The Cholesterol Content of Western Diets Plays a Major Role in the Paradoxical Increase in High-Density Lipoprotein Cholesterol and Upregulates the Macrophage Reverse Cholesterol Transport Pathway

Joan Carles Escolà-Gil, Gemma Llavèrías, Josep Julve, Matti Jauhiainen, Jesús Méndez-González, Francisco Blanco Vaca

Objective—A high–saturated fatty acid– and cholesterol-containing (HFHC) diet is considered to be a major risk factor for cardiovascular disease. The present study aimed to determine the effects of this Western-type diet on high-density lipoprotein (HDL) metabolism and reverse cholesterol transport (RCT) from macrophages to feces.

Methods and Results—Experiments were carried out in mice fed a low-fat, low-cholesterol diet, an HFHC diet, or an HFHC diet without added cholesterol (high–saturated fatty acid and low-cholesterol [HFLC]). The HFHC diet caused a significant increase in plasma cholesterol, HDL cholesterol, and liver cholesterol and enhanced macrophage-derived $[^{3}H]$cholesterol flux to feces by 3- to 4-fold. These effects were greatly reduced in mice fed the HFLC diet. This HFHC diet–mediated induction of RCT was sex independent and was not associated with obesity or insulin resistance. The HFHC diet caused 1.4- and 3-fold increases in $[^{3}H]$cholesterol efflux to plasma and HDL-derived $[^{3}H]$tracer fecal excretion, respectively. Unlike a low-fat, low-cholesterol and HFLC diets, the HFHC diet increased liver ABCG5/G8 expression. The effect of the HFHC diet on fecal macropage-derived $[^{3}H]$cholesterol excretion was totally blunted in ABCG5/G8–deficient mice.

Conclusion—Despite its deleterious effects on atherosclerosis, the HFHC diet promoted a sustained compensatory macrophage-to-feces RCT. Our data provide direct evidence of the crucial role of dietary cholesterol signaling through liver ABCG5/G8 upregulation in the HFHC diet–mediated induction of macrophage-specific RCT. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: Macrophages | ABCG5/G8 | HDL | dietary fat | reverse cholesterol transport

Dietary saturated fat intake has been associated with an increased risk of atherosclerotic cardiovascular disease and metabolic diseases, such as obesity and type 2 diabetes. This effect is thought to be mediated by an increase in plasma cholesterol, mainly low-density lipoprotein cholesterol. However, both dietary saturated fat and cholesterol intake are known to raise plasma high-density lipoprotein cholesterol (HDL-C) levels. Several epidemiological studies and 1 meta-analysis of 60 controlled trials showed a positive correlation between high saturated fat intake and HDL-C. In an attempt to determine the mechanism underlying this paradoxical observation, several studies reported that a low saturated fat and cholesterol intake reduced HDL-C levels by reducing the apolipoprotein A-I secretion rate. However, other studies found this effect to be associated with decreased apolipoprotein A-I fractional catabolic rates. Also, when dietary cholesterol was increased along with total and saturated fat, increases in large high-density lipoprotein (HDL) subpopulations and HDL apolipoprotein E amounts were observed.

Macrophage-specific reverse cholesterol transport (RCT) is thought to be one of the most important HDL-mediated cardioprotective mechanisms. HDL plays a critical role in cholesterol efflux from macrophages, the first step in RCT. However, despite the reported changes in HDL composition and size, the results of several human studies on the effect of dietary fatty acid composition and cholesterol amount on cellular cholesterol efflux to HDL are contradictory. In
the present study, we evaluated the effect of a high–saturated fatty acid, Western-type diet, with or without added cholesterol, on the entire macrophage-specific RCT pathway using a validated mouse assay in which radiolabeled cholesterol from macrophages is traced in plasma, liver, and feces.26,29 Our results demonstrate that a high–saturated fat and cholesterol-containing diet increased macrophage-to-feces RCT and that dietary cholesterol and liver ABCG5/G8 upregulation played a major role in this paradoxical increase.

### Methods

All animal procedures were conducted in accordance with published regulations and reviewed and approved by the Institutional Animal Care Committee of the IIB Sant Pau. A detailed description of methods used in the present study is provided in the Supplemental Data, available online at http://atvb.ahajournals.org. A brief description of the methods is given below.

C57BL/6 and human CETP transgenic mice were obtained from the Jackson Laboratory. ABCG5 and ABCG8 double heterozygous mice were crossed to produce wild-type (ABCG5G8+/−) and double knockout ABCG5G8(−/−) mice.26 All mice were maintained on a low-fat, low-cholesterol (LFLC) chow diet containing 3% fat and 0.02% cholesterol. Mice were fed for 8 additional weeks with the LFLC diet, a high–saturated fatty acid–and cholesterol-containing (HFHC) diet containing 21% fat (saturated fat/total fat ratio = 0.7) and 0.2% cholesterol or the high–saturated fatty acid and low-cholesterol (HFLC) diet. The HFLC diet contained 0.05% cholesterol and was prepared exactly as the HFHC diet was, with the sole difference being a cholesterol supplementation in the latter. A detailed description of dietary fatty acid composition is provided in Supplemental Table I. The methods used for plasma lipid and apolipoprotein analyses have been described in detail elsewhere.27–29 For the in vivo macrophage-specific reverse cholesterol method, [3H]cholesterol-labeled mouse macrophages were injected into the peritoneal cavity, and the rate of RCT was measured after 48 hours of macrophage injection.26–28 Glucose tolerance tests were performed by intraperitoneal administration of glucose (1 mg/g of body mass) and subsequent measurement of plasma glucose at t=0 (baseline) and 20, 60, 120, and 180 minutes. Autologous [3H]cholesteryl oleate–labeled HDL were prepared and injected intravenously into each mouse.26 Blood was collected into tubes at 1, 3, 6, 24, and 48 hours, and the fractional catabolic rate was determined.29 At the end of the experiment, fecal [3H]cholesterol and the [3H]tracer detected in fecal bile acids were determined. Liver and small intestine RNA were isolated and polymerase chain reaction assays were performed on an Applied Biosystems Prism 7000 sequence detection system.28 One-way ANOVA with a Tukey multiple comparison post test was used to compare differences among groups. A probability value <0.05 was considered statistically significant.

### Results

#### Plasma Lipoproteins, Liver Lipids, and Atherosclerosis

Male C57BL/6 mice fed the HFHC diet for 8 weeks showed several previously reported changes, such as increased mean weight, plasma cholesterol, and HDL-C (Table).31,33 HFLC intake resulted in a significant increase in HDL-C, although this change was less pronounced than that in mice fed an HFHC diet (Table). High-fat diets did not affect plasma triglyceride levels. We further studied the effects of diets on HDL apolipoprotein amount and size (Table). Mice fed both the HFHC and HFLC diets showed a significant increase in apolipoprotein A-I compared with the LFLC diet. Plasma and HDL-associated apolipoprotein E were increased in HFHC diet–fed mice compared with the LFLC and the HFLC groups. Nondenaturing gel gradient electrophoresis revealed that both the HFHC and HFLC diets produced a marginal increase on the size distribution of plasma HDL (Supplemental Figure I). On the other hand, the HFHC diet did increase liver cholesterol and triglycerides (Table). This liver cholesterol increase resulted mainly from the increase in cholesteryl esters. The HFLC-induced increase in liver lipids was less pronounced compared with that in HFHC diet–fed mice. Plasma and liver lipid changes were not associated with changes in net intestinal cholesterol absorption (Table).

C57BL/6 mice fed the HFHC diet for 24 weeks developed more early aortic atherosclerosis than LFLC-fed mice (Supplemental Table II). As previously reported, the atherosclerosis area was greater in females than in males.34

### In Vivo Macrophage-Specific RCT

To ascertain whether saturated fat and cholesterol intake affected macrophage-dependent RCT pathway in vivo, radiolabeled macrophages were injected into male C57BL/6 mice fed either the LFLC, HFHC, or LFLC diet. The [3H]tracer recovery was measured in plasma and HDL at 24 and 48 hours and in liver at 48 hours, and feces were collected over 48 hours. Approximately 80% of radiolabeled [3H]cholesterol was bound to plasma HDL of mice at 24 hours and increased significantly in mice fed the HFHC and HFLC diets (2.8±0.2 and 3.0±0.4, respectively, versus 1.7±0.1% of injected dose in the LFLC group; P≤0.05). However, plasma, HDL and liver [3H]cholesterol levels at 48 hours were not affected by the fat content of the diet (Figure 1A and 1B). TLC analyses

### Table. Plasma and Liver Lipids in Male C57BL/6 Mice Fed a Low-Fat, Low-Cholesterol (LFLC) Diet, a High–Saturated Fatty Acid– and Cholesterol-Containing (HFHC) Diet, or a High–Saturated Fatty Acid, Low-Cholesterol (HFLC) Diet

<table>
<thead>
<tr>
<th></th>
<th>LFLC (n=7)</th>
<th>HFHC (n=7)</th>
<th>HFLC (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>28.8±0.6</td>
<td>34.1±1.4</td>
<td>31.5±0.5</td>
</tr>
<tr>
<td>Total plasma cholesterol, mmol/L</td>
<td>2.6±0.1</td>
<td>4.8±0.4*</td>
<td>3.8±0.1†</td>
</tr>
<tr>
<td>Plasma HDL cholesterol, mmol/L</td>
<td>2.1±0.1</td>
<td>3.8±0.3*</td>
<td>3.1±0.1†</td>
</tr>
<tr>
<td>Plasma non-HDL cholesterol, mmol/L</td>
<td>0.5±0.1</td>
<td>1.0±0.1*</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Plasma apoA-I, mg/mL</td>
<td>1.3±0.1</td>
<td>2.3±0.1*</td>
<td>2.1±0.1*</td>
</tr>
<tr>
<td>Plasma apoE, μg/mL</td>
<td>42.2±1.4</td>
<td>54.2±1.8*</td>
<td>45.9±2.2†</td>
</tr>
<tr>
<td>Plasma HDL apoE, μg/mL</td>
<td>19.5±0.6</td>
<td>21.8±0.5*</td>
<td>19.7±0.5†</td>
</tr>
<tr>
<td>Liver weight/mouse weight, %</td>
<td>4.8±0.1</td>
<td>5.1±0.2</td>
<td>4.2±0.2†</td>
</tr>
<tr>
<td>Liver free cholesterol, μmol/g of liver</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Liver cholesterol esters, μmol/g of liver</td>
<td>0.5±0.1</td>
<td>2.7±0.2*</td>
<td>1.2±0.1†</td>
</tr>
<tr>
<td>Liver triglycerides, μmol/g of liver</td>
<td>1.1±0.3</td>
<td>23.1±6.4*</td>
<td>5.6±0.7†</td>
</tr>
<tr>
<td>Intestinal cholesterol absorption, %</td>
<td>57.5±6.7</td>
<td>62.1±3.7</td>
<td>66.5±6.7</td>
</tr>
</tbody>
</table>

Plasma samples were collected in mice fed for 8 wk with the corresponding diet. All animal manipulations began at 12PM with the mice fed ad libitum. Values are expressed as mean±SEM. HDL indicates high-density lipoprotein; apo, apolipoprotein.

*P<0.05 vs LFLC mice.
†P<0.05 vs HFHC mice.

Intestinal cholesterol absorption was determined in 4 individual animals per group.
showed that the HFHC diet caused a significant increase in liver $[^{3}H]$cholesterol esters and a pronounced reduction in liver free $[^{3}H]$cholesterol (Figure 1B). Fecal macrophage-derived $[^{3}H]$tracer excretion in mice given the HFHC diet was significantly higher than in mice given the LFLC and resulted mainly from the increase in radiolabeled $[^{3}H]$cholesterol (Figure 1C). The dietary stimulus on RCT was almost totally absent in mice fed the HFLC diet. The HFHC diet also increased significantly the net fecal $[^{3}H]$cholesterol + bile acid excretion in female C57BL/6 mice compared with mice given either the LFLC or HFLC diet (Supplemental Figure IIA).

Given that CETP plays a crucial role in lipoprotein metabolism in humans, we determined plasma lipoproteins and macrophage RCT in humanized male CETP-transgenic mice fed an LFLC, HFHC, or HFLC diet (Supplemental Table III). The HFHC and HFLC diets induced a HDL-raising effect in these transgenic mice similar to that found in wild-type mice. Also, similar to the findings in wild-type mice, the HFHC diet induced a significant increase in fecal macrophage-derived $[^{3}H]$tracer excretion in human CETP transgenic mice compared with either an LFLC or HFLC diet.

Given that the HFHC diet induces obesity and insulin resistance in C57BL/6 mice and those have been associated with an impaired ability of HDL to induce cholesterol efflux, we aimed to ascertain whether long-term feeding with the HFHC diet could affect fecal excretion of macrophage-derived $[^{3}H]$tracer. As expected, the male C57BL/6 mice gained weight rapidly (Figure 2A). Basal glucose levels and area under the curve of the glucose tolerance test were raised in mice given the HFHC diet for 8, 16, or 24 weeks (Figure 2C and 2E). Female C57BL/6 mice were more resistant, and in vivo reverse cholesterol transport from macrophages to feces. A and B, Average weight of male (A) and female (B) C57BL/6 mice fed an HFHC diet for 8, 16, or 24 weeks. C to F, Fasting glucose levels and the area under the curve of glucose in glucose tolerance tests in males (C and E) and females (D and F). G and H, Fecal $[^{3}H]$tracer over 48 hours in males (G) and females (H). Values are mean ± SEM of 4 to 6 mice per group. LFLC indicates low-fat, low-cholesterol.

Figure 1. In vivo reverse cholesterol transport from macrophages to feces in male C57BL/6 mice fed a low-fat, low-cholesterol (LFLC) diet, a high–saturated fatty acid– and cholesterol-containing (HFHC) diet, or a high–saturated fatty acid and low-cholesterol (HFLC) diet for 8 weeks. Individually housed mice were injected intraperitoneally with $[^{3}H]$cholesterol-labeled P388D1 mouse macrophages (13.9 $\times$ 10$^6$ cells containing 2.0 $\times$ 10$^6$ cpm in 0.5 mL of saline in each mouse; cell viability 97%). A and B, Plasma total and high-density lipoprotein (HDL)-associated $[^{3}H]$cholesterol (A) and liver $[^{3}H]$cholesteryl-esters and free cholesterol (B) at 48 hours. C, Fecal $[^{3}H]$cholesterol and $[^{3}H]$tracer from fecal bile acids over 48 hours. Values are mean ± SEM of 7 individual animals per group. The amount of $[^{3}H]$tracer is expressed as a fraction of the injected dose.
RCT Step Evaluation: Cholesterol Efflux and HDL Catabolism

Cholesterol efflux from P388D1 macrophage cultures to plasma of male C57BL/6 mice fed the HFHC was 1.4-fold higher than that of mice fed the LFLC or HFLC diet (Figure 3A). This variable correlated with HDL-C levels when fit to a linear model ($r = 0.57$, $P < 0.018$). Another key step in macrophage-specific RCT is liver HDL-derived cholesterol uptake and biliary secretion. To determine the fate of $[^{3}H]$cholesterol from the HDL core, mice from each group were injected intravenously with HDL-$[^{3}H]$cholesteryl oleate. None of the diets affected the clearance or fractional catabolic rate of intravenously injected $[^{3}H]$HDL (Figure 3B). Importantly, recovery of HDL-derived $[^{3}H]$cholesterol in feces was 3-fold higher in mice fed the HFHC diet, whereas the ability of HFLC diet-fed mice to increase excretion of fecal HDL-derived $[^{3}H]$cholesterol was completely blunted (Figure 3C).

Liver and Small Intestine Gene Expression Analyses

Liver expression of genes involved in HDL synthesis and biliary cholesterol excretion was evaluated. The HFHC diet strongly upregulated $ABCG1$ and $ABCG5$ gene expression (Figure 4A and Supplemental Figure IIB). Liver $ABCG8$ expression was found to be significantly upregulated in the groups given the HFHC and HFLC diets (Figure 4A). However, the expression of other major HDL synthesis-related genes, such as $apoA-I$, $ABCA1$, and $LCAT$, was not affected by the diet (Figure 4A). No significant changes in liver $SR-BI$ and $CYP7A1$ mRNA expression were found among groups (Figure 4A).

Small intestine $ABCG5$ and $ABCG8$ expression was similar in all groups (Figure 4B). However, the HFHC diet downregulated intestinal $NPC1L1$ and $SR-BI$ expression compared with the LFLC diet.

Figure 3. Effects of the low-fat, low-cholesterol (LFLC), high–saturated fatty acid– and cholesterol-containing (HFHC), and high–saturated fatty acid and low-cholesterol (HFLC) diets on the independent reverse cholesterol transport (RCT) steps. A, In vitro macrophage cholesterol efflux from $[^{3}H]$cholesterol-labeled P388D1 mouse macrophages to plasma at 4 hours. Results are expressed as mean±SEM of 6 individual animals per group. B, In vivo clearance of $[^{3}H]$cholesteryl oleate–radiolabeled high-density lipoprotein (HDL). Plasma $[^{3}H]$HDL-cholesteryl oleate clearance is displayed as fractional catabolic rate (FCR). C, In vivo excretion of HDL-derived $[^{3}H]$cholesterol oleate in fecal cholesterol and bile acids. In B and C, results are expressed as mean±SEM of 5 individual animals per group.

Figure 4. Real-time reverse transcription–polymerase chain reaction quantification of relative mRNA expression in livers (A) and small intestines (B) of male mice. The signal of low-fat, low-cholesterol (LFLC) diet-fed mice was set at a normalized value of 1 arbitrary unit (AU). GAPDH was used as internal control. Results are expressed as mean±SEM of 5 individual animals per group. HFHC indicates high–saturated fatty acid– and cholesterol-containing; HFLC, high–saturated fatty acid and low-cholesterol; Apo, apolipoprotein.
Our in vitro cellular cholesterol efflux results (Supplemental Figure IIC).

The Effects of ABCG5/G8 Expression on HFHC-Induced Macrophage-Specific RCT Pathway

To study the in vivo effect of ABCG5 and ABCG8 on RCT, a separate experiment was conducted to determine macrophage-to-feces RCT in wild-type male (ABCG5G8+/+) and double knockout ABCG5G8−/− mice fed the low-fat, low-cholesterol (LFLC), high–saturated fatty acid– and cholesterol-containing (HFHC), or high–saturated fatty acid and low-cholesterol (HFLC) diet for 8 weeks. Individually housed mice were injected intraperitoneally with [3H]cholesterol-labeled P388D1 mouse macrophages as described in Figure 1. Shown are fecal [3H]cholesterol and [3H]tracer from fecal bile acids over 48 hours. Values are mean±SEM of 5 to 6 individual animals per group. The amount of [3H]tracer is expressed as a fraction of the injected dose.

Figure 5. In vivo reverse cholesterol transport from macrophages to feces in male wild-type (ABCG5G8+/+) and double knockout ABCG5G8−/− mice fed the low-fat, low-cholesterol (LFLC), high–saturated fatty acid– and cholesterol-containing (HFHC), or high–saturated fatty acid and low-cholesterol (HFLC) diet for 8 weeks. Individually housed mice were injected intraperitoneally with [3H]cholesterol-labeled P388D1 mouse macrophages as described in Figure 1. Shown are fecal [3H]cholesterol and [3H]tracer from fecal bile acids over 48 hours. Values are mean±SEM of 5 to 6 individual animals per group. The amount of [3H]tracer is expressed as a fraction of the injected dose.

Discussion

Strong evidence supports a HDL-C-raising effect of diets enriched in saturated fat and cholesterol in humans.4–11 In the current study, we showed that an HFHC diet promotes macrophage-to-feces RCT in vivo that depends mainly on dietary cholesterol. In contrast, our results indicated that saturated fat intake did not affect macrophage-to-feces RCT. However, the differences in the source of fats and proteins do not permit definitive conclusions to be drawn as to whether LFLC and HFLC could promote differences in macrophage-to-feces RCT.

Many studies have attempted to determine whether variations in dietary fat produce changes in cellular cholesterol efflux to plasma. However, dietary saturated fat and cholesterol amount do not appear to affect cholesterol efflux to plasma in most of the studies conducted in humans22–24 and animals.37,38 Our in vitro cellular cholesterol efflux results were more comparable to those reported by Blanco-Molina et al20 where the ability of serum to promote cholesterol efflux was slightly greater in 14 human subjects after a low-fat, high-cholesterol diet compared with the low-fat diet alone. Our results are also consistent with the reported HFHC diet–mediated effects on HDL-C and cholesterol efflux from macrophages and liver ABCG1 expression in mice.39

However, although the HFHC diet exerted a significant stimulatory effect on the transfer of cholesterol from macrophages to feces, cholesterol-derived radioactivity in plasma and liver at 48 hours was similar among groups. This lack of association between [3H]cholesterol levels in plasma and liver and cholesterol mass has been reported previously.26,30,40,41 It should be noted that RCT is a dynamic process and [3H]cholesterol levels in intermediate compartments at any time point may not reflect the flux of [3H]cholesterol through different compartments. To evaluate additional RCT steps downstream from the cholesterol efflux, we carried out an HDL turnover study by injecting [3H]cholesterol olate–labeled HDL. Our studies indicated that the HFHC diet did not affect liver SREBP-1c expression or plasma HDL clearance. These results are comparable to those previously obtained in mice and hamsters with a similar dietary change.33,42 However, the mouse study also found a moderate reduction in HDL fractional catabolic rate.43 The differences might be attributed, at least in part, to differences in the radiolabeled substrate because those authors used [3H]cholesterol olate ether–labeled rat HDL.

Nevertheless, despite no changes in plasma HDL clearance, the HFHC diet significantly promoted fecal HDL-derived cholesterol excretion in mice. Our results indicate that the major effect of the HFHC diet accelerating the rate of fecal [3H]cholesterol excretion involves liver ABCG5/G8 upregulation. We also found that the HFHC diet upregulated other LXR-regulated genes, such as liver ABCG1 and SREBP-1c (data not shown).26 Glucose can activate LXR43; however, the HFHC diet–induced effects on RCT were also found in females, with no modifications in plasma glucose levels. Therefore, one possible mechanism contributing to this effect may be related to the increased flux of cholesterol through the liver and, consequently, the increased generation of oxysterols which can activate LXR.44

Of note, Nishimoto et al45 reported that a fish oil– and cholesterol-enriched diet significantly promoted macrophage-to-feces RCT, which was closely associated with increased liver ABCG5 and ABCG8 expression and reduced liver [3H]cholesterol esters. However, 2 key points should be taken into account: first, all the diets used in that study contained the same amount of cholesterol (1.25%, higher than in the present study)45; second, omega-3 fatty acids are poor substrates for ACAT, and in addition, liver Scd1 mRNA was found to be decreased.45 Both decreased enzymatic activities diminish the liver content of cholesteryl esters.45

Our results rule out the possibility that the HFHC diet differentially inhibited intestinal cholesterol absorption and that this resulted in increased RCT in vivo, as occurred with ezetimibe treatment or specific intestinal LXR activation.46,47 Furthermore, even though a Western-type diet increased transintestinal cholesterol export in mice by 50%,48 this would not explain the substantially higher RCT-raising effect of the HFHC diet, which, in different experiments shown here, was in the range of 3- to 4-fold. Furthermore, transin-
testinal cholesterol export changes were dependent on the increase in dietary fat content rather than that of cholesterol.48

We and others18,25 recently showed macropage-specific RCT to be a good predictor of atherosclerosis susceptibility in mouse models of lipoprotein disorders and atherosclerosis. This would not be the case in mice fed an HFHC diet because they showed both increased macrophage-specific RCT and atherosclerosis susceptibility. This suggests that the observed increased macrophage-specific RCT is an insufficient, compensatory mechanism to protect against atherosclerosis development. Therefore, HFHC diet-mediated effects on HDL antiinflammatory activity,49 non-HDL-C, and fat cells and macrophage inflammation50 appear to be the major contributor to atherosclerosis in these mice.

A recent report showed that diet-induced dyslipidemia impaired macrophage-to-feces RCT in hamsters fed a low-fat diet containing 0.3% cholesterol, although it did cause a pronounced increase in HDL-C.51 Hamsters express CETP, but the HFHC diet also promoted RCT in our CETP transgenic mice. The differences in these results may be attributable to the specific diet-induced changes in the liver gene expression profile of hamsters. Thus, the dyslipidemic diet used in hamsters upregulated liver ABCA1 and ABCG5 expression but caused a severe downregulation in liver SR-BI, a known positive regulator of in vivo macrophage-specific RCT.52

In conclusion, our results demonstrate that an HFHC diet promoted the fecal excretion of macrophage- and HDL-derived cholesterol. This RCT increase was independent of other metabolic HFHC diet-induced complications, such as obesity and insulin resistance. The presence of dietary cholesterol and liver ABCG5 and ABCG8 transporters is required for the HFHC-mediated induction of RCT. Because the HFHC diet also promoted atherosclerosis, this change in RCT may constitute a compensatory mechanism to protect macrophages from cholesterol accumulation.

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Disclosures
None.

References
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Cholesterol in Western Diets Promotes RCT


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Supplementary Methods

Mice and diets

All animal procedures were conducted in accordance with published regulations and reviewed and approved by the Institutional Animal Care Committee of the Hospital de la Santa Creu i Sant Pau. Male and female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME; #000664). Human CETP transgenic mice were obtained from Jackson Laboratories (#003904). ABCG5 and ABCG8 double heterozygous mice (ABCG5G8+/−) were obtained from Jackson Laboratories (#004670; Bar Harbor, ME) and crossed to produce wild-type (ABCG5G8+/+) and double knockout ABCG5G8 (−/−) mice 1. All mice were maintained on a low-fat, low-cholesterol chow diet (LFLC, A04, Safe, Scientific Animal Food & Engineering, Augy, France) containing 3% fat and 0.02% cholesterol. At 8 weeks of age, mice were randomized into three groups. Mice were fed for 8 additional weeks with the LFLC diet, a high saturated fatty acid- and cholesterol-containing (HFHC) diet (Western-type diet TD88137, Harlan Teklad, Madison, WI, containing 21% of fat –saturated fat/total fat ratio = 0.64– and 0.2% cholesterol) or the high-saturated fatty acid and low-cholesterol diet (HFLC). The HFLC diet contained 0.05% cholesterol and was prepared exactly as the HFHC diet with the sole difference being a cholesterol supplementation in the latter. A detailed description of dietary fatty acid composition is provided in supplementary Table 1. Independent experiments were also performed in C57BL/6 mice fed the HFHC diet for 16 and 24 weeks. All animal manipulations began at 12 p.m. with the mice fed ad libitum.

Lipid and apolipoprotein analyses
Mice were euthanized and exsanguinated by cardiac puncture at the end of the study and livers removed after being perfused with saline. Cholesterol, HDL cholesterol and triglycerides were determined enzymatically using commercial kits adapted to an BM/HITACHI 917 autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland) \(^2\). Triglyceride determinations were corrected for the free glycerol present in each fraction (Sigma Diagnostics, St. Louis, MO). Mouse apoA-I assay was performed as reported \(^3\) and mouse apoE was quantified using a commercial method (Uscn Life Science Inc, Wuhan, China). HDL was isolated by sequential ultracentrifugation at 100,000 \(g\) for 24 h at a density of 1.063-1.21 g/ml \(^2\). HDL size was determined by non-denaturing 4-30% polyacrylamide gel gradient electrophoresis stained with Coomassie blue. Liver lipids were extracted from 100 mg of liver with isopropyl alcohol-hexane (2:3, \(v/v\)) and cholesterol, free cholesterol and triglycerides determined using a commercial kit adapted to a BM/HITACHI 917 autoanalyzer \(^4\). Net intestinal cholesterol absorption was measured in mice at the end of the study by a fecal dual-isotope ratio method as previously described \(^5\).

**Aortic atherosclerosis measurement**

Oil red O staining of atherosclerotic lesion areas present in the proximal aorta was measured in each mouse in four 10-\(\mu\)m sections interspaced by 80 \(\mu\)m \(^6\). Video images were captured directly from a color video camera (TK-C621, JVC) attached to an Olympus-BX50 microscope and displayed on an ADI microScan 5V/5V+ monitor. Lesion areas were measured blindly with a PC image analysis software (PC image, version 2.2.02, Foster Findlay Associates Ltd, Newcastle-upon-Tyne, UK).
In vivo macrophage-specific reverse cholesterol transport

Macrophage-like cell line P388D1 (ATCC; Manassas, VA) was cultured in 75-cm tissue culture plates at 5 million cells per plate and grown to 90% confluence in RPMI 1640 supplemented with 10% fetal bovine serum. Mouse macrophages were incubated for 48 hours in the presence of 5 \( \mu \text{Ci/ml} \) of \( [1\alpha,2\alpha(n)^3\text{H}] \) cholesterol (GE Healthcare), 100 \( \mu \text{g/ml} \) of acetylated LDL and 10% lipoprotein-depleted serum. These cells were washed, equilibrated, detached by gently pipetting, resuspended in 0.9% (w/v) saline and pooled before being intraperitoneally injected into mice. All mice were injected intraperitoneally with \( [^3\text{H}] \) cholesterol-labeled mouse macrophages (13.9 x10^6 cells containing 2.05x10^6 cpm in 0.5 mL of saline for each mouse; cell viability 97% was measured by trypan blue staining). Mice were then individually housed in metabolic cages and stools collected over the next two days. Plasma radioactivity was determined at 48 hours by liquid scintillation counting. HDL-associated \( [^3\text{H}] \) cholesterol was measured after precipitation of \( \beta \)-lipoproteins. At that point, mice were euthanized and livers removed. Liver and fecal lipids were extracted with isopropyl alcohol-hexane. The lipid layer was collected, evaporated and \( [^2\text{H}] \) cholesterol radioactivity measured by liquid scintillation counting. The distribution of \( [^3\text{H}] \) cholesterol between free cholesterol and cholesteryl ester was determined by thin-layer chromatography (TLC). The \( [^3\text{H}] \) tracer detected in fecal bile acids was determined in the remaining aqueous portion of fecal material extracts. A known amount of \( [1\alpha,2\alpha(n)^3\text{H}] \) cholesterol (GE Healthcare) and \( [^3\text{H}(G)] \) taurocholic acid (PerkinElmer LAS, Boston, USA) was used as internal control for lipid extraction. The amount of \( [^3\text{H}] \) tracer was also expressed as a fraction of the injected dose.
**Glucose tolerance test**

Glucose tolerance tests were performed by intraperitoneal administration of glucose (1 mg/g of body mass) after a 12-hour fast and subsequent measurement of plasma glucose at $t = 0$ (baseline) and 20, 60, 120 and 180 minutes. The area under the concentration curve (AUC) was calculated to evaluate glucose tolerance.

**RCT step analyses: cholesterol efflux and HDL catabolism**

*In vitro* cellular cholesterol efflux to plasma was determined using $[^3\text{H}]$cholesterol-labeled P388D1 mouse macrophages as described. Autologous $[^3\text{H}]$cholesteryl oleate-labeled HDL (containing 1 million cpm, specific activity of $22.7 \times 10^3$ cpm/nmol) were prepared and injected intravenously into each mouse as described. Blood was collected into tubes at 1, 3, 6, 24 and 48 hours and the radioactivity contained in 20 µl of plasma aliquots determined. This analysis was used to fit a biexponential curve to each set of plasma-decay data, and residence time and fractional catabolic rate (FCR) were determined. At the end of the experiment, liver and fecal $[^3\text{H}]$cholesterol and the $[^3\text{H}]$tracer detected in fecal bile acids were determined as described above.

**Quantitative real-time RT-PCR analyses**

Liver and small intestine RNA were isolated using the trizol RNA isolation method (Gibco/BRL, Grand Island, NY, USA). The whole small intestine was cut into three segments with a length ratio of 1:3:2 (duodenum-jejenum-ileum). From the middle of each intestinal segment, 1.5 cm of the duodenal, jejunal and ileal tissues were extracted and pooled for each mouse. Total RNA samples were repurified, checked for integrity by agarose gel electrophoresis and
reverse-transcribed with Oligo(dT)$_{15}$ using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant to generate cDNA. Predesigned validated primers (Assays-on-Demand, Applied Biosystems, Foster City, CA) were used with Taqman probes. PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described ¹. All analyses were performed in duplicate and relative RNA levels were determined using GAPDH as internal control.

**Statistical Methods**

One-way ANOVA with a Tukey’s multiple comparison post-test was used to compare differences among groups. Mann-Whitney U test was used to compare atherosclerosis susceptibility among groups since the data did not follow a Gaussian distribution. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used for all statistical analyses. A $P$ value $<0.05$ was considered statistically significant.


8. Rader DJ, Gregg RE, Meng MS, Schaefer JR, Zech LA, Benson MD, Brewer HB, Jr. In vivo metabolism of a mutant apolipoprotein, apoA-
Supplementary Table I. Fatty acid profile of fat sources in LFLC and HFHC/HFLC diets.

<table>
<thead>
<tr>
<th>Fatty acid (% total fat)</th>
<th>LFLC</th>
<th>HFHC or HFLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4:0</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>C6:0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>C8:0</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>C10:0</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>C14:1</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>C15:0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>12.9</td>
<td>29.4</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.9</td>
<td>1.7</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>12.6</td>
<td></td>
</tr>
<tr>
<td>C18:1 (Oleic)</td>
<td>20.7</td>
<td>20.7</td>
</tr>
<tr>
<td>C18:1 Isomers</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>C18:2 (Linoleic)</td>
<td>40.1</td>
<td>2.3</td>
</tr>
<tr>
<td>C18:2 Isomers</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>C18:3 (Linolenic)</td>
<td>0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Supplementary Table II. Early atherosclerosis in the proximal aorta of C57BL/6 mice fed a low-fat, low-cholesterol (LFLC) or a high-fat, high-cholesterol (HFHC) diet for 24 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LFLC (n=4)</td>
<td>HFHC (n=4)</td>
</tr>
<tr>
<td></td>
<td>LFLC (n=7)</td>
<td>HFHC (n=7)</td>
</tr>
<tr>
<td>Type of lesions</td>
<td>Early fatty streak</td>
<td>Early fatty streak</td>
</tr>
<tr>
<td></td>
<td>Early fatty streak</td>
<td>Early fatty</td>
</tr>
<tr>
<td>Mean lesion area</td>
<td>54±37 µm²/section</td>
<td>5002±2396*</td>
</tr>
<tr>
<td></td>
<td>25±11</td>
<td>19439±6206*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Each value was obtained from the average lesion area of four proximal aortic sections from each animal stained with Oil red O and counterstained with hematoxylin. *P<0.05 versus LFLC mice. Mann-Whitney U test was used for comparisons.
Supplementary Table III. Plasma and macrophage-derived fecal parameters in male CETP transgenic mice fed a low-fat, low-cholesterol (LFLC) diet, a high-fat, high-cholesterol (HFHC) diet or a high-fat, low-cholesterol (HFLC) diet for 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>LFLC (n=5)</th>
<th>HFHC (n=5)</th>
<th>HFLC (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma cholesterol (mmol/L)</td>
<td>1.5±0.1</td>
<td>3.7±0.2*</td>
<td>3.1±0.2*,†</td>
</tr>
<tr>
<td>Plasma HDL cholesterol (mmol/L)</td>
<td>0.8±0.1</td>
<td>2.4±0.2*</td>
<td>1.9±0.2*</td>
</tr>
<tr>
<td>Plasma non-HDL cholesterol (mmol/L)</td>
<td>0.7±0.1</td>
<td>1.3±0.1*</td>
<td>1.2±0.2*</td>
</tr>
<tr>
<td>Plasma CETP activity (µmol/L h)</td>
<td>160 ± 36</td>
<td>335 ± 27*</td>
<td>270 ± 57</td>
</tr>
<tr>
<td>Fecal [³H]cholesterol (% injected dose)</td>
<td>0.5± 0.1</td>
<td>2.1± 0.4*</td>
<td>0.5 ± 0.1†</td>
</tr>
<tr>
<td>Fecal [³H]bile acids (% injected dose)</td>
<td>0.4± 0.1</td>
<td>0.6± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. * P<0.05 in versus LFLC mice, † P<0.05 vs. HFHC.
Supplementary Figure legends

Supplementary Figure I. Effect of the LFLC, HFHC and HFLC diets on HDL size isolated by ultracentrifugation. HDL separation by non-denaturing 4-30% polyacrylamide gel gradient electrophoresis stained with Coomassie blue. The size of the molecular weight markers is shown on the left. The mean apparent diameters of the HDL were 10.41, 10.64 and 10.58 nm in mice fed the LFLC, HFHC and HFLC diets, respectively.

Supplementary Figure II. In vivo reverse cholesterol transport from macrophages to feces in female C57BL/6 mice fed the LFLC, HFHC or HFLC diets for 8 weeks. Individually-housed mice were injected intraperitoneally with \([^{3}\text{H}]\)cholesterol-labeled P388D1 mouse macrophages as described in Figure 1. 
(A) Fecal \([^{3}\text{H}]\)cholesterol and \([^{3}\text{H}]\)tracer from fecal bile acids over 48 hours. Values are mean ± SEM of 7 mice per group. (B) Real-time RT-PCR quantification of relative mRNA expression in livers of female mice. Values are mean ± SEM of 5 mice per group (C). In vivo reverse cholesterol transport from macrophages to feces in female wild-type (ABCG5G8+/+) and double knockout ABCG5/G8 (−/−) mice fed the HFHC or HFLC diets for 8 weeks. Fecal \([^{3}\text{H}]\)cholesterol and \([^{3}\text{H}]\)tracer from fecal bile acids over 48 hours. Values are mean ± SEM of 4 mice per group.
Supplementary Figure II

A. Fecal [3H]tracer (% injected dose)
- Cholesterol
- Bile acids

B. Liver mRNA levels (AU)
- ABCG5
- ABCG8

C. Fecal [3H]tracer (% injected dose)
- ABCG5G8^{+/+} mice
- ABCG5G8^{-/-} mice

P < 0.01, P < 0.001, P < 0.05, P = 0.06