High-Density Lipoprotein Attenuates Inflammation and Coagulation Response on Endotoxin Challenge in Humans


Objective—Low high-density lipoprotein (HDL) cholesterol is a strong independent cardiovascular risk factor, which has been attributed to its role in reverse cholesterol transport. Whereas HDL also has potent antiinflammatory effects, the relevance of this property remains to be established in humans. In the present study, we evaluated whether there is a relation between HDL and sensitivity toward a low-dose endotoxin challenge.

Methods and Results—Thirteen healthy men with genetically determined isolated low HDL cholesterol (averaging 0.7±0.1 mmol/L) and 14 age- and body weight-matched healthy men with normal/high HDL cholesterol levels (1.9±0.4 mmol/L) were challenged with low-dose endotoxin intravenously (1 ng/kg body weight). The incidence and severity of endotoxin-associated clinical symptoms was increased in the low HDL group. Accordingly, both the inflammatory response (tumor necrosis factor-α, IL-1β, IL-6, IL-8, and monocyte chemoattractant protein-1) as well as thrombin generation (prothrombin activation fragments F1+2) were significantly increased in the low HDL group on endotoxin challenge.

Conclusions—Low HDL in healthy males is associated with increased sensitivity toward inflammatory stimuli as reflected by enhanced inflammatory and coagulation responses on endotoxin challenge. These antiinflammatory effects of HDL in humans may lend further support to HDL-increasing interventions, particularly in proinflammatory conditions, such as acute coronary syndromes. (Arterioscler Thromb Vasc Biol. 2007;27:1153-1158.)

Key Words: cholesterol ■ coagulation ■ inflammation ■ lipoproteins ■ thrombosis

After the first report on the potential antiatherogenic effect of high-density lipoprotein (HDL) almost 60 years ago, HDL is now generally acknowledged as a potent antiatherogenic mediator. The impact of isolated low HDL cholesterol on atherogenesis was recently underscored by the finding that carotid intima-media thickness in patients with genetically determined low apolipoprotein (apo) A-I was comparable to that in patients with familial hypercholesterolemia. In line, HDL increasing drugs now are prime candidates for combined use with statins in high-risk subjects.

Traditionally, the protective effect of HDL was considered to be confined to its role in the reverse cholesterol transport pathway. However, recent evidence supports a wide array of antiatherogenic effects by HDL, comprising antioxidative, antithrombotic, and antiinflammatory effects. The latter has attracted special interest, because inflammation has been acknowledged to underlie atherosclerotic lesion formation. At the same time, HDL cholesterol consistently shows an inverse relation with systemic markers of inflammation. Interestingly, HDL-increasing compounds (eg, reconstituted HDL) have recently been shown to attenuate systemic inflammation in humans, as well as vessel wall inflammation in experimental animal models. However, it remains to be established whether HDL also exerts antiinflammatory effects in the human setting. In the present study, we evaluated the impact of plasma HDL cholesterol level on the sensitivity toward a low-dose endotoxin challenge in subjects with genetically determined low versus normal/high HDL cholesterol levels.

Patients and Methods

Study Participants

Study subjects were recruited from a study designed to identify genes that control HDL cholesterol levels. Healthy male subjects with plasma HDL cholesterol levels <10th percentile (low HDL group, n=13) and healthy male subjects with plasma HDL cholesterol levels >90th percentile (high HDL group, n=7) matched for age and sex were recruited from families in which an autosomal-dominant phenotypic trait for low or high HDL cholesterol was established in at least 3 first-degree relatives. Subjects in the low HDL group with known genetic causes for low HDL cholesterol were excluded from the study, including carriers of the apoA-I (L178P) mutation. Subjects with secondary dyslipidemias, such as familial combined hyperlipidemia, were excluded. We also excluded low HDL as part of the metabolic syndrome or secondary to hypertriglyceridemia.
Unaffected healthy male relatives with normal (40th to 60th percentile) HDL cholesterol levels, matched for age and gender, were also recruited from families included in the database (n=7). Because the primary objective was to evaluate increased sensitivity toward inflammatory challenge in individuals with low HDL cholesterol, data from subjects with normal and high HDL levels (n=14) were combined in the analyses.

Written informed consent was obtained from all subjects. The study protocol was approved by the institutional review board at the Academic Medical Center in Amsterdam. Subjects with cardiovascular disease and risk factors for cardiovascular disease such as impaired fasting glucose, diabetes mellitus, hypertension, hypercholesterolemia, hypertriglyceridemia, C-reactive protein (CRP) levels >5 mg/mL, elevated Lp(a), and smoking were excluded during the screening visit. Other exclusion criteria were a history of alcohol and/or drug abuse, vaccination in the previous 6 months, previous exposure to endotoxin experiments, use of medication such as lipid-modifying drugs (resins, statins, niacin, fibrates), nonsteroidal antiinflammatory drugs, paracetamol, and antioxidants. All study subjects were free from signs of acute infection or febrile illness during the month preceding the study. One subject in the low HDL group was excluded because he had elevated hepatic enzymes (>2-times upper limit of normal) and was suspect of alcohol abuse.

**Study Design**

Study participants were required to refrain from alcohol and caffeine-containing beverages at least 24 hours before the study. The incidence, time, and severity of clinical symptoms associated with endotoxemia were recorded as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. Other clinical parameters such as blood pressure, heart rate, and body temperature were also recorded. Carotid intima-media thickness measurements were performed at baseline as previously described.2

On the morning of the study day at 7:30 AM after an overnight fast, study participants were admitted to the research unit. At 7.45 AM a catheter was inserted in an antecubital vein of each arm. At 8.00 AM (time [t]=0), blood was drawn for baseline measurements. Subsequently, subjects received a bolus infusion of 1 mg/kg body weight of endotoxin (Escherichia coli lipopolysaccharide, catalog number 1235503, lot G2B274; United States Pharmacopeial Convention Inc, Rockville, Md) in the antecubital vein of the contralateral arm. Blood samples were collected at t=0, 1, 2.5, 4, 6, and 8 hours after endotoxin challenge. The next morning at 8:00 AM, 24 hours after endotoxin infusion, study participants returned after an overnight fast for final blood withdrawal.

**Biochemical Analysis**

Blood was collected in EDTA, citrate, and heparin anticoagulated aliquots, as well as serum tubes, which were kept on ice and centrifuged at 1600g for 15 minutes at 4°C, snap-frozen, and stored at −80°C until analysis. Plasma total cholesterol was measured with an enzymatic colorimetric procedure (CHOD-PAP; Boehringer Mannheim, Mannheim, Germany). HDL cholesterol was determined after precipitation of apoB-containing lipoproteins by MnCl₂. Low-density lipoprotein cholesterol was calculated using the Friedewald formula. ApoA-I and apoB were measured using Beckman reagents and array nephelometry (Beckman, Brea, Calif). Triglycerides were measured using an enzymatic colorimetric method using lipase, glycerol kinase, and glycerol-3-phosphate 3 oxidase. Hematology parameters were assessed by standard laboratory techniques. Baseline lipid measurements were repeated at least 3 times. First, general practitioners selected patients on the basis of HDL cholesterol values. Next, genetic field workers collected blood samples from these patients and first-degree relatives, and those were assayed for lipid profiles. Subsequently, eligible patients were invited for a screening visit. Finally, on the study day the research physician took baseline lipid measurements. All samples were obtained after a 12-hour overnight fast.

**Analysis of the Inflammatory Response**

CRP was measured by a high-sensitivity immunoturbimetric assay (Roche Diagnostics Corporation, Basel, Switzerland), while CRP levels in excess of 10 mg/L were assayed by immunonephelometry (P800 analyzer; Roche Diagnostic Corporation). Circulating levels of tumor necrosis factor-α, IL-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 were measured with the lumien method (Bioplex Human Cytokines 1×96 wells, catalog number X500000 FFS; Bio-Rad Laboratories Inc, Hercules, Calif). Lipopolysaccharide binding protein was measured with a commercially available enzyme-linked immunosorbent assay (Human lipopolysaccharide-binding protein ELISA, catalog number HK 315; Cell Sciences Inc, Canton, Mass).

**Paraoxonase-1 Activity**

Serum paraoxonase-1 activity was measured as previously described.7

**Analysis of the Procoagulant Response**

Coagulation activation was assessed by measuring plasma levels of prothrombin fragment 1+2 (F1+2) as a marker of in vivo thrombin generation (ELISA; Dade-Behring, Marburg GmbH, Germany). Enzyme-linked immunosorbent assays were used to measure markers of endogenous fibrinolysis, ie, plasma levels of tissue-type plasminogen activator (Asserachrom tPA; Diagnostic Stago, Asnieres-sur-Seine, France), plasminogen activator inhibitor-1 antigen (Monozyme, Charlottelund, Denmark), and the fibrin split product D-dimer (Asserachrom, D-Di; Diagnostic Stago, Asnieres-sur-Seine, France).

**Statistical Analysis**

Results are expressed as mean±standard deviation. Differences between the low HDL group versus the normal/high HDL group were tested by analysis of variance for repeated measures. Linear regression analysis was used to evaluate correlations between HDL cholesterol, as well as apoA-I levels versus inflammation parameters and coagulation parameters. The SPSS software package for Windows was used for statistical analysis (version 12.0; SPSS Inc, Chicago, Ill).

**Results**

**Baseline Characteristics**

The demographic and biochemical characteristics of study participants in the low HDL group and the normal/high HDL group are listed in Table 1. The 2 groups were carefully matched for age and body mass index, lipids, and lipoproteins, except for plasma HDL cholesterol and apoA-I levels.

**Clinical Symptoms**

Endotoxin infusion caused typical endotoxin-induced symptoms.8 Clinical symptoms such as backache, chills, headache, myalgia, nausea, and vomiting occurred frequently, earlier, and more intensive in the low HDL group (Table 2). In the low HDL group, heart rate increased from 69 beats per minute at baseline to 80 beats per minute at 4 hours versus 67 to 76 beats per minute in the normal/high HDL group (P=0.03, between groups). Blood pressure did not change throughout the experiment in both groups.

**Lipids and Lipoprotein Response**

Endotoxin challenge induced modest decreases in low-density lipoprotein cholesterol from 2.7±0.6 to 2.4±0.5 mmol/L (P<0.01) and from 2.7±0.8 to 2.4±0.9 mmol/L (P<0.01),
Inflammatory Response

Leukocyte Response

After endotoxin infusion, early leukocytopenia and monocytopenia was comparable between both groups. The subsequent increase in leukocytes and monocytes, however, was significantly higher in the low HDL group (Figure 1A and 1B). In addition, endotoxin infusion significantly increased the neutrophil response in the low HDL group (Figure 1C).

Cytokines

Baseline cytokine levels were similar in both groups. The effects on endotoxin infusion on each cytokine are shown in Figure 2. At 1 hour after infusion, tumor necrosis factor-α levels were significantly increased in the low HDL group compared with the normal/high HDL group (Figure 2A). A similar effect was noted for IL-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 levels at 2.5 hours after endotoxin infusion (Figure 2B to 2E).

Acute Phase Proteins

Although the groups had similar baseline high-sensitivity CRP levels (Table 1), CRP was significantly elevated in the low HDL group compared with the normal/high HDL group at 24 hours (43.2±6.5 mg/L versus 27.2±5.9 mg/L; *P<0.01). Baseline lipopolysaccharide binding protein levels were elevated in the low HDL group (15.8±6.0 μg/mL) as opposed to the normal/high HDL group (12.4±5.3 μg/mL; **P=0.03) and remained significantly elevated after endotoxin infusion through the 24-hour period (37.7±11.3 μg/mL versus 29.0±12.0 μg/mL; †P<0.01 respectively).

Paraoxonase-1 Activity

Serum paraoxonase-1 activity was similar at baseline in both groups and was unaffected by endotoxin challenge (data not shown).

Coagulation Response

Starting with similar prothrombin fragments (F1+2) in both groups, F1+2 levels were significantly increased in the low

### TABLE 1. Demographic and Biochemical Characteristics of the Study Subjects

<table>
<thead>
<tr>
<th></th>
<th>Low HDL Group, n=13</th>
<th>Normal/High HDL Group, n=14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>35.4±2.5</td>
<td>35.1±5.2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.9±1.4</td>
<td>24.7±1.5</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>125±6</td>
<td>125±9</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>36.6±0.8</td>
<td>36.7±0.7</td>
</tr>
<tr>
<td>Alcohol use (current), (%)</td>
<td>6/13 (46)</td>
<td>6/14 (43)</td>
</tr>
<tr>
<td>Smoking (previous), (%)</td>
<td>9/13 (69)</td>
<td>10/14 (71)</td>
</tr>
<tr>
<td>Family history of CVD, (%)</td>
<td>6/13 (46)</td>
<td>14/14 (7)</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>2.7±0.6</td>
<td>2.7±0.8</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.1±0.5</td>
<td>1.0±0.6</td>
</tr>
<tr>
<td>ApoA-I, g/L</td>
<td>1.0±0.2</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>ApoB, g/L</td>
<td>1.1±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>hsCRP, mg/L</td>
<td>1.4±1.1</td>
<td>1.3±1.4</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>5.3±0.5</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td>Carotid IMT, mm</td>
<td>0.57±0.07</td>
<td>0.52±0.06</td>
</tr>
</tbody>
</table>

apoA-I indicates apolipoprotein A-I; apoB, apolipoprotein B; BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; LDL, low-density lipoprotein.

Data are expressed as mean±SD.

*P=0.02 by χ² test.
†P=0.001 and ‡P<0.001 by independent Student t test.

### TABLE 2. Effect of HDL Levels on Endotoxin-Induced Clinical Symptoms

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Low HDL Group, n=13</th>
<th>Normal/High HDL Group, n=14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Backache</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.5±0.8</td>
<td>0.2±0.4</td>
</tr>
<tr>
<td></td>
<td>1.8±0.2</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>Chills</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.1±0.9</td>
<td>0.4±0.6</td>
</tr>
<tr>
<td></td>
<td>1.4±0.1</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fever†</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>38.3±0.9</td>
<td>37.8±0.8</td>
</tr>
<tr>
<td></td>
<td>3.5±1.1</td>
<td>4.1±2.0</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.43</td>
</tr>
<tr>
<td>Headache</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.4±0.9</td>
<td>0.5±0.7</td>
</tr>
<tr>
<td></td>
<td>2.0±0.3</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Myalgia</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.6±0.8</td>
<td>0.2±0.4</td>
</tr>
<tr>
<td></td>
<td>3.1±0.2</td>
<td>4.0±0.1</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nausea</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.1±0.8</td>
<td>0.4±0.6</td>
</tr>
<tr>
<td></td>
<td>2.9±0.3</td>
<td>2.9±0.3</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.78</td>
</tr>
<tr>
<td>vomiting</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.7±1.0</td>
<td>0.2±0.6</td>
</tr>
<tr>
<td></td>
<td>2.6±0.2</td>
<td>3.8±0.4</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total AE</td>
<td>55</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>29</td>
</tr>
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</tbody>
</table>

The total number of volunteers with a specific symptom, mean maximum severity (0, absent; 1, mild; 2, moderate; 3, severe) and time of maximum severity (hours, relative to endotoxin infusion) are summarized.

AE indicates adverse events.

Data are expressed as mean±SD. *P indicates difference between the mean maximum severity by independent Student t test between both groups.

†P indicates difference between the time of maximum severity by independent Student t test between both groups.

‡Mean severity for fever is denoted as temperature in °C. Fever is defined as a body temperature of >38°C.
HDL group at time points 4 and 6, but returned to baseline in both groups at 24.

Fibrinolysis
Fibrinolytic markers (D-dimer, tissue plasminogen activator, and plasminogen activator inhibitor-1) were similar between both groups at baseline and after endotoxin infusion, peaking at 2.5 hours after endotoxin infusion and returning to normal levels at 24 hours (data not shown).

Linear Regression Analysis
Linear regression analysis revealed a significant inverse relation between HDL cholesterol and apoA-I levels versus leukocytes (Figure 3A), proinflammatory cytokines (Figure 3B), and prothrombin fragments (data not shown).

Discussion
In the present study, we demonstrate that apparently healthy males with genetically determined isolated low HDL cholesterol levels are characterized by an increased sensitivity toward a low-dose endotoxin challenge compared with subjects with normal/high HDL cholesterol levels. Throughout the whole range of HDL cholesterol levels, there was an inverse relation between apoA-I levels and sensitivity toward this inflammatory challenge. These findings lend further support to the relevance of HDL as an antiinflammatory mediator in vivo.

Study Population
To evaluate the interaction between HDL and inflammation, we recruited participants from a study designed to identify genes that control HDL cholesterol levels, excluding subjects with known genetic causes for low HDL, such as apoA-I mutations. In line with a primary selection on low HDL cholesterol levels, difference in HDL cholesterol was more pronounced than the difference in apoA-I, implying the abundance of smaller HDL particles in the low HDL group. In the control group, both subjects with normal HDL cholesterol levels were included, as well as 7 subjects with genetically determined high HDL cholesterol levels. Importantly, the low and normal/high HDL groups were carefully matched for parameters known to affect HDL cholesterol levels or inflammatory state such as body mass index and smoking.

Clinical Parameters and Lipid Changes
Endotoxin-induced symptoms like backache, chills, body temperature increase, heart rate increase, headache, myalgia, and nausea were increased and noted more severe in the low HDL group compared with the normal/high HDL group. In line with these results, low HDL cholesterol level is associated with an increased mortality and severity of septic disease. The sequential changes in lipids and (apo)lipoproteins after endotoxin challenge were comparable between groups. Within the 24-hour observation period, neither HDL cholesterol nor apoA-I changed significantly on endotoxin challenge, which is in line with previous analyses using a 1-ng/kg body weight lipopolysaccharide infusion in healthy volunteers.

Inflammatory Response
Early leukocytopenia, monocyteopenia, and decreased neutrophil count were comparable between groups (Figure 1). The magnitude of the “early” leukocyte margination has been shown to closely reflect the dose of endotoxin infused. Hence, a similar degree of early leukocytopenia implies comparable exposure to endotoxin in both the low and normal/high HDL groups. In contrast, endotoxin challenge elicited augmented leukocytosis, monocytes, and increased neutrophil count in the low HDL group at later time points (Figure 1). Similarly, the increase of proinflammatory cytokines as well as acute phase reactants was also elevated in the low HDL group compared with the normal/high HDL group. Linear regression analysis revealed a strong inverse relation between HDL cholesterol and apoA-I levels versus leukocyte response, proinflammatory cytokines, and plasma CRP levels, supporting an antiinflammatory effect of HDL throughout a wide concentration range (Figure 3).
Mechanism of the Antiinflammatory Effects of HDL

Theoretically, the increased in vivo antiinflammatory effect of HDL in the normal/high HDL group could be adjudicated solely to increased scavenging of endotoxin, thereby minimizing the amount of endotoxin available to elicit inflammatory activation. However, several other facts must be taken into account. First, the scavenging of endotoxin by HDL does not equal HDL-mediated neutralization of endotoxin bioactivity. Endotoxin triggers the inflammatory cascade by binding of the TLR4 receptor on monocytes and endothelial cells, which occurs within seconds, whereas HDL requires several minutes to scavenge endotoxin within its lipid moiety. Even after endotoxin sequestration, HDL needs hours to fully neutralize the bioactivity of “trapped” endotoxin. Second, endotoxin elicits rapid sequestration of leukocytes and monocytes, the magnitude of which closely mirrors the dose of endotoxin exposure. Because leukocyte and monocyte margination were identical in low and normal/high HDL group, decreased exposure toward endotoxin is less likely. Combined, these data imply that HDL scavenging cannot fully account for the differences in inflammatory and coagulation responses observed in the low versus normal/high HDL group. In this respect, HDL may also have direct antiinflammatory effects, independently from endotoxin scavenging. First, the HDL particle harbors protective enzymes, such as paraoxonase-1, which has been shown to inhibit monocyte migration. However, in the present study, paraoxonase-1 activity was similar in both groups. Other moieties within HDL have also been implicated to exert antiinflammatory effects. Thus, apoA-I itself has a direct inhibitory effect on several proinflammatory loops. In fact, the potent, inverse relation between apoA-I and endotoxin-induced systemic response may highlight a role for apoA-I as antiinflammatory mediator in vivo.

Procoagulant Response

Endotoxin infusion resulted in a significantly larger increase in thrombin generation in the low HDL group as compared with the normal/high HDL group, implying that HDL may also attenuate coagulation activation. Mechanistically, HDL may attenuate coagulation either indirectly by modulating the inflammatory cascade via its associated proteins, but also by directly affecting the coagulation system. Previous in vitro studies have already shown that HDL enhances the activity of the important physiological anticoagulant protein C pathway. In line with these findings, population-based studies have shown an inverse relationship between HDL cholesterol levels and tissue factor pathway inhibitor levels. Furthermore, HDL have been shown to inhibit the expression of tissue factor by endothelial cells. An inverse relation between HDL and coagulation has been supported by clinical observations. Indeed, low levels of HDL have been associated with increased risk for venous thrombosis. HDL may also be entangled with arterial thrombosis in the setting of acute coronary syndromes.

Figure 2. Effect of inflammatory challenge on inflammation and coagulation activation. Tumor necrosis factor-α (A), IL-1β (B), IL-6 (C), IL-8 (D), monocyte chemoattractant protein-1 (E), and F1 Δ F2 (F) curves in the low HDL group (○) and the normal/high HDL group (□). Difference between time points by unpaired Student t test (P<0.05). Probability value indicates difference between the low HDL group vs the normal/high HDL group using analysis of variance repeated measures analysis. Data are expressed as means±standard deviation.
Summary
In the present study we provide evidence that endogenous HDL attenuates the inflammatory and coagulation response toward low-dose endotoxin challenge. These findings provide a further impetus for implementation of HDL-increasing strategies in the acute, often inflammatory, setting of myocardial infarction and acute coronary syndrome.25

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Disclosure
Professor John Kastelein is an established investigator of the Dutch Heart Foundation (2000D039). There is no conflict of interest for all authors listed on this manuscript. The authors have no financial disclosure to report in relation with this manuscript.

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