

SR-BI- and ABCA1-mediated cholesterol efflux to serum from patients with Alagille syndrome

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Abstract Alagille syndrome is associated with bile duct paucity resulting in liver disease. Patients can be divided into mildly and severely icteric groups, with both groups having altered lipoproteins. The incidence of ischemic heart disease is rare in severely cholestatic children despite increased total cholesterol and decreased high density lipoprotein cholesterol (HDL-C). The present studies examine the impact of altered lipid and lipoproteins on scavenger receptor class B type I (SR-BI)- and ABCA1-mediated efflux to serum from both groups. Efflux was compared with serum from 29 patients (15 with normal plasma cholesteryl ester, 14 with low cholesteryl ester). Efflux via SR-BI and ABCA1 was studied using cell systems having either low or high expression levels of these receptors. SR-BI efflux was lower ($P = 0.04$) with serum from severely icteric patients ($3.9 \pm 1.4\%$) compared with serum from mildly icteric patients ($5.1 \pm 1.4\%$) and was positively correlated with HDL-C and its apolipoproteins. SR-BI-mediated efflux was not correlated with any particular mature HDL but was negatively correlated with small lipid-poor pre β -1 HDL. Consistent with severely icteric patients having high pre β -1 HDL levels, the ABCA1 efflux was significantly higher with their serum ($4.8 \pm 2.2\%$) compared with serum from mildly icteric patients ($2.0 \pm 0.6\%$) and was positively correlated with pre β -1 HDL. These studies demonstrated that pre β -1 HDL is the preferred acceptor for ABCA1 efflux, whereas many particles mediate SR-BI efflux.—Yancey, P. G., B. F. Asztalos, N. Stettler, D. Piccoli, D. L. Williams, M. A. Connelly, and G. H. Rothblat. **SR-BI- and ABCA1-mediated cholesterol efflux to serum from patients with Alagille syndrome.** *J. Lipid Res.* 2004. 45: 1724–1732.

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Alagille syndrome is a rare autosomal dominant disorder associated with bile duct paucity resulting in liver dis-

ease. Patients are also characterized by facial, skeletal, and cardiac abnormalities. Increased plasma cholesterol and phospholipid (PL) levels are usually observed in cholestatic children with Alagille syndrome (1–3). Although many patients present with xanthomas and plasma cholesterol levels that rival those of familial homozygote hypercholesterolemic patients, patients with primary biliary cirrhosis, such as Alagille patients, rarely suffer from ischemic heart disease. Besides being hypercholesterolemic, Alagille patients have abnormal lipoprotein patterns, and the lipoprotein abnormalities differ depending on the degree of cholestasis (1–3).

Patients with severe cholestasis are markedly hypercholesterolemic and present with increased levels of plasma free cholesterol (FC) and low density lipoprotein cholesterol (LDL-C) (1–3). The increased plasma FC associates with PL and various exchangeable apolipoproteins, but not apolipoprotein B (apoB), to form lipoprotein X (LpX) (4–6). Studies have shown that LpX contains mainly apoC-III and some apoC-II and apoE (5). The increased plasma FC also leads to an enrichment of all of the lipoprotein fractions with FC and PL (2). The increased FC is the result of decreased plasma LCAT activity, which is also observed in adults with severe cholestasis (2, 7). The low plasma LCAT activity also results in markedly decreased high density lipoprotein cholesterol (HDL-C) levels (8). This lack of maturation of HDL particles correlates with increased levels of pre β HDL particles (1). In contrast to patients with severe cholestasis, patients who are mildly icteric are moderately hypercholesterolemic (1, 2). In addition, these patients maintain plasma LCAT ac-

Abbreviations: apoB, apolipoprotein B; CE, cholesteryl ester; cpt, 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate; CS, calf serum; FC, free cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; LpX, lipoprotein X; LSC, liquid scintillation counting; PL, phospholipid; SR-BI, scavenger receptor class B type I; TC, total cholesterol; TG, triglyceride.

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tivity (2), and the increased plasma cholesterol and PL are incorporated into HDL (2). As a result, these patients have increased HDL-C, apoA-I, and apoA-II but have decreased pre β HDL levels compared with control subjects (1). Both types of Alagille patients have increased levels of apoE, apoC-II, and apoC-III compared with control subjects.

In the general population, HDL cholesterol levels are inversely correlated with the incidence of coronary artery disease (9–12). One mechanism by which HDL is thought to protect against atherosclerosis is the removal of excess FC from peripheral cells and subsequent delivery to the liver for excretion (13–15). HDL is very heterogeneous in nature, and the ability of a particular HDL subpopulation to promote cholesterol efflux may differ depending on the mechanism of efflux. At present, there are three known mechanisms by which HDL and/or its apolipoproteins can remove FC from cells. Aqueous diffusion is a relatively inefficient efflux mechanism that occurs with all cell types (16). In recent years, two proteins have been discovered that mediate efficient cholesterol efflux. The scavenger receptor class B type I (SR-BI) facilitates the bidirectional flux of FC between cells and HDL (17, 18), and ABCA1 (19–21) mediates the unidirectional efflux of cellular FC and PL to lipid-poor apoA-I and other exchangeable apolipoproteins.

The goal of the present studies was to determine the abilities of serum from Alagille patients with either severe or mild cholestasis to promote either ABCA1- or SR-BI-mediated efflux. The efflux potential of the serum via the two mechanisms of efflux was then related to differences in both serum lipid or apolipoprotein parameters and HDL subpopulation distribution.

MATERIALS AND METHODS

Materials

Tissue culture plasticware was obtained through Falcon (Lincoln, NJ). Calf serum (CS), FBS, BSA, penicillin, and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2-³H]cholesterol was purchased from NEN Life Sciences, Inc. All other reagents and organic solvents were purchased from Fisher. The acyl-CoA:cholesterol acyltransferase inhibitor, compound CP113,818, was a generous gift from Pfizer.

Alagille subjects studied

Twenty-nine patients with Alagille syndrome were studied. Serum was obtained with informed consent. For this study, patients were assigned to one of two groups according to their serum cholesteryl ester (CE) levels: >65% of total cholesterol (group 1) or <65% of total cholesterol (group 2). Studies have shown that serum CE levels are indicative of serum LpX levels and LCAT activity in subjects with Alagille syndrome (2, 3). Fourteen subjects had low serum CE levels and 15 patients had high serum CE levels. The age ranges of group 1 and group 2 subjects were 2.2–16.7 and 2.3–12.7, respectively.

Serum lipid and apolipoprotein analyses

Blood was collected into tubes without anticoagulant and serum was isolated as described previously (22). HDL-C and serum

total cholesterol (TC), FC, PL, and triglyceride (TG) were measured on a Cobas Fara (Roche Diagnostic Systems, Inc., Montclair, NJ) using Sigma reagents. The non-HDL-C value was obtained by subtracting HDL-C from serum TC. In group 1 patients, the non-HDL-C fraction contains VLDL-C, intermediate density lipoprotein cholesterol, and LDL-C, whereas in group 2 patients, the non-HDL-C fraction would contain the same plus LpX-C. Human apoA-I, apoA-II, apoB, apoE, and apoCs were measured using turbidometric assays (Sigma) on a Cobas Fara.

Nondenaturing two-dimensional electrophoresis

ApoA-I-containing HDL subspecies were determined by non-denaturing two-dimensional gel electrophoresis, immunoblotting, and image analysis as described earlier (23). Briefly, 4 μ 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 ¹²⁵I-labeled secondary antibodies [immunopurified rabbit F(ab')₂ fraction against goat IgG]. The bound ¹²⁵I-labeled secondary antibody was quantitated in a FluorImager (Molecular Dynamics).

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

Pre β -1 and pre β -2 particles do not overlap with any other HDL particles, so they are easily delineated. Designation of the α -mobility HDL subpopulations is based on the integration of α -migrating HDL. Finally, eight apoA-I-containing HDL subpopulations were encircled, and signals were measured in each area and used to calculate the percentage distribution of HDL subpopulations. Sizes were determined from the molecular weight standard (¹²⁵I-labeled Pharmacia high molecular weight standards) run simultaneously on the same gel. Results are expressed as averages of two parallel separations. ApoA-I concentrations of the subpopulations are calculated by multiplying percentiles by plasma total apoA-I concentrations.

Cell culture and transient transfections

J774 macrophages were maintained on RPMI supplemented with 10% FBS and antibiotics. COS-7 cells were maintained on DMEM containing 10% CS and antibiotics. For transfection, COS-7 cells were seeded on 100 mm plates and incubated for 18 h at 37°C in 10% CS in DMEM. Cells were transfected with 10 μ g of the indicated plasmid and diluted in serum-free DMEM and Fugene 6 (Roche Molecular Biochemicals) as described previously (24). The pSG5 vector (Stratagene, Inc.) with or without murine SR-BI was prepared using endotoxin-free Qiagen Maxi-prep kits.

Measurement of SR-BI- and ABCA1-mediated cholesterol efflux

SR-BI-mediated cholesterol efflux was measured using control and SR-BI-transfected COS-7 cells as previously described (25). After transfection, the cells were removed from the 100 mm plates by trypsinization. The transfected cells were then suspended in 10% CS DMEM containing 2 μ g/ml CP113,818 and plated onto 24-well plates. For labeling, the cells were incubated for 24 h in 0.5 ml of 10% CS DMEM containing 12 μ Ci of

[³H]cholesterol and 2 µg of CP113,818 per milliliter. For cholesterol efflux, the cells were washed once with 0.5 ml of 1% BSA in MEM and once with MEM. Medium containing 1% human serum was then added to the wells. After 2 h, 150 µl aliquots of the medium were removed and filtered through 0.45 µm multi-screen filtration plates to remove any floating cells. The [³H]cholesterol in 100 µl of the filtrate was then measured by liquid scintillation counting (LSC). The percentage efflux is based on the total [³H]cholesterol present in the cells before the efflux incubation. To measure the [³H]cholesterol present in the cells, the cell lipids were extracted by incubating the cell monolayers overnight in isopropanol. After lipid extraction, the total [³H]cholesterol present in the lipid extract was measured by LSC.

ABCA1-mediated FC efflux was measured using control and 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (cpt)-cAMP-treated J774 macrophages (26). J774 macrophages were plated onto 24-well plates at a density of 350,000 cells per well. After 24 h, the cells were labeled by incubation for 24 h in 1% FBS RPMI containing 12 µCi of [³H]cholesterol and 2 µg of CP113,818 per milliliter. The cells were then incubated for 15 h in 0.2% BSA in RPMI containing 2 µg CP113,818/ml alone or plus 0.3 mM cpt-cAMP. The cells were then washed once with 0.5 ml of 1% BSA in MEM and once with MEM. Medium containing the human serum to be tested diluted to 1% was then added to the cells and incubated at 37°C. After 4 h, the [³H]cholesterol content of the medium was measured as described above.

In all SR-BI and ABCA1 FC efflux measurements, the percentage FC efflux was corrected for the small amount of [³H]cholesterol released to medium without acceptors present. The percentage SR-BI-mediated FC efflux was calculated as percentage FC efflux from SR-BI-expressing cells minus the percentage FC efflux from control COS cells. The percentage ABCA1-mediated FC efflux was calculated as the percentage FC efflux from cells upregulated with cpt-cAMP minus the percentage FC efflux from control J774 cells. This calculation controls for the contribution of other efflux mechanisms and yields data that are more specific for the contribution of ABCA1 or SR-BI. It should be noted, however, that in both cases the control cells lack significant levels of either ABCA1 (no cpt-cAMP treatment) or SR-BI (transfected with empty vector) (17, 18, 26). It is possible that cAMP treatment of the J774 cells upregulates other efflux mechanisms that, in this system, would be attributed to ABCA1. As a control, a standard human serum pool was run in parallel with all SR-BI and ABCA1 FC flux assays. Lipid-free apoA-I (20 µg/ml medium) was also tested for efflux in parallel with all ABCA1 efflux assays as a positive control for the upregulation of ABCA1. In the case of J774 cells, efflux to apoA-I from cells not treated with cAMP was only 4% of that obtained with cAMP-treated cells.

Data analysis

The data were analyzed using Stata version 6.0 (Stata Corp., College Station, TX). Descriptive analyses were conducted using proportions for categorical variables or means and standard deviations for continuous variables. To test for the difference between two groups, *t*-tests were used for normally distributed variables and rank-sum tests were used for variables not normally distributed. Differences in proportion between groups were tested using the Chi-square test or Fisher's exact test, as appropriate. Associations between variables were explored using graphic display, then tested using simple linear regressions, and are reported using correlation coefficients. Furthermore, to investigate if some of the correlations observed were attributable to confounding by a known variable or were independent, multiple linear regressions were used in selected cases in which an underlying mechanism could be hypothesized. All statistical tests were

two-tailed, and a value of *P* < 0.05 was considered statistically significant.

RESULTS

Serum lipid and apolipoprotein composition

Because studies have shown that serum CE levels are indicative of serum LpX levels and LCAT activity in subjects with Alagille syndrome (2), the patients were assigned to one of two groups according to their serum CE levels [$\geq 65\%$ of TC (group 1) or $< 65\%$ of TC (group 2)]. Based on this criterion, the average percentage serum CE of group 2 patients ($47 \pm 15\%$) was significantly lower compared with the average of group 1 patients ($70 \pm 3\%$; **Table 1**). Consistent with percentage serum CE being a marker for LCAT activity, HDL-C levels in group 2 patients were 38% lower compared with HDL-C levels in group 1 patients (Table 1). In addition, the serum apoA-I and apoA-II levels were significantly lower in group 2 patients compared with group 1 patients (Table 1).

The percentage serum CE was indicative of cholestasis; thus, the serum bilirubin and FC levels of group 2 patients were 3.8- and 3.7-fold higher compared with the bilirubin and FC levels of group 1 patients (Table 1). However, there was not a perfect overlap between the percentage serum CE and severe cholestasis (bilirubin > 3.5 mg/dl; Table 1). Of the 15 patients with $> 65\%$ CE, 3 had severe cholestasis, and of the 14 patients with $< 65\%$ CE, 11 had severe cholestasis. In addition, two of the group 1 patients and eight of the group 2 patients had xanthomas (Table 1). The serum TC, LDL-C, PL, TG, apoB, and apoE levels were all significantly higher in group 2 patients compared with the same serum parameters of group 1 patients (Table 1). There was no significant difference in apoC-III levels between the two groups. It is noted that the apoE and

TABLE 1. Serum parameters in group 1 and group 2 Alagille patients

Parameter	Group 1 (n = 15)	Group 2 (n = 14)
Cholesteryl ester (% of total cholesterol)	70 ± 3	47 ± 15 ^a
Bilirubin (mg/dl)	1.8 ± 1.9	6.9 ± 4.1 ^a
Bilirubin > 3.5 mg/dl	16 of 29	13 of 29 (NS)
Xanthomas	2 of 15	8 of 14 ^b
Total cholesterol (mg/dl)	277 ± 110	599 ± 389 ^a
Free cholesterol (mg/dl)	86 ± 37	318 ± 199 ^a
Non-HDL-C (mg/dl)	165 ± 71	506 ± 400 ^a
HDL-C (mg/dl)	89 ± 47	55 ± 45 ^a
Triglycerides (mg/dl)	115 ± 33	188 ± 79 ^a
Phospholipids (mg/dl)	333 ± 136	703 ± 344 ^a
apoB (mg/dl)	94 ± 20	122 ± 28 ^a
apoA-I (mg/dl)	151 ± 22	97 ± 47 ^a
apoA-II (mg/dl)	39 ± 5	26 ± 13 ^b
apoE (mg/dl)	14 ± 14	23 ± 15 ^b
apoC-III (mg/dl)	18 ± 8	21 ± 11 (NS)

Data are presented as means ± SD. apoB, apolipoprotein B; HDL-C, high density lipoprotein cholesterol.

^a Significantly different from group 1 (*P* < 0.01).

^b Significantly different from group 1 (*P* < 0.05).

apoC-III levels in both groups of patients were 4- to 5-fold higher compared with what has been reported for children without liver disease (2).

SR-BI- or ABCA1-mediated cholesterol efflux and relationships with serum parameters

Shown in Fig. 1A, B are the SR-BI- and ABCA1-mediated cholesterol efflux values obtained with serum from group 1 and group 2 Alagille patients. Although not statistically significant, the SR-BI-mediated efflux was on average higher with serum from group 1 patients ($5.0 \pm 1.3\%$) compared with serum from group 2 patients ($4.1 \pm 1.7\%$). As already mentioned, there was not a perfect overlap between cholestasis severity and percentage serum CE (Table 1). When the patients were divided into mild (bilirubin < 3.5 mg/dl) and severe (bilirubin ≥ 3.5 mg/dl) cholestasis groups, the SR-BI-mediated efflux was significantly higher with serum from the group with mild cholestasis ($5.1 \pm 1.4\%$, $P = 0.04$) compared with serum from the group with severe cholestasis ($3.9 \pm 1.6\%$). This is consistent with group 1 patients having higher HDL-C than group 2 patients (Table 1). In contrast to SR-BI-mediated cholesterol efflux, the ABCA1-mediated efflux was much lower with serum from group 1 patients ($2.0 \pm 0.6\%$) compared with serum from group 2 patients ($4.8 \pm 2.2\%$).

The SR-BI-mediated efflux with serum from group 1 patients was significantly correlated in a positive manner with a number of serum lipid parameters, including TC, FC, CE, TG, and PL levels (Fig. 2A). The SR-BI-mediated efflux with serum from group 1 patients was also positively correlated with both HDL-C and non-HDL-C. In addition, SR-BI-mediated efflux was positively correlated with a

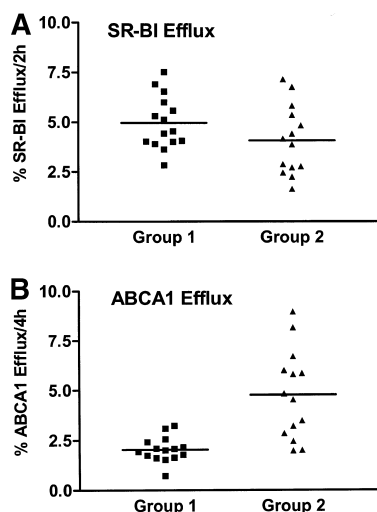


Fig. 1. Scavenger receptor class B type I (SR-BI)- and ABCA1-mediated cholesterol efflux to serum from group 1 and group 2 Alagille patients. SR-BI- (A) and ABCA1- (B) mediated cholesterol efflux to serum from group 1 [high cholesteryl ester (CE)] or group 2 (low CE) was measured as described in Materials and Methods. The data are expressed as percentage SR-BI- or percentage ABCA1-mediated efflux (see Materials and Methods), and each data point is the average of triplicate determinations.

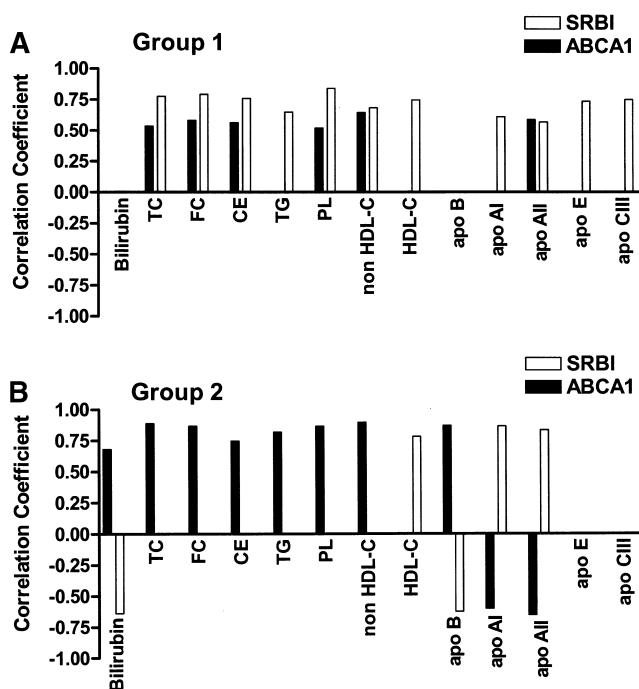


Fig. 2. Correlative data obtained with serum parameters from group 1 and group 2 patients. Shown are the correlation coefficients for the relationships between SR-BI- (closed bars) or ABCA1- (open bars) mediated efflux and parameters measured in serum from either group 1 (A; high CE) or group 2 (B; low CE). Serum parameters, SR-BI, and ABCA1-mediated efflux were measured as described in Materials and Methods. Correlation coefficients that were determined to be statistically significant at $P < 0.05$ are shown. apoB, apolipoprotein B; FC, free cholesterol; HDL-C, high density lipoprotein cholesterol; PL, phospholipid; TC, total cholesterol; TG, triglyceride.

number of the exchangeable apolipoproteins, including apoA-I, apoA-II, apoE, and apoC-III (Fig. 2A), but a significant correlation was not observed with apoB. In contrast to serum from group 1 patients, the SR-BI-mediated efflux with serum from group 2 patients was not significantly correlated with serum TC, FC, CE, TG, and PL (Fig. 2B). Also, unlike the serum from group 1 patients, the SR-BI-mediated efflux with serum from group 2 patients was not significantly correlated with non-HDL-C, and interestingly, there was a strong negative correlation with apoB (Fig. 2B). However, similar to the serum from group 1 patients, the SR-BI-mediated efflux with serum from group 2 patients was positively correlated with HDL-C, apoA-I, and apoA-II levels (Fig. 2B). To test if the negative correlation between SR-BI-mediated efflux with apoB was confounded by the positive association between SR-BI-mediated efflux with apoA-I or apoA-II, we performed multiple linear regression analyses. After adjustment for apoA-I or apoA-II, the association between SR-BI-mediated efflux and apoB was no longer significant ($P = 0.5$ and $P = 0.3$, respectively). In addition, the SR-BI-mediated efflux with serum from group 2 patients was negatively correlated with bilirubin levels (Fig. 2B). Similarly, using multiple linear regression, bilirubin was no longer

associated with SR-BI-mediated efflux ($P = 0.9$ and $P = 0.5$, respectively) after adjustment for apoA-I or apoA-II.

Surprisingly, the ABCA1-mediated efflux with serum from group 1 patients was positively correlated with many of the same parameters as SR-BI-mediated efflux, including TC, FC, PL, non-HDL-C, and apoA-II levels (Fig. 2A). In addition, there was no significant correlation with either HDL-C or apoA-I. The ABCA1-mediated efflux with serum from group 2 patients was positively correlated with a number of parameters, including serum bilirubin, TC, FC, CE, TG, PL, non-HDL-C, and apoB levels (Fig. 2B). Similar to the serum from group 1 patients, there was no significant correlation with HDL-C. Surprisingly, ABCA1-mediated efflux was negatively correlated with both apoA-I and apoA-II in serum from group 2 patients.

HDL subpopulation distribution and relationships with SR-BI- and ABCA1-mediated cholesterol efflux

Shown in Table 2 is the percentage HDL subpopulation distribution of serum from either group 1 or group 2 patients. Of the HDL subpopulations, the percentage distribution of both pre β -1 HDL and α -3 HDL were significantly higher in the serum of group 2 patients compared with the serum of group 1 patients. However, consistent with group 2 patients having lower HDL-C compared with group 1 patients, the percentage distribution of most of the other HDL particles was significantly lower in the serum of group 2 patients compared with group 1 patients. In particular, the percentage distribution of α -1 HDL and pre β -2 HDL were low in serum from group 2 patients. In addition, the percentage distribution of all of the pre α HDL particles was lower in serum from group 2 patients compared with serum from group 1 patients. The same trends in HDL subpopulation distribution were observed for serum from both group 1 and group 2 patients when the data were expressed as milligrams of apoA-I per deciliter (data not shown). Some serum from both group 1 and group 2 patients had very large α -1 and pre α -1 HDL particles.

The statistically significant correlation coefficients for the relationships between the percentage HDL subpopulation distribution and SR-BI- or ABCA1-mediated efflux with serum from group 1 and group 2 patients are shown

TABLE 2. Percentage distribution of apoA-I-containing HDL in group 1 and group 2 Alagille patients

HDL Subpopulation	Group 1	Group 2
Pre β -1 HDL	14.4 \pm 4.7 (12)	37.3 \pm 4.9 (12) ^a
Pre β -2 HDL	1.9 \pm 1.4 (12)	0.4 \pm 0.6 (12) ^a
α -1 HDL	23.1 \pm 9.6 (12)	8.9 \pm 8.8 (10) ^a
α -2 HDL	27.2 \pm 6.4 (12)	24.1 \pm 11.8 (10) (NS)
α -3 HDL	23.3 \pm 8.0 (12)	31.3 \pm 7.8 (12) ^b
Large α -1 HDL	10.9 \pm 4.7 (2)	6.6 \pm 4.4 (2) (NS)
Pre α -1 HDL	3.2 \pm 2.6 (12)	0.7 \pm 0.2 (7) ^b
Pre α -2 HDL	2.9 \pm 1.7 (12)	1.7 \pm 0.7 (7) (NS)
Pre α -3 HDL	2.1 \pm 1.1 (12)	1.8 \pm 0.5 (7) (NS)
Large pre α -1 HDL	1.4 \pm 0.1 (2)	0.5 \pm 0.3 (3) ^b

Data are presented as means \pm SD. The number per group is noted in parentheses.

^a Significantly different from group 1 ($P < 0.01$).

^b Significantly different from group 1 ($P < 0.05$).

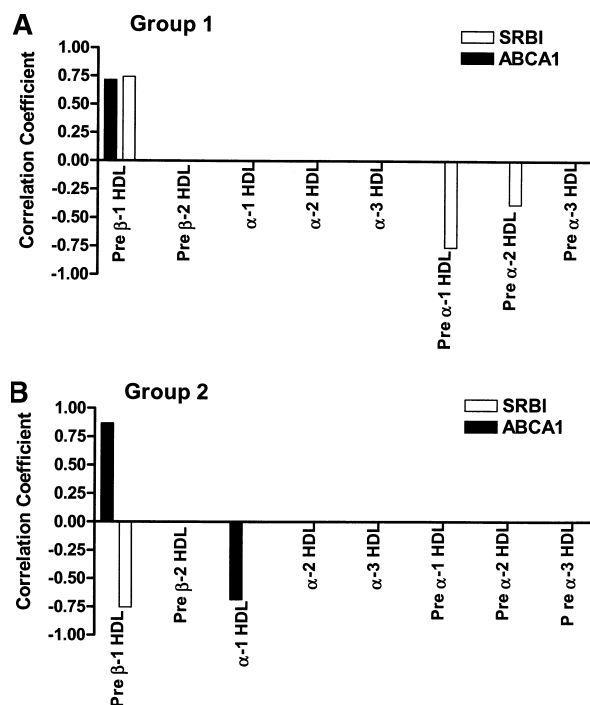


Fig. 3. Correlative data obtained with the HDL subpopulation distribution from group 1 and group 2 patients. Shown are the correlation coefficients for the relationships between SR-BI- (closed bars) or ABCA1- (open bars) mediated efflux and the percentage HDL subpopulation distribution of serum from either group 1 (A; high CE) or group 2 (B; low CE). HDL subpopulation distribution, SR-BI-, and ABCA1-mediated efflux were measured as described in Materials and Methods. Correlation coefficients were obtained using linear regression, and only the correlation coefficients that were determined to be statistically significant at $P < 0.05$ are shown.

in Fig. 3A, B. Similar correlation coefficients were obtained when the analyses were done using the HDL subpopulations expressed as milligrams of apoA-I per deciliter (data not shown). Surprisingly, the SR-BI-mediated cholesterol efflux with serum from group 1 patients was positively correlated with the percentage distribution of pre β -1 HDL (Fig. 3A). In addition, SR-BI-mediated cholesterol efflux was negatively correlated with both pre α -1 and pre α -2 HDL particles. Significant relationships were not observed between any of the other HDL subpopulations and SR-BI-mediated efflux with serum from group 1 patients. In contrast to serum from group 1 patients, the SR-BI-mediated efflux with serum from group 2 patients was negatively correlated with pre β -1 HDL (Fig. 3B). This was the only significant correlation observed with SR-BI-mediated efflux with serum from group 2 patients.

The ABCA1-mediated cholesterol efflux was positively correlated with the percentage distribution of pre β -1 HDL in serum from both group 1 and group 2 patients (Fig. 3A, B). With serum from group 1 patients, no significant relationships were observed between ABCA1-mediated efflux and the other HDL subpopulations. Similarly, the only other significant relationship with serum from group 2 patients was a negative correlation between α -1 HDL particles and ABCA1-mediated cholesterol efflux.

DISCUSSION

The concept of reverse cholesterol transport proposes that excess cell cholesterol in peripheral tissues is removed by serum lipoproteins, particularly HDL, and returned to the liver for excretion as cholesterol and bile acids (13, 15, 27). The first step in this process is the efflux of FC from the cell to the acceptor lipoprotein. Our understanding of this process has advanced greatly in the last few years, and it is now known that there are at least three mechanisms that mediate cell FC efflux (28–30). Cell surface proteins, SR-BI and ABCA1, mediate the two most quantitatively important mechanisms. SR-BI promotes the bidirectional flux of FC between lipoproteins and the cell membrane. HDLs, particularly PL-enriched HDL particles, are believed to be the most efficient in this process (31). In contrast, ABCA1 mediates the release of cell FC and PL to lipid-free or lipid-poor apolipoproteins such as apoA-I, apoA-II, and apoA-IV (26, 32, 33).

An earlier study by Davit-Spraul et al. (1) on the relationships between lipoproteins in Alagille sera and cell cholesterol efflux used Fu5AH hepatoma cells as cholesterol donors. We now know that efflux from these cells is mediated primarily by SR-BI (34). In the study of Davit-Spraul et al. (1), the sera were separated into two groups based on bilirubin levels, and it was demonstrated that there was enhanced efflux from the group with moderately increased bilirubin ($<100 \mu\text{mol/l}$) and efflux was decreased with sera from individuals with highly elevated bilirubin ($>100 \mu\text{mol/l}$). These investigators concluded that the changes in efflux from Fu5AH cells could be explained by an increase in HDL-related parameters in the mildly icteric patients as opposed to an accumulation of particles with very abnormal composition in severely icteric individuals (1).

In the present investigation, we have examined the ability of sera from Alagille children to promote cholesterol efflux specifically by the ABCA1 and SR-BI pathways. SR-BI- and ABCA1-mediated efflux was measured using established cell systems with low and high receptor expression (29, 30). Earlier studies by Davit-Spraul et al. (1) demonstrated that differences in lipoprotein profiles between mildly and severely icteric Alagille patients depended on LCAT activity. Because LCAT activity is markedly decreased in severely cholestatic individuals, and because LCAT plays a fundamental role in lipoprotein synthesis and remodeling, we chose to divide our subjects into two groups based on the percentage of serum cholesterol present as CE. The ratio of FC to CE has been shown to be highly associated with LCAT activity. For our study, the first group had normal percentage CE values of $\geq 65\%$ (average, $70 \pm 3\%$; $n = 15$) and the second group had abnormally low values of $<65\%$ (average, $47 \pm 15\%$; $n = 14$), indicative of reduced LCAT activity and abnormal lipoprotein profiles. A comparison of the lipid and lipoprotein profiles of the two groups (Table 1) demonstrates a pattern very similar to that previously reported (1–3), with the low CE group exhibiting marked increases in all lipid classes, but particularly in FC. HDL-C and the HDL-associ-

ated apolipoproteins apoA-I and apoA-II were lower in the low CE group, whereas apoB particles were increased. These changes are consistent with those reported in subjects with genetic LCAT deficiencies (35–37). HDLs are heterogeneous and consist of a number of subpopulations that differ in composition, size, and charge (10, 38). We also examined the subpopulation distribution of HDL in the Alagille sera using two-dimensional electrophoresis, a system that separates on the basis of both size and charge (39, 40). The subjects with low CE were found to have very high concentrations of pre β -1 HDL, and both their α HDL and pre α HDL levels were low compared with those of patients having normal CE content.

These dramatic differences in serum lipid levels, lipoprotein profiles, and HDL subpopulation distribution between the two groups of Alagille patients provided us a unique opportunity to study the impact of these features on the two mechanisms of efflux. The studies have given insight into which lipoproteins mediate SR-BI and ABCA1 efflux and also into which plasma factors affect the two mechanisms of efflux. The results indicate that the generally accepted model in which lipid-poor lipoprotein particles mediate ABCA1 efflux and PL-rich lipoproteins promote SR-BI-mediated efflux does not always explain the efflux patterns obtained with Alagille sera.

SR-BI-mediated efflux

The average SR-BI-mediated efflux was higher with serum from group 1 Alagille patients compared with serum from group 2 patients (Fig. 1A). An examination of the relationships between SR-BI-mediated efflux and serum parameters for the two groups demonstrated striking differences (Fig. 2A, B). The serum from children with normal CE distributions (group 1) demonstrated strong positive correlations with numerous serum parameters. The positive correlations with HDL and its major apolipoproteins is similar to that previously observed when adult human serum was studied using Fu5AH rat hepatoma cells as cholesterol donors (22). These cells have been shown to efflux cholesterol primarily via the SR-BI pathway and have very little functional ABCA1. Interestingly and in contrast to previous studies with adult serum, the SR-BI efflux with group 1 serum was also positively correlated with non-HDL-C, TG, apoE, and apoC-III, suggesting that the lipoproteins in the non-HDL-C fraction in the normal CE group can be acceptors for SR-BI. The strong correlations between SR-BI efflux and a number of serum parameters in group 1 patients is consistent with SR-BI interacting with, and mediating efflux to, a variety of mature lipoprotein particles, including both HDL and non-HDL particles.

Similar to group 1 patients, SR-BI efflux was positively correlated with HDL-C in serum from patients with low CE. In striking contrast to the SR-BI efflux data obtained with the group 1 sera, however, many of the positive relationships seen with a number of parameters in group 1 sera were lost, and surprising, highly significant negative correlations were obtained between SR-BI-mediated efflux and both bilirubin and apoB levels. This negative re-

relationship between apoB and SR-BI-mediated efflux probably does not reflect a direct link between apoB particles and the receptor but rather is the result of strong negative correlations between apoB levels in these children and the serum concentrations of both apoA-I ($r^2 = 0.64$) and apoA-II ($r^2 = 0.73$). This hypothesis was confirmed using multiple linear regressions. Thus, as bilirubin and apoB increased, the two apolipoproteins associated with functional HDL, apoA-I and apoA-II, decreased. Similar relationships were not observed in the sera from individuals with normal CE ratios. Unlike the normal CE group, SR-BI efflux was not correlated with the non-HDL-C, suggesting that these particles are not adequate acceptors for SR-BI and are different from the particles in the normal CE group. Studies have shown that the non-HDL-C fraction of the low CE group is altered in a number of ways, including the marked enrichment in FC and the decreased exchangeable apolipoprotein content.

Assessment of the relationships between SR-BI-mediated efflux and HDL subpopulations revealed that there were no positive relationships between efflux and any one population of large PL-enriched HDL in serum from both groups of patients. However, the SR-BI-mediated efflux with serum from both groups of patients was positively related to HDL-C. This is consistent with the concept that SR-BI can mediate efflux with a variety of particles. There was a strong negative correlation between SR-BI-mediated efflux and pre β -1 HDL with serum from group 2 patients, suggesting that while pre β -1 HDL may interact with SR-BI, the particles do not stimulate SR-BI efflux. Pre β -1 HDLs contain only apoA-I, and in addition, they are small and contain fewer lipid molecules compared with the other HDL subpopulations (39, 41–43). This is consistent with studies showing that lipid-free apoA-I can bind to SR-BI but does not stimulate efflux (31) and with studies showing that SR-BI efflux is a function of the PL content of HDL (25). In contrast to group 2 patients, there was a strong positive correlation between pre β -1 HDL and SR-BI-mediated efflux with serum from group 1 patients. Rather than pre β -1 HDL of group 1 patients mediating SR-BI efflux, this correlation is probably a reflection of the levels of pre β -1 HDL in these patients being correlated with many of the same serum parameters as SR-BI-mediated efflux, including TC, FC, CE, non-HDL-C, TG, and PL. With normocholesterolemic human adult serum, similar correlations are not observed between pre β -1 HDL and these serum parameters (B. F. Asztalos, unpublished observations).

With serum from group 1 patients, there was also a strong negative correlation between SR-BI-mediated efflux and pre α -1 HDL. A similar negative, but weaker, correlation was also observed with pre α -2 HDL. Similar correlations were not observed with pre α -3 HDL or with any of the pre α HDLs in serum from group 2 patients, probably because of the low levels of these pre α HDLs. These data suggest that the pre α HDLs are able to interact with SR-BI but do not promote efflux. Previous studies have shown that the pre α HDLs are PL poor compared with α HDLs and contain a higher proportion of charged PL (44).

Both factors probably contribute to the pre α HDLs not being efficient acceptors for SR-BI efflux.


ABCA1-mediated cholesterol efflux

The ABCA1-mediated efflux was higher with serum from group 2 patients compared with serum from group 1 patients (Fig. 1B). In both groups of patients, the ABCA1-mediated efflux was positively correlated with pre β -1 HDL, and group 2 patients had much higher levels of pre β -1 HDL compared with group 1 patients. These results are consistent with small, poorly lipidated HDLs mediating ABCA1 efflux and suggest that pre β -1 HDL is the HDL subpopulation that mediates ABCA1 efflux in vivo.

With serum from group 1 patients, ABCA1 efflux was positively correlated with a number of serum lipid parameters, including TC, FC, CE, non-HDL-C, and PL. As already stated, the pre β -1 HDL in these patients was similarly correlated with these same parameters, suggesting that the formation of pre β -1 HDL is related to these parameters. The transfer of lipids from HDL to the apoB-containing fraction by plasma transfer proteins, including both phospholipid transfer protein and cholesteryl ester transfer protein (45, 46), results in the formation of pre β -1 HDL. Relationships of these serum parameters with pre β -1 HDL might be expected under conditions in which these transfer reactions are more pronounced. Because of the combination of the large influx of FC into plasma and the maintenance of LCAT activity, these transfer reactions are probably enhanced in group 1 patients. Consistent with this possibility are studies showing that the VLDL and LDL fractions of Alagille patients are greatly enriched in both CE and PL compared with the same fractions in age-matched controls (3).

The ABCA1-mediated efflux with serum from group 2 patients was also positively correlated with a number of serum parameters, including bilirubin, TC, FC, CE, TG, PL, non-HDL-C, and apoB. These same serum lipid parameters were increased in group 2 patients compared with group 1 patients with mild cholestasis. The severe hypercholesterolemia in these patients is attributable to both the influx of bile FC into plasma and unregulated cholesterol synthesis in the liver (1, 2). In the present studies, all of the serum cholesterol parameters and apoB were positively correlated with serum bilirubin. Similarly, serum PL and TG were positively correlated with bilirubin (data not shown). In addition, studies have shown that the levels of LCAT activity are inversely related to bilirubin levels. Given these relationships with serum bilirubin, these serum factors are not likely directly linked to ABCA1 efflux. Rather, they are a reflection of the severe liver disease in these patients, resulting in both decreased LCAT activity and increased pre β -1 HDL levels.

In summary, the present studies have shown that the serum from Alagille patients with both mild and severe cholestasis has enhanced efflux properties, but by different mechanisms. The serum from patients with mild cholestasis has increased efflux potential via SR-BI, whereas the serum from patients with severe cholestasis has increased cholesterol acceptors for ABCA1. The

present studies have also shown that SR-BI is capable of releasing cholesterol to a wide range of HDL subpopulations and to apoB-containing lipoproteins, whereas ABCA1 efflux is mediated by apoA-I-containing pre β -1 HDL. Adults with severe primary biliary cirrhosis have lipid profiles and low LCAT activity similar to Alagille patients with severe cholestasis (6, 7, 47). Despite the severe hypercholesterolemia, both groups appear not to be at increased risk for atherosclerosis. It is conceivable that the increased pre β -1 HDL plays a role in protecting these patients against the development of coronary artery disease. 

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