

# Oral Recombinant Methioninase Prevents Nonalcoholic Fatty Liver Disease in Mice on a High Fat Diet

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**Abstract.** *Background/Aim:* We have recently shown that oral recombinant methionase (o-rMETase) prevents obesity and diabetes onset in mice on a high-fat (HF) diet. The present study aimed to determine if o-rMETase can inhibit the onset of nonalcoholic fatty liver disease (NAFLD) onset in mice on a high-fat diet. *Materials and Methods:* Male C57BL/6J mice in the control group were fed a normal-fat diet (NFD) (+6.5% fat), and other mice were fed a high-fat (HF) diet (+34.3% fat). Then, the mice on the HF diet were divided into two dietary groups: i) HF+phosphate buffered saline (PBS) group, and ii) HF+o-rMETase group. *Result:* The fatty change score in the livers of mice treated with HF+PBS increased to an average of 2.6 during the experimental period of 8 weeks. In contrast, the fatty change in the livers of mice on the HF+o-rMETase group had an average score of 0.92 ( $p=0.04$ , HF+PBS vs HF+o-rMETase). *Conclusion:* o-rMETase inhibited the onset of NAFLD as well as prevented obesity and the onset of diabetes on a high-fat diet, offering a possibility of a new paradigm to prevent liver cirrhosis or liver cancer via NAFLD.

The population of nonalcoholic fatty liver disease (NAFLD) patients is rapidly increasing worldwide due to the increase of obesity and diabetes (1). NAFLD causes liver cancer without liver cirrhosis (2). Thus, decreasing the onset of NAFLD is a goal to prevent liver cirrhosis and liver cancer worldwide. Methionine restricted (MR) diets in rodents have been shown to prevent obesity and improve hepatic steatosis (3). MR diets also prevent obesity and diabetes in rodent models (4, 5). We have previously shown that oral recombinant methionase (o-rMETase) is a much more effective means of MR than an MR diet to prevent obesity (6) and onset of diabetes (7) in mice on a high-fat diet.

In the present report, we show that o-rMETase inhibits NAFLD onset in mice on a high fat diet, thereby opening a new paradigm to prevent NAFLD.

## Materials and Methods

*Animal studies.* C57BL/6 mice, aged 8 weeks (AntiCancer Inc, San Diego, CA), were used in this study. Mice were housed in a barrier facility on a high efficacy particulate air (HEPA)-filtered rack under standard conditions of 12-h light/dark cycles. Animal studies were performed with an AntiCancer Institutional Animal Care and Use Committee (IACUC)-protocol (AC-HFD-3), specially approved for this study and in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

*Recombinant methioninase.* Recombinant L-methionine  $\alpha$ -deamino- $\gamma$ -mercaptomethane lyase [recombinant methioninase, (rMETase)] (EC 4.4.1.11) from *Pseudomonas putida* has been previously cloned and is produced in *Escherichia coli* (AntiCancer, Inc.). rMETase is a homotetrameric PLP enzyme of a 172-kDa molecular mass (8).

*Study design.* Mice were randomized into three groups: i) standard diet (6.5% fat) without treatment ( $n=3$ ), ii) high fat (HF) diet (34.3% fat), treated with phosphate-buffer saline (PBS) by oral gavage

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**Key Words:** Methioninase, methionine restriction, MR, fatty liver, NAFLD, mice.

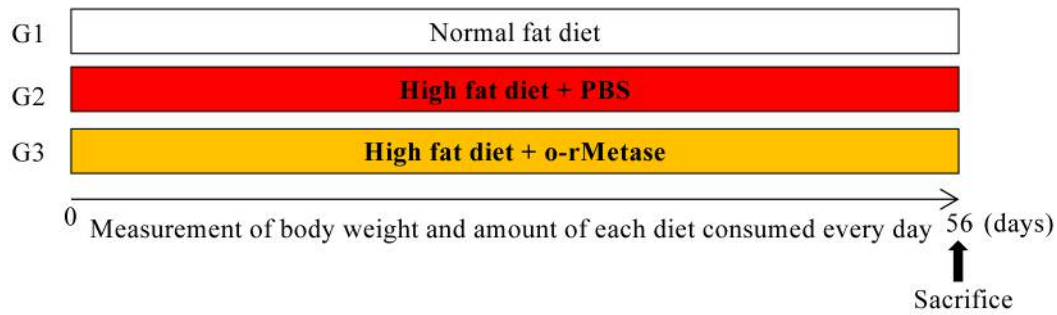


Figure 1. Treatment schematic of the study protocol. G1: Normal fat diet (NFD) (untreated control) (n=3); G2: High fat diet (HFD)+PBS (50  $\mu$ l/day, twice a day, oral gavage, daily) (n=3); G3: HFD+o-rMETase (50 units/dose, twice a day, oral gavage) (n=5).

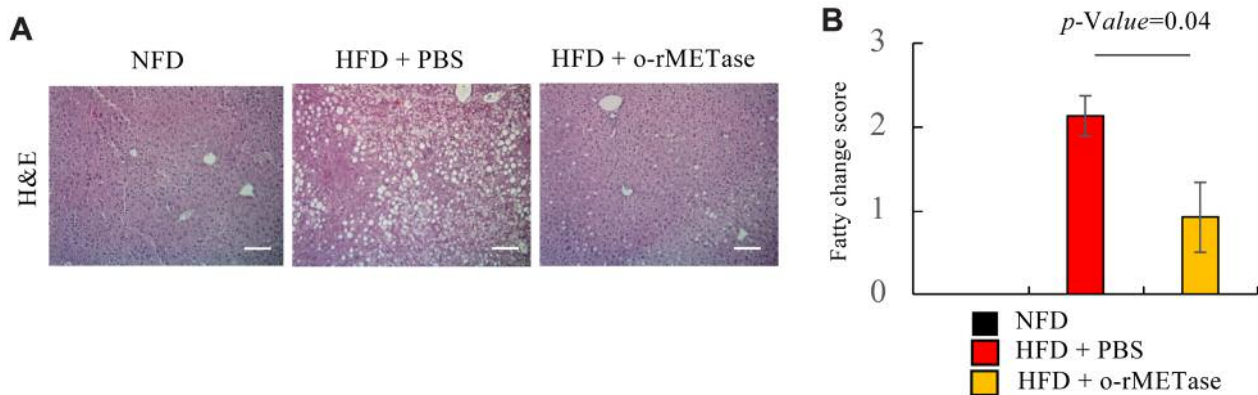


Figure 2. o-rMETase inhibited HF-diet-induced fatty change in the liver. A) Following an 8-wk high fat (HF) diet, mouse liver tissue was stained with hematoxylin & eosin and showed reduced steatosis in the o-rMetase group compared to the HF+PBS group. B) Quantitative display of the o-rMETase group reduced fatty liver. Scale bar=100  $\mu$ m.

(n=3), iii) HF diet with o-rMETase (100 units per dose, twice a day, 56 consecutive days, by oral gavage) n=5) (Figure 1). C57BL/6 mice were fed either a standard global rodent diet (Teklad 2020x, Harlan laboratories, Indianapolis, IN, USA) or HF diet chow containing 60% kcal from fat (Teklad TD.06414, Harlan laboratories, Indianapolis, IN, USA) for 56 days. Each mouse was given the experimental diet in accordance with a pair-feeding protocol. Therefore, daily food intake and energy, protein and fat intake did not differ among the groups.

**Physiological and Biochemical determinations.** Body weight and dietary intake were recorded daily. All examinations were performed on day 56. Serum, separated from orbital eye bleeding, was measured for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) with a Transaminase CII Test kit® (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) (9). Liver tissue was collected and measured after euthanasia.

**Histological assessment of liver tissue.** Whole liver samples were fixed in 10% phosphate-buffered formalin for  $\geq 5$  days. Paraffin sections (4  $\mu$ m) were stained with hematoxylin and eosin (H&E). The NASH clinical research network scoring system was used to

histologically grade NAFLD in the mouse liver (10). Steatosis degree (0-3) was determined as i) 0=5% hepatocytes involved, ii) 1=5-33% of hepatocytes involved, iii) 2=33-66% hepatocytes involved, and iv) 3 $\geq$ 66% hepatocytes involved (10). Hepatocyte ballooning score (0-2) was determined as i) 0=none, ii) 1=few ballooned cells, and iii) 2=many cells or prominent ballooning (10).

Lobular inflammation score (0-3) was determined as i) 0=none, ii) 1=<2 foci per  $\times 200$  field, iii) 2=2-4 foci per  $\times 200$  field, and iv) 3= $\geq 4$  foci per  $\times 200$  field (10). These scores are graded semi-quantitatively. The NAFLD Activity Score (NAS) (0-8) was assessed by a combination of each score (10).

**Statistical analyses.** All data are presented as a mean $\pm$ standard error of the mean (SEM). The Student *t*-test was performed. A *p*-value of  $\leq 0.05$  was considered significant.

## Results

**Efficacy of o-rMETase to inhibit fatty change of the liver in mice on the high-fat diet.** After eight weeks on the high-fat diet, the HF+PBS group showed higher levels of fatty

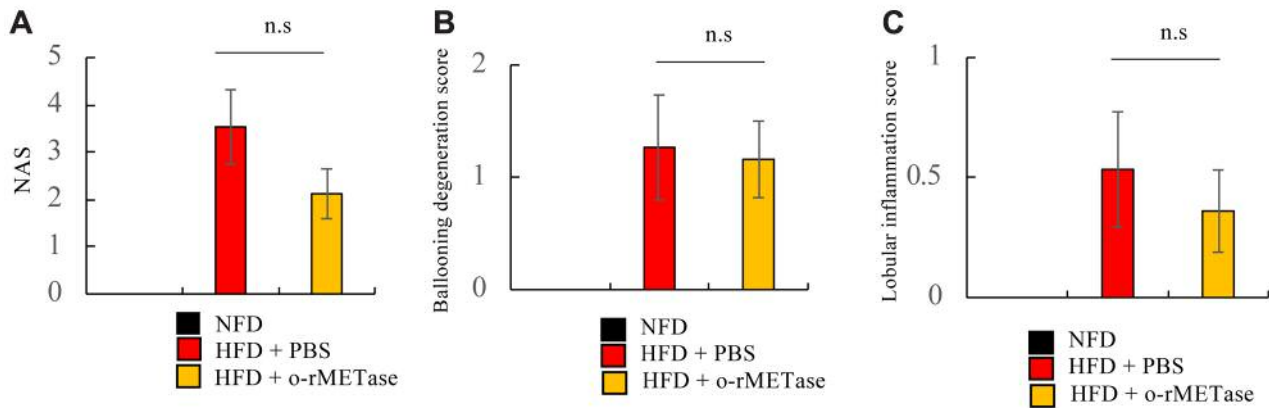


Figure 3. NAS, ballooning degeneration and lobular inflammation. A) Hematoxylin & eosin stained slides from the mice on the normal-fat diet (NFD), high-fat diet (HFD) treated with PBS and the HFD treated with oral recombinant methioninase (o-rMETase) were analyzed for A) non-alcoholic fatty liver disease (NAFLD) activity score (NAS), B) Ballooning degeneration score and (C) Lobular inflammation score. n.s: Non-significant.

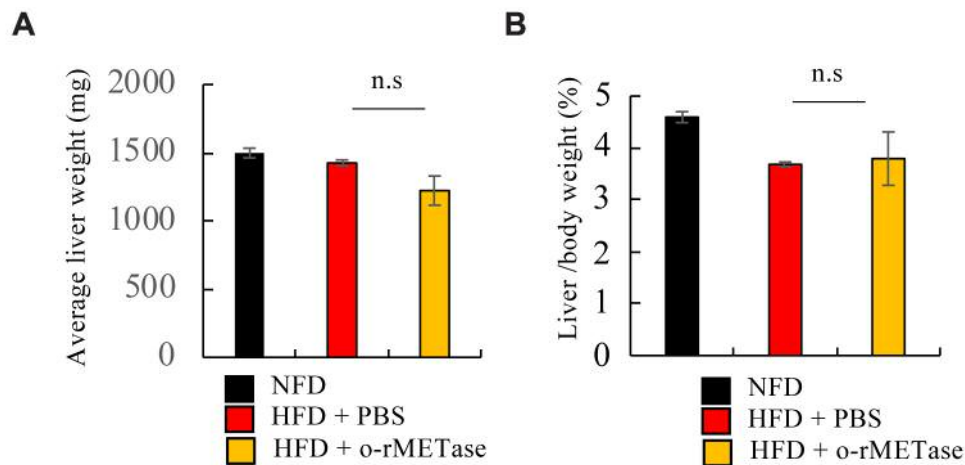


Figure 4. Liver weight of animals in NFD, HFD+PBS and HFD+o-rMETase groups after 8 weeks treatment. A) Average liver weight, B) Ratio of liver weight to body weight. NFD: Normal-fat diet; HFD: High fat diet; n.s: non-significant.

change compared to the HF+o-rMETase group (Figures 2A and B) ( $p=0.04$ ). The NAS score, ballooning degeneration score and lobular inflammation score were not significantly affected by o-rMETase in mice on the high-fat diet compared to the HF+PBS group (Figures 3A-C). Average liver weight and liver/body weight were not different between the other groups (Figures 4A and B).

*Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in o-rMETase-treated and untreated mice on a high fat diet were measured in the serum from mice in each group. AST and ALT were not affected by o-rMETase in mice on the high-fat diet compared to the HF+PBS group (Figures 5A and B), as they were not elevated to a large extent by the high-fat diet. AST and ALT are markers of liver damage (11).*

## Discussion

The global rise of NAFLD is an urgent problem that comes as a result of the rapid increase in the prevalence of obesity, diabetes and metabolic syndrome (1). NAFLD is the greatest risk factor for cirrhosis, liver failure and liver cancer. Currently, specialists are predicting that fatty-liver-related cirrhosis will be leading reason for liver transplants in the next 10-20 years, displacing hepatitis C- and alcohol- related liver transplants (12). However, NAFLD does not have approved therapeutics and interventions that can halt the progression of the disease to steatosis and fibrosis. One of the biggest goals of research in this area has been to identify effective treatment.

NAFLD is more prevalent in patients with obesity. Cognitive behavioral therapy and diet therapy are the most

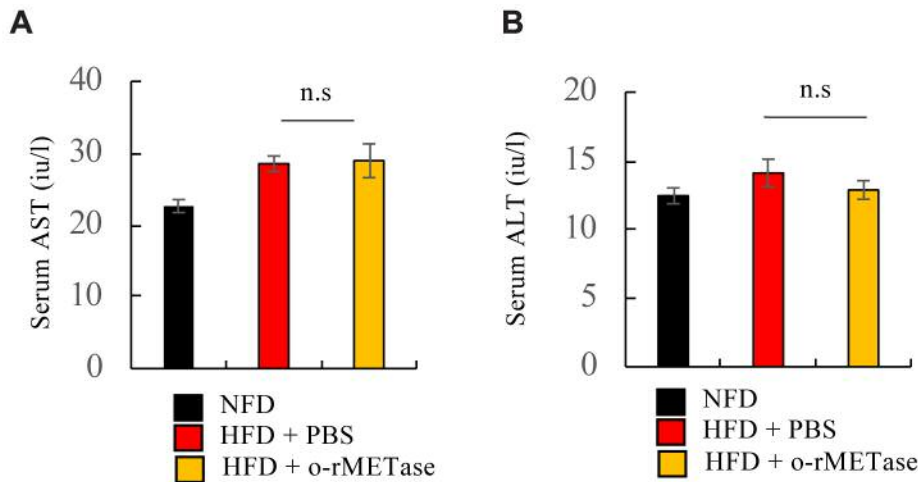


Figure 5. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in NFD, HFD+PBS and HFD+o-rMETase groups after 8 weeks of treatment. A) AST and B) ALT. n.s.: Non-significant.

frequent treatments for NAFLD (13, 14). However, both these therapies are not efficient and they have a high dropout rate (15). Additionally, body weight easily rebounds because it is difficult to maintain these regimens (16). Bariatric surgery has recently become popular in obesity patients, with an improvement in liver histology and function (17); however, bariatric surgery often involves complications, insufficient efficacy and high rates of recurrence (18-22).

Methionine, an essential amino acid, is absorbed in the small intestine and is used for protein synthesis as well as for the production of S-adenosylmethionine, which is the universal methyl donor (23). Dietary MR, such as with a vegan diet, has been demonstrated to prevent the progression of steatosis in rodent models and suggests a potential nutritional strategy of dietary MR to prevent NAFLD (3, 25). However, it is difficult to maintain dietary MR in daily life as it requires severe dietary changes (26).

Our laboratory developed rMETase from *Pseudomonas putida*, cloned in *Escherichia coli*, as a more efficient means of MR compared to dietary MR (8). We have shown that o-rMETase prevents obesity (6), and diabetes onset (7) in mice on a high fat diet. The present study is the first demonstration of the efficacy of o-rMETase to inhibit the progression of steatosis in mice on a high-fat diet. In the present study, fatty change in the liver of the o-rMETase-treated mice on a high-fat diet was significantly suppressed as a result of the o-rMETase treatment compared to the liver of the PBS-treated mice on a high-fat diet. Furthermore, body weight gain in the o-rMETase-treated mice on a high-fat diet was significantly lower compared to the PBS-treated mice on a high-fat diet (6). Thus, o-rMETase may be a new beneficial strategy to prevent steatosis as well as prevent obesity and diabetes..

There are no approved therapeutics for patients with NAFLD (27). Current recommendations involve pioglitazone, an anti-diabetic drug that controls high blood sugar, and vitamin E, both of which are effective (28). However, pioglitazone should not be used to treat NAFLD without the diagnosis of a biopsy-proven nonalcoholic steatohepatitis (NASH) and vitamin E has insufficient evidence for its use in diabetics (14). Dietary active vitamin D supplementation also has potential benefits for NAFLD (29), but it is not yet fully approved. o-rMETase has the potential to possibly eliminate the need for lifestyle modifications or other drugs to prevent NAFLD; however, this will need to be tested in the clinic.

In summary, we demonstrate for the first time the efficacy of o-rMETase to prevent NAFLD in mice on a high fat diet, suggesting a possible new paradigm for prevention of NAFLD.

## Conflicts of Interest

The Authors declare no competing financial interests.

## Authors' Contributions

Y.T. and R.M.H designed and performed experiments, analyzed data and wrote the paper; Q.H., M.Z. and Y.T. provided reagents; N.S., J.Y., H.N., A.T., M.M., M.B. and H.N. gave technical support and conceptual advice.

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