Title: *Lactobacillus casei* strain Shirota protects against non-alcoholic steatohepatitis development in a rodent model

Authors:

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Running Head: Suppression of NASH development by LcS

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Abstract

Gut microbiota alterations are associated with various disorders. In this study, gut microbiota changes were investigated in a methionine-choline deficient (MCD) diet-induced non-alcoholic steatohepatitis (NASH) rodent model, and the effects of administering Lactobacillus casei strain Shirota (LcS) on the development of NASH were also investigated. Mice were divided into three groups, given the normal chow diet (NCD), MCD diet, or the MCD diet plus daily oral administration of LcS for 6 weeks. Gut microbiota analyses for the three groups revealed that lactic acid bacteria such as Bifidobacterium and Lactobacillus in feces were markedly reduced by the MCD diet. Interestingly, oral administration of LcS to MCD diet-fed mice increased not only the L. casei subgroup but also other lactic acid bacteria. Subsequently, NASH development was evaluated based on hepatic histochemical findings, serum parameters and various mRNA and/or protein expression levels. LcS intervention markedly suppressed MCD-diet induced NASH development, with reduced serum lipopolysaccharide concentrations, suppression of inflammation and fibrosis in the liver, and reduced colon inflammation. Therefore, reduced populations of lactic acid bacteria in the colon may be involved in the pathogenesis of MCD diet-induced NASH, suggesting normalization of gut microbiota to be effective for treating NASH.

Key words: lipid metabolism; inflammation; gut microbiota; non-alcoholic steatohepatitis; Lactobacillus casei strain Shirota
Introduction

Gut microbiota species are involved in several intestinal biological functions such as defense against colonization by opportunistic pathogens and intestinal architecture development, thereby contributing to immune homeostasis (26). Whole-body energy metabolism is also affected by the condition of intestinal gut microbiota and deviations were reported to frequently be observed in patients with obesity and associated metabolic disorders (3, 29). In addition, several diseases have been implicated in gut microbiota alterations. For example, reduced microbial diversity, indicative of a dysfunctional ecosystem that leads to decreased microbiota stability, is reportedly associated with both inflammatory bowel disease and obesity (23, 30). Thus, modifying these changes in gut microbiota using probiotics is a potential therapeutic method for certain diseases, including those mentioned above (8, 26).

Non-alcoholic steatohepatitis (NASH) is a serious disease which can lead to liver cirrhosis and hepatic cancer, and its incidence is increasing in many developed countries. However, there are currently no treatment options specifically for NASH, and this disease is usually managed with lifestyle changes such as diet, exercise and weight reduction (10, 27). In this study, we speculated that dietary alterations would affect the composition of gut microbiota, and, in fact, it was revealed that lactic acid bacteria such as Bifidobacterium and Lactobacillus in feces were markedly reduced by a methionine choline deficient (MCD) diet, used as an inducer of NASH. Thus, Lactobacillus casei strain Shirota (YIT9029, LcS) was orally administered to mice fed the MCD diet, and its effects on the development of NASH were investigated.

Regarding the pathogenesis of NASH, the “two-hit theory” has been proposed as a mechanism underlying the development of NASH. The first hit involves simple steatosis, which arises from an excess supply of fatty acids and/or glucose, lipotoxicity, and insulin resistance. The second hit involves aggravating factors such as oxidative stress, inflammatory cytokines and endotoxins that are considered to play an important role in the progression of liver damage (7, 16). Since it was reported that probiotic bacteria prevent...
hepatic damage and maintain colonic barrier function in a murine sepsis model (12), we speculated that gut microbiota might be a potential target for treating NASH development.

Herein, we present evidence that LcS intervention markedly suppressed NASH development in our MCD diet-fed rodent model, suggesting normalization of gut microbiota to be beneficial for treating NASH.
Materials and Methods

Strains and culture conditions
LcS was obtained from the culture collection of the Yakult Central Institute for Microbiological Research (Tokyo, Japan). To prepare bacterial cells for daily oral administration to mice, LcS was cultured in MRS medium as previously reported (25). The cultured cells were collected by centrifugation and washed three times with saline, and then suspended in saline at a concentration of $5 \times 10^9$ colony-forming units /ml.

Animals and treatments
C57BL/6 mice (SLC, Hamamatsu, Japan) were crossbred and kept in the same environment. They were housed in temperature and light controlled rooms with free access to food and water. At 6 weeks of age, the mice were randomly allocated into three groups as described above. After 6 weeks, immediately post-defecation, fecal samples were placed in a tube (76 x 20 mm, SARSTEDT AG & Co., Germany) containing 2 ml of RNAlater (Ambion, Inc., Austin, TX, U.S.A.). After a fecal sample-containing tube was weighed, RNAlater (Ambion) at a 9-fold volume was added to prepare a fecal suspension. For RNA stabilization, fecal homogenate (200 μl) was added to 1 ml of sterilized phosphate buffer solution (PBS), and then centrifuged at 5,000 × g for 10 min. The supernatant was discarded and the pellet was stored at -80 °C until it was used for RNA extraction. RNA was isolated using a modification of the acid guanidinium thiocyanate-phenol-chloroform
bacterial count was determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which has been established as an analytical method for intestinal microbiota (17, 18). A standard curve was generated using RT-qPCR data (using the threshold cycle \( C_T \), the cycle number at which threshold fluorescence was reached) and the corresponding cell count, which was determined microscopically by 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) staining for the dilution series of the standard strains described elsewhere (17, 18). To determine the bacteria present in samples, three serial dilutions of an extracted RNA sample were used for RT-qPCR, and the \( C_T \) values in the linear range of the assay were applied to the standard curve generated in the same experiment to obtain the corresponding bacterial cell count in each nucleic acid sample, which was then converted to the number of bacteria per sample. The specificity of the RT-qPCR assay using group- or species-specific primers was determined as described previously (17, 18). The advantage of using ribosomal RNA rather than DNA is that one bacterial cell usually possesses 5-10 copies of DNA encoding ribosomal RNA, but contains approximately 10,000 copies of ribosomal RNA. This makes the measurement of ribosomal RNA by RT-qPCR far more sensitive than measuring genomic ribosomal DNA, thereby allowing relatively rare bacterial strains to be detected.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from mouse livers and colons using Sepasol reagent (Nakalai Tesche, Kyoto, Japan). First-strand cDNAs were synthesized using PrimeScript reverse transcriptase with oligo (dT). Quantitative real-time transcriptional PCR (qRT-PCR) was performed using SYBR Green PCR master mix (Invitrogen, Tokyo, Japan) on a CFX96 real time PCR system (Bio-Rad, Tokyo, Japan). Relative mRNA gene levels were normalized to the GAPDH mRNA level and relative expressions were determined by the comparative \( C_T \) method.

The designed primers were as follows: tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \) forward:
353 GTAGCCACGTCGTAAGCAAAC; 355 CTGGCACACCTAGTGGTTGTC, sterol regulatory element binding protein 1c (SREBP1c) forward: TAGAGCATATCCCCCAGGTG; SREBP1c reverse: GGTACGGGCCACAAGAAGAAGTA fatty acid synthase (FAS) forward: GCTGCGGAAACTTCAGGAAAT; FAS reverse: AGAGACGTGTCACTCCTGGACTT, tissue inhibitor of metalloproteinase1 (TIMP-1) forward: ATTCAAGGCTGTGGGAAATG; TIMP-1 reverse: CTCAGAGTACGCCAGGGAAC, α-smooth muscle actin (α-SMA) forward: ACCAACTGGGACGACATGGAA; α-SMA reverse: TGTCAGCAGTGTGGGATGCTC. Histochemical studies Paraffin-embedded sections were stained with hematoxylin and eosin (HE). For Oil Red-O staining, livers were frozen in liquid nitrogen and embedded in OTC compound. Colon sections were immunohistochemically stained with NFκBp65. Paraffin-embedded sections were deparaffinized in xylene and rehydrated. Sections were incubated with anti-NFκBp65 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C, followed by the rabbit ABC Staining system (Santa Cruz Biotechnology). Incubation with PBS instead of the primary antibody served as a negative control. Digital images of lesions were obtained with a multifunctional microscope (BZ-9000; KEYENCE Co, Osaka, Japan). In specimens containing positive cells, the positive cells were counted in nine randomly selected areas of 20× fields and analyzed by Image-J (National Institute of Health, MD, USA).

Biochemical analysis Serum alanine aminotransferase (ALT) activity was determined using a Transaminase C-II test Wako kit (Wako, Osaka, Japan). Hepatic total lipid was extracted and then assayed using the Folch method (13). The triglyceride content was assayed with the Triglyceride E test (Wako, Osaka, Japan). Serum lipopolysaccharide (LPS) concentrations were determined using the LAL kit endpoint QCL-1000 (Walkersville, MD, USA), according to
Analysis of the methionine and choline contents of the MCD diet versus those from LcS.

The methionine content of the MCD diet and that of LcS were analyzed by high performance liquid chromatography after performic acid oxidation and acid hydrolysis at the Yakult Central Institute. The choline content of the MCD diet and that of LcS were analyzed by Reinecke's salt precipitation method after acid hydrolysis at the Japan Food Research Laboratories (Tokyo, Japan).

Statistical analysis

Results are expressed as means ± S.E. Statistical significance was assessed using ANOVA followed by the Tukey HSD test. Variables not normally distributed were log-transformed before the analysis. All statistical analyses were performed using SPSS version 12.0 (SPSS, Chicago, IL, USA), and $P < 0.05$ was taken to indicate a statistically significant difference.
Results

Gut microbiota changes in response to MCD diet feeding and oral administration of LcS

Gut microbiota were examined in the three groups of mice fed the NCD, MCD diet and MCD diet plus LcS. The total numbers of bacteria were significantly lower in the MCD diet than in the NCD fed group, and oral administration of LcS did not affect the total amounts of gut bacteria (Fig. 1A).

The ratios of each bacterial subgroup to total bacteria are shown in Fig. 1B and C. Interestingly, the ratios of lactic acid bacteria, such as Bifidobacterium and Lactobacillus, in feces were markedly lower in the MCD than in the NCD fed group, whereas the ratios of Bacteroides fragilis group, Enterobacteriaceae and Enterococcus were higher. Oral administration of LcS to the MCD diet-fed mice increased the L.casei subgroup in feces, as expected. Interestingly, LcS also increased the proportions of other lactic acid bacteria such as Bifidobacterium in the feces of MCD diet fed mice. Amounts of archaean and fungal species were not measured in this study.

Analyses of the methionine and choline contents of the MCD diet and of LcS were performed for NCD, the MCD diet and LcS (Table 1). One gram of NCD, the MCD diet and LcS contains 6.0 mg, 0.053 mg and 0.050 mg of methionine, and 1.8 mg, 0.153 mg and less than 0.00456 mg of choline, respectively. Taking into consideration that one mouse ate 2-3 g of NCD or the MCD diet and 0.2 g of LcS, it was estimated that the MCD plus LcS group consumed more methionine, by 0.010 mg, and choline, by less than 0.000910 mg, than the MCD group (0.106-0.159 mg of methionine and 0.306-0.459 mg of choline, respectively). Given that the control group consumed 12-18 mg of methionine and 3.6-5.4 mg of choline, the amounts of methionine and choline from LcS are considered to be negligible.

LcS intervention suppressed NASH development

To investigate the effects of LcS on the development of NASH, mice...
were fed NCD, the MCD diet or the MCD diet plus oral administration of viable LcS for 6 weeks. Mice fed the MCD diet lost weight in comparison to those fed the NCD, while there was no significant body weight difference between the MCD diet and MCD diet plus LcS groups (data not shown). The livers were harvested and subjected to histological analysis. HE staining revealed marked increases in fat droplets, increased inflammatory cell infiltration and balloon-like structures in the livers of the MCD diet fed mice, while LcS intervention suppressed these abnormalities (Fig. 2A). In addition, Oil-Red-O staining showed highly advanced lipid accumulation in the livers of MCD diet fed mice, while lipid accumulation was minimal with LcS intervention (Fig. 2B). The hepatic triglyceride content data and serum ALT levels support these histological findings (Fig. 2C,D). This series of data showed LcS intervention to markedly attenuate MCD diet-induced NASH development.

Fibrotic change is the final step in the development of NASH, and is not observed in the setting of simple fatty liver. Collagen deposition was remarkable in the livers of the MCD-diet fed mice, while being suppressed in those receiving the LcS intervention (Fig. 3A). Hepatic stellate cell (HSC) activation is reportedly involved in collagen deposition and fibrosis (11). α-smooth muscle actin (α-SMA) is known to be a marker of HSC activation and tissue inhibitor of metalloproteinase1 (TIMP-1) expressed in activated HSCs reportedly plays a critical role in the process of liver fibrosis (14). The mRNA levels of α-SMA and TIMP-1 were revealed to be markedly elevated in the livers of the MCD diet fed mice, and these high expressions were normalized by LcS intervention (Fig. 3B). These data suggest LcS intervention to have suppressed HSC activation and liver fibrosis.

To investigate the molecular mechanisms underlying the resistance to NASH development conferred by LcS intervention, the expressions of genes involved in NASH pathogenesis were evaluated. Azan staining was performed to evaluate fibrotic changes in the liver.

LcS intervention suppressed liver fibrosis
LcS intervention, the mRNA expression levels of lipogenic enzyme genes such as SREBP-1c and FAS, \( \beta \)-oxidation enzyme genes, CPT-1 and CD36 were examined by quantitative real-time PCR. While MCD diet feeding significantly elevated the expression of SREBP-1c but not that of FAS, LcS intervention suppressed these mRNA levels as compared with the MCD diet alone (Fig. 4A). The mRNA expressions of \( \beta \)-oxidation enzymes and CD36 did not differ significantly between the MCD diet and the MCD diet plus LcS groups (data not shown).

Thus, protection from liver steatosis by LcS intervention may, at least partially, be attributable to reduced expressions of lipogenic enzymes.

In addition to lipid accumulation in hepatocytes, increased expressions of inflammatory cytokines play an important role in the pathogenesis of NASH. TNF-\( \alpha \) mRNA levels were upregulated in the MCD diet fed mice, but these increases were suppressed by LcS intervention (Fig.4B). The serum concentration of LPS, a possible inducer of hepatic inflammation, was markedly elevated in the MCD diet-fed mice as previously reported (6), and this MCD diet-induced elevation was significantly suppressed by LcS intervention (Fig. 4C).

As shown in Fig.4C, LcS reduced diet-induced serum LPS elevation. LPS produced in the gut would be absorbed into the blood stream with efficiencies dependent on \( \beta \)-oxidation enzyme genes, CPT-1 and CD36 were examined by quantitative real-time PCR. While MCD diet feeding significantly elevated the expression of SREBP-1c but not that of FAS, LcS intervention suppressed these mRNA levels as compared with the MCD diet alone (Fig. 4A). The mRNA expressions of \( \beta \)-oxidation enzymes and CD36 did not differ significantly between the MCD diet and the MCD diet plus LcS groups (data not shown).

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Thus, protection from liver steatosis by LcS intervention may, at least partially, be attributable to reduced expressions of lipogenic enzymes.
Discussion

Recently, much attention has been paid to the relationship between gut microbiota and various diseases, which raises the possibility of gut microbiota normalization becoming a therapeutic strategy. In fact, probiotic bacteria reportedly prevented hepatic damage and maintained colonic barrier function in a murine sepsis model (12). Lactic acid bacteria have also been recognized as a representative probiotic strain commercially available as a health food in a number of countries and prescribed as medicine for intestinal disorders such as diarrhea. In addition, in murine models, oral administration of LcS has been shown to improve drug-induced small intestinal injury (32), type 2 diabetes (19), and insulin resistance (22).

In this study, it was clearly demonstrated that orally administered LcS protected against MCD diet-induced NASH development in a rodent model. To rule out the effect of LcS being attributable simply to compensation for the methionine and choline deficiencies in the MCD diet, nutrient analyses were performed for comparisons among the control, MCD and MCD plus LcS groups. As shown in Table 1, the amounts of methionine and choline derived from the LcS were very low, such that the protective effect of LcS against MCD diet-induced NASH development is unlikely to be due to compensation for the methionine and choline deficiencies in this diet.

Regarding the pathogenesis of NASH, both hepatic steatosis and inflammation are key factors. In this study, the serum LPS concentration was shown to be elevated by MCD diet feeding, and, importantly, LcS administration suppressed this MCD diet-induced serum LPS elevation (Fig. 4C). LPS, a bacterial component sensed by toll-like receptor 4, is regarded as an inducer of hepatic inflammation. Obesity and high-fat diet feeding reportedly induced changes in gut microbiota and increased intestinal permeability by reducing tight junction protein expressions. These alterations would increase the incorporation of LPS into the bloodstream, leading to hepatic inflammation and metabolic disorders (2, 4). In fact, gut permeability and/or small intestinal bacterial overgrowth were reportedly observed more frequently in NASH
patients than in healthy subjects (20, 28).

Possible mechanisms underlying regulation of the serum LPS concentration by the MCD diet and LcS include gut barrier function as well as the amount of LPS produced in the gut due to the altered microbiota. Interestingly, LcS intervention increased the proportion of not only LcS but also those of other lactic acid bacteria, while fecal populations of lactic acid bacteria such as *Bifidobacterium* and *Lactobacillus* were markedly reduced by the MCD diet (Fig. 1). However, our gut microbiota analysis using RNA provided only the quantity of metabolically active bacteria rather than the semi-absolute number of bacteria.

Taking previous reports showing that some lactic acid bacteria contribute to the normalization of tight junction proteins (1, 21) and that intestinal inflammation induces loss of tight junction proteins (24) into consideration, protection against MCD diet-induced impairment of gut permeability by LcS intervention might be attributable to increased lactic acid bacteria and suppression of inflammation. Anti-inflammatory effects of LcS might be attributable not only to a reduced serum LPS concentration, but also to prevention of endotoxin-triggered inflammatory activation via a peroxisome proliferator-activated receptor-γ dependent mechanism as reported previously (31).

In addition, it should be noted that LcS intervention suppressed triglyceride accumulation (Fig. 2) with reductions in hepatic lipogenic enzyme expressions (Fig. 4A), taking place before the inflammation which occurs during NASH development. Given the previous study showing that mice intraperitoneally injected with LPS developed lipid accumulation in the liver with SREBP1c activation (5), protection from hepatic steatosis by LcS can also be explained as resulting from a reduced serum LPS concentration. Taking these observations together, we speculate that multiple mechanisms impacting lipid accumulation and inflammatory processes eventually lead to the suppression of hepatic inflammation, and the proposed mechanisms by which LcS intervention prevents the pathogenesis of NASH are summarized...
In conclusion, this is the first clear demonstration that LcS intervention suppresses the pathogenesis of NASH in a rodent model. In a future study, we hope to assess whether normalization of gut microbiota might be an effective strategy for treating various disorders.
References


**Figure legends**

**Fig. 1. Gut microbiota changes in response to LcS intervention**
Mice were fed the normal chow diet (NCD), the methionine-choline deficient (MCD) diet, or the MCD diet plus daily oral administration of *Lactobacillus casei* strain Shirota (MCD+LcS) for 6 weeks. We collected the feces and their bacterial contents were analyzed. Numbers of viable bacteria were expressed as cells per gram of feces.

A. Bacteria in feces in each group
B. Bar chart of relative abundances of different bacterial species expressed as the percentage of total bacteria
C. Relative abundances of different bacterial species expressed as the percentage of total bacteria

Data are presented as means ± SE. *Statistical significance P<0.05.

**Fig. 2. LcS intervention suppressed NASH development**
Mice were fed the NCD, the MCD diet, or the MCD diet plus daily oral administration of LcS for 6 weeks and then sacrificed.

A. Liver sections were stained with HE. Magnification, ×20.
B. Liver sections were stained with Oil-Red-O. Magnification, ×20.
C. Hepatic triglyceride levels were measured.
D. Serum ALT levels were measured.

Data are presented as means ± SE. *Statistical significance P<0.05.

**Fig. 3. LcS intervention suppressed liver fibrosis**
Mice were fed the NCD, the MCD diet, or the MCD diet plus daily oral administration of LcS for 6 weeks and then sacrificed.

A. Liver sections were stained with Azan. Magnification, ×20. Positively-stained areas were assessed employing NIH image.
B. Hepatic mRNA levels of α smooth muscle actin (α-SMA) and tissue inhibitor of
metalloproteinase 1 (TIMP-1) were measured by quantitative real-time PCR. Data are presented as means ± SE. *Statistical significance P<0.05.

Fig. 4. LcS intervention suppressed the expressions of genes involved in NASH pathogenesis. Mice were fed the NCD, the MCD diet, or the MCD diet plus daily oral administration of LcS for 6 weeks and then sacrificed.

A. Hepatic mRNA levels of sterol regulatory element binding protein 1c (SREBP1c) and fatty acid synthase (FAS) were measured by quantitative real-time PCR (qRT-PCR).

B. Hepatic mRNA level of tumor necrosis factor α (TNF-α) was measured by qRT-PCR.

C. Serum lipopolysaccharide (LPS) was measured. Data are presented as means ± SE. *Statistical significance P<0.05.

Fig. 5. LcS intervention suppressed colon inflammation

Mice were fed the NCD, the MCD diet, or the MCD diet plus daily oral administration of LcS for 6 weeks and then sacrificed.

Immunohistochemical staining for NFκBp65. Incubation with PBS instead of the primary antibody served as a negative control (NC). Magnification, ×20. Positively-stained cells were counted employing NIH image.

Fig. 6. Schema showing the effects of LcS on the pathogenesis of NASH
Table 1. Analyses of the methionine and choline contents of the NCD, MCD diet and LcS

Methionine and choline contents of the MCD diet and those derived from LcS were analyzed at the Yakult Central Institute Chemical Analysis Center (Tokyo, Japan), and Japan Food Research Laboratories (Tokyo, Japan), respectively.

Then, after considering the daily intakes of one mouse, the methionine and choline intakes from the NCD, MCD diet and LcS were compared with that from the NCD diet taken as 100%.

A. Methionine content per gram, given amount per day and amount of methionine consumed by one mouse per day from the NCD, MCD diet and LcS

B. Choline content per gram, given amount per day and amount of choline consumed by one mouse per day from the NCD, MCD diet and LcS
**Figure 1**

**A**

Log$_{10}$ cells/g feces for various bacterial groups across different conditions. Bars represent the mean with error bars indicating standard error.

**B**

Relative abundances (% of total bacteria) for bacterial groups across different conditions. Bars represent the distribution of each group as a percentage of the total bacterial count.

**C**

Relative abundances (% of total bacteria) for specific bacterial subgroups across different conditions. Bars represent the mean with error bars indicating standard error.
Figure 3

A

NCD  MCD  MCD+LcS

B

α-SMA  TIMP-1

Relative mRNA level

NCD  MCD  MCD+LcS  NCD  MCD  MCD+LcS

*  *
Figure 4

A  

**SREBP1c**

![Bar Chart: SREBP1c levels across NCD, MCD, and MCD+LcS conditions.]

- NCD: Relative mRNA level
- MCD: Higher relative mRNA level
- MCD+LcS: Lower relative mRNA level

B  

**TNF-α**

![Bar Chart: TNF-α levels across NCD, MCD, and MCD+LcS conditions.]

- NCD: Lower relative mRNA level
- MCD: Higher relative mRNA level
- MCD+LcS: Lower relative mRNA level

C  

**FAS**

![Bar Chart: FAS levels across NCD, MCD, and MCD+LcS conditions.]

- NCD: Relative mRNA level
- MCD: Higher relative mRNA level
- MCD+LcS: Lower relative mRNA level

- Additionally, serum LPS (EU/ml) levels are shown:
  - NCD: 0.5 EU/ml
  - MCD: 3 EU/ml
  - MCD+LcS: 1.5 EU/ml

* indicates statistical significance.
Figure 5

- NC
- NCD
- MCD
- MCD+LcS

Bar chart showing NFκBp65 positive cells:
- NCD
- MCD
- MCD+LcS
Figure 6

LcS administration

- Gut microbiota change
  - Inflammation↓
  - Permeability↓

- LPS etc.
  - Translocation↓

- Hepatocyte
  - Steatosis↓

- Kupffer cell
  - Inflammation↓

- Stellate cell
  - Fibrosis↓

↓

NASH↓

Colon

Portal vein

Liver
### Table 1

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