RESEARCH METHODOLOGY/STUDY DESIGN

CURRENT DEVELOPMENTS IN NUTRITION

Basic Science

Considerations When Choosing High-Fat, High-Fructose, and High-Cholesterol Diets to Induce Experimental Nonalcoholic Fatty Liver Disease in Laboratory Animal Models

Sridhar Radhakrishnan,[1] Steven F Yeung,[1] Jia-Yu Ke,[1] Maísa M Antunes,[2] and Michael A Pellizzon[1]

[1]Research Diets, Inc., New Brunswick, NJ, USA and [2]Center for Gastrointestinal Biology, Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil

ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is intricately linked to metabolic disease (including obesity, glucose intolerance, and insulin resistance) and encompasses a spectrum of disorders including steatosis, nonalcoholic steatohepatitis (NASH), and fibrosis. Rodents consuming high-fat (HF; ∼40 kcal% fat including fats containing higher concentrations of saturated and *trans* fats), high-fructose (HFr), and high-cholesterol (HC) diets display many clinically relevant characteristics of NASH, along with other metabolic disorders. C57BL/6 mice are the most commonly used animal model because they can develop significant metabolic disorders including severe NASH with fibrosis after months of feeding, but other models also are susceptible. The significant number of diets that contain these different factors (i.e., HF, HFr, and HC), either alone or in combination, makes the choice of diet difficult. This methodology review describes the efficacy of these nutrient manipulations on the NAFLD phenotype in mice, rats, guinea pigs, hamsters, and nonhuman primates. *Curr Dev Nutr* 2021;5:nzab138.

Keywords: NAFLD, diet, high-fat, high-fructose, high-cholesterol, mice, rats, guinea pigs, hamsters, nonhuman primates

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Address correspondence to MAP (e-mail: michael.pellizzon@researchdiets.com).

Abbreviations used: ALIOS, American Lifestyle-Induced Obesity Syndrome; ALT, alanine aminotransferase; AMLN, Amylin Liver NASH; AST, aspartate aminotransferase; FPC, fructose, palmitate, cholesterol, and *trans* fat diet; GAN, Gubra-Amylin NASH; HC, high-cholesterol; HF, high-fat; HFC, high-fat and high-cholesterol; HFD, high-fat diet; HFr, high-fructose; HFHFr, high in fat and fructose; MAFLD, metabolic (dysfunction) associated fatty liver disease; MCD, methionine and choline deficient; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; NHP, nonhuman primate; PPARγ, peroxisome proliferator–activated receptor γ; collagen type I alpha 1 chain, Col1a1; Sprague Dawley, SD; TG, triglyceride.

Introduction

As the name suggests, nonalcoholic fatty liver disease (NAFLD) is characterized as a spectrum of disorders ranging from excessive fat storage in hepatocytes (steatosis) to nonalcoholic steatohepatitis (NASH) and fibrosis. If untreated, it can also lead to cirrhosis and hepatocellular carcinoma (1–3). The prevalence of NAFLD is rapidly increasing and it is estimated that >30% of the population worldwide has been diagnosed with ≥1 stage of NASH (4). The estimated annual medical costs directly attributable to NAFLD exceed €35 billion in 4 large European countries (the United Kingdom, France, Germany, and Italy) and are >$100 billion in the United States (5). The health and economic burden of NAFLD has led to a wide interest in understanding the etiology of this disease in the past decade. Despite the enormous amount of research in this field, the pathogenesis of NAFLD/NASH has not been completely elucidated. Furthermore, NAFLD is complicated by its link to metabolic syndrome and related comorbidities; thus, there is a need for additional studies and models to elucidate its pathophysiology and to ultimately develop therapeutics for this condition (6). A variety of animal models that mirror both the pathophysiology and the histopathology of each stage of NAFLD/NASH are available, including rodents, but also certain large animals such as pigs and nonhuman primates (NHPs). Researchers have used chemical, genetic, and/or diet-induced models to mimic human disease and to produce different severities of disease along the NAFLD spectrum (6). Genetic models (e.g., ob/ob mice, MATO mice, db/db mice) and chemicals (e.g., streptozotocin, diethylnitrosamine, CCl_4) have also been used either alone or in combination with a dietary model to induce NAFLD (6, 7). However, the focus of this review is to examine the dietary approaches.

There is a wide range of dietary approaches used for studying NAFLD in rodent models. Purified ingredient diets are open to the public and contain 1 main nutrient per ingredient (8), which offers researchers the opportunity to both define and modify the nutrient composition with ease. This in turn allows for testing the effect of a

single nutrient or a combination of nutrients on NAFLD promotion or reversal. Such dietary manipulations have helped in evaluating different therapeutic approaches toward the treatment of NAFLD (6, 9). Variations of high-fat diets (HFDs) and methionine- and choline-deficient (MCD) diets are used to study different stages of NAFLD/NASH, depending on the type of diet/variation and length of feeding (6). There are some differences between these experimental models with respect to clinical and histological features in comparison with human NAFLD; still, these models do help us to gain insight into disease progression in humans. The NAFLD activity score (NAS), which was developed as a clinical tool for scoring the extent of steatosis, inflammation, and hepatocyte ballooning in humans, is used quite often for defining the extent of NAFLD in rodents; a fibrosis score is also used for defining the extent of fibrosis. We have briefly described these scoring systems in our previous review (6), and will use these scoring systems when defining the extent of NAFLD in our current review.

In brief, HFDs contain typically 30–60 kcal% fat (various fat sources) and are appropriate when there is an interest in understanding the beginning stages of disease progression in the context of obesity and other metabolic disease development, including insulin resistance/glucose intolerance. Conversely, MCD diets are commonly able to drive NASH and fibrosis, but fail to promote weight gain and other metabolic diseases. These models are reviewed extensively elsewhere, including in our recent review and others (6, 7, 10). Given most patients with NAFLD also have metabolic disturbances, NAFLD is being called the hepatic manifestation of metabolic syndrome (11, 12). In fact, some recent publications have called for a change of nomenclature such that NAFLD be renamed as MAFLD, which stands for metabolic (dysfunction) associated fatty liver disease (13). Thus, appropriate animal models for this disorder should be able to reflect the whole NAFLD spectrum and present metabolic disturbances (MAFLD). In order to address the full spectrum of NAFLD and associated metabolic diseases that are commonly found in humans with NAFLD, these diets have evolved significantly over time. Certain nutrients have been added or increased to better model the human condition including fructose, different sources of fat (e.g., those high in *trans* fatty acids or SFAs), and cholesterol. Independently, each of these 3 dietary factors added in high concentration can drive certain aspects of the NAFLD spectrum [reviewed in (6)]. Researchers have shown that, when added together, they act synergistically and thus are more effective in promoting the full spectrum of the disease.

One of these factors, the dietary fat source, can be highly variable from one study to the next. Some groups have used a source of *trans* fatty acids (14, 15), which can drive NASH and fibrosis when fed over long time frames (4–6 mo) and can also induce other metabolic phenotypes commonly found in humans with NASH (i.e., weight gain, insulin resistance). The *trans* fat ban by the FDA in 2018 (16) called for the use of non–*trans* fat sources or *trans* fat sources that are not in the food supply, and recent data have suggested that fat sources higher in saturated fat [palmitic acid (16:0) in particular] may drive more weight gain, other metabolic disease (i.e., glucose intolerance, insulin resistance), and NASH/fibrosis than *trans* fat sources do (17, 18). Because of these recent data and the increased number of potential options of high-fat (HF), high-fructose (HFr), and high-cholesterol (HC) diets, the appropriate choice of diet can be a difficult task. Therefore, we reviewed how various modifications to these diets influence the development of NAFLD along with metabolic disturbances in mice, rats, hamsters, and guinea pigs. We have also included data for NHPs, which to our knowledge have not been reviewed previously.

Mice

A landmark publication (14) provided mice with what was called the American Lifestyle-Induced Obesity Syndrome (ALIOS) model, which combined an HFD with fructose. Animals in this study were fed ad libitum a 45 kcal% fat HFD, which also contained 22% (wt:wt) sucrose. Dietary fat came mainly from partially hydrogenated vegetable oil with ∼34% (wt:wt) as *trans* fat. In addition, the drinking water was supplemented with high-fructose corn syrup (42 g/L). C57BL/6 mice consuming such a diet became obese and developed reduced glucose tolerance, hyperinsulinemia, and substantial hepatic steatosis associated with a necroinflammatory and profibrogenic response. The combination of fructose and *trans* fats contributed to the development of obesity and insulin resistance, and hepatic steatosis and injury. After 6 mo, mice develop moderate macrovesicular steatosis and lobular inflammation with a mean NAS score of 2.4. The disease progressed to inflammation and bridging fibrosis (mean NAS score: 5.0) after 12 mo and hepatocellular neoplasms were observed in 60% of mice fed ALIOS (19). Dietary cholesterol is an additional critical factor in the progression of NASH and hepatic inflammation in animal models (20–23). In 1 study, the combination of HF (33 kcal% fat, mainly cocoa butter) and HC (1% wt:wt) interacted synergistically to drive features of NASH. In this study, steatohepatitis with mild fibrosis was only observed in the combination of HC and HF (HFC). In addition, mice fed the HFC diet had greater weight gain, hepatic lipid accumulation, serum alanine aminotransferase (ALT) concentrations, and fibrosis, and decreased adiponectin concentrations, compared with animals fed a control low-fat diet (11 kcal% fat), HFD (33 kcal% fat), or low-fat/HC diet (11 kcal% fat, 1% cholesterol) (24).

This discovery led to the development of a diet with higher fat, cholesterol, and fructose, which was firstly used by the researchers at Amylin Pharmaceuticals (now MedImmune). In this study, C57Bl/6J mice were fed a purified diet containing 40 kcal% fat (of which ∼18% was *trans* fat), 22% fructose (wt:wt), and 2% (wt:wt) cholesterol. The combination of these 3 nutrients synergistically allowed for the development of all 3 stages of the NAFLD spectrum including steatosis, steatohepatitis with fibrosis, and cirrhosis as assessed by histological and biochemical methods in ∼30 wk, as shown by a few different studies (15, 25). This diet (**Table 1**, "Primex shortening"), referred to as the Amylin Liver NASH (AMLN) diet in the NAFLD research community, also induced metabolic dysfunction, because animals had increased total cholesterol, fasting insulin, and HOMA-IR (a marker of insulin resistance) and lower adiponectin compared with animals fed a low-fat (10 kcal% fat) control diet. Another model using a similar diet was recently presented by the Yecuris Corporation (L Foquet, E Henson, F Lime, R Copenhaver, M Grompe, unpublished data, presented at the Liver Meeting, 2020 and at the Keystone meeting NAFLD, 2020). They used humanized FRG® knockout animal models; these models have a knockout of the fumarylacetoacetate hydrolase (*Fah*), recombinant activation gene-2 (*Rag2*), and interleukin 2 receptor common γ chain (*IL2rg*) genes. These knockouts allow repopulation of mouse

TABLE 1 Formulations of Primex® replacement diets

	Primex shortening		Palm oil		Corn-oil shortening		Primex, non–*trans* fat shortening		Matching *trans* fat to Primex		Matched low-fat control	
Product#	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**
Protein	22%	20%	22%	20%	22%	20%	22%	20%	22%	20%	19%	20%
Carbohydrate	45%	40%	45%	40%	45%	40%	45%	40%	45%	40%	67%	70%
Fat	20%	40%	20%	40%	20%	40%	20%	40%	20%	40%	4%	10%
Total	—	100%	—	100%	—	100%	—	100%	—	100%	—	100%
kcal/g	4.5		4.5		4.5		4.5		4.5		3.8	
Ingredient												
Casein	200	800	200	800	200	800	200	800	200	800	200	800
l-Cystine	3	12	3	12	3	12	3	12	3	12	3	12
Corn starch	0	0	0	0	0	0	0	0	0	0	350	1400
Maltodextrin 10	100	400	100	400	100	400	100	400	100	400	85	340
Fructose	200	800	200	800	200	800	200	800	200	800	0	0
Dextrose	0	0	0	0	0	0	0	0	0	0	169	676
Sucrose	96	384	96	384	96	384	96	384	96	384	96	384
Cellulose	50	0	50	0	50	0	50	0	50	0	50	0
Soybean oil	25	225	25	225	25	225	25	225	25	225	25	225
Primex shortening	135	1215	0	0	0	0	0	0	0	0	0	0
Primex shortening, non–*trans* fat	0	0	0	0	0	0	135	1215	45	405	0	0
Corn oil, partially hydrogenated	0	0	0	0	135	1215	0	0	110	990	0	0
Palm oil	0	0	135	1215	0	0	0	0	0	0	0	0
Lard	20	180	20	180	20	180	20	180	0	0	20	180
Mineral mix S10026	10	0	10	0	10	0	10	0	10	0	10	0
Dicalcium phosphate	13	0	13	0	13	0	13	0	13	0	13	0
Calcium carbonate	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, 1 H_2O	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix V10001	10	40	10	40	10	40	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0	2	0	2	0	2	0
Cholesterol	18	0	18	0	18	0	18	0	18	0	0	0
FD&C Yellow Dye #5	0.05	0	0	0	0.025	0	0	0	0	0	0.025	0
FD&C Blue Dye #1	0	0	0.025	0	0.025	0	0	0	0	0	0	0
FD&C Red Dye #40	0	0	0.025	0	0	0	0.05	0	0.05	0	0.025	0
Total	904.05	4056	904.05	4056	904.05	4056	904.05	4056	904.05	4056	1055.05	4057

liver with any donor hepatocytes. When such a model was colonized with human patatin-like phospholipase domain-containing protein 3 (PNPLA3)-148M hepatocytes, the animals developed an LDL cholesterol:HDL cholesterol ratio of >1.6, similar to humans. Introduction of an HF (40 kcal% fat), HFr (22% wt:wt), and HC (2%) diet for 24 wk led to increased blood lipid concentrations (the LDL cholesterol increase was more dramatic than that of HDL cholesterol) and the development of hepatocyte ballooning, fibrosis, and Mallory bodies at week 16; collagen deposition in hepatic sinusoids at week 20; and bridging fibrosis at week 24. The animals also had clear signs of steatosis, with a mean NAS score of 4.25 and fibrosis score of 2.

The main source of fat in the AMLN diet is Primex®, a mixture of partially hydrogenated soybean and palm oils, and this partial hydrogenation process forms ∼28% *trans* fatty acids (wt:wt). However, the FDA banned the use of Primex® and other food sources of *trans* fat in 2018 (16), which prompted the research community studying NASH to investigate alternatives to this fat source. One fat source, a corn-oil shortening, not used in the food industry, has a slightly higher percentage of *trans* fat than Primex® (∼34% compared with ∼28%). When a modified diet with corn-oil shortening (Table 1) was fed for 24 wk to hepatocyte-specific peroxisome proliferator–activated receptor γ (PPARγ) knockout (*Ppar*γ^{Δ}Hep) mice and wild-type (C57BL/6J and 129 background) controls, male control mice developed elevated steatosis, inflammation, ALT concentrations, liver weight, liver triglyceride (TG) content, and bridging fibrosis. The mean NAS score (± SE) in these animals was 6.61 ± 0.36 (26). The impact of this diet on female mice was not as severe, as evidenced by lower plasma ALT, liver weight, and hepatic PPARγ expression than the males. C57BL/6J mice on this diet showed development of steatosis comparable with mice fed the AMLN diet, with progression to NASH (hepatocyte ballooning, massive fatty liver degeneration; NAS score 5–6) after 24 wk (18). Furthermore, this modified diet also increased immune cell infiltration and collagen accumulation in the epididymal white adipose tissue, which was not present in mice fed the AMLN diet (18). The substrain of the animal also plays a role because C57BL/6N mice only developed significant liver injury, hepatic inflammation, lipid accumulation, and collagen deposition on this modified diet compared with a control diet with no added sucrose, and not on the original AMLN diet (27). However, it was not clear whether the difference in phenotype observed was due to the change in the fat source from Primex® to corn-oil shortening or due to the difference in sucrose concentrations in the control diets because the corn-oil shortening diet was compared with a control diet with no added sucrose, whereas the control diet for the Primex® group contained 10% sucrose. One group combined corn-oil shortening with non–*trans* fat Primex-Z® (a mixture of fully hydrogenated soybean oil and palm oil) to match both the palmitic acid and the *trans* fat content of the original AMLN diet (Table 1). SFA accumulation and inefficient disposal of saturated free fatty acids (like palmitic acid) is hepatotoxic (28, 29) and increases NASH risk; thus, increasing palmitic acid in the diet seems to be a reasonable modification to promote NASH development. C57BL/6J mice were fed this diet for 12 wk, and steatosis with mild fibrosis was confirmed, using high resolution physio-chemical analysis and histological observation. After an additional 12 wk on this diet, histological analysis (based on hematoxylin and eosin staining) and a mean NAS score (± SE) of 6.2 ± 0.4 indicated that this modified diet induced histologic features characteristic of NASH with fibrosis (mean score ± SE: 1.40 ± 0.16), including micro- and macrovesicular steatosis, hepatocyte ballooning, and infiltration of inflammatory cells (30). In another study, the same diet was fed to both male and female C57BL/6N mice, and after 31 wk all mice had increased liver damage (hepatocyte ballooning, lobular inflammation) and fibrosis (F3–F4 for ≥50% of mice), but only mild weight gain and insulin resistance (31). Female mice were slightly slower to respond to the diet intervention, with normal concentrations of circulating ALT at week 12, but significantly elevated by week 24. A similar diet, containing slightly lower concentrations of palmitic acid (but similar *trans* fat concentrations), also demonstrated development of steatosis with NASH after 15 wk in C57BL/6 (substrain not reported) animals (32, 33).

Different fat sources (Primex®, palm oil, corn-oil shortening, or Primex-Z®—non–*trans* fat shortening) in the background of the AMLN diet were compared by feeding these diets to C57BL/6 (substrain not reported) mice for 16 or 30 wk (34). The diet containing non–*trans* fat Primex-Z® led to a sustained increase in adiposity index (∼90% increase of epididymal, retroperitoneal, and mesenteric fat pads) after 30 wk when compared with other fat sources. Although mice fed all the different HFDs had several similar disease outcomes, including fat accumulation within hepatocytes (**Supplemental Figure 1**), diets which contained palm oil (or Primex-Z®; Table 1) induced a more severe form of liver inflammation, along with increased inability to arrest bacteria under blood flow (**Supplemental Figure 2**), which could render mice more susceptible to infections. The inability to arrest bacteria is clearly evident in Supplemental Figure 2 where lesser numbers of *Escherichia coli* are trapped by the Kupffer cells in these diets than in the control group (shown by white arrows). Similar results were observed by another group who found diets containing Primex-Z® produced more pronounced NASH and fibrosis (18). This was also true in a choline-deficient amino acid diet background where diets containing Primex-Z® induced a greater extent of hepatocellular apoptosis at week 13 in C57BL/6J mice, and more pronounced proliferative (preneoplastic and nonneoplastic) nodular lesions at week 26, than the diet containing the *trans* fat version of Primex® (35). Together, these studies suggest that the absence of *trans* fat does not impede the large disturbances within the liver that are caused by enhanced fat ingestion. They also allude to the potential hepatotoxic role of palmitic acid, which is present in high concentrations (∼42%) in palm oil and Primex-Z®.

Replacing Primex® with palm oil (Table 1) in the AMLN diet drives a remarkably similar phenotype in C57BL/6J mice (17, 36). Palm oil, which is part of Primex® and Primex-Z®, contains mainly palmitic acid (∼42% of total fat) (for complete fatty acid profiles of the AMLN diet and modified fat versions, see **Supplemental Table 1**). Thus, this substitution increases the concentration of palmitic acid in the palm oil–based diet to ∼37% of total fat, compared with only 17% in the AMLN diet. This diet has been tested by the Danish contract research organization, Gubra, and is also referred to as the Gubra-Amylin NASH (GAN) diet in the literature. They showed that this diet was able to induce similar biopsy-confirmed liver lesions, with hallmarks of fibrotic NASH, in both ob/ob mice (after 16 wk) and C57BL/6J mice (after 28 wk) (17). Steatosis scores were 3 in both groups, and scores of inflammation (average: 2.5) and fibrosis (1.6–1.9) were also quite similar among animals fed either diet. However, C57BL/6J mice showed significantly greater mean weight gain (± SE) when fed the GAN (46.0 ± 0.8 g) diet than those fed the AMLN diet (40.6 ± 0.6 g) (17). In another study by Gubra

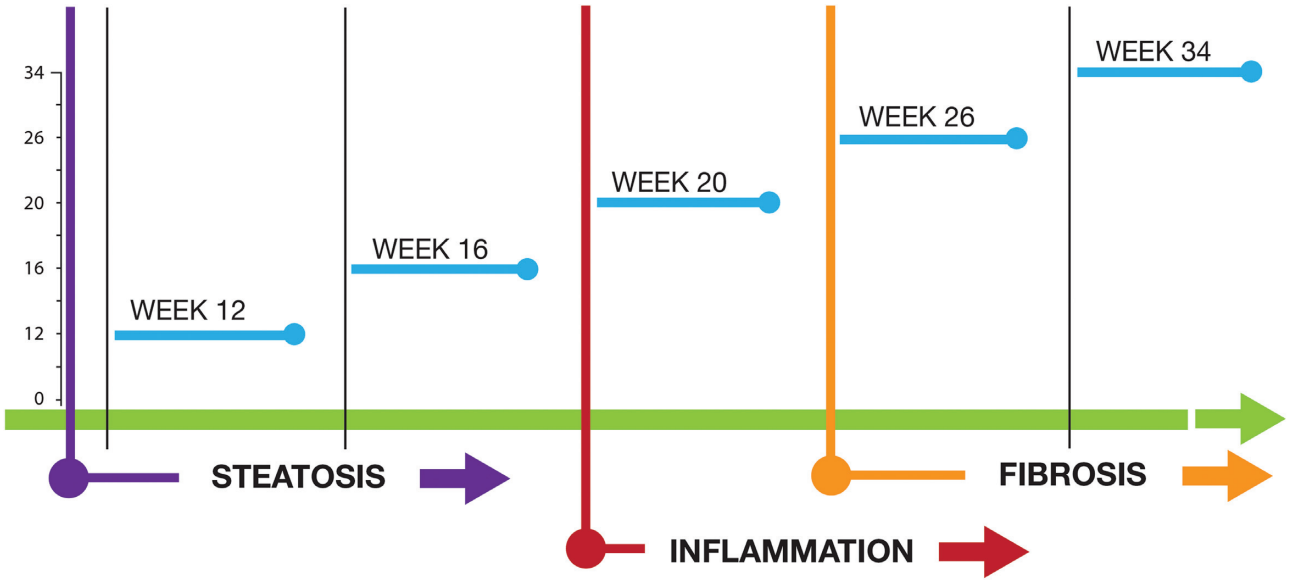

FIGURE 1 Typical timeline of NASH development in C57Bl/6 mice fed a high-fat (40 kcal%), high-fructose (20 kcal%), and high-cholesterol (2% wt:wt) diet. The timeline is adapted from the figure showing disease stages from the website of Taconic's Diet Induced NASH B6: https://www.taconic.com/mouse-model/diet-induced-nash-b6. NASH, nonalcoholic steatohepatitis.

(36), feeding C57BL/6J mice the GAN diet for 38–44 wk confirmed that hepatic histological and transcriptome changes in biopsy specimens from mice were similar to those seen in human NASH patients. In addition, mice consuming the GAN diet developed characteristics of the metabolic syndrome, such as obesity, hypercholesterolemia, and hyperinsulinemia with impaired glucose tolerance. **Figure 1** presents the typical progression of liver disease over time with the AMLN or the GAN diet in the C57BL/6 model. When animals consuming the GAN diet were housed in thermoneutral conditions (29°C), the animals displayed increased adiposity, NASH (characterized by increased liver weight, steatosis, NAS score: 5, hepatocyte ballooning, and elevated expression of liver cytokines), and liver fibrosis (fibrosis score: 1, elevated Collagen Type I Alpha 1 Chain (Col1a1) expression) compared with a group fed a 10 kcal% fat matched control diet at 16 wk (M Morrow, E Tsakiridis, J Lally, J Leroux, P Mundra, J Kwiecien, G Steinberg, unpublished data, presented at Keystone Meeting, NAFLD, 2020). At week 24, thermoneutrality accelerated NASH (NAS score: 6 at 29°C compared with 4 at 22°C, hepatocyte ballooning) and fibrosis development (fibrosis stage: 1C at 29°C compared with 1A at 22°C, increased expression of Col1a1 and Collagen Type IV Alpha 1 Chain); however, housing temperature did not further affect adiposity or parameters of insulin resistance. These robust models have also been shown to be an effective tool for identifying the key cell types that are involved in the pathogenesis of NAFLD (37), therapeutic targets such as hepatic thyroid hormone receptor β (38), and characterizing therapies for NAFLD with drugs and compounds such as nitro-oleic acid (30), rosiglitazone (39), and incretin (40) among others.

Whereas several of the aforementioned diets contained certain types of fat (i.e., palm oil or vegetable shortenings), fructose, and cholesterol at similar compositions, different compositions of these 3 ingredients have also been used for NASH development. Supplementation of an HFD or Western diet (typically containing a high amount of saturated fat and cholesterol at varying amounts) with fructose in the drinking water is another commonly used method to induce NASH in mice. For example, feeding C57BL/6N mice an HFD (45 kcal% fat with mainly lard with no added cholesterol) with 30% fructose in the drinking water induced hepatic steatosis, ballooning, inflammation, and fibrosis after 31 wk (31). However, the degree of inflammation and fibrosis was not as severe as that shown in mice fed the modified AMLN diet with palmitic acid and *trans* fats matched with the original AMLN diet. The combination of a Western diet (41 kcal% fat with mainly milk fat) and 5% fructose in water also has been used to induce NASH in an inbred polygenic and leptin-independent mouse model of obesity, MS-NASH (formerly called FATZO). After 20 wk of feeding, mice exhibited not only metabolic disturbances, like weight gain, obesity, and hypercholesterolemia, but also hepatic steatosis, ballooning, lobular inflammation, and fibrosis, with an average NAS score of 5 (41). Another similar dietary method, a Western diet (42 kcal% fat from milk fat with ~0.2% cholesterol) with an HFr-high-glucose solution (23.1 g D-fructose/L + 18.9 g D-glucose/L), has been used in combination in a stable isogenic cross between C57Bl/6J and 129S1/SvImJ to recapitulate the key features of human metabolic syndrome and NASH. This diet-induced animal model of NAFLD (termed DIAMOND mice) mimics key features of human NASH (42). After 16 wk of feeding, the mice developed weight gain, insulin resistance, and elevated ALT and aspartate aminotransferase (AST). Lobular inflammation, hepatocellular ballooning, Mallory-Denk bodies, and severe bridging fibrosis were observed after 52 wk of feeding. Signaling pathways related to human NASH, like lipogenic, inflammatory, and proapoptotic signaling pathways, were also activated in DIAMOND mice. Moreover, a majority of mice (89%) developed hepatocellular carcinoma between weeks 32 and 52. The same dietary treatment was applied to the parent strains (129S1/SvImJ and C57BL/6J) for 16–22 wk but the parent strains did not exhibit com-

promised glucose homeostasis (determined by insulin tolerance test). Although all 3 strains exhibited a similar degree of ballooning and lobular inflammation, lesser hepatic steatosis was observed in the parent 129S1/SvImJ mice and the degree of fibrosis was lower in the C57BL/6J mice. The fructose, palmitate, cholesterol, and *trans* fat (FPC) diet was another model inspired by the Western diet and ALIOS model (43). Cholesterol (1.25%) and pure palmitic acid (4% wt:wt) were added to a modified Western diet with *trans* fats (the fat source was a combination of butter, pure palmitic acid, and Primex®; total fat was 52 kcal%) and the diet was combined with high-fructose corn syrup [42 g/L glucose and fructose (55%/45%, wt:wt) in water]. By feeding the diet for 16 wk, C57BL/6J mice exhibited weight gain, increased fasting glucose and insulin, elevated ALT and AST, and hepatic steatosis. Markers for inflammation (*Tnfa, Mcp1, f4/80*) and fibrosis (*Tgfb1, Acta2*) were also upregulated in the liver of FPC-fed mice. Histological analysis revealed a score ~3 for steatosis, ~1–1.5 for inflammation, and ~1–2 for fibrosis (44).

Rats

Similarly to mice, rats are commonly used for NAFLD/NASH and can respond to different dietary strategies. Wistar rats fed an HFr diet (73 kcal% fructose) had increased hepatic TG, macrovesicular and microvesicular steatosis (score: 2.6–2.9), and lobular inflammation (score: 2.4). The concentrations of hepatic TG were almost 2–4 times higher than in animals on the high-sucrose diet (73 kcal%), HFD (40 kcal% fat, mixture of lard and soybean oil), or a diet high in fat and fructose (HFHFr; 40 kcal% fat, 41 kcal% fructose) (45). When fructose was provided in the water (20% fructose wt:vol) in combination with an HFD (60 kcal% fat), researchers observed that intrahepatic inflammation and metabolic derangements (glucose intolerance, oral glucose tolerance test) were more prominent in this HFHFr combination model than in either of the singular HF or HFr diet models in Sprague Dawley (SD) rats for 20 wk (46). Cholesterol is another important parameter needed to produce advanced-stage NASH in rats similar to that observed in mice. When SD rats were provided with an HFD (68% grain-based diet, 30% palm oil, 2% cholic acid) or HFC diet (68% grain-based diet, 30% palm oil, 2% cholic acid, and either 1.25% or 2.5% cholesterol) for 9 wk (47), the researchers observed that rats fed the HFD developed mild steatosis and inflammation without fibrosis, whereas all rats given the HFC diet developed NASH with hepatocyte ballooning and fibrosis. NAS scores in the animals consuming the HFC diet were ~6–7 on average, whereas in the HFD group they were ~2 (47). When stroke-prone spontaneously hypertensive rats (SHRSP5/Dmcr) were fed a similar HFC diet (mixture of 68% grain-based diet, 25% palm oil, 5% cholesterol, and 2% cholic acid), the animals developed NASH after 2 wk, including ballooning and fibrosis after 8 and 14 wk, respectively. The average NAS scores were 5.1 at week 8 and 5.7 at week 14, whereas in the control group they were ~1 (48). It is important to note, however, that in these particular modified diets, extra fat has been added to a grain-based diet. This additional fat dilutes other nutrients in the diet, such as protein and micronutrients. Such overzealous addition of fat also makes comparisons with the control diet (unmodified grain-based diet) impossible because this diet is unbalanced nutritionally compared with the original grain-based diet [reviewed in (8)]. Furthermore, these rats gained less weight than those fed the grain-based diet, likely owing to this imbalanced approach and the presence of a significant amount of cholic acid in these diets, which suppresses weight gain and improves insulin sensitivity in rodents fed an HFD (49). Thus, this approach would be less clinically relevant than when using a well-balanced purified diet with a combination of HF, HFr, and cholesterol to study the pathogenesis of NASH.

Researchers have used a combination of fructose/sucrose and cholesterol in an HFD background to enhance the development of NASH in rats. When Wistar rats were fed for 90 d with a diet high in fat, cholesterol, and sucrose [45 kcal% fat as lard + 5% sucrose in water (wt:vol) + cholesterol, concentration not reported], researchers observed that the HFC + sucrose diet group had the highest grade of steatosis (grade 2 of 3) and hepatic TG and cholesterol compared with the other groups fed the grain-based diet, the grain-based diet + 20% sucrose in water, and the HFD + 5% sucrose in water (wt:vol) without added cholesterol. These rats also had elevated body weight and liver weight, and were hyperglycemic compared with animals consuming the diets with no added cholesterol (50). Similarly to mice, SD rats fed the AMLN diet for 16 wk showed extensive hepatic steatosis and inflammation, and significantly elevated hepatic TG and cholesterol concentrations compared with the matched low-fat diet group (10 kcal% fat). These animals also had elevated concentrations of hepatic MCP-1 ($P < 0.01$), increased hepatic macrophage infiltration ($P < 0.001$), and higher plasma concentrations of ALT and AST compared with the mice fed the low-fat diet (51). In another study, male SD rats were fed a grain-based diet or AMLN diet for 4, 8, 12, or 19 wk (52). Preliminary data from this study showed that AMLN diet–fed rats gained more weight and had macrovesicular hepatic steatosis as early as 4 wk compared with the rats fed the low-fat control diet. When fed for 19 wk, animals in the AMLN group showed significant increases in the macrophage markers CD68 and C-C motif chemokine receptor 2, and extensive clusters of hepatic macrophages, indicative of enhanced hepatic inflammation compared with the low fat–fed control group. Interestingly, in the rat model (unlike mice), NAFLD/NASH development (steatosis, inflammation, ALT) using the AMLN diet seems to progress in a relatively shorter time frame (~16–20 wk). However, to our knowledge, there are no studies in rats on NASH development using a fat source other than Primex® (in the context of the AMLN diet).

Hamsters

Hamsters are becoming an increasingly popular choice for modeling lipid metabolism and NAFLD. Unlike wild-type mice and rats, hamsters carry a larger fraction of cholesterol as LDL, and their hepatocytes excrete only apoB-100, which more closely resembles human lipoprotein metabolism (53, 54). Although traditionally fed an HFD and/or HFr diet to promote NAFLD, the addition of cholesterol appears to exacerbate this phenotype and the severity of metabolic disturbances. In golden Syrian hamsters, adding 0.25% cholesterol to an HFHFr diet [30% fat (wt:wt), 40% fructose (wt:wt); 6 wk] increased plasma TG and cholesterol concentrations compared with the control group with no added cholesterol (55). Dietary cholesterol mainly induced VLDL cholesterol, HDL cholesterol, and VLDL TG concentrations. Furthermore, animals on the 0.25% cholesterol diet had worsened glucose tolerance and in-

sulin sensitivity compared with animals fed a 0.05% cholesterol diet (55). Another research group observed an elevation in plasma ALT, TGs, and total cholesterol, HDL cholesterol, and LDL cholesterol in male hamsters fed an HFC [40.8% fat (wt:wt), 0.5% cholesterol] diet with fructose in the drinking water [10% fructose (wt:vol)] for 20 wk relative to hamsters fed a grain-based diet with normal drinking water (56). These hamsters also had higher liver weights and liver cholesterol, TGs, and inflammation than animals fed a grain-based diet and histopathological evaluation revealed portal and bridging fibrosis accompanied with microvesicular steatosis. The NAS scores were closer to 9 in the HFC diet compared with almost 0 in the grain-based diet group. In another study, Syrian hamsters fed the corn-oil shortening–based HFC diet with higher fructose, but with lower cholesterol concentrations (0.3%), had elevated plasma lipids (after week 2), steatosis in the liver (after 4 mo), and hepatic stellate activation and fibrosis (4–8 mo) compared with the hamsters fed the control low-fat diet (10 kcal% fat). In addition, this diet also elevated liver cholesterol and TG, and increased circulating markers of liver dysfunction (AST and haptoglobin) and β-oxidation (3-β-hydroxybutyrate) (57). Compared with mice, hamsters seem to be more responsive to an HFC diet (grain-based diet supplemented with 11.5% coconut oil, 11.5% corn oil, and 1% cholesterol), as manifested by higher plasma ALT and AST, elevated plasma LDL, and more advanced development of hepatic steatosis, inflammation, and fibrosis (58). Again, as discussed previously, this dietary approach when adding fat to a grain-based diet is not recommended because the addition of the significant amount of fat dilutes other dietary nutrients, leading to a nutritionally unbalanced diet.

Guinea pigs

Guinea pigs are another animal model for diet-induced NASH and, like hamsters, share more similarities in cholesterol and lipoprotein metabolism with humans. For example, guinea pigs, like hamsters, possess plasma cholesteryl ester transfer protein (CETP) activity. In addition, guinea pigs and humans share a remarkable similarity in lipoprotein cholesterol distribution, with the majority of the plasma cholesterol carried by LDL (59). After feeding an HFC diet with higher sucrose [20% fat (wt:wt), 15% sucrose (wt:wt), 0.35% cholesterol] for 16 wk, guinea pigs exhibited elevated plasma AST and ALT accompanied with hepatic macro- and microvesicular steatosis and intralobular inflammation, despite no signs of weight gain, obesity, or insulin resistance (60). One group compared the effect of sucrose (0%, 15%, or 25% wt:wt) in an HFC diet [20% fat (wt:wt) and 0.35% cholesterol] on the development and progression of NAFLD/NASH (60). Regardless of the sucrose concentration, HFC diets caused similar accumulation of hepatic TG and cholesterol and elevated plasma AST and ALT. Micro- and macrovesicular steatosis, lobular inflammation, ballooning, and bridging fibrosis were also found in the liver sections after 25 wk of feeding (61). Sampling sites of livers for histological analysis seemed to not change the outcome of how an HF, high-sucrose, and HC diet [20% fat (wt:wt), 15% sucrose (wt:wt), 0.35% cholesterol] influenced fibrosis, but time frame was important (20 wk: fibrosis score = ~1; 24 wk: fibrosis score = ~5) (62). As with other rodent models, the severity of NASH after being fed an HF, high-sucrose, and HC diet [20% fat (wt:wt), 15% sucrose (wt:wt), 0.35% cholesterol] for 16–24 wk can differ from one breeder to the next, as suggested by a retrospective analysis of 5 different studies (63). Although the guinea pig has the capability of developing severe NASH and also elevations in blood cholesterol and LDL-cholesterol concentrations, one caveat with guinea pigs (like hamsters) is that they do not gain weight significantly after HFC diet feeding relative to animals fed a lower-fat diet. Therefore, extrapolation of data from this model clinically may be limited to a nonobese population with NAFLD.

NHPs

The relation between fructose intake and metabolic syndrome has been investigated in NHPs. Rhesus monkeys developed increased adiposity, insulin resistance, and elevated fasting TG concentrations after consuming 30 kcal% energy from fructose in the form of a fructose-sweetened beverage for 12 mo. Four of 29 monkeys even developed type 2 diabetes confirmed by a fasting blood glucose concentration >126 mg/dL (64). Prolonged feeding of an HFr diet (24 kcal% energy) to a cohort of Cynomolgus monkeys for 0.27–6.6 y (mean: 2.75 y) resulted in hepatic steatosis (specifically microvesicular steatosis) and fibrosis (65). However, there was no high-glucose diet as a control in these studies, so it is unknown whether the metabolic effects found in NHPs were due to high dietary fructose per se, or high simple sugars in general. Interestingly, in Cynomolgus and Grivet monkeys, 6 wk of feeding an HFr diet (24 kcal% fructose) in the context of a lower-fat and lower-cholesterol diet (17 kcal% fat with butter and vegetable oils and ~0.007% cholesterol) did not cause fatty liver compared with those fed a grain-based diet, which contained <3 kcal% sucrose and glucose and <0.5 kcal% fructose (66). Even though HFr-fed monkeys did not exhibit fatty liver, the total plasma cholesterol concentration was significantly increased, as were plasma markers for liver injury and inflammation: AST, ALT, γ-glutamyl transpeptidase, and C-reactive protein. Because of the relatively short time frame of feeding, perhaps a longer time frame would have allowed for fatty liver to occur in these NHPs. Addition of fructose to an HFC diet accelerated NASH progression as shown by a study in Cynomolgus monkeys. Aged and obese Cynomolgus monkeys (mean: 16.1 y) fed an HF, HC, and HFr diet for 12 mo developed elevated fasting plasma glucose and LDL cholesterol compared with baseline (67). The baseline NAS score was ~0–2 in ~60% of the population, and 3–5 in ~40% of the population. After 12 mo of dietary treatment, the disease progressed such that 40% of monkeys had an NAS score of 6–8. The fibrosis score also increased from 0–1 to 0–2, such that 20% of monkeys had a fibrosis score of 2. Treatment with pioglitazone, a drug shown to improve NASH and fibrosis, decreased the NAS score by ≥2 with changes in ≥2 components of NAS, in 30% of treated monkeys, with significant reduction in ballooning (67). Recently, an HF, HFr, and HC purified diet was fed to Rhesus and Cynomolgus monkeys to induce NASH. After 8 wk of feeding, histological evaluation suggested increased hepatic steatosis, ballooning, inflammation, and fibrosis, as well as elevated plasma ALT, AST, cholesterol, and TG concentrations, compared with baseline in Cynomolgus monkeys. Hepatic steatosis and fibrosis were also observed in Rhesus monkeys after 12 wk of feeding (K Chng, unpublished data, Crown Biosciences, Inc., https://www.crownbio.com/hubfs/assets/CrownBio-NASH-Day-Diet-Enhanced-NAFLD-NASH-NHP.pdf?hsLang=en).

Conclusion

As described, nutritionally balanced purified ingredient diets containing a combination of high concentrations of fat (high in *trans* fatty acids and/or SFAs), fructose, and cholesterol are capable of driving NASH and associated metabolic disorders in several different animal models. Many diets are commercially available and different laboratories have their own preferences. Their use has been prolific in recent years because they can provide a clinically relevant model owing to the ability of these diets to drive a combination of both metabolic disease and NASH. This is in contrast to other common diets for NASH induction, including the MCD diet, which reduces weight gain and other metabolic disturbances (i.e., insulin resistance, hyperlipidemia). Because there are a variety of HF, HC, and HFr diets that are commercially available to researchers, each of which can cause a different experimental outcome, proper reporting of the complete diet composition in the methods sections of articles is vital to continue advancing our knowledge of NASH. Some diets are not advised, including those where fat, fructose, and/or cholesterol are added to a grain-based diet background, which dilutes the nutritional and nonnutritional components in the diet; thus, data interpretation is severely diminished because it is unknown what is driving NASH (i.e., the addition of fat, fructose, and/or cholesterol or dilution of essential nutrients/nonnutrients, or both?).

Although many use the male C57BL/6 mouse model (including different substrains) for their work owing to their susceptibility to metabolic disease induction and potential to have severe symptoms of NASH including fibrosis, other rodent models such as rats, hamsters, and guinea pigs have certain advantages in terms of their metabolism being more similar to humans (e.g., guinea pigs have a similar lipoprotein profile to humans). Translation of any treatment effect on fibrosis to humans is likely better with NHPs owing to their closer genetic profile, which becomes valuable for evaluating the efficacy of drug candidates designed to reduce fibrosis. That said, there are limitations to how translatable some data are from animals such as guinea pigs, which when fed a higher-fat diet do not exhibit signs of obesity or insulin resistance, both of which would be commonly present in humans with NASH; moreover, differences exist in the lipid metabolism of wild-type mice and rats relative to humans. Other models that we did not discuss which respond to HFC diets and may provide improved translation of NASH data to humans include the minipig, and this has been reviewed elsewhere (68).

Furthermore, careful consideration of the control diet is required. Ideally, matched diets made with similar purified ingredients (except with limited amounts of those that are affecting NASH) should be used as control diets (6). For example, if the main fat source in a NASH-promoting diet is palm oil, then the control diet should contain a smaller amount of palm oil, while being calorically balanced with increased carbohydrate. To limit fructose and metabolic disease promotion, typically corn starch or glucose-derived carbohydrates are used for these calorie-balancing adjustments (see example in Table 1). Nevertheless, it is common to find that a grain-based diet (sometimes called "standard chow" or "regular diet") has been chosen to serve as a "control" diet. Grain-based diets, however, have a completely different ingredient composition to purified ingredient diets, making it impossible to determine what factors contributed to any observed changes (69). Furthermore, biologically active factors that are present in these grain-based diets, such as phytoestrogens, heavy metals, and mycotoxins, may vary significantly from one batch of diet to the next (69, 70). In light of these differences, a grain-based diet can only be viewed as a different experimental diet, and only a properly matched purified ingredient diet can serve as a suitable control for proper data interpretation.

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