ApoE and apoC-III-defined HDL subtypes: A descriptive study of their LCAT and CETP content and activity

CURRENT STATUS: UNDER REVIEW

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DOI:
10.21203/rs.2.20333/v2

SUBJECT AREAS
Endocrinology & Metabolism  Cardiac & Cardiovascular Systems

KEYWORDS
HDL, lecithin cholesterol acyltransferase, cholesterol ester transfer protein, apolipoprotein E, apolipoprotein C-III, reverse cholesterol transport
Abstract

Background High-density lipoproteins (HDL) functionality predicts cardiovascular risk better than HDL concentrations. The apolipoprotein composition of HDL may be a determinant of their function. Lecithin-cholesterol acyl transferase (LCAT) and cholesterol-ester transfer protein (CETP) are key enzymes for HDL-mediated reverse cholesterol transport. We assessed the distribution and activity of LCAT and CETP in HDL subspecies defined by their content of apolipoproteins E (apoE) and C-III (apoC-III) in humans.

Methods We isolated in 18 adult humans of both sexes (mean age 55.6, BMI 26.9 Kg/m2, HbA1c 5.4%), four subspecies of HDL containing respectively: No apoE and no apoC-III (E-C-), apoE but not apoC-III (E+C-), apoC-III but no apoE (E-C+) and both apoE and apoC-III (E+C+). In each HDL subspecies, we measured LCAT and CETP concentration and activity using immunoenzymatic and fluorometric methods. Additionally, we determined the size distribution of HDL in each apolipoprotein-defined fraction using non-denaturing electrophoresis and anti-ApoA-I western blot.

Results Similar to previous studies, HDL in the E-C- fraction was the predominant subtype. The size distribution of HDL was very similar across all four apolipoprotein-defined fractions. LCAT was most abundant in E-C- HDL (3.58 mg/mL, 59.6 % of plasma LCAT mass), while HDL with apoE or apoC-III had much less LCAT (19.8%, 12.2% and 8.37% of plasma LCAT respectively for E+C-, E-C+ and E+C+). LCAT mass was lower in E+C- HDL relative to E-C- HDL, but LCAT activity was similar in both fractions, signaling a greater activity-to-mass ratio associated with the presence of apoE. Both CETP mass and CETP activity showed only slight variations across HDL subspecies. There was an inverse correlation between plasma LCAT activity and both E-C+ pre-beta HDL (r=-0.55, p=0.017) and E-C-alpha 1 HDL (r=-0.49, p=0.041). Conversely, there was a direct correlation between E-C+ alpha 1 HDL and CETP activity in plasma (r=0.52, p=0.025).

Conclusions Our results suggest that LCAT activity in humans is influenced by the presence of small interchangeable apolipoproteins. The presence of apoE in small HDL is correlated with increased LCAT activity and esterification of plasma cholesterol.

Background
A host of observational studies has demonstrated a negative association between plasma concentrations of high-density lipoprotein cholesterol (HDL-C) and the risk of cardiovascular disease (CVD) (1-4). However, medications aimed at raising HDL-C have failed to reduce the incidence of CVD in clinical trials. The groups of HDL-C raising agents that have proven ineffective in preventing CVD includes cholesteryl ester transfer protein (CETP) inhibitors, fibrates and niacin (5-8). This apparent paradox seems to be explained by the fact that HDL functionality, rather than HDL-C concentration, is the relevant measure associated with CVD prevention (9-10). Many aspects of HDL functionality have been evaluated over the recent years, including but not limited to reverse cholesterol transport (RCT), induction of nitric oxide synthesis, reduction in the expression of cell adhesion molecules and antioxidant capacity (11). Lecithin-cholesterol acyl transferase (LCAT) and CETP are key enzymes in HDL metabolism and functionality. LCAT transfers an acyl group from lecithin to free cholesterol, forming cholesterol esters that move to the core of the HDL particle and are eventually delivered to the liver (12,13). Meanwhile, CETP mediates the transfer of cholesterol esters from HDL to apoB lipoproteins, while triglycerides are simultaneously transferred in the opposite direction (14). Subsequently, the apoB-containing lipoproteins that received cholesterol esters are taken up by the liver, completing the indirect pathway of RCT (15).

The concentration and activity of LCAT and CETP can be modulated by different molecules. For example, polyunsaturated fatty acids (PUFA) reduce transcription of the LCAT mRNA and synthesis of the LCAT protein in vitro (16). On the other hand, sphingomyelin (17), oxidized lipids (18,19), n-3 fatty acids (20) and trans-unsaturated fatty acids (21) inhibit LCAT in vitro. Factors associated with upregulation of CETP gene expression are a high dietary intake of cholesterol of omega-3 PUFA, and the use of fibrates (22,23). Meanwhile, plasma CETP activity and mass are increased upon increases in the plasma concentrations of bile acids (24), and decreased in patients with hypothyroidism (25). Nonetheless, the modulation of human LCAT and CETP by components of HDL in vivo is insufficiently understood.

All lipoproteins harbor a repertoire of small apolipoproteins, which are important modulators of their metabolism. Two of these small apolipoproteins are apoE and apoC-III. ApoE mediates the clearance
of VLDL, IDL and chylomicron remnants via the LDL receptor, LDL receptor-related protein-1 (LRP-1) or heparan sulfate proteoglycans (26,27). Contrastingly, apoC-III strongly inhibits the clearance of all apoB-lipoproteins (28,29). ApoC-III also impairs the catabolism of triglyceride-rich lipoproteins and stimulates hepatic VLDL assembly and secretion (28,30). However, the role of apoE and apoC-III on HDL physiology is less understood. It is known that besides apoA-I (the natural cofactor of LCAT) (31), apoE is able to activate LCAT in vitro (32), while an increase in the apoC-III content of synthetic HDL shows the opposite effect (33). In apoA-I knockout mice, apoE is able to partially rescue LCAT activity, albeit in VLDL and LDL, not in HDL (34). Despite their major relevance in lipoprotein metabolism, little is known about the influence of apoE and apoC-III over LCAT and CETP in humans.

With this background, we analyzed the distribution of LCAT and CETP mass and activity in multiple HDL subclasses defined by their content of apoE and apoC-III and molecular size, in normal weight and overweight adult humans of both sexes.

Methods
Study Design and Participants

We studied 18 patients (8 male, 10 female) aged 33 to 76, selected from a project about new biomarkers of insulin resistance (34). We excluded individuals with known diabetes mellitus or use of anti-diabetic medications, other endocrine disorders, diseases of the exocrine pancreas, pregnant women or patients who received oral anticoagulants or lipid-lowering drugs. Patients who had symptoms of an acute viral or bacterial infection, or in whom hsCRP levels were higher than 10 mg/L, were also excluded. We obtained blood samples in EDTA tubes after an 8 hours fast. Plasma was promptly separated, supplemented with a preserving cocktail (benzamidine, phenylmethylsulphonyl fluoride and gentamicin), aliquoted and stored at -80°C for later analyses. We measured plasma concentrations of apoA-I using a nephelometric method (35) and fasting plasma glucose (FPG), plasma lipids and creatinine using conventional colorimetric assays (Biosystems, Spain). Glycated hemoglobin A1c (HbA1c) was determined using a National Glycohemoglobin Standardization Program-certified boronate affinity technique (NycoCard™ Reader II, Alere Technologies, Norway).
**HDL isolation and separation**

Plasma was passed through Acrodisc® 5µm filters (Pall Corporation, Port Washington, NY), in order to remove fibrin impurities. Then, HDL was purified from plasma using immunoaffinity chromatography as follows: Sepharose 4B™ resin bound to goat polyclonal anti-human apoA-I antibodies (Academy Bio-medical Co) was loaded into 10mL Poly-Prep® chromatography columns (Bio-Rad Laboratories), and 1 mL of plasma was incubated overnight in the column. The unbound fraction was collected by gravity flow and stored at -80 °C. The bound fraction was eluted using 3 consecutive washes of 3M sodium thiocyanate (NaSCN) and one last PBS wash followed by concentration and desalting in 10 KDa MW cutoff Amicon® filters (Merck Millipore, Billerica, MA) until 1 mL of fraction was achieved. Then two PBS washes were performed to remove any NaSCN left over in the column before another use. The same process was carried out with columns containing goat polyclonal anti-human apoC-III and anti-human apo-E antibodies, in that order. At the end, HDL from each plasma sample was divided into 4 subfractions: HDL without apoE or apoC-III (E-C-), HDL with apoE but without apoC-III (E+C-), HDL without apoE but with apoC-III (E-C+) and HDL with both apoE and apoC-III (E+C+). The efficiency of the immunoaffinity columns was 95-98% for all study subjects. The final buffer for all HDL subfractions was PBS.

**Determination of enzymatic content and activity in HDL subfractions**

LCAT concentration was determined using an immunoenzymatic, double-sandwich assay (ALPCO Diagnostics, Salem, NH) in which capture is performed by a first monoclonal antibody against LCAT (MoAb 36486) and detection is performed with a different, horseradish peroxidase (HRP)-labeled anti-LCAT monoclonal antibody (MoAb 36487). After incubation with a substrate solution and termination with a stop reagent, the intensity of absorbance at 492 nm was read in a Synergy HT microplate reader using Gen5 software (BioTek, Winooski, VT). CETP concentration was determined using an immunoenzymatic sandwich assay (ALPCO Diagnostics, Salem, NH), with MoAb 3-11D as capture antibody and HRP-labeled MoAb 14-8F as detection antibody.

Measurement of LCAT activity was done using a fluorometric assay (Calbiochem, Darmstadt,
Germany). The assay is based on the incubation of a substrate that fluoresces at 470 nm with the study samples. After LCAT in the samples removes a fatty acid from the substrate and transfers it to cholesterol, the substrate loses fluorescence at 470 nm and gains fluorescence at 390 nm. Thus, LCAT activity was measured as change in 470/390 emission intensity by comparison against a calibration curve based on dilutions of a plasma pool. CETP was also measured with a fluorometric assay (Abcam, Cambridge, UK). The assay principle involves incubating the sample with a mixture that contains a self-quenched fluorescent neutral lipid and an acceptor molecule. Action of CETP in the sample results in transfer of the neutral lipid to the acceptor molecule and an increase in its fluorescence (excitation at 465 nm, emission at 535 nm). For CETP too, activity was measured by comparison against a calibration curve based on dilutions of a plasma pool. Enzymatic activity was expressed as percent of activity in the plasma pool, and was therefore expressed in Arbitrary Units (AU). All measurements were performed in duplicate.

The cholesteryl ester content of plasma and HDL subfractions was measured using the Abcam cholesteryl ester kit (ab65359). The assay is based on two simultaneous reactions for cholesterol determination, one of which includes cholesterol esterase as part of the reaction mixture, while the other does not. Cholesteryl esters are then calculated by subtraction as (total cholesterol - free cholesterol). Phosphatidylcholine in plasma and HDL subfractions was measured using the Abcam fluorometric assay (ab83377). The test is based on an enzyme-coupled reaction that hydrolyzes phosphatidylcholine and releases choline, which in turn reacts with the OxiRed probe and generates fluorescence at 587nm wavelength.

**Determination of HDL size distribution**

HDL contained in each of the four apoC-III and apoE-defined immunofractions were separated by size using non-denaturing polyacrylamide gradient gel electrophoresis (NDPAGGE). Twenty-five microliters of each immunofraction plus 25 microliters of sample buffer were loaded into a 4-30% polyacrylamide gradient gel (Jule Inc., Milford, CT). Wells 1 and 10 were loaded with molecular size standards (Amersham HMW Native Marker Kit, GE Healthcare, Little Chalfont, UK) and gels were run for 24 hours
at constant 70V. Then, contents of the gel were transferred to a 0.45 micrometer pore size polyvinylidene fluoride (PVDF) membrane (Pall Corporation) in a wet transfer apparatus at 30V for 24 hours. The lanes containing the MW markers were cut from the rest of the membrane, stained in 0.2% amido black solution for 20 minutes and stored for later photographing. The rest of the membrane was blocked with 5% powder low-fat milk, incubated with an HRP-conjugated goat anti-human apoA-I antibody (Academy Biomedical, Houston, TX), and revealed using 3,3’,5,5’-tetramethylbenzidine as substrate. Later, the marker lanes and the rest of the membrane were placed side to side and photographed in a Bio-Rad ChemiDoc™ MP gel documenter. Using the molecular size standards as reference, the intensity of the bands/smears in each size range fractions was quantitated in Image Lab™ software. Size fractions were defined as follows: prebeta HDL: <7.1 nm, alpha 3 HDL: 7.1-8.2 nm, alpha 2 HDL: 8.2-9.5 nm and alpha 1 HDL: 9.5-12.2 nm. The concentration of apoA-I in each HDL size subfraction was estimated by multiplying the proportion of apoA-I within that fraction by the directly measured total plasma apoA-I. Laboratory procedures were executed at the Diabetes, Lipids and Metabolism laboratory of Universidad de Los Andes, following current institutional biosafety protocols.

Statistical analyses

Our sample size of eighteen participants gave us 87% power to detect a true difference of at least 8% in LCAT activity between two HDL subtypes, assuming a 5% variation coefficient in LCAT activity (18), at a 5% significance level. The distribution of plasma apoA-I across HDL subfractions was compared in a 2-way ANOVA model in which apoA-I concentration in each subfraction was the dependent variable, and HDL size and immunofraction were fixed factors. Enzyme masses and activity across HDL subtypes were compared using a 1-way ANOVA in which HDL immunofraction was the only fixed factor. When global ANOVA was significant, post-hoc comparisons against the reference fraction (E-C-) were done using Scheffé´s method. Comparisons of quantitative variables between groups were performed using Mann-Whitney´s U test. Differences in categorical variables between groups were
performed using Fisher´s exact test. All tests were done at a 0.05 significance level and all reported p-values are 2-sided.

Results
The study included 8 male and 10 female adults, with mean age 55.6 +/- 11.2 years and mean body-mass index (BMI) 26.9 +/- 4.0 kg/m². Women had higher plasma total cholesterol, HDLc and LDLc. Women also had higher plasma apoA-I levels (121.4 +/- 14.3 mg/dL versus 97.7 +/- 23.0 mg/dL in men, p= 0.016). Total plasma LCAT was 6.3 +/- 2.2 mg/dL in women and 5.7 +/- 2.2 mg/dL in men, while plasma CETP concentrations were 1.9 +/- 0.7 mg/dL in women and 1.7 +/- 0.7 mg/dL in men. Relative plasma CETP activity was very similar in both sexes, 98.7 +/- 13.7 AU in women and 101.7 +/- 6.1 AU in men. Meanwhile, LCAT activity was 96.1 +/- 22.3 AU in women and 96.8 +/- 18.4 AU in men (Table 1).

HDL with apoE or apoC-III are minority
The predominant subfraction of HDL (as reflected by its concentration of apoA-I) was E-C- in most study participants. On average, apoA-I in E-C- HDL was the most abundant HDL subtype, representing 50.1% of plasma apoA-I, followed by E+C- (22.6%), E-C+ (15.2%) and E+C+ (12.1%) (p<0.001 for difference across subfractions) (Figure 1). There was significantly more apoA-I in E-C- than in E+C-, E-C+ or E+C+ HDL (p<0.001 for each of the three pairwise comparisons). Overall, 34.7% of HDL contained any apoE while 27.3% contained any apoC-III.

LCAT concentration and activity vary across HDL subfractions
Most plasma LCAT mass (3.58 microg/mL, 59.6%) was concentrated in the E-C- HDL subfraction. The remaining fractions with either apoE or apoC-III had similarly lower LCAT concentrations: 1.19 microg/mL in E+C-, 0.74 microg/mL in E-C+ and 0.50 microg/mL in E+C+ (p<0.001 for the difference across groups). The difference in LCAT concentration between E-C- and every other HDL subfraction was statistically significant (p<0.001 for each of the three comparisons) (Figure 2, upper panel). Surprisingly, this heterogeneity in LCAT protein distribution was not paralleled by LCAT activity, which
had a different distribution across HDL subfractions. The E-C- fraction had a relative LCAT activity of 24.1 AU (25% of total plasma LCAT activity), while the highest LCAT activity was found in the in E+C- fraction (26.5 AU, 27.4% of total plasma LCAT activity) (p=0.35 for difference across groups). HDL in the E-C+ and E+C+ fractions contained respectively 25.8 and 20.0 AU of LCAT activity (Figure 2, lower panel). The ratio of LCAT activity / LCAT mass was 27.5 AU*mL/microg in HDL containing apoE (E+C- plus E+C+), and 11.6 AU*mL/microg for HDL not containing apoE (E-C- plus E-C+).

**CETP concentration and activity are similar across HDL subfractions**

Most CETP was concentrated in the E+C- subfraction (0.60 mg/mL, 32.5% of total plasma CETP), while the other three types of HDL contained respectively 25.9% (E-C-), 21.1% (E-C+) and 20.4% (E+C+) of plasma CETP (p=0.49 for comparison across fractions) (Figure 3, upper panel). CETP activity showed a very homogeneous distribution across HDL subtypes (p=0.50, Figure 3, lower panel).

**Lipid contents of HDL subtypes**

ApoC-III containing HDL displayed a higher triglyceride-to-apoA1 molar ratio, while E-C- HDL were poorer in triglycerides relative to the other fractions (p=0.01 for global ANOVA, Table 2). Despite numerically higher phosphatidylcholine-to-apoA1 molar ratios in the apoE or apoC-III containing HDL, this difference did not achieve statistical significance. The cholesterol and cholesteryl ester contents of the four HDL subtypes were numerically similar, and not statistically different.

**The concentration of some apolipoprotein and size-defined HDL correlates with plasma LCAT and CETP activity**

The concentration of apoA-I in the E-C+ pre-beta HDL subfraction correlated inversely with total plasma LCAT activity (r=-0.55, p=0.017, Table 3). Similarly, apoA-I in the E-C- alpha 1 HDL subfraction correlated inversely with total plasma LCAT activity (r=-0.49, p=0.041). On the other hand, larger E-C+ HDL (alpha 1) exhibited a positive correlation with plasma CETP activity (r=0.52, p=0.025). For the E-C+ HDL subtype, the correlation between HDL concentration and CETP activity was not
significant for smaller HDL, but tended to increase along with HDL size.

The distribution of LCAT and CETP concentration and activity was not influenced by BMI

As a sensitivity analysis, we examined whether the findings concerning enzymatic concentration and activities were different for participants with a normal body-mass index (BMI<25), compared to those with excess body weight (overweight or obesity, BMI≥25). Just like in the compete study sample, in each of the two groups the concentration of LCAT was significantly higher in E-C- HDL than in the other fractions, but enzymatic activity was similarly distributed across HDL subtypes (Figure 4). Also in each of the two BMI-defined subgroups, CETP concentration and activity were very similar across the four HDL subtypes.

Discussion

In this study of human HDL, we found that HDL subtypes defined by their apoE and apoC-III content have different LCAT mass but comparable LCAT activity. In other words, we encountered that HDL with apoE have a greater ratio of LCAT activity to mass, signaling a greater degree of LCAT activation. Contrastingly, CETP mass and activity were not associated with the apoE or apoC-III composition of HDL. As reported previously, apoE and/or apoC-III-containing HDL were a minority of total plasma HDL (36), yet they proved to be different from E-C- HDL in their enzymatic activity.

LCAT was most abundant in E-C- HDL, while HDL with either apoE or apoC-III contained similar amounts of LCAT. Despite having lower LCAT masses compared to E-C- HDL, subfractions with apoE or apoC-III had very similar LCAT activities, suggesting that the presence of either small apolipoprotein, or some factor closely associated with them, influences LCAT. Of note, the simultaneous presence of apoE and apoC-III was not accompanied by a higher LCAT activity. Unlike apoA-I (the essential cofactor of LCAT), very little is known about the impact of other apolipoproteins of the small, exchangeable type on the activity of this crucial HDL enzyme. Studies in apoA-I/- apoE/- double knockout mice have identified apoE as a potential LCAT activator (37). ApoE is also able to activate LCAT in discoidal reconstituted HDL devoid of apoA-I (38). In contrast, apoC-III dose-dependently
inhibits the LCAT reaction in reconstituted HDL (33), and in synthetic phosphatidylcholine vesicles containing apoA-I (39). Therefore, the finding of no decrease in LCAT activity in apoC-III containing HDL was unexpected.

Contrary to LCAT, CETP concentration and activity did not vary among HDL subfractions. Although some CETP inhibitors have the ability to increase both plasma apoC-III and plasma apoE (40-42), our findings suggest that CETP activity and concentration in HDL are not influenced by these apolipoproteins. CETP activity in humans may be influenced by high plasma cholesterol concentrations, or by cholestasis (43,44).

Concerning the lipid composition of HDL subfractions, we found more phosphatidylcholine in HDL with either apoE or apoC-III. Thus, higher LCAT activity in these HDL subtypes may be due to greater availability of substrate. This explanation has biological plausibility, as the movement of apolipoproteins between lipoproteins sometimes involves phospholipid transfer protein (PLTP), in which cases there may be simultaneous transfer of the surrounding phospholipid-rich membrane (45). We did not find statistically significant differences in cholesteryl ester content among the four HDL subtypes.

We found a significant inverse correlation between total plasma LCAT activity and the concentration of both E-C+ pre-beta HDL and E-C- alpha 1 HDL. These results suggest that the presence of apoC-III in pre-beta HDL may correlate with limited LCAT activity, less maturation and accumulation of pre-beta HDL (46). Interestingly, a study comparing patients with and without CVD found an inverse correlation between LCAT activity and plasma pre-beta HDL concentrations, which was stronger in the CVD group (47). Our finding of a negative correlation between E-C- alpha 1 HDL and plasma LCAT activity suggests that not all size conversion/maturation of HDL requires LCAT. Meanwhile, the positive correlation of plasma CETP activity with the abundance of large E-C+ HDL may just reflect the frequent coexistence of both proteins (CETP and apoC-III) in this HDL type.
The main limitations of our study are its limited sample size and the fact that we interrogated only two specific apolipoproteins, out of the many known to be present in HDL. However, this was a proof-of-concept study in which the core analyses were performed within-individual, so that enzymatic contents and activities were performed for different HDL subtypes belonging to the same individual. The biological antagonism between apoE and apoC-III (48) has also been proven to modulate HDL metabolism and its association with cardiovascular risk (49), making them particularly worthy of investigation, particularly because the influence of apoE on HDL metabolism has been proven to be susceptible of positive modification through dietary interventions (50). Another possible limitation is that the employed LCAT activity assay probably detects only the fatty acid removal from PC and not its successful transfer to free cholesterol. Nonetheless, given the known nature of the LCAT enzymatic process, the ability of LCAT to perform this initial cleavage \textit{in vitro} has been widely employed as a proxy for LCAT activity.

In conclusion, our results showed specific profiles of LCAT mass and activity in HDL subtypes defined by their content of apoE and apoC-III, and suggest that the presence of apoE protein in HDL may be a correlate of increased LCAT activity. Further studies describing the changes in HDL composition, functionality and associated enzymatic activity in the context of CVD would expand on our findings.

List Of Abbreviations

\textbf{apoC-III:} apolipoprotein C-III

\textbf{apoE:} apolipoproteins E

\textbf{AU:} Arbitrary Units

\textbf{BMI:} body-mass index

\textbf{CETP:} cholesterol-ester transfer protein

\textbf{CVD:} cardiovascular disease

\textbf{HDL:} High density lipoproteins

\textbf{HRP:} horseradish peroxidase
**LCAT**: lecithin-cholesterol acyl transferase

**LRP-1**: LDL receptor-related protein-1

**RCT**: reverse cholesterol transport

**PUFA**: Polyunsaturated fatty acids

**PVDF**: polyvinylidene fluoride

**Declarations**

*Ethics approval and consent to participate*

The Internal Review Board (Comité de Ética) of Universidad de los Andes approved the study according to minute 307 of 2014. We complied with all scientific, technical and administrative norms for health research dictated by resolution 008430 – 1993 of the Colombian Ministry of Health and with the principles stated by the Declaration of Helsinki. All study subjects underwent an informed consent procedure and provided written informed consent.

*Consent for publication*

Not applicable

*Availability of data and materials*

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

*Competing interests*

The authors declare that they have no competing interests

*Funding*

This study was funded by Vicerrectoría de Investigaciones, Universidad de los Andes, Colombia

*Authors' contributions*
MA-M, JAP-C, LSS-B and COM participated in the study conception, MA-M, JAP-C, LSS-B, DR-M, MCPM, MAJM and COM executed study experiments and data collection, MA-M, DR-M and COM participated in data analysis and manuscript writing, COM directed the project and participated in all phases of its conception, execution, and in the manuscript writing.

Acknowledgements

We want to thank the study participants for their patience and collaboration.

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Tables

Table 1. Characteristics of study participants. eGFR: Estimated glomerular filtration rate. Data are mean +/- SD unless stated otherwise.

| Characteristic                          | Mean ± SD   |
|----------------------------------------|-------------|
| Sex (F:M)                              | 10:8        |
| Age (years)                            | 55.6 ± 11.2 |
| Weight (kg)                            | 72.0 ± 12.9 |
| Height (m)                             | 163.5 ± 9.2 |
| Body-mass index (kg/m²)                | 26.9 ± 4.0  |
| Percent body fat (%)                   | 33.1 ± 7.1  |
| Percent abdominal fat (%)              | 9.4 ± 3.9   |
| Percent lean mass (%)                  | 63.6 ± 6.7  |
| Systolic blood pressure (mmHg)         | 117.8 ± 14.6|
| Diastolic blood pressure (mmHg)        | 73.8 ± 11   |
| Fasting plasma glucose (mg/dl)         | 96.1 ± 10.6 |
| Glycated hemoglobin (HbA1c) (%)        | 5.4 ± 1.0   |
| eGFR (ml/min)                          | 93.2 ± 18.0 |
| Total cholesterol (mg/dl)              | 195.5 ± 52.4|
| Triglycerides (mg/dl)                  | 154.2 ± 58.4|
| HDL cholesterol (mg/dl)                | 43.8 ± 15.8 |
| LDL cholesterol (mg/dl)                | 126.3 ± 44.4|
| C-reactive protein (mg/l)              | 1.9 ± 2.6   |
| Plasma apoA-I (mg/dl)                  | 110.9 ± 21.8|
| Plasma CETP (microg/ml)                | 1.8 ± 0.7   |
| Plasma LCAT (microg/ml)                | 6.0 ± 2.1   |
| Plasma CETP activity (AU)              | 100.0 ± 10.8|
| Plasma LCAT activity (AU)              | 96.4 ± 20.1 |

Table 2. Molar ratios of lipids to apoA-I in the four HDL subfractions. Data in the first row are mean lipid to apoA-I molar ratios +/- SD. The second row of each analyte shows the significance values from a post-hoc pairwise comparison versus the E-CIII- fraction. The third row of each analyte shows the proportion of each lipid in that fraction as a percentage of the total mass of such lipid in HDL. The last row shows the significance value from a global one-way ANOVA for the analysis of lipid to apoA-I molar ratios by subfractions.
|                  | E-CIII-            | E+CIII-           | E-CIII+           |
|------------------|--------------------|------------------|------------------|
| Triglycerides    | to apoA-I molar ratio | 0.47 +/- 0.22    | 1.09 +/- 1.41    | 2.73 +/- 3.48    |
| p-value versus E-CIII- | -                 | 0.88             | 0.035            |
| % of total mass in HDL | 25.2%             | 17.2%            | 29.8%            |
| p-value from ANOVA |                   |                  | 0.01             |
| Cholesterol      | to apoA-I molar ratio | 26.6 +/- 18.7    | 51.5 +/- 68.4    | 48.1 +/- 32      |
| p-value versus E-CIII- | -                 | 0.43             | 0.55             |
| % of total mass in HDL | 40.7%             | 22.5%            | 20.8%            |
| p-value from ANOVA |                   |                  | 0.26             |
| Phosphatidylcholine| to apoA-I molar ratio | 109.2 +/- 69.3   | 317.6 +/- 367.7  | 455.7 +/- 547.3  |
| p-value versus E-CIII- | -                 | 0.65             | 0.21             |
| % of total mass in HDL | 27.1%             | 27.0%            | 26.0%            |
| p-value from ANOVA |                   |                  | 0.088            |
| Cholesterol esters| to apoA-I molar ratio | 14.6 +/- 16      | 23 +/- 29.9      | 16.3 +/- 37.9    |
| p-value versus E-CIII- | -                 | 0.98             | 0.99             |
| % of total mass in HDL | 48.6%             | 28.8%            | 12.3%            |
| p-value from ANOVA |                   |                  | 0.86             |

**Table 3. Correlation between ApoA1 within HDL size-defined subfractions and plasma enzymatic activity.** Data are Spearman linear correlation coefficients. Asterisks denote correlations significantly different from zero.

|                  | E-CIII- | E+CIII- | E-CIII+ | E+CIII+ |
|------------------|---------|---------|---------|---------|
| Alpha 1 HDL      | -0.49*  | -0.30   | -0.35   | -0.10   |
| Alpha 2 HDL      | -0.01   | -0.29   | -0.37   | 0.02    |
| Alpha 3 HDL      | 0.33    | 0.13    | -0.30   | 0.04    |
| Pre-Beta HDL     | 0.24    | -0.08   | -0.55*  | 0.06    |
| Correlations with CETP activity | E-CIII- | E+CIII- | E-CIII+ | E+CIII+ |
| Alpha 1 HDL      | 0.16    | -0.10   | 0.52*   | 0.46    |
| Alpha 2 HDL      | 0.04    | -0.07   | 0.39    | 0.34    |
| Alpha 3 HDL      | -0.03   | 0.11    | 0.28    | 0.28    |
| Pre-Beta HDL     | 0.11    | -0.01   | 0.13    | 0.05    |

**Figures**
Figure 1

Distribution of plasma apoA-I across HDL subfractions defined by size, apoE and apoC-III content. HDL size ranges are as follows: Prebeta: <7.1 nm, alpha 3: 7.1-8.2 nm, alpha 2: 8.2-9.5 nm, alpha 1: 9.5-12.2 nm (n=18, 10 women and 8 men).
LCAT concentrations and activities in HDL subfractions defined by their apoE and apoC-III content. (A-Top) LCAT concentrations. (B-bottom) LCAT activities (n=18, 10 women and 8 men).

Figure 2
CETP concentrations and activities in HDL subfractions defined by their apoE and apoC-III content. (A-top) CETP concentrations. There was no significant difference across subfractions (overall ANOVA p=0.48). (B-bottom) CETP activities. There was no significant
difference across subfractions (overall ANOVA p=0.50) (n=18, 10 women and 8 men).

**Figure 4**

LCAT concentrations and activities in HDL subfractions defined by their apoE and apoC-III content, in patients with body-mass index below 25 Kg/m2 (n=7, left side, panels A and C) or equal to or greater than 25 Kg/m2 (n=11, right side, panels B and D).
Figure 5

CETP concentrations and activities in HDL subfractions defined by their apoE and apoC-III content, in patients with body-mass index below 25 Kg/m² (n=7, left side, panels A and C) or equal to or greater than 25 Kg/m² (n=11, right side, panels B and D).