Association Between Cholesterol Efflux Capacity and Atherosclerotic Cardiovascular Disease in Patients With Familial Hypercholesterolemia

Masatsune Ogura, Mika Hori, Mariko Harada-Shiba

Objective—Patients with familial hypercholesterolemia (FH) are at high risk for premature atherosclerotic cardiovascular disease (ASCVD), especially because of long-term exposure to high low-density lipoprotein cholesterol levels. It has been reported that low-density lipoprotein–lowering therapy delays the onset of ASCVD. However, it still remains difficult to prevent it. Therefore, novel biomarkers and therapeutic targets are necessary to evaluate and prevent atherosclerosis in FH. The aim of this study was to investigate associations of cholesterol efflux capacity with the presence of ASCVD and clinical features in patients with heterozygous FH.

Approach and Results—We measured cholesterol efflux capacity in 227 patients with heterozygous FH under pharmaceutical treatment. Seventy-six (33.5%) of them were known to have ASCVD. In a logistic-regression analysis adjusted for risk factors, increased efflux capacity was associated with decreased risk of ASCVD even after the addition of high-density lipoprotein cholesterol level as a covariate (odds ratio per 1-SD increase, 0.95; 95% confidence interval, 0.90–0.99; \( P<0.05 \)). Decreased cholesterol efflux capacity was associated with the presence of corneal arcus after adjusting for age and sex. In addition, inverse relationships between cholesterol efflux capacity and Achilles tendon thickness, as well as carotid intima-media thickness, were observed after adjustment for age, sex, and traditional cardiovascular risk factors.

Conclusions—Cholesterol efflux capacity was independently and inversely associated with the presence of ASCVD in heterozygous FH. In view of residual risks after treatment with statins, cholesterol efflux capacity might be a novel biomarker and a therapeutic target for preventing atherosclerosis in patients with FH. (Arterioscler Thromb Vasc Biol. 2016;36:181-188. DOI: 10.1161/ATVBAHA.115.306665.)

Key Words: Achilles tendon • arcus senilis • cardiovascular diseases • carotid intima-media thickness • cholesterol, HDL • macrophages
been reported that the ability of HDL to promote cholesterol efflux from macrophages, the first step in the reverse cholesterol transport pathway, is inversely associated with risk for ASCVD even after adjusting for HDL-C, providing support for the importance of HDL functionality over the simple measurement of HDL-C levels.

To date, a few studies have helped clarify the mechanism of reverse cholesterol transport in patients with FH. Nenseter et al revealed that cholesterol efflux capacity in patients with homozygous FH was reduced when compared with healthy controls, and Bellanger et al demonstrated that large HDL2 particles isolated from 12 patients with FH displayed reduced cholesterol efflux capacity when compared with 12 healthy normolipidemic subjects. However, it had not been investigated whether cholesterol efflux capacity is a risk marker of ASCVD in patients with FH.

Accordingly, the present study was performed to investigate relationships between cholesterol efflux capacity and clinical features, including corneal arcus, Achilles tendon thickness, subclinical atherosclerosis, and presence of ASCVD, in patients with heterozygous FH under treatment.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**Study Population**

The characteristics of the 227 FH patients with or without ASCVD are shown in Table 1. Seventy-six (33.5%) patients among them were known to have ASCVD. ASCVD was considered to be present if subjects met one of the following criteria: (1) myocardial infarction proven by ECG abnormalities and enzyme changes; (2) symptomatic ischemic stroke identified by computed tomogram or magnetic resonance imaging; (3) diagnosis of angina pectoris with significant stenosis >75% on coronary angiogram; (4) coronary bypass surgery or percutaneous coronary interventions. There were 36 patients with myocardial infarction, 12 patients with symptomatic ischemic stroke, 33 patients with angina who had undergone either coronary bypass surgery or percutaneous coronary interventions, and 4 patients with coronary stenosis without any revascularization. Nine of the patients with ischemic stroke previously had coronary heart diseases.

Among the 227 patients with FH, 88 (38.8%) had loss-of-function mutation in LDL receptor, 7 (3.1%) had gain-of-function mutation in proprotein convertase subtilisin/kexin type 9 (PCSK9), 11 (4.8%) had both, 80 (35.2%) patients were genotype absent, 26 (11.5%) patients had not undergone genetic testing, and 15 (6.6%) patients were under examination. The genotype absent patients were clinically diagnosed as heterozygous FH using diagnostic criteria for adult heterozygous FH described in Japanese guidelines for the management of FH.

Patients with ASCVD were older and had, on average, higher body mass indexes, in addition to a higher prevalence of hypertension and diabetes mellitus, than those without ASCVD. There were many more patients with smoking history in the ASCVD group. Mean levels of HDL-C and ApoAI were lower in patients with ASCVD, whereas the median triglycerides level was significantly higher. Furthermore, prevalence of corneal arcus, mean Achilles tendon thickness, and mean carotid intima-media thickness (IMT) were greater in patients with ASCVD.

**Cholesterol Efflux Capacity in Patients With FH**

The mean values for cholesterol efflux capacity after normalization to the value of the pooled sample were 0.88±0.14 (range, 0.40–1.22) in patients with FH. Scatterplot analyses revealed significant correlations between cholesterol efflux capacity and levels of both HDL-C (r = 0.83; P<0.0001) and ApoAI (r = 0.83; P<0.0001; Figure I in the online-only Data Supplement). We conducted a linear regression with all risk factors to clarify correlations with cholesterol efflux capacity in aggregate. Probucol use, Log triglycerides, and having both LDL receptor and proprotein convertase subtilisin/kexin type 9 mutations were associated with reduced cholesterol efflux capacity, whereas genotype absence was related to increased cholesterol efflux capacity (Table 2, model 1). After the addition of the presence of corneal arcus, Achilles tendon thickness, mean carotid IMT, and the presence of ASCVD as covariates, probucol use, Log triglycerides, and the presence of ASCVD were associated with reduced cholesterol efflux capacity (Table 2, model 2).

**Association Between ASCVD and Cholesterol Efflux Capacity**

In patients with ASCVD, the mean cholesterol efflux capacity after normalization to the value of the pooled sample was significantly lower than in patients without ASCVD (0.80±0.14 versus 0.92±0.11, respectively; P<0.0001; Table 1). In a logistic-regression analysis adjusted for age, sex, and traditional cardiovascular risk factors (smoking history [current and past], the presence of hypertension, the presence of diabetes mellitus, the presence of obesity [body mass index, ≥25 kg/m²], LDL-C, and Log triglycerides), increased efflux capacity was associated with decreased risk of ASCVD even after the addition of HDL-C level as a covariate (odds ratio [OR] per 1-SD increase, 0.95; 95% confidence interval [CI], 0.90–0.99; P=0.0260; Table 3). The results were similar when the ApoAI level was substituted for the HDL-C level (OR per 1-SD increase, 0.95; 95% CI, 0.90–1.00; P=0.0470). Moreover, this relationship remained robust even after additional adjustment for probucol use (Table I in the online-only Data Supplement). Among known cardiovascular risk factors, the presence of hypertension was also associated with presence of ASCVD (Table 3).

**Association Between Corneal Arcus and Cholesterol Efflux Capacity**

Patients with corneal arcus had lower cholesterol efflux capacity than those without (0.84±0.15 versus 0.91±0.13;
respectively; \( P=0.0002 \); Table 4). Because it has been reported that the prevalence of corneal arcus increases with advancing age and is lower in women, we conducted the logistic analysis adjusting for age and sex. As a result, decreased cholesterol efflux capacity was associated with the presence of corneal arcus (OR per 1-SD increase, 0.98; 95%
for traditional cardiovascular risk factors, HDL-C, or ApoAI levels attenuated this relationship. In the logistic regression analysis, age (OR per 1-SD increase, 1.05; 95% CI, 1.02–1.08; P=0.0001), male sex (OR, 3.11; 95% CI, 1.33–7.52; P=0.0089), smoking history (OR, 2.78; 95% CI, 1.25–6.32; P=0.0124), and LDL-C levels (OR per 1-SD increase, 1.01; 95% CI, 1.00–1.02; P=0.0206) were associated with presence of corneal arcus.

**Association Between Achilles Tendon Thickness and Cholesterol Efflux Capacity**

Patient characteristics according to tertiles of Achilles tendon thickness are presented in Table 5. Cholesterol efflux capacity was inversely associated with Achilles tendon thickness (P<0.0001 for linear trend across tertiles). A significant inverse relationship between cholesterol efflux capacity and Achilles tendon thickness was observed even in multivariate analysis after adjustment for age, sex, and traditional cardiovascular risk factors. However, subsequent adjustment for HDL-C or ApoAI levels attenuated this relationship (Table III in the online-only Data Supplement). In the linear regression analysis, male sex (β=0.82; 95% CI, 0.01–1.63; P=0.0472), and LDL-C levels (β=0.02; 95% CI, 0.0006–0.04; P=0.0411) were associated with an increase in Achilles tendon thickness.

**Association Between Subclinical Atherosclerosis and Cholesterol Efflux Capacity**

Patient characteristics according to tertiles of mean carotid IMT (mean-IMT) are presented in Table 6. Cholesterol efflux capacity was inversely associated with mean-IMT (P<0.0001 for linear trend across tertiles). A significant inverse relationship between cholesterol efflux capacity and mean carotid IMT was observed even in multivariate analysis after adjustment for age, sex, and traditional cardiovascular risk factors. However, subsequent adjustment for HDL-C or ApoAI levels attenuated this relationship in analogy with Achilles tendon thickness (Table IV in the online-only Data Supplement). In the linear regression analysis, we found that ApoAI levels were independently and inversely associated with mean IMT (β=−0.005; 95% CI, −0.01 to −0.001; P=0.0157). In addition, age (β=0.18; 95% CI, 0.013–0.023; P<0.0001) and the presence of hypertension (β=0.08; 95% CI, 0.003–0.16; P=0.0412) were associated with mean carotid IMT.

**Discussion**

The main finding of the present study was that decreased cholesterol efflux capacity was associated with increased risk of ASCVD even in statin-treated patients with FH. In addition, we found that patients with corneal arcus had lower cholesterol efflux capacity after adjustment for age and sex. There was also an inverse association of cholesterol efflux capacity with Achilles tendon thickness as well one with carotid IMT after adjustment for age, sex, and traditional cardiovascular risk factors. However, these associations diminished after additional adjustment for HDL or ApoAI.

We and other researchers have reported that the presence of corneal arcus or tendon xanthomas is associated with higher risk for ASCVD in patients with FH,\(^9,24,25\) suggesting...
that corneal arcus, xanthomas, and atherosclerosis are related and have similar mechanisms of cholesterol accumulation. However, it had not been evident previously whether capacity for cholesterol removal from cornea and Achilles tendon xanthomas is associated with their presence and thickness. In the present study, we observed both reduced cholesterol efflux capacity in patients with corneal arcus and an inverse relationship between cholesterol efflux capacity and Achilles tendon thickness in patients with FH. Importantly, decreased cholesterol efflux capacity was associated with the presence of corneal arcus even after adjustment for age and sex. In addition, the inverse association between Achilles tendon thickness and cholesterol efflux capacity remained robust after adjustment for age, sex, and traditional cardiovascular risk factors. Although these relationships were attenuated after additional adjustment for HDL-C levels, we consider our finding to be clinically important and provide a novel insight into our current understanding of corneal arcus and Achilles tendon xanthomas.

We also demonstrated that there was an inverse relationship between cholesterol efflux capacity and mean carotid IMT in the 227 patients with FH. This reinforces the finding of a recent study that there was a significant inverse relationship between cholesterol efflux capacity and carotid IMT in 12 patients with FH. The relationship in our study was attenuated after additional adjustment for HDL-C or ApoAI levels. Furthermore, we found that ApoAI levels remained a strong predictor for max- and mean IMT. In this regard, Junyent et al. have reported that the HDL-C level and total cholesterol/HDL ratio are strong predictors of preclinical carotid atherosclerosis in patients with heterozygous FH. Therefore, our observations strengthen the evidence for the quantities of HDL and ApoAI being important risk markers for preclinical atherosclerosis. On the contrary, Doonan et al. have reported an inverse association between cholesterol efflux capacity and carotid stenosis after adjusting for HDL-C or ApoAI levels in patients with high-grade carotid stenosis. In their study, only modest correlations between cholesterol efflux capacity and HDL-C ($r = 0.27$) and ApoAI ($r = 0.361$) were observed, whereas we found significant relationships between cholesterol efflux capacity and both HDL-C ($r = 0.83$) and ApoAI ($r = 0.83$; Figure I in the online-only Data Supplement).

**Table 5. Characteristics of Study Subjects Presented as Tertiles of Achilles Tendon Thickness**

<table>
<thead>
<tr>
<th></th>
<th>ATT Tertile 1</th>
<th>ATT Tertile 2</th>
<th>ATT Tertile 3</th>
<th>P Value for Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATT cut-offs, mm</td>
<td>&lt;9.5</td>
<td>9.5–12.7</td>
<td>&gt;12.7</td>
<td></td>
</tr>
<tr>
<td>ATT, mm</td>
<td>8.0±1.0</td>
<td>10.9±1.0</td>
<td>17.3±4.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>n</td>
<td>74</td>
<td>76</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>56±17</td>
<td>52±17</td>
<td>63±14</td>
<td>0.0006</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>22 (29.7)</td>
<td>37 (48.7)</td>
<td>42 (54.5)</td>
<td>0.0067</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>20 (27.0)</td>
<td>23 (30.3)</td>
<td>39 (50.6)</td>
<td>0.0015</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>12 (16.2)</td>
<td>11 (14.5)</td>
<td>17 (22.1)</td>
<td>0.3467</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td>22 (29.7)</td>
<td>25 (32.9)</td>
<td>39 (50.6)</td>
<td>0.0112</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.0±3.3</td>
<td>22.8±3.4</td>
<td>22.8±3.3</td>
<td>0.2717</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>206±43</td>
<td>211±51</td>
<td>195±45</td>
<td>0.1176</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dL</td>
<td>129±38</td>
<td>137±47</td>
<td>132±33</td>
<td>0.4875</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dL</td>
<td>58±17</td>
<td>52±15</td>
<td>43±16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>86 (60–121)</td>
<td>95 (66–127)</td>
<td>85 (66–135)</td>
<td>0.3061</td>
</tr>
<tr>
<td>ApoAI, mg/dL</td>
<td>142±27</td>
<td>136±24</td>
<td>118±31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>89±23</td>
<td>97±27</td>
<td>96±21</td>
<td>0.1395</td>
</tr>
<tr>
<td>ApoE, mg/dL</td>
<td>3.8±1.1</td>
<td>3.9±1.4</td>
<td>3.9±1.0</td>
<td>0.9111</td>
</tr>
<tr>
<td>Cholesterol efflux capacity</td>
<td>0.93±0.13</td>
<td>0.88±0.12</td>
<td>0.81±0.16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Corneal arcus, n (%)</td>
<td>29 (39.2)</td>
<td>34 (44.7)</td>
<td>51 (66.2)</td>
<td>0.0026</td>
</tr>
<tr>
<td>Max IMT, mm</td>
<td>1.7±0.9</td>
<td>1.8±0.9</td>
<td>2.6±1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mean IMT, mm</td>
<td>0.9±0.5</td>
<td>1.0±0.5</td>
<td>1.4±0.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Presence of ASCVD, n (%)</td>
<td>13 (17.6)</td>
<td>22 (28.9)</td>
<td>41 (53.2)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

ASCVD indicates atherosclerotic cardiovascular disease; ATT, Achilles tendon thickness; BMI, body mass index; HDL, high-density lipoprotein; IMT, intima-media thickness; and LDL, low-density lipoprotein.
Recently, Rohatgi et al. have shown that cholesterol efflux in patients with FH under aggressive lipid-lowering treatment suggests that cholesterol efflux capacity might be a novel biomarker of atherosclerosis. Despite the fact that all subjects were treated with statins, cholesterol efflux capacity was robust even after adjustment for HDL-C or ApoAI levels, with those without ASCVD. Importantly, this relationship was significantly less cholesterol efflux capacity when compared to patients with ASCVD. Therefore, ApoAI quantity was a stronger predictor than cholesterol efflux capacity in our study.

In addition, our findings strongly support the concept that cholesterol efflux capacity was a stronger predictor for the presence of ASCVD than HDL quantity remain to be elucidated. We speculate that considerable heterogeneity in protein and lipid composition of HDL might have an influence. Recently, Shao et al. have shown that there was a significant correlation between levels of post-translationally modified ApoAI, including that in which there has been chlorination of the tyrosine residue at position 192 and oxidation of methionine 148 (Met (O) 148), and HDL-C efflux capacity, and that levels of Met (O) 148 had the highest OR for cardiovascular disease status. In addition, HDL phospholipid composition has been positively associated with cholesterol efflux capacity. Therefore, identification of ApoAI modification and HDL phospholipid composition in patients with FH would provide important information for predicting ASCVD risks.

The hypolipidemic drug probucol is generally prescribed to patients who cannot be effectively treated with statins and ezetimibe only. It is known to reduce plasma HDL by inhibiting ATP-binding cassette transporter A1–mediated LDL biogenesis. Therefore, we observed that patients who had been treated with probucol had significantly lower cholesterol efflux capacity than those who had not, which leads us to speculate that patients with severe ASCVD might have received higher doses of probucol. On the contrary, probucol has been reported to reduce atherosclerosis in animal models and xanthomas in humans. Therefore, in our multivariate analysis, we added probucol as a covariate and it did not affect the relationship between cholesterol efflux capacity and the presence of ASCVD (Table I in the online-only Data Supplement) or Achilles tendon thickness (data not shown).

Our study has several limitations. First, it had a cross-sectional design. Therefore, we could only demonstrate associations and not causal relationships. Second, the cholesterol efflux capacity assay quantifies not only one component of the reverse cholesterol transport pathway but also one property of HDL-C levels and LDL-C levels before any lipid-lowering therapy was initiated, and the duration of the untreated period was not considered. Therefore, pretreatment levels of LDL-C might have been underestimated. Finally, we could not exclude the possibility that other medications might have affected lipids and cholesterol efflux as well.

In conclusion, our findings strengthen the concept that assessment of HDL function may provide more information about HDL-mediated atheroprotection in patients with FH. In addition, our findings strongly support the concept that enhancement of HDL functions could be a promising strategy.

Table 6. Characteristics of Study Subjects Presented as Tertiles of Mean Carotid Intima-Media Thickness

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean-IMT, mm</td>
<td>10.0±2.3</td>
<td>11.5±4.2</td>
<td>14.5±5.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Max IMT, mm</td>
<td>1.3±0.6</td>
<td>1.8±0.7</td>
<td>2.9±0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Presence of ASCVD, n (%)</td>
<td>10 (13.1%)</td>
<td>27 (36.0%)</td>
<td>39 (52.0%)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

ASCVD indicates atherosclerotic cardiovascular disease; ATT, Achilles tendon thickness; BMI, body mass index; HDL, high-density lipoprotein; IMT, intima-media thickness; and LDL, low-density lipoprotein.

differences might be the reason why ApoAI quantity was a stronger predictor than cholesterol efflux capacity in our study.
in the treatment of patients with FH by inducing removal of cholesterol from peripheral tissues, including that in the cornea, xanthomas, and the arterial wall.

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Disclosures
None.

References


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**Significance**

Familial hypercholesterolemia is an autosomal dominant disorder characterized by an extremely elevated plasma concentration of low-density lipoprotein cholesterol and increased risk of premature atherosclerotic cardiovascular disease. We have shown that cholesterol efflux capacity in patients with familial hypercholesterolemia was independently and inversely associated with the presence of atherosclerotic cardiovascular disease. It remains to be investigated whether therapies targeting quality of high-density lipoprotein could be effective for preventing atherosclerosis in familial hypercholesterolemia.
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Materials and Methods

Study subjects

Among patients followed up in the outpatient clinic of National Cerebral and Cardiovascular Center Hospital, 227 consecutive ethnic Japanese patients diagnosed as heterozygous FH were enrolled in this cross-sectional study. All patients had been previously treated with lipid lowering therapy, including statins. In view of the poor prognosis of FH, statin therapy was initiated at the time of diagnosis. Ninety-nine (43.6%) patients also received ezetimibe, and 46 (20.3%) patients received probucol. Resin (cholestimide), niacin, eicosapentaenoic acid, and fibrate were additionally prescribed for 37 (16.3%), 6 (2.6%), 8 (3.5%), and 3 (1.3%) of the patients, respectively. The procedures and possible risks of the study were explained to all patients and they gave written informed consent. This study was performed in conformity with the Declaration of Helsinki and approved by the ethical committee of National Cerebral and Cardiovascular Center, Osaka, Japan.

Clinical and laboratory characteristics

Serum total cholesterol, triglycerides, HDL cholesterol, and apolipoprotein levels were measured enzymatically with a commercial kit (Daiichi Pure Chemicals Co., Tokyo, Japan) using an automated analyzer (Hitachi model 704; Hitachi, Tokyo, Japan) in the clinical laboratory of National Cerebral and Cardiovascular Center. LDL cholesterol was calculated by the Friedewald formula. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m²). Hypertension was defined as use of antihypertensive drugs or blood pressure more than 140 mmHg systolic or more than 90 mmHg diastolic, or both, at the time of collecting blood samples. Diabetes mellitus was defined according to the Evidence-based Practice Guideline for the Treatment for Diabetes in Japan 2013.

Achilles tendon thickness

Achilles tendon thickness was measured using soft X-ray radiography according to the method previously described. To ensure reproducibility, the following imaging conditions were employed. The angle between lower leg bone and sole was ninety degrees. An angle of incidence with respect to the fibular lateral malleolus was established and lateral projection was used. The imaging distance was 120 cm. The imaging voltage-current characteristics were 50 kV.
and 5.0 mA, respectively.

**Carotid ultrasonography**
Carotid atherosclerosis was evaluated by high-resolution ultrasonography with a 7.5-MHz transducer that produced an axial resolution of 0.1 mm (SSA-780A, TOSHIBA, Tokyo, Japan) by 2 examiners certified by the Japan Society of Ultrasonics in Medicine. We measured the carotid arteries from the superior border of the collarbone to the inferior margin of the mandible. Each index of carotid atherosclerosis was defined as follows. Mean-IMT was defined as the mean of the proximal and distal walls for both sides of the common carotid arteries at a point 10 mm proximal to the beginning of each carotid artery bulb. Max-IMT was defined as the maximum IMT in the entire scanned area. To investigate inter-examiner reliability, the max-IMT and mean-IMT of 10 patients were measured, and high inter-examiner correlations were observed (0.91 and 0.93, respectively).

**DNA analysis**
Genomic DNA was extracted from whole white blood cells of patients using an automated DNA extraction machine (NA-3000; KURABO, Osaka, Japan). Primers covering all the exons and exon-intron boundary sequence of PCSK9 (12 exons) and LDLR (18 exons) were designed. Primers including 100-150 bases upstream or downstream of each exon sequence were also designed. The polymerase chain reaction (PCR) was carried out in a reaction mixture containing 20 ng genomic DNA, the forward and reverse primers (5 pmol) and 0.5 U HotStarTaq® Master Mix Kit (Qiagen) with the corresponding buffer. The amplification products were purified by ExoSAP-IT® (GE healthcare, Waukesha, WI). The M-13 sequence (5’-AAAACGACGGCCAGT-3’) was added to the designed primers for convenience of sequencing analysis. Direct sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies, Foster, CA) on an ABI Prism 3130 DNA Analyzer (Life Technologies).

**Assessment of cholesterol efflux capacity**
Cholesterol efflux capacity in the patients with FH was quantified using the slightly modified method previously reported. Blood was collected from FH and control subjects and after centrifugation, serum was frozen at -70°C until use.
The apolipoprotein B (apoB)-depleted serum was obtained from the whole serum by precipitating the apoB-containing lipoproteins with a polyethylene glycol solution as described previously. J774.1 cells (National Institute of Biomedical Innovation, Osaka, Japan) derived from a murine macrophage cell line were plated and radiolabeled with 0.33 μCi of 3H-cholesterol (Perkin-Elmer Analytical Sciences, MA, US) per milliliter for 24 hours. Then, the cells were washed with PBS and efflux mediums containing 2.8% apolipoprotein B–depleted serum were added, and left for 4 hours. All steps were performed in the presence of the acyl–coenzyme A: cholesterol acyltransferase inhibitor Sandoz 58-035 (Santa Cruz Biotech, 2 μg per milliliter). Liquid scintillation counting (Perkin-Elmer Analytical Sciences, MA, US) was used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated by means of hexane and isopropanol extraction of control wells not exposed to patient serum. Percent efflux was calculated by the following formula:

\[
\text{Percent efflux} = \left( \frac{\text{μCi of } ^3\text{H}-\text{cholesterol in mediums containing } 2.8\% \text{ apoB--depleted serum} - \text{μCi of } ^3\text{H}-\text{cholesterol in serum-free mediums}}{\text{μCi of } ^3\text{H}-\text{cholesterol in cells extracted before the efflux step}} \right) \times 100.
\]

All assays were performed in duplicate. To correct for inter-assay variation across plates, a pooled serum control from eleven healthy volunteers was included on each plate, and values for serum samples from patients were normalized to the value of the pooled sample in subsequent analyses.

**Statistical analysis**

Categorical variables are presented as frequencies and percentages, and continuous variables as means and standard deviations, or medians and interquartile ranges for variables with skewed distributions. The association of cholesterol efflux capacity with clinical and HDL-related variables was assessed with the use of Pearson’s correlation coefficients, with partial correlation coefficients after adjustment for HDL cholesterol level also reported. Linear regression was used to characterize the relationship between efflux capacity and both Achilles tendon thickness and carotid intima–media thickness. Age, sex, smoking history (current and past), presence of hypertension, presence of diabetes, and levels of low-density lipoprotein (LDL) cholesterol, HDL cholesterol, and ApoAI were included as covariates. Beta coefficients are reported for a 1-standard deviation (SD) increase for continuous variables.
Logistic regression was used to estimate the association between cholesterol efflux capacity and cardiovascular disease status after adjustment for age, sex, smoking history, presence or absence of diabetes, presence or absence of hypertension, and LDL cholesterol level. HDL cholesterol and ApoAI levels were added in subsequent models. Adjusted odds ratios for CVD are reported for a 1-SD increase in efflux capacity.

All reported P values are two-tailed, with a P value of 0.05 indicating statistical significance. Analyses were performed with the use of JMP software, version 12.0 (SAS Institute).

References