



ATP binding cassette family A protein 1 determines hexosylceramide and sphingomyelin levels in human and mouse plasma^S

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Abstract Sphingolipids, including ceramide, SM, and hexosylceramide (HxCer), are carried in the plasma by lipoproteins. They are possible markers of metabolic diseases, but little is known about their control. We previously showed that microsomal triglyceride transfer protein (MTP) is critical to determine plasma ceramide and SM, but not HxCer, levels. In human plasma and mouse models, we examined possible HxCer-modulating pathways, including the role of ABCA1 in determining sphingolipid plasma concentrations. Compared with control samples, plasma from patients with Tangier disease (deficient in ABCA1) had significantly lower HxCer (−69%) and SM (−40%) levels. Similarly, mice deficient in hepatic and intestinal ABCA1 had significantly reduced HxCer (−79%) and SM (−85%) levels. Tissue-specific ablation studies revealed that hepatic ABCA1 determines plasma HxCer and SM levels; that ablation of MTP and ABCA1 in the liver and intestine reduces plasma HxCer, SM, and ceramide levels; and that hepatic and intestinal MTP contribute to plasma ceramide levels, whereas only hepatic MTP modulates plasma SM levels. **Key words:** These results identify the contribution of ABCA1 to plasma SM and HxCer levels and suggest that MTP and ABCA1 are critical determinants of plasma sphingolipid levels.—Iqbal, J., M. T. Walsh, S. M. Hammad, M. Cuchel, D. J. Rader, and M. M. Hussain. **ATP binding cassette family A protein 1 determines hexosylceramide and sphingomyelin levels in human and mouse plasma.** *J. Lipid Res.* 2018. 59: 2084–2097.

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Low density apoB-containing lipoproteins (B-lps) and HDLs carry the bulk of lipids in the blood circulation. These lipoproteins carry triacylglycerols, phospholipids, cholesterol, and sphingolipids (1–5). The major sphingolipids in the plasma are SM, ceramide (Cer), hexosylceramide (HxCer), and lactosylceramide (LactCer). Sphingolipids consist of a common 18-carbon amino-alcohol backbone, sphingosine. These molecules are structurally diverse and regulate significant physiologic functions associated with several metabolic diseases such as insulin resistance, progression of nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH), atherosclerosis, and coronary artery disease (6–13). Earlier, we showed that microsomal triglyceride transfer protein (MTP) is a critical determinant of plasma Cer levels and partially contributes to plasma SM by investigating sphingolipid concentrations in the plasma of MTP-deficient abetalipoproteinemia subjects as well as in liver- and intestine-specific MTP-deficient mice (14). MTP deficiency in humans and mice was associated with ~80% reductions in plasma Cer levels and ~40% reduction in plasma SM levels. Mechanistic studies showed that MTP does not play a role in sphingolipid biosynthesis but is important for sphingolipid secretion. Furthermore, the data

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Abbreviations: B-lps, low-density apoB-containing lipoproteins; Cer, ceramides; HE, heterozygous Tangier subjects; HO, homozygous Tangier subjects; HxCer, hexosylceramide; LactCer, lactosylceramide; LXR, liver X receptor; MTP, microsomal triglyceride transfer protein

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suggested that MTP might be involved in the intracellular transfer of Cer and SM to B-lps to facilitate their secretion. These studies also showed that MTP deficiency had no effect on plasma HxCer levels. This was surprising, as most, if not all, of these sphingolipids are present in B-lps (1, 15).

Here, we examined pathways that might modulate plasma HxCer levels. We hypothesized that, instead of B-lps, HDL might be involved in bringing these glycosphingolipids to the plasma compartment. Plasma HxCer species have been identified as possible biomarkers of disease progression. It has been shown that plasma HxCer species were high in chronic hepatitis C virus patients with severe fibrosis and hepatic necroinflammation (16, 17). Furthermore, HxCer levels were closely associated with severe fibrosis in these patients following adjustment for several confounding variables in multivariate analysis (17). It has been suggested that HxCer (d18:1/12:0) could be a potential diagnostic marker for severe hepatic steatosis. The reasons for high plasma HxCer in fibrosis are not known (17). HxCer (16:1) has also been suggested to be a possible biomarker for disease progression in relapsing multiple sclerosis patients (18) and in drug-induced hepatic phospholipidosis (19).

Plasma membrane-anchored ABCA1 actively participates in concert with extracellular acceptors (mainly apoAI) to generate HDL (20–24). This process is regulated by liver X receptors (LXRs) (25, 26). ABCA1 deficiency in Tangier disease significantly lowers plasma HDL and increases tissue sterols and premature coronary atherosclerosis in some individuals (27, 28). ABCA1 deficiency in mice reduces plasma HDL and increases atherosclerosis (29). Tissue-specific ablation studies in mice showed that hepatic and intestinal ABCA1 contribute to ~70% and 30% of plasma HDL cholesterol, respectively (30, 31). The effect of ABCA1 deficiency on plasma sphingolipids is unknown. Studies presented here show that ABCA1 is critical for modulating plasma HxCer and SM levels in humans and mice.

MATERIALS AND METHODS

Materials

The [³H]dihydrospingosine (60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. C6-NBD-Cer (catalog no. 6224) was purchased from Setareh Biotech, and NBD-SM (catalog no. 229573) was from Avanti Polar Lipids.

Human plasma samples from heterozygous abetalipoproteinemia (ABL, normal, n = 4) were treated as normal controls, as abetalipoproteinemia is an autosomal recessive disorder, and obligate heterozygous parents of these subjects have normal plasma lipids levels (32, 33). The same values have been used as controls in our previous study (14). Heterozygous (HE; n = 5) and homozygous (HO; n = 6) Tangier subjects were collected at the University of Pennsylvania, after obtaining approvals by the university's Institutional Review Board and consents from subjects and/or parents. Frozen samples were shipped to SUNY Downstate Medical Center, where different analyses were performed. Additionally, appropriate aliquots of these samples were shipped from SUNY Downstate to the Lipidomics Shared Resource at the Medical University of South Carolina for sphingolipid analyses using LC/MS/MS as described previously (1, 14).

Animals

Chow-fed male, 10-week-old mice on a C57BL/6J background were used in these studies. Abca1 floxed (*Abca1^{fl/fl}*) mice (30) were crossed with *ER^{T2}-villin-Cre* mice to obtain *ER^{T2}-villin-Cre;Abca1^{fl/fl}* mice as described previously (34). To generate liver- and intestine-specific ABCA1 ablated (*L,I-Abca1^{-/-}*) mice, 10-week-old *ER^{T2}-villin-Cre;Abca1^{fl/fl}* mice were injected once intravenously with 10⁹ pfu of either AAV-TBG-Luc (Adeno-associated virus expressing luciferase under the control of human thyroxin binding-globulin promoter; control) or AAV-TBG-Cre viruses (to ablate hepatic floxed genes) in 100 μl of sterile PBS. Additionally, mice injected with AAV-TBG-Luc were injected intraperitoneally with 200 μl of corn oil for three alternate days to serve as controls. Mice injected with AAV-TBG-Cre were injected with 0.5 mg of tamoxifen (induces Cre-recombinase expression in the intestine to ablate floxed genes) in 200 μl of corn oil for three alternate days to obtain *L,I-Abca1^{-/-}* mice. To generate individual liver-specific (*L-Abca1^{-/-}*) or intestine-specific (*I-Abca1^{-/-}*) KO mice, *ER^{T2}-villin-Cre;Abca1^{fl/fl}* mice (34) were either injected once intravenously with 10⁹ pfu of AAV-TBG-Cre virus or intraperitoneally with 0.5 mg of tamoxifen in 200 μl of corn oil for three alternate days, respectively. To generate liver- and intestine-specific KO mice for both *Mttp* and *Abca1* genes, we crossed *Mttp^{fl/fl}* and *ER^{T2}-villin-Cre;Abca1^{fl/fl}* mice to obtain *ER^{T2}-villin-Cre;Abca1^{fl/fl}Mttp^{fl/fl}* mice. These mice were injected with AAV-TBG-Cre and tamoxifen as described above. Tissue-specific liver (*L-Mttp^{-/-}*) or intestine (*I-Mttp^{-/-}*) KO mice were generated by injecting *ER^{T2}-villin-Cre;Mttp^{fl/fl}* mice (34) either once intravenously with 10⁹ pfu of AAV-TBG-Cre virus or intraperitoneally with 0.5 mg of tamoxifen in 200 μl of corn oil for three alternate days, respectively. Plasma and tissues were harvested 7 days after the last tamoxifen injection. All experiments were approved by the Institutional Animal Care and Use Committee at SUNY Downstate Medical Center.

Quantification of lipids and apolipoproteins

Kits were used to measure triglycerides (Thermo Fisher Scientific, catalog no. TR22421), phospholipids (Wako Diagnostics, catalog no. 433-36201), and cholesterol (Thermo Fisher Scientific, catalog no. TR13421) in plasma, liver, and intestine according to the manufacturer's instructions. Human apoB and apoAI were quantified by ELISA (35, 36).

Quantification of sphingolipids

Plasma (100 μl) from heterozygous abetalipoproteinemia (normal; n = 4) and HE (n = 5) and HO (n = 6) Tangier subjects, as well as plasma (150 or 100 μl) from different WT and KO mice (n = 3), was used for the quantification of different species of Cer, SM, HxCer, and LactCer using HPLC-MS/MS (1, 14). Amounts of different sphingolipid species were combined to obtain total plasma levels. Lipids were extracted from 2 mg of liver or intestinal tissue homogenates from mice and used to quantify intracellular sphingolipids.

Western blot

For detection of MTP or ABCA1 in the liver, liver homogenates were homogenized in Buffer K. They (20 μg of protein) were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, blocked with 50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% Tween 20 and 5% milk (TBS plus Tween 20), probed with either mouse anti-mouse MTP (BD Transduction LaboratoriesTM, catalog no. 612022; 1:3,000 dilution) or anti-mouse ABCA1 followed by Alexa Fluor[®] 633-labeled anti-IgG (Life Technologies, Inc.; 1:10,000 dilution). The membranes were stripped and probed with anti-vinculin (Sigma, catalog no. V9131; 1:2,000 dilutions) or anti-GAPDH (Santa Cruz, catalog no. sc-48167; 1:2,000 dilution).

antibodies as a control. To detect apoB and apoA1, plasma (0.5 μ l) was run on a 5–14% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was cut in half. One-half was probed with rabbit anti-mouse apoB (Meridian Life Science, catalog no. K23300R; 1:1,000 dilution) and then incubated with Alexa Fluor[®] 633 goat anti-rabbit IgG (Life Technologies, Inc., catalog no. A-21070; 1:10,000 dilution). The other half was probed with goat anti-apoA1 (Academy Bio-Medical Co., Inc., catalog no. 11S-G2; 1:2,000 dilution) followed by Alexa Fluor[®] 633 donkey anti-goat IgG (Life Technologies, Inc., catalog no. A-21082; 1:10,000 dilutions). Bands were visualized in a phosphorimager.

Cell culture and transfection

Huh-7 cells (ATCC) were grown in DMEM supplemented with 10% FBS, L-glutamine, and antibiotics. Cells were reverse-transfected with siRNAs using RNAiMAX (ThermoFisher, catalog no. 13778150) according to the manufacturer's instructions. Briefly, siABCA1, scrambled siRNA, and RNAiMAX were diluted individually in serum-reduced Opti-MEM, combined, and incubated at room temperature for 30 min. Next, Huh-7 cells were trypsinized, centrifuged, and plated in a 96-well plate (Cer synthesis) or 6-well plates (Cer and SM synthesis) containing the complexed siRNA and RNAiMAX. Cells were incubated in reduced serum medium for 24 h. Cells were rinsed and incubated for 24 h in 10% FBS DMEM before analysis.

Cer synthesis

Liver homogenates (250 μ g of protein) from WT and KO mice were incubated with [³H]dihydro-sphingosine (0.05 μ Ci), dihydro-sphingosine (15 μ M), FA-free BSA (20 μ M), and lignoceroyl-CoA (50 μ M) in the presence or absence of Fumonisin B1 (50 μ M; an inhibitor of sphingolipids) in a total volume of 200 μ l for 30 min at 37°C. The reaction was stopped by adding 200 μ l of CHCl₃:CH₃OH (2:1, v/v). Lipids were extracted and separated on thin-layer silica plates (catalog no. 44931, Analtech, Inc.) using a CHCl₃:CH₃OH:C₆H₅CH₃:NH₄OH:H₂O (40:40:20:0.4:1.6, ratios by volume) solvent system (14). Radioactivities in the dihydro-sphingosine and dihydroceramide bands were quantified using a scintillation counter.

SM synthesis

Liver tissues from WT and KO mice were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5% sucrose (pH 7.4), and a cocktail of protease inhibitors. The homogenate was centrifuged in a tabletop Centrifuge 5415D (Eppendorf) at 2,320 *g* for 10 min at 4°C. The supernatant (250 μ g of protein) was added to assay buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, C6-NBD Cer (3.3 μ g/ml), and phosphatidylcholine (100 μ g/ml) in a total volume of 200 μ l reaction and incubated for 2 h at 37°C. The reaction was stopped by adding 200 μ l of CHCl₃:CH₃OH (2:1, v/v). Lipids were extracted (37), dried, and resuspended in 100 μ l of isopropanol for the separation of sphingolipids on TLC as above. The TLC plates were visualized with the phosphorimager, and bands were quantified.

Sphingolipid efflux

Huh-7 cells or hepatocytes isolated from different mice were incubated with 2 μ M C6-NBD Cer in 10% FBS containing DMEM at 37°C for 3 h and washed three times with DMEM plus 0.1% BSA, and the efflux was initiated by the addition of fresh DMEM containing 40 μ g/ml either BSA, human serum LDL (catalog no. MBS173145, MyBioSource), human serum ApoA1 (catalog no. MBS173253, MyBioSource), or human serum HDL (catalog no. MBS173147, MyBioSource). In some experiments, efflux was performed in the presence or absence of either 2 μ M LXR agonist

(T0901317, catalog no. T2320, Sigma-Aldrich) or 500 nM MTP inhibitor (Lomitapide) or 250 μ M ABCA1 inhibitor (glyburide, catalog no. 2539, Sigma-Aldrich). For LXR agonist experiments, cells were pretreated with 2 μ M LXR agonist for 17 h before the efflux. Culture media were harvested after 6 h of incubation and centrifuged at 580 *g* for 15 min at 4°C to pellet-detached cells. Lipids in the media were extracted, separated on TLC, and visualized with the phosphorimager as described above.

Statistics

Comparisons between WT and different KO mouse models were made by Student *t*-test. Comparisons among HE ABL (normal), HE Tangier, and HO Tangier subjects were evaluated using the Student *t*-test using GraphPad Prism. Significance among different treatments in supplemental tables was determined by one-way ANOVA.

RESULTS

Tangier patients have low plasma SM and HxCer

MTP plays a critical role in determining plasma concentrations of Cer and SM, but plasma levels of HxCer and LactCer were found not to be affected by MTP deficiency (14), even though these sphingolipids are also found to be associated with B-lps (1–5). Here, we hypothesized that HxCer and LactCer arrive to the blood circulation via apoB-independent pathways. We have previously shown that apoB-dependent and -independent pathways contribute to cholesterol absorption (38, 39) and that MTP and ABCA1, respectively, are the major determinants of these pathways (34, 39). Hence, we investigated whether ABCA1 might contribute to plasma glycosylceramide levels.

To investigate whether ABCA1 in humans is important for plasma sphingolipids, we measured these lipids in the plasma of HE and HO Tangier patients that carry codominant mutations in the *ABCA1* gene (27, 40–42) and compared them with those found in the plasma of heterozygous abetalipoproteinemia subjects that have normal plasma levels. Plasma total triglyceride, cholesterol, Cer, dihydroceramide, LactCer, sphingosine, and sphingosine-1P levels were similar in all three groups (Fig. 1; supplemental Table S1). Total phospholipids, HDL-cholesterol, apoA1, and dihydro-sphingosine levels were significantly lower in HE and HO Tangier subjects. In addition, SM and HxCer levels were significantly reduced in HO Tangier subjects compared with controls. However, plasma levels of dihydro-sphingosine-1P were increased in Tangier subjects (~260%) (Fig. 1N; supplemental Table S2). These studies indicate that ABCA1 deficiency in Tangier subjects significantly reduces plasma SM and HxCer levels. Thus, ABCA1 might participate in the control of plasma SM and HxCer levels in humans.

Although total Cer levels were not significantly different in the three groups, there was a trend toward lower levels with *P* = 0.053 and *P* = 0.06 in HE and HO subjects, respectively, when compared with controls (supplemental Table S1). Analyses of individual species revealed that C14-Cer, C18-Cer, C20-Cer, C20:1-Cer, and C24-Cer levels were significantly reduced in HE and HO Tangier subjects compared

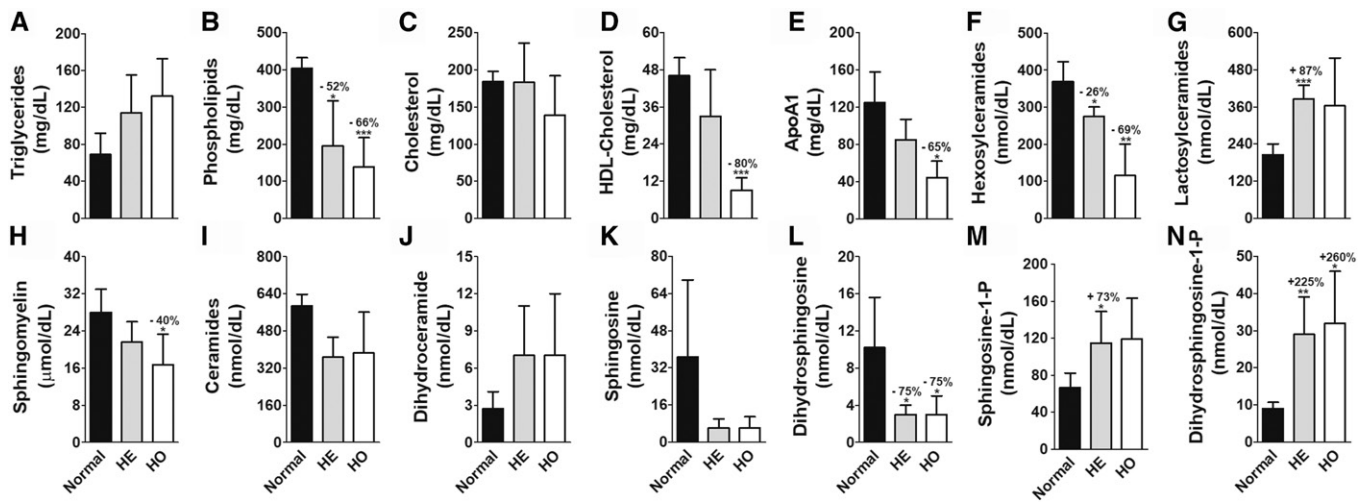


Fig. 1. Total plasma lipids and apolipoproteins in normal, HE, and HO Tangier subjects. A–C: Plasma from normal ($n = 4$), HE ($n = 5$), and HO ($n = 6$) subjects were used to measure triglyceride, phospholipids, and cholesterol using commercial kits. D: HDL from these subjects was isolated after the precipitation of apoB-containing lipoproteins by $MgCl_2$:phosphotungstic acid and subsequently used to determine HDL-cholesterol (HDL-C) levels by a commercial kit. E: apoA1 was measured by ELISA. G–N: Various species of Cer, SM, HxCer, and LactCer were measured in the plasma of different subjects using HPLC-MS/MS. Values for individual species are shown in supplemental Table S1. Amounts of different species were combined to obtain total plasma levels. *P* values are after two-tailed Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

with controls (supplemental Table S2). Thus, ABCA1 deficiency appears to reduce plasma concentrations of several Cer species, but does not affect overall plasma Cer levels.

The major species of HxCer present in control plasma were C16-HxCer, C22-HxCer, C24-HxCer, and C24:1-HxCer (supplemental Table S3). All these species, except for C16-HxCer, were significantly reduced in HO Tangier subjects. C16-HxCer was reduced (~39–43%) but was not statistically significant. All major species of LactCer were increased in the plasma of HE and HO Tangier subjects (supplemental Table S4). Significant reductions in SM in HO Tangier subjects (Fig. 1) were due to reductions in C14-SM, C16-SM, C18-SM, C20-SM, C20:1-SM, C22:1-SM, and C24:1-SM (supplemental Table S5). Thus, ABCA1 deficiency in humans is associated with significant reductions in plasma HxCer and SM levels.

Plasma sphingolipids are significantly lower in combined liver- and intestine-specific ABCA1-deficient ($L, I-Abca1^{-/-}$) mice

The studies described above in Tangier subjects suggest that ABCA1 plays a role in determining plasma SM and HxCer. To study the effect of ABCA1 deficiency in the liver and intestine on sphingolipid levels, we generated mice with combined liver- and intestine-specific ABCA1 deficiency. ABCA1 expression was reduced by 88% and 89% in the intestine (Fig. 2A) and liver (Fig. 2D) of these mice, respectively, with no effect on MTP mRNA (Fig. 2B, E), activity (Fig. 2C, F) and protein levels (Fig. 2G). Plasma apoA1 levels were significantly reduced by 98% along with a small decrease of 45% in apoB100 levels in $L, I-Abca1^{-/-}$ mice without a significant change in apoB48 levels (Fig. 2H). Plasma concentrations of triglycerides were not different in $L, I-Abca1^{-/-}$ mice compared with controls (Fig. 3A). However, $L, I-Abca1^{-/-}$ mice had significantly reduced plasma levels of phospholipids, cholesterol, HxCer, LactCer, and

SM. The plasma levels of dihydroceramide, dihydrospingosine, dihydrospingosine-1P, sphingosine, and spingosine-1P were not significantly affected by ABCA1 deficiency. In general, all major species of Cers, SM, HxCer, and LactCer were reduced in the plasma of $L, I-Abca1^{-/-}$ mice (supplemental Tables S6–S9). Thus, combined intestinal and hepatic ABCA1 deficiency significantly reduces plasma phospholipids, cholesterol, SM, HxCer, and LactCer in mice.

Except for significant increases in hepatic HxCer, other hepatic lipids were not affected by combined hepatic and intestinal deficiency of ABCA1 (Fig. 3B). Analyses of individual Cer species showed that, except for increases in hepatic C16-Cer and C24:1-Cer, other Cer species were not significantly different in control and $L, I-Abca1^{-/-}$ mice (supplemental Table S6). The major HxCer species (C16-HxCer) in the mouse livers was significantly increased (supplemental Table S7). There were no significant differences in different LactCer species in the livers of control and $L, I-Abca1^{-/-}$ mice (supplemental Table S8). There were no significant differences in hepatic SM species in the two groups, except for C24:1-SM, that showed significant increases (supplemental Table S9). Thus, ABCA1 deficiency increases hepatic HxCer and has no effect on other lipids.

The intestines of $L, I-Abca1^{-/-}$ mice had higher phospholipids and lower HxCer (Fig. 3C). Other lipids were not significantly different from controls. The intestines of $L, I-Abca1^{-/-}$ mice showed significant reductions in C18-Cer, C20-Cer, and C20:1-Cer (supplemental Table S6). Significant reductions in intestinal HxCer were due to reductions in C20-HxCer, C22-HxCer, C22:1-HxCer, C24-HxCer, C24:1-HxCer, and C26:1-HxCer. All LactCer species showed nonsignificant reductions (supplemental Table S8). Different SM species levels in the intestines of WT and $L, I-Abca1^{-/-}$ mice were similar (supplemental Table S9). Thus, except for significant reductions in intestinal

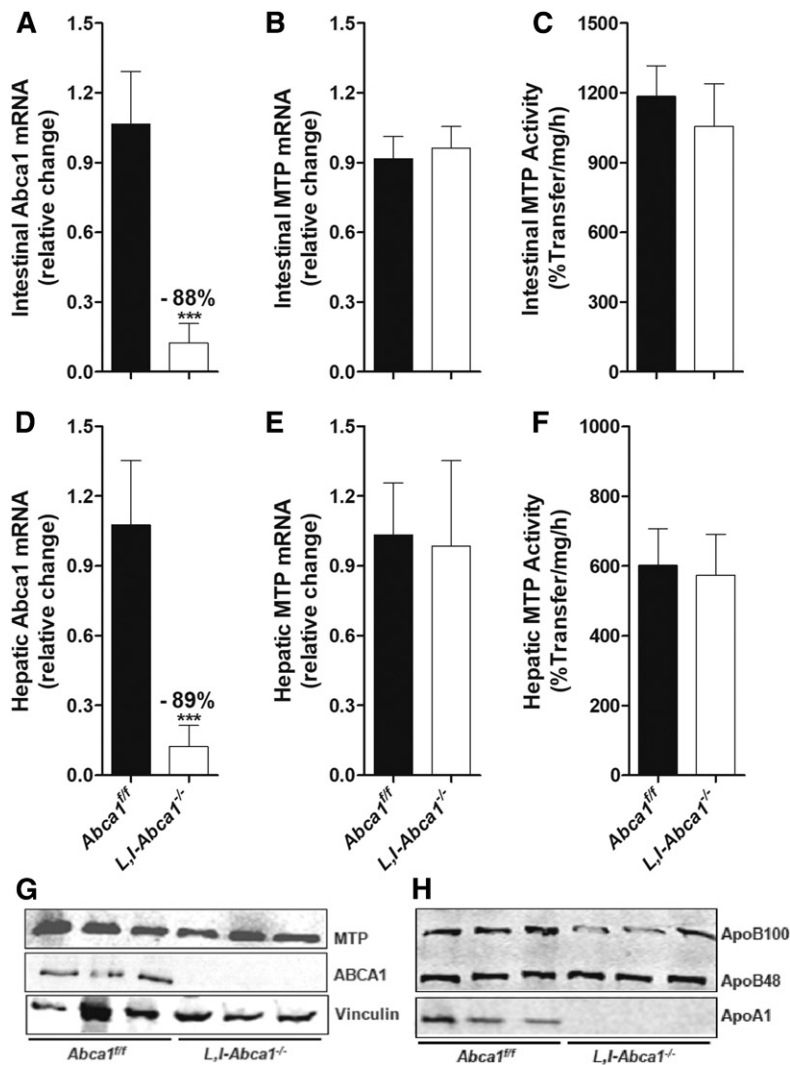


Fig. 2. ABCA1, apoA1, MTP, and apoB levels in the liver, intestine, and plasma of *L, I-Abca1^{-/-}* mice. Chow-fed, male, 10-week-old *Abca1^{fl/fl}* mice ($n = 3$) were injected once intravenously with 109 pfu of either AAV-TBG-Luc or AAV-TBG-Cre viruses in 100 μ l of sterile PBS. Next, mice injected with AAV-TBG-Luc (*Abca1^{fl/fl}*) were injected intraperitoneally with 200 μ l of corn oil for three alternate days. On the other hand, mice injected with AAV-TBG-Cre were injected with 0.5 mg of tamoxifen in 200 μ l of corn oil for three alternate days to obtain *L, I-Abca1^{-/-}* mice. Seven days after the last injection, mice were fasted for 16 h and used to collect plasma and tissues. A–F: Intestinal and liver homogenates from *Abca1^{fl/fl}* and *L, I-Abca1^{-/-}* mice were used to measure ABCA1 (A, D) and MTP (B, E) mRNA levels. Tissue homogenates were also used to measure MTP activity levels in the intestine (C) and liver (F). P values are after two-tailed Student's t -test. *** $P < 0.001$. G: Western blot of liver homogenates (20 μ g of protein) for MTP and ABCA1. Vinculin was used as a control. H: To detect apoB and apoA1, plasma (0.5 μ l) was run on a 5–14% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was cut in half. One-half was probed with anti-apoB antibody, and one-half was probed with anti-apoA1 antibody. Bands were visualized in a phosphorimager.

HxCer levels, ABCA1 deficiency does not appear to affect intestinal sphingolipid contents.

These studies suggest that the combined intestinal and hepatic ABCA1 deficiency significantly reduces both plasma and tissue HxCer levels, but their deficiency only reduces plasma LactCer and SM with no effect on their tissue levels. These studies show that both plasma and tissue HxCer levels are more sensitive to ABCA1 deficiency than other lipids. Thus, ABCA1 perhaps plays a significant role in controlling plasma and tissue HxCer as well as plasma SM and LactCer levels in mice.

Plasma sphingolipids are significantly lower in liver-specific ABCA1-deficient (*L-Abca1^{-/-}*) mice, but not in intestine-specific ABCA1-deficient (*I-Abca1^{-/-}*) mice

The above results showed that the combined liver and intestine deficiency of ABCA1 decreases plasma SM and HxCer in mice. To assess the contribution of ABCA1 in individual tissues to plasma sphingolipid levels, we studied liver-specific (*L-Abca1^{-/-}*) or intestine-specific (*I-Abca1^{-/-}*) ABCA1 KO mice. Hepatic (Table 1) ablation of ABCA1 had no effect on plasma triglycerides, but reduced plasma cholesterol by $\sim 67\%$ and phospholipids by $\sim 37\%$ in

agreement with other studies (30, 31). Analyses of sphingolipids showed that plasma Cers, SM, and HxCer and sphingosine-1-P were significantly reduced. However, LactCer was not (Table 1). Analyses of individual species revealed that C22-Cer, C22:1-Cer, C24-Cer, C24:1-Cer, and C26-Cer were significantly reduced in the plasma of *L-Abca1^{-/-}* mice (supplemental Table S10). Significant reductions in plasma HxCer were due to lower levels of C16-HxCer, C20-HxCer, C22-HxCer, C22:1-HxCer, C24-HxCer, and C24:1-HxCer (supplemental Table S11). There were no significant differences in different species of LactCer in the plasma of *L-Abca1^{-/-}* and *L-Abca1^{fl/fl}* mice (supplemental Table S12). Furthermore, all SM species, except C20-SM, C26-SM, and C26:1-SM, were significantly lower in the plasma of *L-Abca1^{-/-}* mice (supplemental Table S13). These studies show that hepatic ABCA1 contributes to plasma SM, Cer, and HxCer levels.

Intestine-specific ablation of ABCA1 significantly reduced plasma cholesterol levels by $\sim 30\%$ and is consistent with our earlier studies (31). Intestinal ABCA1 deficiency had no effect on plasma triglyceride, phospholipid, Cer, SM, HxCer, LactCer, sphingosine, dihydrosphingosine, and sphingosine-1-P levels (Table 2). Furthermore,

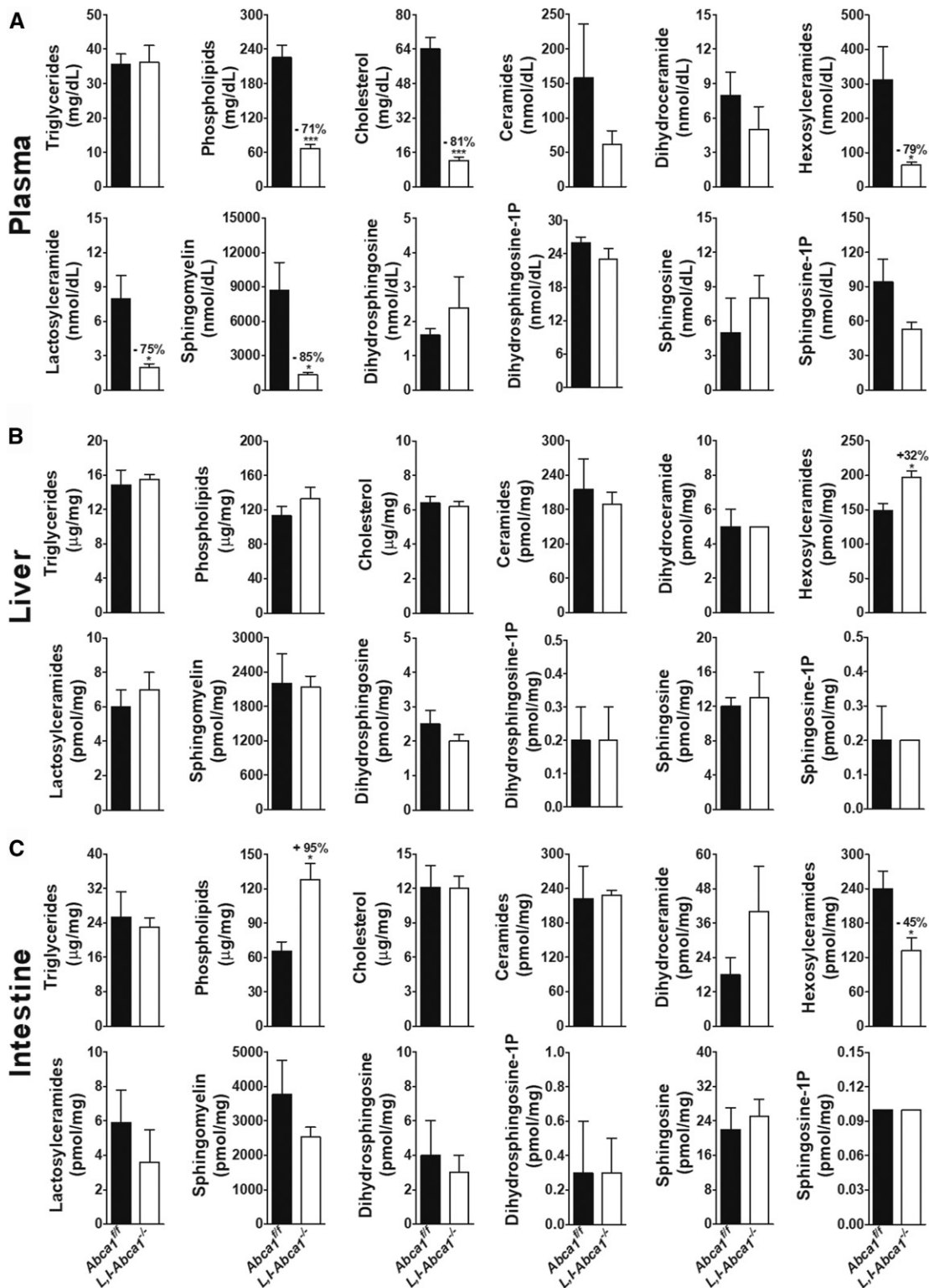


Fig. 3. Effect of hepatic and intestinal ABCA1 deficiency on plasma and tissue lipids in mice. In *L1-Abca1^{-/-}* mice, the *Abca1* gene was conditionally deleted in the liver and intestine as described in Materials and Methods and in Fig 2. On day 12 after the first injection, mice were fasted for 16 h, and lipids were measured in plasma (A), liver (B), and intestine (C). *P* values are after two-tailed Student's *t* test. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

intestine-specific ABCA1 deficiency did not significantly affect various intestinal sphingolipid species (supplemental Tables S10–S13). These studies indicate that intestinal ABCA1 is critical for plasma cholesterol levels and probably related to its recognized role in the production of

intestinal HDL, but not for plasma sphingolipid levels. Combined results from these individual KO mice models suggest that liver ABCA1 plays a more significant role in the regulation of plasma sphingolipid levels than intestinal ABCA1.

TABLE 1. Effect of liver-specific ABCA1 deficiency in mice on plasma lipids

Plasma components	<i>Abca1^{fl/fl}</i>	<i>L-Abca1^{-/-}</i>	<i>P</i>	% Change
Triglycerides (mg/dl)	36.6 ± 7.7	34.8 ± 6.1	0.772	-5
Phospholipids (mg/dl)	161.8 ± 13.7	102.2 ± 16.9	0.018	-37
Cholesterol (mg/dl)	80.1 ± 6.4	26.2 ± 4.7	0.001	-67
Cers (nmol/dl)	170 ± 34	63 ± 9	0.026	-63
Dihydroceramide (nmol/dl)	BDL	0.8 ± 0.2	N/A	N/A
SM (nmol/dl)	9,399 ± 1418	1,202 ± 90	0.010	-87
HxCer (nmol/dl)	627 ± 114	84 ± 13	0.014	-87
LactCers (nmol/dl)	9.1 ± 4.8	3.6 ± 1.5	0.177	-61
Dihydrosphingosine (nmol/dl)	0.6 ± 0.1	0.7 ± 0.1	0.221	-17
Sphingosine (nmol/dl)	0.6 ± 0.3	1.0 ± 0.3	0.271	67
Sphingosine-1-P (nmol/dl)	107 ± 17	49 ± 9	0.013	-54

BDL, below detectable levels; N/A, not available.

Combined deficiency of intestinal and hepatic MTP and ABCA1 reduces plasma glycerolipids, sterols, and sphingolipids

The data presented above and in our previous publication (14) showed that MTP and ABCA1 contribute to plasma Cer, SM, and HxCer levels. To study the combined contributions of these proteins on sphingolipid metabolism, we generated mice (*L, I-Mttp^{-/-}, Abca1^{-/-}*) deficient in intestinal and hepatic *Mttp* and *Abca1* genes. These mice had ~60–70% lower mRNA levels of ABCA1 and MTP in their intestines (Fig. 4A, B) and livers (Fig. 4D, E). Furthermore, MTP activity was reduced by 78–87% in the intestine (Fig. 4C) and liver (Fig. 4F) of *L, I-Mttp^{-/-}, Abca1^{-/-}* mice compared with controls. Ablation of MTP and ABCA1 in the liver and intestine significantly reduced the levels of plasma apoB and apoA1 (Fig. 4G). Combined deficiency of these two proteins in the liver and intestines of mice significantly reduced all plasma lipids except for dihydroceramide, LactCer, dihydrosphingosine, dihydrosphingosine-1P, and sphingosine (Fig. 5A). Analyses of different sphingolipid species showed that all the species of Cers (supplemental Table S14), HxCer (supplemental Table S15), and SM (supplemental Table S16) were reduced. There were no significant differences in different species of sphingosines (supplemental Table S14) and LactCers (supplemental Table S16). Thus, the expressions of these two proteins in the livers and intestines of mice are the major determinants of plasma glycerolipids, cholesterol, Cer, SM, and HxCer, but not for LactCer and sphingoid bases and their phosphates.

Livers of *L, I-Mttp^{-/-}, Abca1^{-/-}* mice had significantly lower triglyceride, phospholipids, cholesterol, and LactCer, and

higher levels of dihydroceramide and HxCer compared with controls (Fig. 5B). Hepatic Cer, SM, dihydrosphingosine, dihydrosphingosine-1P, sphingosine, and sphingosine-1P levels were not affected by their deficiency. Analyses of different species of Cers revealed that hepatic C16-Cer, C18-Cer, C18:1-Cer, and C24:1-Cer were significantly increased, whereas C22-Cer and C24-Cer levels were significantly decreased (supplemental Table S14). Except for C22-HxCer and C24-HxCer, all HxCer species were increased in the livers of *L, I-Mttp^{-/-}, Abca1^{-/-}* mice (supplemental Table S15). All species of LactCer were decreased in the plasma of *L, I-Mttp^{-/-}, Abca1^{-/-}* mice (supplemental Table S16). C20-SM, C20:1-SM, C22:1-SM, C24:1-SM, and C26-SM were significantly reduced in *L, I-Mttp^{-/-}, Abca1^{-/-}* mice (supplemental Table S17). Thus, deficiency of these two proteins significantly reduces hepatic content of various sphingolipid species. We had anticipated that their deficiency would increase various sphingolipids, indicating more complex roles of these proteins in sphingolipid metabolism.

Intestinal lipid analysis revealed significant increases in triglycerides, phospholipids, and Cers, but no difference in cholesterol, dihydroceramide, HxCer, SM, dihydrosphingosine, dihydrosphingosine-1P, sphingosine, and sphingosine-1P in *L, I-Mttp^{-/-}, Abca1^{-/-}* mice (Fig. 5C). Analyses of different species of Cers revealed that intestinal C20-Cer, C20:1-Cer, C22:1-Cer, and C26-Cer levels were significantly increased by 3- to 15-fold (supplemental Table S14). There were no significant differences in different species of HxCer, LactCer, and SM (supplemental Table S15-17). Thus, MTP and ABCA1 deficiency mainly increases intestinal Cers without affecting other sphingolipids. It is likely that

TABLE 2. Effect of intestine-specific ABCA1 deficiency in mice on plasma lipids

Plasma components	<i>Abca1^{fl/fl}</i>	<i>I-Abca1^{-/-}</i>	<i>P</i>	% Change
Triglycerides (mg/dl)	43.0 ± 5.5	43.9 ± 5.2	0.850	2
Phospholipids (mg/dl)	152.9 ± 13.5	133.5 ± 6.7	0.155	-13
Cholesterol (mg/dl)	72.4 ± 5.5	52.7 ± 8.3	0.041	-27
Cers (nmol/dl)	136 ± 20	107 ± 2	0.122	-22
Dihydroceramide (nmol/dl)	1.4 ± 0.5	3.7 ± 0.4	0.023	164
SM (nmol/dl)	11,405 ± 2303	9,569 ± 805	0.300	-16
HxCer (nmol/dl)	746 ± 53	648 ± 125	0.310	-13
LactCers (nmol/dl)	13.5 ± 5.7	11.2 ± 6	0.672	-18
Dihydrosphingosine (nmol/dl)	5.0 ± 5.3	1.8 ± 0.5	0.404	-64
Sphingosine (nmol/dl)	17.9 ± 12.8	7.9 ± 5.2	0.310	-56
Sphingosine-1-P (nmol/dl)	154 ± 14	155 ± 16	0.990	0.01

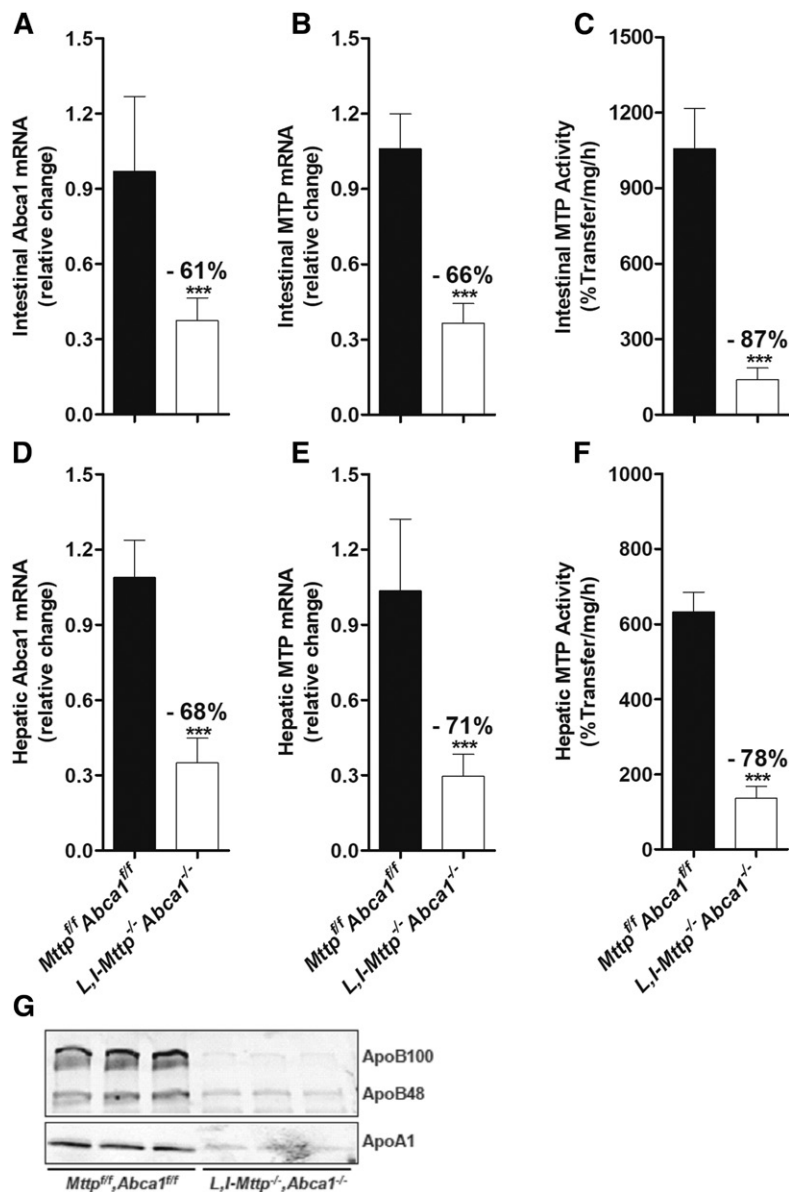


Fig. 4. MTP, ABCA1, apoB, and apoA1 levels in the liver, intestine, and plasma of MTP- and ABCA1-deficient mice. Chow-fed, male, 10-week-old *Mtp^{fl/fl} Abca1^{fl/fl}* mice (n = 3) were injected once intravenously with 109 pfu of either AAV-TBG-Luc (Control) or AAV-TBG-Cre viruses in 100 μ l of sterile PBS. Next, mice injected with AAV-TBG-Luc were injected intraperitoneally with 200 μ l of corn oil for three alternate days. On the other hand, mice injected with AAV-TBG-Cre were injected with 0.5 mg of tamoxifen in 200 μ l of corn oil for three alternate days to obtain *L,I-Mtp^{-/-} Abca1^{-/-}* mice. Seven days after the last injection, mice were fasted for 16 h and used to collect plasma and tissues. A–F: Intestinal and liver homogenates from *Mtp^{fl/fl} Abca1^{fl/fl}* and *L,I-Mtp^{-/-} Abca1^{-/-}* mice were used to measure ABCA1 (A, D) and MTP (B, E) mRNA levels. Tissue homogenates were also used to measure MTP activity levels in the intestine (C) and liver (F) of MTP- and ABCA1-deficient and control mice. P values are after two-tailed Student's *t*-test. *** *P* < 0.001. G: Liver homogenates (25 μ g of protein) were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, probed with either mouse anti-mouse MTP followed by Alexa Fluor® 633-labeled goat anti-mouse IgG. For control, an identical blot was probed with anti-GAPDH (Santa Cruz; # sc-48167; 1:2,000 dilution). G: To detect apoB and apoA1, plasma (0.5 μ l) was run on an SDS-PAGE gel and probed for apoB and apoA1. Bands were visualized in a phosphorimager.

intestinal MTP and ABCA1 play a major role in mobilizing intestinal Cer.

These studies indicate that the source of the majority of sphingolipids in the plasma is the liver and intestine, and their levels are regulated by the expression of both ABCA1 and MTP in these tissues. Furthermore, combined deficiency of intestinal and hepatic MTP and ABCA1 has differential effects on glycerolipid and sphingolipid metabolism.

Plasma sphingolipids are significantly low in liver-specific MTP-deficient (*L-Mtp^{-/-}*) mice, but they are unaffected by intestine-specific MTP deficiency (*I-Mtp^{-/-}*)

We have previously shown that combined deficiency of MTP in the liver and intestine significantly reduced plasma concentrations of Cers and SM, but not HxCer and LactCer (14). However, these studies did not address specific roles of hepatic and intestinal MTP on plasma Cer and SM. Hence, to determine the tissue-specific contribution of MTP to plasma sphingolipid levels, we

created either liver-specific (*L-Mtp^{-/-}*) or intestine-specific (*I-Mtp^{-/-}*) MTP KO mice. Hepatic ablation of MTP decreased plasma triglycerides, phospholipids, and cholesterol by 58%, 75%, and 73%, respectively, as compared with control mice (Table 3). Analyses of sphingolipids showed that plasma Cers and SM were significantly reduced by 71% and 58%, respectively, in the liver-specific MTP KO mice. Reductions in the levels of plasma dihydroceramide, dihydrosphingosine, sphingosine and sphingosine-1-P in *L-Mtp^{-/-}* mice did not reach statistical significance (Table 3). Analyses of individual species revealed that C20-Cer, C22-Cer, C24-Cer, C24:1-Cer, C26-Cer, C24-SM, and C24:1-SM were significantly reduced in the plasma of *L-Mtp^{-/-}* mice (supplemental Tables S18 and S19). We did not measure HxCer and LactCer in these mice, as we have previously shown that MTP deficiency has no effect on their plasma levels (14). These studies showed that hepatic MTP contributes significantly to plasma Cers and SM levels.

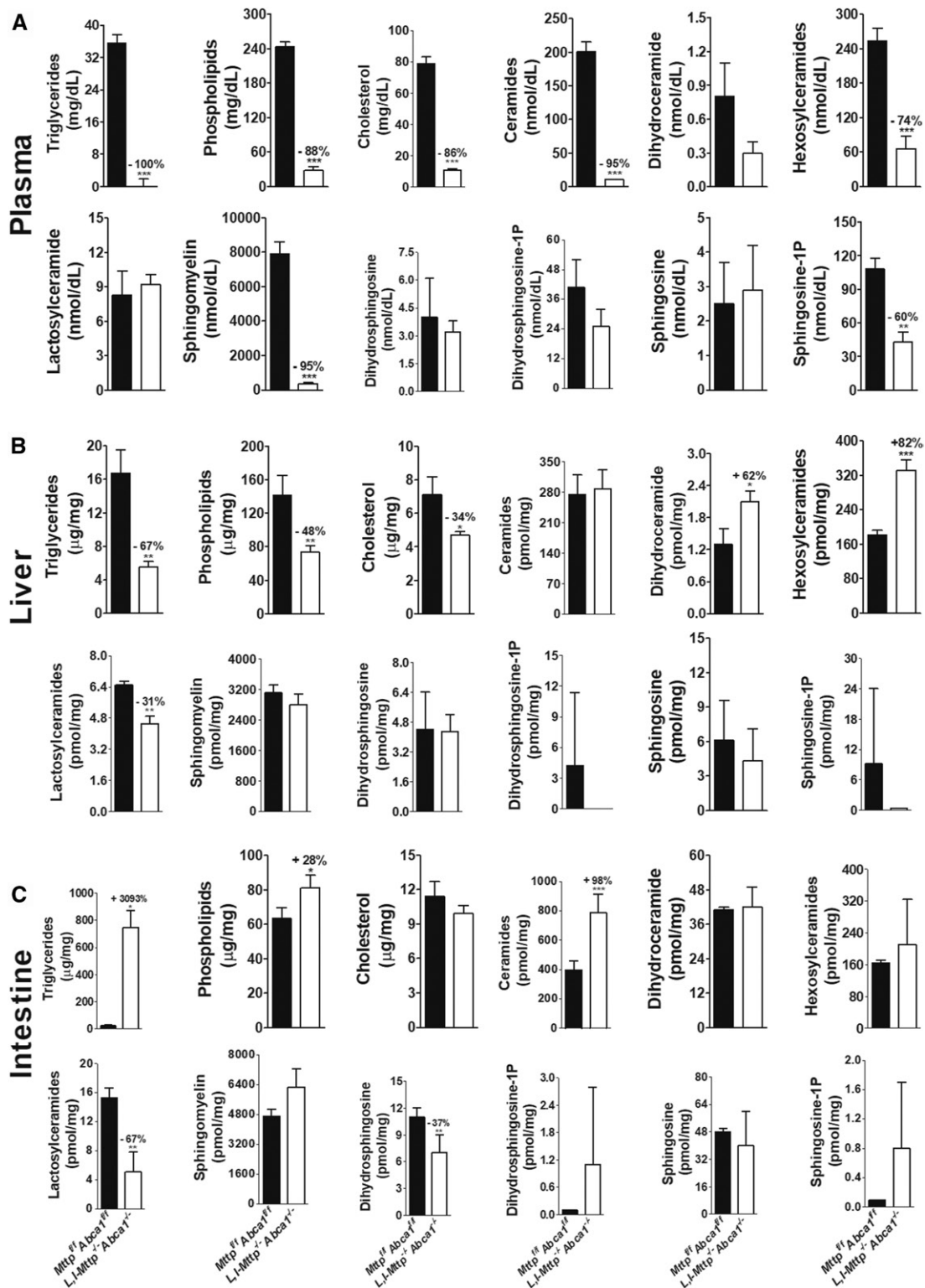


Fig. 5. Effect of combined deficiency of intestinal and hepatic MTP and ABCA1 on plasma, liver, and intestinal lipids in mice: In *L, I-Mttp^{-/-} Abca1^{-/-}* mice, the *Mtp* and *Abca1* genes were conditionally deleted in the liver and intestine, as described in Materials and Methods. On day 12 after the first injection, mice were fasted for 16 h, and lipids were measured in plasma (A), liver (B), and intestine (C). *P* values are after two-tailed Student's *t*-test. * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

Intestine-specific ablation of MTP significantly reduced plasma triglycerides, phospholipids, cholesterol, and Cer levels, but had no effect on dihydroceramide, SM, dihydrospingosine, sphingosine, and sphingosine-1-P levels (Table 4). The decrease in plasma Cers was mainly due

to reductions in C20-Cer, C20:1-Cer, C22-Cer, C22:1-Cer, C24-Cer, and C26-Cer levels (supplemental Table S18). Analyses of different species of SM uncovered that C24-SM and C24:1-SM levels were significantly decreased, whereas C16-SM and C18-SM levels were significantly increased in

TABLE 3. Effect of liver-specific MTP deficiency in mice on plasma lipids

Plasma components	<i>Mttp</i> ^{f/f}	<i>L-Mttp</i> ^{-/-}	<i>P</i>	% Change
Triglycerides (mg/dl)	65.8 ± 8.4	27.7 ± 3.8	0.002	-58
Phospholipids (mg/dl)	311.2 ± 20.1	76.4 ± 7.4	0.000	-75
Cholesterol (mg/dl)	86.8 ± 6.8	23.7 ± 3.4	0.000	-73
Cers (nmol/dl)	155 ± 20	44 ± 19	0.002	-71
Dihydroceramide (nmol/dl)	2.3 ± 0.9	1.0 ± 0.2	0.062	-57
SM (nmol/dl)	4,662 ± 248	1,957 ± 126	0.000	-58
Dihydrosphingosine (nmol/dl)	2.7 ± 0.5	1.5 ± 1.4	0.209	-46
Sphingosine (nmol/dl)	2.4 ± 0.7	2.1 ± 0.5	0.652	-12
Sphingosine-1-P (nmol/dl)	128 ± 18	93 ± 16	0.065	-27

the plasma of *I-Mttp*^{-/-} mice (supplemental Table S19). Overall, total SM levels in the plasma were unchanged between the WT and *I-Mttp*^{-/-} mice. Thus, intestinal MTP does play an important role in determining plasma levels of various species of Cers, but it may play a differential role in the metabolism of medium- and long-chain SM species.

Besides the known importance of MTP in the mobilization of triglycerides, phospholipids, and cholesterol, the current studies suggest that intestinal and hepatic MTP is critical for plasma Cer levels. The combined data from our previous study (14) and this study suggest that effects of intestine and liver MTP on plasma Cer levels are additive and contribute to almost 95% of plasma Cer. On the other hand, only liver MTP determines total plasma SM levels, whereas intestinal MTP deficiency has differential effects on medium- and long-chain plasma SM species.

ABCA1 is not involved in sphingolipid synthesis

To understand why plasma sphingolipid levels were low in the plasma of ABCA1-deficient mice, we studied the effect of ABCA1 deficiency on the synthesis of dihydroceramides from dihydrosphingosine as well as synthesis of SM from Cers (Fig. 6). Liver homogenates from ABCA1-deficient mice had no effect on the synthesis of dihydroceramide or SM (Fig. 6A, B). Furthermore, deficiency of both MTP and ABCA1 in the liver did not affect dihydroceramide or SM synthesis (Fig. 6A–C). These data suggest that neither ABCA1 nor MTP deficiency affects early steps common to sphingolipid synthesis.

ABCA1 is not directly involved in sphingolipid efflux

Next, we studied whether ABCA1 is involved in the efflux of these sphingolipids. Huh-7 cells were labeled with C6-NBD Cers and then incubated with different putative acceptors to induce efflux (Fig. 7A). BSA, LDL, and apoAI showed similar basal efflux. In contrast, HDL increased

efflux of Cer, HxCer, and SM by 150%, 80%, and 40%, respectively (Fig. 7A; supplemental Table S20). Furthermore, incubation of cells with an LXR agonist, T0901317 that is known to increase ABCA1 expression, did not increase the efflux of Cer, HxCer, and SM to HDL or apoAI (Fig. 7B; supplemental Table S20). Next, we studied efflux of these sphingolipids in siABCA1-treated cells. Again, ~75% decreased ABCA1 mRNA had no effect on the efflux of Cer, HxCer, and SM to HDL and apoAI (Fig. 7C; supplemental Table S20). Further, hepatocytes isolated from *Abca1*^{f/f} and *L,I-Abca1*^{-/-} mice did not show any difference in the efflux of these sphingolipids to HDL (Fig. 7D). When the C6-NBD Cer-labeled Huh-7 cells were incubated with BSA or HDL in the presence of glyburide, an inhibitor of ABCA1 activity, there was no significant difference between the treated and untreated cells (Fig. 7E). Similarly, MTP inhibition had no effect on the efflux of these sphingolipids to HDL (Fig. 7F). Thus, HDL, but not free apoAI, is able to efflux Cer, HxCer, and SM from cells more than basal efflux with BSA. Increasing, decreasing and ablation of ABCA1 expression do not affect this efflux. We interpret these data to suggest that ABCA1 is not directly involved in the efflux of Cer, HxCer, and SM to HDL but plays an important role in determining their plasma concentrations via unknown novel mechanism(s). An unknown protein may be involved in their efflux to HDL. An alternate explanation is that the cell model used that is largely based on cholesterol efflux studies is not suitable to study sphingolipid efflux.

DISCUSSION

It is becoming clear that plasma sphingolipids could be markers for several metabolic disorders such as insulin resistance, progression of NAFLD to NASH, atherosclerosis,

TABLE 4. Effect of intestine-specific MTP deficiency in mice on plasma lipids

Plasma components	<i>Mttp</i> ^{f/f}	<i>I-Mttp</i> ^{-/-}	<i>P</i>	% Change
Triglycerides (mg/dl)	74.6 ± 7.5	33.8 ± 5.5	0.001	-55
Phospholipids (mg/dl)	325.8 ± 35.0	122.3 ± 16.6	0.000	-62
Cholesterol (mg/dl)	88.9 ± 8.5	33.8 ± 4.2	0.000	-62
Cers (nmol/dl)	143 ± 31	57 ± 12	0.011	-60
Dihydroceramide (nmol/dl)	0.40 ± 0.1	0.36 ± 0.1	0.703	-10
SM (nmol/dl)	4,768 ± 113	4,882 ± 241	0.490	2
Dihydrosphingosine (nmol/dl)	2.5 ± 0.6	2.4 ± 0.4	0.805	-4
Sphingosine (nmol/dl)	2.7 ± 0.9	3.2 ± 0.6	0.383	24
Sphingosine-1-P (nmol/dl)	136 ± 20	101 ± 30	0.172	-26

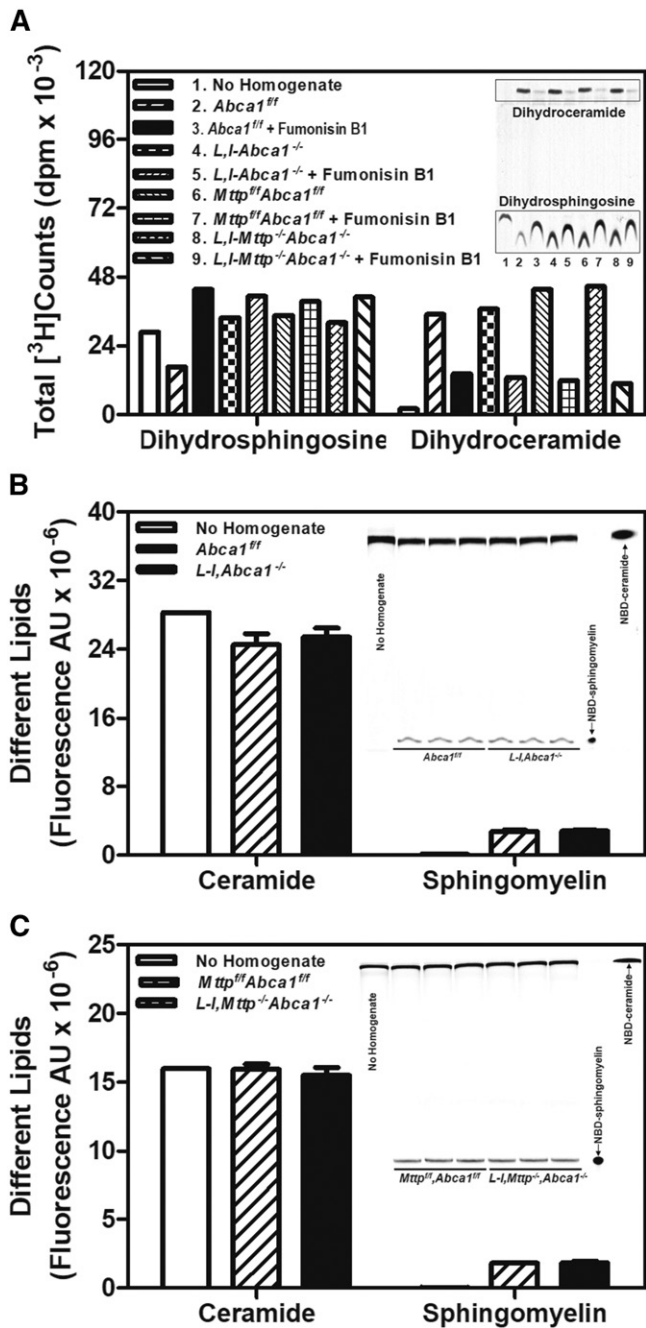


Fig. 6. ABCA1 deficiency does not alter dihydroceramide or SM synthesis. **A:** Liver homogenates (250 μ g of protein) from WT and KO mice were incubated with [³H]dihydrosphingosine (0.05 μ Ci), dihydrosphingosine (15 μ M), FA-free BSA (20 μ M), and lignoceroyl-CoA (50 μ M) in the presence or absence of Fumonisin B1 (50 μ M) in a total volume of 200 μ l reaction for 30 min at 37°C. The reaction was stopped by adding 200 μ l of CHCl₃:CH₃OH (2:1, v/v). Lipids were extracted and subjected to TLC. Radioactivities in the dihydro-sphingosine and dihydroceramide bands were quantified using a scintillation counter. No homogenate data show that the precursor dihydrosphingosine used for synthesis has very little dihydroceramide. Radioactivity in dihydroceramide bands in different conditions refers to unused precursor. The radioactive counts in dihydroceramide represent new synthesis. **B, C:** In vitro SM synthesis was performed in liver homogenates obtained either from *Abca1*^{fl/fl} and *L-I-Abca1*^{-/-} mice (**B**) or *Mttp*^{fl/fl} *Abca1*^{fl/fl} and *L-I-Mttp*^{-/-} *Abca1*^{-/-} mice (**C**). Liver homogenates (250 μ g of protein) from these mice were incubated in triplicate with C6-NBD

and coronary artery disease (6–13). However, very little is known about the mechanisms that control plasma sphingolipids. In this study, we report that mechanisms involved in the arrival of sphingolipids from the liver and intestine is more complex than those involved in glycerolipid and sterol transport. Our previous (14) and current studies show that arrival of Cer to the plasma is critically dependent on MTP, similar to those of triglycerides. Here, we demonstrate for the first time that hepatic ABCA1 is a critical determinant of plasma HxCer. Tissue-specific ABCA1 and MTP ablation studies showed that the liver, and not the intestine, is the major contributor to plasma SM. Our studies further show that ABCA1- and MTP-dependent pathways are not universal for all sphingolipids. In fact, LactCer, dihydroceramide, dihydrosphingosine, dihydro-sphingosine-1P, sphingosine, and sphingosine-1P arrive in the plasma independent of both MTP and ABCA1. However, in the absence of MTP and ABCA1, plasma concentrations of these sphingolipids are low, suggesting that plasma lipoproteins synthesized with the help of these proteins are important. Thus, these studies provide evidence for the existence of multiple pathways for the transport of sphingolipids from tissues to the plasma compartment.

HxCer

A consistent finding of this study is that ABCA1 deficiency is associated with reduced plasma HxCer in humans and mice. In Tangier subjects, HxCer levels were reduced by ~70%, possibly suggesting the existence of additional mechanisms in the transport of HxCer from tissues to the plasma compartment in humans. In *L-I-Abca1*^{-/-} mice HxCer were reduced by ~80%. Tissue-specific ABCA1 ablation studies indicate that hepatic ABCA1 is responsible for the majority of the plasma HxCer in mice. Thus, hepatic ABCA1 plays a crucial and predominant role in determining plasma HxCer levels in mice.

Our studies show that HDL can, but LDL and apoA1 cannot, efflux HxCer from cells when BSA was used as a control to study efflux. However, increasing, decreasing and ablation of ABCA1 had no effect on HxCer efflux from cells to HDL. Further, an LXR agonist had no effect on the efflux. ApoA1, which is directly involved in ABCA1-mediated cholesterol efflux, did not act as an acceptor for HxCer. There could be several explanations for these negative results. First, ABCA1 may not be directly involved in HxCer efflux. Second, reductions in plasma HxCer levels in ABCA1-deficient humans and mice are secondary to low levels of HDL. Third, HDL may interact with a different receptor on the cell surface to pick up HxCer. Because HDL levels are low in ABCA1-deficient humans and mice, there is less HDL available to pick up HxCer from plasma membranes of cells. Fourth, cell culture studies designed based on cholesterol efflux studies may not be suitable to recapitulate

Cer (3.3 μ g/ml) and phosphatidylcholine (100 μ g/ml) for 30 min at 37°C. Lipids were extracted, separated on TLC, and visualized with the phosphorimager. Arbitrary units (AU) in C6-NBD-Cer represent amounts left at the end of the reaction.

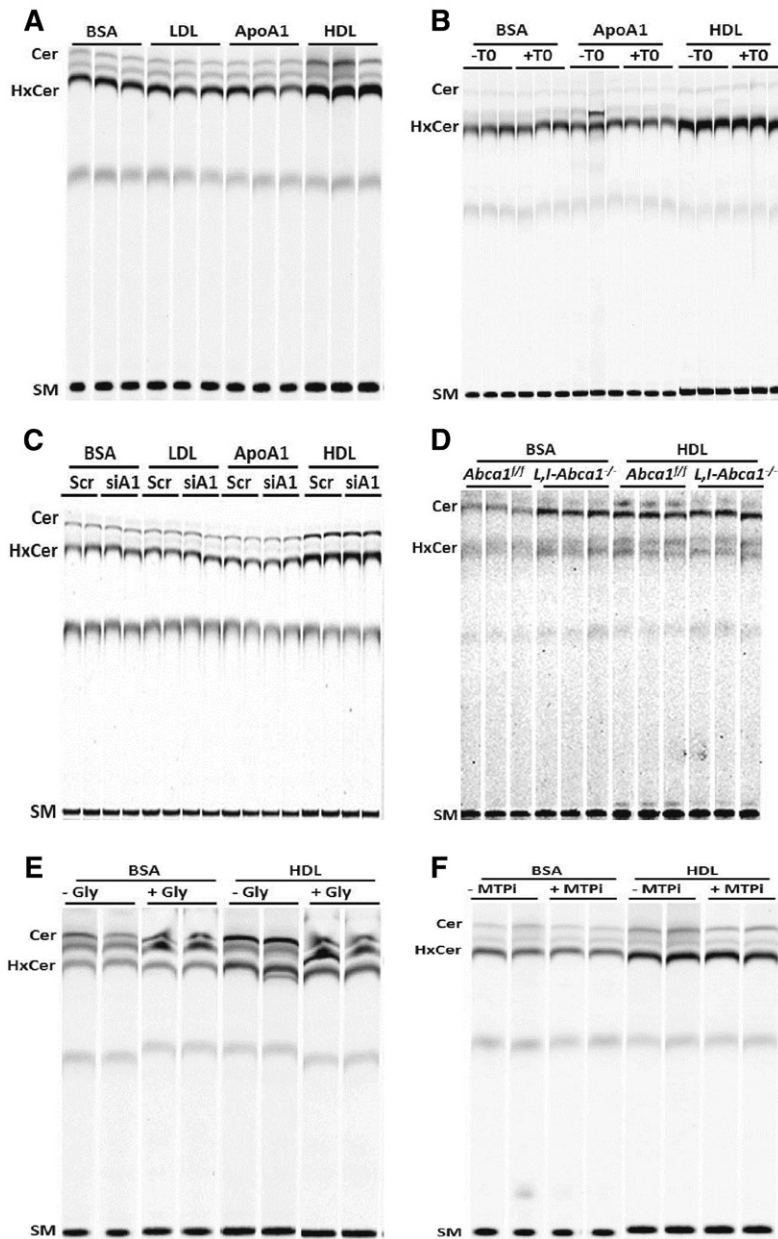


Fig. 7. ABCA1 is not directly involved in the efflux of HxCer and SM. A: Efflux of sphingolipids was performed in Huh-7 cells incubated with 2 μM C6-NBD Cer in 10% FBS for 3 h. Cells were washed three times with 0.1% BSA-containing DMEM. After washing, cells were incubated with either 40 μg/ml BSA, LDL, ApoA1, or HDL for 6 h. Lipids were separated on TLC, and fluorescence in Cer and SM bands was quantified. B: Huh-7 cells were preincubated for 17 h with or without 2 μM LXR agonist, T0901317. Cells were then labeled with 2 μM C6-NBD Cer to study efflux as described above. C: Huh-7 cells were transfected with 50 nM siControl (Scr) or siABCA1 (siA1) for 72 h and labeled with 2 μM C6-NBD Cer to study efflux as before. D: Hepatocytes isolated from *Abca1^{fl/fl}* and *L, I-Abca1^{-/-}* mice were used to study efflux. E, F: Efflux in Huh-7 cells was performed in the presence or absence of either 250 μM ABCA1 inhibitor, glyburide (E) or 500 nM MTP inhibitor, Lomitapide (F). Lipids in the media were extracted, separated on TLC, and visualized with the phosphorimager.

sphingolipid efflux. More mechanistic studies are needed to explain how ABCA1 modulates plasma HxCer levels.

SM

Absence of ABCA1 was associated with ~40% reductions in plasma SM levels in Tangier subjects. Previously, we showed that MTP deficiency in abetalipoproteinemia reduces plasma SM levels by ~41% (14). Thus, both MTP and ABCA1 independently and significantly modulate plasma SM levels in humans. In mice, ABCA1 ablation decreased plasma SM by ~85%. The liver appears to be the major source of plasma SM, as their levels were decreased by ~90% in *L-ABCA1^{-/-}* mice. Similarly, hepatic ablation of MTP also reduced plasma SM levels by ~58%. These studies suggest that hepatic MTP and ABCA1 are the major determinants of plasma SM levels in both humans and mice.

Next, we tried to understand how MTP and ABCA1 modulate plasma SM levels. Our studies show that neither MTP

nor ABCA1 is involved in Cer or SM synthesis. We hypothesized that ABCA1 might be involved in SM efflux, similar to its role in cholesterol efflux. Overexpression, downregulation, and ablation of ABCA1 did not reduce SM efflux to HDL. The LXR agonist did not affect SM efflux to HDL. Thus, ABCA1 may not directly participate in SM efflux, and this efflux may not be regulated by LXR. We postulate that ABCA1 is indirectly involved in bringing SM to plasma compartment. Absence of ABCA1 reduces HDL cholesterol levels, thereby indirectly diminishing SM efflux and plasma SM levels. ABCA1 may act to bring intracellular SM to the plasma membrane and/or to retain SM in the plasma membrane. Another unknown membrane-embedded protein may efflux SM to HDL.

Cer

Tissue-specific ablation studies revealed that both intestinal and hepatic MTP modulate plasma Cer levels. The

present study indicates that ABCA1 deficiency tends to associate with lower plasma Cer levels that did not reach statistical significance. It is likely that the contributions of ABCA1 will become statistically significant when the number of cases (n values) is increased. Thus, MTP is the major determinant of plasma Cer levels, and ABCA1 is also involved to some extent.

LactCer

Our studies show that there are significant differences in plasma concentrations of LactCer in humans and mice. Humans contain significantly higher concentrations of plasma LactCer compared with mice. Second, ABCA1 deficiency in humans has no effect on plasma LactCer levels, but hepatic and intestinal ABCA1 deficiency in mice reduces LactCer ~75%, and liver-specific ABCA1 deficiency reduces plasma LactCer by 55%. These data indicate that LactCer levels may be regulated by different mechanisms in humans and mice.

In mice, hepatic and intestinal ABCA1 deficiency, as well as hepatic-specific ABCA1 ablation, decreases plasma LactCer (Fig. 3 and Table 1). However, intestine-specific ablation as well as combined intestinal and hepatic ablation of ABCA1 and MTP has no effect on plasma LactCer. These conflicting observations might be due to low plasma concentrations of LactCer in mice. Previous (14) and current studies, however, consistently show that MTP deficiency in mice and humans has no effect on plasma LactCer levels. Based on these observations, we surmise that ABCA1 and MTP probably do not play a significant role in regulating plasma LactCer levels. Thus, other proteins, other than MTP and ABCA1, and mechanisms independent of B-lps and HDL could be more important for the transport of LactCer to the plasma compartment. Additionally, it is likely that LactCer transport mechanisms are different in humans and mice.


Sphingoid bases and their phosphates

In our study, Tangier plasma had higher levels of dihydrosphingosine-1P than controls; however, it was not significantly different between control and *LjAbca1*^{-/-} mice. In ABCA1-deficient mice, there was a trend toward reduced sphingosine-1P levels compared with controls. However, we did not see a trend toward reductions in plasma sphingosine-1P levels in our Tangier subjects. A published study showed significantly reduced sphingosine-1P levels in the plasma of older Tangier subjects with very low levels of HDL and apoA1 (99% and 97% reduction, respectively) (43). It is known that sphingosine-1P transport to plasma involves apoM and spinster 2 and resides mainly on HDL₃ (6, 44–46). Longitudinal, prospective studies in larger cohorts are needed to determine plasma apoM, sphingoid bases, and their phosphate levels in Tangier subjects.

It can be assumed that the hydrophobic nature of sphingolipids requires amphiphilic vehicles, such as lipoproteins, for their transport. Surprisingly, we show that the dependence of sphingolipids on lipoproteins as transporters is different for different sphingolipids. Therefore, their arrival to plasma is not dependent on the availability of a

hydrophobic carrier for secretion. Instead, our studies indicate the presence of specific mechanisms and carriers for different sphingolipids. There appear to be at least three different mechanisms for the transport of sphingolipids. Previously, we showed that intracellular transfer of Cer and SM to B-lps by MTP may contribute to their plasma levels (14). Here, we show that HDL accepts Cer, HxCer, and SM from cells. In contrast to these sphingolipids, LactCer and sphingosines arrive to plasma-independent of B-lps and HDL. Thus, these sphingolipid transport mechanisms are more complex and diverse than the mechanisms known for glycerolipids and sterol transport that mainly involve MTP and ABCA1-mediated B-lps and HDL. The reasons for these different mechanisms for sphingolipids might be related to their diverse functions.

A drawback of our studies is the use of heterozygous abetalipoproteinemia as a control. We used heterozygous ABL as control because it is known that MTP heterozygotes do not differ from controls (32, 33). Another shortcoming of this study is the low number of control and Tangier subjects used in the study. This is mainly due to the rare occurrence of this disease. To alleviate this deficiency, we performed supporting experiments in mice deficient in *Mtp* and *Abca1* genes. Because of similar phenotypes observed in patients and mouse models, our approaches provide extensive evidence that MTP and ABCA1 and lipoproteins assembled with their help are critical determinants of different sphingolipids. Furthermore, different sphingolipids use different cargo and proteins for their transport, and there are specific mechanisms and proteins that carry out these processes.

In summary, these studies have identified two specific proteins and the liver as a major organ involved in determining plasma Cer, SM, and HxCer levels. These studies identified ABCA1 as a major protein involved in the efflux of hepatic SM and HxCer to plasma. It is known that ABCG1 also plays a role in the efflux of cholesterol to HDL. More studies are needed to examine the role of ABCG1 in the transport of sphingolipids to HDL. These studies point to the possible existence of novel pathways and proteins controlling plasma LactCer and sphingosines and highlight the complexities involved in the transport of various sphingolipids from hepatic and possible other cells to the plasma compartment. 

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