Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition

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Charlton M, Krishnan A, Viker K, Sanderson S, Cazanave S, McConico A, Masuoko H, Gores G. Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. Am J Physiol Gastrointest Liver Physiol 301: G825–G834, 2011. First published August 11, 2011; doi:10.1152/ajpgi.00145.2011.—Although there are small animal platforms that recapitulate some of the histological features of nonalcoholic fatty liver disease, there are no small animal models of nonalcoholic steatohepatitis (NASH) with consistent hepatocellular ballooning and progressive fibrosis that also exhibit fidelity to the human condition physiologically. We examined the metabolic and histological effects of a diet on the basis of the composition of “fast food” (high saturated fats, cholesterol, and fructose). Mice (n = 8 in each group) were assigned to diets as follows: 1) standard chow (SC), i.e., 13% energy as fat [1% saturated fatty acids (SFA)], 2) high fat (HF), i.e., 60% energy as fat (1% SFA), and 3) fast food (FF), i.e., 40% energy as fat (12% SFA, 2% cholesterol). All three diets were supplemented with high fructose. All diets produced obesity. The HF and FF diets produced insulin resistance. Liver histology was normal in animals fed the SC diet. Steatohepatitis with pronounced ballooning and progressive fibrosis (stage 2) was observed in mice fed the FF diet. Although the HF diet produced obesity, insulin resistance, and some steatosis; inflammation was minimal, and there was no increase in fibrosis. The FF diet produced a gene expression signature of increased fibrosis, inflammation, and endoplasmic reticulum stress and lipopoapoptosis. A diet based on high cholesterol, high saturated fat, and high fructose recapitulates features of the metabolic syndrome and NASH with progressive fibrosis. This represents a novel small animal model of fibrosing NASH with high fidelity to the human condition. These results highlight the contribution of dietary composition to the development of nonalcoholic fatty liver disease and NASH.

nonalcoholic steatohepatitis; hepatocellular ballooning; nonalcoholic fatty liver disease

ON THE BASIS OF CURRENT PREVALENCES of obesity and type 2 diabetes mellitus, nonalcoholic fatty liver disease (NAFLD) can conservatively be estimated to affect >30,000,000 people in the United States, of which >600,000 are likely to have cirrhosis (4, 13, 17, 22, 23, 25, 44, 46). It is estimated that 3% of patients with NAFLD will develop liver-related complications (e.g., hepatocellular carcinoma) within 10 yr (2). Liver disease secondary to nonalcoholic steatohepatitis (NASH) is already a common indication for liver transplantation (3). The scale of the public health burden of NAFLD is likely to increase in parallel with increases in the prevalence and severity of obesity in the United States and globally. There are no approved pharmacotherapies for NAFLD and NASH. NAFLD, for the great majority of affected individuals, is one of many consequences of chronic overnutrition and obesity. Hepatic histological findings in chronically overnourished individuals range from entirely normal, to simple steatosis, to steatohepatitis with progressive fibrosis (48, 62). The various histological features of NAFLD are thus not inevitable consequences of overnutrition, obesity, or insulin resistance but are based on the balance between biological mechanisms for hepatic susceptibility and the physiological consequences of overnutrition. Human studies of the pathophysiology of NAFLD and NASH have been limited, in part, by difficulties in distinguishing primary cause(s) from secondary effects and epiphenomena related to obesity and liver disease. A fuller understanding of the physiology of NAFLD and NASH has been impeded by the absence of an animal model that closely recapitulates the human condition. Although there are an increasing number of animal models of NAFLD, none of these models fully produces the metabolic profile in concert with the histological patterns seen in humans (20, 32, 47, 49, 57, 63). The widely employed methionine-choline-deficient mouse (33) produces steatohepatitis and fibrosis, but in a metabolic context that is distinct from that of humans with NASH. A recent murine model incorporating prolonged administration of a “Western diet,” with high saturated fat and cholesterol content, was able to reproduce NASH with some increase in fibrosis markers, but not ballooning (19). The lack of a substantial content of fructose in the Western diet may have been important physiologically, as the addition of high fructose content to a diet high in saturated fat and cholesterol has been seen to reproduce all the features of NASH, including ballooning in large animals (36), and is a typical feature of the diet of humans with NASH (1, 45, 65). Recreation of NAFLD through genetic manipulation, such as leptin receptor deficiency, can result in the loss of an important component of fibrosis signaling (39, 67). More recent overnutrition-based models have demonstrated substantial metabolic similarity to humans with NAFLD and NASH but incompletely reproduce the histological features of NASH (53, 58, 59). Most importantly, none of the small animal models consistently produces NASH with hepatocellular ballooning and progressive hepatic fibrosis in a context of high fidelity to the physiological profile seen in humans with fibrosing NASH.

We sought to develop a new rodent model of fibrosing NASH by reproducing the physiological milieu seen in humans with NASH, i.e., physical inactivity and chronic overnutrition with a high caloric intake rich in saturated fats and fructose. Because fibrosis in patients with NASH typically evolves over
prolonged periods of time, we planned to continue overnutrition and physical inactivity for longer than dietary interventions in previous studies in overnutrition rodent models of NASH. The availability of a more “accurate” mouse model of NASH would greatly facilitate studies of the pathobiology of NAFLD and NASH and also the screening of potential therapies.

**MATERIALS AND METHODS**

**Animals.** Genetically unaltered C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained in individual cages (1 mouse per cage to promote sedentary movement patterns) and had free access to standard rodent chow and water for 1 wk until the start of the experiment. The animals were then randomly assigned to three groups receiving different diets (Table 1) for 25 wk: 1) fast food (FF) diet (relatively rich in saturated fats and cholesterol and fructose), providing 40% of energy as fat (milk fat, 12% saturated) with 2% cholesterol (AIN-76 Western Diet, Test Diet), 2) high-fat (HF) diet (rich in nonsaturated fats and fructose), providing 60% of energy as fat (milk fat, 0.8% saturated; DIO Basal Purified Diet, Crisco, Test Diet), and 3) standard chow (SC) diet, providing 13% of energy as fat (milk fat, 0.9% saturated; PicoLab Rodent Diet 20, Lab Diet).

High-fructose corn syrup (HFCS, 42 g/l final concentration) was also administered in the drinking water of all mice. Detailed diet compositions are shown in Table 1. At 6 mo (174 days), mice were weighed and euthanized by carbon dioxide inhalation, blood was drawn by cardiac puncture, and the liver was excised, weighed, and apportioned for RNA/DNA extraction or tissue histochemistry as flash-frozen tissue or preserved in 10% buffered formalin. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Mayo Clinic, which reviewed and approved all protocols. The gender makeup of each group is as follows: 5 males and 3 females in the SC diet group, 4 males and 3 females in the FF diet group, and 4 males and 4 females in the HF diet group. Caloric intake was not recorded.

**Biochemistry and lipids.** Serum glucose was measured using a blood glucose monitor (Assure 4, Arkray). Serum aspartate aminotransferase (AST) levels were measured using standardized and automated procedures of the diagnostic laboratory of the Mayo Clinic. Commercial ELISA kits were used to measure levels of insulin, growth hormone, adiponectin (EZRM113K, EZRMG145K, and EZMD160K, Millipore), cholesterol, and insulin-like growth factor 1 (MGI100, R & D Systems) following the manufacturers’ instructions. Histology, immunohistochemistry, and digital image analysis. Formalin-preserved liver tissue samples were embedded in paraffin and sectioned (4–6 μm thick). Deparaffinized, hydrated serial liver tissue sections were stained with hematoxylin-eosin, Masson’s trichrome, and picrosirius red using standardized protocols of the Pathology Department of the Mayo Clinic. Tissue sections stained with hematoxylin-eosin and Masson’s trichrome were analyzed for symptoms of FNASH by a pathologist (S. Sanderson) who was blinded to the study. The classification of Brunt et al. (9) was used to assign numerical scores to steatosis, inflammation, and fibrosis. Quantitative analysis of collagen deposition was based on digitally acquired images of picrosirius red-stained slides. Digital images were acquired on a Leica DMR microscope mounted with an Olympus DP70 camera. A series of images covering >90% of the total tissue section were generated at uniform settings of magnification, light, and exposure time. Quantitative analysis was performed using KS4000 software (Carl Zeiss). Data are presented as percentage of total tissue area. Additional serial sections were stained with antibody against lamincn (AF2846, R & D Systems) and anti-smooth muscle actin (ASMA; ASMA; NB11-55432, Novus Biologicals) at dilutions that were optimized for each stain. Bound antibodies were detected using diaminobenzidine, and sections were counterstained with hematoxylin.

Apoptotic cells were visualized by TdT dUTP nick end labeling of DNA strand breaks with fluorescein using a commercially available kit following the manufacturer’s instructions (11684795910, Roche). Tissue sections were counterstained with 4′,6-diamidino-2-phenylindole for visualization of nuclei. Four to six images were generated per section. Nuclei were quantified using KS4000 software. Fluorescing apoptotic nuclei were individually verified for colocalization with nuclear staining, and the count was normalized to total nuclei count.

8-Hydroxydeoxyguanosine. Genomic DNA was extracted from ~150 mg of flash-frozen liver tissue (Wako Chemicals) and diluted to a uniform concentration of 1.5 mg/ml DNA was hydrolyzed/oxidized, and 8-hydroxydeoxyguanosine was measured using a competitive ELISA according to the manufacturer’s instructions (Wako Chemicals).

**RNA isolation and quantitative RT-PCR.** Total RNA was isolated from frozen liver tissue using the RNeasy Plus kit (Qiagen) according to the manufacturer’s instructions. Equal quantities of total RNA were reverse-transcribed into cDNA using random hexamers (Transcriptor High Fidelity cDNA synthesis kit, Roche). Real-time PCR for collagen, ASMA, transforming growth factor-beta1 (TGFβ1), Tnfrα, fatty acid-binding protein (FABP), SOD1, and specificity protein 1 (Sp1) was performed on an iCycler (Bio-Rad) in a total volume of 20 μl using LightCycler 480 SYBR Green 1 Master Mix (Roche). Genomic DNA was extracted and purified. Genomic DNA was extracted using LightCycler 480 SYBR Green 1 Master Mix (Roche, Indianapolis, IN). Eukaryotic translation initiation factor 2α (eIF2α), 8-Hydroxydeoxyguanosine.

**Statistics.** Values are means ± SE representing replications within an experiment. Statistic significance was determined by Student’s t-test using two-tailed analyses. P < 0.05 was considered significant.

**RESULTS**

Animals reared on the FF diet recapitulate the clinical phenotype of NASH: physical characteristics and serum profile. Animals reared on the FF and HF diets were significantly more obese, averaging 42 and 44 g, respectively, than those raised on the SC diet (29 g; Table 2). The initial rate of increment in weight was greatest for the FF animals, although...
by 6 mo the weights of the FF and HF groups were statistically and numerically similar. Weight of the SC animals did not increase significantly during the experiments (Fig. 1). FF animals developed hepatomegaly, with significantly higher mean liver weights as a proportion of total body weight (8.5%) than animals fed the HF (4.2%) or SC (4.4%) diet ($P < 0.0003$). Serum cholesterol was significantly higher in FF than SC and HF animals ($P < 0.0001$). Serum AST was more elevated in FF animals ($P < 0.001$) animals. AST

<table>
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<tr>
<th>Table 2. Phenotype and serum biochemical profile</th>
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<tr>
<td><strong>Diet</strong></td>
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<td><strong>Body weight, g</strong></td>
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<td><strong>Liver-to-body weight ratio, %</strong></td>
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<td><strong>Serum AST, IU/l</strong></td>
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<td><strong>Glucose, mg/dl</strong></td>
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<td><strong>HOMA-IR</strong></td>
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<td><strong>Serum cholesterol, mg/dl</strong></td>
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<td><strong>Total IGF-I, ng/ml</strong></td>
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<td><strong>Growth hormone, ng/ml</strong></td>
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<td><strong>Adiponectin, ng/ml</strong></td>
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Values are means ± SE. AST, alanine aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance; IGF-I, insulin-like growth factor I.

*Statistical significance.

Evidence of perisinusoidal and pericellular fibrosis (stage 2 of 4; Fig. 3). Picrosirius red-stained tissue sections were analyzed for collagen distribution. The increase in collagen-stained area was significantly greater (~2-fold, $P < 0.05$) in tissue sections from FF than HF or SC mice. Gene expression studies indicated the same directional differences between study groups, i.e., 17-fold higher in FF than SC or HF animals. Since the major sources of collagen in the liver are hepatic stellate cells, tissue sections were immunostained for ASMA, a known indicator of their activation. Morphometric analysis of digital images indicated that ASMA was significantly overexpressed in FF mice ($P < 0.05$; Fig. 4). In addition to collagen, lumican, an extracellular matrix protein involved in collagen fibrogenesis, has been previously shown to be upregulated in NASH (8, 15). Lumican gene expression was significantly increased in FF compared with SC and HF animals ($P < 0.01$; Table 4). Immunohistochemical analysis also showed that lumican was overex-

**FF diet induces steatosis, pronounced hepatocellular ballooning, and steatofibrosis of the liver.** Hematoxylin-eosin-stained sections of liver tissue of all animals were scored for symptoms of NASH by a pathologist (S. Sanderson) who was blinded to the study. Scores for steatosis, inflammation, and fibrosis were assigned according to the classification of Brunt et al. (9). There was no evidence of steatosis in SC mice (Fig. 1). In contrast, all FF animals developed cellular ballooning, paracinar steatosis, and intra-acinar inflammation commonly associated with severe NASH (Fig. 2). The average score for steatosis for FF mice was 2.71 (Table 3). Although HF animals also showed evidence of steatosis, with an average score of 2.13, steatosis was largely microvesicular, and there was little or no evidence of inflammation (Table 3, Fig. 1). Hepatic triglyceride levels were $3.48 \pm 0.40$, $3.53 \pm 0.33$, and $3.99 \pm 0.18$ nmol in SC, FF, and HF animals, respectively. Although hepatic triglycerides were numerically higher in HF than FF and SC animals, the difference was not significantly different.

Hepatocellular ballooning, which has been difficult to recreate in animal models of NAFLD/NASH, was a pronounced histological feature in FF animals (Fig. 2). Furthermore, there was no evidence of fibrosis in SC or HF animals. By comparison, in six of the seven FF animals, there was evidence of perisinusoidal and pericellular fibrosis (stage 2 of 4; Fig. 3). Picrosirius red-stained tissue sections were analyzed for collagen distribution. The increase in collagen-stained area was significantly greater (~2-fold, $P < 0.05$) in tissue sections from FF than HF or SC mice. Gene expression studies indicated the same directional differences between study groups, i.e., 17-fold higher in FF than SC or HF animals. Since the major sources of collagen in the liver are hepatic stellate cells, tissue sections were immunostained for ASMA, a known indicator of their activation. Morphometric analysis of digital images indicated that ASMA was significantly overexpressed in FF mice ($P < 0.05$; Fig. 4). In addition to collagen, lumican, an extracellular matrix protein involved in collagen fibrogenesis, has been previously shown to be upregulated in NASH (8, 15). Lumican gene expression was significantly increased in FF compared with SC and HF animals ($P < 0.01$; Table 4). Immunohistochemical analysis also showed that lumican was overex-

Fig. 1. Weight change over time in animals fed standard chow (SC), fast food (FF), and high-fat (HF) diets. Although initial rate of increment in weight was greatest for FF animals, by 6 mo, weights of FF and HF animals were statistically and numerically similar. Weight did not increase significantly in SC animals. *Statistically significantly ($P = 0.02–0.001$) greater for FF and HF than SC.
pressed (2-fold, \( P < 0.05 \)) in FF compared with HF and SC mice.

**Profibrotic and proinflammatory pathways are activated in FF animals.** Expression levels of the profibrotic and proinflammatory genes were similar between SC and HF animals. Hepatic expression was fivefold higher for TGF\( \beta \) (\( P = 0.0009 \)) and ninefold higher for TNF\( \alpha \) (\( P = 0.0001 \)) in FF than HF animals (Table 4). FF animals also demonstrated increased hepatic expression of tissue inhibitor of metalloproteinase 1 (TIMP1, \( \sim 30 \)-fold, \( P = 0.0002 \)) compared with HF and SC animals. Hepatocyte growth factor was also upregulated in FF mice compared with HF and SC animals.

**Indexes of cellular stress, apoptosis, and unfolded protein response are differentially expressed in FF animals.** Oxidative stress, free radicals, and endoplasmic reticulum (ER) disturbances have been associated with the onset and development of NASH in clinical investigations. Hepatic gene expression of PERK, CHOP, and ATF6, proteins that are upregulated during ER stress, was significantly (\( P < 0.05 \)) increased in FF animals compared with SC animals (Table 4). However, expression of

Table 3. Hematoxylin-eosin scores of liver from SC, FF, and HF mice

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>FF</th>
<th>HF</th>
<th>( P ) Value</th>
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<tr>
<td>Steatosis</td>
<td>1.38 ± 0.53</td>
<td>2.71 ± 0.29</td>
<td>2.13 ± 0.48</td>
<td>0.33</td>
<td>0–3</td>
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<tr>
<td>Microvesicular steatosis</td>
<td>0.5 ± 0.19</td>
<td>1.0 ± 0</td>
<td>0.63 ± 0.20</td>
<td>0.07</td>
<td>0–3</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.0 ± 0</td>
<td>1.57 ± 0.30</td>
<td>0.0 ± 0</td>
<td>0.00008</td>
<td>0–4</td>
</tr>
<tr>
<td>Inflammation lobular</td>
<td>0.0 ± 0</td>
<td>2.51 ± 0.20</td>
<td>0.38 ± 0.18</td>
<td>0.000002</td>
<td>0–3</td>
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<tr>
<td>Hepatocellular ballooning</td>
<td>0.0 ± 0</td>
<td>1.57 ± 0.20</td>
<td>0.38 ± 0.18</td>
<td>0.0007</td>
<td>0–3</td>
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Mallory’s hyaline was not observed. No nuclei were glycogenated.
PUMA, a protein associated with ongoing lipoapoptosis in clinical NASH (12), was similar between groups. SOD1, an enzyme associated with removal of free radicals from the cytoplasmic milieu, has been previously shown to be down-regulated in clinical NASH (56). Gene expression of SOD1 was similar between all experimental groups (Table 4). Abundance of 8-hydroxydeoxyguanosine, an indicator of oxidative DNA damage induced by oxidative radicals, was similar in all three groups (Table 4). FF animals experienced a greater degree of hepatic apoptosis activity as measured by the number of TdT duTTP nick end label-positive cells per 1,000 cells (1.55 ± 0.21, 10.34 ± 1.73, and 4.90 ± 2.14 in SC, FF, and HF, respectively, \( P = 0.002 \) for FF vs. SC and \( P = 0.08 \) vs. HF). GRP78, a key regulator of the unfolded protein response, was similarly expressed in all three groups.

The transcription factor Sp1 is upregulated in FF mice. Sp1 regulates and interacts with a number of other transcription factors, including Smad, which regulates TGFβ1 signaling (21, 37). Sp1 can also regulate collagen and lumican gene transcription (38), both of which were overexpressed in FF mice. We therefore looked at gene expression levels of Sp1 in these groups of mice. Sp1 gene expression was significantly (\( P < 0.05 \)) upregulated in the FF mice compared with SC or HF mice.

A small animal model that produces NASH with hepatocellular ballooning and fibrosis in a physiological environment with fidelity to the condition in humans with NASH has been a longstanding need. While an increasing number of animal models have been reported to develop features of NAFLD and NASH, none consistently and simultaneously recapitulates the combined metabolic, physical, and histological features in humans with NASH with progressive fibrosis. We had hypothesized that recreating the nutritional and physical environment seen in NASH, with progressive fibrosis and a sedentary lifestyle in conjunction with chronic overnutrition with a diet high in calories and enriched with saturated fats and fructose, might produce the clinical and histological phenotype of NASH with fibrosis. The primary result of this study is that the FF mouse exhibits all the hallmarks of fibrosing NASH most commonly observed in humans: obesity, metabolic syndrome, steatohepatitis, hepatocellular ballooning, and progressive fibrosis. The most significant new feature of this model of NASH is the presence of ballooning with the frequent development of fibrosis. An approximately twofold increase in collagen-stained area was observed in FF mice compared with HF and SC animals. Evidence of increased hepatic fibrosis in the FF mouse model was present histologically by Masson’s trichrome and picrosirius red staining (with digital analysis) and also by expression levels of the profibrotic and proinflammatory gene TGFβ1, with associated increased stellate cell activation and increased abundance of mRNA for procollagen.

It is, of course, important to consider how this model is distinct from other animal models of NASH and what the potential significance of this model might be. Although the prevalence of NAFLD is indisputably high, \( \sim 25-50\% \) among obese individuals (10, 24, 28, 54, 62), the prevalence of NASH with progressive fibrosis is proportionally low. Two recent large, prospective cross-sectional studies reported the frequency of stage 2 or higher fibrosis among obese individuals to be 2.5–5%, with <1% developing stage 3 or higher fibrosis. Liver-related clinical consequences of NAFLD and NASH (e.g., related to portal hypertension and cirrhosis) are unlikely in patients without progressive fibrosis (48). Therapeutic modalities for NASH will need to prevent or reverse progressive fibrosis to confer clinical benefit in terms of preventing liver-related morbidity and mortality. Given the inherent limitations and complexities of studying the biology of NAFLD and NASH in humans, a fuller understanding of the mechanistic basis of steatofibrosis and the identification of methods to prevent/reverse steatofibrosis are likely to be expedited by the availability of a small animal model that also exhibits steatofibrosis. Existing models of NAFLD/NASH are characterized by (1) production of steatohepatitis (with or without fibrosis), but without features of the metabolic syndrome, or (2) reproduction of the metabolic syndrome, but with incomplete histological features of NASH. The methionine-choline deficiency model, for example, produces NASH and even fibrosis but is associated with substantial weight loss, low serum leptin levels, and lack of insulin resistance (34, 49). The ob/ob mouse develops obesity but is leptin-deficient and lacks the inflammatory and fibrosis components of NASH (31, 35). Overfeeding models, while fairly efficient in producing obesity, have been highly variable in resulting hepatic histology (1- to 7-mo...
Overfeeding with fat in a mouse strain susceptible to obesity and insulin resistance has produced encouraging results. A model of a high-fat diet in male C57BL/6J mice led to the development of features of the metabolic syndrome and steatohepatitis but only mild fibrosis after 50 wk (32). A model utilizing male C57BL/6 mice that were fed high-fat chow containing trans fats and a HFCS for up to 16 wk produced obesity, features of the metabolic syndrome, and hepatic steatosis with associated necroinflammatory changes. Although signals for hepatic fibrosis were increased, hepatic fibrosis was not. An important observation of this study was that HFCS promoted food consumption (58). In our current study, we also used C57BL/6 mice and a high-fat, HFCS diet. The basis of development of NASH and progression to stage 2 fibrosis (7) in animals fed a FF diet merits consideration. The diet chosen for our study included a relatively high abundance of saturated fats, 12% of total calories, with 2.2% as cholesterol, and 23 g/l HFCS. In addition, we housed the mice singly (1 mouse per cage) to promote sedentary behavior. Because FF mice were exposed to greater amounts of saturated fats/cholesterol in addition to higher carbohydrate intake, it is impossible to know the relative contributions of high carbohydrate and high saturated fat exposure to the response; it is only possible to know that the combination of the two produced the observed phenotype. In addition, because HFCS was included in all three diets but caloric intake was not reported, defining a specific contribution of HFCS would require further study. The fact

![Collagen staining with picrosirius red (left), anti-smooth muscle actin (ASMA, middle), and lumican (right) in SC, FF, and HF animals. On digital image analysis, collagen-stained area was significantly more abundant (2-fold, \( P < 0.05 \)) in FF than HF and SC animals. Staining for ASMA (\( P < 0.05 \)) and lumican (\( P < 0.01 \)) was also significantly more abundant in FF than HF or SC animals.*Statistically significant compared with HF and SC.](G830.jpg)
that our animals fed the HF diet, in which saturated fats were only minimally present, developed obesity, insulin resistance, and steatosis, but not NASH or fibrosis, highlights the importance of dietary fat type in the development of NASH and fibrosis. Dietary cholesterol has been identified as an essential determinant of progression from NAFLD to NASH in a murine model of NASH (66). The inflammatory effects of dietary cholesterol are thought to be mediated by cholesterol oxidation products sensitizing the liver to TNFα and nuclear factor-κB signaling (18, 41). In addition, excess dietary cholesterol has been shown to induce TGFβ1 (52) and TIMP1 expression (60).

We observed relative increases in TGFβ1 and TIMP1 in FF animals. The effect of cholesterol appears to be relatively specific, as mitochondrial loading of free cholesterol, but not triglycerides and free fatty acids, decreases mitochondrial glutathione and sensitizes it to the TNFα-mediated apoptosis of hepatocytes (41). Our model is similar in many respects to that recently reported by Matsuzawa et al. (42), who fed C57BL/6 mice a diet containing 1.25% cholesterol and 0.5% cholate for 24 wk. The model of Matsuzawa et al. also produced NASH with fibrosis. The fibrosis was, however, not characterized/ staged. The use of HFCS, a common element of the Western diet and ubiquitous component of fast food products, also distinguishes our model from that reported by Matsuzawa et al. Because the endocrinologic profile of the mice used by Matsuzawa et al. was also not reported, the fidelity of the model to the human condition is unknown. HFCS is thought to be important in the pathogenesis of NAFLD and NASH in mice (58, 61) and humans (1, 45), promoting food consumption, obesity, increased TNFα activation (55), and impaired insulin sensitivity (58). This difference may account for the much greater mean body weight at 24 wk in our FF model (44.9 ± 1.14 g) than in the HFCS-free high cholesterol/atherogenic diet model of Matsuzawa et al. (26.4 ± 1.1 g). Insulin resistance, as measured by homeostasis model assessment of insulin resistance, was also numerically much greater in our FF model than in the model described by Matsuzawa et al. Similarly, a recent murine model incorporating prolonged administration of a Western diet, containing high levels of saturated fat and cholesterol without a high level of fructose, was able to reproduce NASH with some increase in fibrosis markers but not hepatocellular ballooning (19). Lack of a substantial content of fructose in the Western diet is also suggested to have been important physiologically, as addition of a high level of fructose to a diet high in saturated fat and cholesterol has been seen to reproduce all the features of NASH, including ballooning in large animals (36), and is a typical feature of the diet of humans with NASH (1, 45, 65). Thus, although atherogenic, high-fat/high-cholesterol diets replicate human histopathology, the metabolic status appears to be distinct. Hebbard and George (26) commented that “Further studies are required to address whether alterations in fat composition or the addition of other dietary factors associated with metabolic status, such as fructose, can lead to this model attaining the relevant human metabolic parameters.” The specific contribution of HFCS to the metabolic and histological features observed in our mice remains to be determined.

Histological features other than fibrosis that define NASH in humans, assessed according to the classification of Brunt et al. (9), were also seen in FF mice. All FF animals developed cellular ballooning, paracinematic steatosis, and intra-acinar inflammation. Although animals reared on the HF diet also showed evidence of steatosis, this was largely microvesicular, and there was little or no evidence of inflammation (Table 3, Fig. 2).
Since hepatic fibrosis is characterized by aberrant collagen deposition, picrosirius red-stained tissue sections were analyzed for collagen distribution. Gene expression studies indicated the same trends: 17-fold higher collagen expression in FF than SC or HF animals. Since the major source of collagen in the liver is hepatic stellate cells, tissue sections were immunostained for ASMA, a known indicator of their activation. Morphometric analysis of digital images indicated that ASMA was significantly overexpressed in HF mice (Fig. 4). In addition to collagen, lumican, an extracellular matrix protein involved in collagen fibrogenesis, has been previously shown to be upregulated in NASH (8, 15). Lumican gene expression was significantly increased in FF animals compared with SC and HF animals. Immunohistochemical analysis also showed that lumican was overexpressed (2-fold) in FF mice compared with HF and SC mice.

In addition to a histological picture similar to the human condition, it is desirable that small animal models of NASH would also have a metabolic profile that has fidelity to that of humans who develop NASH with fibrosis, specifically, insulin resistance (16, 51), high systemic leptin levels (5, 43), and low levels of growth hormone (14, 29, 30), dehydroepiandrosterone (14), and adiponectin (27). Circulating cytokines are also abnormal in NASH, with increased circulating levels of TNFα and markers of apoptosis (43, 64). The FF diet model recreates each of these features. Although the link(s) between hepatic steatosis, inflammation, and fibrosis is not fully known, increased oxidative stress is a feature of animal models of steatohepatitis (63) and humans with NAFLD (22, 64) with free fatty acid sensitization of hepatocytes to TNFα-related apoptosis-inducing ligand (40). Hepatic gene expression of PERK and CHOP, two proteins that are upregulated during ER stress in NASH (12), was significantly increased in FF animals. Similarly, ATF6 was relatively overexpressed in FF animals compared with HF animals. Expression of GRP78/BiP, a key regulator of the unfolded protein response, was greatest in HF animals. SOD levels, shown to be reduced in humans with NASH with progressive fibrosis (56), were also comparatively low in FF animals. NASH with progressive fibrosis in humans has recently been reported to be associated with increased intracellular expression of lumican and decreased FABP1 (15). These proteins were similarly differentially abundant in the livers of the FF mice, suggesting that the increased lumican and attenuated FABP1 expression in NASH expression may be mediated by dietary cholesterol and hypercholesterolemia.

Several technical aspects of the study merit consideration. Reproducibility is a key determinant of the success of any animal model of disease. The strain of mice used, C57BL/6, is, of course, commercially available. The FF diet comprised a standard, commercially available chow (AIN-76 Western Diet, Test Diet) with added fructose. While many variables may have contributed to the net effect of the FF diet model, the conditions are not difficult to reproduce. We housed animals singly to encourage sedentary behavior. Being housed singly may also have provoked a stress response and contributed to the overall observed changes in the FF group. As animals in all the study groups were housed singly, we do not believe that the stress of being housed singly could explain the relative differences between the three groups, however.

In conclusion, a diet based on the composition of “fast food” (high cholesterol, high saturated fat, and high fructose) administered for 6 mo recapitulates features of the metabolic syndrome and NASH with progressive fibrosis in C57BL/6 mice. This represents a novel small animal model of fibrosing NASH with high fidelity to the human condition. These results highlight the contribution of dietary composition in the development of NAFLD and NASH.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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