Preferential Sphingosine-1-Phosphate Enrichment and Sphingomyelin Depletion Are Key Features of Small Dense HDL3 Particles
Relevance to Antiapoptotic and Antioxidative Activities

Anatol Kontush, Patrice Therond, Amal Zerrad, Martine Couturier, Anne Négre-Salvayre, Juliana A. de Souza, Sandrine Chantepie, M. John Chapman

Objective—The purpose of this study was to define heterogeneity in the molecular profile of lipids, including sphingomyelin and sphingosine-1-phosphate, among physicochemically-defined HDL subpopulations and potential relevance to antiatherogenic biological activities of dense HDL3.

Methods and Results—The molecular profile of lipids (cholesteryl esters, phospholipids, sphingomyelin, and sphingosine-1-phosphate) in physicochemically-defined normolipidemic HDL subpopulations was determined by high-performance liquid chromatography and gas chromatography. As HDL particle size and molecular weight decreased with increment in density, molar lipid content diminished concomitantly. On a % basis, sphingomyelin abundance diminished in parallel with progressive increase in HDL density from HDL2b (12.8%) to HDL3c (6.2%; \( P<0.001 \)); in contrast, sphingosine-1-phosphate was preferentially enriched in small HDL3 (40 to 50 mmol/mol HDL) versus large HDL2 (15 to 20 mmol/mol HDL; \( P<0.01 \)). Small HDL3c was equally enriched in LpA-I particles relative to LpA-I:A-II. The sphingosine-1-phosphate/sphingomyelin ratio correlated positively with the capacities of HDL subspecies to attenuate apoptosis in endothelial cells (\( r=0.73, P<0.001 \)) and to retard LDL oxidation (\( r=0.58, P<0.01 \)).

Conclusions—An elevated sphingosine-1-phosphate/sphingomyelin ratio is an integral feature of small dense HDL3, reflecting enrichment in sphingosine-1-phosphate, a key antiapoptotic molecule, and depletion of sphingomyelin, a structural lipid with negative impact on surface fluidity and LCAT activity. These findings further distinguish the structure and antiatherogenic activities of small, dense HDL. (Arterioscler Thromb Vasc Biol. 2007;27:0-0.)

Key Words: sphingolipids ■ phospholipid molecular species ■ cholesteryl esters ■ HDL particle remodeling

Low circulating levels of high density lipoprotein (HDL)-cholesterol (HDL-C) constitute a major independent and predictive cardiovascular (CV) risk factor; in contrast, elevated HDL-C concentrations may be atheroprotective.1 Indeed, HDL particles possess multiple antiatherogenic properties, including cellular cholesterol efflux capacity as well as antiapoptotic, antioxidative, antiinflammatory, and vasodilatory activities.1 Such diversity in biological function is intimately related to the marked heterogeneity of HDL particles, which present as a continuum of subpopulations distinct in physicochemical properties, structure, and intravascular metabolism.

The biological activities and atheroprotective function of HDL are inseparably linked to the physicochemical properties of both lipid and protein moieties, and equally to particle structure. Indeed, small, dense, lipid-poor HDL particles possess elevated capacities to accept cellular cholesterol,2 to inhibit cellular expression of adhesion molecules,3 and to protect LDL from oxidation4 as compared with large, light, lipid-rich HDL. The intravascular metabolism of lipid components of HDL is regulated by cholesteryl ester transfer protein (CETP), lecithin:cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP), lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase (EL). As a result, HDL lipids represent complex mixtures of multiple molecular species of phospholipids (PL), cholesteryl esters (CE), triacylglycerols, and partial glycerides which differ in their fatty acid composition, in addition to free cholesterol (FC) and lipophilic vitamins.1 The abundance of individual molecular species of CE in HDL reflects esterification of FC.

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by LCAT, but equally removal of HDL CE by cellular receptors, primarily by scavenger receptor class B type I (SR-BI), and by CETP, which transfers CE to apoB-containing particles, including very low density lipoprotein (VLDL), VLDL remnants, and low density lipoprotein (LDL), in exchange for triglycerides (TG). As key determinants of core lipid abundance and composition, LCAT and CETP activities regulate the maturation of nascent HDL to spherical particles, and thereby modulate HDL heterogeneity and function. Similarly, the content of PL molecular species in the HDL particle surface can be modulated by transfer or exchange with cell membranes and lipoproteins facilitated by PLTP, and by the actions of plasma and lipoprotein-associated phospholipases, HL, and EL.

Among HDL sphingolipids and their metabolites, sphingosine-1-phosphate (S1P) functions as both an extracellular and intracellular signaling mediator in the regulation of diverse biological processes, whereas sphingomyelin (SM) is a major structural PL in HDL, but equally a determinant of CE content by virtue of its action as an inhibitor of cholesterol esterification by LCAT via diminished fluidity of the surface monolayer. These bioactive lipids may therefore directly impact not only the atheroprotective activities but also the intravascular metabolism of HDL particles. Given our lack of knowledge of the potential relationships between the lipid components of HDL particles and their antiatherogenic activities, we evaluated molecular species of major lipids, including S1P and SM, in normolipidemic HDL subpopulations. These studies identified small dense HDL3 as preferentially S1P-enriched, SM-poor particles, thereby providing a plausible structural basis for their potent antiapoptotic and antioxidative activities.

### Methods

Lipoproteins were preparatively fractionated by isopycnic density gradient ultracentrifugation from normolipidemic human serum or EDTA plasma as previously described. Five major subfractions of HDL were isolated, ie, large light HDL2b (d 1.063 to 1.090 g/mL) and HDL2a (d 1.090 to 1.120 g/mL) and small dense HDL3a (d 1.120 to 1.150 g/mL), HDL3b (d 1.150 to 1.180 g/mL), and HDL3c (d 1.180 to 1.210 g/mL). The validity and reproducibility of this density fractionation of HDL particle subspecies has been extensively documented. Details of blood samples and characterization of lipoproteins and statistical analysis are available online at http://atvb.ahajournals.org.

### Results

#### Plasma Concentrations and Physicochemical Characteristics of HDL Particle Species

On a molar basis, large light HDL2a and small dense HDL3a subclasses predominated in normolipidemic subjects; HDL subclass concentrations decreased in the order HDL2a > HDL3a > HDL2b > HDL3b > HDL3c (supplemental Table II). Molecular weights and particle diameters diminished in parallel with increment in HDL density from HDL2b to 3c (supplemental Table II). Progressive reduction in HDL particle size with increase in hydrated density was associated with progressive elevation in protein content and in surface/core ratio; reduction in size was equally accompanied by progressive reduction in HDL subclass concentrations decreased in the order HDL2a > HDL3a > HDL2b > HDL3b > HDL3c (supplemental Table II). Molecular weights and particle diameters diminished in parallel with increment in HDL density from HDL2b to 3c (supplemental Table II). Progressive reduction in HDL particle size with increase in hydrated density was associated with progressive elevation in protein content and in surface/core ratio; reduction in size was equally accompanied by reduction in core neutral lipid content (<50 mol CE and TG per HDL particle in HDL3b and 3c), consistent with the marked predominance of surface components in small dense HDL (supplemental Tables II and III).

#### Molecular Lipid Species in HDL Particles

Molar particle content of CE, FC, and of PL subclasses including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, SM, and lysophosphatidylcholine showed a marked tendency to decrease progressively in parallel with increase in hydrated density from HDL2b to HDL3c. Indeed, particle content of CE, FC, and all PL subclasses on a molar basis was significantly lower (P<0.05) in small HDL3b and 3c versus large HDL2b and 2a particles (Table). Furthermore, small HDL3c contained significantly less phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, SM, lysophosphatidylcholine, and FC relative to HDL3b on a molar basis. Interestingly, no such differences were evident between HDL subclass species when data for CE, phosphatidycholine, phosphatidylethanolamine, phosphatidylserine, and lysophosphatidylcholine were expressed as a percentage of total lipids (Table). Indeed, CE content was maintained in the range 37.9 to 50.5% across HDL subclasses; with respect to PL subclasses, phosphatidylcholine varied from 33.8 to 40.8%, phosphatidylethanolamine from 33.8 to 40.8%, and phosphatidylserine from 2.49 to 4.0%, respectively.
lamine from 0.7 to 1.0%, phosphatidylinositol from 2.8 to 5.1%, and lysophosphatidylcholine from 0.5 to 0.6%. SM constituted an exception, however, as the proportion of this lipid decreased progressively in parallel with HDL density from 12.8% in HDL2b to 6.2% in HDL3c (Figure 1A). Consequently, the SM/phosphatidylcholine ratio decreased from 0.62 in HDL2b to 0.14 in HDL3c, consistent with earlier data11 in total HDL2 and HDL3. The depletion of SM in small HDL was not associated with elevated sphingomyelinase activity, as no such activity was detected in HDL subfractions (data not shown), consistent with earlier data.12

As for SM, FC content decreased 2-fold from 8.2% in HDL2b to 4.2% in HDL3c (Table). Interestingly, the CE/FC ratio significantly increased with HDL density from 4.3±0.6 in HDL2b to 10.2±2.1 in HDL3c (P<0.001).

When HDL particle contents of molecular species of CE and phosphatidylcholine were evaluated, cholesteryl linoleate predominated among CE, with a tendency to enrichment in HDL3c (supplemental Table IV). Similarly, cholesteryl arachidonate, the minor ester, tended to be more abundant in HDL3b and 3c. As in the case of PL subclasses, the absolute molar content of each CE species diminished in parallel with
diminution in the molecular weight and particle size from HDL2 to HDL3 subfractions (supplemental Table IV).

The 18:2/16:0, 18:2/18:0 and 20:4/16:0 species of phosphatidylcholine predominated in all HDL particle subclasses, representing 17.3 to 20.3, 6.7 to 8.3, and 4.3 to 6.0%, respectively of total lipids (supplemental Table IV). Similarly, percentage content of minor PL species containing arachidonic (20:4/16:0 and 20:4/18:0) and docosahexaenoic (22:6/16:0 22:6/18:0) acids were relatively constant across the HDL particle spectrum (6.8 to 9.3% and 2.6 to 3.1%, respectively).

When lipid moieties of HDL particle subspecies were analyzed on the basis of their total fatty acid content, thereby including all PL, CE, and TG fatty acid residues, the % distribution of saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) n-6 and n-3 fatty acids was indistinguishable between HDL particle subfractions (supplemental Figure II). n-6 PUFAs preponderated in all subfractions (45% to 50% of total), with lesser abundance of SFA (30% to 35%), MUFA (12% to 15%), and n-3 PUFA (<5%). Notwithstanding such marked similarities in overall fatty acid profile among HDL subfractions, absolute molar contents of fatty acids were significantly lower in small HDL3b and 3c (P<0.05) as compared with the larger HDL2b, 2a, and 3a subfractions (supplemental Figure II).

S1P
Among the minor bioactive lipid components, the abundance of S1P per HDL particle was asymmetrical across the HDL spectrum, with preferential enrichment (40 to 50 mmol/mol HDL) in HDL3 as compared with HDL2 subfractions (15 to 20 mmol/mol; Figure 1). For example, S1P was 3-fold enriched in HDL3c (approximately 1 mole per 17 HDL particles) as compared with HDL2b (approximately 1 mole per 50 HDL particles). By contrast, no considerable difference was detected in the content of S1P between HDL3a, 3b, and 3c subfractions. HDL molar content of S1P negatively correlated with weight % content of SM (r=-0.73, P=0.002; Figure 2A) and positively correlated with that of apoA-I (r=0.63, P<0.001; Figure 2B) and total protein (r=0.70, P<0.001) as well as with the HDL surface-to-core ratio (r=0.70, P<0.001). As a result, the S1P/SM molar ratio increased (P<0.001 for trend) from 0.28±0.96 mmol/mol in HDL2b to 11.0±0.86 mmol/mol in HDL3c.

**Protein Moieties of HDL Particle Species**
The particle content of apoA-I fell progressively from a maximal level of approximately 4 mol/mol in HDL2 to a particle average of 2.5 mol/mol in HDL3c (supplemental Table V); HDL subfraction content of LpA-I decreased in a similar manner. By contrast, maximal particle contents of apoA-II occurred in HDL2a and 3a (1.1 to 1.4 mol/mol), a finding consistent with the predominance of LpA-I:A-II particles in the HDL2a and 3a subfractions, in which the abundance of LpA-I:A-II was 2- to 3-fold greater than that of LpA-I (supplemental Table V). Interestingly, and consistent with earlier published data,13 the molar ratio of apoA-I to apoA-II was highest in the largest and smallest HDL particles, respectively (HDL2b, 5.59±2.09; HDL3c, 6.89±4.23),

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**Figure 2.** Correlations between HDL molar content of S1P and wt% content of SM (A), between HDL molar content of S1P and wt% content of apoA-I (B), and between the capacity of HDL subfractions to inhibit HMEC-1 apoptosis and the S1P/SM molar ratio (C). In panel A, SM is expressed as a weight% of the sum of all molecular species of lipids measured by HPLC (PL, CE, FC, lysophosphatidylcholine, and S1P).
thereby attesting to their preferential apoA-I enrichment. Consistent with these data, the highest ratio of LpA-I/LpA-I:LpA-II was seen in the HDL2b and HDL3c particles (supplemental Table V), confirming an earlier report.\textsuperscript{14} LpA-I:LpA-II particles were more abundant than LpA-I in all HDL subfractions (LpA-I:LpA-I:LpA-II ratio <0.67).

Quantitatively minor HDL apolipoproteins including apoC-II, apo C-III, and apoE are key determinants of HDL metabolism. Although apoC-II was detected in all HDL subclasses, it was preferentially enriched (up to 3-fold) in large light HDL2b (0.85 mol/mol versus 0.24 to 0.37 mol/mol in other subclasses; supplemental Table V). ApoC-III was equally enriched in HDL2b (1.5 mol/mol); in contrast to apoC-II, however, apoC-III content fell markedly with increase in density, resulting in an elevated apoC-II/apoC-III ratio in small dense HDL3b and 3c subfractions (1.23 and 3.68 mol/mol respectively; supplemental Table V). By contrast, no significant difference in the content of apoE was found between HDL subfractions.

**Enzymatic Activities**

PON1 activity of HDL subfractions with phenyl acetate as substrate increased in the order HDL2b <HDL2a <HDL3a <HDL3b <HDL3c (supplemental Table VI). PON1 activity to paraoxon was similarly distributed among HDL subfractions (data not shown), consistent with earlier data.\textsuperscript{15} PAF-AH activity was significantly elevated in the large HDL2b and small dense HDL3c subfractions; LCAT activity tended to be elevated in the HDL3c subfraction (supplemental Table VI), consistent with our recent data.\textsuperscript{4}

**Antioxidative Activity of HDL Subfractions**

The capacity of isolated HDL subfractions to inhibit LDL oxidation by AAPH increased in the order HDL2b <HDL2a <HDL3a <HDL3b <HDL3c on a particle mass basis, consistent with earlier data.\textsuperscript{4} Small dense HDL3b and 3c subfractions (but not HDL2) decreased the oxidation rate of LDL in the propagation phase (−27 and −25%, respectively) and equally prolonged this phase (+37 and +39% respectively).

The capacity of HDL subfractions to inhibit LDL oxidation was significantly correlated with the S1P/SM molar ratio ($r = -0.43$, $P<0.05$, versus LDL oxidation rate and $r = 0.58$, $P<0.01$, versus duration of the propagation phase).

**Antiapoptotic Activity of HDL Subfractions**

Death of HMEC-1 endothelial cells treated with mildly oxLDL (200 μg apoB-100/mL; 4 to 13 mol of conjugated dienes/mol LDL) occurred mainly through an apoptotic process, as suggested by the number of cells exhibiting characteristic morphological nuclear changes, such as chromatin condensation and nuclear fragmentation (data not shown). By contrast, the level of primary necrosis was very low (<2%) as shown by flow cytometry using the annexin V-fluorescein isothiocyanate (FITC)/PI test.

Apoptosis and necrosis were quantified as cellular binding of annexin V and staining by PI; indeed, annexin V specifically interacts with phosphatidylserine in the extracellular membrane leaflet of apoptotic cells, whereas PI stains necrotic nuclei.\textsuperscript{16} Preincubation with small dense HDL3b and 3c (25 μg protein/mL) significantly diminished (−54%, $P<0.01$, and −148%, $P<0.001$, respectively) annexin V binding to HMEC-1 induced by oxLDL, whereas large HDL2a and 3a particles were not effective (data not shown). By contrast, large HDL2b was able to significantly inhibit apoptosis in this assay (−47%, $P<0.001$), an observation potentially related to their elevated content of neutral phospholipids (PL); such PL might replace phosphatidylserine in the outer plasma membrane, thereby attenuating binding of annexin V.

The capacity of HDL subfractions to inhibit HMEC-1 apoptosis was strongly and positively correlated with the S1P/SM molar ratio ($r=0.73$, $P<0.001$; Figure 2C). By contrast, no significant correlation between the antia apoptotic and antioxidative activities of HDL subfractions measured as above was found (data not shown).

In a separate experiment, normolipidemic human plasma was preincubated without or with S1P (5 μmol/L). HDL isolated from S1P-supplemented plasma were enriched in S1P (from 1.8-fold in HDL3c to 2.9-fold in HDL2b, n=3). Such S1P enrichment of HDL subfractions consistently enhanced their antia apoptotic activity. Indeed, S1P-enriched HDL2b, 2a, and 3a decreased annexin V binding to HMEC-1×30, 35% and 24%, respectively as compared with their nonenriched counterparts; S1P-enriched small dense HDL3b and 3c provided slightly weaker effects (annexin V binding was reduced by 23% and 9%, respectively), consistent with their lower levels of S1P-enrichment (data not shown).

**Discussion**

Accumulating evidence from both in vivo and in vitro studies involving recombinant as well as authentic HDL particles has revealed that small HDL3 of elevated hydrated density (>1.125 g/mL) express a spectrum of biological activities which include protection of LDL against oxidative stress, attenuation of inflammation, and elevated cellular cholesterol efflux potential.\textsuperscript{8} The mechanistic basis of the antiatherogenic and vasculoprotective actions of HDL particles, and notably the role of their lipid components, is largely indeterminate.\textsuperscript{4}

The present lipidomic investigations have revealed that small HDL3 particles are enriched in S1P but poor in SM; the S1P/SM molar ratio increased 4.3-fold from HDL2b to HDL3c. Moreover, small HDL3 potently attenuated apoptosis in endothelial cells and delayed LDL oxidation. The S1P/SM molar ratio was strongly positively correlated with the antia apoptotic and antioxidative activities of HDL subfractions, thereby identifying elevated S1P/SM ratio as an integral feature of antiatherogenic small HDL3 particles.

S1P, a bioactive lipid, plays key roles in vascular biology and can be generated from membrane sphingolipids and their metabolites, including ceramide, sphingosine, and SM.\textsuperscript{17} S1P functions as a ligand for the family of G protein–coupled S1P receptors present on endothelial and smooth muscle cells, which regulate cell proliferation, motility, apoptosis, angiogenesis, wound healing, and immune response.\textsuperscript{17} The origin of HDL–associated S1P is indeterminate as it is primarily formed by the action of intracellular sphingosine kinase on sphingosine. Hydrolysis of SM to ceramide and thence to
Sphingosine by sphingomyelinase and ceramidase, respectively, could provide sphingosine for this pathway.\textsuperscript{18} Sphingomyelinase activity is however undetectable in human HDL subfractions, consistent with earlier data;\textsuperscript{22} HDL S1P may thus be derived from platelets which do not contain S1P lyase and could release S1P into the circulation,\textsuperscript{19} or from red blood cells which display minimal S1P lyase activity.\textsuperscript{20} Enrichment of small HDL in S1P might be mechanistically related to the potent capacity of such particles to acquire polar lipids of cellular origin.\textsuperscript{23} Alternatively, sphingosine kinase, which may be released from endothelial cells, can convert sphingosine to S1P at the lipoprotein surface.\textsuperscript{18} Degradation of S1P to sphingosine by an endothelial cell-derived phosphatase represents another mechanism for potential modulation of HDL S1P content.\textsuperscript{22} By contrast, dietary origin of S1P is unlikely because there is no evidence for transport of S1P from the gut into plasma lipoproteins.\textsuperscript{23}

Mechanistically, antitherogenic action of S1P presumes initial interaction of HDL with cellular surfaces. Cell tethering could be mediated by SR-BI; subsequently, S1P can exert intracellular effects after either interaction with membrane S1P receptors\textsuperscript{5} or internalization as a component of HDL particles. The low abundance of SM in small HDL may enhance the fluidity of its surface monolayer,\textsuperscript{24} thereby facilitating selective cellular uptake of lipids, including S1P. Indeed, the positive correlation between HDL S1P content and HDL surface-to-core ratio suggests that S1P is predominantly located in the surface monolayer of HDL and should therefore be accessible for selective uptake. Consistent with this pathway, inhibition of S1P/SIP, receptors decreases the protective action of HDL3c on oxLDL-induced apoptosis (A. Négre-Salvayre et al, unpublished observation, ●●).

HDL-associated S1P has been proposed to account for protection of endothelial cells from apoptosis, induction of NO-dependent vasorelaxation, and stimulation of the anti-inflammatory expression of transforming growth factor (TGF)-β.\textsuperscript{5} S1P may equally be implicated in potent anti-inflammatory activities of small HDL3, such as inhibition of vascular adhesion molecule expression in endothelial cells.\textsuperscript{3} Our data indicate that S1P may be significant in the antiapoptotic activity of small HDL; indeed, in vitro enrichment of HDL subfractions with S1P enhanced their cytoprotective properties. Earlier investigations have established that other lysolipids, primarily sphingosylphosphorylcholine and lysosulfatide, may equally contribute HDL-mediated atheroprotection.\textsuperscript{3} By contrast, lysophosphatidic acid (LPA) does not appear to be implicated in the antiapoptotic activity of HDL subfractions because of low LPA abundance, as LPA was undetectable in our HDL subfractions (P. Therond et al unpublished observation, ●●), as reported earlier.\textsuperscript{25}

The antiapoptotic activity of HDL may be distinct from its capacity to delay LDL oxidation. Whereas the latter activity involves removal of oxidized lipids from LDL on direct contact between HDL and LDL,\textsuperscript{26} the cytoprotective action of HDL does not necessitate direct contact between HDL and oxLDL but rather is dependent on preincubation of HDL with cells and ensuing protein synthesis.\textsuperscript{22} Moreover, we found no significant correlation between the antiapoptotic and antioxidative activities of HDL subfractions. These data discriminate between cellular antiapoptotic effects of HDL subfractions and their direct effect on oxLDL, strongly arguing for a mechanism of antiapoptotic action independent of the direct antioxidative action of HDL on LDL.

In contrast to the asymmetrical distribution of S1P across HDL subfractions, the quantitative distribution of molecular species of major polar PL was uniform, suggesting that their molecular species are in dynamic equilibrium between HDL subpopulations. LCAT and CETP activity are essential factors in HDL metabolism; the relative preferential enrichment of small dense HDL3c in CE and depletion of FC supports the contention that small HDL may be a major site of cholesterol esterification within the HDL particle spectrum.\textsuperscript{28} The diminished SM/phosphatidylcholine ratio in HDL3 is consistent with this proposal, as SM functions as a physiological inhibitor of LCAT. Elevated HDL content of SM might therefore contribute to low HDL-C states; SM-mediated attenuation of lipoprotein lipase activity in TG-rich lipoproteins may further contribute to a low HDL-C phenotype, resulting in reduced release of lipolytic surface fragments to the HDL pool.\textsuperscript{29}
The distinctly low SM content (as wt%) in small HDL3c suggests that this pool is not in equilibrium with that of other HDL subpopulations, consistent with the slow rate of exchange of SM between lipoproteins and cell membranes. TG-rich lipoproteins may represent a major source of HDL SM which is principally transported to HDL by PLTP as a component of surface remnants. Preferential transfer of SM to large HDL together with its high affinity for free cholesterol might account for SM accumulation in large HDL2 particles (Figure 3). By contrast, preferential SM hydrolysis in HDL3 does not appear to contribute to SM depletion, because of the lack of sphingomyelinase activity. Alternatively, the low SM/PC ratio may reflect a distinct cellular origin(s) of small HDL as suggested by low SM content of small nascent HDL secreted by J774 macrophages, which originate from the exofacial leaflet of the plasma membrane.

SM content constitutes a critical factor in determining surface pressure in lipid membranes and lipoproteins, enhancing rigidity. Such action is consistent with the capacity of SM to inhibit selective uptake of CE by SR-BI and exchange of FC between plasma membranes and lipoproteins, potentiating by inhibiting cholesterol desorption. SM is therefore a key player in cellular cholesterol homeostasis; its depletion in small HDL is thus consistent with the elevated cellular cholesterol efflux capacity of these particles. Moreover, reduced surface pressure in SM-poor small HDL may enhance their capacity to integrate oxidized lipids from other lipoproteins or arterial wall cells, thereby contributing to potent antioxidative activity. Together, these pathways might contribute to the plausible prophagocytic activity of SM and to the antiatherogenic properties of inhibitors of SM biosynthesis.

Small HDL3c particles were selectively enriched in LpA-I reflecting enrichment in apoA-I relative to apoA-II. ApoA-I is a key component of HDL cholesterol efflux capacity and antiinflammatory and antioxidative activities, a finding consistent with elevated activities of antiatherogenic PON1, PAF-AH, and LCAT in these particles. Clearly then, small HDL3c particles display unique compositional features.

Finally, small dense HDL3b and 3c were equally characterized by an elevated apoC-II/apoC-III ratio. ApoC-II is an activator of LPL and facilitates lipolysis of TG-rich lipoproteins, whereas apoC-III inhibits this process. Enrichment of small HDL in apoC-II paralleled their depletion in SM, an inhibitor of LPL. Because lipolysis of TG-rich lipoproteins with release of surface constituents is critical for maintenance of the HDL pool, apoC-II enrichment of small HDL particles and low SM content might accelerate their maturation to large HDL, an essential step in HDL metabolism.

In conclusion, our data add a new dimension to the physicochemical and functional heterogeneity of HDL particles and reveal that small dense HDL3c are S1P-, apoA-I-, and LpA-I-enriched, but SM-poor, and that such components can contribute to their potent antiapoptotic and antioxidative activities.

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Disclosures
None.

References


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**Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small, dense HDL3 particles: Relevance to antiapoptotic and antioxidative activities**

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**Online Supplement**

**Methods**

**Blood samples**

Serum and EDTA plasma (final EDTA concentration 1 mg/ml) were prepared from venous blood collected into sterile evacuated tubes (Vacutainer) from nine healthy male volunteers after an overnight fast. Donors were normolipidemic, non-obese, normotensive, normoglycemic and displayed normal levels of hsCRP and 8-isoprostanes\(^1\, ^2\) (Online Table I, please see www.ahajournals.org). The study was approved by the Institutional Review Committee; all subjects gave their informed consent and all procedures were in accordance with institutional guidelines. None of our blood donors was receiving antioxidant vitamin
Small HDL3 enriched in S1P but sphingomyelin-poor

supplementation or drugs known to affect lipoprotein metabolism; all subjects were non-smokers and either abstainers or only moderate alcohol consumers. After blood collection, serum and EDTA plasma were immediately separated by centrifugation at 4°C; plasma and serum were each mixed with sucrose (final concentration 0.6%) as a cryoprotectant for lipoproteins and frozen at -80°C under nitrogen for less than 3 months.

Fractionation of lipoproteins

Lipoproteins were preparatively fractionated by isopycnic density gradient ultracentrifugation as previously described. Four major subfractions of HDL were isolated, i.e. large, light HDL2b (d 1.063–1.090 g/ml) and HDL2a (d 1.090–1.120 g/ml), and small, dense HDL3a (d 1.120–1.150 g/ml), HDL3b (d 1.150–1.180 g/ml), and HDL3c (d 1.180–1.210 g/ml). The validity and reproducibility of this density fractionation of HDL particle subspecies has been extensively documented. Lipoproteins were stored at 4°C and analysed within 10 days. As LCAT activity rapidly decreases in HDL subfractions upon storage at 4°C (Nobecourt E, Kontush A, Chapman MJ, unpublished data), LCAT was assayed not later than 48h after HDL isolation. Before use, KBr (and, in the case of plasma, EDTA) was removed from LDL and HDL solutions by exhaustive dialysis for 24 h at 4°C.

Physicochemical characterisation of lipoproteins

Total protein, lipids and apolipoproteins. Total protein, total cholesterol (TC), FC, PL and TG contents of isolated lipoprotein subfractions were determined using commercially available enzymatic assays (coefficients of variation <7%). Cholesteryl esters (CE) were calculated by multiplying the difference between total and free cholesterol by 1.67. Total HDL mass was calculated as a sum of total protein, FC, PL, TG and CE. ApoA-I, apoA-II, apoC-II, apoC-III and apoE were measured using immunonephelometry. Plasma levels of LpA-I and LpA-I:A-II were measured by immunoelectrophoresis (Sebia, Issy-les-Moulineaux, France; coefficient of variation, 3%).
Molecular weights of HDL subfractions were calculated by transforming concentration data (mg/dl) into absolute molar units using molecular weights of CE, FC, PL and TG of 650, 387, 750 and 850 Da, respectively\(^9\); the HDL protein moiety was considered to consist of two apolipoproteins, apoA-I and apoA-II, and the molecular weight of the protein moiety in each HDL subfraction was calculated using the total protein content (mg/dl) converted to molarity on the basis of relative mass content of apoA-I and apoA-II.

**Enzymatic activities.** PON1 activity of HDL subfractions (100 µg protein/ml) isolated from serum was determined photometrically in the presence of CaCl\(_2\) (1 mM) using phenyacetate or paraoxon as a substrate.\(^{10,11}\) Activity of PAF-AH was assessed using C\(_6\)NBD phosphatidylcholine as a fluorescent substrate.\(^{11,12}\) LCAT activity was measured using a fluorescent LCAT activity kit (Roar Biomedical, New York, NY, USA).\(^{11}\) Sphingomyelinase activity was assayed using the Amplex Red fluorescence kit (Invitrogen, Carlsbad, CA, USA).

**Molecular species of cholesteryl esters and phosphatidylcholine.** Lipids were extracted with methanol/hexane (4/10 v/v) from aliquots of HDL subfractions as previously described.\(^{13}\) Briefly, the hexane layer (upper phase containing CE) and the methanol/water layer (lower phase containing phosphatidylcholine) were separated by centrifugation at 1500 g for 5 min and evaporated to dryness under nitrogen. The dried residue corresponding to phosphatidylcholine was dissolved in methanol and, after loading onto the HPLC system, separation of molecular species of phosphatidylcholine was performed as previously described\(^{14}\) with a 250 x 4.6 mm C18 Kromasil column with 6% ammonium acetate (10 mM, pH5.0)/ 94% methanol as mobile phase. The dried residue corresponding to CE was dissolved in methanol containing 1% hexane and separation of CE was performed with a 150 x 4.6 mm C18 Spherisorb column and methanol as mobile phase as previously described.\(^{14}\)

**Major classes of CE, PL and lysophosphatidylcholine.** Lipids were extracted from HDL subspecies using the method of Folch et al.\(^{15}\) Organic extracts were evaporated to dryness...
under nitrogen and the dried lipids were dissolved in isopropanol: hexane (60: 40, v/v).

Normal phase HPLC separation was performed on a Kromasil silica 5 µm (2.1 mm i.d. x 250 mm) column with elution using a mobile phase of isopropanol: hexane: 25 mM potassium acetate (pH 7.0) (57.5: 36:6.5, v/v/v) at 50°C with a gradient from 36 to 50% of hexane in 30 minutes and a flow rate of 0.4 ml/minute. Chromatographic peaks were identified using UV absorbance at 205 nm. All PL were clearly separated into phosphatidylcholine, phosphatidylethanolamine, sphingomyelin (two peaks corresponding to two molecular species, 16:0 sphingomyelin and 18:0 sphingomyelin), phosphatidylinositol and lysophosphatidylcholine (Online Fig. I). Individual PL and lysophosphatidylcholine peaks were identified by comparison of retention time to known standards (16:0/18:2 phosphatidylethanolamine, 16:0 sphingomyelin, 16:0/18:2 phosphatidylinositol, 18:0 lysophosphatidylcholine). Calibration curves for each lipid standard were established for quantification in HDL subspecies (coefficients of variation, <5%). The extraction efficacy was similar for all lipids between large and small HDL particles. Indeed, recoveries of minor lipids (phosphatidylinositol, sphingomyelin and lysophosphatidylcholine) from large HDL2 vs. small HDL3 were as follows (n=4): phosphatidylinositol, 90.1±2.8 vs. 89.5±1.9%; sphingomyelin, 91.1±5.5 vs. 91.6±3.5%; lysophosphatidylcholine, 95.3±3.0 vs. 90.3±7.6%.

Recoveries of major lipids (phosphatidylcholine and phosphatidylethanolamine) ranged from 91 to 95% and did not differ between large and small HDL either.

*Sphingosine-1-phosphate (SIP).* SIP was determined in HDL subspecies as described by Nofer et al.\textsuperscript{16} Methanol (1 ml) containing 2.5 µl concentrated HCl was added to 100 µL of HDL solution (0.5 to 2.0 mg HDL per ml buffer as a function of HDL subspecies). C_{17}-SIP (15 pmoL) was added as internal standard and lipids were extracted by addition of 1 ml chloroform, 200 µl NaCl (4 M) and 100 µl NaOH (3 M). The alkaline aqueous SIP-containing phase devoid of other sphingoid bases and of the majority of hydrophobic PL was
transferred to a clean tube and the organic phase re-extracted with 0.5 ml methanol, 0.5 ml of 1M NaCl and 3N NaOH (50 µl). The alkaline aqueous phases were combined, acidified with 100 µl concentrated HCl and extracted twice with 1.5 ml chloroform. The organic phases were evaporated and the dried lipids were dissolved in a mixture (50 µl) of methanol and 0.07 M K₂HPO₄ (9:1 v/v). A derivatization mixture of 10 mg o-phthaldialdehyde, 200 µl ethanol, 10 µl 2-mercaptoethanol and 10 ml boric acid (3% v/w) was prepared and adjusted to pH 10.5 with KOH. Five µl of the derivatization mixture was added to the lipids and the solution incubated for 15 minutes at room temperature. The derivatives were analyzed with a Hewlett Packard HPLC system using an RP 18 Kromasil column (2.1 mm i.d. x 150 mm) maintained at 45°C. Separation was performed with the isocratic eluent containing methanol: K₂HPO₄ (0.07 M) (78:22 v/v) at a flow rate of 0.25 mL/min. The derivatives were detected selectively using a Hewlett Packard spectrofluorometer with an excitation wavelength of 340 nm and an emission wavelength of 456 nm. S1P was quantified by comparison of its fluorescent signal with that of the derivative of the internal standard (coefficients of variation, <5%).

**Fatty acids.** Fatty acids were analysed by gas-liquid chromatography (GLC) using a Shimadzu instrument (Kyoto, Japan) equipped with a 30-m Supelcowax 10 glass capillary column (Supelco, Bellefonte, PA, USA) and connected to a Delsi Nermag integrator (Argenteuil, France). Total lipids were extracted according to the method of Bligh and Dyer from 0.1 ml of each HDL subspecies. The chloroform phase was evaporated to dryness under nitrogen and total lipids were transesterified with 1 ml of methanolic KOH-BF₃ at 70°C for 5 minutes. After extraction by 1 ml of hexane and evaporation under nitrogen, the residue was reconstituted in isooctane (25 µl). Two to four µl of this organic solution of fatty acid methyl esters were separated and measured by GLC. Peaks were identified by comparing the retention time with those of the corresponding standards and quantified by using an internal standard (heptadecanoic acid; coefficient of variation, <5%).
Antioxidative activity of HDL subfractions

Antioxidative activity of HDL subfractions was assessed towards normolipidemic reference LDL\(^1,2\). LDL (10 mg TC /dl) was oxidised in the absence or presence of HDL particles at 37\(^\circ\)C in Dulbecco’s PBS (pH 7.4) by 1 mM 2,2’-azobis-(2-amidinopropane) hydrochloride (AAPH); HDL subfractions were added to LDL immediately before oxidation at a final concentration of 10 mg total mass/dl.\(^1,2\) Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm.\(^1,2\) Absorbance kinetics were corrected for the absorbance of AAPH itself run in parallel as a blank. The kinetics of diene accumulation revealed two characteristic phases, the lag and propagation phases. For each curve, the duration of each phase, average oxidation rates within the propagation phase and amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated.

Antiapoptotic activity of HDL subfractions

Human microvascular endothelial cells-1 (HMEC-1) were grown in MCDB 131 medium supplemented with 10% fetal calf serum, glutamine (2 mM), 100 U/ml penicillin and 100 µg/ml streptomycin. At 100% confluence, cells were plated at a concentration of 10,000 to 40,000 cells/ml and subsequently (24 or 48 h later) preincubated in the absence (control) or in the presence of each HDL subfraction (25 µg protein/ml) in a serum-free RPMI medium for the next 24 h. The medium containing HDL was removed, cells washed and incubated with oxidised LDL (oxLDL) for up to 24 hours. LDL (d 1.018–1.065 g/ml; 200 mg apoB-100/dl corresponding to 4 µM LDL) was oxidised by UV irradiation in the presence of Cu\(^{2+}\) (5 µM). The LDL preparation was irradiated for 2h as a thin film (5 mm) in an open beaker placed 10 cm under the UV-C source (HNS 30W OFR Osram UV-C tube, \(\lambda_{max}\) 254 nm, 0.5 mW/cm\(^2\)). This approach produces mildly oxLDL that selectively induces apoptosis rather than necrosis in endothelial cells.\(^18\) The level of LDL oxidation was evaluated by the absorbance increment
at 234 nm (Δ234 nm), which reflects the accumulation of lipid hydroperoxides possessing a conjugated diene structure; diene concentration in the samples was calculated using $\varepsilon = 29,500 \text{ M}^{-1}\text{cm}^{-1}$. The absorbance increment at 234 nm of 0.050-0.150 was used, corresponding to 4-13 mol of conjugated dienes/mol LDL. After irradiation, oxLDL preparations were sterilised using 0.2 µm Millipore filters.

Apoptosis and necrosis in HMEC-1 were quantified using the Annexin V- FITC / propidium iodide (PI) kit (Beckman Coulter, Roissy, France). Cell medium was collected and cells were washed twice with PBS at 4°C, trypsinised for 15 min at 37°, centrifuged, washed and stained for 15 min at 4°C in the dark. The level of primary apoptosis was specifically determined by flow cytometry analysis at excitation and emission wavelengths of 492 and 520 nm respectively for annexin V - FITC and 550 and 680 nm respectively for PI; cells displaying primary apoptosis were identified by a combination of high FITC and low PI signals. Non-stained controls were used to correct for cellular auto-fluorescence in the presence of oxLDL.

To prepare S1P-enriched HDL subfractions, EDTA plasma from a normolipidemic donor was incubated overnight with S1P under constant stirring at 4°C. S1P was added as a methanolic solution to an empty tube (final concentration 5 µM), methanol evaporated and plasma added. Plasma from the same subject incubated in parallel was used as a control. Subsequently, S1P-enriched and control HDL subfractions were isolated and their S1P content measured by HPLC as described above. HMEC-1 (20,000-30,000 cells/well) were plated in 12-well plates and incubated in the absence or presence of each HDL subfraction (25µg of total protein/ml) in a serum-free RPMI 1634 medium for 24h; oxLDL (Δ234 nm, 0.050-0.150 corresponding to 4-13 mol of conjugated dienes/mol LDL) was added for 16 to 24h and flow cytometry performed as described above.

Statistical analysis
Significance of differences in chemical composition between HDL subfractions was analysed with a one-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni correction for multiple group comparisons or with Student’s t-test for dependent samples when applicable. Using the correction for multiple comparisons, the p-level for a given comparison was adjusted down in such a way that the alpha level of 0.05 can be still considered significant.

Pearson’s moment-product correlation coefficients were calculated to evaluate relationships between variables. All results are expressed as means ± SD unless otherwise indicated. Statistical analysis of differences between HDL subfractions was carried out by Wilcoxon’s test. Values of $P < 0.05$ were considered significant. Calculations were performed using STATISTICA 6.1 (StatSoft Inc., www.statsoft.com). or StatView (SAS Institute Inc., www.statview.com/) software packages.
**References**


12. Gowri MS, Van der Westhuyzen DR, Bridges SR, Anderson JW. Decreased protection by HDL from poorly controlled type 2 diabetic subjects against LDL oxidation may be due to the abnormal composition of HDL. *Arterioscler Thromb Vasc Biol.* 1999;19:2226-2233.


Online Figure Legends

Online Fig. I. Typical chromatograms of molecular species of PL in HDL subfractions. Lipids were extracted from HDL subspecies and separated using normal phase HPLC on a Kromasil silica 5 µm (2.1 mm i.d. x 250 mm) column with isopropanol: hexane: 25 mM potassium acetate (pH 7.0) (57.5: 36:6.5, v/v/v) as a mobile phase at 50°C with a gradient from 36 to 50% of hexane in 30 minutes and a flow rate of 0.4 ml/minute. Chromatographic peaks were identified using UV absorbance at 205 nm. All PL were clearly separated into phosphatidylethanolamine, phosphatidylcholine, sphingomyelin (two peaks corresponding to two molecular species, 16:0 sphingomyelin and 18:0 sphingomyelin), phosphatidylinositol and lysophosphatidylcholine. Individual PL and lysophosphatidylcholine peaks were identified by comparison of retention time to known standards (16:0/18:2 phosphatidylethanolamine, 16:0 sphingomyelin, 16:0/18:2 phosphatidylinositol, 18:0 lysophosphatidylcholine).

Online Fig. II. Major classes of fatty acids in HDL subfractions from normolipidemic subjects (n=6). HDL content of saturated (dotted bars), monounsaturated (filled bars), n-6 (hatched bars) and n-3 polyunsaturated (open bars) fatty acids is shown as mol/mol HDL (A) or as a percentage of total fatty acids in each HDL subfraction (B). For superscripts, see Online Table II.
**Online Table I. Biological characteristics of normolipidemic subjects (n=9)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>48±16</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>9/0</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>81±6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.8±1.5</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>130±12</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>86±8</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>170±25</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>83±14</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>100±24</td>
</tr>
<tr>
<td>ApoB-100 (mg/dl)</td>
<td>86±19</td>
</tr>
<tr>
<td>VLDL-C (mg/dl)</td>
<td>17±4</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>53±9</td>
</tr>
<tr>
<td>ApoA-I (mg/dl)</td>
<td>155±14</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>95±14</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.8 (0.2; 1.5)</td>
</tr>
<tr>
<td>8-Isoprostanes (ng/l)</td>
<td>39±22</td>
</tr>
</tbody>
</table>
Online Table II. Plasma concentrations, molecular weight, particle diameter and surface/core ratio of HDL particle subfractions from normolipidemic subjects

<table>
<thead>
<tr>
<th></th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar concentration, µmol/l</td>
<td>1.44±0.51</td>
<td>1.81±0.36&lt;sup&gt;d,j&lt;/sup&gt;</td>
<td>1.60±0.36&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>1.39±0.45&lt;sup&gt;b,h,o&lt;/sup&gt;</td>
<td>1.01±0.37&lt;sup&gt;g,h,n&lt;/sup&gt;</td>
</tr>
<tr>
<td>Calculated molecular weight, kDa</td>
<td>499±102&lt;sup&gt;c,n,o&lt;/sup&gt;</td>
<td>413±58&lt;sup&gt;c,n,o&lt;/sup&gt;</td>
<td>370±38&lt;sup&gt;a,b,n,o&lt;/sup&gt;</td>
<td>198±12&lt;sup&gt;k,l,m,o&lt;/sup&gt;</td>
<td>163±19&lt;sup&gt;k,l,m,n&lt;/sup&gt;</td>
</tr>
<tr>
<td>Particle diameter, nm</td>
<td>10.1±0.3&lt;sup&gt;g,h,d,j&lt;/sup&gt;</td>
<td>8.2±0.1&lt;sup&gt;f,e&lt;/sup&gt;</td>
<td>7.8±0.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.6±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7±0.1&lt;sup&gt;b,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Surface/core ratio</td>
<td>2.18±0.25&lt;sup&gt;b,m,n,o&lt;/sup&gt;</td>
<td>2.49±0.30&lt;sup&gt;a,h,n,o&lt;/sup&gt;</td>
<td>2.69±0.26&lt;sup&gt;k,g,n,j&lt;/sup&gt;</td>
<td>3.41±0.46&lt;sup&gt;k,l,m&lt;/sup&gt;</td>
<td>3.92±0.67&lt;sup&gt;k,l,h&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are shown for healthy male donors (n=9). HDL surface/core ratio was calculated as the ratio of surface components (PL, FC, protein) to core (CE, TG) components (wt/wt). Superscripts are as follows: <sup>a</sup>p < 0.05 vs. HDL2b, <sup>b</sup>p < 0.05 vs. HDL2a, <sup>c</sup>p < 0.05 vs. HDL3a, <sup>d</sup>p < 0.05 vs. HDL3b, <sup>e</sup>p < 0.05 vs. HDL3c, <sup>f</sup>p < 0.01 vs. HDL2b, <sup>g</sup>p < 0.01 vs. HDL2a, <sup>h</sup>p < 0.01 vs. HDL3a, <sup>i</sup>p < 0.01 vs. HDL3b, <sup>j</sup>p < 0.01 vs. HDL3c, <sup>k</sup>p < 0.001 vs. HDL2b, <sup>l</sup>p < 0.001 vs. HDL2a, <sup>m</sup>p < 0.001 vs. HDL3a, <sup>n</sup>p < 0.001 vs. HDL3b, <sup>o</sup>p < 0.001 vs. HDL3c.
Online Table III. Chemical composition (weight %) of HDL subfractions from normolipidemic subjects

<table>
<thead>
<tr>
<th></th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl esters</td>
<td>25.7 ± 2.6</td>
<td>24.8 ± 3.2</td>
<td>23.5 ± 2.6</td>
<td>19.4 ± 2.4</td>
<td>16.9 ± 4.0</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>5.3 ± 1.2</td>
<td>3.3 ± 0.8</td>
<td>2.6 ± 0.7</td>
<td>2.2 ± 0.7</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>28.5 ± 4.4</td>
<td>31.7 ± 4.6</td>
<td>28.1 ± 4.2</td>
<td>21.5 ± 2.8</td>
<td>15.8 ± 3.3</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>6.0 ± 2.2</td>
<td>4.1 ± 1.4</td>
<td>3.7 ± 1.0</td>
<td>3.5 ± 1.1</td>
<td>3.8 ± 1.6</td>
</tr>
<tr>
<td>Total protein</td>
<td>34.6 ± 2.5</td>
<td>36.1 ± 3.3</td>
<td>42.0 ± 3.3</td>
<td>53.4 ± 3.2</td>
<td>61.6 ± 5.8</td>
</tr>
</tbody>
</table>

Data are shown for 9 healthy male donors. The surface/core ratio was calculated as a ratio of surface (PL, FC, total protein) over core (CE, TG) HDL components (wt/wt); for superscripts, see Online Table II.
**Online Table IV. Molecular species of cholesteryl esters and phosphatidylcholine in HDL particle subfractions from normolipidemic subjects**

<table>
<thead>
<tr>
<th>Lipid component</th>
<th>Composition of HDL subspecies (mol/mol HDL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL2b</td>
</tr>
<tr>
<td>Cholesteryl esters</td>
<td></td>
</tr>
<tr>
<td>20:4</td>
<td>12.44±4.98&lt;sup&gt;n,o&lt;/sup&gt; (4.8)</td>
</tr>
<tr>
<td>18:2</td>
<td>92.1±34.1&lt;sup&gt;n,o&lt;/sup&gt; (35.2)</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td></td>
</tr>
<tr>
<td>22:6/16:0</td>
<td>4.62±2.38&lt;sup&gt;n,o&lt;/sup&gt; (1.8)</td>
</tr>
<tr>
<td>20:4/16:0</td>
<td>11.25±4.26&lt;sup&gt;n,o&lt;/sup&gt; (4.3)</td>
</tr>
<tr>
<td>18:2/16:0</td>
<td>45.66±18.21&lt;sup&gt;n,o&lt;/sup&gt; (17.4)</td>
</tr>
<tr>
<td>22:6/18:0</td>
<td>2.20±0.78&lt;sup&gt;n,o&lt;/sup&gt; (0.8)</td>
</tr>
<tr>
<td>20:4/18:0</td>
<td>6.44±2.06&lt;sup&gt;n,o&lt;/sup&gt; (2.5)</td>
</tr>
<tr>
<td>18:2/18:0</td>
<td>18.40±7.30&lt;sup&gt;n,o&lt;/sup&gt; (7.0)</td>
</tr>
</tbody>
</table>
Data are shown for healthy male donors (n=6). The % composition of the lipid moiety of each HDL particle subfraction as measured by HPLC (CE, FC, PC, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and lysophosphatidylcholine but excluding TG and partial glycerides) is given in parentheses; for superscripts, see Online Table II.
**Online Table V.** Lipoprotein A-I, lipoprotein A-I:A-II and apolipoprotein contents of HDL particle subfractions from normolipidemic subjects

<table>
<thead>
<tr>
<th></th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>LpA-I:A-II, mg/dl</td>
<td>10.05±5.33</td>
<td>19.61±7.98</td>
<td>17.34±3.40</td>
<td>8.26±0.67</td>
<td>4.94±1.14</td>
</tr>
<tr>
<td>LpA-I/LpA-I:A-II, wt/wt</td>
<td>0.91±0.23</td>
<td>0.50±0.18</td>
<td>0.30±0.03</td>
<td>0.44±0.15</td>
<td>0.67±0.11</td>
</tr>
<tr>
<td>ApoA-I, mol/mol HDL</td>
<td>4.07±0.90</td>
<td>4.09±0.67</td>
<td>3.05±0.86</td>
<td>2.73±1.01</td>
<td>2.53±1.11</td>
</tr>
<tr>
<td>ApoA-II, mol/mol HDL</td>
<td>0.79±0.23</td>
<td>1.41±0.26</td>
<td>1.14±0.32</td>
<td>0.64±0.23</td>
<td>0.42±0.19</td>
</tr>
<tr>
<td>ApoA-I/apoA-II, mol/mol</td>
<td>5.59±2.09</td>
<td>2.93±0.37</td>
<td>2.77±0.66</td>
<td>4.64±1.88</td>
<td>6.89±4.23</td>
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<tr>
<td>ApoE, mol/mol HDL</td>
<td>0.12±0.05</td>
<td>0.03±0.01</td>
<td>0.14±0.07</td>
<td>0.08±0.04</td>
<td>0.04±0.03</td>
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<tr>
<td>ApoC-II, mol/mol HDL</td>
<td>0.85±0.38</td>
<td>0.37±0.15</td>
<td>0.24±0.05</td>
<td>0.30±0.06</td>
<td>0.34±0.09</td>
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<tr>
<td>ApoC-III, mol/mol HDL</td>
<td>1.46±0.53</td>
<td>0.85±0.37</td>
<td>0.54±0.28</td>
<td>0.30±0.15</td>
<td>0.15±0.12</td>
</tr>
<tr>
<td>ApoC-II/apoC-III, mol/mol</td>
<td>0.56±0.07</td>
<td>0.43±0.02</td>
<td>0.50±0.21</td>
<td>1.23±0.63</td>
<td>3.68±3.23</td>
</tr>
</tbody>
</table>

Data are shown for 3-9 healthy male donors. For superscripts, see Table 1.
**Online Table VI. Enzymatic activities of HDL subfractions from normolipidemic subjects**

<table>
<thead>
<tr>
<th></th>
<th>HDL2b</th>
<th>HDL2a</th>
<th>HDL3a</th>
<th>HDL3b</th>
<th>HDL3c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PON1, nmol/min/mg protein</strong></td>
<td>$45 \pm 25^{b,h,i,o}$</td>
<td>$95 \pm 63^{a,h,i,o}$</td>
<td>$419 \pm 257^{f,g,i,o}$</td>
<td>$1648 \pm 755^{f,g,h,j}$</td>
<td>$3788 \pm 1374^{k,l,m,i}$</td>
</tr>
<tr>
<td><strong>PAF-AH, nmol/min/mg protein</strong></td>
<td>$2.89 \pm 1.17^{b,c}$</td>
<td>$1.01 \pm 0.19^{a,d,j}$</td>
<td>$0.98 \pm 0.14^{a,d,j}$</td>
<td>$1.96 \pm 0.56^{h,c,j}$</td>
<td>$3.17 \pm 0.81^{g,h,i}$</td>
</tr>
<tr>
<td><strong>LCAT, % hydrolysed substrate</strong></td>
<td>$5.7 \pm 4.1$</td>
<td>$6.4 \pm 3.9$</td>
<td>$6.5 \pm 3.7$</td>
<td>$7.4 \pm 5.9$</td>
<td>$21.6 \pm 9.8$</td>
</tr>
</tbody>
</table>

Data are shown for 5 healthy male donors; for superscripts, see Online Table II.
Online Figure I
Online Figure IIA
Online Figure IIB