

Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux

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Abstract Our objective was to evaluate the associations of individual apolipoprotein A-I (apoA-I)-containing HDL subpopulation levels with ABCA1- and scavenger receptor class B type I (SR-BI)-mediated cellular cholesterol efflux. HDL subpopulations were measured by nondenaturing two-dimensional gel electrophoresis from 105 male subjects selected with various levels of apoA-I in pre β -1, α -1, and α -3 HDL particles. ApoB-containing lipoprotein-depleted serum was incubated with [³H]cholesterol-labeled cells to measure efflux. The difference in efflux between control and ABCA1-upregulated J774 macrophages was taken as a measure of ABCA1-mediated efflux. SR-BI-mediated efflux was determined using cholesterol-labeled Fu5AH hepatoma cells. Fractional efflux values obtained from these two cell systems were correlated with the levels of individual HDL subpopulations. A multivariate analysis showed that two HDL subspecies correlated significantly with ABCA1-mediated efflux: small, lipid-poor pre β -1 particles ($P = 0.0022$) and intermediate-sized α -2 particles ($P = 0.0477$). With regard to SR-BI-mediated efflux, multivariate analysis revealed significant correlations with α -2 ($P = 0.0004$), α -1 ($P = 0.0030$), pre β -1 ($P = 0.0056$), and α -3 ($P = 0.0127$) HDL particles. These data demonstrate that the small, lipid-poor pre β -1 HDL has the strongest association with ABCA1-mediated cholesterol even in the presence of all other HDL subpopulations. Cholesterol efflux via the SR-BI pathway is associated with several HDL subpopulations with different apolipoprotein composition, lipid content, and size.—Asztalos, B. F., M. de la Llera-Moya, G. E. Dallal, K. V. Horvath, E. J. Schaefer, and G. H. Rothblat. **Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux.** *J. Lipid Res.* 2005. 46: 2246–2253.

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The inverse relationship between plasma levels of HDL, either cholesterol or apolipoprotein A-I (apoA-I) content,

and coronary heart disease (CHD) has been demonstrated in many epidemiological, prospective, and intervention studies (1–6). The cardioprotective effect of HDL has been largely attributed to its role in reverse cholesterol transport (RCT), in which cholesterol that has been synthesized or deposited in peripheral tissues is returned to the liver for either recycling or excretion in the bile. This pathway involves several identifiable steps: 1) ApoA-I is synthesized in the liver or small intestine. 2) Phospholipids and cholesterol from cell membranes are transferred to apoA-I in the extracellular space in an ABCA1-dependent process that results in the formation of discoidal HDL (7–9). 3) Free cholesterol (FC) is esterified by the action of LCAT, and the small, discoidal particles are converted to larger, spherical HDL (10). 4) The interaction of spherical HDL with cholesteryl ester transfer protein transfers HDL cholesteryl esters (CEs) to apoB-containing lipoproteins (11). 5) Cholesterol is transported to the liver, either via the selective uptake of HDL CEs by scavenger receptor class B type I (SR-BI) (direct RCT) or via the uptake of whole LDL by the LDL receptor in the liver (indirect RCT) (12).

Experiments with transgenic animals suggest that disturbances in one or more steps in RCT result in accelerated atherosclerosis, whereas the overexpression of key factors involved in RCT is atheroprotective (13). Moreover, humans who do not secrete apoA-I and have defective ABCA1 have premature CHD (14, 15).

Some investigators believe that HDL has no significant role in RCT, because the amount of cholesterol that HDL carries back to the liver is insignificant compared with the amount carried by apoB-containing lipoproteins (16, 17). Concentrations of HDL even in the peripheral lymph are far higher than required for any step in RCT (18–21). Several investigators have suggested that HDL protects against atherosclerosis as a result of its anti-inflammatory and antioxidant functions as well as its actions in downreg-

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ulating adhesive molecules on the surface of vascular endothelium and inhibiting platelet aggregation (22, 23). Although these properties are well documented in vitro, these HDL functions have not yet been unequivocally established in vivo.

HDL is a heterogeneous lipoprotein class with different subspecies that vary in apolipoprotein and lipid composition, in size and charge, and in physiological functions (8, 24–26). We hypothesize that the diverse HDL subpopulations have different roles in RCT. Understanding the role of HDL in RCT is important, especially in light of epidemiological studies demonstrating significant correlations between specific HDL subspecies and the incidence of CHD (27–29). HDL₂ separated by ultracentrifugation contains mainly the α -1 HDL subpopulation, whereas HDL₃ contains all of the other apoA-I-containing HDL subpopulations (27).

Studies in recent years have identified cell surface receptors, ABCA1 and SR-BI, as significant factors in HDL metabolism (30–32). Consequently, the interaction between HDL and these cell surface proteins is under intense investigation. Early studies demonstrated that SR-BI could mediate the selective transfer of CE from HDL into cells and also promote the bidirectional flux of FC between HDL and the plasma membrane (33, 34). Despite intensive investigations, many aspects of the mechanism of SR-BI-mediated lipid flux (e.g., FC efflux and CE-selective uptake) between HDL and the plasma membrane remain unclear. With respect to the selective uptake of CE, it has been proposed that SR-BI functions in a two-step process in which the binding of HDL to the receptor is coupled to the flux of lipids (35–37). Studies using both synthetic reconstituted HDL (rHDL) and rat HDL showed that smaller HDLs promote more selective CE uptake, whereas other studies concluded that larger HDLs are able to deliver more CE compared with smaller HDLs (38, 39). HDL

size affects SR-BI-mediated selective CE uptake, although the mechanism is poorly understood. Similar to CE selective uptake, SR-BI-mediated FC efflux is also size-dependent. Thus, large rHDL particles stimulated more cell cholesterol efflux than small particles when present at the same particle concentration (39). It has also been shown that differences in apoA-I conformation in different sized rHDL particles influence binding to SR-BI (39).

Almost all studies of cell cholesterol efflux as mediated by SR-BI or ABCA1 have used purified preparations of native lipoproteins or reconstituted particles; yet, in vivo, cells are exposed to a mixture of HDL particles. We exposed cells to the entire HDL fraction from individual serum specimens. These were prepared by the removal of apoB-containing lipoproteins using a polyethylene glycol (PEG) precipitation protocol, which does not dissociate apolipoproteins from mature HDL particles. Using this approach, we could establish the relative efflux efficiencies of each type of HDL particle by establishing the correlations between these subpopulations and ABCA1- and SR-BI-mediated cholesterol efflux. Our hypothesis was that different HDL subpopulations vary significantly in their ability to elicit cellular cholesterol efflux.

METHODS

Subjects

Plasma samples of 105 healthy male individuals (aged 18–65 years) were selected from a large pool of samples based on their specific HDL subpopulation levels and grouped accordingly: 1) low pre β -1 (4.5 ± 1.1 mg/dl) and low α -1 (7.5 ± 1.7 mg/dl); 2) low pre β -1 (4.1 ± 1.0 mg/dl) and high α -1 (32.6 ± 4.5 mg/dl); 3) very low pre β -1 (2.3 ± 0.9 mg/dl); 4) high pre β -1 (20.5 ± 2.2 mg/dl) and low α -1 (5.7 ± 1.0 mg/dl); 5) high pre β -1 (19.9 ± 1.0 mg/dl) and high α -1 (32.7 ± 6.1); 6) low α -3 (18.2 ± 2.1 mg/dl); and 7) high α -3 (58.2 ± 1.3 mg/dl) (Table 1).

TABLE 1. Major characteristics of all subjects (n = 105) and subgroups of subjects (n = 15) selected with various pre β -1, α -1, and α -3 levels

Characteristic	All Subjects	Low Pre β -1, Low α -1	Low Pre β -1, High α -1	Very Low Pre β -1	High Pre β -1, Low α -1	High Pre β -1, High α -1	Low α -3	High α -3
Plasma lipid and apoA-I levels [mg/dl (SD)]								
LDL-cholesterol	115 (32)	124 (32)	94 (24)	97 (21)	131 (27)	128 (27)	106 (33)	125 (36)
HDL-cholesterol	47 (17)	32 (6)	58 (14)	43 (12)	36 (9)	68 (10)	47 (20)	46 (13)
Triglyceride	116 (78)	112 (39)	57 (15)	82 (27)	224 (92)	85 (32)	93 (52)	162 (99)
ApoA-I	126 (27)	101 (14)	132 (18)	115 (20)	120 (25)	160 (17)	114 (25)	138 (19)
ApoA-I levels in HDL subpopulations [mg/dl (SD)]								
Pre β -1	10.7 (8.0)	4.5 (1.1)	4.1 (1.2)	2.3 (0.6)	20.5 (1.8)	19.9 (1.1)	6.3 (3.2)	17.2 (6.2)
Pre β -2	2.2 (1.5)	1.3 (0.9)	3.1 (1.7)	2.2 (1.7)	1.6 (1.3)	2.8 (1.6)	2.9 (1.3)	1.9 (0.9)
α -1	20.0 (12.8)	7.5 (1.7)	32.6 (4.7)	22.3 (8.7)	5.7 (1.1)	32.7 (6.4)	28.3 (11.5)	10.8 (6.2)
α -2	39.2 (11.4)	34.1 (9.6)	41.9 (9.6)	37.4 (7.7)	33.4 (12.4)	52.0 (9.4)	36.5 (8.2)	38.9 (12.4)
α -3	36.6 (14.6)	40.3 (8.7)	26.2 (6.6)	30.2 (10.6)	48.7 (9.8)	34.4 (5.8)	18.2 (2.1)	58.2 (1.0)
Pre α -1	6.3 (4.9)	2.6 (0.8)	10.9 (3.4)	8.3 (4.4)	1.7 (1.0)	7.8 (3.1)	10.1 (5.9)	2.6 (1.8)
Pre α -2	6.6 (3.5)	5.1 (1.7)	9.3 (3.3)	8.6 (4.2)	3.5 (1.6)	7.2 (1.7)	8.1 (4.0)	4.2 (1.8)
Pre α -3	4.0 (1.9)	5.3 (2.6)	3.6 (1.3)	4.3 (1.9)	4.3 (2.4)	2.9 (0.8)	3.1 (1.1)	4.4 (1.5)
Cholesterol efflux (4 h) mediated by (%)								
ABCA1	2.5 (1.5)	1.9 (1.0)	1.9 (1.1)	1.8 (1.1)	2.7 (1.6)	4.0 (1.3)	3.0 (1.8)	2.6 (1.5)
SR-BI	4.6 (1.3)	3.6 (0.8)	5.2 (1.2)	3.9 (0.6)	4.2 (1.0)	5.9 (1.4)	4.2 (1.1)	5.2 (1.3)

apoA-I, apolipoprotein A-I; SR-BI, scavenger receptor class B type I.

Serum lipid and apolipoprotein analyses

EDTA plasma was collected from all individuals after an overnight fast. Plasma was stored at -80°C until use. Total cholesterol, FC, HDL-cholesterol, and triglyceride (TG) concentrations were measured using standard automated enzymatic assays. LDL-cholesterol was calculated using the Friedewald formula. ApoA-I concentrations were measured with a turbidimetric immunoassay from Wako Diagnostics.

ApoA-I-containing HDL subpopulations were determined by nondenaturing two-dimensional gel electrophoresis, immunoblotting, and image analysis, as described previously (40). Briefly, $4\ \mu\text{l}$ of plasma was applied and electrophoresed on a vertical-slab agarose gel (0.7%) in the first dimension at 250 V until the α -mobility front moved 3.5 cm from the origin. The agarose gel was sliced, and the strips were applied onto 3–35% nondenaturing concave gradient polyacrylamide gels. In the second dimension, gels were electrophoresed to completion at 250 V for 24 h at 10°C followed by electrotransfer to nitrocellulose membranes at 30 V for 24 h at 10°C . ApoA-I was immunolocalized on the membrane with monospecific goat anti-human primary and ^{125}I -labeled secondary antibodies [immunopurified rabbit F(ab')₂ fraction against goat IgG]. The bound ^{125}I -labeled secondary antibody was quantified in a FluorImager (Molecular Dynamics).

Quantitative image analysis of the apoA-I-containing HDL subpopulations

Pre β -mobility particles did not overlap with any other HDL particles, so they were easily delineated. Designation of the α -mobility HDL subpopulations was based on the integration of α -migrating HDL. Particles with faster than α -mobility in the same size ranges were designated as pre α particles. Eleven apoA-I-containing HDL subpopulations were encircled (pre β -1_{a,b}, pre β -2_{a,b,c}, α -1₋₃, and pre α -1₋₃), and signals were measured in each area and used to calculate the percentage distribution of HDL subpopulations. Sizes were determined from the molecular mass standard (^{125}I -labeled Pharmacia high molecular mass standards) run simultaneously on the same gel (Fig. 1). Results were expressed as averages of two parallel separations. ApoA-I concentrations of the subpopulations were calculated by multiplying percentiles by plasma total apoA-I concentrations. Because subjects were selected on the basis of total pre β -1 concentration, in the analyses we calculated with eight apoA-I-containing HDL subpopulations. However, in some analyses, we calculated with pre β -1_a and pre β -1_b separately.

Routine maintenance of cells and assay of cell cholesterol efflux

BSA, HEPES, and all reagents, solvents, and chemicals were obtained from Fisher Scientific or as specified in the text. PBS, MEM, and RPMI were purchased from Mediatech. FBS, bovine calf serum, enzymes, and antibiotics for cell culture were obtained from Sigma. Compound CP113,818 was a gift from Pfizer. Tissue culture flasks were obtained from Corning. All filtration products were obtained from Millipore. Other materials and reagents were obtained as noted.

Fu5AH cells were maintained in MEM supplemented with 5% bovine calf serum and antibiotics. J774 macrophages were maintained in RPMI supplemented with 10% FBS and antibiotics. For efflux experiments, 350,000 cells per well were plated on 24-well plates using 0.5 ml of the corresponding growth medium. After 24 h, the growth medium was replaced with 0.5 ml of labeling medium containing $2\ \mu\text{Ci}$ [^3H]cholesterol/ml and $2\ \mu\text{g}/\text{ml}$ of the ACAT inhibitor CP113,818, and the cells were incubated in this medium for another day. Fu5AH cells were labeled with radioactive cholesterol in MEM containing 0.5% bovine calf serum. J774 cells were labeled using RPMI containing 1% FBS.

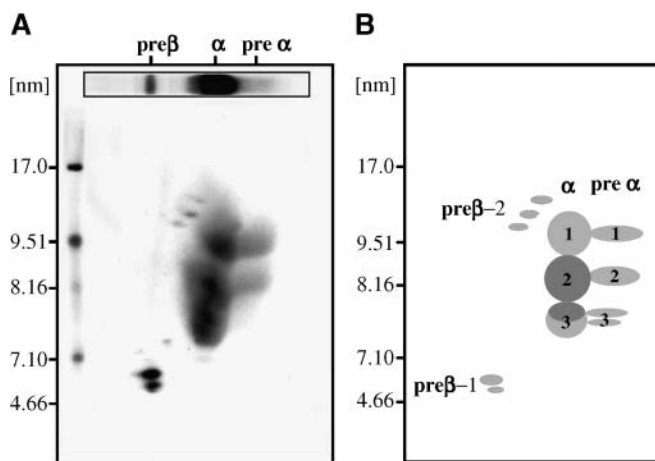


Fig. 1. The left panel shows the apolipoprotein A-I (apoA-I)-containing HDL subpopulations separated by two-dimensional nondenaturing agarose PAGE of a normolipidemic, healthy male subject. The rectangular inset represents the first-dimensional separation on an agarose gel (separation based on charge). A duplicate of that agarose strip was applied on the top of a concave gradient polyacrylamide gel (3–35%) and electrophoresed in the second dimension (separation based on size). Molecular mass standards are shown at left. The right panel shows a scheme of the apoA-I-containing HDL subpopulations in human plasma. The lighter shading indicates apoA-I-containing subpopulations; the darker shading represents apoA-I- and apoA-II-containing subpopulations.

Control HDL₃ (1.125–1.21 g/ml) was isolated by sequential centrifugation from human serum obtained from healthy, normolipidemic volunteers. Control human serum pools were also prepared from healthy normolipidemic volunteers. ApoA-I was obtained from human HDL. The apoprotein was isolated by anion-exchange chromatography using a fast-protein liquid chromatography method adapted from Weisweiler, Friedl, and Ungar (41). [^3H]cholesterol was purchased from New England Nuclear Life Science Products.

Just before the initiation of the efflux assays, the plasma was rapidly thawed and serum was prepared from the test plasmas as follows. Using a glass tube, 1 M CaCl₂ was diluted to a final concentration of 25 mM (1:40 dilution) in the plasma to be clotted, the mixture was incubated at room temperature for 1 h, and the plasma clot was removed by low-speed centrifugation to obtain the serum supernatants. Aliquots from these serum specimens were then treated with PEG solution to precipitate apoB-containing lipoproteins by adding 40 parts PEG solution (20% PEG in 200 mM glycine buffer, pH 7.4) to 100 parts serum. After a 20 min incubation, the precipitate was removed by high-speed centrifugation (10,000 rpm, 30 min, 4°C) to obtain the PEG supernatant containing the HDL lipoprotein fraction. SR-BI-mediated cell cholesterol efflux was measured as the fraction of radioactive cholesterol released from Fu5AH cells to 0.2 ml/well of MEM-HEPES containing either the test serum diluted to 2% or the same serum diluted to 2.8% after removal of apoB-containing lipoproteins as described below. ABCA1-mediated efflux was measured as the difference in release of radioactive cellular cholesterol to these same media from J774 control macrophages and J774 macrophages expressing ABCA1 (ABCA1 efflux = FC efflux from upregulated cells – FC efflux from control cells). To upregulate ABCA1 in J774 macrophages, half of the wells, plated with labeled J774 cells, were incubated for 16–18 h with 0.5 ml/well RPMI containing 0.2% BSA, $2\ \mu\text{g}/\text{ml}$ CP113,818, and 0.3 mM cpt-cAMP. Labeled control J774 cells were also incubated for

TABLE 2. Univariate and multivariate regression analyses of ABCA1-mediated cholesterol efflux from J774 macrophages on apoA-I-containing HDL subpopulations

Subpopulation	Univariate Analysis				Multivariate Analysis			
	B	se(B)	t Value	P Value	B	se(B)	t Value	P Value
Pre β -1	0.0617	0.0174	3.54	0.0006	0.0686	0.0218	3.15	0.0022
Pre β -2	0.1039	0.0987	1.05	0.2953	0.0229	0.1145	0.20	0.8422
α -1	0.0157	0.0115	1.36	0.1758	-0.0131	0.0274	-0.48	0.6341
α -2	0.0359	0.0125	2.88	0.0048	0.0402	0.0200	2.01	0.0477
α -3	0.0068	0.0101	0.67	0.5050	-0.0188	0.0173	-1.08	0.2810
Pre α -1	0.0185	0.0300	0.62	0.5384	0.0703	0.0736	0.95	0.3420
Pre α -2	0.0044	0.0427	0.10	0.9190	-0.0818	0.0931	-0.88	0.3815
Pre α -3	-0.0620	0.0777	-0.80	0.4265	0.0484	0.0947	0.51	0.6103

B, regression coefficient; se(B), standard error (B); t, t-test [B/se(B)].

16–18 h with the same medium without cpt-cAMP. Before the addition of efflux medium containing the samples to be tested, all cell monolayers were gently washed once with 0.5 ml/well MEM supplemented with 1% BSA and once with 0.5 ml/well MEM. All efflux assays were performed in triplicate. The fraction of the total radiolabel incorporated into cell lipids released during incubation with the test samples was measured as follows. Radioactive cholesterol incorporated into cell lipids was extracted by incubating a set of triplicate wells of Fu5AH, control J774, and upregulated J774 cells not exposed to the test serum (T0 cells) overnight with 1 ml isopropanol/well. Total cellular cholesterol label was measured by liquid scintillation counting in aliquots of the isopropanol extracts after drying and resuspension in toluene. Radioactive cholesterol released to test samples after incubation with Fu5AH cells for 2 h or with control and upregulated J774 cells for 4 h was measured by liquid scintillation counting of 100 μ l aliquots of efflux medium that had been filtered through 0.45 μ m multiscreen filtration plates. All efflux values were corrected by subtracting the small amount of radioactive cholesterol released from triplicate wells of Fu5AH, control, and upregulated J774 cells incubated with serum-free medium.

To correct for interassay variability, control lipid-free human apoA-I and a pool of human serum were tested in parallel with the test samples in every assay. These internal controls were used to normalize the efflux values to control for the assay variation. Efflux from Fu5AH cells (SR-BI) was normalized on the basis of the efflux obtained with the human serum pool (2%). HDL₃ was used as a control for monitoring SR-BI-mediated efflux. ABCA1-mediated efflux from the J774 cells used the efflux obtained with lipid-free apoA-I (20 μ g/ml) for normalization.

Data analysis

The data were examined graphically and numerically to determine whether transformations were needed before formal analy-

sis. Simple and multiple regression models were fitted to assess each particle's contribution to efflux. Also, all 255 possible regression models, defined by all possible subsets of the eight particles, were fitted and their adjusted R^2 and Mallows' C_p statistics (42) were examined. Calculations were performed using the SAS package for Windows, version 8.2 (SAS Institute, Cary, NC), and SYSTAT, version 10.2.01 (Systat Software, Inc., Point Richmond, CA).

RESULTS

To evaluate the association of different HDL subpopulations with cellular cholesterol efflux, each serum sample was treated with PEG according to the protocol described in Methods to remove the apoB-containing lipoproteins that can contribute to efflux to varying degrees. Previous studies using a large number of specimens (>75) demonstrated that this procedure eliminated 97% of the apoB-containing lipoproteins while retaining >90% of apoA-I and apoA-II-containing HDL particles (G. H. Rothblat, unpublished observations).

Major characteristics of all subjects (n = 105) and subgroups of subjects (n = 15) selected with various pre β -1, α -1, and α -3 levels are presented in Table 1. The mean apoA-I and HDL-cholesterol levels were highest in the group with high pre β -1 and high α -1 levels. The mean LDL-cholesterol and TG levels were highest in the high pre β -1/low α -1 group. HDL from subjects in the group with high pre β -1 and high α -1 promoted the highest level of ABCA1-mediated cholesterol efflux. HDL from subjects

TABLE 3. Univariate and multivariate regression analyses of SR-BI-mediated cholesterol efflux from Fu5AH cells on apoA-I-containing HDL subpopulations

Subpopulation	Univariate Analysis				Multivariate Analysis			
	B	se(B)	t Value	P Value	B	se(B)	t Value	P Value
Pre β -1	0.0545	0.0151	3.60	0.0005	0.0377	0.0133	2.83	0.0056
Pre β -2	0.2716	0.0822	3.31	0.0013	0.0392	0.0700	0.56	0.5772
α -1	0.0448	0.0091	4.95	<0.0001	0.0511	0.0168	3.05	0.0030
α -2	0.0744	0.0086	8.68	<0.0001	0.0452	0.0122	3.69	0.0004
α -3	0.0133	0.0088	1.52	0.1305	0.0256	0.0106	2.42	0.0174
Pre α -1	0.0644	0.0254	2.54	0.0127	0.0121	0.0450	0.27	0.7890
Pre α -2	0.0785	0.0363	2.16	0.0330	-0.0224	0.0569	-0.39	0.6951
Pre α -3	0.0412	0.0677	-0.61	0.5444	0.0966	0.0579	1.67	0.0981

B, regression coefficient; se(B), standard error (B); t, t-test [B/se(B)].

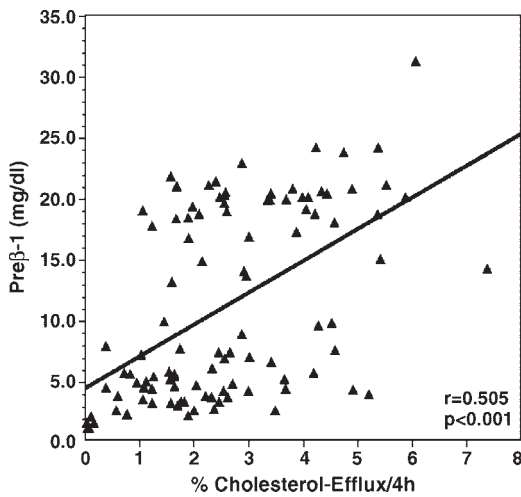


Fig. 2. Linear regression analysis of pre β -1 HDL subpopulation levels and ABCA1-mediated cholesterol efflux from J774 macrophage cells determined after 4 h of incubation.

in the groups with low pre β -1 (low pre β -1/low α -1, low pre β /high α -1, and very low pre β -1) promoted the three lowest levels of cholesterol efflux via the ABCA1 pathway. HDL from subjects selected with high α -1 (high α -1/high

pre β -1, high α -1/low pre β -1) promoted the highest levels of cholesterol efflux via the SR-BI pathway.

In a univariate analysis, pre β -1 ($P = 0.0006$) and α -2 ($P = 0.0048$) were significantly related to ABCA1-mediated cholesterol efflux (Table 2). In multivariate analysis, the relationships between ABCA1-mediated efflux and pre β -1 ($P = 0.0022$) and α -2 ($P = 0.0477$) were significant. We ran analyses with all possible (255) combinations of HDL particles. All of the analyses suggested a role for pre β -1 and α -2 in ABCA1-mediated cholesterol efflux. The model that included all HDL particles as predictors of ABCA1-mediated efflux had an adjusted R^2 of 0.1194 ($P = 0.0086$). The regression model containing pre β -1 alone had an adjusted R^2 of 0.1003. Moreover, every model that included pre β -1 had an adjusted R^2 of 0.0924 or greater, whereas every model that excluded pre β -1 had an adjusted $R^2 < 0.0691$. The model containing pre β -1 and α -2 had an adjusted R^2 of 0.1440. When pre β -1_a and pre β -1_b were calculated separately, only the larger pre β -1_a correlated significantly with the cholesterol efflux via the ABCA1 pathway (data not shown).

SR-BI-mediated cholesterol efflux was handled in a similar manner. In univariate analyses, we observed significant associations between efflux levels and pre β -1, pre β -2,

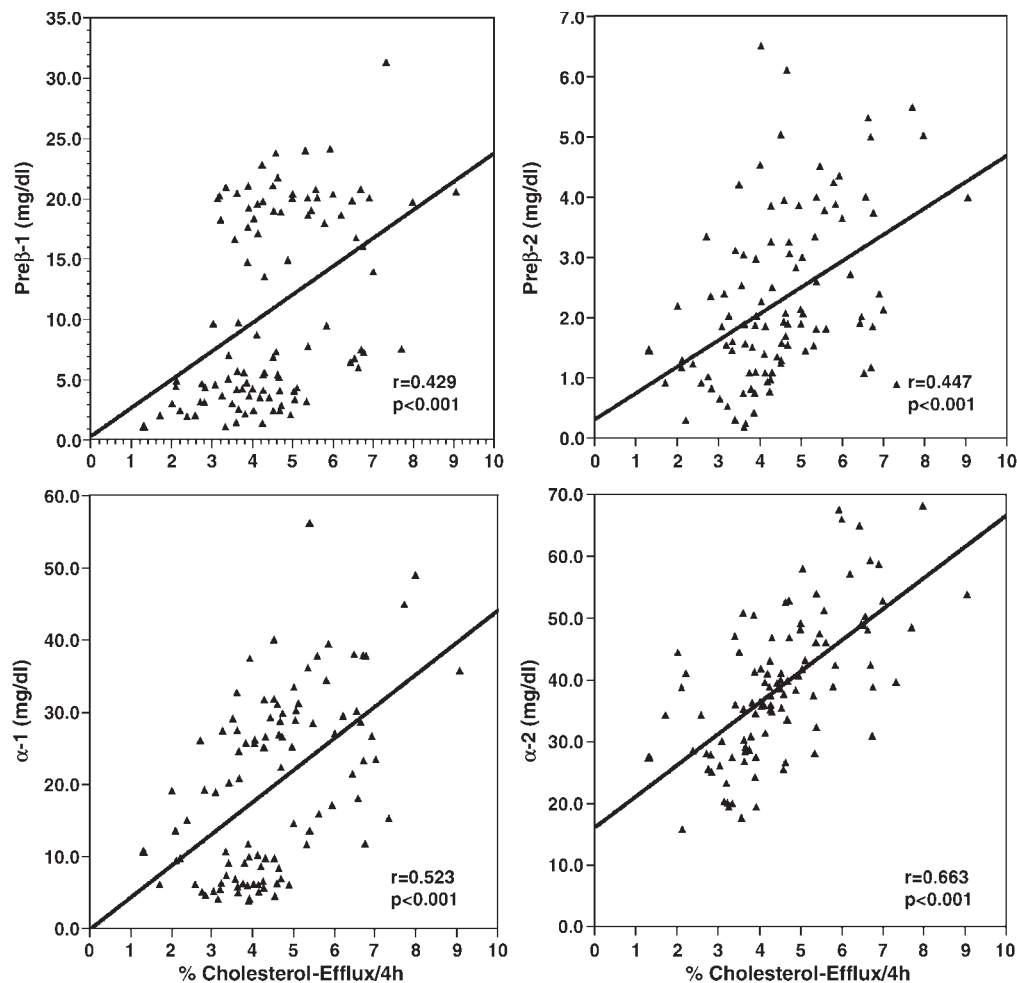


Fig. 3. Linear regression analysis of scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux from Fu5AH hepatoma cells and levels of various HDL subpopulations.

α -1, α -2, pre α -1, and pre α -2 concentrations (Table 3). In multivariate analyses, the associations between SR-BI-mediated cholesterol efflux and pre β -1, α -1, α -2, and α -3 levels remained significant. There were many regression models that were equally effective in terms of the adjusted R^2 statistic. However, Mallows' C_p statistic did not obtain acceptable values until all of pre β -1, α -1, α -2, and α -3 were included in the model. When pre β -1_a and pre β -1_b were calculated separately, we found that only the smaller pre β -1_b correlated significantly with the cholesterol efflux via the SR-BI pathway (data not shown).

In a subsequent analysis, the levels of each HDL particle were plotted against ABCA1- and SR-BI-mediated cholesterol efflux and the fitness of the scatterpoints (r) was evaluated. Mean ABCA1-mediated cholesterol efflux showed a significant positive relationship with the pre β -1 level only (Fig. 2). In contrast, SR-BI-mediated cholesterol efflux values showed significant positive associations with four HDL subpopulations, pre β -1, pre β -2, α -1, and α -2 (Fig. 3). It is interesting that even though α -3 did not have statistically significant predictive capability on its own, it significantly increased the association between α -1 levels and SR-BI-mediated cholesterol efflux. α -1 and α -3 had adjusted R^2 values of 0.1840 and 0.0126, respectively, when they were in a two-predictor regression model. However, for these two HDL particles combined, the adjusted R^2 (0.4620) became higher than that for α -2 (0.4170), which had the highest single value. To determine how α -1 and α -3 together fit SR-BI-mediated efflux better than suggested by their individual contributions, we studied the three-dimensional scatterplots of α -1, α -3, and SR-BI-mediated efflux data. We rotated the chart to see the surface of scatter from different angles of the axes. This analysis indicated that including α -3 into the model made α -1 scatter tighter, improving the R^2 (Fig. 4).

DISCUSSION

The role of HDL in RCT has been studied extensively, but it is not completely understood. RCT involves all HDL particles, HDL-modifying factors (LCAT, cholesteryl ester transfer protein, phospholipid transfer protein, lipases, etc.), and two cell surface proteins, ABCA1, which promotes the unidirectional flux of cholesterol out of cells, and SR-BI, which enhances the bidirectional flux of FC and the unidirectional influx of CE. There are numerous reports describing how whole plasma, whole HDL fraction, or specific HDL subclasses (HDL₂, HDL₃, LpA-I, LpA-I:A-II, pre β -1, and reconstituted HDL particles with a variety of compositions) promote cholesterol efflux from cells. However, there is no information on correlations of individual HDL particles and cholesterol efflux when they are present as a mixture, as in serum.

In this study, we investigated how the entire HDL fraction promoted cholesterol efflux via the ABCA1 and SR-BI pathways and related these efflux data to the concentration of individual HDL particles, determined quantitatively by two-dimensional nondenaturing gel electrophoresis, immunoblotting, and image analysis. Our objective was to

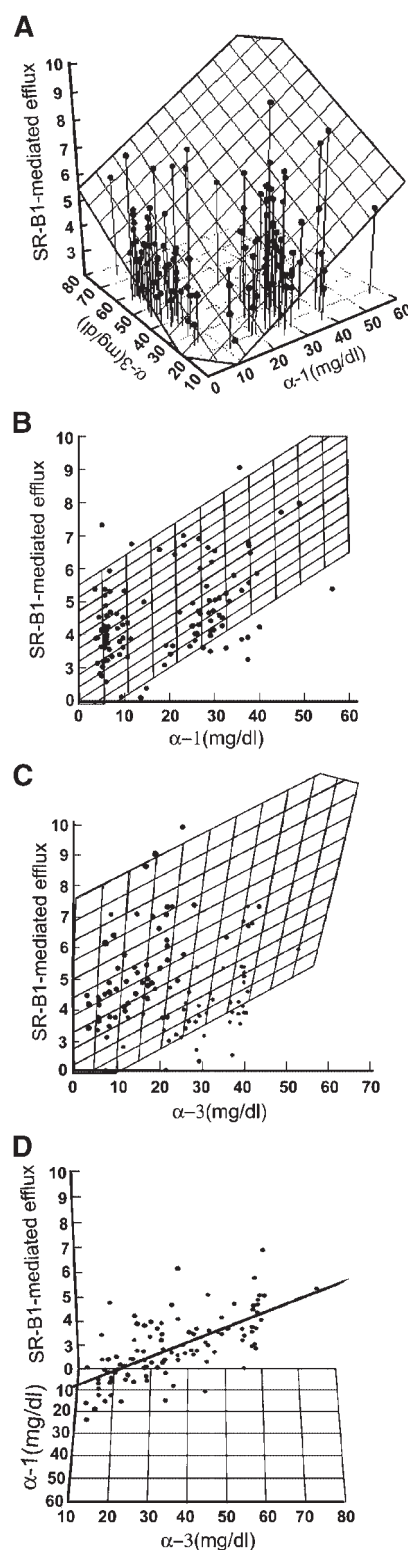


Fig. 4. Association between SR-BI-mediated cholesterol-efflux from Fu5AH hepatoma cells and levels of HDL particles (pre β -1, pre β -2, α -1, and α -2). A: Regression plane from the view of all three axes (SR-BI-mediated efflux, α -1 levels, and α -3 levels). B: Regression plane from the perspective of SR-BI-mediated efflux and α -1 levels. C: Regression plane from the perspective of SR-BI-mediated efflux and α -3 levels. D: Regression plane from the perspective of SR-BI-mediated efflux and α -1 levels when the α -3 surface is turned to horizontal. The comparison between B and D indicates that α -3 levels influence the fitness between α -1 level and SR-BI-mediated efflux.

evaluate the associations between levels of individual HDL subpopulations and ABCA1- and SR-BI-mediated cholesterol efflux.

Interestingly, HDL fractions obtained from subjects with high pre β -1 and high α -1 levels promoted the greatest ABCA1-mediated cholesterol efflux. However, the HDL fraction of subjects with low pre β -1 and high α -1 levels did not enhance cholesterol efflux via the ABCA1 pathway.

We hypothesize that factors other than HDL particle levels influence ABCA1-mediated cholesterol efflux as well. These factors may include the lipid and/or apolipoprotein composition of HDL particles, which can influence the conversion of pre β -1 to larger, more lipidated α -mobility particles. Recent data indicate that TG-rich lipoproteins inhibit ABCA1-mediated cholesterol efflux from macrophages (43). Subjects with high pre β -1 and high α -1 levels usually have lower than average total TG levels; consequently, they have lower than average TG-rich lipoprotein and HDL-TG levels. Data on ABCA1-mediated cholesterol efflux to whole serum showed the same trend: plasma samples with high pre β -1 and high α -1 levels promoted more cholesterol efflux than other samples via the ABCA1 pathway (data not shown).

In contrast to ABCA1-mediated cholesterol efflux, several individual HDL subpopulations (pre β -1, pre β -2, α -1, α -2, and α -3) were significantly associated with cholesterol efflux via the SR-BI pathway. The highest level of SR-BI-mediated cholesterol efflux was also observed in subjects with high pre β -1 and high α -1 levels. In addition to a high α -1 level, this group had the highest mean level of α -2 (33% more than the average of all subjects). Both α -1 and α -2 levels were highly correlated with SR-BI-mediated cholesterol efflux; therefore, the highest combined levels of α -1 and α -2 might be the explanation for the highest efflux level.

SR-BI has a bidirectional function by promoting both selective cholesterol uptake from HDL and cholesterol efflux to HDL. However, the method we used allowed us to determine only cholesterol efflux to HDL via the SR-BI pathway. We do not know how these data are applicable to selective cholesterol uptake from HDL. The two small, lipid-poor HDL particles (pre β -1 and α -3) probably do not contribute significant amounts of cholesterol to cells via SR-BI. We hypothesize that the two large HDL subpopulations α -1 and α -2, containing the bulk of HDL neutral lipids, are more than adequate substrate for SR-BI-mediated selective cholesterol uptake. In contrast to α -1, α -2 is an LpA-I:A-II particle, and apoA-II was reported to modulate the binding and selective lipid uptake from rHDL by SR-BI (44). This recent report indicated that A-I/A-II-rHDL had decreased binding but increased selective uptake compared with apoA-I-rHDL. However, these rHDL particles do not mirror the lipid and apolipoprotein composition of mature α -1 and α -2 particles. We believe that α -2 level is a very important factor in RCT because α -2 levels are two to six times higher than α -1 levels in normal human plasma and even higher in certain pathological conditions in which α -1 levels are negligible but α -2 levels are still close to normal (27–29).

Because the potential for selectively increasing HDL subpopulations with different drug classes is increasing, there is an urgent need for information about the role of these HDL subpopulations in modifying CHD risk. Statins and niacin primarily increase α -1 levels (28, 45). Preliminary data on apoA-I and HDL mimetics indicate that these compounds primarily increase pre β -1 levels. Our preliminary data indicate that in the Veterans Affairs HDL Intervention Trial, gemfibrozil primarily increased α -2 levels (unpublished data).

We conclude that SR-BI-mediated cholesterol efflux from hepatoma cells is significantly associated with the cholesterol-rich LpA-I α -1 and LpA-I:A-II α -2 HDL particles, whereas the ABCA1-mediated FC efflux from macrophages is significantly associated with the lipid-poor pre β -1 HDL. In this study, we measured associations between HDL particle levels and cholesterol efflux. The cause-effect relationship and the mechanism still have to be elucidated by incubating isolated HDL subpopulations with the cells.

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REFERENCES

1. Miller, N. E., D. S. Thelle, O. H. Forde, and O. D. Mjos. 1977. The Tromso Heart Study. High-density lipoprotein and coronary heart disease: a prospective case-control study. *Lancet*. **1**: 965–968.
2. Gordon, D. J., J. L. Probstfield, R. J. Garrison, J. D. Neaton, W. P. Castelly, J. D. Knoke, D. R. Jacobs, S. Bangdiwala, and H. A. Tyroler. 1989. High-density lipoprotein cholesterol and cardiovascular disease: four prospective American studies. *Circulation*. **79**: 8–15.
3. Assmann, G., and H. Shulte. 1992. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). *Am. J. Cardiol*. **70**: 733–737.
4. Barter, P., and K. Rye. 1996. High density lipoprotein and coronary heart disease. *Atherosclerosis*. **121**: 1–12.
5. Montali, A., G. L. Vega, and S. M. Grundy. 1994. Concentrations of apolipoprotein A-I-containing particles in patients with hypoalphalipoproteinemia. *Arterioscler. Thromb. Vasc. Biol*. **14**: 511–517.
6. Robins, S. J., D. Collins, J. T. Wittes, V. Papademetriou, P. C. Deedwania, E. J. Schaefer, J. R. McNamara, M. L. Kashyap, J. M. Hershman, L. F. Wexler, et al. 2001. Veterans Affairs High-Density Lipoprotein Intervention Trial. Relation of gemfibrozil treatment and lipid levels with major coronary events. VA-HIT: a randomized controlled trial. *J. Am. Med. Assoc*. **285**: 1585–1591.
7. Fielding, C. J., and P. E. Fielding. 1995. Molecular physiology of reverse cholesterol transport. *J. Lipid Res*. **36**: 211–228.
8. von Eckardstein, A., Y. Huang, and G. Assmann. 1994. Physiological role and clinical relevance of high-density lipoprotein subclasses. *Curr. Opin. Lipidol*. **5**: 404–416.
9. Young, S. G., and C. J. Fielding. 1999. The ABCs of cholesterol efflux. *Nat. Genet*. **22**: 316–318.
10. Francone, O. L., A. Gurakar, and C. Fielding. 1989. Distribution and functions of lecithin:cholesterol acyltransferase and cholesteryl ester transfer protein in plasma lipoproteins. Evidence for a functional unit containing these activities together with apolipoproteins A-I and D that catalyzes the esterification and transfer of cell-derived cholesterol. *J. Biol. Chem*. **264**: 7066–7072.
11. Barter, P. J. 2002. Hugh Sinclair Lecture. The regulation and remodelling of HDL by plasma factors. *Atheroscler. Suppl*. **3**: 39–47.
12. Quintao, E. C. R. 1995. Is reverse cholesterol transport a misnomer for suggesting its role in the prevention of atherome formation? *Atherosclerosis*. **116**: 1–14.

13. von Eckardstein, A., J. R. Nofer, and G. Assman. 2000. Acceleration of reverse cholesterol transport. *Curr. Opin. Cardiol.* **15**: 348–354.
14. Ordovas, J. M., D. K. Cassidy, F. Civeira, C. L. Bisgaier, and E. J. Schaefer. 1989. Familial apolipoprotein A-I, C-III, and A-IV deficiency and premature atherosclerosis due to deletion of a gene complex on chromosome 11. *J. Biol. Chem.* **264**: 16339–16342.
15. Asztalos, B. F., M. E. Brousseau, J. R. McNamara, K. V. Horvath, P. S. Roheim, and E. J. Schaefer. 2001. Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease. *Atherosclerosis.* **156**: 217–225.
16. Osono, Y., L. A. Woollett, K. R. Marotti, G. W. Melchior, and J. M. Dietschy. 1996. Centripetal cholesterol flux from extrahepatic organs to the liver is independent of the concentration of high density lipoprotein-cholesterol in plasma. *Proc. Natl. Acad. Sci. USA.* **93**: 4114–4119.
17. Jolley, C. D., L. A. Woollett, S. D. Turley, and J. M. Dietschy. 1998. Centripetal cholesterol flux to the liver is dictated by events in the peripheral organs and not by the plasma high density lipoprotein or apolipoprotein A-I concentration. *J. Lipid Res.* **39**: 2143–2149.
18. Reichl, D. 1990. Lipoproteins of human peripheral lymph. *Eur. Heart J.* **11**: 230–236.
19. Hara, H., and S. Yokoyama. 1991. Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol. *J. Biol. Chem.* **266**: 3080–3086.
20. Sviridov, D., A. Hoang, W. H. Sawyer, and N. H. Fidge. 2000. Identification of a sequence of apolipoprotein A-I associated with the activation of lecithin:cholesterol acyltransferase. *J. Biol. Chem.* **275**: 19707–19712.
21. Wong, L., B. Sivok, E. Kurucz, C. H. Sloop, P. S. Roheim, and B. Asztalos. 1995. Lipid composition of HDL subfractions in dog plasma and lymph. *Arterioscler. Thromb. Vasc. Biol.* **15**: 1875–1881.
22. Cockerill, G. W., K. A. Rye, J. R. Gamble, M. A. Vadas, and P. J. Barter. 1995. High-density lipoproteins inhibit cytokine-induced expression of endothelial cell adhesion molecules. *Arterioscler. Thromb. Vasc. Biol.* **15**: 1987–1994.
23. Watson, A. D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman, and M. Navab. 1995. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96**: 2882–2891.
24. Mowri, H. O., W. Patsch, L. C. Smith, A. M. Gotto, and J. R. Patsch. 1992. Different reactivities of high-density lipoprotein2 subfractions with hepatic lipase. *J. Lipid Res.* **33**: 1269–1279.
25. Castro, G. R., and C. J. Fielding. 1988. Early incorporation of cell-derived cholesterol into pre- β -migrating high-density lipoprotein. *Biochemistry.* **27**: 25–29.
26. Miida, T., M. Kawano, C. J. Fielding, and P. E. Fielding. 1992. Regulation of the concentration of pre β high-density lipoprotein in normal plasma by cell membranes and lecithin-cholesterol acyltransferase activity. *Biochemistry.* **31**: 11112–11117.
27. Asztalos, B. F., P. S. Roheim, R. L. Milani, M. Lefevre, J. R. McNamara, K. V. Horvath, and E. J. Schaefer. 2000. Distribution of apoA-I-containing HDL subpopulations in patients with coronary heart disease. *Arterioscler. Thromb. Vasc. Biol.* **20**: 2670–2676.
28. Asztalos, B. F., M. Batista, K. V. Horvath, C. E. Cox, G. E. Dallal, J. S. Morse, G. B. Brown, and E. J. Schaefer. 2003. Change in α_1 HDL concentration predicts progression in coronary artery stenosis. *Arterioscler. Thromb. Vasc. Biol.* **23**: 847–852.
29. Asztalos, B. F., L. A. Cupples, S. Demmissie, K. V. Horvath, C. E. Cox, M. C. Batista, and E. J. Schaefer. 2004. High-density lipoprotein subpopulation profile and coronary heart disease prevalence in male participants of the Framingham Offspring Study. *Arterioscler. Thromb. Vasc. Biol.* **24**: 2181–2187.
30. Rigotti, A., H. E. Miettinen, and M. Krieger. 2003. The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. *Endocr. Rev.* **24**: 357–387.
31. Williams, D. L., M. A. Connelly, R. E. Temel, S. Swarnakar, M. C. Phillips, M. de la Llera-Moya, and G. H. Rothblat. 1999. Scavenger receptor BI and cholesterol trafficking. *Curr. Opin. Lipidol.* **10**: 329–339.
32. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271**: 518–520.
33. Ji, Y., B. Jian, N. Wang, Y. Sun, M. L. Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272**: 20982–20985.
34. Rodriguez, W. V., S. T. Thuahnai, R. E. Temel, S. Lund-Katz, M. C. Phillips, and D. L. Williams. 1999. Mechanism of scavenger receptor class B type I-mediated selective uptake of cholesteryl esters from high density lipoprotein to adrenal cells. *J. Biol. Chem.* **274**: 20344–20350.
35. Thuahnai, S. T., S. Lund-Katz, G. M. Anantharamaiah, D. L. Williams, and M. C. Phillips. 2003. A quantitative analysis of apolipoprotein binding to SR-BI: multiple binding sites for lipid-free and lipid-associated apolipoproteins. *J. Lipid Res.* **44**: 1132–1142.
36. Gu, X., R. Lawrence, and M. Krieger. 2000. Dissociation of the high density lipoprotein and low density lipoprotein binding activities of murine scavenger receptor class B type I (mSR-BI) using retrovirus library-based activity dissection. *J. Biol. Chem.* **275**: 9120–9130.
37. Liu, T., M. Krieger, H. Y. Kan, and V. I. Zannis. 2002. The effects of mutations in helices 4 and 6 of ApoA-I on scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux suggest that formation of a productive complex between reconstituted high density lipoprotein and SR-BI is required for efficient lipid transport. *J. Biol. Chem.* **277**: 21576–21584.
38. Pittman, R. C., C. K. Glass, D. Atkinson, and D. M. Small. 1987. Synthetic high density lipoprotein particles. Application to studies of the apoprotein specificity for selective uptake of cholesterol esters. *J. Biol. Chem.* **262**: 2435–2442.
39. Thuahnai, S. T., S. Lund-Katz, P. Dhanasekaran, M. de la Llera-Moya, M. A. Connelly, D. L. Williams, G. H. Rothblat, and M. C. Phillips. 2004. Scavenger receptor class B type I-mediated cholesteryl ester-selective uptake and efflux of unesterified cholesterol. Influence of high density lipoprotein size and structure. *J. Biol. Chem.* **279**: 12448–12455.
40. Asztalos, B. F., M. Lefevre, T. A. Foster, R. Tulley, M. Windhauser, L. Wong, and P. S. Roheim. 1997. Normolipidemic subjects with low HDL cholesterol levels have altered HDL subpopulations. *Arterioscler. Thromb. Vasc. Biol.* **17**: 1885–1893.
41. Weisweiler, P., C. Friedl, and M. Ungar. 1987. Isolation and quantitation of apolipoproteins A-I and A-II from human high density lipoproteins by fast-protein liquid chromatography. *Clin. Chim. Acta.* **169**: 249–254.
42. Mallows, C. L. 1973. Some comments on C_p . *Technometrics.* **15**: 661–675.
43. Palmer, A. M., N. Murphy, and A. Graham. 2004. Triglyceride-rich lipoproteins inhibit cholesterol efflux to apolipoprotein (apo) A1 from human macrophage foam cells. *Atherosclerosis.* **173**: 27–38.
44. de Beer, M. C., L. W. Castellani, L. Cai, A. J. Stromberg, F. C. de Beer, and D. R. van der Westhuyzen. 2004. SR-BI and CD36. ApoA-II modulates the association of HDL with class B scavenger receptors SR-BI and CD36. *J. Lipid Res.* **45**: 706–715.
45. Asztalos, B. F., K. V. Horvath, J. R. McNamara, P. S. Roheim, J. J. Rubinstein, and E. J. Schaefer. 2002. Effects of atorvastatin on the HDL subpopulation profile of coronary heart disease patients. *J. Lipid Res.* **43**: 1701–1707.