Diminished gene expression of ileal apical sodium bile acid transporter explains impaired absorption of bile acid in patients with hypertriglyceridemia

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Abstract **Patients with type IV hyperlipoproteinemia, particularly those with familial hypertriglyceridemia (FHT), have impaired absorption of bile acid, a defect that may contribute to the hypertriglyceridemia (*J. Lipid Res.* 1995. 36: 96–107). To determine whether this absorption defect is a result of abnormal expression of the ileal apical sodium bile acid transporter (ASBT) gene, we biopsied the terminal ileum at colonoscopy in 28 subjects, 13 with hypertriglyceridemia and 15 control subjects. Of the 13 hypertriglyceridemic subjects, 10 had lipid profiles compatible with FHT (elevated very low density lipoprotein [VLDL] triglycerides with normal LDL cholesterol). ASBT mRNA levels were measured in these biopsies by RNase protection assay, using glyceraldehyde dehydrogenase mRNA as a reference. ASBT protein was quantitated by Western blotting with an antibody to the carboxy-terminal 20 amino acids of the protein. The mean ± SEM ASBT mRNA level in the control group was 205.7 ± 19.9 (arbitrary units) compared with 138.7 ± 19.1 for all 13 hypertriglyceridemics ($P = 0.03$) and 141.7 ± 20.8 in the 10 FHT patients ($P = 0.05$). Commensurate with these mRNA levels, the mean ASBT protein level in the control group was 126.2 ± 22.6 versus 58.8 ± 13.8 in hypertriglyceridemics ($P = 0.02$) and 61.8 ± 15.2 in the FHT patients ($P = 0.05$). We conclude that impaired absorption of bile acid in type IV hypertriglyceridemia results from diminished expression of the ASBT gene in terminal ileum.—** Duane, W. C., L. A. Hartich, A. E. Bartman, and S. B. Ho. **Diminished gene expression of ileal apical sodium bile acid transporter explains impaired absorption of bile acid in patients with hypertriglyceridemia. *J. Lipid Res.* 2000.** 41: **1384–1389.**

Supplementary key words bile acids and salts • cholesterol • triglycerides • atherosclerosis • cholelithiasis

Type IV hypertriglyceridemia is associated with abnormal absorption of bile acid. This possibility was first suggested by Angelin et al. (1), after they observed that the postprandial rise in serum bile acids was strikingly blunted in type IV patients, specifically those with familial hypertriglyceridemia (FHT). Subsequently we measured absorption of bile acid in type IV patients, mainly those with the FHT phenotype, by measuring the mass of bile acid secreted into bile and excreted in the feces. Those studies demonstrated that the fraction of bile acid not absorbed was nearly twice as high in type IV patients as in control subjects (2).

Although the cause-and-effect relationship of this association remains to be established, the most interesting and plausible hypothesis is that reduced absorption of bile acid actually contributes to the hypertriglyceridemia. That conjecture is supported by the increase in serum triglycerides observed to follow induction of bile acid malabsorption by either ileal bypass (3) or administration of bile acid-binding resins (4–7). It is also consistent with the observation that administration of chenodeoxycholic acid to human subjects lowers serum triglyceride levels (8–12), apparently by reducing hepatic production of very low density lipoprotein (VLDL) triglycerides (4, 9).

Despite the potential contribution of impaired bile acid absorption to the pathogenesis of hypertriglyceridemia, the mechanism for this absorption defect is unknown. Although some bile acid is absorbed over the length of the small intestine, most bile acid absorption occurs by active transport in the terminal ileum. The transporter for the initial active uptake of bile acid is termed the apical sodium-dependent bile acid transporter (ASBT). It is a 348-amino acid protein located on the brush border of the ileocyte (13). The gene for the human ASBT (SLC10A2) is located on chromosome 13q33 (14) and has been fully sequenced in the laboratory of P. Dawson (15, 16).

In the present article we set out to determine whether impaired absorption of bile acid in type IV hypertriglyceridemia, particularly FHT, is a result of diminished expression of the ASBT in human ileum. To test this hypothesis

Abbreviations: ASBT, apical sodium bile acid transporter; BMI, body mass index; FCHL, familial combined hyperlipidemia; FHT, familial hypertriglyceridemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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TABLE 1. Characteristics of study subjects

	Control	Hypertriglyceridemia	FHT Profile
Number	15	13	10
Age (years)	66 ± 4	67 ± 4	66 ± 5
BMI (kg/m^2)	30 ± 1	33 ± 2	31 ± 3
Triglyceride (mg/dL)	125 ± 9	351 ± 62	382 ± 79
Cholesterol (mg/dL)	210 ± 13	232 ± 7	226 ± 8
LDL cholesterol (mg/dL)	142 ± 12	124 ± 12	111 ± 13

Values given as means ± SEM; BMI, body mass index.

we measured levels of both ASBT mRNA and protein in biopsies of human ileum obtained at colonoscopy.

MATERIALS AND METHODS

Subjects and biopsy procedure

The study protocol was approved by the Institutional Review Board of the Veterans Affairs Medical Center (Minneapolis, MN). Informed consent was obtained from all subjects before enrollment.

We studied 28 subjects (**Table 1**) who were undergoing colonoscopy for reasons related to their own health, primarily follow-up of colonic adenomas or evaluation of hemoccult positive stools. Subjects were included provided that *a*) neither the indication for the colonoscopy nor the findings at colonoscopy would be expected to affect ASBT expression, *b*) there was no indication in the patient's history or medical record of major medical problems or colonic surgery and no clinical indication of impaired nutritional status, *c*) the subject was taking no medications expected to affect lipid or bile acid metabolism, including fiber supplementation (restricting recruitment to patients taking no medications would have made it impossible to find adequate numbers of subjects), and *d*) the subject's terminal ileum could be intubated and appeared normal at colonoscopy. Fifteen of the selected subjects had normal triglycerides (<200 mg/dL). Thirteen of the subjects had hypertriglyceridemia. Ten of these had lipid profiles compatible with FHT (elevated VLDL triglycerides with a low density lipoprotein [LDL] cholesterol level below 160 mg/dL). The other three hypertriglyceridemic subjects had LDL cholesterol levels above 160 mg/dL. Because we did not measure serum lipid levels of family members of study subjects, we did not unequivocally establish a diagnosis of FHT in the 10 subjects with lipid profiles typical of FHT.

Using standard pinch forceps, three to five biopsies were obtained from the ileum within 10 cm of the ileocecal valve, placed immediately in liquid nitrogen, and stored at −70°C until use.

RNase protection assay

A cDNA template for the human ASBT was generated by methods similar to those of Wong et al. (13). Briefly, RNA was isolated from a sample of human ileum with Trizol (GIBCO-BRL, Grand Island, NY) according to the manufacturer instructions, except that the procedure was done at 4°C instead of room temperature. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on this RNA with sense oligonucleotide primer 5′-CAGTTTGGAATCATGCCCCTC-3′ and antisense primer 5′-TGTTCTGCATCCCCGTTTCAA-3′, which correspond, respectively, to amino acids 75–81 and 261–266 of the ASBT gene. After heating to 94°C for 5 min, the reaction was run for 30 cycles at a denaturing temperature of 94°C for 1 min, an annealing temperature of 55°C for 1 min, and an extending temperature of 72°C for 2 min. A product of the expected size, 576 base pairs, was separated by electrophoresis, ligated into a pGEM-T-Easy vector (Promega, Madison, WI), and transformed into JM109 competent *Escherichia coli* (Promega). The plasmid was subsequently sequenced with the original primers and the Promega fmol DNA sequencing system to confirm that the cDNA clone was identical to the targeted segment of the human ASBT, the complete sequence of which has been published (16).

After linearizing our cDNA template with a restriction enzyme appropriate to its orientation to produce an antisense RNA using the SP6 RNA polymerase promoter contained on the vector, the preparation was cleaned with proteinase K and an aliquot checked by gel electrophoresis. The linearized probe was used to generate a ^{32}P-labeled RNA probe with the SP6 riboprobe kit (Promega). The product was separated on a 6% denaturing polyacrylamide gel and eluted with 0.5 M ammonium acetate, 1 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS) overnight at 37°C. An antisense RNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was made in similar fashion, using a cDNA template purchased from Ambion (Austin, TX) (17).

ASBT was quantitated by RNase protection, using a method previously described (17). Briefly, 5 μg of ileal RNA and 20 μg of yeast transfer RNA were ethanol precipitated with about 0.1 μCi each of the labeled ASBT and GAPDH RNA probes. The pellet was washed with 70% ethanol, air dried, and resuspended in hybridization buffer [80% formamide, 1 mM EDTA, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.2 M sodium acetate, pH 6.4]. The mixture was heated to 85°C for 5 min and then cooled to 55°C overnight. RNase One digestion buffer (180 μl; 10 mM Tris-HCl, 5 mM EDTA, 200 mM sodium acetate, pH 7.5) containing 1 unit of RNase One (Promega) was added and incubated at room temperature for 30 min. RNase digestion was stopped by adding 20 μl of stop buffer (10% SDS, yeast RNA [1 mg/mL]). The undigested RNA was precipitated with ethanol and run on a 6% denaturing acrylamide gel. The gel was exposed to X-ray film and the bands quantitated by densitometry. After background subtraction, each ASBT mRNA band was normalized to the corresponding GAPDH mRNA band by determining for each subject the ratio of the ASBT mRNA band to the GAPDH mRNA band and multiplying by the densitometry measurement of GAPDH mRNA in a standard sample of ileum. This normalization procedure, which takes advantage of the relative constancy of GAPDH as a constituitively expressed housekeeping gene (18), has been used previously in work from our laboratory (17) and serves to correct for losses during the workup of samples.

Immunoblot analysis

An antibody to adjuvant-linked peptide identical to the carboxyl-terminal 20 amino acids of the human ASBT was raised in rabbits and affinity purified by Genosys (Houston, TX). Brush border membrane protein was prepared from ileal biopsies in standard fashion (19). Known amounts of this protein were separated on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel coupled to a 4% polyacrylamide stacking gel and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 10 mL of milk buffer (5% Carnation instant milk in Tris-buffered saline–Tween 20) for 1 h at room temperature and then probed overnight with 2 μL of rabbit anti-ASBT (0.15 μg/mL) in the same milk buffer. After three 20-min washes with Tris-buffered saline–Tween 20, the membranes were exposed for 1 h to goat anti-rabbit IgG conjugated with horseradish peroxidase (Dako, Carpinteria, CA) in 10 mL of milk buffer. After washing three times with Tris-buffered saline–Tween 20, membranes were exposed to a chemiluminescent reagent (ECL Plus; Amersham, Arlington Heights, IL) for 1 min in the dark, according to the manufacturer procedure. Bands were visualized and quantitated on

a Storm 860 PhosphorImager equipped with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA). Preliminary studies with varying protein concentrations were performed to determine the linear range of the immunoblot assay. Immunospecificity was established by exposure of duplicate samples run identically except that the primary antibody was blocked with the original synthetic peptide antigen in a weight ratio of 3:2 peptide:antibody. In addition, performing the assay with rabbit serum obtained prior to immunization with adjuvant–peptide (preimmune serum) showed no bands.

Statistical analysis

Statistical testing was by unpaired *t*-test.

RESULTS

Figure 1 shows a typical gel from the RNase protection assay. The left lane of this gel is a control without added RNA and shows two bands that correspond to the two full-length protected antisense RNA probes. The other four lanes represent four subjects selected because they typified the overall results. In most subjects both the ASBT mRNA band and the GAPDH band appeared as doublets. According to the manufacturer of the Riboprobe kit this is typical and likely a result of rehybridization of a portion of the antisense probe to its complementary sequence subsequent to denaturation in the loading buffer. In any case,

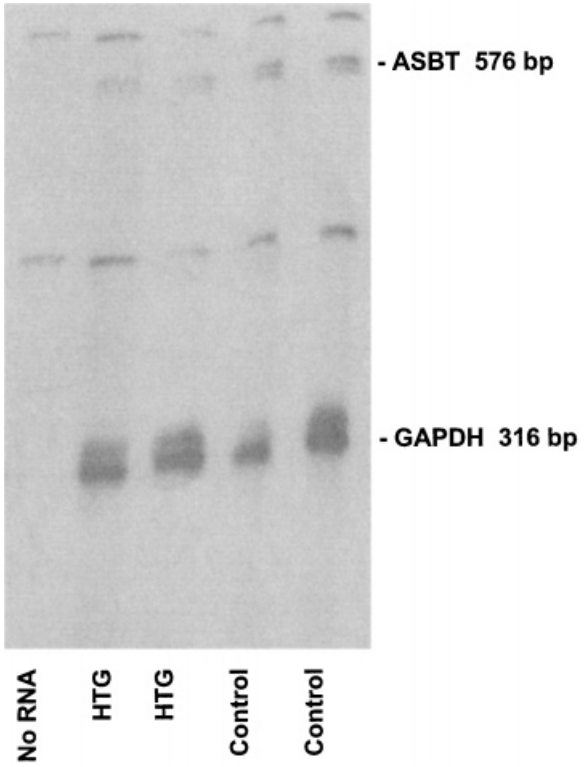

Fig. 1. Gel from nuclease protection assay for human ASBT mRNA. Far left lane is a control without RNA. The other lanes are from four subjects, two with hypertriglyceridemia (HTG) and two control subjects with normal lipids, selected because they typify the overall results. In most subjects both the ASBT mRNA band and the GAPDH band appeared as doublets (see text for explanation).

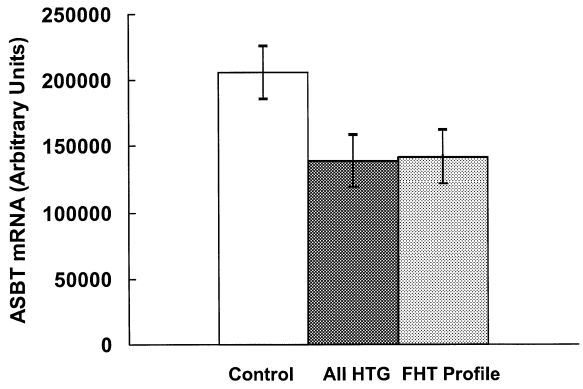

Fig. 2. Mean (and SEM) levels of ASBT mRNA (in arbitrary densitometry units) for control subjects, all hypertriglyceridemic subjects (HTG), and the subset of hypertriglyceridemic patients with a lipid profile compatible with familial hypertriglyceridemia (FHT). Compared with control subjects, the mean level of ASBT mRNA was significantly lower in the group of all hypertriglyceridemic patients ($P = 0.03$) and in the group of hypertriglyceridemic patients with an FHT lipid profile ($P = 0.05$).

both doublet bands were absent in negative control subjects (run without RNA). Therefore both components of each doublet were included in the densitometry analysis

Means ± SEM of the densitometry results (in arbitrary units) from the nuclease protection assays are shown in **Fig. 2**. The 13 patients with hypertriglyceridemia had mean levels of ASBT mRNA of 138.7 ± 19.1 compared with 205.7 ± 19.9 for the 15 control subjects ($P = 0.03$). Eliminating the three hypertriglyceridemic subjects with lipid profiles suggesting familial combined hyperlipidemia (FCHL) did not substantially change the comparison. Thus the 10 patients with lipid profiles compatible with FHT had mean levels of ASBT mRNA of 141.7 ± 20.8 ($P = 0.05$ compared with control).

Figure 3A shows an immunoblot of 5 μg of protein from each of three tissues: *a*) colon from a colonoscopic biopsy, *b*) proximal ileum from a surgical specimen, and *c*) terminal ileum from a colonoscopic biopsy. A single band of the expected molecular mass (about 45 kDa) is seen in the sample of terminal ileum, but in neither of the other two tissues. Blocking the antibody with authentic peptide (Fig. 3B) eliminated this band, confirming that it corresponded to ASBT protein. Immunoblots from three subjects (two control subjects and one with hypertriglyceridemia) showed a faint band at about 90 kDa, consistent with a dimeric form of the protein. Because this band was so faint and infrequent, we did not include it in the PhosphorImager analysis.

Figure 4 shows an immunoblot from four subjects, again selected because they typified the overall results. As suggested by visual inspection of this gel, the hypertriglyceridemic patients had lower levels of ASBT protein than did the normal control subjects.

Quantitation of these results for all subjects is shown in **Fig. 5**, in which protein band density is expressed as a percentage of a reference sample carried throughout all assays. For two of the hypertriglyceridemic subjects and four

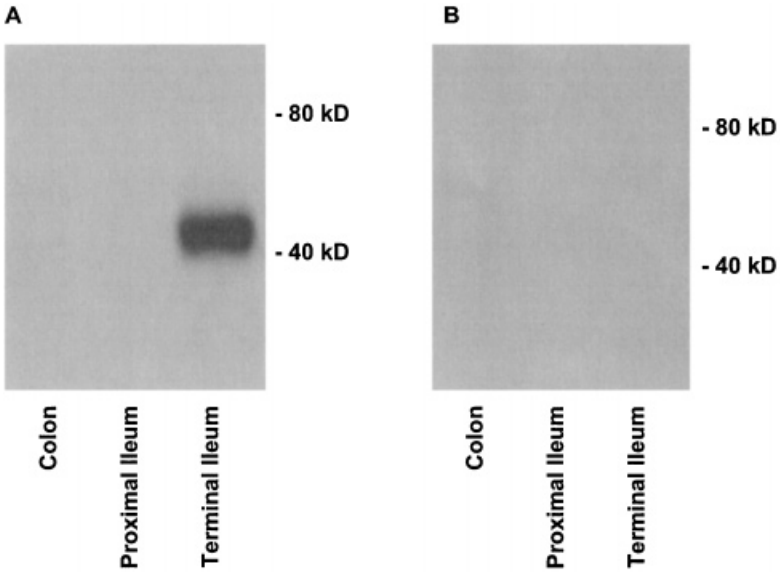

Fig. 3. (A) Gel from Western blot assay for ASBT protein in three tissues: colon, proximal ileum (PI), and terminal ileum (TI). A single band of the expected molecular weight is seen in terminal ileum, but not in colon or proximal ileum. (B) Same three tissues, but with the primary antibody blocked with authentic peptide used in production of the antibody.

of the control subjects we did not have sufficient tissue to perform Western blotting. In these 11 control subjects the mean ± SEM level of ASBT protein was 126.2 ± 22.6% compared with 58.8 ± 13.8% in the 11 hypertriglyceridemics ($P = 0.02$). For those eight hypertriglyceridemic subjects with an FHT profile and sufficient tissue to measure protein levels, the mean ASBT protein level was 61.8 ± 15.2% ($P = 0.05$ compared with control).

DISCUSSION

Studies from our laboratory (2), as well as from the laboratory of Angelin et al. (1) have provided evidence that patients with type IV hyperlipidemia, particularly those with FHT, have abnormally low rates of bile acid absorption. The present study provides strong support for that conclusion and goes further to demonstrate the mechanism for this absorption abnormality. Thus the lower level of ASBT protein in hypertriglyceridemics compared with control subjects (Fig. 5) would be expected to result in substantially less active absorption of bile acid in the terminal ileum of these hypertriglyceridemic subjects. Presumably this reduced level of active transport is responsible for the impaired absorption of bile acid previously documented in hypertriglyceridemic subjects (1, 2).

Although regulation of tissue protein levels is complex, in a broad sense the level of ASBT protein in ileal mucosa could be reduced by either accelerated removal or diminished production. Our finding of a concomitantly lower level of ASBT mRNA in hypertriglyceridemic subjects compared with control subjects (Fig. 2) supports dimin-

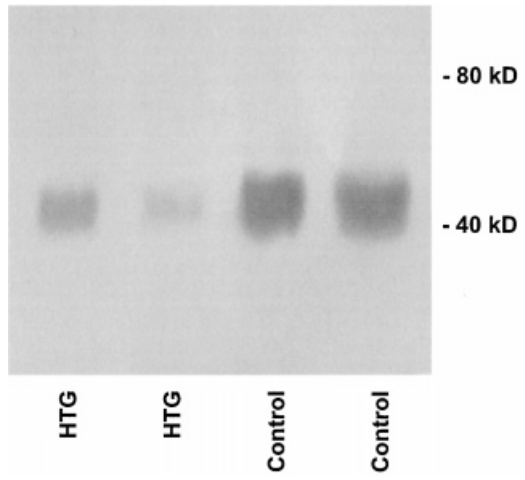

Fig. 4. Western blot gel of four subjects selected because they typify the overall results, two with hypertriglyceridemia (HTG) and two control subjects with normal lipids.

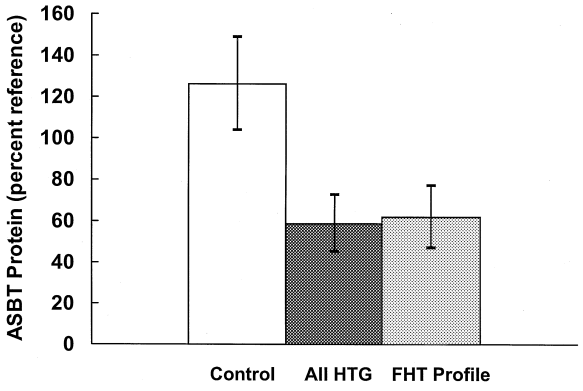

Fig. 5. Mean (and SEM) levels of ASBT protein (expressed as a percentage of a reference sample carried through all assays) in control subjects, all hypertriglyceridemic subjects (HTG), and the subset of hypertriglyceridemic patients with a lipid profile compatible with familial hypertriglyceridemia (FHT). Compared with control subjects, the mean level of ASBT protein was significantly lower in the group of all hypertriglyceridemic patients ($P = 0.02$) and in the group of hypertriglyceridemic patients with an FHT lipid profile ($P = 0.05$).

ished production as the explanation for lower levels of ASBT protein in these hypertriglyceridemic subjects. These data strongly suggest that at least some patients with hypertriglyceridemia have impaired absorption of bile acid because of diminished expression of the ASBT gene.

The association of hypertriglyceridemia with reduced expression of the ASBT gene does not establish a cause-and-effect relationship. Strictly speaking it is possible that the hypertriglyceridemia, or something associated with hypertriglyceridemia, caused reduced expression of the ASBT gene. However, there is no known mechanism by which hypertriglyceridemia might affect ASBT gene expression. Moreover, at least one subset of hypertriglyceridemia patients, those with FCHL, does not manifest impaired absorption of bile acid (1). Thus, it seems more likely that the association is explained by impaired absorption of bile acid contributing to the hypertriglyceridemia. That conclusion is strongly supported by the fact that serum triglycerides increase in response to decreasing bile acid absorption with ileal bypass (3) or administration of cholestyramine (5–7) and that triglyceride levels fall in response to administration of chenodeoxycholic acid (8–12).

The mechanism by which changes in bile acid absorption affect serum triglyceride levels has been illuminated by in vitro studies. In studying human hepatocyte cultures, Lin et al. (20) have shown that addition of bile acid to the cultures lowers production of VLDL. Del Pozo and Barth (21) reported similar findings in studies of rat hepatocytes. These data suggest that the reduced flux of bile acid through the liver, resulting from impaired absorption of bile acid, would stimulate production of VLDL resulting in increased serum levels of triglyceride.

In conjunction with these earlier observations, the findings of the present study strongly support the hypothesis that reduced expression of ASBT contributes to hypertriglyceridemia. However, it is unlikely that this absorption defect is the entire explanation of elevation of triglycerides in type IV patients. Thus, while reducing bile acid absorption by ileal bypass increases serum triglycerides by a mean of about 30% (3), type IV patients often have triglyceride levels more than 30% above the upper limit of normal. It seems more likely, therefore, that subjects predisposed to hypertriglyceridemia for other reasons, either genetic or environmental, may be particularly sensitive to upward pressure put on VLDL synthesis as a result of reduced bile acid returning to the liver. In the present study it was not feasible to assess the interaction of such predisposing factors with expression of the ASBT gene. It is perhaps worth noting, however, that the control and hypertriglyceridemic groups were similar with respect to sex (all males) and age (Table 1). Of interest is that degree of obesity, as measured by body mass index (BMI), was slightly greater in the group of all hypertriglyceridemics compared with control subjects. Nevertheless, it seems unlikely that interaction of obesity with ASBT gene expression played an important role in the hypertriglyceridemia of these subjects. Thus, compared with control subjects, the difference in triglyceride, ASBT mRNA, and ASBT protein levels were all similar for the FHT patients versus the group of all hypertriglyceridemic subjects despite the fact that the mean BMI in the FHT patients was virtually identical to that of the control group (Table 1). The complex and difficult task of unraveling the interactions of ASBT gene expression with other factors predisposing to hypertriglyceridemia will require sophisticated future studies.

It should be noted that diminished levels of ASBT mRNA in ileal tissue from these hypertriglyceridemic subjects do not necessarily imply a mutation in the ASBT gene. Indeed, we believe this is unlikely to be the case. Thus, studies in the laboratory of P. Dawson, with collaboration from our laboratory and others, have uncovered (with one heterozygous exception) no functionally important mutations of the ASBT gene in about 39 FHT subjects (P. A. Dawson, unpublished data). Rather, it seems likely that subjects with hypertriglyceridemia have an abnormality in the regulation of ASBT gene expression. Presumably either the ASBT gene in these subjects is particularly sensitive to downward regulation or one of the factors that normally inhibit ASBT gene expression is more pronounced in hypertriglyceridemia. What factors influence ASBT gene expression are largely unknown. Animal studies suggest that lumenal bile acid levels may be one such regulatory factor (22–26), but the data are quite inconsistent and no studies are available in human subjects. Studies are currently underway in our laboratory to determine, in human subjects, the importance of lumenal bile acids for regulation of ASBT gene expression.

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