Attenuation of the hepatoprotective effects of ileal apical sodium dependent bile acid transporter (ASBT) inhibition in choline-deficient L-amino acid-defined (CDAA) diet-fed mice

Ivo P. van de Peppel  Paul A. Dawson
Anuradha Rao
Sanjeev Gumber
Saul J. Karpen

In preparation
Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is a major growing worldwide health problem. We previously reported that interruption of the enterohepatic bile acid (BA) circulation using a non-absorbable apical sodium-dependent BA transporter inhibitor (ASBTi; SC435) reduced the development of NAFLD in high fat diet fed mice. However, fibrosis was not observed using this dietary model. Therefore, the ability of ASBTi treatment to impact the progression of NAFLD to fibrosis using a diet induced model remains untested.

In the current study, we assessed whether ASBTi treatment is hepatoprotective in the choline-deficient, L-amino acid-defined (CDAA) dietary model of NASH-induced fibrosis.

Methods: Male C57Bl/6 mice were fed for 22 weeks with: A) choline-supplemented L-amino acid defined diet (CSAA) (31 kcal% fat), B) CSAA diet plus ASBTi (SC-435; 60 ppm), C) CDAA, or D) CDAA diet plus ASBTi. Body weight and food intake were monitored. Liver effects were assessed by histology, biochemistry and gene expression. Additionally, fecal excretion of BAs and intestinal fatty acid absorption were measured.

Results: ASBTi treatment reduced bodyweight gain in both mice fed either the CSAA and CDAA diet. The ASBTi prevented the increase in liver to body weight ratio observed in CDAA mice. ASBTi-associated reductions in hepatic triglyceride levels and steatosis scores were observed in CSAA but not CDAA mice. Interestingly, intestinal fat absorption was significantly reduced with ASBTi treatment in mice fed a CSAA (85% vs 95%, \(P < 0.001\)) but not CDAA (94% vs 94%, \(P = \text{ns}\)) diet. The CDAA diet induced hepatic fibrosis which was increased upon ASBTi treatment, as quantified by the Ishak Scoring system.

Conclusion: ASBT inhibition reduced intestinal fat absorption, body weight gain and hepatic steatosis in CSAA fed mice. The effects of the ASBTi on steatosis and fat absorption were attenuated in the context of choline-deficiency. Inhibition of intestinal absorption of fatty acids may be involved in the therapeutic effects of ASBTi treatment.
Introduction

Parallel to the global rise in obesity, the disease burden related to nonalcoholic fatty liver disease (NAFLD) is emerging as a major worldwide problem. NAFLD prevalence increases steadily every year with the current global prevalence estimated at 24% (1,2). NAFLD comprises a spectrum of disease states, from non-symptomatic hepatic steatosis to non-alcoholic steatohepatitis (NASH) and varying degrees of liver fibrosis. NAFLD activity also increases morbidity and mortality due to other diseases such as type 2 diabetes mellitus, cardiovascular disease and chronic kidney disease (3). Unfortunately, an incomplete understanding and a lack of experimental NAFLD/NASH models that faithfully reproduce the human pathophysiology has slowed the development of new therapies (4).

Recently, bile acid (BA) homeostasis has emerged as an important therapeutic target for disorders of glucose and lipid metabolism (5). Under physiological conditions, BA synthesis and enterohepatic cycling are tightly regulated to maintain relatively a constant whole-body BA pool size and restrict the systemic distribution of BAs. After their secretion along with bile into the duodenum, about 95% of the BAs are reabsorbed by the apical sodium-dependent bile acid transporter (ASBT) in the distal small intestine. This system is tightly controlled by BA signaling via the farnesoid-X receptor (FXR) in the liver and intestine. Intestinal microbiota harbor enzymes to deconjugate and biotransform primary BAs into secondary BAs, changing the BA pool size and composition, and thereby altering FXR signaling and activation of other BA activated receptors, modulating metabolic responses (6).

Several BA based treatments are being studied for their effect on metabolic disorders including NAFLD. Obeticholic acid, a derivative of the naturally occurring FXR agonist chenodeoxycholic acid, improved liver biochemistry and histology scores in NASH patients (7,8). Conversely, we have previously shown that interruption of the enterohepatic circulation by the ASBT inhibitor SC-435 (ASBTi) prevented hepatic lipid accumulation in mice fed a high fat diet (9). The ASBT inhibitor Volixibat is currently in phase 2 clinical trials for treatment of NASH (ClinicalTrials.gov Identifier: NCT02787304).

Although previous data showed a robust effect of ASBT inhibition on hepatic lipid accumulation, the effects of ASBT inhibition on the progression from steatosis to steatohepatitis and fibrosis remained unclear (9). Feeding a Western type diet (high fat, high sucrose and high cholesterol) to mice typically models the human condition and successfully induces hepatic lipid accumulation. However, progression from steatosis to NASH and fibrosis is generally limited and highly
variable (10). Therefore, various other dietary, genetic or toxic models, or combinations thereof, are required to study NASH progression. One widely used model of dietary induction of NASH and subsequent fibrosis is the MCD diet. Depriving mice of dietary choline impairs hepatic secretion of very low density lipoproteins (VLDL) and results in hepatic steatosis, oxidative stress, cell death and changes in cytokines (11). Combined with methionine deficiency, mice develop extensive inflammation after 2 weeks and significant fibrosis after 6 weeks (10). However, when fed the MCD diet, mice generally lose considerable bodyweight and show no insulin resistance (or even increased sensitivity), conditions that poorly correlated with development of NASH in humans (12,13).

In this current study we assessed the effects of ASBTi treatment on development of NAFLD induced fibrosis using a choline deficient L-amino acid defined (CDAA) diet which has been shown to successfully induce hepatic steatosis and subsequent fibrosis after 22 weeks without bodyweight loss (14,15).
Methods

Animals

Male C57Bl/6J mice aged 10 weeks were obtained from Jackson Laboratories. Animals were group housed with 4 mice in laboratory cages at 23°C and a 12-hour light/dark cycle. Mice were fed either a choline deficient L-amino acid defined diet (CDAA, Catalog # 518753; Dyets Inc., Bethlehem, PA, USA) containing 36% fat by calories or a choline sufficient L-amino acid defined control diet (CDAA, Catalog # 518754; Dyets Inc., Bethlehem, PA, USA) with or without 0.006% (w/w) of an ASBT inhibitor (SC-435) ad libitum for 22 weeks. During the final week mice were individually housed for 1 week during the fat absorption measurements. The Emory University Institutional Animal Care and Use Committee approved these experiments.

Animal experiments

During the final week of CSAA/CDAA (+/- ASBTi) diet feeding, mice received powdered diet containing 0.7% sucrose polybehenate (w/w) (16). Sucrose polybehenate is not absorbed in the intestine. Therefore, by using the ratio of behenic acid to fatty acids in the diet and comparing this to the ratio in the feces, one can calculate absorption without the being reliant on often less accurate food intake data. Powdered diet was placed in a feeding jar in the cage and replaced every 2-3 days. Feces were collected for individual mice during the final 3 days of the experiment and used for fatty acid, neutral sterol and bile acid measurements. After, mice were sacrificed and tissues collected for further analysis. Mice were anaesthetized using isoflurane. Blood was obtained via cardiac puncture, centrifuged and obtained plasma stored at -80°C. Livers were excised, weighed and pieces collected for subsequent histology analysis. Remaining liver tissue was snap frozen in liquid nitrogen. The small intestines were excised, measured, cut into five equal segments, flushed with ice cold phosphate buffered solution (PBS) and snap frozen for gene expression analysis. The length of the colon was measured, divided into proximal (60%) and distal (40%) segments, flushed with ice cold PBS and immediately snap frozen in liquid nitrogen.

Fatty acid measurements

Weighed samples of the synthetic diet and feces were saponified with methanolic NaOH, extracted with hexane, converted to methyl esters. Samples were analyzed via gas chromatography (GC) for fatty acid methyl esters as previously described (17–19). Each chromatogram was examined for correct identification of constituent fatty acids and quality control.
Histology

The livers were removed and weighed, and a portion was formalin-fixed, embedded in paraffin, and stained with hematoxylin and eosin. Sirius red staining was performed on paraffin-embedded liver sections (method adapted from Picosirius Red Stain Kit, Polysciences, Inc.). The liver histology was blindly assessed by S.G. for steatosis, lobular inflammation, and hepatocellular ballooning to derive the NAS scale as described (20). Sirius red stained sections were blindly assessed by S.G. for Ishak Stage using the scores in table 1 adapted from (21).

| Ishak Stage, Categorical description | Score |
|-------------------------------------|-------|
| No fibrosis                         | 0     |
| Expansion of some portal areas with or without short fibrous septa | 1     |
| Expansion of most portal areas with or without short fibrous septa | 2     |
| Expansion of most portal areas with occasional portal to portal bridging | 3     |
| Expansion of portal areas with marked bridging (portal-portal and/or portal-central) | 4     |
| Marked bridging with occasional nodules (incomplete cirrhosis) | 5     |
| Cirrhosis, probable or definitive   | 6     |

Table 1. Ishak stage scoring system adapted from (21)

Hepatic lipids

Hepatic lipids were extracted according to a protocol based on the Folch method (22). Briefly, lipids were extracted from ~60mg liver tissue using 3 ml of chloroform:methanol (2:1) and incubated at 55°C for at least 2h. Phases were split by adding 0.05% (v/v) sulfuric acid in water and centrifugation at 1500rpm for 15 minutes. Part of the bottom layer was transferred, dried down under nitrogen and dissolved in 2% (v/v) TritonX-100 in water. Hepatic concentrations of total cholesterol (Pointe Scientific, C7510-01-906), free cholesterol (Wako Diagnostics, Cat# 993-02501) and triglyceride (Wako Diagnostics, Cat#994-02891 and 990-02991) were subsequently measured by enzymatic assays.

Gene expression

Total RNA was isolated from proximal colon and liver using miRNeasy kit (Qiagen, Cat# 74106). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with 1µg RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Cat# 4368814). Real-time quantitative PCR was performed with a Sybr Green master mix (Applied Biosystems, Cat# 4309155) using a
StepOne Plus real time PCR system (Applied Biosystems). Gene expression levels were normalized to *Cyclophilin*.

**Fecal bile acids and neutral sterols**

Fecal pellets were sorted, air-dried, weighed and mechanically homogenized. Neutral sterols and bile acids were extracted from 50mg feces and diet as described (23). Briefly, samples were heated for two hours at 80ºC with a mixture of 1M sodium hydroxide and methanol (3:1). Neutral sterols were then extracted 2 times with 2ml petroleum ether and derivatised with BSTFA-pyridine-TMCS (5:5:0.1). Bile acids were quantitatively extracted from feces, isolated on Sep-Pak C-18 columns, methylated with methanol/acetyl chloride (20:1) and derivatised with BSTFA-pyridine-TMCS (5:5:0.1). Both neutral sterols and bile acids were measured by gas chromatography (GC) (24). The total amount of bile acids or neutral sterols was calculated as the sum of the individual species.

**Statistical analyses**

Data are presented as means ± standard deviation (SD), unless stated otherwise. Statistical analyses were performed and graphs were created using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Differences between groups were assessed by a two-tailed ANOVA with Tukey’s post-hoc test except for Ishak Stage which was tested using a Chi-Squared test. Different lowercase letters indicate statistical significant differences *(P<0.05)* between groups.
Results

The effect of ASBT inhibitor treatment on bodyweight gain in mice fed CSAA and CDAA diets

To determine whether ASBT inhibition could prevent development of NASH and fibrosis, male C57Bl/6J mice were fed a choline-deficient L-amino-defined diet (CDAA) or a choline-sufficient control diet (CSAA) with or without ASBTi for 22 weeks (Fig. 1A). ASBTi treatment was successful in increasing total fecal BA excretion by about 5-fold in CSAA fed mice (4.1 vs 21.4 µmol/24h/100gBW, P<0.001) and in CDAA fed mice by about 4-fold (5.3 vs 19.1 µmol/24h/100gBW, P<0.001) (Fig. 1B). Analysis of the BA composition of the feces revealed that ASBTi treatment was associated with increased excretion of the secondary BAs, deoxycholic acid (DCA) and lithocholic acid (LCA), as well as the primary BA, α-muricholic acid (MCA) (Fig. S1). Interruption of the enterohepatic circulation of BAs was confirmed by increased levels of hepatic BA synthesis, assessed by gene expression of cholesterol 7 alpha-hydroxylase (Cyp7a1), and increased levels of colonic gene expression of the intestinal bile acid-binding protein (Ibabp) suggesting ileal BA malabsorption (Fig. 1C-D). Together these data indicate that the ASBTi treatment was successful in interrupting the enterohepatic circulation of BAs in both CSAA and CDAA diet fed mice.
To assess whether choline deficiency and/or ASBTi treatment affected body mass, we recorded bodyweight weekly (Fig. 2A). Bodyweight gain was similar for mice fed the CSAA and the CDAA diets. After 22 weeks, mice on the CSAA diet gained weight similar to mice on the CDAA diet (54% vs 49%, $P=0.8$, Fig. 2B). ASBTi treated groups gained less weight over the course of 22 weeks, irrespective of the diet, and significantly lowered bodyweight gain to about 38% on the CSAA diet and 39% on the CDAA diet. Caloric intake was slightly lower in the CDAA group treated with ASBTi (15.1 vs 15.9 kcal/day/mouse in the untreated CDAA group, $P=0.02$, Fig. 2C). Hepatomegaly due to steatosis and inflammation is a common feature of NAFLD. After 22 weeks both absolute liver weight (2.4 vs 3.4 g, $P=0.001$, Fig. 2D) and liver to bodyweight ratio (0.06 vs 0.08, $p<0.001$, Fig. 2E) were increased in mice on the CDAA diet compared to the CSAA diet. On the
CSAA diet, ASBTi treatment did not significantly affect liver weight (1.9 vs 2.4 g, \( P=0.2 \), **Fig. 2D**) and liver to bodyweight ratio (0.05 vs 0.06, \( P=0.5 \), **Fig. 2E**) in mice as compared to the CSAA controls. On the CDAA diet, liver weight (2.4 vs 3.4, \( P=0.001 \), **Fig. 2D**) and liver to bodyweight ratio (0.07 vs 0.08, \( P=0.002 \), **Fig. 2E**) was lower upon ASBTi compared to the untreated CDAA diet group and were similar to CSAA control values.

Figure 2. The effect of ASBTi treatment on bodyweight gain and liver weight in mice fed a CSAA and CDAA diet. (A) Bodyweight gain over time in *ad libitum* fed mice, (B) Bodyweight gain in percentage of initial bodyweight after 22 weeks, (C) Calories consumed per mouse per day, (D) Absolute liver weights after 22 weeks, (E) Liver to bodyweight ratio; *n=9-12 per group*
The effect of ASBT inhibitor treatment on hepatic cholesterol and triglyceride accumulation on a CSAA and CDAA diet

To assess the key features of the pathophysiology and progression of NAFLD we examined the liver histology of the diet-fed mice. Untreated mice on both the CSAA and CDAA diet showed clear features of NAFLD, including steatosis, lobular inflammation and hepatocyte ballooning (Fig. 3A, B). Treatment with an ASBTi reduced visible lipid accumulation in mice fed the CSAA diet (Fig. 3C) whereas the ASBTi effects on lipid accumulation were attenuated in mice fed the CDAA diet (Fig. 3D). Slides were assessed by a certified pathologist (author S.G.), who was blinded to the 4 groups, to determine a NAFLD activity (NAS) and steatosis scores (Fig. 3E-F). NAFLD activity (6.1 vs 6.3, \(P=0.9\), Fig. 3E) and steatosis (2.6 vs 2.8, \(P=0.7\), Fig. 3F) were similar for mice fed the CSAA and CDAA diets. ASBTi treatment reduced the NAS and steatosis in mice fed the CSAA but not on a CDAA diet. To quantify hepatic lipid accumulation, we measured hepatic triglyceride and cholesterol content biochemically. In agreement with the NAS results, hepatic triglyceride levels were similar in mice fed the CSAA and CDAA diets (201 vs 236 µg/mg, \(P=0.6\), Fig. 3G). ASBTi treatment significantly reduced hepatic triglyceride accumulation significantly for mice fed the CSAA diet (90 vs 201 µg/mg, \(P=0.002\), Fig. 3G), but the ASBTi-associated reduction in hepatic triglyceride was attenuated in mice fed the CDAA diet (183 vs 236 µg/mg, \(P=0.2\), Fig. 3G). Total hepatic cholesterol content in the liver was similar between the mice fed the CSAA and CDAA diets (5.2 vs 7.0 µg/mg, \(P=0.1\), Fig. 3H). Upon ASBTi treatment, both total (3.6 vs 7.0 µg/mg, \(P<0.001\)) and free liver cholesterol (2.3 vs 3.9 µg/mg, \(P=0.01\)) levels in the CDAA diet were reduced and a similar trend was observed for the CSAA diet (Fig. 3H, I). Cholesteryl esters were significantly reduced upon ASBTi treatment for both CSAA and CDAA fed mice (Fig. 3J).

Altogether, these show that ASBTi treatment lowers hepatic steatosis on the CSAA diet, in agreement with earlier observations (9). However, choline deficiency partially attenuated these effects.
The effect of ASBT inhibitor treatment on development of hepatic inflammation and fibrosis on a CDAA diet

Although it was previously shown that administration of an ASBTi reduces hepatic steatosis, the effects this treatment on the progression to NASH and fibrosis remained unclear (9). The CDAA diet has been shown to successfully induce fibrosis after 22 weeks of diet (14,15,25). To visualize fibrosis on histology, collagen was stained with Sirius Red. The CSAA diet did not visually induce fibrosis, whereas hepatic fibrosis was readily apparent in the mice fed the CDAA diet (Fig. 4A, 4C). On the CSAA diet, treatment with the ASBTi had no visual impact on histologic Sirius Red staining (Fig. 4B). On the CDAA diet ASBTi treatment showed visible fibrosis comparable to the CDAA diet alone (Fig. 4D). To quantify the degree of fibrosis, slides were analyzed and scored by a certified pathologist (author S.G., blinded to diet/treatment) and quantified using the Ishak Scoring system (Table 1) (21,26). Livers of mice on the CSAA diet with and without ASBTi did not show any signs of fibrosis resulting in an Ishak score of 0. Mice on the CDAA diet had significantly higher fibrosis compared to the CSAA diet quantified as an average Ishak score of 1 (Fig. 4E, \( P<0.001 \)), corresponding to a Sirius Red stained proportion of 3.0%, fibrous expansion of some of the portal areas with or without fibrous septa. Treatment with the ASBTi on a CDAA diet resulted in a significantly higher Ishak score of 2 (Fig. 4E, \( P=0.02 \)), corresponding
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to a Sirius Red stained proportion of 3.6% Sirius with fibrous expansion to most of the portal areas with or without short fibrous septa.

Figure 4. The effects of ASBTi treatment on hepatic fibrosis in mice fed a CSAA and CDAA diet. Sirius red stained liver sections from (A) CSAA, (B) CSAA+ASBTi, (C) CDAA and (D) CDAA+ASBTi fed mice after 22 weeks; Scale bars= 100µm, representative samples; (E) Fibrosis score (Ishak stage); n=9-12 per group

Hepatic gene expression of a panel of fibrosis related genes, shown before to be upregulated in fibrosis caused by a CDAA diet (25), was measured to assess whether the changes in histology were reflected on the mRNA level. Collagen type I alpha 1 (Col1a1) encoding the main component of collagen type 1 fibers was upregulated in the CDAA diet and unaffected by SC-435 treatment (Fig. 5A). Hepatic expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), a protein involved in degradation of extracellular matrix and promoting proliferation, was similarly increased in the mice fed the CDAA diet and CDAA diet plus the ASBTi (Fig. 5B). Interestingly, alpha-smooth muscle actin (α-SMA), a marker used for hepatic stellate cell activation (27), was unaffected by diet or treatment (Fig. 5C). Connective tissue growth factor (CTGF) and transforming growth factor beta (TGF-β) play an important role in fibrogenesis, both together and separately. CTGF expression was not significantly increased in the CDAA diet compared to the CSAA diet (Fig. 5D). However, ASBTi treatment significantly lowered CTGF expression in the CDAA diet compared to the corresponding control. TGF-β was similar between the CSAA and CDAA diet (Fig. 5E). ASBTi treatment significantly lowered TGF-β expression on the CSAA diet but not on the CDAA diet.
As progression of liver steatosis to fibrosis generally involves inflammation, we measured hepatic gene expression of several important inflammatory genes. Expression of the proinflammatory genes tumor necrosis factor α (TNFα), inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein 1 (MCP1), were upregulated on a CDAA diet compared to the CSAA diet and unaffected by ASBTi treatment (Fig. 6A-C). Hepatic cell damage and increases in inflammation are often paired with oxidative stress. Therefore, we measured glutathione S-transferase A1 (GSTα1) expression, the gene encoding a key enzyme in the antioxidative glutathione pathway. GSTα1 expression was increased upon feeding a CDAA diet compared to a CSAA diet but unaffected by ASBTi treatment (Fig. 6D).

Altogether, these data suggest that ASBTi treatment was not able to prevent inflammation, oxidative stress or fibrosis in this dietary choline deficiency model of NAFLD/NASH.
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Figure 6. The effects of ASBTi treatment on hepatic gene expression related to inflammation in mice fed a CSAA and CDAA diet. (A-D) Relative hepatic mRNA expression of genes related to inflammation, normalized to cyclophilin; n=9-12 per group

The effects of ASBT inhibitor treatment on fat absorption on a CSAA and CDAA diet

We previously reported that genetic ASBT knockout or pharmacological inhibition in mice lowers fat absorption, particularly for saturated longer chain fatty acids (Van de Peppel IP et al. In preparation). This partially explained the lower bodyweight gain and metabolic benefits observed in ASBT knockout mice compared to wildtype mice when feeding a high fat diet. Therefore, we measured fat absorption using the sucrose polybehenate method (16). On the CSAA diet, ASBTi treatment lowered total fatty acid absorption by about 10% (85.3% vs 94.6%, P<0.001, Fig. 7A). We found no difference in total fat absorption between
the CSAA and the CDAA diet (94.6% vs 93.6%, \( P=0.9 \), Fig. 7A). Additionally, total fat absorption was comparable between the CDAA diet with and without ASBTi treatment (93.5 vs 93.6%, \( P=1.0 \), respectively, Fig. 7A). In line with previous observations by our laboratory (Van de Peppel et al. In preparation), the decrease in fat absorption by the ASBTi on the CSAA diet was the greatest for saturated fatty acids (C14:0, C16:0, C17:0 and C18:0) and difference increased with longer chain lengths which in turn corresponds to a higher hydrophobicity (28), followed by the trans fatty acid, C18:1 (Fig. 7B). Reductions in fat absorption for the mono- and polyunsaturated fatty acids (C18:1\( \omega_9 \), C18:1\( \omega_7 \), C18:2\( \omega_6 \)) by ASBTi treatment on the CSAA diet were statistically significant but remained preserved in terms of the absolute differences (Fig. 7B).

Previous data has shown that \textit{Asbt} knockout mice have severely impaired intestinal absorption of cholesterol (Van de Peppel et al. in preparation). While we did not directly assess cholesterol absorption in the current study, we measured fecal excretion of neutral sterols (NS), cholesterol and its fecal metabolites (Fig S2A,B). Fecal NS excretion was significantly higher in CSAA mice treated with an ASBTi compared to CSAA control mice (12.0 vs 7.5 \( \mu \)mol/24h/100gBW, \( P=0.02 \), Fig. S3A). On the CDAA diet, fecal NS excretion was comparable to the CSAA diet (9.7 vs 7.5 \( \mu \)mol/24h/100gBW, \( p=0.4 \), Fig. S2A). ASBTi treatment did not significantly affect fecal NS excretion on the CDAA diet (8.0 vs 9.7 \( \mu \)mol/24h/100gBW, \( P=0.6 \), Fig. S3A). It is suggested that upon choline deficiency, triglycerides translocated into the enterocyte accumulate because they are not efficiently secreted into chylomicrons (29–31). However, triglyceride and total cholesterol content were not different in small intestinal tissue (Fig. S3A,B). We previously suggested that the effects of ASBT inactivation on fat absorption partially modulated the beneficial effects on high fat diet induced obesity (Van de Peppel et al. in preparation). Correlation analysis showed that hepatic triglyceride content highly correlated to intestinal fatty acid absorption the CSAA (Spearman \( R=0.8, P<0.001 \), Fig. S4A) but not in the CDAA diet (Spearman \( R=0.0, P=0.9 \), Fig. S4B).

Altogether, these data suggest that choline deficiency attenuates the previously consistent effects of ASBT inhibition on fat and cholesterol absorption (32,33) (Van de Peppel et al. in preparation).
Figure 7. The effects of ASBTi treatment on intestinal fatty acid absorption in mice fed a CSAA and CDAA diet. (A) Percentage of total intestinal fat absorption, (B) percentage of intestinal absorption of individual fatty acids, normalized to cyclophilin; n=9-12 per group.
Discussion

In the current study, we showed that treatment with an ASBTi did not prevent development of hepatic fibrosis in a CDAA diet model. Additionally, under choline deficient conditions, previously reported effects of the ASBT inhibition on fat absorption (33) were nullified, possibly contributing to the phenotype.

Dietary mouse models to induce NASH and fibrosis are limited. The CDAA diet was used in the current study because, contrary to the MCD diet, it was shown to successfully induce hepatic fibrosis without significantly lowering bodyweight and plasma glucose levels (14,15). About 95% of choline in animal tissues is present as phosphatidylcholine (PC), a class of phospholipid, which is a major component of membranes (34). The main mechanism believed to be involved in the effects of choline deficiency on hepatic steatosis is impairment of VLDL secretion due to PC reduction (11). Interestingly, bile acid binding sequestrants work similar to ASBT inhibition, by interrupting the enterohepatic circulation of BAs, and have been shown to increase plasma VLDL and triglyceride levels (35,36). Therefore, one of the anti-steatotic benefits of the ASBTi could be the result of increased VLDL and triglyceride clearance from the liver which is prevented due to choline deficient conditions.

Furthermore, choline deficiency also alters mitochondrial function (37), fatty acid beta oxidation (38), epigenetics (39), and intestinal microbiota (40) which are possibly involved in NAFLD development. It was previously shown that treatment with the ASBTi for 16 weeks on a high fat diet did not change total BA concentrations in the liver, while composition shifted towards a more hydrophobic and thus potentially cytotoxic profile (9). In vitro studies have shown that PC protects hepatocytes from BA induced cytotoxicity (41). Therefore, the combined effects of choline deficiency with a more hydrophobic BA profile upon ASBTi treatment could have potentially aggravated effects on fibrosis. Additionally, both choline deficiency and BAs affect the intestinal microbiota profile (40,42). Bacterial toxicity of high colonic levels of BAs upon ASBT inhibition combined with choline deficiency possibly resulted in a more unfavorable microbiome affecting NAFLD development.

Anti-steatotic effects of the ASBTi have been consistent in our laboratory on high fat diets (including the CSAA diet in this study) (9). Future studies using different models are needed for definitive conclusions of the role of ASBTi treatment in development of NASH and fibrosis. Fortunately, recent murine NAFLD
models have shown promising results in mimicking human NAFLD/NASH pathophysiology (43,44).

We have previously observed robust effects of ASBT inactivation, both genetic and via inhibitor treatment, on lowering intestinal absorption of fatty acids (Van de Peppel et al. In preparation). In our current study, ASBTi treatment lowered fatty acid absorption on a CSAA but not CDAA diet. Moreover, there was no difference in intestinal fat absorption between mice fed a CSAA and CDAA diet. Intestinal fat absorption positively correlated with hepatic triglyceride levels in the CSAA diet but not in the CDAA diet suggesting the possibility that intestinal fat absorption was involved in the anti-steatotic effects of the ASBTi.

In rats, dietary choline deficiency was shown to impair chylomicron secretion and alter intestinal cell morphology and physiology, resulting in impairments of dietary fat absorption (29–31). Intraluminal PC concentrations contribute to mixed micelle formation, transport across the unstirred water layer, and subsequent translocation to the brush border membrane (45). However, luminal PC also affects hydrolysis of lipids. In vitro studies showed that PC inhibited lipase and colipase activity and subsequent cholesterol and fatty acid absorption (46). The mechanism underlying our observations with CDAA feeding remains unclear. One could speculate that decreased biliary PC might be involved in intraluminal effects on lipolytic and post-lipolytic events related to intestinal lipid absorption.

Additionally, in vivo studies supported the idea that PC in the intestine protects enterocytes from BA induced toxicity (47). In the absence of sufficient luminal PC concentrations, more free BAs are present in the intestinal lumen, which could damage the gut epithelium and impair gut barrier function (48). Subsequently, it could be speculated that this resulted in more passive transport of BAs into enterocytes resulting in a higher return BAs in the enterohepatic circulation explaining differential effects of the ASBTi under CDAA compared to CSAA fed conditions. While this theory is not supported by changes in fecal BA concentrations, colonic gene expression of the BA activated Fxr target gene Ibabp was significantly higher in ASBTi treated mice on a CDAA compared to a CSAA diet suggesting possible higher concentrations of intra-enterocyte BA concentrations in the colon.

In conclusion, this study showed that ASBTi treatment did not affect intestinal fat absorption and could not prevent hepatic fibrosis in a dietary choline deficient model of NASH. These results should be interpreted with caution in regard to
human NAFLD pathophysiology, as this is different and rarely involves choline deficiency.

Nevertheless, this data adds to our understanding to the benefits and limitations of ASBTi treatment and highlights opportunities to further explore underlying mechanisms. Indirectly modulating intestinal fat absorption via ASBTi treatment is an interesting potential therapeutic target. Future studies using different models for NASH are warranted to establish the exact role and mechanism of ASBTi treatment in hepatic steatosis progression to NASH and fibrosis.

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Supplementary figures

Figure S1. The effects of ASBTi treatment on fecal bile acid secretion of individual bile acid species in mice fed a CSAA and CDAA diet. \( n=9\)-12 per group

Figure S2. The effects of ASBTi treatment on fecal neutral sterol excretion in mice fed a CSAA and CDAA diet. (A) Total fecal neutral sterol excretion, (B) Fecal excretion of different individual neutral sterol species; \( n=9\)-12 per group
Figure S3. The effects of ASBTi treatment on intestinal lipid content in mice fed a CSAA and CDAA diet. (A) Intestinal triglyceride content, (B) Intestinal total cholesterol content; n=9-12 per group.

Figure S4. Correlation between intestinal fat absorption and hepatic triglyceride levels of mice fed a CSAA and CDAA diet with or without ASBTi. (A) correlation for CSAA with and without ASBTi, Spearman R: 0.8, p<0.001, n=9-12 per group (B) correlation for CDAA with and without ASBTi, Spearman R: 0.0, p=0.9, n=11-12 per group.
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