Enhanced HDL Functionality in Small HDL Species Produced Upon Remodeling of HDL by Reconstituted HDL, CSL112

Effects on Cholesterol Efflux, Anti-Inflammatory and Antioxidative Activity

Svetlana A. Didichenko, Alexei V. Navdaev, Alexandre M.O. Cukier, Andreas Gille, Patrick Schuetz, Martin O. Spycher, Patrice Thérond, M. John Chapman, Anatol Kontush, Samuel D. Wright

Rationale: CSL112, human apolipoprotein A-I (apoA-I) reconstituted with phosphatidylcholine, is known to cause a dramatic rise in small high-density lipoprotein (HDL).

Objective: To explore the mechanisms by which the formation of small HDL particles is induced by CSL112.

Methods and Results: Infusion of CSL112 into humans caused elevation of 2 small diameter HDL fractions and 1 large diameter fraction. Ex vivo studies showed that this remodeling does not depend on lipid transfer proteins or lipases. Rather, interaction of CSL112 with purified HDL spontaneously gave rise to 3 HDL species: a large, spherical species composed of apoA-I from native HDL and CSL112; a small, disc-shaped species composed of apoA-I from CSL112, but smaller because of the loss of phospholipids; and the smallest species, lipid-poor apoA-I composed of apoA-I from HDL and CSL112. Time-course studies suggest that remodeling occurs by an initial fusion of CSL112 with HDL and subsequent fission leading to the smaller forms. Functional studies showed that ATP-binding cassette transporter 1–dependent cholesterol efflux and anti-inflammatory effects in whole blood were carried by the 2 small species with little activity in the large species. In contrast, the ability to inactivate lipid hydroperoxides in oxidized low-density lipoprotein was carried predominantly by the 2 largest species and was low in lipid-poor apoA-I.

Conclusions: We have described a mechanism for the formation of small, highly functional HDL species involving spontaneous fusion of discoidal HDL with spherical HDL and subsequent fission. Similar remodeling is likely to occur during the life cycle of apoA-I in vivo. (Circ Res. 2016;119:751-763. DOI: 10.1161/CIRCRESAHA.116.308685.)

Key Words: antioxidant ■ apolipoprotein A-I ■ cholesterol ■ high-density lipoproteins ■ inflammation

High-density lipoprotein (HDL) cholesterol (HDL-C) levels poorly predict the ability of plasma to promote cholesterol efflux from cells and to reduce inflammatory responses of cells ex vivo. For example, plasma samples from patients with median levels of HDL-C may have cholesterol efflux values either well above or below the median.1 Importantly, efflux values to apolipoprotein B–depleted plasma are much stronger predictors of risk of coronary heart disease than HDL-C values.2–4 This illustrates both the disconnection of functional measures of HDL-C and the potential value of functional measures in understanding human disease. This point is further emphasized by observations on the cholesterylester transfer protein (CETP) inhibitor, dalcetrapib. Treatment with dalcetrapib elevated HDL-C by ≈30% but increased total cholesterol efflux (primarily ATP-binding cassette transporter [ABCA1]–independent efflux) by <10%.5

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Recent work has described a mechanism by which measures of HDL-C and cholesterol efflux may diverge. The largest source of patient-to-patient variation in cholesterol efflux is the ABCA1-dependent fraction of efflux.1 The ABCA1 transporter...
efficiently moves cellular cholesterol exclusively to small, lipid-poor, protein-rich HDL species but is practically inactive with large, cholesterol-rich HDL species. In keeping with these findings, ABCA1-dependent efflux capacity in human plasma samples correlates closely with levels of the smallest HDL form, which is known as preβ1-HDL, very small-HDL (VS-HDL), or lipid-poor apolipoprotein A-I (apoA-I). Thus, the HDL species that make the largest contribution to cholesterol efflux carry the least cholesterol. The factors that regulate formation of these highly functional but low-cholesterol species of HDL are key to understanding the relationship of HDL to cardiovascular risk. About 5% of plasma apoA-I is present as a lipid-poor species as measured with 2-dimensional (2D) gel electrophoresis or with a specific ELISA.10

The steps by which lipid-poor apoA-I acquires cholesterol and grows from discs to spheres through the action of lecithin:cholesterol acyltransferase (LCAT) are relatively well understood. In contrast, the process by which apoA-I is recycled from mature HDL to become lipid-poor is less well studied. One proposed process involves lipolysis of chyomicrons and generation of lipid-poor apoA-I. In a similar fashion, lipolysis of HDL by hepatic and endothelial lipase can lead to particle remodeling with the release of lipid-poor apoA-I. In another proposed process, CETP catalyzes interaction of HDL with triglyceride-rich lipoproteins to yield lipid-poor apoA-I and HDL of a larger size, whereas phospholipid transfer protein (PLTP) may catalyze interaction of HDL particles to yield both lipid-poor apoA-I and lipoproteins of a larger size. A common feature of all these processes is that all are catalyzed by specific lipid transfer proteins or lipases.

We have studied formation of lipid-poor apoA-I in blood and plasma with a novel agent. CSL112 is human apoA-I reconstituted into disc-shaped lipoproteins with phosphatidylcholine in a form suitable for intravenous infusion. CSL112 is currently in phase II clinical development for the treatment of acute coronary syndrome. We have previously observed that infusion of CSL112 into healthy volunteers causes HDL remodeling with an extraordinary rise in lipid-poor apoA-I measured by ELISA with levels rising ≤36-fold. Here we describe the mechanism behind this elevation and characterize the composition and biological activities of the products of HDL remodeling. To our surprise, we found that generation of lipid-poor apoA-I seems not to require triglyceride-rich lipoproteins, CETP, PLTP, or lipases. Rather, the lipid-poor apoA-I is formed on the spontaneous interaction of CSL112 with HDL2 or HDL3, deriving equally from the mature HDL and from CSL112. We found that the small HDL species resulting from this remodeling mediate the majority of the ABCA1-dependent cholesterol efflux and exert potent anti-inflammatory effects.

**Methods**

An expanded Methods section is available in Material section Online Data Supplement.

**Reconstituted HDL**

CSL112 particles contain 2 molecules of apoA-I and 110 molecules of phosphatidylcholine per particle as described. HDL labeling, lipoprotein isolation, and in vitro incubations are described in the Online Data Supplement.

**Electron Microscopy**

Negative stain electron microscopy was performed as described previously.

**Cholesterol Efflux Assay**

The capacity of the HDL to efflux cholesterol was assessed using [3H]-cholesterol-loaded RAW264.7 macrophages as previously described.

**Work With Human Subjects**

Plasma samples of the healthy subjects from a completed phase I clinical trial (NCT01281774) were obtained at multiple time points for assessment of apoA-I by nephelometry (Pacific Biomarkers Inc) and 2D-gel (3%–35% concave gradient) electrophoresis (Boston Heart Diagnostics).

Western blotting of 2D-gradient gels was conducted using polyclonal anti-apoA-I and diluted anti-albumin antibodies. Relative apoA-I content in HDL subfractions on blots was quantified by densitometry as a percentage of the total combined signal (100% per blot).

**Stimulation of Peripheral Blood Mononuclear Cells, Cytokine Measurements, and Cell Lysis**

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation using Ficoll-Pague Plus (GE Healthcare). PBMCs were cultured in RPMI 1640 supplemented with 5% fetal calf serum (Gibco), 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at a density of 1 to 1.5×10⁶ cells per well in 48-well plates. Cells were stimulated with 1 µg/mL phytohemagglutinin M (Calbiochem) in the presence or absence of different HDL preparations and incubated for 20 hours at 37°C in a CO₂ incubator. To prevent potential effects of endotoxin contamination, cell culture medium was supplemented with 1 µg/mL polymyxin B (Sigma-Aldrich); HDL and phytohemagglutinin M were preincubated with polymyxin B for 15 minutes before being added to PBMC. Tumor necrosis factor-α, interleukin-1β (IL-1β), IL-6, and macrophage inflammatory protein-1β were measured in cell-free supernatants using Human Cytokine Magnetic 4-Plex Panel (R&D Systems).

**Phospholipid Hydroperoxide Inactivating Capacity of HDL**

Reference low-density lipoprotein (LDL) obtained from a healthy normolipidemic subject was preoxidized at 40-mg total cholesterol/dL with 4-mmol/L 2,2′-Azobis(2-amidinopropane) dihydrochloride treatment.
for 6 hours at 37°C. Oxidative modification was terminated by addition of EDTA (10 µmol/L) and butylated hydroxytoluene (10 µmol/L). Oxidized LDL (oxLDL) was dialyzed against PBS at 4°C to remove EDTA and excess butylated hydroxytoluene, and incubated at a concentration of 20 mg total cholesterol/dL for 4 hours in the presence or absence of HDL (8 mg total protein/dL) in PBS at 37°C. EDTA (100 µmol/L) was present to inhibit lipid peroxidation during the incubation. Phospholipid hydroperoxides (PLOOH) were quantified in the reaction mixture before and after incubation by high-performance liquid chromatography with chemiluminescent detection as described elsewhere. To measure apoA-I content of native and oxidized methionine (Met) residues, HDL was subjected to high-performance liquid chromatography with ultraviolet detection at 214 nm as previously described.

Statistics

Values are presented as mean±SDs. All results were analyzed for statistical significance using 1-way ANOVA followed by Dunnet post hoc test. Statistical significance was set at P<0.05.

Results

CSL112 Is Rapidly Remodeled on Infusion into Human Subjects

HDL remodeling was studied in selected healthy subjects receiving infusions of 6.8 g of CSL112 or placebo (n=4 per group) from a completed phase I clinical trial (NCT01281774). Infusion with CSL112 resulted in immediate elevation of plasma apoA-I (Figure 1A) and profound changes in concentration and size distribution of plasma HDL subclasses (Figure 1C) as assessed by 2D-gel electrophoresis and Western blotting with anti–apoA-I antibody. Individual HDL subpopulation, α-migrating (α1, α2, α3, and α4), and preβ-migrating (lipid-poor apoA-I) HDL particles were identified, and their concentrations were quantified by densitometry as described in Materials and Methods section of this article (Figure 1D). Figure 1B and 1C demonstrate that at 2- to 4-hour postinfusion parent CSL112 was no longer seen in plasma, and several HDL species became elevated: lipid-poor apoA-I (preβ1-HDL), small (α3 and α4) HDL, and large (α1 and α2) HDL. The most prominent increase in concentration was observed for lipid-poor apoA-I and α1 HDL (Figure 1D). These data suggest that CSL112 caused HDL particle remodeling and was incorporated into the endogenous HDL pool. CSL112 does not fuse with large apolipoprotein B (apoB)-containing lipoproteins or affect the distribution of apolipoprotein E (apoE; Online Figure I). Concentrations of apoA-II did not change immediately after the infusion of CSL112. However at 24 hours post infusion, there was a modest dose-dependent increase in apoA-II not exceeding 10% of the baseline apoA-II concentration (Online Figure II).

HDL Remodeling Induced by CSL112 in Plasma Ex Vivo Is a Time- and Temperature-Dependent Process

The early effects of the infusion of CSL112 into human subjects, including a rapid increase in concentration of lipid-poor apoA-I, apparent changes in the HDL2 and HDL3 particle size distribution and disappearance of parent CSL112 (Figure 1B and 1C; Online Figure III), were nearly identical to those observed on short-term incubation of CSL112 with human plasma ex vivo. Additional studies, therefore, used ex vivo incubations to identify factors underlying these changes. First, we examined whether inhibition or stimulation of the plasma factors LCAT, CETP, PLTP, and secretory phospholipase 2 could affect the observed formation of lipid-poor apoA-I after the addition of CSL112 to human plasma. Incubations were carried out for 1 hour at 37°C in the presence
of LCAT inhibitor (5,5-dithiobis(2-nitrobenzoic acid), a CETP inhibitor (Torcetrapib), inhibitors of secretory phospholipase 2 (LY 311727) or a PLTP stimulator (4-(2-aminoethyl)-benzenesulphonyl fluoride). We observed no significant differences in levels of lipid-poor apoA-I (measured by ELISA) and in the size distribution of HDL population after incubation of CSL112 with control plasma or plasma treated with different inhibitors (Online Figures IVA and IVB). Importantly, addition of CSL112 to plasma dramatically elevated both ABCA1-dependent and ABCA1-independent efflux, and this elevation was similar under all conditions tested (Online Figure IVC). Additional confirmatory studies showed normal remodeling in plasma from mice deficient in PLTP or LCAT (Online Figure V).

To evaluate the temperature dependence of HDL remodeling, CSL112 was incubated with plasma for different periods of time at 0°C or at 37°C, and plasma lipoproteins were separated on nondenaturing gradient gels followed by the detection of apoA-I-containing particles by Western blotting using an anti–apoA-I antibody. Incubation at 37°C led to changes in HDL particle size distribution and progressive accumulation of lipid-poor apoA-I with clear effects already visible by 15 to 30 minutes, whereas incubation at 0°C resulted in no remodeling (Figure 2A). Heating of the plasma for 1 hour at 56°C before incubation with CSL112 for another hour did not inhibit the HDL remodeling suggesting that heat-sensitive factors present in normal plasma are not required (Figure 2B). However, when lipoproteins (very low-density lipoprotein/LDL and HDL) were depleted from normal plasma by density gradient centrifugation, even prolonged incubation with CSL112 for 4 hours at 37°C led to no increase in levels of lipid-poor apoA-I (Figure 2C). Moreover, trace amount of lipid-poor apoA-I present in very low-density lipoprotein/LDL and HDL-depleted plasma appeared to be fully incorporated into CSL112 particles during incubation. These data suggest that a direct interaction between CSL112 and plasma lipoproteins is necessary for remodeling in the plasma compartment.

Interaction Between HDL and CSL112 Is Sufficient for HDL Particle Remodeling

To determine whether interaction with HDL alone is sufficient for particle remodeling by CSL112, we used purified HDL2 or HDL3 for the incubation with CSL112 at 37°C for various periods of time and assessed HDL particle size by nondenaturing polyacrylamide gradient gel electrophoresis followed by Western blotting with anti–apoA-I antibody. In this reconstituted system, HDL is progressively converted into larger particles (L-HDL<sub>rem</sub>, ≈10–12 nm; Figure 3A). Simultaneously, large amount of lipid-poor apoA-I (≈5.4 nm) are produced. Comparable changes were seen with either HDL3 or HDL2. These changes were accompanied by simultaneous, progressive reduction in the size of parent CSL112 particles (≈7.6–7.9) and accumulation of smaller species (S-HDL<sub>rem</sub>, ≈7.3–7.6 nm). In contrast, incubation of CSL112, HDL2, or HDL3 by themselves lead to no changes in size distribution or amount of lipid-poor apoA-I (Online Figure VI). Thus, addition of CSL112 induces remodeling of purified HDL and the changes seen after the admixture of these purified species recapitulated the remodeling in whole plasma.

Origin and Destination of apoA-I During Remodeling

Protein labeling of CSL112 or HDL with biotin or with DyLight 488 fluorophore was undertaken to follow the distribution of specifically labeled apoA-I among the 3 products of particle remodeling. When biotinylated CSL112 was incubated with purified LDL for 1 hour at 37°C, we observed no remodeling of biotinylated CSL112 particles and no binding of biotinylated apoA-I to LDL (Figure 3B). However, after the incubation of biotinylated CSL112 with HDL2 or HDL3,
all 3 products of remodeling (L-HDLrem, S-HDLrem, and lipid-poor apoA-I) were found to incorporate biotin label and could be removed from incubation mixtures with streptavidin sepharose (Figure 3B, right). These data suggest that CSL112-derived apoA-I moves to all species during remodeling.

Consistently, on incubation of CSL112 labeled with DyLight 488 fluorophore and biotinylated HDL3, fluorescently labeled apoA-I derived from CSL112 was also detected in all products of the remodeling (Figure 3C, middle). In contrast, placement of the biotin label on HDL3 showed that HDL3-derived biotinylated apoA-I was primarily found in L-HDLrem and lipid-poor apoA-I, but not in S-HDLrem (Figure 3C, left). Precipitation with streptavidin beads depleted fluorescently labeled L-HDLrem and lipid-poor apoA-I from the incubation mixtures (Figure 3C, middle) but did not deplete S-HDLrem.

These findings indicate that during remodeling apoA-I moves from HDL3 to L-HDLrem and to the lipid-poor apoA-I fraction but not to S-HDLrem. The ability of streptavidin to precipitate both biotinylated and fluorescently labeled apoA-I in L-HDLrem indicates that apoA-I originating from HDL3 and CSL112 comes to reside on the same particle. We further presume that under the conditions of our experiments, lipid-poor apoA-I reaches a concentration sufficient to drive the well-characterized, concentration-dependent self-association of apoA-I to form dimers and multimers, and this oligomerization accounts for the coprecipitation.

The movement of apoA-I from CSL112 and HDL3 to the 3 products of remodeling is consistent with a model involving transient fusion of CSL112 with HDL3 and subsequent fission to lead to the products (Online Figure VII).
Dynamics of Transfer of Protein and Lipid Content During Particle Remodeling

To closely follow the dynamics of the particle remodeling, we used CSL112 labeled with DyLight 488 fluorophor and HDL3 labeled with DyLight 680 fluorophor for the incubation, and monitored the distribution of specifically labeled apoA-I among the products of the remodeling over time, from 45 minutes to 3 hours. Fluorescence imaging revealed that both DyLight 488 apoA-I and DyLight 680 apoA-I derived from CSL112 and HDL3, respectively, progressively accumulated in lipid-poor apoA-I in a continuous, time-dependent manner (Figure 4A). Formation of large HDL subspecies (L-HDL^rem) was characterized by a rapid association of parent HDL3 with DyLight 488 apoA-I from CSL112, but its abundance in large HDL subspecies remained unchanged. S-HDL^rem particles were primarily composed of DyLight 488 apoA-I after 45 minutes of incubation confirming that they originate from CSL112. During prolonged incubation (between 90 minutes and 3 hours), DyLight 488 apoA-I was partially displaced by DyLight 680 apoA-I from HDL3 in a time-dependent manner, suggesting ongoing interaction between S-HDL^rem and L-HDL^rem. Such ongoing interaction is also suggested by the continued growth in the size of L-HDL^rem during this period (Figure 4A, right).

Next, we examined whether phospholipid transfer between CSL112 and HDL may contribute to the decline of CSL112 size during conversion to S-HDL^rem and increase in size of HDL3 during conversion to L-HDL^rem. CSL112 was labeled with a fluorescent phospholipid analog (18:1–06:0 NBD-PC). After labeling, the molar ratio of phosphatidylcholine/apoA-I in NBD-PC–labeled CSL112 was increased compared with that in unlabeled CSL112 (72:1 and 50:1, respectively). Incubation of HDL3 with NBD-PC–labeled CSL112 resulted in a rapid movement of the major portion of CSL112-derived phospholipid to HDL on remodeling.
fluorescent lipid into L-HDLrem (Figure 4B, right). S-HDLrem showed low levels of the fluorescent lipid deriving from CSL112. Thus, remodeling of CSL112 to S-HDLrem involves retention of apoA-I (Figure 4A, left) but loss of phospholipid (Figure 4B, right) and transfer of the phospholipid to L-HDLrem.

These data further support remodeling by a transient fusion of CSL112 and HDL (Online Figure VII). Subsequent fission yields lipid-poor apoA-I composed of apoA-I from both CSL112 and HDL3, and enlarged HDL3 (L-HDLrem) particles made larger by receipt of both apoA-I and phospholipid from CSL112. The third product, S-HDLrem, seems to be CSL112, which has lost some of its phospholipid to HDL3. This model was further tested by compositional analysis of the isolated products of remodeling.

**Lipid Composition, Morphology, and Structure of the Individual Products of HDL Remodeling**

The 3 individual products of remodeling, L-HDLrem (=10–11 nm), S-HDLrem (=7.3–7.6 nm), and lipid-poor apoA-I (=5.4 nm), were isolated out of the incubation mixture of CSL112 and HDL3 by preparative ultracentrifugation. Particle purity was analyzed by gradient gel electrophoresis (Online Figure VIII), particle content of choline-containing phospholipids was measured enzymatically and by high-performance liquid chromatography, and data were expressed as the lipid/total protein ratio (Online Table I). We observed that the phospholipid/total protein ratio of L-HDLrem was significantly increased (2-fold) compared with parent HDL3, whereas for the S-HDLrem, this ratio was decreased (2.3-fold) compared with parent CSL112. The amount of phospholipid measured in lipid-poor apoA-I was low and close to the detection limit (Online Table I).

Next, phospholipid content of CSL112, HDL3 and purified products of the remodeling was analyzed by mass spectroscopy. The fatty acid chain lengths of the phosphatidylcholine in CSL112 differ from those in HDL3 (Online Table II), in particular, phosphatidylcholine (18:2/18:2) is abundant in CSL112 but rare in HDL3. This fatty acid signature of phospholipid originally present in CSL112 was found at high levels in L-HDLrem (Table), confirming studies with NBD-phosphatidylcholine (Figure 3B) showing that HDL particle remodeling is accompanied by phospholipid transfer from CSL112 to HDL3. In a similar fashion, phospholipid species, such as phosphatidylinositol, that are present in HDL but not in CSL112 allowed us to document movement of phospholipids from HDL3 into S-HDLrem (Table; Online Table II). Despite the movement of HDL3-derived phospholipid to S-HDLrem during remodeling, the net movement of phospholipid is from CSL112 into HDL3 and contributes to the increase in size of the large remodeled product (L-HDLrem). The net loss of phospholipid by CSL112 seems to account for its reduction in size and transformation into S-HDLrem.

To examine morphology and structure of the products of HDL particle remodeling, we used an optimized electron microscopy method that minimizes rouleau formation and enables better visualization and measurements of individual lipoprotein particles. We measured the geometric mean of longest diameter (representing particle size), its orthogonal diameter, and their aspect ratio (representing particle shape). On electron micrographs, HDL3 and L-HDLrem seem as spherical particles with contiguous high densities near the particle’s edge and center. In contrast, CSL112 and S-HDLrem seem as flattened ovoid discs without rouleau (Figure 5A). Computed geometric means for HDL3 and L-HDLrem showed that the L-HDLrem particles were larger compared with HDL3. The majority of L-HDLrem particles were between 9 and 14 nm in diameter, with the peak population (>12%) at ±11.0 nm, whereas HDL3 particles were 7 to 13 nm in diameter, with the peak population (>16%) at ±9.8 nm (Figure 5B). The peak population of L-HDLrem and HDL3 had aspect ratios of 1.15 and 1.14, respectively, confirming their spherical morphology (Figure 5C).

In contrast with HDL3 and L-HDLrem, CSL112 and S-HDLrem particles appear as contiguous irregular cable-like, high-density rings, with their central regions appearing as a lower density hollow (Figure 5A). The geometric means for CSL112 particles were between 7 and 11 nm and the peak population (>12.4%) at 8.4 nm. S-HDLrem particles were smaller, with the majority of particles being between 5 and 10 nm in diameter and the peak population (>14.9%) at 7.3 nm (Figure 5B). Comparison of aspect ratios showed similar features for S-HDLrem and CSL112 particles. The peak population of S-HDLrem and CSL112 had aspect ratios of 1.39 and 1.33, respectively (Figure 5C), suggesting a discoidal morphology. Electron microscopy analysis of lipid-poor apoA-I did not reveal any particular structures, globular or discoidal (data not shown).

**Enhanced Cholesterol Efflux and Anti-Inflammatory Effects Mediated by CSL112 Are Linked to HDL Particle Remodeling**

We have previously shown that CSL112 improves HDL functionality in plasma, increasing the capacity to support cholesterol efflux and to inhibit proinflammatory cytokine production. To evaluate the contribution of HDL remodeling induced by CSL112 to the increase in HDL functionality, we examined the functional properties of the individual remodeled HDL subspecies using CSL112 and HDL3 for direct comparison. Two products of HDL remodeling, lipid-poor apoA-I and the S-HDLrem, exhibited a

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<th>Table. Phospho- and Sphingolipid Composition of Native, Reconstituted, and Remodeled HDL Species</th>
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<tr>
<td>PC 18:2/18:2</td>
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<tr>
<td>HDL3 (n=2)</td>
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<tr>
<td>CSL112 (n=2)</td>
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<tr>
<td>L-HDLrem (n=2)</td>
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The mean (min–max) from 2 independent measurements performed with 2 different HDL3 samples are shown. Similar patterns as phosphatidylinositol were seen with sphingomyelin, phosphatidic acid, phosphatidyglycerol, and ceramide (Online Table I). HDL indicates high-density lipoprotein; n.d., not detected; PC, phosphatidylcholine; PI, phosphatidylinositol; PL, phospholipid; and SL, sphingolipid.
much higher capacity to efflux cholesterol via ABCA1 compared with the original CSL112 and HDL3 or L-HDL<sub>rem</sub>. In sharp contrast, almost no ABCA1-independent efflux was promoted by lipid-poor apoA-I. S-HDL<sub>rem</sub> enhanced ABCA1-independent efflux to a similar degree compared with other lipidated HDL particles tested (CSL112, HDL3,
or L-HDLrem; Figure 6A). Additional studies using a different cell system confirmed these findings about ABCA1 (Online Figure IX) and further showed that both S-HDLrem and L-HDLrem are good acceptors of cholesterol via ATP-binding cassette subfamily G member 1 while, as expected by their low lipid content, S-HDLrem and lipid-poor apoA-I were poor acceptors of cholesterol via scavenger receptor class B member 1. Taken together, these data indicate that the process of remodeling leads to the formation of 2 HDL subpopulation, lipid-poor apoA-I and S-HDLrem, which are extremely efficient acceptors of cholesterol via ABCA1 pathway while efflux via ATP-binding cassette subfamily G member 1 and scavenger receptor class B member 1 is maintained. Importantly, this process seems driven by the admixture of lipoprotein particles because incubation of CSL112, HDL2, or HDL3 alone did not lead to increases in efflux activity (Online Figure VI). Thus, an increase in concentration of these HDL subpopulation driven by CSL112 infusion in vivo may account for the elevated capacity of plasma to induce cholesterol efflux via ABCA1.

Next, we examined the ability of remodeled HDL subtypes to inhibit phytohemagglutinin-induced proinflammatory cytokine production in human PBMC. Lipid-poor apoA-I, S-HDLrem, and CSL112 exerted the strongest inhibitory effects on secretion of proinflammatory mediators (tumor necrosis factor-α, IL-1β, IL-6, and macrophage inflammatory protein-1β), whereas HDL3 and L-HDLrem were much less effective (Figure 6B). The extent of inhibition by the products of HDL remodeling in PBMC correlated positively with the induction of activating transcription factor 3 (ATF3), a known negative regulator of the macrophage transcriptional response to inflammatory stimuli. Lipid-poor apoA-I and S-HDLrem induced higher protein levels of ATF3 in phytohemagglutinin-stimulated PBMC compared with medium control, HDL3 or L-HDLrem (Figure 6C).

Antioxidative Activity of the Products of HDL Remodeling
HDL has been shown to protect LDL from oxidative damage by inhibiting accumulation of primary and secondary peroxidation products in LDL. To evaluate antioxidative activity of the products of HDL remodeling, we compared their capacity

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![Figure 6](http://circres.ahajournals.org/)

**Figure 6. Enhanced cholesterol efflux and anti-inflammatory effects mediated by CSL112 are linked to high-density lipoprotein (HDL) particle remodeling.** A, ATP-binding cassette transporter 1 (ABCA1)–dependent and ABCA1-independent cholesterol efflux from RAW264.7 cells to isolated individual products of HDL remodeling (large [L-HDLrem] and small HDL species [S-HDLrem]) and lipid-poor apolipoprotein A-I [apoA-I]. Final concentration of cholesterol acceptors in efflux medium was 20-μg protein/mL. Each fractional efflux value represents the mean±SD for 3 independent experiments performed with different samples measured in triplicate. B, Small HDL species inhibit proinflammatory cytokine production in peripheral blood mononuclear cells (PBMCs). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and macrophage inflammatory protein-1β (Mip-1β) were measured in cell-free supernatants at 20 h after phytohemagglutinin (PHA) stimulation. The mean values±SD are shown and are derived from triplicate cell cultures of at least 4 donors. **P<0.01, *P<0.05 vs control PHA-stimulated cells. C, Small HDL species stimulate activating transcription factor 3 (ATF3) in PHA-M–stimulated PBMC. PBMC were stimulated or not with PHA-M (1 μg/mL) in the presence of different lipoproteins (at final protein concentration of 1 mg/mL). Cellular lysates were subjected to SDS-PAGE followed by Western blotting with an anti-ATF3 antibody.
to inactivate PLOOH in LDL preoxidized by 2,2’-Azobis(2-amidinopropane) dihydrochloride (oxLDL). HDL3, L-HDL<sub>rem</sub>, and S-HDL<sub>rem</sub> equally well attenuated PLOOH content in incubation mixtures with oxLDL (47.5±9.5%, 45.9±2.6%, and 47.4±9.5% PLOOH inactivated, respectively), whereas CSL112 and lipid-poor apoA-I were markedly less effective (33.4±10.5% and 23.1±2.6% PLOOH inactivated, respectively, Figure 7A). HDL-mediated inactivation of linoleic acid 9-mono hydroperoxide (LOOH) was associated with the formation of oxidized forms of apoA-I characterized by the conversion of apoA-I Met 112 and Met 86 residues into Met(O) (Figure 7B). Oxidation of apoA-I in different HDL species after incubations with oxLDL decreased in the order HDL3=S-HDL<sub>rem</sub>≥L-HDL<sub>rem</sub>>CSL112>lipid-poor apoA-I, thereby mirroring their protective activity during LDL oxidation (Figure 7A).

As absolute concentrations of apoA-I in HDL samples tested were similar, low antioxidative activity of lipid-poor apoA-I suggests that because of structural characteristics of this particle, oxidation-sensitive Met residues were poorly accessible for the interaction with PLOOH. Overall, a positive correlation observed between the levels of oxidized apoA-I and levels of inactivated PLOOH supported a role for Met residues of apoA-I as the principal mechanism of PLOOH inactivation by HDL.

Figure 7. On remodeling, antioxidative activity was predominantly associated with large and small remodeled high-density lipoprotein (HDL) species, L-HDL<sub>rem</sub> and S-HDL<sub>rem</sub>, paralleled by the increase in oxidation of apolipoprotein A-I (apoA-I) Met residues. A, Inactivation of phospholipid hydroperoxide (PLOOH) in oxidized LDL (oxLDL) incubated with HDL3, CSL112, or individual products of HDL remodeling. LDL (40 mg total cholesterol [TC/dL]) was oxidized by 2,2’-Azobis(2-amidinopropane) dihydrochloride (4 mol/L) for 6 h, dialyzed and incubated at 20 mg/TC/dL alone or in the presence of individual lipoproteins (each at a final concentration of 8 mg total protein/dL) for 4 h at 37°C. PLOOH was evaluated in incubation mixtures by high-performance liquid chromatography (HPLC) with chemiluminescent and ultraviolet (UV) detection. Inactivation of PLOOH in oxLDL+HDL incubation mixtures is presented as percentage change from the PLOOH levels detected in oxLDL incubated alone. Means±SDs for at least 3 independent experiments are shown; ***P<0.001, **P<0.01, *P<0.05 vs nonmodified HDL3 (HDL3). B, Oxidation of apoA-I Met residues in different HDL species incubated alone or with oxLDL. Oxidized at Met residues and nonoxidized apoA-I were measured by HPLC using UV detection. ApoA-I<sub>1,32</sub> and apoA-I<sub>16,1</sub> refer to oxidized forms 16 and 32 U greater, respectively, than nonoxidized apoA-I as shown by mass spectrometry. ApoA-I<sub>1,32</sub>, apoA-I<sub>16,1</sub> oxidized at Met112 and Met86; ApoA-I<sub>1,32</sub>, apoA-I oxidized at Met112 (1) or Met86 (2).

Discussion

In earlier work, we showed that addition of CSL112 to plasma dramatically elevates cholesterol efflux capacity of human serum. Careful quantitation showed that CSL112 elevated efflux capacity of plasma much more strongly than an equivalent amount of HDL3. Similarly, the anti-inflammatory effects of addition of CSL112 to whole human blood were substantially stronger than those caused by addition of HDL3. Here, we show that this enhancement of HDL function by CSL112 may be ascribed to particle remodeling with formation of small, highly functional species. The small forms, lipid-poor apoA-I and S-HDL<sub>rem</sub>, produced during remodeling, exhibit both an ABCA1-dependent efflux capacity and an anti-inflammatory capacity that are higher than that of HDL3 or large remodeled HDL (L-HDL<sub>rem</sub>). This increased activity may thereby explain the rise in functional HDL, that is greater than the rise in HDL-C or apoA-I. Such remodeling occurs on infusion of CSL112 into human subjects, addition of CSL112 to plasma, and on incubation of CSL112 with purified HDL. The correlation of CSL112-driven remodeling with cholesterol efflux capacity observed here is consistent with the findings of Borja et al who found a strong correlation between the exchangeability of apoA-I on plasma HDL and the cholesterol efflux capacity of plasma.

We have purified and analyzed the function of 2 small species: the lipid-poor apoA-I and S-HDL<sub>rem</sub> and found that both...
species are individually active in ABCA1-mediated cholesterol efflux and in reducing the cytokine response of blood leukocytes to an inflammatory stimulus (Figure 6A and 6B). To our knowledge, this is the first functional analysis of these species in isolation. The potent capacity of small, dense HDL subspecies to efflux cellular cholesterol through ABCA1 is fully consistent with recent demonstration of HDL particle size being a critical determinant of ABCA1-mediated cholesterol export from macrophages, with a HDL particle size equal to 8.0 nm or lower being required for efficient cholesterol efflux through ABCA1.6 The correlation of anti-inflammatory activity with ABCA1-dependent efflux activity is consistent with the view that cholesterol depletion may cause the anti-inflammatory effects.30 These findings are in keeping with published reports demonstrating that cholesterol efflux is required to prevent cell activation.31,32

We have also confirmed the findings of De Nardo et al33 that reconstituted HDL causes induction of ATF3, a negative transcription regulator of toll-like receptor-induced cytokine production in macrophages29 and have expanded the findings by showing that while large HDL species have low ability to induce ATF3, it is the smaller species that have the strongest anti-inflammatory and ATF3-inducing capacity (Figure 6B and 6C). ATF3 has been shown to regulate lipid body formation in macrophages, at both basal and high levels of cellular lipid loading, representing an intersection point for inflammatory and metabolic pathways in macrophages.33 Thus, infusion of CSL112 and the resulting increase of small HDL species, S-HDLrem and lipid-poor apoA-I, capable of inducing ATF3 may be beneficial to moderate both foam cell formation and inflammation via ATF3.

Studies of the antioxidant activity of the remodeled HDL species showed that while S-HDLrem was capable of reducing the PLOOH content of oxLDL, the lipid-poor apoA-I displayed low activity. This finding is consistent with previous data documenting potent capacity of small, dense HDL subspecies from human plasma to protect LDL from oxidative stress,34 and to inactivate PLOOH derived from oxLDL.23 Mechanistically, the antioxidative effects of HDL on LDL lipids involve transfer to HDL of their oxidized species, primarily those derived from phospholipids, with subsequent inactivation.34 The transfer process depends on the fluidity of surface HDL lipids and may require the presence of an organized lipid surface in HDL; as the latter is most likely not the case in lipid-free apoA-I, this structural feature can account for its low capacity to inactivate PLOOH. Together, the present data on the potent biological activities of remodeled small, dense HDL particles provide further support to the notion of distinct role of particle subspecies in the protection from atherosclerosis.28,35

Contrary to our expectations, HDL remodeling induced by CSL112 was independent of plasma lipid transfer proteins or lipases, as heat inactivation of plasma, inhibition of CETP, LCAT, secretory phospholipase 2 with specific inhibitors or stimulation of PLTP, or genetic deletion of PLTP or LCAT neither prevented nor accelerated the formation of lipid-poor apoA-I (Online Figures IV and V). Rather, remodeling appeared to arise from a spontaneous interaction of CSL112 with HDL2 and HDL3. We speculate that this interaction involves first a fusion of CSL112 with native HDL and a subsequent fission to yield 3 remodeling products. An initial intimate interaction between CSL112 and HDL is indicated first by the observation of bulk movement of phospholipid from CSL112 to the larger remodeled product and by the smaller inverse movement of HDL3-derived phospholipid to the smaller remodeled product (Table; Online Tables I and II). Phospholipid has little ability to diffuse in aqueous solutions, thus favoring the notion of initial particle fusion. The suggestion of fusion between CSL112 and native HDL and subsequent fission is further supported by analysis of the size, and protein origin, and lipid constituents of the reaction products (Figures 3 and 4).

Fusion of particles with subsequent release of lipid-poor apoA-I has also been postulated to be the mechanism by which PLTP promotes the formation of lipid-poor apoA-I.18 Although these authors proposed a direct bridging role for PLTP in the fusion, the data reported here suggest an alternative path: PLTP-mediated exchange of lipids among lipoprotein particles may give rise to HDL species with structure similar to CSL112, which spontaneously fuse with HDL2 or HDL3. Indeed, Ji et al18 have shown that pretreatment of apoA-I with PLTP enables it to bind rapidly to HDL2 or HDL3.

The fission of a fused CSL112-HDL particle may be a physical consequence of its composition. Because the disc-shaped CSL112 contains only surface lipids and no core lipids (neither triglycerides nor cholesterol ester), then the products of fusion will of necessity have an increased ratio of surface to core. This relative excess of surface may promote dissociation of apoA-I by a process analogous to that which may occur during lipolysis of a chyomicron. Nevertheless, the initial interaction of CSL112 and HDL3 seems to have more than 1 potential outcome (Online Figure VII). During the course of remodeling, we observed formation not only of lipid-poor apoA-I but also of a species smaller than CSL112 and with less phospholipid (S-HDLrem). We speculate that S-HDLrem is mainly a product of a net transfer of phospholipids from CSL112 to HDL3. This is consistent with the finding that both CSL112 and S-HDLrem particles seem to be of a discoidal morphology, but particle size of S-HDLrem is smaller than that of CSL112 (Figure 5). Supporting this deduction is also the observation that while CSL112 seems to rapidly donate phospholipid to HDL3, the movement of apoA-I from HDL3 into S-HDLrem occurs much more slowly (Figure 4). Similar observations have been made by Settasation et al18 who showed that a fusion product of spherical HDL particles may release daughter particles of either small or intermediate size. Finally, we note that S-HDLrem particles seem to retain the ability to fuse with endogenous HDL as they gradually exchange apoA-I with L-HDLrem and eventually disappear during longer incubation in plasma (Figure 2A).

The main limitation of the current work is that the detailed studies of remodeling were done in vitro under cell-free conditions and, thus, the contribution of cell-bound enzymes, such as lipases, cannot be taken into account. In addition, it is expected that in vivo, cholesterol from blood cells and other tissues may join the lipoprotein pool and affect this remodeling. Nevertheless, we saw no change in the kinetics of remodeling in plasma versus whole blood (Online Figure X) versus in man (Online Figure III). CSL112 did promote remodeling of HDL2 and HDL3 with slightly different kinetics of lipid-poor apoA-I formation (Figure 3A), suggesting that differences in particle
size may have potential to influence HDL particle remodeling. Protein composition is also likely to affect fusion, and previous work has shown that apoA-II inhibits CETP-mediated remodeling of reconstituted HDL into large and small particles in a process that leads to lipid-poor apoA-I formation. Analysis of apoA-II distribution among the products of CSL12-induced remodeling of HDL3 revealed that apoA-II is present in remodeled small HDL particles with its time-dependent distribution pattern identical to that of apoA-I, but it is absent in remodeled small HDL and lipid-poor apoA-I (Online Figure XI), and infusion of CSL12 did not significantly change apoA-II blood levels (Online Figure II). These data suggest that while apoA-II did not inhibit fusion with CSL12, it did not join apoA-I in forming smaller fission products. Finally, ApoE seems unlikely to play a role in the remodeling described here because neither CSL12 nor HDL3 bear apoE (Online Figure XII), yet CSL12 drives remodeling of HDL3. Furthermore, CSL12 did not affect the size distribution of apoE-containing particles in plasma (Online Figure I).

We have used an exogenously supplied source of apoA-I, CSL12, to document a novel mechanism for formation of small forms of HDL with high biological activity. We speculate that this process may occur not only after infusion of a reconstituted HDL but also during the normal lifecycle of HDL. After synthesis, lipid-poor apoA-I is lipidated by ABCA1 and gives rise to discoidal nascent HDL. Our work suggests that one fate of such particles may involve fusion with spherical HDL and production of smaller daughter products. Such a process of fusion of nascent discs with circulating spheres may underlie the recent findings of Mendivil et al who observed direct hepatic secretion of all size classes of HDL in the venous blood of human volunteers. Our data suggest that fusion of nascent discs made by the liver with native HDL in the space of Disse or hepatic sinusoids would lead to rapid appearance of newly synthesized apoA-I in all size classes, as observed by Mendivil et al.

It has been long recognized that HDL may exist in forms with higher or lower functional activity. Recent observations now strongly connect HDL function with coronary heart disease outcomes and make understanding the factors that change HDL functionality a vital topic. Here we characterize highly active HDL species and describe a novel mechanism for their formation in response to CSL12, a product currently in clinical development for prevention of early recurrent atherothrombotic events in acute coronary syndrome. Importantly, ongoing clinical evaluation of CSL12 holds the hope of validating the connection between functional assays such as those studied here and clinical disease.

Acknowledgments
We would like to thank Drs Rye and Remaley for careful reading of the article and critical comments. We gratefully acknowledge Dr. Jiang for providing plasma samples of wild-type and PLTP knockout mice, B. Haenni, M. Kaminek, and Dr B. Zuber for electron microscopy analysis performed at the Microscopy Imaging Center (MIC) of the University of Bern and Dr Asztalos for 2-dimensional gel analysis. Editorial assistance with the preparation of this article was provided by Meridian HealthComms Ltd, funded by CSL Behring.

Sources of Funding
This work was funded by CSL Behring.


Enhanced HDL Functionality in Small HDL Species Produced Upon Remodeling of HDL by Reconstituted HDL, CSL112: Effects on Cholesterol Efflux, Anti-Inflammatory and Antioxidative Activity

Svetlana A. Didichenko, Alexei V. Navdaev, Alexandre M.O. Cukier, Andreas Gille, Patrick Schuetz, Martin O. Spycher, Patrice Thérond, M. John Chapman, Anatol Kontush and Samuel D. Wright

Circ Res. 2016;119:751-763; originally published online July 19, 2016; doi: 10.1161/CIRCRESAHA.116.308685

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIAL

Methods

HDL labeling

Biotinylation of CSL112 or high-density lipoprotein 3 (HDL3) was performed as described by Lund-Katz et al.1 with modifications. Briefly CSL112 or HDL3 were dialyzed into PBS (pH 7.4) prior to biotinylation. The EZ-link sulfo-NHS-LC-biotinylation kit (Pierce Biotechnology) was used for attaching biotin molecules through a 2.24 nm spacer arm to lysine residues on the surface of HDL particles. CSL112 or HDL3, each at 10 mg protein/mL, were mixed with 10 mmol/L sulfo-NHS-LC-biotin solution at a 30-fold molar excess of biotin. The lipoproteins were incubated at 4°C for 30 min, reaction was terminated by adding glycine (pH 7.5) up to final concentration of 100 mM for 30 min. Unbound biotin was removed by ultrafiltration using Amicon Ultra Centrifugal Filter (Ultracel 50K; Merck Millipore). The degree of biotinylation of the particles was determined using conditions recommended by Pierce Biotechnology. For fluorescence imaging, CSL112 or HDL3 were labeled with DyLight 488 or DyLight 680 Amine-Reactive Dyes (Pierce Biotechnology) following the manufacturer's recommendations. CSL112 or HDL3, each at 5 mg protein/mL, were mixed with DyLight fluorophore solution at a 5.5-fold molar excess of fluorophore. The labeling procedures performed yielded one to two biotin or DyLight molecules per apoA-I molecule in CSL112 or HDL particles.

CSL112 was labeled with a fluorescent phospholipid analog (18:1-06:0 NBD PC [1-oleoyl-2-(6-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino)hexanoyl]-sn-glycero-3-phosphocholine], Avanti Polar Lipids) as previously described.2 CSL112 was diluted in PBS (final concentration of 2 mg protein/mL) and incubated with the NBD-PC-precoated glass beads with agitation at room temperature for 3 h. After pelleting the beads, NBD-PC-labeled CSL112 was subjected to ultracentrifugation at a density of 1.125 g/mL for 20 h at 10°C (55,000 rpm, TFT 70.38 rotor). Unbound NBD-PC micelles concentrated in a layer at the top of the tube were removed. The density of the bottom fraction containing NBD-PC-labeled CSL112 was readjusted and centrifugation was repeated to ensure complete removal of unbound NBD-PC micelles. NBD-PC-labeled CSL112 was concentrated and dialyzed against PBS. Due to labeling, the ratio of PC/apoA-I (mol/mol) in NBD-PC-labeled CSL112 increased compared to the unlabeled CSL112, 72:1 and 50:1, respectively.

In vitro incubations

For the incubation of CSL112 with human whole blood or plasma, blood was collected from healthy donors into EDTA vacutainer tubes. Plasma was prepared by centrifugation of blood at 700 g for 20 min at 4°C. Before incubation with CSL112, plasma was diluted with 0.9% NaCL to adjust for the volume of pelleted red blood cells. CSL112 (stock concentration of 10 mg protein/mL) was added to 190 µL of whole blood or plasma to a final concentration of 1 mg protein/mL and incubation was carried out for the different periods of time (10 min, 30 min, 1 h and 4 h) at 37°C. At the end of incubation, blood samples were briefly centrifuged to obtain cell-free supernatants. All samples were snap frozen in liquid nitrogen and stored at -70°C until required for analysis. To remove apo-B-containing lipoproteins, plasma or blood–derived samples were precipitated with polyethylene glycol (PEG). Briefly, samples were mixed with 0.4 volumes of 20% PEG 8000 MW (Sigma P-2139) in 200 mM glycine pH 7.4 and centrifuged at 14 000 rpm for 5 min at 4°C. Supernatants containing HDL fraction were collected and used for electrophoretic analysis and for the measurements of cholesterol efflux from RAW264.7 cells.
Plasma samples of wild-type (WT), LCAT KO and PLTP KO mice in a C57BL/6 background were used for the incubation with CSL112. CSL112 was incubated with mouse plasma at a final concentration of 0.75 mg protein/mL for 1 h at 37°C. For electrophoretic analysis, amount of plasma protein loaded on a gel was equivalent to 2 µL of undiluted plasma (wild-type mice), and 4 µL of undiluted plasma (PLTP KO and LCAT KO mice).

Lipoprotein isolation

LDL, HDL2 and HDL3 were isolated from cryo-depleted plasma (CSL Behring) by sequential ultracentrifugation. Briefly, plasma was adjusted to appropriate densities by adding solid KBr. Lipoproteins were isolated using a TFT 70.38 rotor at a speed of 55,000 rpm. LDL (d = 1.019-1.055 g/mL), HDL2 (d = 1.065-1.121 g/mL) and HDL3 (d = 1.13-1.18 g/mL) were isolated with a single spin of 24 h at the lower density followed by successive spin for the same period at the higher density. The isolated lipoproteins were dialyzed against endotoxin-free phosphate buffered saline (PBS) [pH 7.4].

Incubation of CSL112 with individual lipoproteins was carried out in PBS or in PBS supplemented with 0.5% fatty acid free bovine serum albumin (BSA, Sigma Aldrich), at a concentration of 1 mg protein/mL of each lipoprotein.

For preparative isolation of the products of HDL remodeling, CSL112 was incubated with HDL3 in PBS for 1 hour at 37°C. Two lipoprotein fractions were purified from the incubation mixtures by sequential ultracentrifugation in the density intervals: remodeled large HDL subspecies (Fr. 1; d = 1.125-1.224 g/mL) and remodeled small HDL subspecies (Fr. 2; d = 1.22-1.24 g/mL). In each case samples were subjected to two successive spins for 20-22 h at 10°C (55,000 rpm, TFT 70.38 rotor), first spin at the higher density followed by the spin at the lower density. Lipid-poor apoA-I (Fr. 3) was collected at the bottom of the tube following centrifugation at the density d = 1.24 g/mL. The final preparations were dialyzed against endotoxin-free PBS (pH 7.4) using Mini Dialysis Kit (molecular weight cut off 8 kDa; GE Healthcare). Amicon Ultra Centrifugal Filters (Ultracel 30K and 100K; Merck Millipore) were used to concentrate the samples. Protein concentration in the samples was measured using the DC Protein Assay Kit II (Bio-Rad Laboratories Inc).

For measurements of antioxidative activity, remodeled HDL species were separated by single-spin density gradient ultracentrifugation for 44 h according to Chapman et al.

Non-denaturing polyacrylamide gradient gel electrophoresis (ND-PAGGE), western blotting and fluorescence imaging

The HDL particle size distribution was determined by non-denaturing gradient gel electrophoresis (ND-PAGGE) on 4–30% polyacrylamide gradient gels (LPE System, CBS Scientific Company Inc., Del Mar, CA). Molecular size markers were from GE Healthcare, (Amersham High Molecular Weight Calibration Kit for Electrophoresis, range: 17 nm thyroglobulin, 12.2 nm ferritin, 9.5 nm catalase, 8.4 nm lactate dehydrogenase, 7.1 nm BSA). Following separation by ND-PAGGE, proteins were transferred on to polyvinylidene difluoride membranes using the semi-dry blotting procedure (iBlot Gel Transfer Stacks, Invitrogen). For immunodetection of apoA-I, blots were probed with goat anti-human apoA-I polyclonal antibody (Rockland Immunochemicals Inc, # 600-101-109). ApoE was detected using apoE mAb (D6E10) [Abcam, ab 1906]. Fluorescence imaging of blots was performed using a Luminescent Image Analyzer (LAS4000, GE Healthcare) equipped with EPI-BGR light set 4000 (filters: Y515
BP Green LED and R670 Cy5 Red LED, to detect apoA-I labeled with DyLight 488 or DyLight 680 fluorophores, respectively).

**Pull-down assay**

Incubation mixtures (100 µL) containing biotinylated CSL112 or biotinylated HDL3 were diluted with PBS (1:1.5 v/v). Each diluted sample was divided into two equal aliquots. One aliquot was incubated with streptavidin sepharose and another one with sepharose 4B (both from GE Healthcare) with manual gentle mixing. To an aliquot of 120 µL, 50 µL of beads (50% slurry in PBS supplemented with 0.5% fatty acid free BSA) were added. After incubation for 15 min at room temperature, beads were removed by centrifugation.

**Phospholipid analysis**

Phospholipid contents were evaluated using an enzymatic assay (LabAssay, Wako Diagnostics). For the analysis of PC content of HDL samples by high performance liquid chromatography (HPLC), phospholipids were separated using a silica column (Ascentis Si 3µm, 2.1x150mm, Sigma-Aldrich). Isocratic elution was performed at a flow rate of 0.5 mL/min at 40°C. Elution buffer contained 87% (vol/vol) methanol and 13% (vol/vol) formic acid (10 mM ammonium formate, 0.125% formic acid). Analytes were detected by a charged aerosol detector (Dionex Corona Ultra RS). A standard curve made with soy L-α-phosphatidylcholine (Avanti Polar Lipids) was used for quantification.

Phospholipid and sphingolipid profiling of HDL samples was performed using an original liquid chromatography–mass spectrometry/mass spectrometry (LC/MS/MS) methodology as described by Camont et al. In brief, HDL subfractions were pooled to provide total HDL (30 µg total phospholipid mass) and added to 4 mL of cold CHCl3/acidified CH3OH (5:2 v/v) containing seven internal lipid standards (Avanti Polar Lipids). A blank and quality controls (one every 5 samples) were extracted in parallel with each batch to ensure for quality control. K4EDTA (200 mmol/L) solution was added (1:5 v/v) and the mixture was vortexed and centrifuged. The organic phase was dried under nitrogen and lipids were reconstituted into isopropanol/hexane/water (10:5:2 v/v), transferred into LC/MS vials, dried under nitrogen and resuspended in isopropanol/hexane/water (10:5:2 v/v). Eight principal phospholipid (PL) subclasses [Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylinositol (PI), phosphatidyglycerol (PG), phosphatidyserine (PS), and phosphatidic acid (PA)] and two principal sphingolipid (SL) subclasses [sphingomyelin (SM) and ceramide (Cer)] were assayed by LC/MS/MS. Lipids were quantified using a QTrap 4000 mass spectrometer (AB Sciex), an LC20AD HPLC system, and the Analyst 1.5 data acquisition system (AB Sciex). Quantification of PLs and SLs was performed in positive-ion mode, except for PI species that were detected in negative-ion mode. Sample (4 µL) was injected onto a Symmetry Shield RP8 3.5µm 2.1x50mm reverse phase column (Waters Corporation) using a gradient from 85:15 to 91:9 (v/v) methanol/water containing 5mmol/L ammonium formate and 0.1% formic acid at a flow rate of 0.1mL/min for 30 min. Lipid species were detected using multiple reaction monitoring reflecting the headgroup fragmentation of each lipid class and quantified using calibration curves specific for the ten individual lipid classes with up to 12 component fatty acid moiety; 17 calibration curves were generated in non-diluted and 10-fold diluted matrices to correct for matrix-induced ion suppression effects. More abundant lipid species which displayed a non-linear response in non-diluted extracts were quantified from a 10- or 100-fold diluted sample.
Electron microscopy

Negative stain electron microscopy (EM) was performed as described previously. A glow-discharged thin carbon-coated 300-mesh copper grid was placed on a 100 µL drop of lipoprotein solution on Parafilm (0.005 mg/mL protein). After 1 min, the excess solution was removed by blotting with filter paper. The grid was washed by briefly touching the surface of the grid with a drop (200 µL) of water on Parafilm and then blotted dry with filter paper. The touching and blotting steps were repeated 3 times, each with a clean drop of water. The grid was placed on a drop (200 µL) of 1% (w/v) uranyl acetate solution for 1 min, then the excess solution was removed by blotting and the sample was air dried at room temperature. Specimens were examined with a FEI Tecnai F20 (FEI, Hillsboro, OR).

Cholesterol efflux assay

The capacity of the HDL to efflux cholesterol was assessed using [³H]cholesterol-loaded RAW264.7 macrophages as previously described. Briefly, RAW264.7 cells (mouse macrophages, ATCC) were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mmol/L glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin in a humidified 37 °C incubator in the presence of 5% CO₂.

On day 1, the cells used for efflux experiments were seeded into 24-well plates at a density of 0.35 x 10⁶ cells per well, in 1 mL of medium. On day 2, cell culture medium was removed, and cells were labeled with cholesterol by incubation for 36 h in DMEM supplemented with 5% (v/v) FCS, 2 mmol/L glutamine and [1,2-³H]cholesterol (1 µCl/mL; 0.5 µCl/0.5 mL of medium per well). Following the labeling period, cells were gently washed with PBS (on day 3) and then incubated in DMEM/2 mmol/L glutamine/0.2% fatty-acid-free BSA medium alone or stimulated with 0.3 mmol/L 8Br-cAMP (8-bromoadenosine 3’,5’-cyclic monophosphate sodium salt) for 16 h to up-regulate ABCA1. Efflux was promoted by incubating the [³H]cholesterol- labeled RAW264.7 cells with each individual acceptor for 5 h. The difference in efflux between stimulated and non-stimulated cells is taken as a measure of ABCA1-mediated efflux.

Cholesterol efflux studies with BHK cells stably transfected with human ABCA1 cDNA or ABCG1 cDNA or SR-B1 were performed as described previously with the following modifications. Cells were seeded into 24-well plates at a density of 0.15 x 10⁶ cells per well, in 1 mL of DMEM containing 10% FCS. Next day, cells were labeled for 20 h with 1 Ci/ml of 3H-cholesterol in DMEM containing 10% FCS. Transporters were induced with 10 nM mifepristone in Dulbecco's modified Eagle's medium plus 0.2 mg/mL of fatty acid-free BSA for 18 h. Cholesterol efflux was measured after the addition of an acceptor in DMEM containing 10 nM mifepristone and 0.2 mg/mL of fatty acid-free BSA for 6 h. The difference in efflux between mifepristone-stimulated and non-stimulated cells is taken as a measure of transporter-mediated efflux.

Preparation of cellular extracts, detection of ATF3 by western blotting

To prepare cellular protein extracts, PBMC were washed three times in PBS and cellular proteins were precipitated with trichloroacetic acid at a final concentration 10%. Following centrifugation for 10 min, 14,000 rpm at 4°C, protein precipitates were washed twice in cold acetone, resuspended in electrophoresis sample buffer, separated on 4-12% pre-cast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex,
Invitrogen) and blotted as described above. For immunodetection of ATF3, blots were probed with rabbit anti-ATF3 polyclonal antibody (sc-188, Santa Cruz Biotechnology).

**Phospholipid hydroperoxide inactivating capacity of HDL**

Reference LDL obtained from a healthy normolipidemic subject was preoxidized at 40 mg total cholesterol/dL with 4 mmol/L AAPH for 6 hours at 37°C. Oxidative modification was terminated by addition of EDTA (10 µmol/L) and BHT [10 µmol/L]. Oxidized LDL was dialyzed against PBS at 4°C to remove EDTA and excess BHT, and incubated at a concentration of 20 mg total cholesterol/dL for 4 hours in the presence or absence of HDL (8 mg total protein/dL) in PBS at 37°C. EDTA (100 µmol/L) was present to inhibit lipid peroxidation during the incubation. Phospholipid hydroperoxides were quantified in the reaction mixture before and after incubation by HPLC with chemiluminescent detection as described elsewhere. To measure apoA-I content of native and oxidized methionine (Met) residues, HDL was subjected to HPLC with ultraviolet detection at 214 nm as previously described.
Supplemental Figures

Supplementary Figure I. Addition of CSL112 to human pooled plasma ex vivo does not influence the size distribution of apoE-containing complexes. Human pooled cryo-depleted plasma (CSL Behring) was incubated alone or in the presence of CSL112 (1 mg protein/mL) for the indicated periods of time at 37°C. Samples of whole plasma or apoB-depleted plasma obtained after precipitation of apoB lipoproteins with polyethylene glycol were subjected to ND-PAGGE and analysed by western blotting with anti-apoA-I antibody (left panel) or with anti-apoE antibody (right panel). Sample of CSL112 (C) was included in the analysis as control.

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L-HDL
S-HDL
Lipid-poor apoA-I

ApoAI
ApoE
Supplementary Figure II. Apolipoprotein A-II concentrations following infusion of CSL112 in the Phase 1 single ascending dose study (Clinical Trial Registration: NCT1129661).\textsuperscript{14, 15} (A) Unadjusted means (standard deviation [SD]) of cohorts as indicated (B) Baseline-corrected means (SD) of cohorts as indicated (C) Baseline-corrected means (SD) at t=2 hours (D) Baseline-corrected means (SD) at t=24 hours. Concentrations of apoA-II did not change immediately following the infusion of CSL112. However at 24 h following the infusion of CSL112 there was a modest dose-dependent increase in apoA-II not exceeding 10% of the baseline apoA-II concentration. This change could be explained by a slight decrease in apoA-II catabolism due to the overall increase of HDL.
Supplementary Figure III. CSL112 promotes particle remodeling similarly in vivo and in vitro. In vivo: shown are plasma samples derived from healthy subjects infused over a 2 h period with CSL112 as part of completed phase I clinical trial NCT0128177414, 15. Donor 1, infusion dose of 75 mg/kg; Donor 2, infusion dose of 105 mg/kg; and Donor 3, infusion dose of 135 mg/kg. Blood was collected before the infusion (P) or at 2 and 8 h post infusion with CSL112. In vitro: CSL112 was incubated with normolipidemic human plasma at a final concentration of 1 mg protein/mL for the indicated periods of time at 37°C (Donor 4). Plasma samples were subjected to ND-PAGGE and analysed by western blotting with anti-apoA-I antibody. The positions of migration of HDL3, HDL2 subclasses and lipid-poor apoA-I are indicated.
Supplementary Figure IV. Inhibition/stimulation of the plasma factors, CETP, LCAT, PLTP and sPLA2-IIA, does not affect the CSL112-induced remodeling of native HDL in human plasma in vitro.

Human fresh EDTA-plasma was preincubated at 4°C for 30 min in the presence or absence of either 10 µmol/L torcetrapib (CETP inhibitor), 2 mmol/L DTNB (LCAT inhibitor), 10 mmol/L 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF, a PLTP stimulator), or 100 µmol/L LY311727 (3-(3-acetamide-1-benzyl-2-ethyl-indolyl-5-oxy)propane phosphonic acid, a sPLA2-IIA inhibitor). CSL112 (final concentration of 1 mg protein/mL) was added to control plasma or plasma pretreated with individual inhibitors, and incubation continued at 37°C for 1 hour (A, C) or for the indicated time periods (B). A, Lipid-poor apoA-I levels were measured in plasma samples by a specific preβ1-HDL ELISA (Sekisui/ American Diagnostica GmbH). Mean concentrations ± standard deviation (SD) are derived from duplicate measurements. B, HDL fractions were subjected to non-denaturing gel electrophoresis on gradient 4-30% polyacrylamide gels followed by western blot analysis with an anti-apoA-I antibody. Data are shown for a representative experiment performed several times. C, HDL fractions were obtained after precipitation of plasma apolipoprotein B lipoproteins with polyethylene glycol16 and used for the measurements of cholesterol efflux from RAW264.7 cells. Final concentration of CSL112 in efflux medium was 5 µg/mL; serum was at 0.45%. Each fractional cholesterol efflux value is the mean ± SD of triplicate measurements.
Supplementary Figure V. CSL112 causes the remodelling of endogenous HDL in plasma of wild-type, PLTP knockout or LCAT knockout mice ex vivo. Plasma samples of wild-type (WT), PLTP knockout (KO) and LCAT KO mice in a C57BL/6 background or normolipidemic human plasma (hPL) were incubated alone or in the presence of CSL112 for 1 h at 37°C. Final concentration of CSL112 was 0.75 mg protein/mL. Plasma samples were subjected to ND-PAGGE and analysed by western blotting with rabbit anti-mouse apoA-I antibody (Acris, #BP2052) (left panels). After stripping, membranes were reprobed with goat anti-human apoA-I antibody (Academy Bio-Medical Co, #11A-G2b) (right panels). (A) WT (n=2); PLTP KO (n=3). (B) WT (n=2); LCAT KO (n=3). Sample of CSL112 was included in the analysis as control (C). Plasma IDs are shown in square boxes below the lanes. The positions of migration of L-HDL$_{rem}$, S-HDL$_{rem}$, and lipid-poor apoA-I are indicated.
Supplementary Figure VI. Stability of parent lipoprotein preparations. CSL112, HDL3 or HDL2 were incubated in PBS for the indicated periods of time at 37°C at a final concentration of 1 mg protein/mL of each lipoprotein (A) Lipoproteins were subjected to ND-PAGGE and analysed by western blotting with anti-apoA-I antibody. (B) ABCA1-dependent and ABCA1-independent cholesterol efflux from RAW264.7 macrophages. Cells were loaded with free [³H]cholesterol for 36 h, and stimulated with 0.3 mM 8Br-cAMP for 16 h to up-regulate ABCA1. Lipoproteins were used as cholesterol acceptors at a final protein concentration of 5 μg protein/mL in efflux medium, incubation was carried out for 5 h. Data of at least 3 independent experiments are shown. Values are presented as mean ± standard deviation.
Supplementary Figure VII. Model for the interaction of CSL112 with HDL.

In the first step, CSL112 spontaneously fuses with plasma HDL generating a large unstable fusion product. The decay of this unstable fusion product may proceed along either of two paths. In path A, further remodeling leads to exchange of apoA-I between the particles and release of lipid-poor apoA-I. The resulting stable large HDL particles (L-HDL\textsuperscript{rem}) contain apoA-I and phospholipid from both parents while the lipid-poor apoA-I contains principally protein, also from both parents. In path B, further remodeling leads to the lipid exchange, but not to exchange of apoA-I before release of daughter particles. Rather, the parent HDL particle acquires only the lipid component from CSL112. The resulting remodeled large HDL (L-HDL\textsuperscript{rem}) contains apoA-I from HDL and additional phospholipid from CSL112 while small HDL (S-HDL\textsuperscript{rem}) particles contain apoA-I from CSL112 and a small amount of lipid, drawn from both parents.
Supplementary Figure VIII. Analysis of isolated individual products of HDL remodeling by non-denaturating 1D gel electrophoresis.

CSL112 and HDL3 were incubated in PBS for 1 hour at 37°C, and products of the particle remodeling were isolated by sequential density gradient ultracentrifugation. Lipoproteins were separated by ND-PAGGE and visualized by Coomassie stain. Shown are isolated large HDL subspecies (Fr. 1; L-HDL\textsuperscript{rem}), small HDL subspecies (Fr. 2, S-HDL\textsuperscript{rem}), lipid poor apoA-I (Fr. 3). The positions of migration of remodeled HDL subspecies, L-HDL\textsuperscript{rem} and S-HDL\textsuperscript{rem}, and lipid poor apoA-I are indicated.
Supplementary Figure IX. Effects of the products of HDL remodeling on efflux cholesterol via ABCA1, ABCG1 or SR-B1 transporter. L-HDL$_{rem}$, S-HDL$_{rem}$ and lipid-poor apoA-I, were purified from CSL112 and HDL3 incubation mixtures as described in Supplementary Figure VIII and used to efflux cholesterol from ABCA1-transfected (upper panel) or ABCG1-transfected (middle panel) or SR-B1-transfected BHK cells. Before the efflux, cells were labeled with free [$^{3}$H]-cholesterol for 20 h, and then stimulated with 10 nM mifepristone for 18 h induce the transporters. Cholesterol acceptors were added on the basis of their protein content at a final concentration of 20 $\mu$g/mL and incubation was carried out for 6 h. Contribution of cholesterol efflux from the transporter was calculated as the difference in efflux between mifepristone-stimulated and non-stimulated cells. Each fractional efflux value represents the mean ± standard deviation for two independent experiments performed with different samples measured in triplicate. Phospholipid-poor acceptor particles, lipid-poor apoA-I and S-HDL$_{rem}$, were most efficient to efflux cholesterol from BHK cells expressing ABCA1. S-HDL$_{rem}$ and L-HDL$_{rem}$ showed comparable activities to efflux cholesterol via ABCG1 which, unlike ABCA1, prefers phospholipid-rich acceptor particles. The most lipidated particle, L-HDL$_{rem}$, was found to mediate efflux via SR-B1 and by an aqueous diffusion process, which also requires phospholipid-rich acceptor particles.
Supplementary Figure X. Similar effects of CSL112 on HDL remodeling and cholesterol efflux in whole blood and plasma ex vivo. CSL112 (1 mg protein/mL) was incubated with nonmolipidemic whole EDTA-blood (blood) or EDTA-plasma (plasma) for the indicated periods of time at 37°C. After the incubation, blood samples were briefly centrifuged to obtain cell-free supernatants. (A) Blood-derived and plasma samples were subjected to ND-PAGGE and analysed by western blotting with anti-apoA-I antibody. Samples of CSL112 and plasma alone (B) were included in the analysis as controls. (B) ApoB-depleted plasma was used for the measurements of ABCA1-dependent and ABCA1-independent cholesterol efflux from RAW264.7 cells. Final concentration of CSL112 in efflux medium was 5 μg/mL; plasma was at 0.45%. Each fractional cholesterol efflux value is the mean ± standard deviation of triplicate measurements. Data for one representative experiment out of three independent experiments are shown.
Supplementary Figure XI. Analysis of apoA-II distribution among the products of CSL112-induced remodeling of HDL3.

CSL112 was incubated with purified HDL3 for the indicated periods of time at 37°C. Lipoprotein mixtures were subjected to ND-PAGGE and analyzed by western blotting with an anti-apoA-II antibody (Abcam) (left panel) and an anti-apoA-I antibody (Rockland) (right panel). The position of migration of remodeled HDL subspecies, L-HDL\textsuperscript{rem} and S-HDL\textsuperscript{rem}, and lipid-poor apoA-I is indicated.
Supplementary Figure XII. Western blot analysis of apoE in CSL112 and purified HDL3 and HDL2 preparations. CSL112 and different samples of purified HDL2 (n=2) and HDL3 (n=3) were separated with 4-12% SDS-PAGE under reducing conditions. For lipoproteins, approximately 5 $\mu$g of total protein was loaded per lane. Amount of plasma protein loaded on a gel was equivalent to 2 $\mu$L of undiluted plasma (cryo-depleted plasma, CSL Behring). ApoE was detected using anti-human apoE antibody. After stripping, membranes were re-probed with anti-human apoA-I antibodies (right panel).
## Supplemental Tables

### Supplementary Table I. HDL particle content of choline-containing phospholipids

<table>
<thead>
<tr>
<th></th>
<th>Ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(Phospholipid : protein, mg/mg)</td>
</tr>
<tr>
<td>HDL3</td>
<td>0.44 (0.42–0.45)</td>
</tr>
<tr>
<td>CSL112</td>
<td>1.40 (1.33–1.37)</td>
</tr>
<tr>
<td>L-HDL\text{rem}</td>
<td>0.97 (0.92–1.02)</td>
</tr>
<tr>
<td>S-HDL\text{rem}</td>
<td>0.37 (0.29–0.45)</td>
</tr>
<tr>
<td>Lipid-poor apoA-I</td>
<td>0.06 (0.05–0.07)</td>
</tr>
</tbody>
</table>

* The mean (min–max) from two independent measurements performed with two different samples are shown.
† Data are shown as mean ± standard deviation (n≥3).
## Supplementary Table II. Phospho- and sphingolipid composition of native, reconstituted and remodeled HDL

<table>
<thead>
<tr>
<th></th>
<th>HDL3 (n=2)</th>
<th>CSL112 (n=2)</th>
<th>L-HDL\textsuperscript{rem} (n=2)</th>
<th>S-HDL\textsuperscript{rem} (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mol of % PC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC 18:2/18:2</td>
<td>4.1 (3.1–5.2)</td>
<td>36.8 (36.2–37.5)</td>
<td>24.3 (23.5–25.2)</td>
<td>24.4 (22.9–25.9)</td>
</tr>
<tr>
<td><strong>Mol % of PL + SL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LysoPC</td>
<td>2.8 (2.6–3.0)</td>
<td>1.9 (1.8–2.0)</td>
<td>4.06 (3.4–4.5)</td>
<td>3.78 (3.7–3.9)</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.071 (0.066–0.076)</td>
<td>n.d.</td>
<td>0.030 (0.028–0.032)</td>
<td>0.069 (0.065–0.073)</td>
</tr>
<tr>
<td>PA</td>
<td>0.051 (0.00–0.101)</td>
<td>0.015 (0.013–0.017)</td>
<td>0.045 (0.00–0.09)</td>
<td>0.067 (0.000–0.135)</td>
</tr>
<tr>
<td>PC</td>
<td>73.9 (7.05–77.3)</td>
<td>98.1 (97.9–98.2)</td>
<td>84.7 (84.6–84.9)</td>
<td>84.7 (82.2–87.3)</td>
</tr>
<tr>
<td>PE</td>
<td>2.5 (1.6–3.5)</td>
<td>n.d.</td>
<td>0.98 (0.92–1.04)</td>
<td>1.4 (1.3–1.5)</td>
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<tr>
<td>PG</td>
<td>0.012 (0.008–0.016)</td>
<td>n.d.</td>
<td>0.008 (0.007–0.008)</td>
<td>0.012 (0.007–0.017)</td>
</tr>
<tr>
<td>PI</td>
<td>5.3 (4.5–6.0)</td>
<td>n.d.</td>
<td>2.9 (2.6–3.1)</td>
<td>2.3 (2.1–2.5)</td>
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<tr>
<td>PS</td>
<td>0.025 (0.013–0.037)</td>
<td>0.001 (0.001–0.001)</td>
<td>0.001 (0.000–0.002)</td>
<td>0.021 (0.000–0.042)</td>
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<tr>
<td>SM</td>
<td>15.3 (12.4–18.2)</td>
<td>n.d.</td>
<td>7.3 (7.1–7.7)</td>
<td>7.6 (5.1–10.0)</td>
</tr>
<tr>
<td>Ceramide</td>
<td>0.035 (0.027–0.044)</td>
<td>n.d.</td>
<td>0.015 (0.012–0.019)</td>
<td>0.007 (0.005–0.009)</td>
</tr>
</tbody>
</table>

The mean (min–max) from two independent experiments performed with two different HDL samples are shown.

n.d., not detected; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidyglycerol; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; SL, sphingolipid; SM, Sphingomyelin
Supplementary References


