High-fat, high-fructose and high-cholesterol diets to induce NASH

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Non-alcoholic fatty liver disease (NAFLD) is intricately linked to metabolic disease (inluding obesity, glucose intolerance, and insulin resistance) and encompasses a spectrum of disorders including steatosis, non-alcoholic steatohepatitis (NASH) and fibrosis. A variety of animal models that mirror both the histopathology and the pathophysiology of each stage of NAFLD/NASH are available, including rodents, but also certain large animals such as pigs and non-human primates. 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 [1]. Purified ingredient diets, which are essential diets for these studies, are open to the public and contain one main nutrient per ingredient [2], offering 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. Given most patients with NAFLD also have metabolic disturbances [3, 4], appropriate diet-induced animal models for this disorder should be able to reflect the whole NAFLD spectrum and present metabolic disturbances.

The AMLN Diet-induced NASH Model

Rodents consuming a diet containing higher fat (HF, around 40 kcal% fat), high fructose (HFr, 22%) and high cholesterol (HC, 2%) diets display many clinically relevant characteristics of NASH, along with other metabolic disorders. Primex®, the main fat source in this diet, is a vegetable shortening high in saturated and trans-fats (~28% trans-fatty acids), and is a combination of palm oil and partially hydrogenated soybean oil. One diet, formulated by researchers at Amylin Pharmaceuticals (now Medimmune),was termed the AMLN diet (D09100301, Table 1) and allowed for the development of all three stages of the NAFLD spectrum NAFLD spectrum including steatosis, steatohepatitis with fibrosis, and cirrhosis in ~30 weeks with a NAS score of 5.2 [5, 6]. This diet also induced metabolic dysfunction, as assessed by increased total cholesterol and markers of insulin resistance (increased fasting insulin and HOMA-IR, and reduced adiponectin) compared to animals on a low-fat control diet (10 kcal% fat, D09100304, Table 1). Similar to mice, SD rats fed the AMLN diet for 16 weeks showed elevated body weight, extensive hepatic steatosis and inflammation, and significantly elevated hepatic TG and cholesterol levels compared to the matched low-fat diet group (10 kcal% fat). These rats also had elevated levels of hepatic MCP-1 ($p < 0.01$), increased hepatic macrophage infiltration ($p < 0.001$), and higher plasma levels of ALT and AST compared to the low-fat diet group [7]. Interestingly, in the rat model, NAFLD/NASH development (steatosis, inflammation, ALT) using the AMLN diet seems to progress in a relatively shorter time-frame (~16-20 weeks, [8]).

Primex Replacement Diets

Due to the FDA ban on the use of Primex® and other food sources of trans-fat in 2018 [9], the NASH research community investigated 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% vs. ~28%). This corn-oil shortening based AMLN diet (D16010101, Table 1) was fed for 24 weeks to C57BL/6J mice. The animals on this diet developed steatosis comparable to mice fed the original AMLN diet, with progression to NASH (hepatocyte ballooning, massive fatty liver degeneration; NAS score 5-6) and mild fibrosis [10]. Furthermore, this modified diet also increased immune cell infiltration and collagen accumulation in the epididymal white adipose tissue [10]. Another group combined the corn oil shortening

Main Fat Source	Primex Shortening		Palm Oil		Corn Oil Shortenining		Primex, Non Trans Fat Shortening		Matching Transfat and Palmitic Acid to D09100301		Matching Transfat to D09100301		Matched Low Fat Control	
Diet ID	**D09100301**		**D09100310**		**D16010101**		**D16022301**		**D17010103**		**D17010102**		**D09100304**	
	gm%	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***	**gm%**	***kcal%***
Protein	**22**	***20***	**22**	***20***	**22**	***20***	**22**	***20***	**22**	***20***	**22**	***20***	**19**	***20***
Carbohydrate	**44**	***40***	**44**	***40***	**44**	***40***	**44**	***40***	**44**	***40***	**44**	***40***	**67**	***70***
Fat	**20**	***40***	**20**	***40***	**20**	***40***	**20**	***40***	**20**	***40***	**20**	***40***	**4**	***10***
Saturated (% Fat)	**27**		**45**		**21**		**44**		**27**		**25**		**23**	
Monounsaturated (% Fat)	**34**		**35**		**39**		**36**		**38**		**38**		**30**	
Polyunsaturated (% Fat)	**17**		**20**		**13**		**19**		**12**		**14**		**47**	
n6:n3 ratio	**11**		**7**		**9**		**12**		**10**		**10**		**8**	
Trans fat (gm)	**39.6**		**0.0**		**48.1**		**0.9**		**39.5**		**39.4**		**0.0**	
kcal/gm	**4.5**		**4.5**		**4.5**		**4.5**		**4.5**		**4.5**		**3.8**	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	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*	3	*12*
Corn Starch	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	350	*1400*
Maltodextrin 10	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*	85	*340*
Fructose	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	200	*800*	0	*0*
Dextrose	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	169	*676*
Sucrose	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*	100	*400*
Cellulose	50	*0*	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*	25	*225*
Primex Shortening	135	*1215*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Primex Shortening, Non Transfat	0	*0*	0	*0*	0	*0*	135	*1215*	45	*405*	25	*225*	0	*0*
Corn Oil, Partially Hydrogenated	0	*0*	0	*0*	135	*1215*	0	*0*	110	*990*	110	*990*	0	*0*
Palm Oil	0	*0*	135	*1215*	0	*0*	0	*0*	0	*0*	0	*0*	0	*0*
Lard	20	*180*	20	*180*	20	*180*	20	*180*	0	*0*	20	*180*	20	*180*
Mineral Mix S10026B	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*	50	*0*
Vitamin Mix V10001C	1	*4*	1	*4*	1	*4*	1	*4*	1	*4*	1	*4*	1	*4*
Choline Bitartrate	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*	2	*0*
Cholesterol	18	*0*	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	*0*	0.025	*0*
FD&C Blue Dye #1	0	*0*	0.025	*0*	0.025	*0*	0	*0*	0	*0*	0.05	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0.025	*0*	0	*0*	0.05	*0*	0.05	*0*	0	*0*	0.025	*0*
Total	**904.05**	***4036***	**904.05**	***4036***	**904.05**	***4036***	**904.05**	***4036***	**904.05**	***4036***	**904.05**	***4036***	**1055.05**	***4037***

TABLE 1: Formulations of Primex® replacement diets.

with non-trans-fat Primex-Z® (a mixture of fully hydrogenated soybean oil and palm oil, contains ~42% palmitic acid) to match both the palmitic acid and the trans-fat content of the original AMLN diet (D17010103, Table 1). C57BL/6J mice fed this diet for 12 weeks developed steatosis with mild fibrosis and at 24 weeks this diet induced histologic features characteristic of NASH (NAS score 6.2 ± 0.4) with fibrosis (score 1.40 ± 0.16) [11]. A similar diet, containing slightly lower levels of palmitic acid (but similar trans-fat levels, D17010102, Table 1), also demonstrated development of steatosis with NASH after 15 weeks in C57BL/6 mice [12, 13].

Palm Oil as a Suitable Primex® Replacement.

Replacing Primex® in the AMLN diet with palm oil (contains 42% palmitic acid), one of the fat sources in Primex®, drives a remarkably similar phenotype in C57BL/6J mice [14, 15]. This diet (D09100310, Table 1) 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. This diet was able to induce similar biopsy-confirmed liver lesions, with hallmarks of fibrotic NASH, in both ob/ob mice (after 16 weeks) and C57BL/6J mice (after 28 weeks) [14] with steatosis scores of 3, inflammation score of ~2.5 and fibrotic scores of 1.6-1.9. However, the GAN diet drove significantly greater weight gain in C57BL/6J mice (46.0 ± 0.8 g) diet compared to animals on the AMLN diet (40.6 ± 0.6 g) [14]. In another study [15], C57BL/6J mice fed the GAN diet for 38-44 weeks confirmed that hepatic histological and transcriptome changes from biopsied mice were similar to those seen in human NASH patients. In addition, mice consuming the GAN diet developed other characteristics of the metabolic syndrome, such as elevated weight gain, hypercholesterolemia, and hyperinsulinemia with impaired glucose tolerance. The typical progression of liver disease over time with the AMLN or the GAN diet in the C57BL/6 model is presented in Figure 1.

We compared these different fat sources (Primex®, palm oil, corn oil shortening, or Primex-Z® – non-trans-fat shortening) in the background of the AMLN diet, by feeding these diets to C57BL/6 mice [16]. The diet containing non-trans-fat Primex-Z®, D16022301, led to a sustained increase in adiposity index (~90% increase of epididymal, retroperitoneal, and mesenteric fat pads) after 30 weeks when compared to other fat sources. While mice fed all the different HFDs had several similar disease outcomes, including fat accumulation within hepatocytes; the diets D09100310 (palm oil)

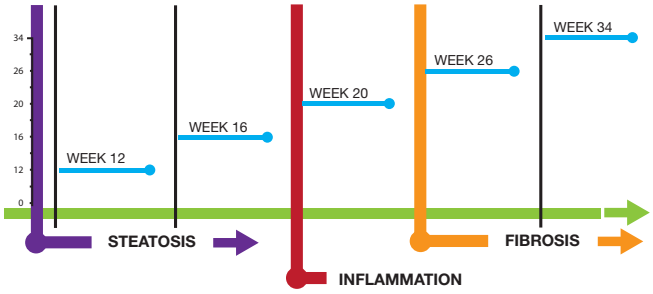

FIGURE 1: Typical timeline of NASH development in C57Bl/6 mice on a high-fat (40 kcal%), high-fructose (22% w/w) and high-cholesterol (2% w/w) diet. The timeline was adapted from the Figure of Disease stages from the website of Taconic's Diet Induced NASH B6. https://www.taconic.com/mouse-model/diet-induced-nash-b6

and D16022301 (Primex-Z®) induced a more severe form of liver inflammation, along with increased inability to arrest bacteria under blood flow, which could render mice more susceptible to infections. Similar results were observed by another group who found diets containing Primex-Z® (D16022301, Table 1) produced more pronounced NASH and fibrosis [10]. 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 non-neoplastic) nodular lesions at week 26 compared to the diet containing the trans-fat version of Primex® [17]. Together, these studies suggest that the absence of trans-fat does not impede the development of NASH and fibrosis, but affirms that the palmitic acid from palm oil or Primex-Z® is important for driving this phenotype.

Conclusion

While several of the diets discussed above contained certain types of fat (i.e. palm oil or vegetable shortenings), fructose, and cholesterol at similar compositions, different compositions of these three ingredients have also been used for NASH development. Given there are a variety of these 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 papers is vital to continue advancing our knowledge of NASH. 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, e.g. fat, fructose and cholesterol, e.g. D09100304, Table 1) should be employed as control diets [1]. We have reviewed this in detail elsewhere [18, 19] and are happy to assist researchers with proper control diet selection.

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" If you want additional details on these diets, please refer to our recently published paper. Considerations When Choosing High-Fat, High-Fructose, and High-Cholesterol Diets to Induce Experimental Nonalcoholic Fatty Liver Disease in Laboratory Animal Models, Sridhar Radhakrishnan, Steven F Yeung, Jia-Yu Ke, Maísa M Antunes, and Michael A Pellizzon. Current Developments in Nutrition. 2021;5:nzab138."

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