at 200, 800, and 3200 \text{ mg/} \mu\text{g/min} ; (3) \( N,N,N,N\text{-tetramethyl-L-arginine} \) (L-NMMA), to test basal production and release of NO, at 1, 2, and \( 4 \text{ mg/} \mu\text{mol/min} \); and (4) simultaneous infusion of L-NMMA (4 \text{ mg/} \mu\text{mol/min} and acetylcholine (12, 24, and \( 48 \text{ mg/} \mu\text{g/min} \)) to test whether any improvement in endothelium-dependent vasodilatation could be blocked by this NO synthase inhibitor. The dose of L-NMMA used (4 \text{ mg/} \mu\text{mol/min} ) is supposed to be at the top of the dose-response curve, to ensure almost complete inhibition of NO synthesis. Before each intervention, saline was infused for 15 minutes to enable forearm blood flow to return to resting levels. Baseline forearm blood flow was obtained from an average of 3 measurements. No significant changes in blood pressure or heart rate were observed during drug administration, which confirmed the local administration of each drug.

**Statistical Analysis**

The differences between treatment groups in clinical characteristics, lipid profiles, and changes of forearm blood flow were analyzed by use of the Student's t test. Vascular reactivity data are expressed as the percent change±SE from the corresponding baseline. Multivariate ANOVA (MANOVA) for repeated measurements was applied to test differences in dose-response curves between groups and treat-
TABLE 2. Comparison of Changes in Lipid Profiles After 24-Week Treatment Period (Mean±SD, n=27) With Fluvastatin or Placebo

<table>
<thead>
<tr>
<th>Lipid Profile</th>
<th>Fluvastatin (n=16)</th>
<th>Placebo (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (%)</td>
<td>−61±62</td>
<td>8±50</td>
<td>0.007</td>
</tr>
<tr>
<td>HDL cholesterol (%)</td>
<td>14±10</td>
<td>9±10</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol (%)</td>
<td>−75±75</td>
<td>−3±45</td>
<td>0.015</td>
</tr>
<tr>
<td>Triglycerides (%)</td>
<td>−12±118</td>
<td>23±130</td>
<td>NS</td>
</tr>
<tr>
<td>Thrombomodulin (%)</td>
<td>−2.2±8.6</td>
<td>−1.8±5.5</td>
<td>NS</td>
</tr>
<tr>
<td>Fibrinogen (%)</td>
<td>18±84</td>
<td>14±73</td>
<td>NS</td>
</tr>
<tr>
<td>Apolipoprotein B (%)</td>
<td>−35±26</td>
<td>−1±19</td>
<td>0.001</td>
</tr>
<tr>
<td>Apolipoprotein A1 (%)</td>
<td>4.8±18.2</td>
<td>3.5±14.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Comparisons by Student's t test. All values are mg/dL.

Results

Changes in Lipid Profiles

After 24 weeks of lipid-lowering therapy with fluvastatin, there was a significant decrease in the treatment group in total cholesterol levels (282±45 versus 221±45 mg/dL; \( P = 0.003 \)), LDL cholesterol (224±56 versus 149±50 mg/dL; \( P = 0.005 \)), and apolipoprotein B levels (142±38 versus 107±25 mg/dL; \( P = 0.001 \)). HDL cholesterol increased significantly (33.9±12.5 versus 48.0±11.3 mg/dL; \( P = 0.001 \)). There were no significant changes in lipid profiles in the placebo group. When changes in lipid profiles were compared between the fluvastatin group and the placebo group after the treatment period, we observed a significant difference in total cholesterol level changes (\( P = 0.007 \)), LDL cholesterol level changes (\( P = 0.015 \)), and apolipoprotein B changes (\( P = 0.001 \)) (Table 2).

Forearm Blood Flow Responses to Acetylcholine

In the healthy control group, baseline forearm blood flow was 4.29±0.24 mL·min\(^{-1}\)·100 mL\(^{-1}\), whereas it was 4.39±0.24 mL·min\(^{-1}\)·100 mL\(^{-1}\) in the total group of hypercholesterolemic patients. Intra-arterial administration of acetylcholine caused a significant increase in forearm blood flow in both groups but with significantly lower blood flow responses (MANOVA \( P < 0.02 \)) in hypercholesterolemic patients for all doses of acetylcholine (Figure 1).

Baseline forearm blood flows in the fluvastatin group and the placebo group before and after the treatment period are shown in Table 3 (differences between all groups were not significant). Intra-arterial administration of acetylcholine caused a significant increase in forearm blood flow with increasing doses in the placebo and treatment groups before and after therapy (MANOVA \( P < 0.001 \)).

In the fluvastatin group, the acetylcholine-induced increase in forearm blood flow was significantly enhanced after 24 weeks of lipid-lowering therapy compared with baseline evaluation (MANOVA \( P < 0.05 \)). In contrast, in the placebo group, no significant changes of forearm blood flow could be demonstrated after the 24-week treatment period (MANOVA \( P = NS \)). Figure 2 (left side) shows dose-response curves to acetylcholine and the subsequent increases in forearm blood flow in all 4 groups (fluvastatin before and after and placebo before and after treatment).

Table 3 shows absolute values of forearm blood flow, and Figure 2 shows percent changes (±SE) from baseline for the different doses of acetylcholine before and after medication in the fluvastatin and placebo groups, as well as corresponding \( P \) values. Again, a significant difference in the vasodilator response to acetylcholine could be demonstrated between before and after therapy in the fluvastatin group for each single dose of acetylcholine. In contrast, we found no differences before and after therapy in the placebo group.

To further analyze the effects in the fluvastatin group versus those in the placebo group, we subtracted the percent change of forearm blood flow from baseline in response to acetylcholine after therapy from that before therapy. Again, fluvastatin significantly improved the vasodilator response compared with placebo at doses of 3 μg/min (42±13% versus 4±13%; \( P < 0.05 \)) and 24 μg/min (108±39% versus 26±32%; \( P \leq 0.05 \)), with a trend toward significance at doses of 12 and 48 μg/min (\( P = 0.1 \)).

A significant correlation (\( r = -0.25, P < 0.05 \)) was found between plasma cholesterol levels and maximum response to acetylcholine before therapy in all subjects examined (Figure 3). Low plasma cholesterol levels induced high vasodilator responses and vice versa. In patients with high LDL cholesterol, there was a significant relation between the maximum percentage increase in blood flow in response to acetylcholine and the decrease in serum cholesterol levels after 24 weeks of lipid-lowering therapy (\( r = -0.49, P < 0.02 \); Figure 4). The more cholesterol decreased, the more endothelium-dependent forearm blood flow increased. Finally, we found a significantly higher increase in forearm blood flow after lipid-lowering therapy in those patients who had lower acetylcholine-induced vasodilatory responses at baseline (\( r = -0.43, P < 0.03 \)).

Forearm Blood Flow Responses to Nitroprusside and L-NMMA

Administration of the endothelium-independent vasodilator sodium nitroprusside caused similar increases in forearm blood flow in the placebo group before and after therapy as
well as in the fluvastatin group before and after therapy (MANOVA P<0.001). In addition, no differences could be found between the fluvastatin and placebo groups before and after the treatment period, respectively (Table 3).

After infusion of the NO synthase inhibitor L-NMMA, forearm blood flow decreased progressively with increasing doses (MANOVA P<0.001) but did not significantly differ between the fluvastatin and placebo groups before and after treatment for all doses of L-NMMA given. Baseline blood flows and changes from baseline in all 4 groups and for the 3 subsequent doses of L-NMMA are given in Table 3.

**Forearm Blood Flow Responses to Acetylcholine With Simultaneous L-NMMA Infusion**

Before the simultaneous intra-arterial infusion of L-NMMA (4 μmol/min) and 3 increasing doses of acetylcholine, baseline forearm blood flow before and after treatment with fluvastatin was 5.09±0.33 and 5.79±0.58 mL·min⁻¹·100 mL⁻¹, respectively (P=NS). The significant improvement in acetylcholine-induced vasodilation after treatment with fluvastatin (see above) was no longer observed if L-NMMA was coinfused (MANOVA P=NS; Figure 2, right side). Increases in forearm blood flow from baseline before and after treatment with fluvastatin, respectively, were 244±46% and 195±53% with acetylcholine 12 μg/min, 170±61% and 222±53% with acetylcholine 24 μg/min, and 317±62% and 351±95% with acetylcholine 48 μg/min (all differences not significant). Figure 5 shows these results together with the results of acetylcholine-induced vasodilation without coinfusion of L-NMMA for the 3 doses of acetylcholine given. In the placebo group, we observed no differences in vasodilator responses to acetylcholine before and after treatment if L-NMMA was coinfused (data not given).

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Left, Increases in forearm blood flow for different doses of intra-arterial acetylcholine as a measure of endothelium-dependent vasodilation. ○, Fluvastatin group before therapy; ●, fluvastatin group after therapy; □, placebo group before therapy; ■, placebo group after therapy. *P<0.05 and **P<0.01 after versus before treatment in the fluvastatin group. Right, The same vasodilator responses for the same groups with coinfusion of the NO synthesis inhibitor L-NMMA (4 μmol/min).
Discussion

In this placebo-controlled, randomized, double-blind trial, we have demonstrated that lipid-lowering therapy with fluvastatin can improve disturbed endothelium-dependent vasodilation in peripheral arteries in hypercholesterolemic patients. This improvement in endothelial function was not only significant compared with baseline endothelial function before treatment but also when compared with a placebo control group (Figure 2, left side). Moreover we have demonstrated that this improvement in endothelial function can be blocked almost completely by coinfusion of the NO synthase inhibitor L-NMMA (Figure 2, right side), indicating that an increase in bioavailability of NO mediates this improvement in endothelium-dependent vasodilation observed after treatment of elevated LDL cholesterol with fluvastatin.

The endothelium, as well as its pathway for NO, is an important modulator of vasodilation through the release of endothelium-derived NO. The integrity of this tissue is impaired in patients with atherosclerosis or hypercholesterolemia, presumably because of direct injurious effects of elevated levels of LDL cholesterol on the endothelium. Lipid-lowering therapy has been shown not only to improve endothelial function but also to precede structural regression of atherosclerotic lesions. Lipid-lowering therapy that is suggested by our data could be explained by an increased synthesis of NO by less-injured endothelial cells.

Moreover, hypercholesterolemia has been suggested to stimulate the generation of superoxide radicals by the endothelium. Superoxide directly inactivates NO and may also increase the subsequent oxidation of LDL particles by the formation of peroxynitrite. A reduction in serum cholesterol is associated with the normalization of oxygen-derived free radical production. The antioxidant vitamin C consistently improves impaired endothelial function in non–insulin-dependent diabetics by diminishing NO inactivation by oxygen-derived free radicals. Thus, a decrease of free radical production and consecutively less degradation together with an increased synthesis of NO could explain the observed improvement in the bioavailability of NO and thus in endothelium-dependent vasodilation in our patients.

Previous trials of cholesterol-lowering therapy have demonstrated beneficial effects on the coronary endothelium and coronary vasomotion in patients with coronary artery disease. Those findings in coronary arteries are consistent with our results in peripheral arteries. It has been suggested that this improvement of endothelium-dependent vasodilation is mediated by an increased bioavailability of NO, but this has never been demonstrated, mainly because of the potential risk of an intra-arterial infusion of NO synthase inhibitors into the coronary arteries, with its subsequent vasoconstriction, which might be harmful in patients with hypercholesterolemia and possible coronary artery disease. A close relationship between coronary artery endothelium-dependent vasomotor responses to acetylcholine and flow-mediated vasodilation in the brachial artery has been shown recently, suggesting that
our results demonstrating an increased bioavailability of NO may also be true for the coronary circulation.

Endothelium-dependent vasodilation was significantly impaired in our hypercholesterolemic patients compared with our healthy control subjects (Figure 1), as was also shown in previous studies. Improvement of lipid profiles resulted in a significant improvement in acetylcholine-mediated, endothelium-dependent vasodilation of the forearm vasculature compared with baseline values before lipid-lowering therapy. This improvement exceeded 80% of the maximal possible vasodilation in our healthy control group. These results confirm previous findings by other investigators. Moreover, our findings were controlled by a placebo group in a double-blind, randomized fashion, ruling out any effect on endothelial function other than the lipid-lowering effect of fluvastatin.

In all hypercholesterolemic patients, endothelium-dependent vasodilation was related to serum LDL cholesterol levels (Figure 3). Higher cholesterol concentrations impair endothelial function more than lower concentrations. Moreover, we could demonstrate a relation between the decrease in cholesterol and the improvement in endothelial function after lipid-lowering therapy (Figure 4) and also could demonstrate that those patients who had the worst endothelial function at baseline had the greatest improvement after lipid-lowering therapy. Therefore, it seems that the more efficient lipid-lowering therapy is, the more endothelial function could recover.

No differences in endothelium-independent vasodilator responses in hypercholesterolemic patients were found before or after lipid-lowering therapy or in comparison with the placebo group. These results indicate that structural wall properties of the forearm vasculature remain unaffected by lipid-lowering therapy. There were also no significant differences in vasoconstrictor responses to L-NMMA in hypercholesterolemic patients before or after lipid-lowering therapy or in comparison with the placebo group. These results indicate that structural wall properties of the forearm vasculature remain unaffected by lipid-lowering therapy. Therefore, it seems that the more efficient lipid-lowering therapy is, the more endothelial function could recover.

This double-blind, randomized, placebo-controlled study has demonstrated that lipid-lowering therapy with fluvastatin can significantly improve endothelial function of the forearm vasculature compared with placebo. Increased bioavailability of NO seems to mediate this improvement.

Acknowledgment
We wish to thank Anja Friedrich (study nurse) for her tremendous help in performing the study and collecting the data.

References
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Circulation. 1998;98:211-216
doi: 10.1161/01.CIR.98.3.211

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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