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# Influence of age in weeks on the development and progression of nonalcoholic steatohepatitis in a diet-induced Sprague-Dawley rat model

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**Background:** Understanding the pathogenesis of nonalcoholic steatohepatitis (NASH) in humans has been hampered by the lack of a comprehensive and physiological small animal model of NASH. We previously reported a dietary (high-fat and high-cholesterol; HFC diet) -induced NASH model that developed advanced fibrosis within a relatively short period (9 weeks) using Sprague-Dawley (SD) rats (age, 9 weeks).
**Methods:** In this study, we evaluated the age-related alterations of NASH in 9-, 18-, and 27-week-old male SD rats that were fed an HFC diet (30% fat, 1.25% cholesterol, and 0.5% sodium cholate, w/w) for 9 weeks (six rats/group).
**Results:** Age-dependent increases in serum transaminases, insulin, and insulin resistance index were observed with or without a significant difference after the 9-week rearing period. Histopathological findings such as hepatic steatosis, lobular inflammation, and hepatocyte ballooning were similar regardless of age, but hepatic fibrosis was more evident in the older groups. Rats in all three groups developed NASH at a high rate (83.3% or higher in each group). The mRNA levels of fibrosis-related genes encoding transforming growth factor-*β* (TGF-*β*) and α-smooth muscle actin (*α*-SMA) in the liver were low in the youngest group and high in the older groups, although this difference was not statistically significant.
**Conclusion:** These results and those from our previous study indicate that a 9-week HFC diet-induced NASH model using SD rats can be applied a relatively wide range of ages (5-27 weeks of old), and that the risk of NASH-related fibrosis increases with age.

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**Key words:** Nonalcoholic steatohepatitis, Animal model, High-fat high-cholesterol diet, Age

## Introduction

Nonalcoholic steatohepatitis (NASH) is the inflammatory subtype of nonalcoholic fatty liver disease (NAFLD), which is one of the most common causes of liver disease worldwide, and has a potentially progressive course that can lead to liver cirrhosis and/or hepatocellular carcinoma.[1,2] Despite its high clinical and socioeconomic burden, the underlying mechanisms of NASH progression have not been completely elucidated; therefore, no efficient pharmacological treatment strategies for NASH are currently available.[3,4]

Liver fibrosis is the main determinant of mortality in patients with NASH.[5] Results of animal studies analyzing the role of diet in the development of NASH suggest that a high-fat and high-cholesterol (HFC) diet is crucial for the progression of NASH.[6] We recently established a dietary (HFC diet)-induced model of NASH that developed advanced fibrosis within a relatively short period (9 weeks) using 9-week-old Sprague-

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Dawley (SD) rats.[7] Having a rodent NASH model that rapidly develops fibrosis would help clarify the mechanisms of disease, aid in the development of therapeutic strategies, and assist in the evaluation of the effects of drugs in vivo.

Aging is associated with a physiological increase in lipid accumulation in non-adipose tissues, including the liver,[8] and therefore, age is strongly associated with the development and progression of NAFLD/NASH.[9,10] In this study, we evaluated the age-related alterations of NASH in 9-, 18-, and 27-week-old male SD rats that were fed an HFC diet for 9 weeks by examining the serology, histopathology, and expression levels of cholesterol or lipid metabolism-, inflammation-, fibrosis-, and oxidative stress-related genes in the liver.

## Materials and methods

### *Animals and experimental design*

Eight-week-old (S group, n=6), 17-week-old (M group, n=6), and 26-week-old (L group, n=6) male SD rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed individually in a temperature- and humidity-controlled room (22-24℃ and 50-60% relative humidity) with a 12-hour light/dark cycle. After 1 week of acclimation with standard rodent chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*, the rats were fed an HFC diet for 9 weeks. The HFC diet was prepared by mixing the MF with 30% (w/w) palm oil, 1.25% (w/w) cholesterol, and 0.5% (w/w) sodium cholate.[8] The proximate dietary composition of the HFC diet is shown in Table 1. Daily energy intake and body weight were monitored throughout the study. After the 9-week rearing period, the rats at 18, 27, and 36 weeks of age were fasted for at least 6 hours and sacrificed under anesthesia with isoflurane. Blood was collected from the inferior vena cava or heart. The epididymal fat pad and liver were removed, washed in cold saline, and weighed. Liver tissues were either placed in 10% neutral buffered formalin or snap frozen in liquid nitrogen and stored at -80˚C. All procedures performed on the animals were approved by the Animal Use Committee of University of Nagasaki (Approval No. R01-13), and the animals were maintained in accordance with the University of Nagasaki guidelines for the care and use of laboratory animals.

**Table 1.** Proximate composition of HFC diet

| Constituents | |
|---|---|
| Water (g) | 5.49 |
| Crude protein (g) | 16.05 |
| Crude lipid (g) | 3.54 |
| Crude ash (g) | 4.03 |
| Crude fiber (g) | 1.95 |
| Nitrogen free extract (g) | 38.43 |
| Palm oil (g) | 28.75 |
| Cholesterol (g) | 1.25 |
| Sodium cholate (g) | 0.50 |
| Total (g) | 100.00 |
| Protein energy ratio (%) | 12.63 |
| Lipid energy ratio (%) | 57.15 |
| Carbohydrate energy ratio (%) | 30.23 |
| Energy (kcal/100 g) | 508.60 |

HFC, high-fat and high-cholesterol.

### *Serum biochemical analysis*

Serum triglyceride (TG), total cholesterol (TC), free fatty acid (FFA), and glucose levels were determined using Triglyceride E-Test Wako, Cholesterol E-Test Wako, NEFA C test Wako, and Glucose C II Test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively. Serum insulin was measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan). Insulin resistance index was calculated using the homeostasis model of assessment (serum glucose [mg/dL] × serum insulin [ng/mL] / 405), and the relative levels were evaluated among groups. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using Transaminase C II test Wako (FUJIFILM Wako Pure Chemical Corporation). Serum leptin and adiponectin levels were measured using a mouse/rat leptin ELISA kit (Morinaga Institute of Biological Science) and a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan), respectively.

### *Hepatic lipid analysis*

Hepatic lipids were extracted from the frozen liver using the method described by Folch et al.[11] The extract was dissolved in isopropanol and analyzed for TG and TC with a kit, as described above.

### *Histopathological assessment of the liver*

After fixation in neutral buffered formalin, the liver tissues were embedded in paraffin, sectioned, and stained with Azan as well as hematoxylin and eosin. All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental data. Histological findings

from the liver were scored using the NASH Clinical Research Network Scoring System based on the following four semi-quantitative factors: steatosis (0-3), lobular inflammation (0-3), hepatocyte ballooning (0-2), and fibrosis (0-4). The NAFLD activity score (NAS) was defined as the unweighted sum of scores for steatosis, lobular inflammation, and hepatocyte ballooning. NAS scores ≥5 and ≤2 were considered to be diagnostic and not diagnostic, respectively, for steatohepatitis.[12] The scores for fibrosis were further classified as a score of 0.5, representing scores between 0 and 1.

*Quantification of mRNA using real-time reverse transcription polymerase chain reaction*

Total RNA from the liver was extracted using RNAiso Plus (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA templates using a commercial kit (PrimeScript RT Master Mix, Takara Bio). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as described previously.[7] Specific primers were designed using the Primer-BLAST primer-designing tool (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) and were synthesized by Greiner Bio-One Japan (Tokyo, Japan) (Table 2). The levels of mRNA relative to those of the internal control acidic ribosomal phosphoprotein (36B4) mRNA (*Rplp0*) were determined using the 2-ΔΔCt method. For studies in rats, the hepatic expression of genes involved in cholesterol or lipid metabolism (*Nr1h3* encoding liver X receptor-*α* [LXR-*α*], *Mttp* encoding microsomal triglyceride transfer protein [MTP], *Fasn* encoding fatty acid synthase [FAS], *Gpam* encoding glycerol-3-phosphate acyltransferase 1 [GPAT1], *Cpt1a* encoding carnitine palmitoyltransferase-1 [CPT-1], *Acox1* encoding acyl-CoA oxidase [AOX], *Srebf1* encoding sterol regulatory element-binding protein-1c [SREBP1c], and *Nr1h4* encoding farnesoid X receptor [FXR]), inflammation (*Tnf* encoding tumor necrosis factor-*α* [TNF-*α*], *Ccl2* encoding monocyte chemoattractant protein-1 [MCP-1], *Il1b* encoding interleukin-1*β* [IL-1*β*], *Il6* encoding interleukin-6 [IL-6], and *Nfkb* encoding nuclear factor-*κ*B [NF-*κ*B]), fibrosis (*Col1a1* encoding collagen type I alpha 1 [COL1A1], *Tgfb1* encoding transforming growth factor-*β* [TGF-*β*], and *Acta2* encoding *α*-smooth muscle actin [*α*-SMA]), and oxidative stress (*Cyp2e1* encoding cytochrome P450 family 2 subfamily E polypeptide 1 [CYP2E1], *Hmox1* encoding heme oxygenase-1 [HO-1], *Gpx1* encoding glutathione peroxidase-1 [GPX-1], and *Sod2* encoding manganese superoxide dismutase [MnSOD]), were quantified. All data were expressed as the fold-change relative to the S group.

**Table 2.** Primer sequences for real-time reverse transcription polymerase chain reaction

| Primer | Sequence (5' to 3') |
|---|---|
| *Nr1h3* | Forward: CAGGACCAGCTCCAAGTAGA |
| | Reverse: GAACATCAGTCGGTCGTGG |
| *Mttp* | Forward: CAAGCTCAAGGCAGTGGTTG |
| | Reverse: AGCAGGTACATCGTGGTGTC |
| *Fasn* | Forward: CAACATTGACGCCAGTTCCG |
| | Reverse: TTCGAGCCAGTGTCTTCCAC |
| *Gpam* | Forward: GCTACCTGAAGGTGAGCCAG |
| | Reverse: AGGTACTCAGACTCCGGGAC |
| *Cpt1a* | Forward: AACCTCGGACCCAAATTGC |
| | Reverse: GGCCCCGCAGGTAGATATATT |
| *Acox1* | Forward: CCACTGAACAAAACAGAGGTCC |
| | Reverse: GTCCCAGGGAAACTTCAAAGC |
| *Srebf1* | Forward: CATGGACGAGCTACCCTTCG |
| | Reverse: GAAGCATGTCTTCGATGTCGG |
| *Nr1h4* | Forward: TGGGAATGTTGGCTGAATGTTTG |
| | Reverse: TGCTAGCTTGGTCGTGGAG |
| *Tnf* | Forward: TGATCGGTCCCAACAAGGA |
| | Reverse: TGGGCTACGGGCTTGTCA |
| *Ccl2* | Forward: TCTGTCACGCTTCTGGGCCTGT |
| | Reverse: GGGGCATTAACTGCATCTGGCTGAG |
| *Il1b* | Forward: CTCCAGTCAGGCTTCCTTGTG |
| | Reverse: GGTCATTCTCCTCACTGTCGAAA |
| *Il6* | Forward: GATACCACCCACAACAGACCAGTA |
| | Reverse: TGCACAACTCTTTTCTCATTTCCA |
| *Nfkb1* | Forward: TGACATCATCAACATGAGAAACGA |
| | Reverse: CCCCAACCCTCAGCAAGTC |
| *Col1a1* | Forward: GCGTAGCCTACATGGACCAA |
| | Reverse: AAGTTCCGGTGTGACTCGTG |
| *Tgfb1* | Forward: CTTTGTACAACAGCACCCGC |
| | Reverse: TAGATTGCGTTGTTGCGGTC |
| *Acta2* | Forward: GCCAAGAAGACATCCCTGAAGT |
| | Reverse: TGTGGCAGATACAGATCAAGCAT |
| *Cyp2e1* | Forward: CCCATCCTTGGGAACATTTTT |
| | Reverse: GCCAAGGTGCAGTGTGAACA |
| *Hmox1* | Forward: CACAGGGTGACAGAAGAGGCTAA |
| | Reverse: GGGACTCTGGTCTTTGTGTTCCT |
| *Gpx1* | Forward: GCTGCTCATTGAGAATGTCG |
| | Reverse: GAATCTCTTCATTCTTGCCATT |
| *Sod2* | Forward: GACCTGCCTTACGACTATG |
| | Reverse: TACTTCTCCTCGGTGACG |
| *Rplp0* | Forward: GGTGTTTGACAATGGCAGCAT |
| | Reverse: ATTGCGGACACCCTCTAGGA |

*Statistical analysis*

All values were expressed as the mean ± standard error (SE). Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA),

followed by Bonferroni's post hoc test, or the chi-squared test. Correlations between two variables were determined by Spearman's rank correlation coefficient. All analyses were performed using IBM SPSS statistics software, version 26 (IBM, Chicago, IL, USA) on a computer with a Windows operating system. A p value <0.05 was considered to indicate statistical significance.

## Results

*Cumulative energy intake, body weight and relative organ weights*

The initial and final body weights were lowest in the S group and highest in the L group. Although cumulative energy intake during the 9-week rearing period did not differ significantly among the three groups, body weight gain and food efficacy, which was calculated using the following formula: [body weight gain (g) / cumulative energy intake (kcal)] × 100, were highest in the S group and lowest in the L group. After the 9-week rearing period, the liver weight/body weight ratio was significantly higher in the S group than in the M and L groups (p=0.002 and p=0.003, respectively), whereas the epididymal fat pad weight/body weight ratio did not differ significantly among the groups (Table 3).

*Serum biochemical parameters and hepatic lipid concentrations*

After the 9-week rearing period, the serum levels of TG, TC, FFA, glucose, insulin, ALT, and leptin were not significantly different among the three groups; insulin resistance index was also similar among groups. The serum AST level was significantly higher in the L group than in the S and M groups (p<0.001 and p=0.042, respectively). The serum adiponectin level was significantly higher in the M group than in the L group (p=0.009). The hepatic TG concentration was not significantly different among the three groups, whereas the hepatic TC concentration was significantly higher in the S group than in the M group (p=0.039, Fig. 1).

*Histopathological findings of the liver*

Figure 2 shows representative histopathological findings after the 9-week rearing period in the liver of the S, M, and L groups. The histopathological assessments after the 9-week rearing period are shown in Table 4. Severe steatosis (score of 3) was observed in all six rats in the S group, and in five of six rats in both the M and L groups. The remaining two rats (one M group and one L group rat) showed moderate steatosis (score of 2). Moderate or severe lobular inflammation (score of 2 or 3) was observed in all 12 rats in the S and L groups, and in five of six rats in the M group. The remaining one rat in the M group showed mild lobular inflammation. Hepatocyte ballooning was not observed in two of six rats and a few ballooning hepatocytes (score of 1) were observed in four of six rats in each of the three groups. According to the NAS score,[12] all six rats in the S group and five (83.3%) of the six rats in both the M and L groups were diagnosed with NASH. The NAS score for the remaining two rats (one M group and one L group rat) was 4, representing "borderline NASH". Hepatic fibrosis was not observed in four (66.7%) of six rats in the S group, two (33.3%) of six rats in the M group, and one (16.7%) of six rats in the L group, whereas mild perisinusoidal and/or portal/periportal fibrosis (score of 1) was observed in one (16.7%) of six rats in the S group and

**Table 3.** Cumulative energy intake, body weight and relative organ weights after 9-week rearing period.

| Item / Group | S (n=6) | M (n=6) | L (n=6) |
|---|---|---|---|
| Initial body weight (g) | 306 ± 3[a] | 442 ± 6[b] | 552 ± 8[c] |
| Final body weight (g) | 497 ± 11[a] | 554 ± 13[b] | 608 ± 9[c] |
| Body weight gain (g) | 191 ± 8[a] | 112 ± 11[b] | 56 ± 7[c] |
| Cumulative energy intake (kcal) | 6341 ± 135 | 6534 ± 190 | 6619 ± 70 |
| Food efficacy* | 3.00 ± 0.08[a] | 1.69 ± 0.11[b] | 0.84 ± 0.10[c] |
| Liver weight / body weight (%) | 5.30 ± 0.14[a] | 4.44 ± 0.13[b] | 4.49 ± 0.15[b] |
| Epididymal fat pad weight / body weight (%) | 1.74 ± 0.14 | 2.07 ± 0.23 | 2.02 ± 0.19 |

Values are expressed as mean ± standard error.
[abc] Values with different lowercase letters within a row are significantly different at p< 0.05, as determined by one-way analysis of variance and Bonferroni's post hoc test.
*Food efficacy was calculated using the following formula: (body weight gain (g) / cumulative energy intake [kcal]) × 100.
S, rats fed a high-fat and high-cholesterol (HFC) diet from 9 to 18 weeks of age; M, rats fed an HFC diet from 18 to 27 weeks of age; L, rats fed an HFC diet from 27 to 36 weeks of age.

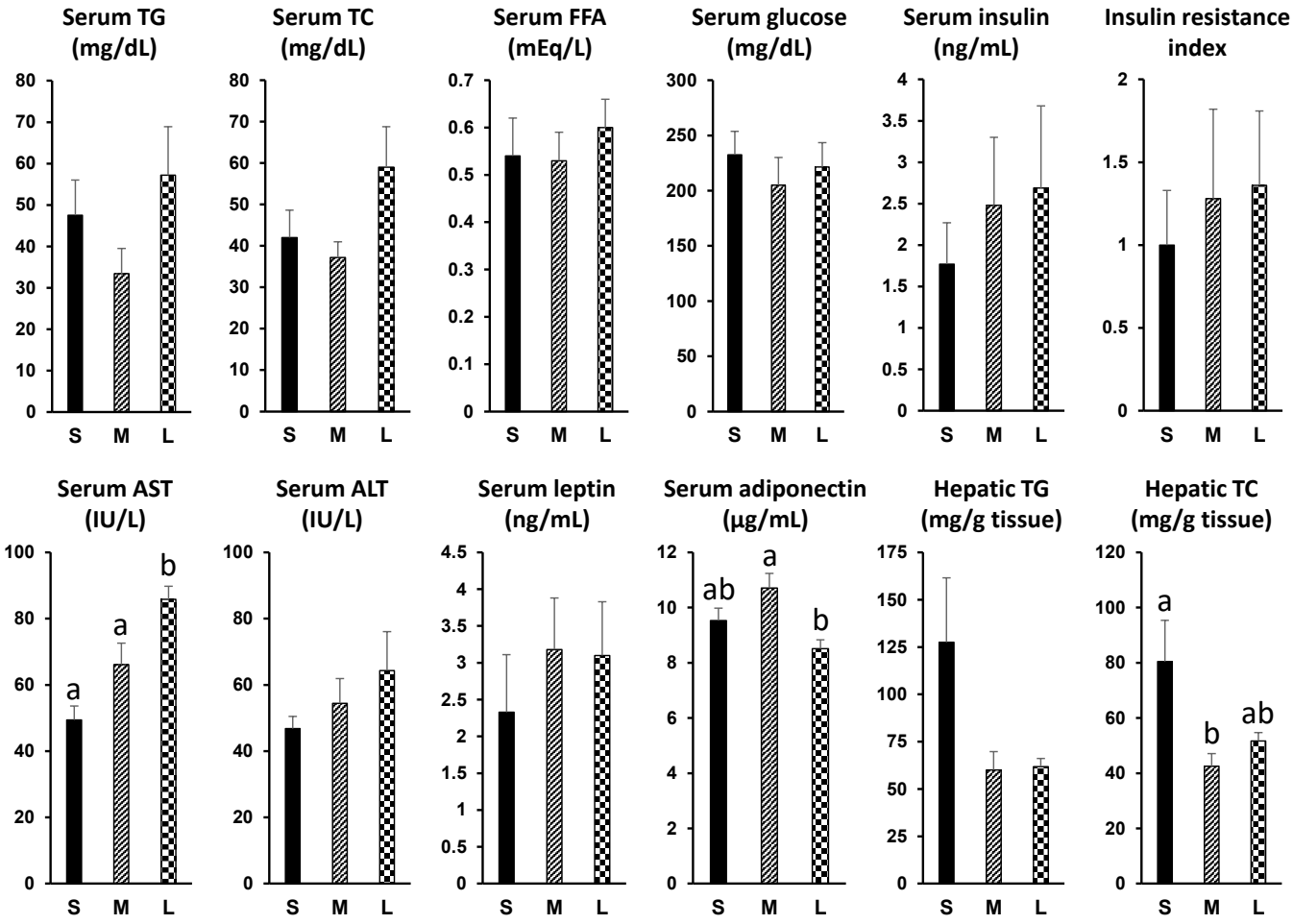



**Figure 1.** Serum parameters and hepatic lipid concentrations after the 9-week rearing period.
[ab] Values with different letters within a graph indicate significant differences at $p < 0.05$.
TG, triglyceride; TC, total cholesterol; FFA, free fatty acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
The insulin resistance index was calculated using the homeostasis model of assessment: serum glucose (mg/dL) × serum insulin (ng/mL) / 405.
S, rats fed a high-fat and high-cholesterol (HFC) diet from 9 to 18 weeks of age; M, rats fed an HFC diet from 18 to 27 weeks of age; L, rats fed an HFC diet from 27 to 36 weeks of age.

three (50%) of six rats in both the M and L groups. The remaining four rats (one S group, one M group, and two L group rats) showed a fibrosis score of 0.5. None of the above parameters were significantly different among the three groups (Table 4).

*Hepatic mRNA expression*

The mRNA level of GPAT1 (*Gpam*), which catalyzes the initial and committing step in glycerolipid biosynthesis, was significantly higher in the S group than in the M group (p=0.042). The mRNA level of FXR (*Nr1h4*), which regulates the synthesis, transport, and detoxification of bile acid, was significantly higher in the S group than in the L group (p=0.025). The other hepatic expressions of genes involved in cholesterol or lipid metabolism, inflammation, fibrosis and oxidative stress were not significantly different among the three groups (Fig. 3).

## Discussion

The life-span of rats is 2.5-3.5 years. Male rats are weaned at an average age of 21 days (3 weeks) after birth, and begin to undergo sexual maturation at an average age of 45-48 days (6-7 weeks) after birth. Adulthood, characterized by behaviors such as increased risk-taking and social play, begins approximately 8 weeks after birth, and skeletal growth tapers

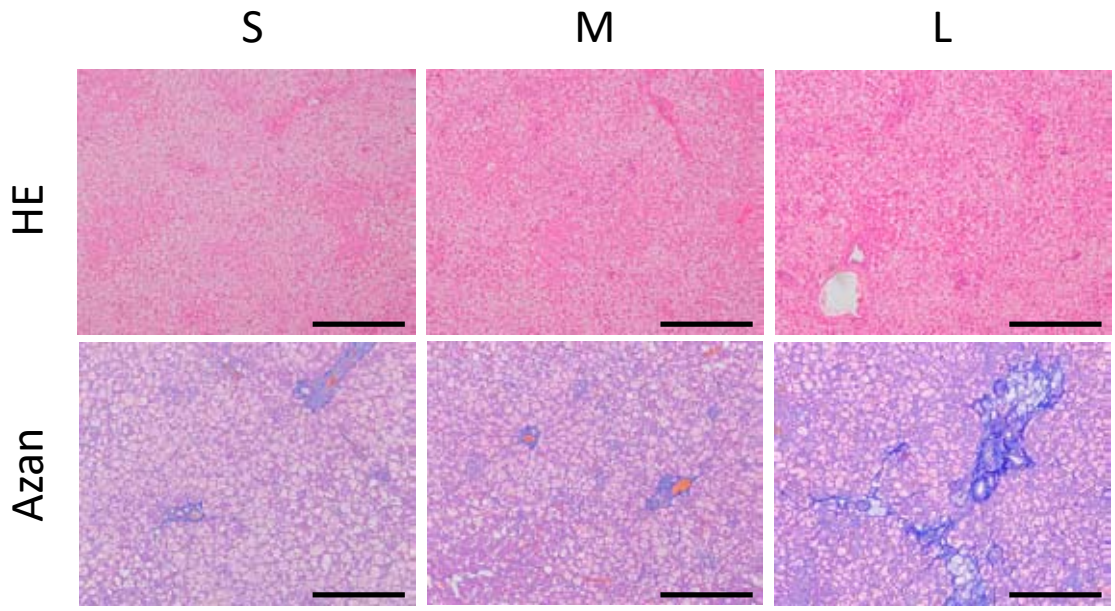
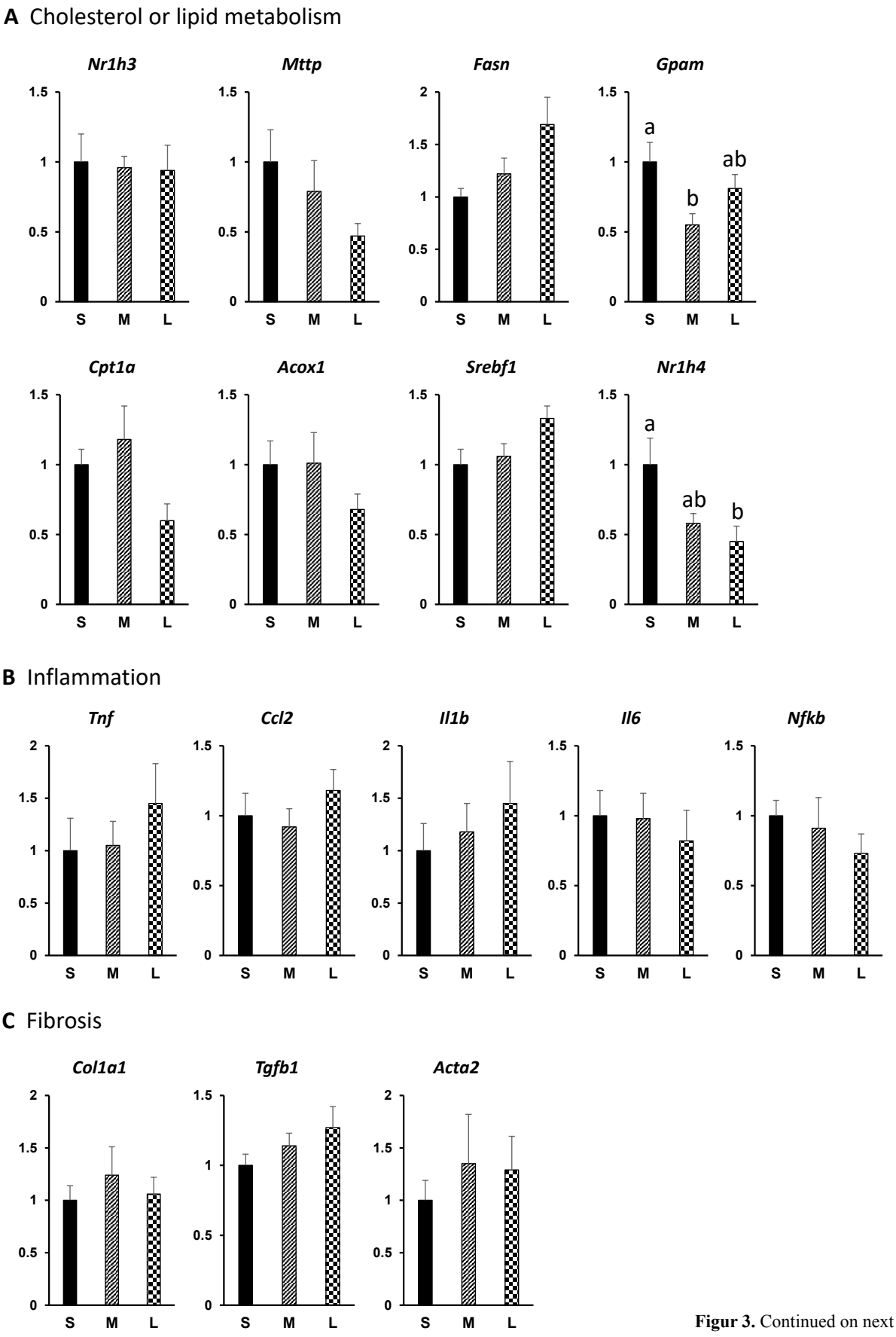



**Figure 2.** Representative histopathological findings in the liver of the S, M and L groups. In one rat in the S group, severe steatosis (score of 3), moderate lobular inflammation (score of 2), no hepatocyte ballooning, and no fibrosis were observed. The NAS score was 5. In one rat in the M group, severe steatosis (score of 3), moderate lobular inflammation (score of 2), a few ballooning hepatocytes (score of 1), and no fibrosis were observed. The NAS score was 6. In one rat in the L group, severe steatosis (score of 3), moderate lobular inflammation (score of 2), a few ballooning hepatocytes (score of 1), and mild fibrosis (score of 1) were observed. The NAS score was 6.
Hematoxylin and eosin-stained section; original magnification, 100 × ; scale bars=400$\mu$m. Azan-stained section; original maginification, 200 × ; scale bars=200$\mu$m.
S, rats fed a high-fat and high-cholesterol (HFC) diet from 9 to 18 weeks of age; M, rats fed an HFC diet from 18 to 27 weeks of age; L, rats fed an HFC diet from 27 to 36 weeks of age.

**Table 4.** Histopathological assessment of the liver after the 9-week rearing period according to the NASH Clinical Research Network Scoring System[12]

| Item / Group | Score | S | M | L |
|---|---|---|---|---|
| Steatosis | 0 | 0 | 0 | 0 |
| | 1 | 0 | 0 | 0 |
| | 2 | 0 | 1 | 1 |
| | 3 | 6 | 5 | 5 |
| Lobular inflammation | 0 | 0 | 0 | 0 |
| | 1 | 0 | 1 | 0 |
| | 2 | 5 | 4 | 6 |
| | 3 | 1 | 1 | 0 |
| Hepatocyte ballooning | 0 | 2 | 2 | 2 |
| | 1 | 4 | 4 | 4 |
| | 2 | 0 | 0 | 0 |
| NAFLD activity score (NAS)* | 0-2 | 0 | 0 | 0 |
| | 3-4 | 0 | 1 | 1 |
| | 5-8 | 6 | 5 | 5 |
| Fibrosis | 0 | 4 | 2 | 1 |
| | 0.5 | 1 | 1 | 2 |
| | 1 | 1 | 3 | 3 |

Values indicate the number of rats.
*NAS scores of 5-8 were considered to be diagnostic for NASH, and scores of 0-2 were considered not to be diagnostic for NASH.
S, rats fed an HFC diet from 9 to 18 weeks of age; M, rats fed an HFC diet from 18 to 27 weeks of age; L, rats fed a HFC diet from 27 to 36 weeks of age.
NASH, nonalcoholic steatohepatitis; HFC, high-fat and high-cholesterol; NAFLD, nonalcoholic fatty liver disease.




**Figur 3.** Continued on next page

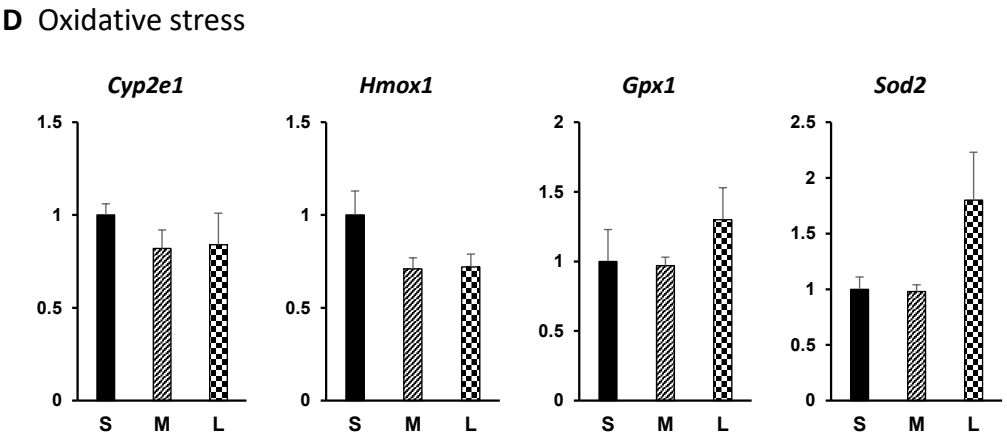



**Figure 3.** Hepatic expression of genes involved in (A) cholesterol or lipid metabolism, (B) inflammation, (C) fibrosis and (D) oxidative stress in the S, M, and L groups (n=6/group) after the 9-week rearing period. The mRNA levels are expressed relative to the levels of the S group (mean ± standard error).
[ab] Values with different letters within a graph indicate significant differences at $p< 0.05$.
S, rats fed a high-fat and high-cholesterol (HFC) diet from 9 to 18 weeks of age; M, rats fed an HFC diet from 18 to 27 weeks of age; L, rats fed an HFC diet from 27 to 36 weeks of age.

off at approximately 7-8 months (28-32 weeks) of age. During the adolescent phase, 10.5 rat days equals one human year.[13] Therefore, in the present study, aged between 9 and 27 weeks, which can be considered equivalent to between young adulthood and adulthood in humans, respectively, were fed an HFC diet for 9 weeks to evaluate age-related alterations in NASH.

Understanding the pathogenesis of NASH in humans has been hampered by the lack of a comprehensive and physiological small animal model of NASH. An ideal clinical animal model of NASH should mimic the pathophysiology of the human disease as closely as possible, but to our knowledge, no model comprising all desirable features and suited for all possible types of studies currently exists. Hitherto, the most commonly used NASH models are genetic, nutrient-deficient, and obesogenic dietary-induced models.[4] In our dietary (HFC diet)-induced NASH model that developed advanced fibrosis within a relatively short period (9 weeks) using SD rats of 9 weeks of age without genetic manipulation,[7] 56 (86.2%) of 65 rats developed histopathologically proven NASH.[14] In our previous study, 5- and 13-week-old SD rats fed the same diet also developed NASH at high rates (66.7% and 83.3% of six rats, respectively).[15] The results of the present study further showed that 18- and 27-week-old SD rats fed the same diet also developed NASH at a high rate (83.3% in each group). These findings indicate that our NASH model of SD rats can be applied for a relatively wide range of ages (5-27 weeks of age). Moreover, 9-week-old Wistar/ST rats fed the same diet also developed NASH at a high rate (83.3% of six rats).[16]

To compare groups and interpret the nutritional effect in interventional studies using adult rats, cumulative energy intake and/or body weight gain should be kept equivalent among groups via dietary restriction. In the present study, however, body weight gain and food efficacy during the 9-week rearing period were the highest in the S group and the lowest in the L group, despite the cumulative energy intakes not being significantly different among groups. This result may be due to the rats' age-related rapid body growth. In male SD rats, the rate of body weight gain has been observed to be fastest between birth and 10 weeks of age, and then, to slow down gradually (personal communication from the manufacturer). Because this study was designed to examine age-related (including eating habits) alterations in NASH in SD rats that were fed the same (HFC) diet for the same period (9 weeks), all rats had *ad libitum* access to food and water.

Aging results in irreversible physiological changes affecting all organs, including the liver.[10] In the present study, age-dependent increases in serum AST, ALT, and insulin, as well as insulin resistance index, were observed after the 9-week rearing period with or without a significant difference. The reason for the significant increase in serum AST in the L group was not clear because the histopathological lobular inflammation score was not different among groups. In our previous study, the serum AST levels were not different among 5, 9, and 13 week of age rat groups.[15] Moreover, the serum ALT levels were not different among S, M, and L groups in the present study. Therefore, the increase in serum AST in the L group may not be due to the hepatic inflammation. The histopathological finding of hepatic fibrosis showed a

similar trend; the fibrosis score was more evident in the M and L groups than in the S group. The fibrosis score was not correlated with cumulative energy intake during the 9-week rearing period nor final body weight after the 9-week rearing period (r=-0.231 and p=0.357, r=0.243 and p=0.332, respectively, data not shown). The mRNA levels of fibrosis-related genes encoding TGF-$\beta$ and $\alpha$-SMA were lowest in the S group and highest in the M and L groups, although this difference was not statistically significant. The mRNA levels of oxidative stress-related genes encoding GPX-1 and MnSOD were higher in the L group than in the S or M group, although this difference was not statistically significant. By contrast, hepatic TG and TC were higher in the S group than in the M and L groups with or without a significant difference. The histopathological finding of steatosis showed a similar trend; severe steatosis score (score of 3) was observed in all six rats in the S group, but in five of six rats in both the M and L groups. These results corroborated those of previous reports, asserting the effect of age on the progression and severity of NAFLD, and suggest that the risk of NASH or NAFLD-related fibrosis increases with age.[8,15,17] In the present study, the fibrosis score was 1 or less in all rats. Further studies using more cholesterol-rich diet, older SD rats or longer rearing period would be needed to elucidate a precise association between hepatic fibrosis and age.

In conclusion, the results of this study indicated that 9-, 18-, and 27-week-old SD rats fed an HFC diet for 9 weeks developed NASH at a high rate. Serological, histopathological, and mRNA expression data suggested that aging is likely associated with NASH-related fibrosis. Taken together with our previous study,[7,14,15] a 9-week dietary (HFC diet)-induced NASH model of SD rats can be applied for a relatively wide range of ages (5-27 weeks of age). However, these ages are relatively young because rats have an average life span of 2.5-3.5 years.[13] In humans, post-menopausal women with or without diabetes mellitus have been reported to have an increased risk of developing NASH with more advanced fibrosis.[18] Therefore, further studies regarding this dietary-induced NASH model using older and/or female SD rats are needed to elucidate fully the association between NASH progression and aging including the occurrence of hepatocellular carcinoma.

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## Disclosure statement

The authors declare no conflicts of interest.

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