A rapid and precise method for measuring plasma apoE-rich HDL using polyethylene glycol and cation-exchange chromatography: a pilot study on the clinical significance of apoE-rich HDL measurements

Toru Ikeda[a], Ryoko Shinohata[a], Masaaki Murakami[b], Kazuyoshi Hina[b], Shigeshi Kamikawa[b], Satoshi Hirohata[a], Shozo Kusachi[b], Arisa Tamura[a], Shinichi Usui[a,*]

[a] *Department of Medical Technology, Okayama University Graduate School of Health Sciences. 2-5-1, Shikata-cho, Kita-ku, Okayama-shi, Okayama 700-8558, Japan*

[b] *Okayama Heart Clinic. 54-1, Takeda, Naka-ku, Okayama-shi, Okayama 703-8251, Japan*

Corresponding author at:

Department of Medical Technology, Okayama University Graduate School of Health Sciences, 2-5-1, Shikata-cho, Kita-ku, Okayama-shi, Okayama 700-8558, Japan.

Phone: +81-86-235-6882; Fax: +81-86-222-3717

E-mail address: usuis@md.okayama-u.ac.jp (S. Usui)

ABSTRACT

Background: High-density lipoprotein (HDL) containing apolipoprotein E (apoE-rich HDL) represents only a small portion of plasma HDL. Reliable methods for determining and isolating apoE-rich HDL have not been well studied.

Methods: We established a novel analytical method for apoE-rich HDL using polyethylene glycol and a cation-exchange column (PEG-column method). Furthermore, we examined biochemical correlates of apoE-rich HDL-cholesterol (HDL-C) in 36 patients who underwent coronary computed tomographic angiography.

Results: Our PEG-column method demonstrated high reproducibility (coefficient of variation <3.52%) and excellent linearity up to 15 mg/dl for apoE-rich HDL-C levels. Isolated apoE-rich HDL exhibited a larger diameter (14.8 nm) than apoE-poor HDL (10.8 nm) and contained both apoE and apoA-I. ApoE-rich HDL-C levels correlated significantly with triglycerides ($r=-0.646$), LDL size ($r=0.472$), adiponectin ($r=0.476$), and other lipoprotein components. No significant correlation was obtained with the coronary calcium score. Multiple regression analysis revealed that plasma triglycerides and adiponectin levels remained significant independent predictors of apoE-rich (adjusted $R^2=0.486$) but not apoE-poor HDL-C.

Conclusions: The PEG-column method demonstrated, to various degrees, significant correlations between HDL subfractions and several lipid-related biomarkers involved in an atherogenic lipoprotein profile. Our separation technique for apoE-rich HDL is useful to clarify the role of apoE-rich HDL in atherosclerosis.

Keywords: HDL subfraction, Atherogenic lipoprotein, Chromatography

1. Introduction

Numerous clinical and epidemiological studies have shown that decreased levels of HDL-cholesterol (HDL-C) represent an independent risk factor for coronary artery disease [1–3]. HDL has been recently proposed to possess multiple biological functions including anti-inflammatory, anti-oxidative, and vasodilatory activities, as well as its traditional role in reverse cholesterol transport [4–9]. HDLs are, however, a highly heterogeneous group and differ in lipid and protein composition, size, hydrated density, and electrophoretic mobility [3,6,10]. Therefore, individual HDL subpopulations might play specific roles in the multiple anti-atherogenic activities associated with HDL overall.

HDLs containing apolipoprotein E (apoE-rich HDL) represent an HDL subclass that accounts for up to 10% of the total HDL in adult human plasma [11,12]. In animal models, apoE-rich HDL is referred to as HDL_1 or HDLc induced by dietary cholesterol and can deliver cholesterol to the liver directly via interaction with the LDL receptor [10,13,14]. ApoE-rich HDL also exhibits several atheroprotective properties including promotion of cholesterol efflux [5], stimulation of endothelial heparin sulfate synthesis [15], inhibition of platelet aggregation [16,17], and maintenance of arterial elasticity [18]. These fundamental findings suggest that apoE-rich HDL might serve as a useful biomarker for cardiovascular risk estimation.

In a clinical study reported by Wilson et al., the level of plasma apoE-rich HDL was decreased in patients with coronary heart disease compared to control subjects [19]. Proteomic analysis revealed an increased level of apoE in an HDL_3 subfraction isolated from subjects with established coronary artery disease [20]. However, there are relatively few studies that have investigated the clinical significance of apoE-rich HDL levels, mainly because no conventional analytical methods are currently available for its

assessment. Simple and rapid methods are therefore required for lipoprotein analysis in clinical settings.

A method used widely for the separation and fractionation of plasma lipoproteins involves ultracentrifugation; however, it has been noted that some apoE could easily dissociate from lipoprotein particles during this process [21,22]. In addition, ultracentrifugation is time-consuming and requires a large sample volume as well as great care to precisely recover lipoproteins from the centrifuge tube. Another critical problem with ultracentrifugation is that the density range of apoE-rich HDL overlaps that of dense LDL, lipoprotein(a), and other HDLs [23]. These findings indicate that ultracentrifugation has marked limitations as a tool for plasma lipoprotein analyses.

In contrast, Chiba et al. reported a useful method for determining the serum concentration of apoE-rich HDL-C, which is calculated as the difference in the cholesterol levels of total and apoE-deficient HDL [11]. This method is based on double precipitation procedures wherein the total HDL fraction is separated from whole serum using polyethylene glycol (PEG) precipitation and the apoE-deficient HDL is prepared using a dextran sulfate-phosphotungstate-Mg^{2+} reagent. More recently, Takahashi et al. established a homogeneous assay [12] allowing sequential assessment of total, apoE-containing, and apoE-deficient HDL that might potentially promote clinical and epidemiological studies on apoE-rich HDL. However, the double precipitation and the homogeneous methods are unlikely to be useful for investigating biological functions and pathophysiological roles of apoE-rich HDL because they lack the ability to isolate apoE-rich HDL.

Based on these considerations, we aimed to develop a novel method for determining the cholesterol levels of apoE-rich HDL and a technique for isolating apoE-rich HDL from serum or plasma using a combination of PEG precipitation with cation-exchange

chromatography (PEG-column method). To further validate the utility of our method, we examined clinical and biochemical correlates of apoE-rich HDL-C levels in patients who underwent coronary computed tomographic (CT) angiography.

2. Materials and methods

2.1. Precipitation method for total HDL separation

To eliminate apoB-containing lipoproteins from whole serum or plasma samples, we used the PEG method described by Chiba et al. [11]. In brief, a 13% PEG6000 (Wako Pure Chemical, Osaka, Japan) solution was mixed with the same volume of serum or plasma. The mixed solutions were left for at least 10 min at room temperature and centrifuged at 8,000 × *g* for 5 min to obtain supernatants containing total HDL (PEG supernatant). Each of the PEG supernatants was passed through a 0.20-μm filter (Sartorius Stedim Biotech, Goettingen, Germany) before being applied to a cation-exchange column.

2.2. Cation-exchange chromatography

The high performance liquid chromatography (HPLC) system (Tosoh Bioscience, Tokyo, Japan) consisted of an AS-8020 sampler, CCPM-II and CCPS pumps, and a UV-8020 detector. A SC-8020 system controller (Tosoh Bioscience) was used for instrument regulation. A μ7 Data Station (System Instruments, Tokyo, Japan) was used for data collection and analysis.

Separation of apoE-rich HDL and apoE-poor HDL was accomplished using a cation-exchange column (HiTrap SP HP, 1 mL, GE Healthcare, Uppsala, Sweden). The column was equilibrated with buffer A (10 mM MOPS, pH 7.2) containing 40 mM magnesium acetate prior to sample injection. Increasing concentrations of Mg^{2+} in

buffer A were used to determine the effect of Mg^{2+} on the separation of HDL subfractions. The retained fraction, corresponding to apoE-rich HDL, was eluted with buffer B (10 mM MOPS, 1.0 M sodium acetate, and 0.01% Triton X100, pH 7.2). Buffers were pumped at a flow rate of 1.0 ml/min; 100% buffer A for 2 min, 100% buffer B for 3 min, and then 100% buffer A for 4 min. Typically, 8 µl of total HDL, isolated by the PEG precipitation method, were injected into the column at intervals of 9 min per sample, and the column effluent was mixed with an enzymatic reagent for cholesterol at a flow rate of 0.3 ml/min. The absorbance at 550 nm was monitored continuously after the enzymatic reaction at 37°C in a reactor coil (Teflon, 30 m × 0.4 mm id). For the determination of apolipoprotein distribution, the column effluent was collected in 0.4-ml fractions with a fraction collector (Bio-Rad Laboratories, Berkeley, CA, USA), and analyzed by an enzyme-linked immunosorbent assay (ELISA).

2.3. Isolation and purification of apoE-HDL

A total HDL fraction containing no other serum proteins was prepared by ultracentrifugation. Briefly, the PEG supernatant was adjusted to a density of 1.21 kg/l by adding solid NaBr, and 3.2 ml of the resulting solution was placed in a polycarbonate centrifuge tube. After centrifugation in an Optima MAX Ultracentrifuge (Beckman Coulter, Brea, CA, USA) with a fixed-angle TLA 110 rotor at 543,000 × *g* for 15 h at 16°C, 1.0 ml of the top layer was recovered, pooled, and dialyzed against 10 mM MOPS (pH 7.2) at 4°C. The dialyzed top fraction was adjusted to a final 40 mM Mg^{2+} concentration by adding solid magnesium acetate, diluted with buffer A, and 30 ml (10 mg/dl total HDL-C) was applied to a cation-exchange column (HiTrap SP HP, 5 mL, GE Healthcare). After washing the column with buffer A, bound HDL (apoE-rich HDL) was eluted with buffer B containing no detergent, collected, and concentrated using an Amicon Ultra

centrifugal filter with a molecular weight cutoff of 30 kDa (Merck Millipore, Darmstadt, Germany). In some experiments, PEG in the unbound HDL (apoE-poor HDL) was removed using an anion-exchange column (HiTrap DEAE FF, 1 mL, GE Healthcare) equilibrated with buffer A. The purified apoE-rich and apoE-poor HDL fractions were dialyzed against 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl, and used for subsequent analyses of apolipoproteins and particle size.

2.4. Determination of LDL and HDL mean particle sizes

The particle size distribution of lipoproteins was analyzed using the gel-permeation HPLC method. Briefly, the separation of lipoproteins was carried out with a Superose 6HR column (300 × 10 mm, GE Healthcare) at a flow rate of 0.5 ml/min with 10 mM Tris-HCl (pH 7.2) containing 0.15 M NaCl, followed by an on-line enzymatic cholesterol detection [24,25]. Elution times of the observed peaks on a chromatogram were converted to particle sizes using a linear calibration curve of the logarithm of the particle sizes plotted against the elution times corresponding to LDL and HDL in a calibrator [26]. The mean particle sizes of LDL and HDL in the calibrator were determined at Skylight Biotech (Tokyo, Japan) to be 25.3 and 11.3 nm, respectively.

2.5. ELISA

ApoA-I, apoA-II, and apoE were measured by a noncompetitive sandwich ELISA. Polystyrene 96-well microtiter plates (Corning Inc., Corning, NY, USA) were coated with 100 μl/well anti-apoA-I (1:2000, BM2577, Acris Antibodies, San Diego, CA, USA), anti-apoA-II (1:1000, 12A-G1b, Academy Bio-Medical Company, Houston, TX, USA), or anti-apoE (1:2000, GtxHu-039-D, ImmunoReagents, Raleigh, NC, USA) antibodies dissolved in 10 mM phosphate-buffered saline (PBS) (pH 7.2) for 12 h at 4°C. The coated

plate was washed with washing buffer (10 mM PBS, 0.01% Tween 20, pH 7.2). Then, the calibrators and samples diluted with 5 mM Tris-HCl (pH 7.2) containing 0.1% casein (Wako) and 0.02% Tween 20 were added to each well (100 μl). The plate was incubated on a microplate shaker (Mikura, Lower Beeding, UK) for 1.5 h at room temperature and washed with washing buffer. For detection, anti-apoA-I (11A-G2b), anti-apoA-II (12A-G1b), and anti-apoE (50A-G1b, Academy Bio-Medical Company) antibodies were labeled with biotin using a Biotin Labeling Kit-SH (Dojindo, Kumamoto, Japan). Each of the biotin-labeled detection antibodies (0.5 μg/ml), dissolved in the reaction buffer containing 5 mM Tris-HCl (pH 7.3), 0.1% casein, 0.01% Tween 20, and 0.15 M NaCl, was added to each well (100 μl) and the plate was incubated for 1.5 h. After washing the plate, 0.5 μg/ml of a streptavidin-horseradish peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) diluted with the reaction buffer was added to each well (100 μl), and the plate was incubated for 0.5 h. After washing the plate, color was developed by the addition of 100 μl/well SureBlue TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), followed by 100 μl/well of 1.0 M HCl to stop the reaction. Absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad Laboratories).

2.6. Precision and linearity

For the reproducibility study, two PEG supernatants with high (88 mg/dl) and low (39 mg/dl) levels of HDL-C were analyzed in 10 replicates to calculate within-day imprecision values. To test the range of linearity for our method, a PEG supernatant separated from the whole serum of a healthy normolipidemic subject [214 mg/dl total cholesterol (TC), 46 mg/dl triglycerides (TG)] was serially diluted with buffer A and 24 μl of the diluted sample was applied to the column.

2.7. Method comparison study

For comparison with the PEG-column method, the double precipitation method was carried out according to the procedure reported previously [11]. First, total HDL was separated from whole serum using PEG precipitation as described in Section 2.1. Next, apoE-deficient HDL was prepared from whole serum using the DS-PT-Mg reagent (0.18% dextran sulfate (Wako), 0.3% sodium phosphotungstate (Nacalai Tesque, Kyoto, Japan), and 0.1 M $MgCl_2$ (Wako)) instead of the 13% PEG solution. The serum apoE-rich HDL-C concentration was calculated as the difference in cholesterol levels of total and apoE-deficient HDL.

2.8. Study subjects

For comparison of apoE-rich HDL-C obtained from human subjects by the double precipitation and the PEG-column methods, 31 apparently healthy Okayama University students (male/female, 7/24), aged 21–23 years, were recruited. In addition, we evaluated the clinical significance of apoE-rich HDL-C level measurements using plasma samples from 36 patients who underwent coronary CT angiography at the Okayama Heart Clinic (Okayama, Japan). Coronary CT scanning was performed using a 64-slice multi-detector instrument (Siemens Healthineers, Erlangen, Germany), and the coronary calcium score (CCS) was calculated automatically. The total CCS, which was calculated as the sum of the CCS values in each lesion of four coronary arteries (left main, left anterior descending, left circumflex, and right coronary artery), was used for data analysis. The presence of coronary artery stenosis was defined as 50% or greater narrowing in luminal diameter. The plasma levels of TC, HDL-C, and TG were measured routinely at the hospital laboratory using standard enzymatic methods.

LDL-cholesterol (LDL-C) was calculated from the formula of Friedewald et al. [27]. Plasma adiponectin and leptin were measured using the Human Total Adiponectin/Acrp30 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and the Human Leptin Assay kit (Immuno-Biological Laboratories, Fujioka, Japan), respectively. Other lipoprotein-associated measures were determined at our laboratory using methods described in Section 2.

The examination procedure complied with the rules of the Declaration of Helsinki [28] and the study was approved by the Institutional Ethics Committee for Human Research of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences. All subjects provided their informed consent before entering the study.

2.9. Statistics

Data analyses were performed using SPSS version 17.0 software (IBM, Armonk, NY, USA). Continuous variables are presented as means ± SD. Correlations between variables were assessed by Pearson's or Spearman's correlation coefficients (r value). Multiple regression analysis was used to identify the factors influencing HDL subfractions. The Shapiro-Wilk test was used to evaluate the normality of distribution of the variables. Skewed data were log-transformed to create a more normal distribution. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of Mg^{2+} concentration in buffer A on HDL separation

We first studied the effect of Mg^{2+} concentration in the running buffer A on HDL separation. For cholesterol detection, almost all HDL passed through the

cation-exchange column when using buffer A without Mg^{2+}, whereas the amount of bound HDL increased gradually and reached a plateau at 20–40 mM Mg^{2+} (Fig. 1A, C). For apoE detection, 70% of the apoE in HDL (HDL-apoE) passed through the column at 0 M Mg^{2+} and bound HDL reached a plateau at 20–40 mM Mg^{2+} (Fig. 1B, D). Approximately 90% of the HDL-apoE was eluted in peak 2 using buffer A containing 40 mM Mg^{2+}. Therefore, 40 mM Mg^{2+} was considered sufficient and suitable to separate the apoE-rich HDL subfraction from total HDL.

3.2 Apolipoproteins and particle size of purified apoE-rich HDL

Purified apoE-rich HDL from a healthy and normolipidemic subject (70 mg/dl HDL-C) was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were stained by CBB R-250. As shown in Fig. 2A, bound HDL (purified apoE-HDL) contained mainly both apoE and apoA-I whereas unbound HDL contained apoA-I but lacked apoE. The bound HDL particles were larger in size compared to unbound HDL (14.2 vs. 11.2 nm), as demonstrated by gel-permeation HPLC analysis (Fig. 2B). The lipid and apolipoprotein composition of apoE-rich HDL from 4 healthy and normolipidemic subjects are summarized in Table 1. The ratio of apoE (mg/dl) to apoA-I (mg/dl) was 1.09 ± 0.315 for apoE-rich HDL and 0.010 ± 0.011 for apoE-poor HDL.

3.3. Precision and linearity

Our PEG-column method produced good within-day imprecision values (coefficients of variation, CVs) of 0.29% for total HDL-C (88.2 mg/dl), 0.33% for apoE-poor HDL-C (81.6 mg/dl), and 0.61% for apoE-rich HDL-C (6.6 mg/dl) in the sample with high HDL-C. In the sample with low HDL-C, CVs for total HDL-C (39.3 mg/dl), apoE-poor HDL-C

(37.6 mg/dl), and apoE-rich HDL-C (1.7 mg/dl) were 0.53, 0.45, and 3.52%, respectively.

To define the assay linearity for HDL subfractions, the chromatographic peak area was plotted as a function of the expected cholesterol values of diluted samples. Good linear correlations were obtained up to at least 200 mg/dl for total HDL-C, 180 mg/dl for apoE-poor HDL, and 15 mg/dl for apoE-rich HDL-C (Fig. 3).

3.4. Comparison between the PEG-column and double precipitation methods

We compared our PEG-column method with the double precipitation method for determining apoE-rich HDL-C in 31 serum samples from healthy volunteers. The scatter plot in Fig. 4A shows that the cholesterol levels of apoE-rich HDL obtained by our method correlated with those generated by the double precipitation method ($r = 0.724$), albeit significantly underestimated their content (3.2 ± 1.3 vs. 5.7 ± 2.8 mg/dl). Conversely, the levels of HDL-apoE were more strongly correlated with those of apoE-rich HDL-C using our method ($r = 0.894$) than by the double precipitation method ($r = 0.525$) (Fig. 4B).

3.5. Clinical and biochemical correlates of apoE-rich HDL-C

Clinical and biochemical characteristics of the studied patients are listed in Table 2. HDL size and leptin levels were significantly higher in females compared to males. There was no significant difference in apoE-rich HDL-C levels between patients with and without >50% stenosis (3.4 ± 1.5 and 3.5 ± 1.4 mg/dl, respectively). Simple correlations of apoE-poor and apoE-rich HDL-C levels with various clinical and biochemical variables are summarized in Table 3. Both HDL subfractions were similarly correlated with several variables owing to a strong positive correlation ($r = 0.901$) between apoE-poor and apoE-rich HDL-C. CCS was not significantly correlated with

either HDL fraction. However, plasma TG and adiponectin levels remained significant independent predictors of apoE-rich HDL, but not apoE-poor HDL, in multiple regression models (Table 4). Only LDL size remained an independent predictor of the apoE-rich/apoE-poor HDL-C ratio.

4. Discussion

Our novel PEG-column method successfully isolated plasma apoE-rich HDL and determined its cholesterol level precisely and rapidly. This meets quality specifications for the clinical evaluation of apoE-rich HDL as well as for basic research on plasma lipoproteins.

Several previous studies reported separation techniques of apoE-rich HDL using approaches based on heparin- [14-19] or immunoaffinity [22,29], reliant upon the well-known apoE heparin-binding domain [30]. For example, Weisgraber and Mahley separated apoE-HDL from total HDL (density: 1.063-1.21 kg/l) using a heparin-sepharose affinity column equilibrated with NaCl-Tris buffer (50 mM NaCl, 5 mM Tris, pH 7.4) containing 25 mM $MnCl_2$ [14]. However, we considered that a Mn^{2+}-free buffer might avoid the interference of Mn^{2+} with enzymatic detection of cholesterol [31], as well as the issues of generating waste containing a heavy metal ion. Although apoE-rich HDL has the ability to bind to a heparin column in the presence of buffer containing Mg^{2+} or Ca^{2+}, in a preliminary study we found that the amount of bound HDL increased dependent upon the divalent cation concentration. Therefore, we were unable to identify a suitable analytical condition for the selective separation of apoE-rich HDL using the heparin column (unpublished data). Alternatively, cation-exchange columns exhibit some similar properties to heparin, which functions as a cation exchanger owing to its high content of anionic sulfate groups. Hirowatari et al.

reported previously a separation method for major serum lipoproteins (HDL, LDL, and very low density lipoprotein (VLDL)) but not HDL subfractions using a cation-exchange column with eluents containing Mg^{2+} [32]. Therefore, we selected and used cation-exchange chromatography to isolate the apoE-rich HDL subfraction from total HDL.

In the present study, the amount of HDL bound to the cation-exchange column increased dependent on the Mg^{2+} concentration in buffer A and reached a plateau at 20–40 mM Mg^{2+} (Fig. 1C, D). Under these analytical conditions, approximately 90% of the HDL-apoE was eluted in the bound fraction (peak 2 in Fig. 1B). Analysis of the apoE-rich HDL fraction (bound HDL) by SDS-PAGE confirmed the presence of apoE as well as apoA-I, a major protein component of HDL (Fig. 2A). The ratio of apoE to apoA-I was higher in apoE-rich HDL compared to apoE-poor HDL (Table 1). The gel-permeation HPLC analysis also revealed that the particle size of isolated apoE-rich HDL was larger compared to that of apoE-poor HDL (Table 1 and Fig. 2B). These results indicate that HDL-apoE distributes predominantly to larger-sized HDL particles containing apoA-I, consistent with previous reports [14,29]. These biochemical characteristics of apoE-rich HDL support the validity of our method for the specific separation of apoE-rich HDL. Therefore, our method is more reliable and useful than the recently published homogeneous method [12] that lacks the ability to isolate apoE-rich HDL particles and to clarify their biological functions.

With respect to analytical precision, our PEG-column method produced good within-day precision with CVs less than 3.52 and 0.45% for apoE-rich and apoE-poor HDL-C, respectively. Good linearity was observed in apoE-rich HDL-C levels from 1.0 to 15 mg/dl (Fig. 3), indicating a marked advantage in the analysis of samples with low HDL-C concentrations. However, our method considerably underestimated apoE-rich

HDL-C levels compared to the double precipitation method, although significant correlations were produced by both methods (Fig. 4A). Notably, our method did not measure all of the apoE-containing HDL because approximately 10% of the HDL-apoE passed through the cation-exchange column (Fig. 1B, D). This finding may partially account for this disagreement. However, the apoE-rich HDL-C levels determined by the PEG-column method correlated more highly with the HDL-apoE levels compared to the relationship observed with the double precipitation method (Fig. 4B), indicating that the double precipitation method values might include a portion of the apoE-deficient HDL content as well as apoE-rich HDL. These comparisons indicate that the PEG-column method may have clinically important advantages compared with the previously reported methods.

We found that apoE-rich HDL-C levels obtained by the PEG-column method correlated strongly with total HDL-C levels in accordance with a previous report [12]. A question thereby arises regarding whether apoE-rich HDL-C levels might represent a clinical biomarker independent of total HDL-C. However, several other clinical and biochemical variables also showed strong correlations with apoE-rich HDL-C (Table 3). It should be noted that plasma TG and adiponectin levels were found to be significant independent predictors of apoE-rich HDL, but not apoE-poor HDL, in multiple regression models (Table 4). Although total HDL-C levels often decrease under conditions of obesity and correlate positively with plasma adiponectin [33], the contribution of HDL subfractions with such variables has been little studied. Our current findings may provide a novel hypothetical concept of HDL subfractions whereby apoE-rich HDL might play more critical roles in TG metabolism and/or adipocyte dysfunctions as compared with apoE-poor HDL.

ApoE-rich HDL particles obtained using the PEG-column method were larger in size

and their cholesterol levels exhibited a significant positive correlation with adiponectin, which is derived from adipose tissue, supporting our previous study that showed a negative correlation of the large-size HDL subclass with the visceral fat area [34]. Maeda et al. reported that the level of small, dense LDL-C showed a negative correlation with the large, buoyant HDL_2 subfraction but not with small, dense HDL_3 [35]. Our data may partially support this study because apoE-rich HDL, that has an overlapping density with HDL_2, showed a strong positive correlation with LDL size as compared to apoE-poor HDL in a simple regression analysis. After adjustment for TG, adiponectin, gender, and age, the positive association disappeared between apoE-rich HDL-C and LDL size but remained significant between the apoE-rich/apoE-poor HDL-C ratio and LDL size (Table 3). These results indicate that the compositional distribution of HDL subfractions might exhibit a more meaningful linkage to LDL size than the amount of total HDL.

There was no significant difference in apoE-rich HDL-C levels between patients with and without >50% stenosis. However, the present study disclosed a significant correlation between apoE-rich HDL-C and other lipid markers of coronary risk. The reason for the insignificant difference in terms of stenosis may be related to the small number of patients studied. In addition, HDL fractions, including apoE-rich HDL-C, did not significantly correlate with CCS. CCS is a prognostic factor of coronary artery disease independent from other risk factors, including lipid markers of coronary risk.

In conclusion, the PEG-column method established in our laboratory demonstrated high reproducibility and reliability for the determination of cholesterol in two HDL subfractions, apoE-rich and apoE-poor HDL. The two HDL subfractions displayed various significant correlations with several other lipid-related biomarkers involved in an atherogenic lipoprotein profile including plasma TG and adiponectin. These findings

indicate potential roles of adipose tissue in modulating HDL heterogeneity and of the distinct involvement of HDL subfractions in energy metabolism. Our separating technique for apoE-rich HDL particles will, therefore, likely be useful to clarify their biological functions and metabolism in human, animal, and cell culture studies.

Conflict of interest

None

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Abbreviations

Apo, apolipoprotein; BMI, body mass index; CCS, coronary calcium score; CT, computerized tomography; CV, coefficient of variation; eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; HDL-C, high-density lipoprotein-cholesterol; HPLC, high performance liquid chromatography; LDL-C, low-density lipoprotein-cholesterol; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein

References

[1] N.E. Miller, D.S. Thelle, O.H. Forde, O.D. Mjos, The Tromsø heart-study. High-density lipoprotein and coronary heart-disease: a prospective case-control study, Lancet. 1 (1977) 965–968.

[2] W.P. Castelli, R.J. Garrison, P.W. Wilson, R.D. Abbott, S. Kalousdian, W.B. Kannel, Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study, JAMA. 256 (1986) 2835–2838.

[3] P.P. Toth, P.J. Barter, R.S. Rosenson, W.E. Boden, M.J. Chapman, M. Cuchel, R.B. D'Agostino, M.H. Davidson, W.S. Davidson, J.W. Heinecke, R.H. Karas, A. Kontush, R.M. Krauss, M. Miller, D.J. Rader, High-density lipoproteins: a consensus statement from the National Lipid Association, J. Clin. Lipidol. 7 (2013) 484–525.

[4] J.A. Glomset, The plasma lecithin:cholesterol acyltransferase reaction, J. Lipid Res. 9 (1968) 155–167.

[5] R.S. Rosenson, H.B. Brewer, W.S. Davidson, Z.A. Fayad, V. Fuster, J. Goldstein, M. Hellerstein, X.C. Jiang, M.C. Phillips, D.J. Rader, A.T. Remaley, G.H. Rothblat, A.R. Tall, L. Yvan-Charvet, Cholesterol efflux and atheroprotection: Advancing the concept of reverse cholesterol transport, Circulation. 125 (2012) 1905–1919..

[6] R. Movva, D.J. Rader, Laboratory assessment of HDL heterogeneity and function, Clin. Chem. 54 (2008) 788–800.

[7] P.J. Barter, S. Nicholls, K.A. Rye, G.M. Anantharamaiah, M. Navab, A.M. Fogelman, Antiinflammatory properties of HDL, Circ. Res. 95 (2004) 764–772.

[8] L. Calabresi, M. Gomaraschi, G. Franceschini, Endothelial protection by high-density lipoproteins: From bench to bedside, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1724–1731.

[9] L. Camont, M.J. Chapman, A. Kontush, Biological activities of HDL subpopulations

and their relevance to cardiovascular disease, Trends Mol. Med. 17 (2011) 594–603.

[10] R.S. Rosenson, H.B. Brewer, M.J. Chapman, S. Fazio, M.M. Hussain, A. Kontush, R.M. Krauss, J.D. Otvos, A.T. Remaley, E.J. Schaefer, HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events, Clin. Chem. 57 (2011) 392–410.

[11] H. Chiba, K. Akizawa, S.I. Fujisawa, T. Osaka-Nakamori, N. Iwasaki, H. Suzuki, S. Intoh, K. Matsuno, T. Mitamura, K. Kobayashi, A rapid and simple quantification of human apolipoprotein E-rich high-density lipoproteins in serum, Biochem. Med. Metab. Biol. 47 (1992) 31–37.

[12] Y. Takahashi, Y. Ito, N. Wada, A. Nagasaka, M. Fujikawa, T. Sakurai, R. Shrestha, S.-P. Hui, H. Chiba, Development of homogeneous assay for simultaneous measurement of apoE-deficient, apoE-containing, and total HDL-cholesterol, Clin. Chim. Acta. 454 (2016) 135–142.

[13] R.W. Mahley, Atherogenic hyperlipoproteinemia. The cellular and molecular biology of plasma lipoproteins altered by dietary fat and cholesterol, Med. Clin. North Am. 66 (1982) 375–402.

[14] K.H. Weisgraber, R.W. Mahley, Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography, J. Lipid Res. 21 (1980) 316–325.

[15] L. Paka, Y. Kako, J.C. Obunike, S. Pillarisetti, Apolipoprotein E containing high density lipoprotein stimulates endothelial production of heparan sulfate rich in biologically active heparin-like domains. A potential mechanism for the anti-atherogenic actions of vascular apolipoprotein E, J. Biol. Chem. 274 (1999) 4816–4823.

[16] K. Desai, K.R. Bruckdorfer, R.A. Hutton, J.S. Owen, Binding of apoE-rich high density lipoprotein particles by saturable sites on human blood platelets inhibits

agonist-induced platelet aggregation, J. Lipid Res. 30 (1989) 831–840.

[17] M. Higashihara, M. Kinoshita, T. Teramoto, S. Kume, K. Kurokawa, The role of apoE in inhibitory effects of apoE-rich HDL on platelet function, FEBS Lett. 282 (1991) 82–86.

[18] D. Kothapalli, S.-L. Liu, Y.H. Bae, J. Monslow, T. Xu, E.A. Hawthorne, F.J. Byfield, P. Castagnino, S. Rao, D.J. Rader, E. Puré, M.C. Phillips, S. Lund-Katz, P.A. Janmey, R.K. Assoian, Cardiovascular protection by ApoE and ApoE-HDL linked to suppression of ECM gene expression and arterial stiffening, Cell Rep. 2 (2012) 1259–1271.

[19] H.M. Wilson, J.C. Patel, D. Russell, E.R. Skinner, Alterations in the concentration of an apolipoprotein E-containing subfraction of plasma high density lipoprotein in coronary heart disease, Clin. Chim. Acta. 220 (1993) 175–187.

[20] T. Vaisar, S. Pennathur, P.S. Green, S. a Gharib, A.N. Hoofnagle, M.C. Cheung, J. Byun, S. Vuletic, S. Kassim, P. Singh, H. Chea, R.H. Knopp, J. Brunzell, R. Geary, A. Chait, X. Zhao, K. Elkon, S. Marcovina, P. Ridker, J.F. Oram, J.W. Heinecke, Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL, J. Clin. Invest. 117 (2007) 746–756.

[21] A. Mackie, M.J. Caslake, C.J. Packard, J. Shepherd, Concentration and distribution of human plasma apolipoprotein E, Clin. Chim. Acta. 116 (1981) 35–45.

[22] G.R. Castro, C.J. Fielding, Evidence for the distribution of apolipoprotein E between lipoprotein classes in human normocholesterolemic plasma and for the origin of unassociated apolipoprotein E (Lp-E), J. Lipid Res. 25 (1984) 58–67.

[23] S.W. Weidman, J.B. Ragland, S.M. Sabesin, Plasma lipoprotein composition in alcoholic hepatitis: accumulation of apolipoprotein E-rich high density lipoprotein and preferential reappearance of “light”-HDL during partial recovery, J. Lipid Res.

23 (1982) 556–569.

[24] S. Usui, Y. Hara, S. Hosaki, M. Okazaki, A new on-line dual enzymatic method for simultaneous quantification of cholesterol and triglycerides in lipoproteins by HPLC, J. Lipid Res. 43 (2002) 805–814.

[25] S. Usui, M. Ichimura, S. Ikeda, M. Okamoto, Association between serum retinol-binding protein 4 and small dense low-density lipoprotein cholesterol levels in young adult women, Clin. Chim. Acta. 399 (2009) 45–48.

[26] S. Usui, M. Nakamura, K. Jitsukata, M. Nara, S. Hosaki, M. Okazaki, Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol, Clin. Chem. 46 (2000) 63–72.

[27] W.T. Friedewald, R.I. Levy, D.S. Fredrickson, Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge, Clin. Chem. 18 (1972) 499–502.

[28] World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, JAMA. 284 (2000) 3043–3045.

[29] M.L. Hannuksela, M.E. Brousseau, S.M. Meyn, H. Nazih, G. Bader, R.D. Shamburek, P. Alaupovic, H.B. Brewer, In vivo metabolism of apolipoprotein E within the HDL subpopulations LpE, LpE:A-I, LpE:A-II and LpE:A-I:A-II, Atherosclerosis. 165 (2002) 205–220.

[30] H. Saito, P. Dhanasekaran, D. Nguyen, F. Baldwin, K.H. Weisgraber, S. Wehrli, M.C. Phillips, S. Lund-Katz, Characterization of the heparin binding sites in human apolipoprotein E, J. Biol. Chem. 278 (2003) 14782–14787.

[31] B.W. Steele, D.F. Koehler, M.M. Azar, T.P. Blaszkowski, K. Kuba, M.E. Dempsey, Enzymatic determinations of cholesterol in high-density-lipoprotein fractions

prepared by a precipitation technique, Clin. Chem. 22 (1976) 98–101.

[32] Y. Hirowatari, H. Kurosawa, H. Yoshida, K.I. Doumitu, N. Tada, Analysis method for lipoproteins by high-performance liquid chromatography with sulfopropyl-ligand column and magnesium ion-containing eluents, Anal. Biochem. 308 (2002) 336–342.

[33] F.C. McGillicuddy, M.P. Reilly, D.J. Rader, Adipose modulation of high-density lipoprotein cholesterol: implications for obesity, high-density lipoprotein metabolism, and cardiovascular disease, Circulation. 124 (2011) 1602–1605.

[34] M. Okazaki, S. Usui, M. Ishigami, N. Sakai, T. Nakamura, Y. Matsuzawa, S. Yamashita, Identification of unique lipoprotein subclasses for visceral obesity by component analysis of cholesterol profile in high-performance liquid chromatography, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 578–584.

[35] S. Maeda, S. Nakanishi, M. Yoneda, T. Awaya, K. Yamane, T. Hirano, N. Kohno, Associations between small dense LDL, HDL subfractions (HDL2, HDL3) and risk of atherosclerosis in Japanese-Americans, J. Atheroscler. Thromb. 19 (2012) 444–452.

Figure legends

Fig.1. Effect of the Mg^{2+} concentration in running buffer A on HDL separation. Cholesterol (A) and apoE (B) distributions separated by cation-exchange chromatography of the PEG supernatant using buffer A containing 0 or 40 mM magnesium acetate. Bound HDL was eluted with buffer B containing 1.0 M sodium acetate. The unbound and the bound HDL fractions correspond to peaks 1 and 2, respectively. Percentage areas of peak 2 in the cholesterol (C) and the apoE (D) patterns are plotted as a function of Mg^{2+} concentrations in buffer A.

Fig.2. Apolipoproteins and particle size of purified apoE-HDL. (A) Ten microliters of purified apoE-rich HDL (20 mg/dl cholesterol), apoE-poor HDL (20 mg/dl cholesterol), or VLDL (30 mg/dl cholesterol) were analyzed on 12% SDS-PAGE under reducing conditions. Proteins were stained by CBB R-250. (B) Ten microliters of purified apoE-rich HDL (8.2 mg/dl cholesterol), apoE-poor HDL (37 mg/dl cholesterol), or whole serum (161 mg/dl cholesterol) were analyzed by gel-permeation HPLC. The mean particle size was 14.2 nm for apoE-rich HDL (with the peak eluted at 32.5 min) and 11.2 nm for apoE-poor HDL (with the peak eluted at 34.8 min).

Fig.3. Linearity of the PEG-column method for total HDL-C (peak 1 + peak 2), apoE-poor HDL-C (peak 1), and apoE-rich HDL-C (peak 2). Chromatographic peak areas are plotted as a function of the expected cholesterol values of serially diluted PEG supernatants. Mean values are plotted from duplicate experiments.

Fig.4. Scatter plots for apoE-rich HDL-C. (A) Comparison of apoE-rich HDL-C obtained

by the double precipitation and the PEG-column methods. (B) Correlations between apoE contents in HDL (HDL-apoE) and apoE-rich HDL-C obtained by the double precipitation (○) and the PEG-column (●) methods.

Table 1. Chemical composition of apoE-rich and apoE-poor HDL

		apoE-rich HDL			apoE-poor HDL		
Lipid (%)		75.4	±	1.8	53.6	±	2.6
	Free cholesterol (%)	8.1	±	0.6	3.3	±	0.2
	Cholesterol ester (%)	22.9	±	2.8	20.0	±	1.4
	Phospholipid (%)	38.1	±	2.3	27.3	±	1.1
	Triglyceride (%)	6.2	±	1.8	3.0	±	0.5
Protein (%)		24.6	±	1.8	46.4	±	2.6
	apoA-I (%)	9.9	±	2.0	35.1	±	2.1
	apoA-II (%)	4.4	±	1.0	10.9	±	0.8
	apoE (%)	10.4	±	1.3	0.3	±	0.4
Particle size (nm)		14.8	±	0.4	10.8	±	0.2

ApoE-rich and apoE-poor HDL were purified from 4 healthy and normolipidemic subjects. Composition is expressed as weight percentage of major lipids and proteins. Values are means ± SD (n = 4).

Table 2. Clinical and biochemical characteristics of the study population

	All	Female	Male	*P* value
number of patients	36	21	15	
Age (years)	71.5 ± 6.0	72.4 ± 5.9	70.3 ± 6.1	0.317
BMI (kg/m^2)	23.5 ± 3.7	23.8 ± 4.3	23.2 ± 2.7	0.689
TG (mg/dl)	130.9 ± 53.5	128.6 ± 62.0	134.1 ± 40.5	0.768
TC (mg/dl)	210.0 ± 47.8	219.1 ± 50.3	197.2 ± 42.3	0.178
LDL-C (mg/dl)	116.4 ± 41.5	121.5 ± 42.7	109.2 ± 40.1	0.387
HDL-C (mg/dl)	67.4 ± 17.1	71.9 ± 17.6	61.2 ± 14.7	0.063
apoE- rich HDL-C (mg/dl)	3.5 ± 1.4	3.8 ± 1.5	3.0 ± 1.0	0.067
HDL-apoA-I (mg/dl)	144.0 ± 30.4	147.5 ± 26.8	139.3 ± 35.3	0.433
HDL-apoA-II (mg/dl)	30.7 ± 6.0	30.2 ± 4.9	31.4 ± 7.5	0.544
LDL size (nm)	27.1 ± 0.9	27.3 ± 0.9	26.9 ± 0.9	0.188
HDL size (nm)	11.1 ± 0.6	11.3 ± 0.5	10.9 ± 0.6	0.022
Leptin (ng/ml)	8.3 ± 12.7	12.5 ± 15.4	2.5 ± 1.8	0.008
Adiponectin (μg/ml)	8.6 ± 7.8	10.2 ± 9.3	6.3 ± 4.5	0.140
Total calcium score	178.6 ± 274.7	134.0 ± 230.4	240.9 ± 325.2	0.256
> 50% stenosis	12 (33.3%)	6 (28.6%)	6 (40.0%)	0.499
Smoking	4 (11.1%)	1 (4.8%)	3 (20.0%)	0.287
Diabetes mellitus	6 (16.7%)	3 (14.3%)	3 (20.0%)	0.677
Hypertension	19 (52.8%)	9 (42.9%)	10 (66.7%)	0.192
Hyperlipidemia	19 (52.8%)	9 (42.9%)	10 (66.7%)	0.192
Statin	10 (27.8%)	5 (23.8%)	5 (33.3%)	0.709
Oral hypoglycemic or insulin	5 (13.9%)	3 (14.3%)	2 (13.3%)	>0.999
Antihypertensive	14 (38.9%)	7 (33.3%)	7 (46.7%)	0.499

BMI, body mass index.
P value (male vs. female) judged by the Student's t or Chi-squared test. Values are means ± SD.

Table 3. Correlation between HDL subfractions and clinical and biochemical variables.

	apoE-poor HDL-C (mg/dl)	apoE-rich HDL-C (mg/dl)	apoE-rich / apoE-poor HDL-C ratio
Age (years)	−0.093	−0.075	−0.080
BMI (kg/m^2)	−0.152	−0.180	−0.151
eGFR (ml/min/1.73m^2)	0.168	0.204	0.142
Total calcium score	−0.040	−0.038	−0.022
apoE-poor HDL-C (mg/dl)	1.000	0.901**	0.433**
apoE-rich HDL-C (mg/dl)	0.901**	1.000	0.752**
apoE-rich/apoE-poor HDL-C ratio	0.433**	0.752**	1.000
TG (mg/dl)	−0.523**	−0.646**	−0.511**
TC (mg/dl)	0.435**	0.384*	0.200
HDL-C (mg/dl)	0.968**	0.904**	0.467**
LDL-C (mg/dl)	0.258	0.259	0.198
HDL size (nm)	0.598**	0.630**	0.391*
LDL size (nm)	0.257	0.472**	0.508**
Leptin (ng/ml)	−0.007	−0.079	−0.081
Adiponectin (μg/ml)	0.440**	0.476**	0.358*
HDL-apoA-I (mg/dl)	0.784**	0.652**	0.208
HDL-apoA-II (mg/dl)	0.537**	0.381*	0.054
HDL-apoE (mg/dl)	0.718**	0.884**	0.815**

BMI, body mass index; eGFR, estimated glomerular filtration rate.

Values are Spearman's correlation coefficients (n = 36).

*P<0.05, **P<0.01.

Table 4. Multiple regression analysis

	variable	beta coefficient	*P*	adjusted R^2
apoE-rich HDL-C	TG (mg/dl)	−0.395	0.030	
	log adiponectin (μg/ml)	0.349	0.040	
	gender	−0.184	0.175	
	age (years)	−0.128	0.350	
	LDL size (nm)	0.077	0.617	
				0.486
apoE-poor HDL-C	log adiponectin (μg/ml)	0.359	0.063	
	TG (mg/dl)	−0.326	0.109	
	age (years)	−0.219	0.164	
	gender	−0.179	0.247	
	LDL size (nm)	−0.017	0.923	
				0.330
log (apoE-rich/apoE-poor HDL-C ratio)	LDL size (nm)	0.343	0.048	
	TG (mg/dl)	−0.291	0.137	
	log adiponectin (μg/ml)	0.172	0.346	
	gender	−0.081	0.584	
	age (years)	−0.043	0.774	
				0.376

Figure(s)

Fig.1

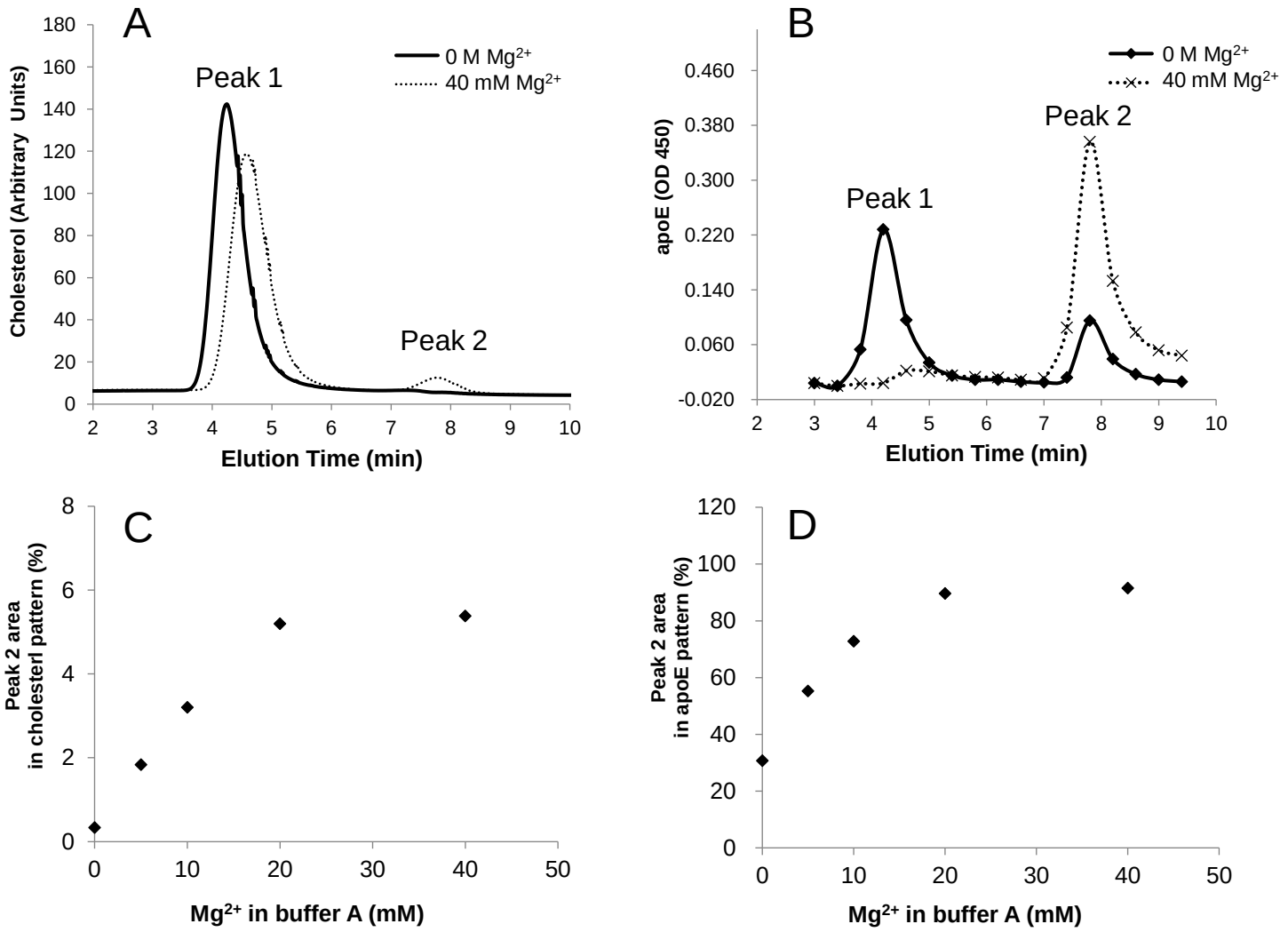


Fig.2

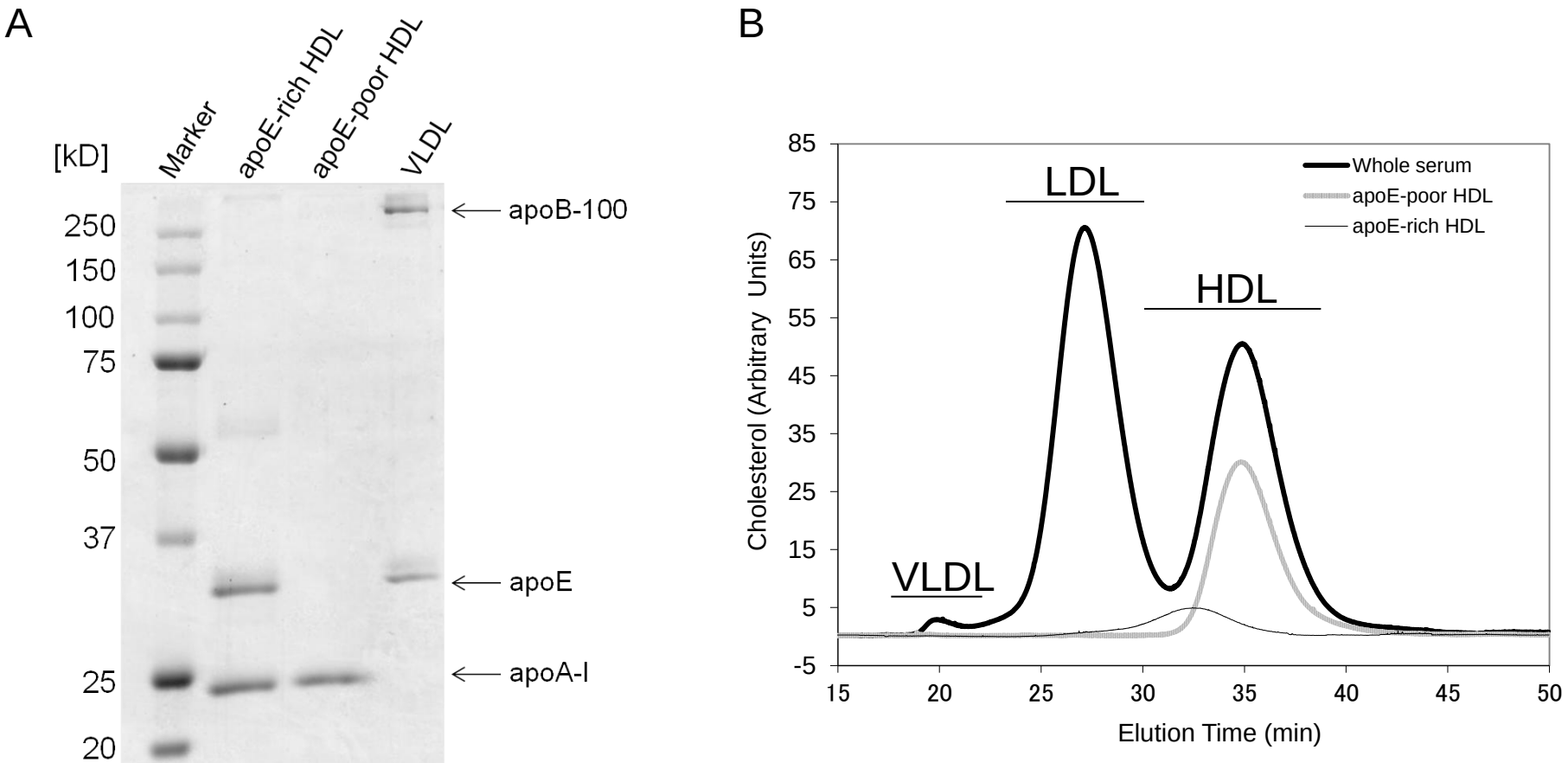
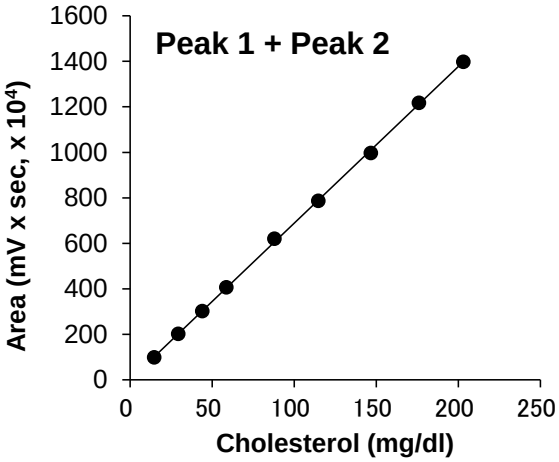
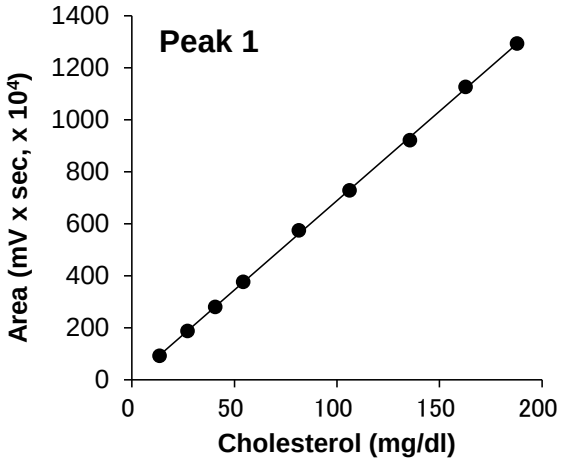

A
[kD]
Marker
apoE-rich HDL
apoE-poor HDL
VLDL
250
150
100
75
50
37
25
20
apoB-100
apoE
apoA-I
B
Cholesterol (Arbitrary Units)
85
75
65
55
45
35
25
15
5
-5
LDL
HDL
VLDL
Whole serum
apoE-poor HDL
apoE-rich HDL
15
20
25
30
35
40
45
50
Elution Time (min)

Fig.3


Peak 1 + Peak 2
Area (mV x sec, x 10⁴)
Cholesterol (mg/dl)
0
200
400
600
800
1000
1200
1400
1600
0
50
100
150
200
250


Peak 1
Area (mV x sec, x 10⁴)
Cholesterol (mg/dl)
0
200
400
600
800
1000
1200
1400
0
50
100
150
200

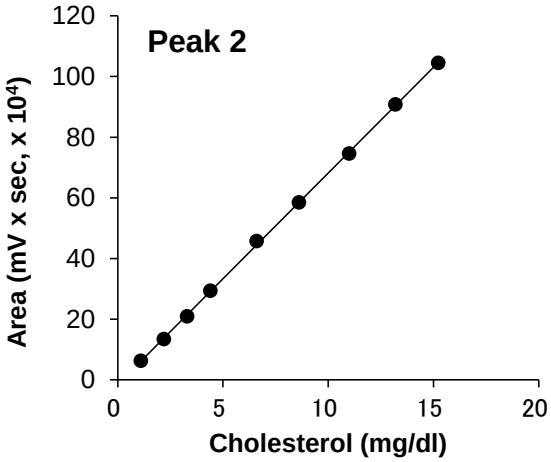
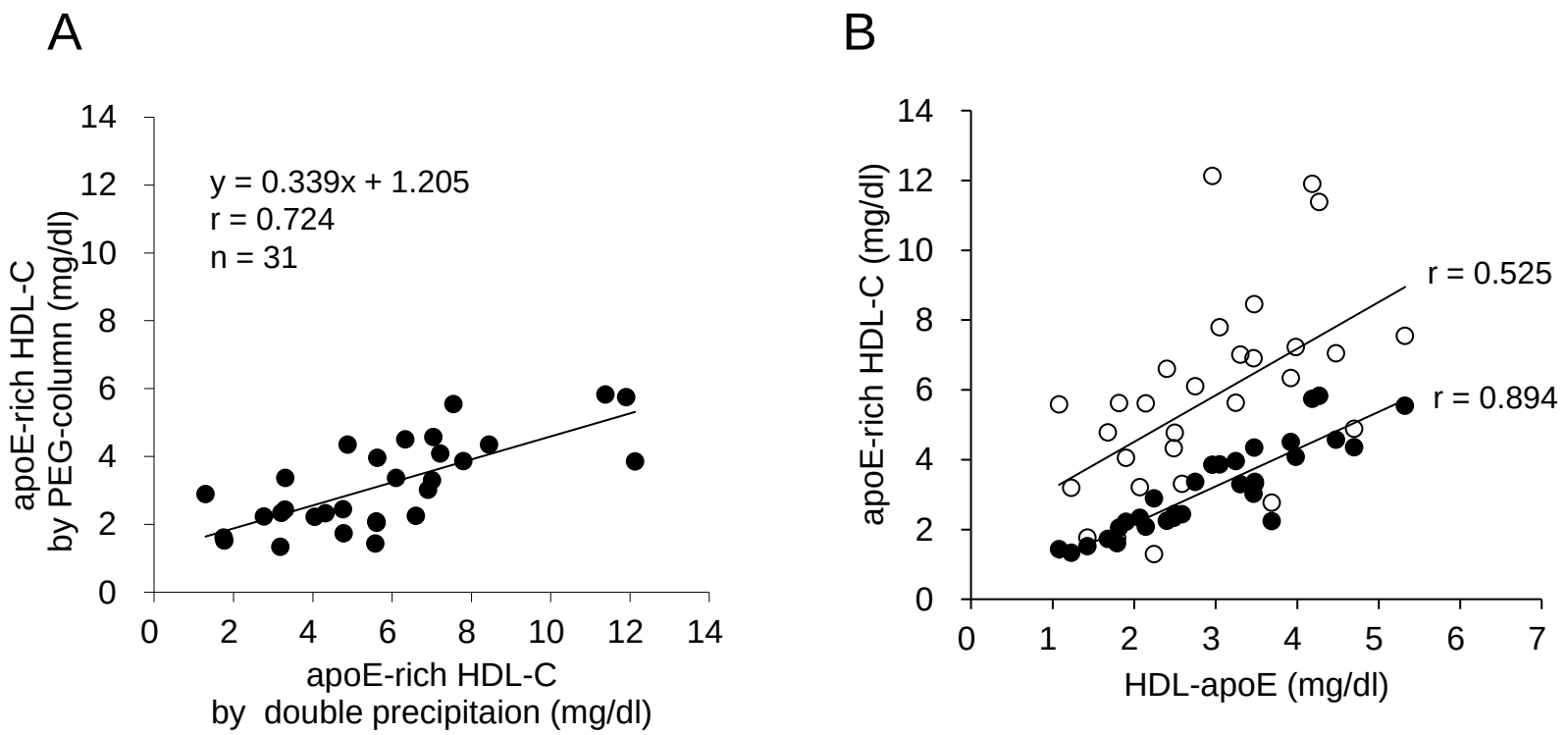

Peak 2
Area (mV x sec, x 10⁴)
Cholesterol (mg/dl)
0
20
40
60
80
100
120
0
5
10
15
20

Fig.4


A
y = 0.339x + 1.205
r = 0.724
n = 31
apoE-rich HDL-C
by PEG-column (mg/dl)
apoE-rich HDL-C
by double precipitaion (mg/dl)
B
r = 0.525
r = 0.894
apoE-rich HDL-C (mg/dl)
HDL-apoE (mg/dl)