Atheroprotective Reverse Cholesterol Transport Pathway Is Defective in Familial Hypercholesterolemia


Objective—Low high-density lipoprotein (HDL) cholesterol levels are frequently observed in familial hypercholesterolemia (FH) and might be associated with functional alterations of HDL particles that may influence their efficaciousness in the reverse cholesterol transport pathway.

Methods and Results—We evaluated key steps of the reverse cholesterol transport, ie, cellular free cholesterol efflux, cholesteryl ester transfer protein-mediated cholesteryl ester (CE) transfer from HDL to apolipoprotein B–containing lipoproteins, and hepatic HDL-CE uptake, in patients displaying FH (n = 12) and in healthy normolipidemic control subjects (n = 12). Large HDL2 particles isolated from FH patients displayed a reduced capacity to mediate free cholesterol efflux via both scavenger receptor-BI– and ABCG1–dependent pathways. A significant inverse relationship between scavenger receptor-BI–dependent HDL2 efflux capacity and carotid intima-media thickness (r = −0.473; P = 0.0186), as well as between ABCG1-dependent HDL2 efflux capacity and carotid intima-media thickness (r = −0.485; P = 0.0212), was detected. We also observed an elevated cholesteryl ester transfer protein-mediated CE transfer from HDL2 and HDL3 particles to low-density lipoprotein and a reduced capacity of HDL particles to deliver CEs to the liver.

Conclusion—We demonstrated that the centripetal movement of cholesterol from peripheral tissues, including the vessel wall, to feces is defective in FH, thereby emphasizing its atherogenicity. (Arterioscler Thromb Vasc Biol. 2011;31:1675-1681.)

Key Words: ABC transporter ■ lipoproteins ■ macrophages ■ metabolism ■ receptors

Familial hypercholesterolemia (FH) is a common inherited dominant autosomal disorder caused by mutations in the gene encoding the low-density lipoprotein (LDL) receptor.1 These mutations lead to a reduced number of functional LDL receptors, resulting in diminished cellular uptake of LDL particles, LDL accumulation, and increased plasma levels of total cholesterol, LDL cholesterol, and premature atherosclerosis.2-3 Within the arterial wall, macrophages take up modified LDL through a variety of scavenger receptors, mainly scavenger receptor (SR)-A and CD36, accumulate cholesteryl esters (CEs), and are progressively converted into lipid-rich foam cells that represent the hallmark of the atherosclerotic plaque.4 Excess cholesterol in macrophages is eliminated via the process of reverse cholesterol transport (RCT), a pathway by which cholesterol from peripheral tissues is transported to the liver for biliary excretion.5 During the past few years, the atheroprotective role of RCT has been clearly demonstrated in humans.6 The first step in this pathway is the efflux of cholesterol from cells to extracellular acceptors, such as high-density lipoprotein (HDL) and apolipoprotein AI (apoAI). Cellular cholesterol efflux to lipid-free or lipid-poor apoAI occurs through a transporter belonging to the ATP binding cassette (ABC) family, ABCA1,7 whereas free cholesterol efflux to mature HDL particles involves both ABCG18 and SR-BI.9 The low HDL cholesterol levels frequently observed in FH patients represent an independent risk factor for the premature development of cardiovascular disease. However, the metabolic basis underlying this low-HDL phenotype has not been entirely elucidated to date. An increased apoAI fractional catabolic rate associated with reduced apoAI synthesis has been previously reported.10,11 It has also been suggested

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Drs Bellanger and Orsoni contributed equally to this work. Drs Le Goff and Guerin contributed equally to this work.

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Correspondence to Maryse Guerin, PhD, INSERM UMRs 939, Hôpital de la Pitié Pavillon Benjamin Delessert, 83 boulevard de l’Hôpital, 75651 Paris cedex 13, France. E-mail maryse.guerin@upmc.fr
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that APOE gene polymorphism might affect HDL levels in heterozygous FH patients.12

Antatherogenic properties of HDL are frequently compromised in metabolic diseases associated with a high cardiovascular risk.13 It has been reported that FH patients with elevated plasma triglyceride levels (>1.5 g/L) display HDL3 particles that possess a reduced antiinflammatory capacity and a decreased ability to promote cholesterol efflux from macrophages in comparison with control subjects.14 In this context, we have recently reported that the major HDL subfractions, HDL2 and HDL3, exhibit functional anomalies in patients displaying a mixed hyperlipidemia that significantly alter efficaciousness of the RCT.15

To date, only a few studies have investigated the mechanism of RCT in FH patients. Compared with normolipidemic subjects, both cholesteryl ester transfer protein (CETP) plasma levels and activity are elevated in FH patients.16–18 In addition, CE transfer from HDL is preferentially enhanced toward small dense LDL particles.17 In consequence, CETP appears to play a proatherogenic role in FH by increasing the cholesterol load in apoB-containing lipoproteins. In the present study, we evaluated whether a potential dysfunction of HDL particles, in particular in their capacity to mediate key steps of the RCT pathway, might contribute significantly to accelerate atherosclerosis in FH patients.

Methods

Patients

Twelve patients aged 13 to 29 years and displaying a FH typical of the type IIa lipid phenotype (5 men and 7 women) were selected for the study. Twelve healthy normolipidemic subjects matched for age (mean, 23±0.6 years) and sex (5 men, 7 women) served as control subjects. Major clinical and biological characteristics of FH patients before and after cholesterol-lowering therapy and of normolipidemic control subjects are presented in Supplemental Table I, available online at http://atvb.ahajournals.org. Carotid and femoral artery atherosclerosis was determined by high-resolution B-mode ultrasound as previously described.19

The study was performed in accordance with the ethical principles set forth in the Declaration of Helsinki. Written informed consent was obtained from all patients.

Lipoprotein Fractionation and Pre-β HDL Quantification

Plasma lipoproteins were isolated from plasma by density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40 000 rpm for 48 hours in a Beckman XL70 at 15°C as previously described.20 The quantification of pre-β-HDL in plasma was performed as previously described.21 The amount of pre-β-HDL was expressed as the percentage of total apoAI (relative concentration) and as absolute concentration (mg/L apoAI) by multiplying its percentage by plasma apoAI levels. Total HDL mass was calculated by adding plasma concentrations of HDL2, HDL3, and pre-β-HDL.

Lipid and Protein Analysis

The lipid contents of plasma and isolated lipoprotein fractions, total protein, apoAI, apoAI, and apoB were quantified with an Autoanalyzer (Konelab 20). Details are given in the Supplemental Methods.

Free Cholesterol Efflux Assays

Lipid efflux assays using Fu5AH, Raw264.7, CHO-K1, Chinese hamster ovary (CHO)-human ABCG1, human THP-1 monocytic cells, and human monocyte-derived macrophages were performed as described previously.16,17,24 More details are given in the Supplemental Methods.

Statistical Analysis

All data are presented in mean±SE. Experimental data were analyzed using the SAS software (SAS/STAT User’s Guide, version 8, SAS Institute Inc., Cary, NC). Differences between patients displaying FH and healthy normolipidemic control subjects were analyzed using Mann-Whitney nonparametric analysis. The results were considered to be statistically significant at P<0.05.

Details of procedures for determination of endogenous plasma CETP activity, plasma phospholipid transfer protein (PLTP) activity, in vitro determination of selective uptake of HDL-CE, and in vivo metabolic studies are given in the Supplemental Methods.

Results

Plasma Lipid and Apolipoprotein Levels

Despite aggressive cholesterol-lowering therapy, type IIa hypercholesterolemic patients still displayed marked elevation in plasma total cholesterol (+140%; P<0.0001) and LDL cholesterol (+238%; P<0.0001) levels in comparison with healthy normolipidemic control subjects. FH patients were also characterized by significantly reduced plasma HDL cholesterol (−54%; P<0.0001) levels (Supplemental Table I). In addition, as shown in Table 1, treated FH patients still developed atherosclerosis. Indeed, mean carotid intima-media thickness (CIMT) for FH patients was 0.78±0.12 mm (ranging from 0.44 to 1.80), whereas mean CIMT for normolipidemic control subjects was 0.47±0.02 mm (ranging from 0.39 to 0.56). In addition, 3 of the 12 IIa hypercholesterolemic patients, representing 25% of our study population, displayed arterial plaques.

Table 1. CIMT and Femoral IMT in Type IIa FH Patients and Healthy NLS

<table>
<thead>
<tr>
<th></th>
<th>NLS (n=12)</th>
<th>FH (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIMT</td>
<td>0.47±0.02</td>
<td>0.78±0.12</td>
<td>0.0035</td>
</tr>
<tr>
<td>Right</td>
<td>0.47±0.02</td>
<td>0.78±0.12</td>
<td>0.0061</td>
</tr>
<tr>
<td>Left</td>
<td>0.47±0.02</td>
<td>0.77±0.12</td>
<td>0.002</td>
</tr>
<tr>
<td>Femoral IMT</td>
<td>0.47±0.02</td>
<td>0.51±0.07</td>
<td>NS</td>
</tr>
<tr>
<td>Right</td>
<td>0.48±0.03</td>
<td>0.53±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Left</td>
<td>0.47±0.02</td>
<td>0.49±0.07</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SE. IMT indicates intima-media thickness; NLS, normolipidemic control subjects; NS, not significant.

Characterization of Plasma HDL Subspecies

The total HDL mass concentration was significantly lower (−25%; P<0.0002) in type IIa hypercholesterolemic patients
compared with normolipidemic controls (277.9±16.9 and 374.2±11.0 mg/dL in FH and control subjects, respectively). This difference reflected a significantly lower (-34%; P<0.001) total plasma HDL2 (133.3±7.1 and 201.2±13.3 mg/dL in FH and control subjects, respectively), as well as a reduced (-20%; P<0.0003) plasma concentration of HDL3 (132.7±4.5 and 164.9±3.8 mg/dL in FH and control subjects, respectively) in FH patients compared with normolipidemic controls. The major HDL subfractions, ie, HDL2 and HDL3, from type IIa hypercholesterolemic patients were distinct in their chemical composition compared with their counterparts in normolipidemic control subjects (Supplemental Table II). Indeed, we observed an increase in HDL2-CE content (+12%; P<0.008) in type IIa patients compared with normolipidemic subjects. In addition, both HDL2 and HDL3 subfractions from type IIa patients were significantly depleted in phospholipids (HDL2: -18%, P=0.0001; HDL3: -10%, P<0.02) compared with their normolipidemic counterparts. To further investigate the observed difference in the HDL phospholipids content between FH and controls subjects, we identified molecular phospholipid species in HDL2 and HDL3 subfractions (Table 2). Interestingly, we observed a significant reduction in PC content in both HDL2 (-25%; P<0.02) and HDL3 (-13%; P<0.05) from FH patients compared with controls, whereas no modification in the SM content was detected in HDL particles between the 2 groups of subjects. The reduction in HDL-PC content resulted primarily from a significant decrease in PC 16:0/18:2 (-37% and -28%, in HDL2 and HDL3, respectively; P<0.007) and in PC 18:0/18:2 (-23% and -24%, in HDL2 and HDL3, respectively; P<0.03). In consequence, the SM/PC ratio was significantly increased in both HDL2 (+28%; P<0.02) and HDL3 (+21%; P<0.02) from type IIa hypercholesterolemic patients in comparison with their counterparts from healthy normolipidemic subjects.

### Table 2. Molecular Lipid Species in HDL Subfractions in Type IIa FH Patients and NLS

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>NLS (n=12)</th>
<th>FH (n=12)</th>
<th>P</th>
<th>NLS (n=12)</th>
<th>FH (n=12)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM 16:0</td>
<td>13.2±1.5</td>
<td>12.5±1.3</td>
<td>NS</td>
<td>9.7±0.8</td>
<td>8.1±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>SM 18:0</td>
<td>10.4±0.5</td>
<td>10.4±0.9</td>
<td>NS</td>
<td>8.5±0.5</td>
<td>10.1±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>PC 16:0/18:2</td>
<td>38.7±1.4</td>
<td>24.3±2.5</td>
<td>0.0039</td>
<td>31.0±2.6</td>
<td>21.1±1.5</td>
<td>0.0071</td>
</tr>
<tr>
<td>PC 18:0/18:2</td>
<td>16.7±2.0</td>
<td>12.8±1.3</td>
<td>0.0253</td>
<td>13.3±0.8</td>
<td>10.1±0.9</td>
<td>0.0209</td>
</tr>
<tr>
<td>PC 16:0/20:4</td>
<td>17.4±2.6</td>
<td>16.0±1.7</td>
<td>NS</td>
<td>15.6±1.8</td>
<td>15.0±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>PC 18:0/20:4</td>
<td>9.6±0.3</td>
<td>9.8±1.2</td>
<td>NS</td>
<td>7.6±0.4</td>
<td>9.2±1.2</td>
<td>NS</td>
</tr>
<tr>
<td>PC 16:0/22:6</td>
<td>10.2±0.7</td>
<td>6.7±0.9</td>
<td>0.0223</td>
<td>8.0±0.5</td>
<td>6.2±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>PC 18:0/22:6</td>
<td>2.9±0.3</td>
<td>2.4±0.3</td>
<td>NS</td>
<td>2.2±0.2</td>
<td>2.2±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>SM</td>
<td>23.6±1.7</td>
<td>22.9±1.4</td>
<td>NS</td>
<td>18.3±1.1</td>
<td>18.2±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>PC</td>
<td>95.5±3.8</td>
<td>72.0±5.1</td>
<td>0.0163</td>
<td>77.7±5.2</td>
<td>63.7±4.7</td>
<td>0.0433</td>
</tr>
<tr>
<td>SM/PC</td>
<td>0.25</td>
<td>0.32</td>
<td>0.0163</td>
<td>0.24</td>
<td>0.29</td>
<td>0.0141</td>
</tr>
</tbody>
</table>

Values are mean±SE. NLS, healthy normolipidemic subjects; NS, not significant.

Plasma Efflux Capacities

To determine whether quantitative and qualitative alterations observed on HDL particles from FH patients might influence the capacity of whole plasma to mediate the cellular free cholesterol efflux, we used three other cellular models, each representative of a specific cholesterol efflux pathway (Supplemental Table III).

### Determination of Cellular Free Cholesterol Efflux

#### Plasma Efflux Capacities

As shown in Figure 1, HDL2 particles obtained from hypercholesterolemic plasmas displayed a markedly reduced capacity to mediate cholesterol efflux through both SR-BI (-50%; P<0.0001; Figure 1A) and ABCG1 (-21%; P=0.0009; Figure 1B) compared with their counterparts from healthy normolipidemic subjects.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Bar graph showing the capacity of HDL2 or HDL3 subfractions isolated from type IIa hypercholesterolemic patients (n=12; black columns) and healthy normolipidemic control subjects (n=12; white columns) to mediate free cholesterol efflux through the SR-BI (A) and ABCG1 (B) pathways or from cholesterol-loaded THP-1 macrophages (C). Fractional free cholesterol efflux from cells was determined after 4 hours of incubation in the presence of isolated HDL particles: 10 μg phospholipids (PL)/mL for Fu5AH (SR-BI-dependent efflux), 5 μg PL/mL for CHO:human ABCG1 cells (ABCG1-dependent efflux), and 15 μg PL/mL for cholesterol-loaded THP-1 macrophages. Values are mean±SE. *P<0.05 vs controls.
isolated from normolipidemic control plasmas. In addition, we observed that the capacity of HDL3 particles to mediate cellular free cholesterol efflux via the SR-BI pathway was reduced significantly in FH patients (−24%; \(P<0.0001\); Figure 1A), whereas those via the ABCG1 pathway were not significantly different between the 2 subject groups (Figure 1B). Similar results were obtained when fractional cholesterol efflux to HDL particles was standardized on HDL apoAI concentrations (data not shown). We then evaluated the capacity of HDL particles to mediate cellular free cholesterol efflux from human cholesterol-loaded THP-1 macrophages (Figure 1C). We observed that HDL2 particles isolated from hypercholesterolemic plasmas displayed a significantly reduced capacity for cholesterol efflux from human-like macrophages (−27%; \(P=0.0006\)) compared with HDL2 particles from normolipidemic control subjects, whereas those of HDL3 were not altered.

It is of particular interest that our present study reveals an association between efflux capacity of large HDL2 particles and premature development of atherosclerosis, as shown by a significant inverse relationship between SR-BI-dependent HDL2 efflux capacity and CIMT (\(r=−0.473; P=0.0186\)), as well as between ABCG1-dependent HDL2 efflux capacity and CIMT (\(r=−0.485; P=0.0212\)). In addition, we also observed an association between SR-BI dependent efflux capacity of HDL3 particles and CIMT; however, this relationship did not reach statistical significance (\(r=−0.388; P=0.0606\)).

**Macrophage Efflux Capacities**

To evaluate whether macrophages isolated from hypercholesterolemic patients might display an alteration in their capacity to promote cholesterol efflux, we performed cholesterol efflux assays using human monocyte-derived macrophages isolated from FH and normolipidemic control subjects in the presence of various extracellular cholesterol acceptors (Supplemental Figure). We observed that human monocyte-derived macrophages isolated from FH patients or from control subjects displayed an equivalent capacity to promote cholesterol efflux to either lipid free apoAI, isolated HDL particles (HDL2 or HDL3), or 40-fold diluted plasma, indicating that SR-BI-, ABCA1-, or ABCG1-mediated efflux, as well as the overall efflux capacity of macrophages from FH patients, was not impaired.

**Determination of CE and Phospholipid Transfer Rates**

Plasma CETP activity, expressed as a percentage of CE transferred from HDL to apoB-containing lipoproteins, was significantly increased (+68%; \(P<0.0001\)) in FH patients compared with CE transfer activity determined in healthy normolipidemic control subjects (55.7±1.8% and 33.2±3.7% in FH and controls, respectively). This increase resulted primarily from a significant 3.5-fold increase (\(P<0.0001\)) in the rate of CE transfer from HDL to LDL, whereas rates of CE transfer from HDL to very low density lipoprotein+intermediate density lipoprotein were not significantly distinct between FH patients and control subjects (Supplemental Table IV). Expression of CE mass transferred from HDL to LDL as a function of plasma LDL concentration allows us to estimate the capacity of LDL particles to accept CE from HDL. We observed that the capacity of LDL particles to accept CE from HDL was not significantly different between the 2 groups (13.9±2.1 and 10.7±0.1 \(\mu\)g CE·h\(^{-1}\)·ml\(^{-1}\) LDL in FH patients and controls, respectively). Consistent with this latter observation, total CE transfer rates from HDL to apoB-containing lipoproteins were significantly correlated with plasma LDL levels (\(r=0.908; P<0.0001\)).

In addition, CE transfer rates from both HDL2 and HDL3 were considerably increased (2-fold and 5-fold from HDL2 and HDL3, respectively) in FH patients compared with normolipidemic controls. Interestingly, in normolipidemic subjects, HDL2 particles represented the major CE donor and accounted for 61% of the total CE transferred from HDL. By contrast, in FH patients, HDL3 particles appeared to be the major CE donor, accounting for 58% of the total CE transferred from HDL.

Plasma PLTP activity was significantly decreased (−37%; \(P=0.001\)) in FH patients compared with PLTP activity determined in healthy normolipidemic control subjects (25.2±1.8 and 40.0±3.2 pmol transferred/min in FH and controls, respectively).

**In Vitro Determination of Selective HDL-CE Uptake**

The capacity of HDL particles isolated from type IIa or from normolipidemic subjects to deliver CEs to hepatic cells was...
In Vivo Determination of HDL Capacity to Promote RCT

The capacity of HDL particles isolated from type IIa or from normolipidemic subjects to deliver CEs to the liver was evaluated in vivo using 2 distinct cellular models of human origin (HepG2) and of murine origin (Fu5AH) (Figure 2). HDL particles from FH patients displayed a significantly reduced capacity to deliver CE to hepatic cells compared with those isolated from normolipidemic subjects (−45% and −50% in Fu5AH and HepG2 cells, respectively).

In Vivo Determination of HDL Capacity to Promote RCT

We detected a significant increase in pre-β-HDL particle levels in plasma from FH patients compared with normolipidemic controls. This observation is entirely consistent with previous studies showing increased levels of pre-β1-HDL particles in coronary artery disease and in metabolic disorders, such as hypercholesterolemia and hypertriglyceridemia, or increased levels of pre-β-HDL concentrations in obese patients. Both CETP and PLTP have been shown to favor intravascular remodeling of HDL particles through different mechanisms. Indeed, CETP involves the conversion of large HDL particles into pre-β HDL, whereas PLTP favors the formation of both larger HDL and smaller HDL particles similar to pre-β HDL.

Our in vivo and in vitro observations are consistent in suggesting that HDL particles formed in FH patients displayed a defective capacity to mediate selective hepatic uptake of CE to the liver, leading to a reduced cholesterol elimination from the body. Indeed, it is important to keep in mind that mice do not have CETP, and thus SR-BI represents the unique way for hepatic HDL-CE uptake, whereas in humans, even in FH patients, alternative routes exist, such as via LDL-related receptor-1 and heparan sulphate proteoglycan following intravascular redistribution of CEs through the action of CETP.

Discussion

In the present study, we demonstrated that FH is associated with quantitative and qualitative modifications of HDL particles that significantly alter their functions in the RCT pathway. Indeed, we observed reduced plasma HDL2 and HDL3 levels in FH patients compared with controls. These quantitative modifications occurred concomitantly with a reduction in the capacity of large HDL2 particles to mediate cellular free cholesterol efflux via both SR-BI and ABCG1 pathways. In addition, our study reveals for the first time an inverse relationship between HDL efflux capacity and development of atherosclerosis in FH patients. We also observed an elevated CETP-mediated CE transfer from HDL2 and HDL3 particles to LDL and a reduced capacity of HDL particles to deliver CEs to the liver for cholesterol excretion. Taken together, these observations demonstrate that the centripetal movement of cholesterol from peripheral tissues to feces is defective in FH. Our present observations strengthen a very recent study showing that patients with coronary artery disease displayed an impaired efflux capacity of HDL by using an ex vivo measurement system. However, our present work adds significantly to this integrated/functional approach by addressing not only the cholesterol efflux capacity of plasma HDL but also the CETP-mediated CE transfer and the hepatic HDL-CE uptake.

We detected a significant increase in pre-β-HDL particle levels in plasma from FH patients compared with normolipidemic controls. This observation is entirely consistent with previous studies showing increased levels of pre-β-HDL particles in coronary artery disease and in metabolic disorders, such as hypercholesterolemia and hypertriglyceridemia, or increased levels of pre-β-HDL concentrations in obese patients. Both CETP and PLTP have been shown to favor intravascular remodeling of HDL particles through different mechanisms. Indeed, CETP involves the conversion of large HDL particles into pre-β HDL, whereas PLTP favors the formation of both larger HDL and smaller HDL particles similar to pre-β HDL.

Thus, it is likely that the increased levels of pre-β HDL observed in FH patients in this study primarily reflect the predominant action of CETP.

It has been previously demonstrated that ABCA1-dependent efflux is closely related to plasma pre-β-HDL levels. An increased capacity of whole plasma to mediate free cholesterol efflux via an ABCA1-dependent mechanism was therefore expected in FH patients. However, we observed a slight but not significant increase (+15%) in the ABCA1-dependent efflux of free cholesterol in FH patients compared with controls. This observation is consistent with previous reports showing that FH patients have an increased ABCA1-dependent efflux of free cholesterol. However, these studies were performed using whole plasma, whereas in our study, we used HDL particles isolated from FH patients.
dependently efflux capacity of plasma from FH patients compared with normolipidemic controls. Both apoAI synthesis and the fractional catabolic rate of apoAI are altered in hypercholesterolemia. Indeed, it has been previously demonstrated that apoAI production is reduced whereas the fractional catabolic rate of apoAI is accelerated in FH patients compared with control subjects. Because ABCA1 is known to mediate the export of cellular cholesterol to both lipid-free apoAI and to pre-β-HDL particles, it is likely that in FH patients, ABCA1-dependent cellular cholesterol efflux to free apoAI is reduced but concomitantly increased toward pre-β-HDL, resulting in an overall normal capacity of whole plasma to mediate cellular cholesterol efflux via the ABCA1 pathway. As mentioned above ABCA1 is responsible for the initial lipidation of apoAI and thus represents a critical molecule that regulates the initial step of the RCT pathway. Our observations might also suggest that levels of lipid free apoAI control ABCA1-dependent cholesterol efflux in a more efficient manner than those of pre-β-HDL.

In agreement with earlier studies, we observed here that CETP-mediated CE transfer from HDL to LDL is accelerated in FH patients compared with normolipidemic controls. This increase in plasma CETP activity is associated with an increase both in CETP mass concentration and in the number of lipoprotein acceptors, mainly LDL particles. Consistent with that observation, LDL particles isolated from FH patients displayed a significant enrichment in their CE content (Supplemental Table V).

The reduced capacity of HDL2 particles isolated from FH patients to mediate cellular cholesterol efflux via the SR-BI and ABCG1 pathways is entirely consistent with the observed modifications in lipid content, and more precisely, in phospholipid content, of HDL particles. It is well established that SR-BI- and ABCG1-dependent efflux preferentially involves large HDL particles and occurs in a phospholipid-dependent manner. In addition, PC represents an important phospholipid in HDL particles that determines the magnitude of cellular cholesterol efflux. Moreover, in good agreement with our present observations, it has recently been demonstrated that specifically increasing PC content in HDL particles leads to improve cholesterol efflux from SR-BI-overexpressing cells, as well as from human THP-1 cholesterol–loaded macrophages.

SR-BI mediates bidirectional flux of cholesterol between cell membranes and plasma lipoproteins. On the one hand, SR-BI is responsible for selective HDL lipid uptake in various tissues, including the liver, and on the other hand, SR-BI can mediate free cholesterol efflux from peripheral cells to HDL acceptors. Our data indicate that the reduction of both hepatic HDL-CE uptake and cellular free cholesterol efflux to HDL particles may contribute significantly to development of premature atherosclerosis in FH. The determinant atheroprotective role of SR-BI through its action on HDL- lipid uptake is well established, and so far SR-BI represents the only receptor that has been demonstrated to mediate selective HDL-CE uptake. The first human genetic variant of SR-BI has recently been reported, strengthening the major contribution of SR-BI in hepatic HDL-CE uptake. However, we cannot exclude the possibility that another receptor, as yet unidentified, might contribute to HDL-CE uptake in addition to SR-BI. Nevertheless, the implication of such a receptor likely represents a minor contribution in this mechanism. By contrast, the contribution of SR-BI to free cholesterol efflux from macrophages to HDL for protection against atherosclerosis in humans remains controversial. In this context, it is relevant to consider that SR-BI/CD36 and LIMPII analogous-1 is highly expressed in fully differentiated human macrophages and has recently been shown to contribute significantly to the elimination of free cholesterol from cholesterol-loaded human macrophages.

Our present observations allow us to propose an integrated mechanism of the RCT pathway in patients displaying FH (Figure 4). Key steps of the RCT, i.e., cellular free cholesterol efflux, CETP-mediated CE transfer between plasma lipoproteins, and hepatic CE delivery, are altered in type Ia patients compared with the normolipidemic situation. Indeed, the capacity of HDL particles to mediate free cholesterol from peripheral cells with subsequent CE delivery to the liver is impaired. The CE transfer rates from HDL to LDL are accelerated as a result of an increase in the circulating number of LDL particles. The elevated CETP-mediated CE transfer from HDL to LDL favors the formation of CE-enriched LDL particles. In conclusion, we have demonstrated in this study that HDL particles in patients displaying FH are dysfunctional in their capacity to mediate RCT, thus further enhancing the progression of atherosclerosis in these subjects. Interestingly, partial CETP inhibition has been shown to normalize defective atherogenic functions of HDL particles, and notably their capacity to deliver CE to the liver and to mediate cellular free cholesterol efflux via both SR-BI and ABCG1 pathways, in type IIIb hyperlipidemia. Together, these observations strongly support the contention that the normalization of HDL functions may constitute a promising strategy in the treatment of FH patients to restore a physiological removal of cholesterol from peripheral tissues, including the arterial wall, and to favor the direct return of cholesterol to the liver.
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Disclosures
None.

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Supplemental File Material

SUPPLEMENTAL METHODS

PATIENTS

Diagnostic for familial hypercholesterolemia was validated on the basis of DNA analysis. Most of the patients were homozygous (n=8) or heterozygous (n=3) for a single point mutation on the \textit{LDLR} gene. The remaining patient corresponded to a compound heterozygous displaying a single point mutation on both \textit{LDLR} and \textit{APOB} genes. All patients were treated daily with lipid-lowering drugs in combination (Atorvastatin/Ezetimibe, 80mg/10mg) and 10 patients regularly underwent LDL-apheresis at intervals of 2 to 3 weeks.

The Intima-Media Thickness (IMT) was measured on the far wall as the distance between the lumen–intima interface and the media–adventitia. All measurements of IMT were made at a site free of any discrete plaques. Near and far walls of all arterial segments were scanned longitudinally and transversally to assess the presence of plaques.

Blood samples were collected by venipuncture from the antecubital vein into sterile EDTA-containing tubes (final concentration of EDTA, 1mg/ml), and plasma separated immediately by low-speed centrifugation (2500 rpm) for 20 min at 4°C and stored at -80°C until use.

LIPID AND PROTEIN ANALYSIS

Reagent kits from Roche diagnostics and ThermoElectron were used for determination of total cholesterol and triglyceride levels, respectively. The levels of unesterified cholesterol and phospholipids were determined with reagent kits (Wako Diagnostics). Cholesteryl ester (CE) mass was calculated as (TC-FC) x 1.67 and thus represents the sum of the
esterified cholesterol and fatty acids moieties. BCA assay reagent (Pierce) was utilized for total protein quantification. Fasting plasma LDL-C was calculated using the Friedewald formula. HDL-C levels were determined after dextran sulphate-magnesium precipitation of apolipoprotein B-containing lipoproteins. Plasma ApoAl, ApoB and ApoAII concentrations were determined using immunoturbidimetric assays (ThermoElectron reagents and calibrators for apoAl and apoB determinations; Wako Diagnostics reagents and calibrators for apoAII determinations).

**FREE CHOLESTEROL EFFLUX ASSAYS**

All cells were maintained at 37°C in 5% CO₂. All media were supplemented with 1% L-glutamine and 0.75% penicillin-streptomycin.

Fu5AH cells were maintained in Eagle's MEM containing 5% new-born calf serum. After plating, cells were labelled by incubation with [³H]-cholesterol (1µCi/ml) in culture medium for 48h. Subsequently, Fu5AH cells were incubated for 24h in serum-free medium supplemented with BSA (0.5%). After equilibration, cholesterol acceptors were added in serum-free medium and incubated with cells for 4 hours at 37°C.

Raw264.7 cells were maintained in DMEM supplemented with 10% foetal bovine serum. The day after cell plating, cells were cholesterol loaded for 24 hours with 50µg/ml [³H]-cholesterol labelled acetylated LDL (0.5 µCi/ml) in serum-free DMEM containing glucose (50mM) and BSA (0.2%) (DGGB). After incubation, Raw264.7 cells were incubated with DGGB in the absence or presence of cAMP (0.3 mM) for 16 hours to induce ABCA1 expression. Cholesterol acceptors were added to Raw264.7 cells in serum-free DMEM for 4 hours at 37°C in the presence or absence of 0.3 mM 8-Br cAMP. The ABCA1-dependent efflux was calculated as the difference between fractional cholesterol efflux to cells in the presence or absence of 8-Br cAMP.
CHO-K1 cells (wild type and hABCG1 transfected cells) were maintained in Ham’s F-12 medium containing 10% foetal bovine serum. Two days after plating, cellular cholesterol was labelled by incubation of cells with culture medium and 1 µCi/ml [³H]-cholesterol for 24h. Equilibration of the label was performed for 90 min in serum-free medium and BSA (0.1%). After equilibration of labelling, acceptors were added to the cells in serum-free medium containing BSA (0.1%) for 4 hours at 37°C. The ABCG1-dependent efflux was calculated as the difference between efflux to hABCG1-transfected CHO-K1 cells and efflux to wild type CHO-K1 cells.

Monocytes were isolated from blood of individual subjects on Ficoll gradients (Ficoll-Paque Plus, GE Healthcare) and subsequently differentiated into human macrophages (HMDM) by adhesion on plastic Primaria plates (Falcon) over a period of 10 days of culture in RPMI 1640 medium supplemented with 10% heat-inactivated human serum and 20 ng/ml human-macrophage colony-stimulating factor (hM-CSF). THP-1 were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum and were differentiated into macrophage-like cells with 50ng/ml phorbol 12 myristate 13-acetate (PMA) for 3 days. Human THP-1 macrophages and HMDM were cholesterol loaded for 24 hours with 50µg/ml [³H]-cholesterol labelled acetylated LDL (1µCi/ml) in serum free RPMI 1640 supplemented with 50mM glucose, 2mM glutamine and 0.2% BSA (RGGB). The labelling medium was removed and human macrophages were then equilibrated in RGGB for an additional 24h-period. After equilibration, free cholesterol acceptors diluted in serum-free medium were added for 4hours at 37°C.

Lipid efflux assays were performed in the presence of the following cholesterol acceptors: 40-fold diluted plasma, 40-fold diluted apoB-depleted plasma, isolated HDL subfractions (5 to 15µg/PL) or lipid-free apoAI (5µg/ml; Biodesign). Optimal plasma dilutions of HDL
concentrations were determined on the basis of dose response curves for the release of free cholesterol from each cellular model as previously described.

Fractional cholesterol efflux was calculated as the amount of the label recovered in the medium divided by the total label in each well (radioactivity in the medium+radioactivity in the cells). The background cholesterol efflux obtained in the absence of any acceptor was subtracted from the efflux values obtained with the test samples. All efflux experiments were performed in triplicate for each sample.

**DETERMINATION OF PLASMA CETP AND PLTP ACTIVITIES**

Determination of endogenous CE transfer from HDL to apolipoprotein B-containing lipoproteins was assayed by modification of the method of Guerin et al. as previously described. Radiolabeled $^3$H-HDL-CE were prepared from the plasma of each subject as previously described. Cholesteryl ester transfer was determined after incubation of whole plasma (500 µl) from individual subjects at 37°C or 0°C for 3 hours in the presence of radiolabeled HDL (25 µg HDL-CE) and iodoacetamide (final concentration 1.5 mmol/l) for inhibition of Lecithin Cholesterol Acyltransferase (LCAT). Following incubation, plasma lipoproteins were fractionated by isopycnic density gradient ultracentrifugation and the radioactive content of each isolated lipoprotein fraction was quantified by liquid scintillation spectrometry with a Trilux 1450 (Perkin Elmer). The CETP-dependent CE transfer was calculated from the difference between the radioactivity transferred at 37°C and 0°C. The rate of CE transfer was calculated from the known specific radioactivity of radiolabelled HDL-CE and expressed as µg CE transferred.h$^{-1}$.ml$^{-1}$ plasma.

PLTP Activity Assay Kit were obtained from Roar Biomedical (New York, NY, USA). Plasma PLTP activity was measured according manufacturer’s instructions.
**In vitro Selective Hepatic Uptake of HDL-CE**

*In vitro* selective HDL-CE liver uptake via SR-BI was performed by using Fu5AH and HepG2 cell models as previously described. HepG2 cells were maintained in DMEM supplemented with 10% foetal bovine serum, 1% L-glutamine and 0.75% penicillin-streptomycin. Confluent cells were incubated in the presence of $^3$H-CE labelled HDL (60µg protein/ml) at 37°C for 5 hours. At the end of incubation, the medium was removed and cells were washed 4 times with PBS and incubated in the presence of an excess of unlabelled HDL (100µg protein/ml) for 30 minutes. Cells were then washed 4 times with PBS and solubilised with 200µl of NaOH 0.2N for 15 minutes at room temperature with gentle mixing. The radioactive content of 100µl of each cell lysate was measured by liquid scintillation counting and the protein content in each well was determined. Selective uptake was calculated from the known specific radioactivity of radiolabelled HDL-CE and is expressed in µgHDL-CE/µg cell protein.

**In vivo Metabolic Studies**

*In vivo* studies of the selective hepatic uptake of HDL-CE were performed as previously described. Wild type C57Bl/6 mice and *ldlr* -/- mice, aged 9 weeks, were housed in a temperature-controlled room under a 12-hour light-dark cycle with free access to standard chow diet and water. Radiolabeled HDL were prepared from the plasma fraction (d>1.063 g/ml) as previously described. Radiolabeled HDL preparations obtained from normolipidemic subjects or from FH patients displayed a specific radioactivity of approximately 20000 cpm/µg. $^3$H-CE labelled HDL (2.10$^6$ cpm/animal) was administered intravenously by retro-orbital injection. Blood samples (100µl) were drawn by retro-orbital puncture into heparinised capillary tubes at 3min, 2h, and 6h after injection. Feces were collected continuously over a 24 hour-period. At 24h after injection, mice were
anesthetized, exsanguinated and perfused with saline buffer through the left ventricle. Plasma samples were used for liquid scintillation counting. The liver was collected and stored at -80°C. Liver (approximately 100 mg) were minced, transferred into counting vials and liquid scintillation solution was added for radioactivity measurement. Total feces were dried at 50°C for 5h, weighed and rehydrated overnight with water (57mg/ml). Feces were then homogenised and 300µl of samples were combined with 10 ml of liquid scintillation for counting. All counts were expressed as a percentage of the total injected $^3$H tracer dose.
SUPPLEMENTAL RESULTS

**Plasma efflux capacities:** The capacity of whole plasma from FH patients to mediate cellular cholesterol efflux via the SR-BI pathway was significantly reduced (-22%; \( p=0.0015 \)) as compared to plasma from normolipidemic healthy control subjects. By contrast, no significant difference was detected between plasmas from the two groups in their capacity to mediate cholesterol efflux via ABCA1 or ABCG1 pathways. However, the ABCG1-dependent efflux measured in the presence of 40-fold diluted apoB-depleted plasma was significantly reduced (-18%; \( p=0.0003 \)) in FH patients as compared to control, thereby suggesting that the ABCG1-dependent efflux to HDL particles is equally reduced in FH patients in comparison with normolipidemic subjects.

It is relevant to note that efflux experiments performed by using whole plasma allow us to evaluate the overall capacity of given plasma to mediate cellular cholesterol efflux. Indeed, in addition to HDL particles, apoB-containing lipoproteins have been shown to represent potential cellular cholesterol acceptors for both the SR-BI and the ABCG1-mediated cholesterol efflux.\(^7\)\(^-\)\(^9\) Therefore, it is likely that modifications in both quantitative and qualitative features of various potential cholesterol acceptors within the plasma presently observed between FH patients and normolipidemic control subjects directly influence total plasma efflux capacity. When SR-BI dependent efflux experiments performed by using 40-fold diluted plasma are corrected according to plasma apoAI or HDL-C levels we observed that total efflux capacity of plasma from FH patients is significantly reduced by 15% (\( p<0.05 \)) as compared to those from control normolipidemic subjects. Correction of the ABCG1-dependent efflux to apoB depleted plasma according apoAI or HDL-C levels revealed a reduction of the ABCG1 dependent efflux to HDL particles from FH patients as compared to controls by 13%, however this latter reduction did not reach statistical significance.
SUPPLEMENTAL DISCUSSION

Analysis of neutral lipid core content of HDL particles failed to detect any depletion in CE content of HDL particles. It is necessary to consider the overall mechanism of action of CETP to interpret these apparent conflicting observations. In plasma, CETP facilitates the homo- or hetero-exchange of neutral lipids, cholesterol esters and triglycerides, between plasma lipoproteins particles. Homo-exchange of neutral lipids corresponds to bidirectional exchange of CE or TG between lipoprotein particles. By contrast, hetero-exchange involves transfer of CE from one lipoprotein to another with a simultaneous reciprocal TG transfer in the opposite direction. The relative lipid content of CE and TG within lipoprotein particles constitutes a major determinant of its specific contribution to neutral lipid transfer. Thus, neutral lipid transfer occurs primarily among lipoproteins with the greatest differences in core lipid composition. A net transfer of TG is frequently observed from VLDL to LDL and HDL particles with a reciprocal transfer of CE. By contrast, depending of the metabolic context, LDL particles may represent either an acceptor of CE transferred from HDL or a donor of CE to HDL. We presently observed that HDL and LDL particles isolated from FH patients displayed similar CE/TG ratio thereby indicating that homo-exchange of neutral lipids between HDL and LDL are probably favoured. By contrast, in normolipidemic subjects, CE/TG ratio of HDL particles was markedly higher than those observed in LDL particles probably reflecting hetero-exchange of CE from HDL to LDL.

Despite low plasma HDL2 levels and reduced capacity of these latter particles for the ABCG1 dependent efflux, the overall capacity of total plasma to remove cellular cholesterol via ABCG1 was not significantly altered in patients displaying Familial Hypercholesterolemia as compared to control subjects. It is important to note that in
addition to HDL particles, apoB-containing lipoproteins represent equivalent potential cellular free cholesterol acceptors for ABCG1-mediated cholesterol efflux. Efflux experiments performed in the presence of apoB-depleted plasma support the possibility that elevated plasma levels of LDL particles observed in FH patients might counterbalance deficient ABCG1-dependent cholesterol efflux towards HDL particles. Equally, we can not exclude the possibility that esterification of cholesterol and its transfer from HDL to apoB-containing lipoproteins as a result of the action of both LCAT and CETP might maintained cholesterol flux from cells to extracellular.

FH patients included in the present study were under lipid-lowering combination atorvastatin/ezetimibe therapy to reduce both de novo hepatic cholesterol synthesis and cholesterol absorption by the enterocyte. Therefore, we cannot exclude the possibility that some defective properties of HDL particles presently observed in FH patients might result in part from the action of the lipid-lowering therapy. However, simvastatin/ezetimibe association has been shown to moderately increase both plasma HDL-C and apoAI levels by up to 10% in patients with heterozygous familial hypercholesterolemia indicating that abnormal low HDL-C and apoAI levels presently observed in FH patients probably not result from statin/ezetimibe combination therapy (Supplemental Table I). In addition, it is relevant to note that atorvastatin directly affects both quantitative and qualitative features of apoB-containing lipoproteins but appears to lack impact on structural and functional atheroprotective activities of HDL particles, particularly their capacity to mediate cellular free cholesterol efflux and to deliver CE to the liver.

In the present study, we did not measured lysophosphatidylcholine in HDL subspecies. However, within the plasma, lysophosphatidylcholine is produced during hydrolysis of phosphatidylcholine by the enzymatic action of LpPLA2. It has been previously reported
that FH patients displayed a significant higher total plasma PLA2 activity as compared to normolipidemic control subjects \textsuperscript{13}. However, no significant difference in the HDL-associated PLA2 activity was observed between FH and control subjects therefore suggesting that a similar lysoPC content in HDL particles in either patient group \textsuperscript{13}.
REFERENCES


Supplemental Table I:

Major clinical and biological characteristic of type IIa hypercholesterolemic patients (FH) and healthy normolipidemic control subjects (NLS)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Duration of therapy (y): Ator+Eze/LDLapheresis</th>
<th>TC</th>
<th>TG</th>
<th>LDL-C</th>
<th>HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH 1</td>
<td>23</td>
<td>M</td>
<td>3/19</td>
<td>Initial</td>
<td>Current</td>
<td>726</td>
<td>98</td>
</tr>
<tr>
<td>FH 2</td>
<td>26</td>
<td>M</td>
<td>6/13</td>
<td>Initial</td>
<td>Current</td>
<td>722</td>
<td>131</td>
</tr>
<tr>
<td>FH 3</td>
<td>26</td>
<td>M</td>
<td>6/16</td>
<td>Initial</td>
<td>Current</td>
<td>535</td>
<td>80</td>
</tr>
<tr>
<td>FH 4</td>
<td>13</td>
<td>F</td>
<td>6/11</td>
<td>Initial</td>
<td>Current</td>
<td>1450</td>
<td>133</td>
</tr>
<tr>
<td>FH 5</td>
<td>26</td>
<td>F</td>
<td>4/8</td>
<td>Initial</td>
<td>Current</td>
<td>1140</td>
<td>190</td>
</tr>
<tr>
<td>FH 6</td>
<td>26</td>
<td>F</td>
<td>6/16</td>
<td>Initial</td>
<td>Current</td>
<td>950</td>
<td>135</td>
</tr>
<tr>
<td>FH 7</td>
<td>29</td>
<td>M</td>
<td>6/9</td>
<td>Initial</td>
<td>Current</td>
<td>900</td>
<td>103</td>
</tr>
<tr>
<td>FH 8</td>
<td>20</td>
<td>F</td>
<td>5/0</td>
<td>Initial</td>
<td>Current</td>
<td>640</td>
<td>62</td>
</tr>
<tr>
<td>FH 9</td>
<td>21</td>
<td>F</td>
<td>6/17</td>
<td>Initial</td>
<td>Current</td>
<td>1140</td>
<td>93</td>
</tr>
<tr>
<td>FH 10</td>
<td>26</td>
<td>F</td>
<td>6/17</td>
<td>Initial</td>
<td>Current</td>
<td>950</td>
<td>86</td>
</tr>
<tr>
<td>FH 11</td>
<td>13</td>
<td>F</td>
<td>5/0</td>
<td>Initial</td>
<td>Current</td>
<td>608</td>
<td>75</td>
</tr>
<tr>
<td>FH 12</td>
<td>21</td>
<td>M</td>
<td>3/12</td>
<td>Initial</td>
<td>Current</td>
<td>800</td>
<td>195</td>
</tr>
<tr>
<td>FH (n=12) Mean ± SE</td>
<td>22±1.5</td>
<td>5M/7F</td>
<td>-</td>
<td>Initial</td>
<td>Current</td>
<td>880±77</td>
<td>115±12</td>
</tr>
<tr>
<td>NLS (n=12) Mean ± SE</td>
<td>23±0.6</td>
<td>5M/7F</td>
<td>-</td>
<td>186±6</td>
<td>77±6</td>
<td>121±6</td>
<td>50±3</td>
</tr>
</tbody>
</table>

Initial plasma lipid values refer to lipid determination performed at the moment of FH diagnosis before any lipid-lowering therapy.
Supplemental Table II:
Percentage Weight Chemical composition of HDL subfractions in type IIa hypercholesterolemic patients (FH) and healthy normolipidemic control subjects (NLS)

<table>
<thead>
<tr>
<th></th>
<th>HDL2</th>
<th></th>
<th></th>
<th>HDL3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NLS (n=12)</td>
<td>FH (n=12)</td>
<td>p value</td>
<td>NLS (n=12)</td>
<td>FH (n=12)</td>
<td>p value</td>
</tr>
<tr>
<td>Free Cholesterol</td>
<td>5.3±0.1</td>
<td>5.7±0.3</td>
<td>ns</td>
<td>2.4±0.2</td>
<td>2.9±0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>22.8±0.5</td>
<td>25.5±0.7</td>
<td>0.0081</td>
<td>13.0±0.4</td>
<td>13.0±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>3.2±0.4</td>
<td>3.0±0.3</td>
<td>ns</td>
<td>2.4±0.3</td>
<td>2.4±0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>31.0±0.5</td>
<td>25.5±0.8</td>
<td>0.0001</td>
<td>17.6±0.4</td>
<td>15.8±0.5</td>
<td>0.0138</td>
</tr>
<tr>
<td>Total Protein</td>
<td>37.7±0.6</td>
<td>40.3±1.4</td>
<td>ns</td>
<td>64.7±0.7</td>
<td>66.0±1.4</td>
<td>ns</td>
</tr>
<tr>
<td>CE/TG</td>
<td>7.12</td>
<td>8.50</td>
<td>ns</td>
<td>5.42</td>
<td>5.42</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are mean±SE.
Supplemental Table III:
Plasma Cellular Free Cholesterol Efflux capacities in type IIa hypercholesterolemic patients (FH) and healthy normolipidemic control subjects (NLS)

<table>
<thead>
<tr>
<th>Efflux pathway</th>
<th>NLS (n=12)</th>
<th>FH (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-BI Fu5AH cells</td>
<td>19.1±0.8</td>
<td>14.8±0.5</td>
<td>0.0015</td>
</tr>
<tr>
<td>ABCA1 Raw264.7 cells</td>
<td>6.7±0.2</td>
<td>13.1±0.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>cAMP-pretreated RAW264.7 cells</td>
<td>24.8±0.6</td>
<td>33.9±0.3</td>
<td>0.0282</td>
</tr>
<tr>
<td>ABCA1-dependent efflux</td>
<td>18.1±1.8</td>
<td>20.8±2.2</td>
<td>ns</td>
</tr>
<tr>
<td>ABCG1 Total plasma</td>
<td>22.9±0.4</td>
<td>22.6±0.4</td>
<td>ns</td>
</tr>
<tr>
<td>ApoB-depleted plasma</td>
<td>30.7±0.9</td>
<td>25.2±0.6</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Values are mean ± SE and are expressed in fractional efflux (%). Efflux were measured following 4h incubation in the presence of 40-fold diluted plasma or apoB-depleted plasma. Efflux to apoB depleted plasma was measured after dextran-sulphate precipitation of apoB containing lipoproteins.
**Supplemental Table IV:**

**Cholesteryl ester transfer rates in type IIa hypercholesterolemic patients (FH) and healthy normolipidemic control subjects (NLS)**

<table>
<thead>
<tr>
<th></th>
<th>NLS (n=12)</th>
<th>FH (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rates of CE transfer to</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL+IDL</td>
<td>5.6±1.0</td>
<td>8.4±1.4</td>
<td>ns</td>
</tr>
<tr>
<td>LDL</td>
<td>31.6±2.3</td>
<td>111.2±10.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Rates of CE transfer from</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL2</td>
<td>22.8±1.3</td>
<td>49.9±6.4</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>HDL3</td>
<td>14.2±2.5</td>
<td>69.9±7.5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Rates of CE transfer are expressed in µg CE transferred.h⁻¹.ml⁻¹ plasma. Values are mean±SE.
Supplemental Table V:
Total Mass Concentration and mean weight percent chemical composition of VLDL and LDL particles in Type IIa Hypercholesterolemic patients (FH) and healthy normolipidemic control subjects (NLS)

<table>
<thead>
<tr>
<th></th>
<th>VLDL</th>
<th>LDL</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NLS (n=12)</td>
<td>FH (n=12)</td>
<td></td>
</tr>
<tr>
<td>Total Mass, mg/dl</td>
<td>68±9.2</td>
<td>56.0±8.8</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>298.7±17.0</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>5.5±0.2</td>
<td>5.4±0.6</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.0±0.3</td>
<td>11.1±0.5</td>
</tr>
<tr>
<td>Cholesteryl Esters</td>
<td>12.0±0.6</td>
<td>17.2±1.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34.9±0.8</td>
<td>41.7±0.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>52.4±1.2</td>
<td>45.1±1.9</td>
<td>&lt;0.007</td>
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<tr>
<td></td>
<td></td>
<td>10.8±0.6</td>
<td>7.1±0.5</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>17.0±0.2</td>
<td>16.7±0.3</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22.7±0.4</td>
<td>21.7±0.2</td>
</tr>
<tr>
<td>Total Protein</td>
<td>13.1±0.8</td>
<td>15.6±1.1</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.6±1.0</td>
<td>18.4±0.9</td>
</tr>
<tr>
<td>CE/TG</td>
<td>0.23</td>
<td>0.40</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.23</td>
<td>5.87</td>
</tr>
</tbody>
</table>

Values are mean±SE.
Supplemental Figure: Fractional free cholesterol efflux from human macrophage derived monocytes (HMDM) isolated from type IIa hypercholesterolemic patients (n=12; closed bar) and healthy normolipidemic control subjects (=12; open bar) determined after 4h incubation in the presence of various acceptors: HDL2 or HDL3 particles (30µgPL/ml), lipid-free apoAl (5µg/ml; Biodesign) and 40-fold diluted plasma. Plasma coming from a single normolipidemic subject not include in the study was used to isolate HDL2 and HDL3. Values are mean±SE.