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A Systematic Review of Animal Models of NAFLD Finds High-Fat, High-Fructose Diets Most Closely Resemble Human NAFLD

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BACKGROUND AND AIMS: Animal models of human disease are a key component of translational hepatology research, yet there is no consensus on which model is optimal for NAFLD.

APPROACH AND RESULTS: We generated a database of 3,920 rodent models of NAFLD. Study designs were highly heterogeneous, and therefore, few models had been cited more than once. Analysis of genetic models supported the current evidence for the role of adipose dysfunction and suggested a role for innate immunity in the progression of NAFLD. We identified that high-fat, high-fructose diets most closely recapitulate the human phenotype of NAFLD. There was substantial variability in the nomenclature of animal models: a consensus on terminology of specialist diets is needed. More broadly, this analysis demonstrates the variability in preclinical study design, which has wider implications for the reproducibility of *in vivo* experiments both in the field of hepatology and beyond.

CONCLUSIONS: This systematic analysis provides a framework for phenotypic assessment of NAFLD models and highlights the need for increased standardization and replication. (Hepatology 2021;0:1-18).

NAFLD is a slowly progressive condition characterized by accumulation of excess hepatic lipids.[1] Some individuals go on to develop NASH that drives fibrosis, which in turn can lead to cirrhosis and HCC.[2] The burden and mortality associated with NAFLD are increasing rapidly, especially in developed countries.[3]

NAFLD is causally associated with insulin resistance, typically through obesity.[4] Individuals with the most severe insulin resistance and metabolic risk factors are at greatest risk of progressive liver disease, which has been reflected in the recently coined term "metabolic dysfunction–associated fatty liver disease."[5] On the other hand, some common genetic variants (e.g., p.Ile148Met in patatin-like phospholipase domain containing 3 [*PNPLA3*]) may lead to the development of NAFLD with lower levels of insulin resistance.[6] Almost all such variants perturb hepatic lipid metabolism,[7] and there has been little human genetic evidence for the inflammatory or fibrotic aspects of NASH.

Histologically, fatty liver disease manifests with a spectrum of histological features on liver biopsy in humans, including steatosis, lobular and periportal inflammation, hepatocellular ballooning, and fibrosis.[8] Simple steatosis can be distinguished from NASH through assessment by pathologists as the latter typically requires the presence of lobular inflammation and hepatocellular ballooning.

Abbreviations: ApoE, apolipoprotein E; HFD, high-fat diet.

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Potential conflict of interest: Dr. Rowe advises Roche.

Animal models of human diseases are an integral part of preclinical research, allowing researchers to facilitate mechanistic and therapeutic studies that are not possible in humans. Studies of NAFLD predominantly use rodent models of obesity (or insulin resistance) to induce hepatic steatosis.[9] However, it remains unclear which rodent models most closely reflect the human disease phenotype.

The theoretical "ideal" NAFLD model should reflect the full human spectrum of hepatic disease clinically, biochemically, and histologically, plus features of the associated metabolic syndrome. It would pass through these stages without taking an unacceptably long duration (such as over 1 year).[10]

We have recently found that variation in study design (e.g., percentage kilocalories from fat in diet, age of mice) has a significant effect on response to therapeutic pharmacological agents in rodent models of NAFLD.[11] Therefore, detailed appraisal of preclinical study design is vitally important for reproducibility, particularly given that some recent high-profile studies could not be replicated in the field.[12]

To address these questions, we systematically reviewed and categorized NAFLD models from over 4,500 published studies. We interrogated features of the human metabolic phenotype, histological features, and gene expression in the animal models to identify the rodent model(s) most resembling human NAFLD.

Materials and Methods

PROTOCOL AND SEARCH STRATEGY

The systematic review protocol was prospectively registered with the Systematic Review Facility in August 2017 and is available from https://drive.google.com/file/d/0B7Z0eAxKc8ApQ0p4OG5SblRlRTA/view. A partial analysis of this data set has been reported elsewhere.[11]

PubMed on MEDLINE, EMBASE, and the National Center for Biotechnology Information's Gene Expression Omnibus were searched for published articles of experimental rodent models of fatty liver, NAFLD, or NASH. The search was completed in January 2019.

STUDY SELECTION AND ELIGIBILITY CRITERIA

Our inclusion criteria were primary research articles using mice or rats to model NAFLD (to include hepatic steatosis, NASH, and NASH-fibrosis) and evidence of hepatic steatosis in model rodents through either increased hepatic triglyceride content or histological assessment. Studies were excluded if they did not model NAFLD/NASH; they were in humans or any animal other than mice and rats; they were reviews, comments, letters, editorials, meta-analyses, or ideas; or they were not in English (unless there was an available translation).

Abstracts and titles were screened to identify relevant studies using Rayyan.[13] Potentially relevant studies had their full text extracted and were assessed against inclusion/exclusion criteria independently by two reviewers, with discrepancies settled by discussion with J.P.M.

DATA COLLECTION

The variables extracted were as follows: phenotypic characteristics of animal model used (e.g., sex, dietary composition, rodent age, genetic alterations,

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background animal strain, chemical agents, genetic manipulations), features of metabolic syndrome (obesity, dyslipidemia, insulin resistance), presence of lipodystrophy, features of NAFLD (elevated aminotransferases, lobular inflammation, hepatocellular ballooning, portal inflammation, NASH, fibrosis [stage, if stated], HCC), and age (in weeks). Where data had been deposited in the Gene Expression Omnibus, the accession number was extracted. Mouse Genome Informatics was used to find human orthologues for murine genes perturbed in genetically modified animals.[14]

The presence or absence of phenotypic characteristics was based on the reported description in the included article. Lipodystrophy was defined as the presence of reduced fat mass plus increased insulin resistance and where the animals were described as lipodystrophic. Obesity was defined as a significantly higher body weight (or fat mass) than control animals. Insulin resistance was defined as a significant elevation in any of the following: fasting or postprandial glucose or insulin, homeostatic model assessment of insulin resistance, greater AUC on glucose or insulin tolerance test, or lower glucose infusion rate during hyperinsulinemic–euglycemic clamp studies. Dyslipidemia was defined by any of the following: higher triglycerides, lower HDL, higher cholesterol, or higher LDL. Elevated aminotransferases were defined by significantly higher alanine or aspartate aminotransferase compared to control animals. Presence or absence of NASH was assessed dichotomously based on description in the article. Many models were dichotomously described to show the presence or absence of fibrosis without further details; therefore, this was collected in addition to fibrosis stage (where available), which was extracted according to Kleiner staging.[15]

Genetically modified animals were included as separate models where they were shown to exacerbate the liver phenotype of NAFLD. If a study reported a "negative control" animal, a model with NAFLD ("positive control"), and a genetically modified animal with less severe NAFLD than the "positive control," the genetically modified animal would not be included as a separate model of NAFLD.

Studies frequently included multiple model designs (or treatment arms for interventional studies). Data were extracted for each model or interventional arm separately. Where data from male and female animals were reported separately, they were included as separate models.

The complete raw data set has been deposited as a Dryad database (https://doi.org/10.5061/dryad.pnvx0k6kq).

COMPARISON OF MODELS FOR SIMILARITY

Models were compared for similarity based on study design. Criteria for considering two models to be the same were chosen manually given the recent finding that most outcomes in rodent models of NAFLD could be modeled in a continuous fashion[11] and included identical genetic background, sex of animals studied, age diet initiated ±1 week, kilocalories of diet from fat ±2%, weight of cholesterol in diet ±0.1%, weight of choline in diet ±0.1%, kilocalories of diet from sucrose ±2%, fructose–glucose in diet ±2% kcal (or ±2 g/L where dissolved in drinking water), age given chemical/virus/surgery ±1 week, dose of chemical ±10% (chemical-specific), and identical route of administration of chemical. Models could be combined using variables that were not reported (e.g., studies not reporting sex, but otherwise identical models, would be considered the same).

Models were organized into three levels: category, main group, and subgroup. This was based on the similarity of model design after data extraction, rather than on terminology used in the original article. For example, a model using 45% kcal fat plus 20% kcal fructose would be categorized as "diet only" > "high fat, high carbohydrate" > "high fat, high fructose," even if the authors described the model as a "high-fat diet" (HFD). The article's original description used for each model was retained for comparison of nomenclature with study design.

COMPARISON OF MODELS WITH HUMAN PHENOTYPE

A metabolic syndrome score (range 0-3) and a liver histology score (range 0-11) were used to assess the similarity of each model to human NAFLD (Supporting Table S1). For the metabolic syndrome score, models scored 1 for each of the following: obesity, insulin resistance, and dyslipidemia. For liver histology, models scored 1 for each of the following:

raised aminotransferases, lobular inflammation, portal inflammation, hepatocellular ballooning, NASH, fibrosis (presence/absence), fibrosis stages 1-4 (1 point for each), and HCC. The scoring of histological features was based upon the author's reported presence or absence of each feature, with the definition of each histological characteristic according to that of the (human) NASH Clinical Research Network.[15] Presence or absence of NASH was based on the author's reported global histological assessment (i.e., no score cutoff was used). These two scores were combined to give an overall phenotype score (range 0-14). Scores were only recorded where suitable data were reported.

RISK OF BIAS ASSESSMENT

Each paper was assessed in the following four areas: reporting the presence of a protocol, reporting use of randomization, reporting use of blinding, and a power calculation for sample size estimation. These were each given a score of 1, and each paper was assigned an overall "risk of bias score."

STATISTICAL ANALYSIS

For comparison of phenotype scores by subgroup, models were filtered for those that had been used by at least one study, and then subgroups were filtered for those that contained at least three models. Gene lists were analyzed using the EnrichR package for R.[16] Histograms and descriptive statistics were extracted using R 3.6.2 for Mac.[17,18] The code used in the analysis is available from https://doi.org/10.5281/zenodo.4656980.

Results

THE LANDSCAPE OF ANIMAL MODELS OF NAFLD

In order to understand the spectrum of animal models used to study fatty liver disease, we screened 8,727 published articles and included 4,540 articles in our final analysis (Fig. 1). From this, we built a database of 3,920 unique rodent models of NAFLD, which we grouped into nine broad categories of model (Table 1; Supporting Tables S2 and S3). These could be further subdivided into 29 main groups (e.g., "high-fat, high-cholesterol diet") and 771 subgroups (e.g., "apolipoprotein E [ApoE] mutant with dietary manipulation").

The most common category of model was dietary (1,927/3,920), followed by genetically altered rodents combined with dietary manipulation (826/3,920). The majority of models were conducted using only male animals (2,777/3,920, 82%); therefore, a direct comparison of female versus male animals was limited except for the three subgroups where sufficient data were available, and no consistent trend was observed (Supporting Fig. S1).

Of the models, 27% (1,055/3,920) were performed using C57BL/6J mice, with hundreds of other genetic backgrounds represented (Supporting Table S4). Twelve weeks was the modal duration of model study (mean = 15 weeks) (Fig. 2A). Study duration was similar for the assessment of all histological features except for the presence of HCC, which was reported at up to 2 years of age (Supporting Fig. S2). Where described, studies were almost uniformly performed at room temperature (20-22°C); and therefore, this was not included as a variable in subsequent analyses. A single study[19] assessed the effect of housing at thermoneutrality (30°C) and found this to exacerbate the HFD phenotype, resulting in a phenotype that more closely mirrored human NASH.

REPLICATION OF MODEL DESIGN

Due to differences in model design (e.g., proportion of fat in diet, age diet initiated, genetic background), models were used by a mean of just 1.6 studies (median 1 study). Genetic models fed standard chow were used by a mean 2.2 studies, compared to only 1.1 for genetic models with dietary manipulation. There was a trend that more complex models (e.g., offspring, chemical plus dietary) were more likely to be used by a single study only. The most frequently used model was male leptin-deficient (*ob/ob*) mice on a pure C57BL/6J background fed standard chow (Table 2).

COMPARABILITY OF MODELS TO HUMAN NAFLD

In order to compare the rodent models to that of human NAFLD, we examined the metabolic

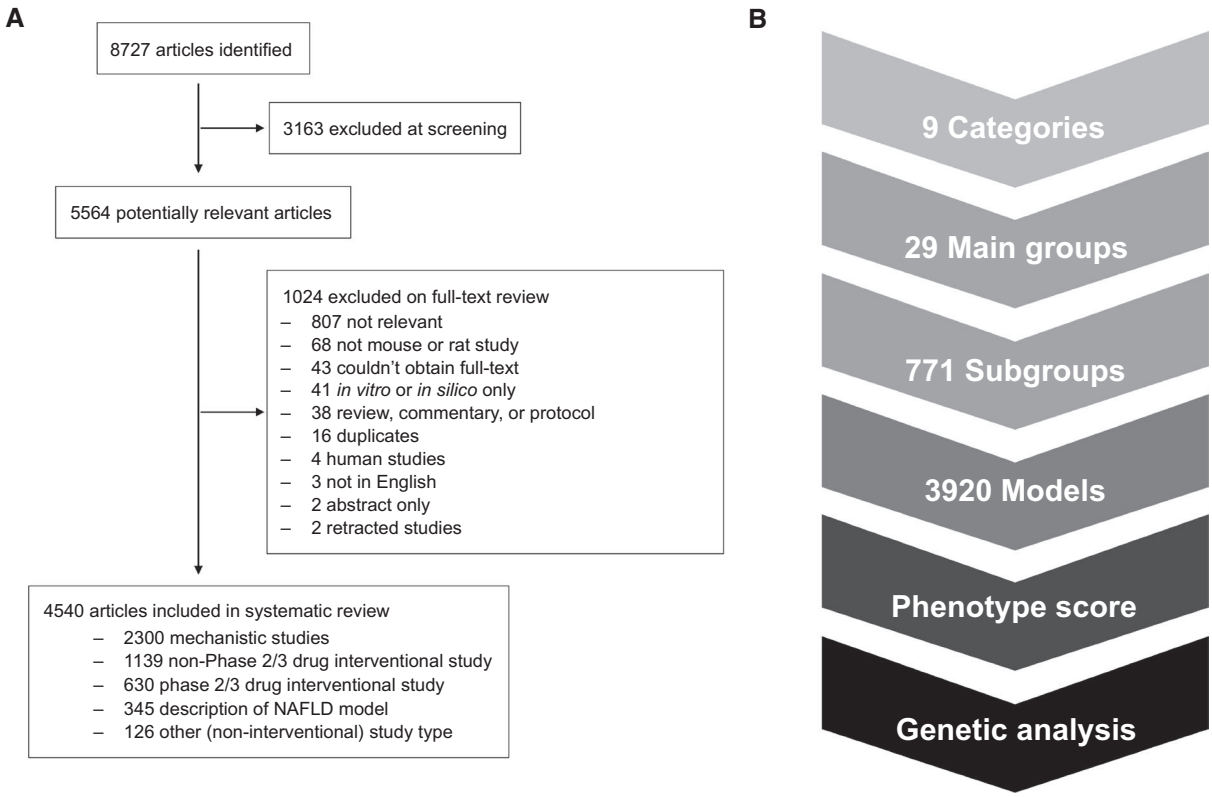

FIG. 1. Study design. (A) Article inclusion and exclusion flowchart. (B) Overview of the categorization hierarchy used in analysis.

phenotype, histology, and gene expression in the animal models.

METABOLIC PHENOTYPE

Of the models, 44% were reported to show insulin resistance, 47% were obese, and 51% had dyslipidemia. Of the models, 47% (1,839/3,920) had at least two features of the metabolic syndrome (Fig. 2D). Chemically induced models were less frequently reported to show metabolic features of NAFLD (e.g., 26% with obesity).

HISTOLOGY

Of the models, 93% (3,655/3,920) reported liver histology. Overall, 35% of models were reported to show NASH and 23% to show some stage of fibrosis, whereas the presence or absence of periportal inflammation was described in only 9% of models (Supporting Fig. S1).

No model was reported to recapitulate all possible features of NAFLD liver histology and the metabolic syndrome. Three models had an overall NAFLD model score of 13 (out of 14). Only one of these three models had been used in at least two studies, i.e., replicated (Table 3). All models, their NAFLD phenotype scores, and studies using each model can be viewed in Supporting Tables S2 and S3. The three highest-scoring models were 10-week-old male C57BL/6J-129/SvlmJ mice fed a 42% calorie fat, 0.1% cholesterol diet and given 55% fructose of 42 g/L water *ad libitum* for 8 weeks, used by two studies[20,21]; 7-week-old male C57BL/6?-129 mice fed a 45% calorie fat diet and given 55% fructose of 42 g/L water *ad libitum* for 24 weeks, used in only one study[22]; and 9-week-old C57BL/6J mice fed a "Western diet" (fed a diet with 42% calories due to fat, 1.25% weight due to cholesterol, 37.3% calories due to sucrose, and given 55% fructose of 42 g/L water *ad libitum*) for 12-24 weeks, used by one study.[23] Hence, two of the models were based on a high-fat, high-fructose

TABLE 1. Characteristics of rodent models of NAFLD

Category		Overall	Dietary	Dietary + Other	Genetic	Genetic + Chemical	Genetic + Dietary	Genetic + Other	Chemical	Offspring	Other
Number of models		3,920	1,927	68	477	65	826	26	391	91	49
Main groups		29	14	9	8	7	8	6	3	4	4
Subgroups		771	44	16	275	32	359	23	28	15	14
Sex	Both	175 (5.2%)	81 (4.7%)	2 (3%)	14 (4.3%)		47 (6.7%)	1 (4.8%)	10 (2.7%)	19 (22.1%)	1 (2.3%)
	Female	444 (13.1%)	180 (10.4%)	32 (48.5%)	47 (14.6%)	7 (13.7%)	78 (11.1%)	5 (23.8%)	52 (14.2%)	26 (30.2%)	17 (38.6%)
	Male	2,777 (81.8%)	1,476 (85%)	32 (48.5%)	261 (81.1%)	44 (86.3%)	578 (82.2%)	15 (71.4%)	304 (83.1%)	41 (47.7%)	26 (59.1%)
Age diet started	Median (SE)	7.0 (0.1)	7.0 (0.1)	7.8 (0.3)		8.0 (0.8)	8.0 (0.2)	8.0 (0.8)	6.0 (0.2)	4.0 (0.5)	8.0 (0.6)
	Range	(1.0-101.0)	(1.0-101.0)	(2.0-14.0)		(3.0-36.0)	(2.0-62.0)	(4.0-16.0)	(3.0-32.0)	(3.0-21.0)	(3.0-21.0)
Fat in diet (%kcal)	Median (SE)	45.0 (0.3)	44.5 (0.4)	45.0 (1.8)		45.0 (1.8)	45.0 (0.5)	42.5 (2.9)	53.0 (0.9)	43.0 (1.5)	34.7 (3.1)
	Range	(0.0-100.0)	(0.0-95.1)	(0.1-82.0)		(12.0-60.0)	(0.0-100.0)	(16.7-60.0)	(5.0-82.0)	(14.2-60.0)	(11.1-60.0)
Cholesterol in diet (%weight)	Median (SE)	1.0 (0.03)	1.2 (0.05)	0.5 (0.30)		0.5 (0.06)	0.2 (0.05)		2.0 (0.14)	0.3 (0.07)	2.0 (0.23)
	Range	(0.0-15.0)	(0.0-15.0)	(0.0-10.0)		(0.2-1.2)	(0.0-12.5)		(0.0-10.0)	(0.1-2.0)	(0.0-4.0)
Sucrose in diet (%kcal)	Median (SE)	29.0 (0.3)	26.2 (0.4)	32.0 (1.8)		17.4 (1.5)	30.4 (0.6)		18.2 (1.3)	30.2 (1.9)	
	Range	(1.0-88.9)	(1.0-88.9)	(21.7-42.4)		(10.3-34.0)	(6.7-74.0)		(6.5-73.0)	(27.0-65.2)	
Fructose in diet (%weight)	Median (SE)	21.0 (0.3)	20.0 (0.5)	13.8 (1.4)			30.0 (0.8)		26.0 (0.9)	20.0 (0.0)	37.5 (4.5)
	Range	(2.3-79.0)	(2.3-79.0)	(2.3-30.0)			(2.3-70.0)		(2.3-66.0)	(20.0-20.0)	(15.0-60.0)
Lipodystrophy		48 (1.2%)			33 (6.9%)		15 (1.8%)				
Metabolic syndrome features	Obesity	1,855 (47.3%)	1,011 (52.5%)	41 (60.3%)	154 (32.3%)	31 (47.7%)	440 (53.3%)	10 (38.5%)	100 (25.6%)	47 (51.6%)	21 (42.9%)
	Insulin-resistant	1,709 (43.6%)	846 (43.9%)	24 (35.3%)	212 (44.4%)	23 (35.4%)	408 (49.4%)	11 (42.3%)	132 (33.8%)	36 (39.6%)	17 (34.7%)
	Dyslipidemia	1,993 (50.8%)	1,025 (53.2%)	27 (39.7%)	234 (49.1%)	35 (53.8%)	442 (53.5%)	15 (57.7%)	146 (37.3%)	46 (50.5%)	23 (46.9%)
Liver histology features	Raised transaminases	1,663 (42.4%)	880 (45.7%)	30 (44.1%)	139 (29.1%)	33 (50.8%)	350 (42.4%)	9 (34.6%)	177 (45.3%)	27 (29.7%)	18 (36.7%)
	Lobular inflammation	772 (19.7%)	408 (21.2%)	16 (23.5%)	58 (12.2%)	12 (18.5%)	165 (20.0%)	5 (19.2%)	93 (23.8%)	7 (7.7%)	8 (16.3%)
	Hepatocellular ballooning	745 (19.0%)	411 (21.3%)	10 (14.7%)	48 (10.1%)	11 (16.9%)	146 (17.7%)	3 (11.5%)	91 (23.3%)	16 (17.6%)	9 (18.4%)
	Steatohepatitis	1,369 (34.9%)	718 (37.3%)	19 (27.9%)	100 (21.0%)	26 (40.0%)	323 (39.1%)	5 (19.2%)	144 (36.8%)	21 (23.1%)	13 (26.5%)
	(Peri-)Portal inflammation	167 (4.3%)	106 (5.5%)	4 (5.9%)	18 (3.8%)		21 (2.5%)		15 (3.8%)	1 (1.1%)	2 (4.1%)
	Fibrosis	891 (22.7%)	455 (23.6%)	17 (25.0%)	75 (15.7%)	12 (18.5%)	215 (26.0%)	4 (15.4%)	97 (24.8%)	8 (8.8%)	8 (16.3%)
	HCC	177 (4.5%)	34 (1.8%)	4 (5.9%)	50 (10.5%)	15 (23.1%)	19 (2.3%)	4 (15.4%)	50 (12.8%)		1 (2.0%)
Citations per model	Mean	1.59	1.81	1.04	2.16	1.03	1.09	1	1.19	1.03	1.24
	Range	(1.0-90.0)	(1.0-78.0)	(1.0-2.0)	(1.0-90.0)	(1.0-2.0)	(1.0-6.0)	(1.0-1.0)	(1.0-12.0)	(1.0-2.0)	(1.0-4.0)

Data from 4,540 studies reporting 3,920 unique NAFLD models, divided into nine categories.

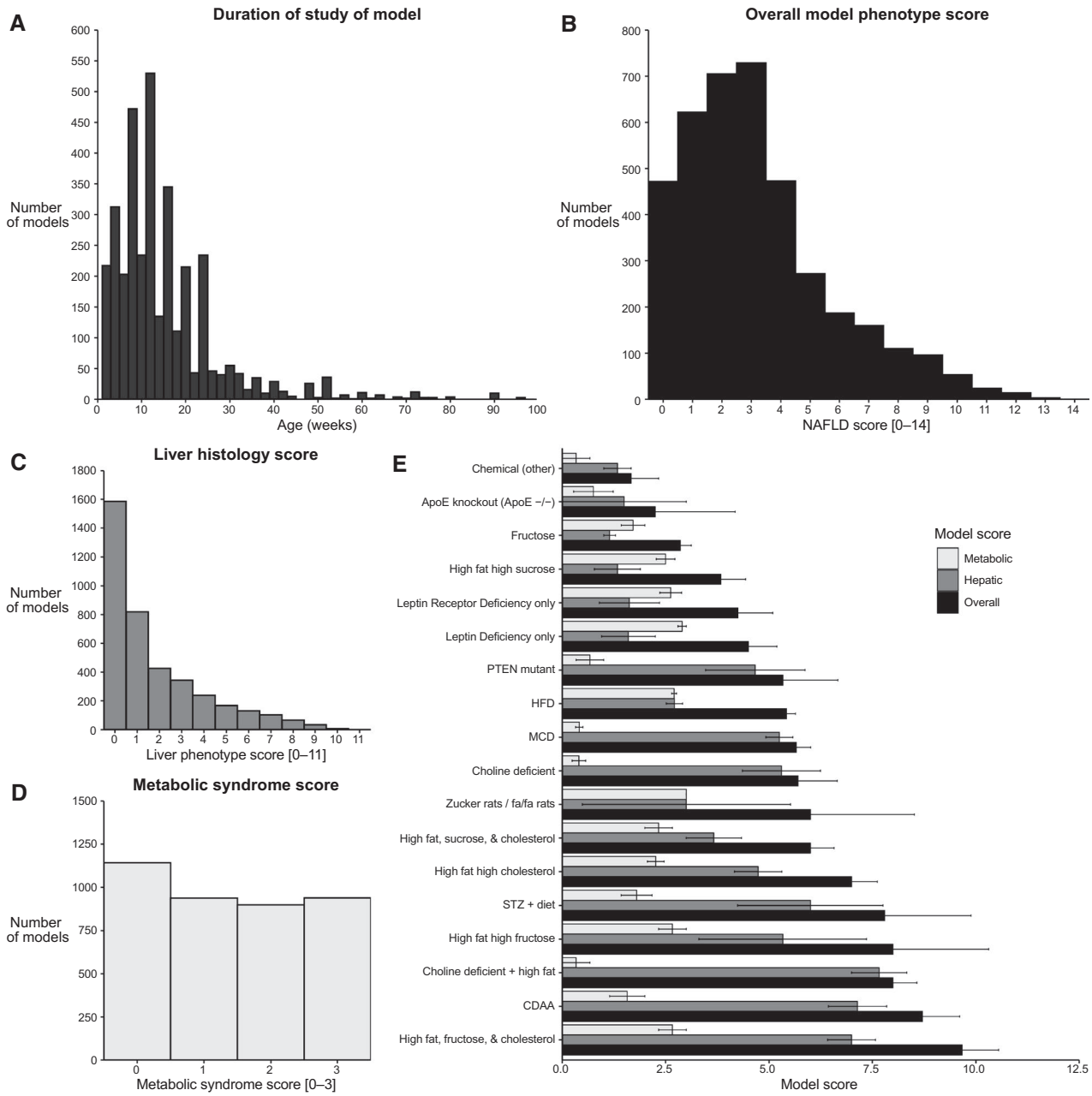

FIG. 2. Phenotype score and duration of rodent models of NAFLD. (A) Histogram illustrating the maximum duration each model (n = 3,920) was studied. (B) Overall phenotype score for each model (0-14) generated from a combination of liver histology score and metabolic syndrome score. (C) Liver histology score (0-11) for each model, where 1 point is given for the presence of each histological feature of human NAFLD, including fibrosis stages 1-4 and HCC, plus raised aminotransferases. (D) Metabolic syndrome score (0-3) for each model was calculated as the presence of obesity, insulin resistance, and dyslipidemia, with 1 point for each. (E) Comparison of mean (±SE) scores for subgroups of models with at least three models, which had all been replicated at least once. Abbreviations: CDAA, choline-deficient, amino acid–defined diet; MCD, methionine-/choline-deficient diet; STZ, streptozocin.

and added cholesterol diet, and all three included a high-fat, high-fructose diet, suggesting that a high-fat, high-fructose diet yields a metabolic and histological phenotype that resembles human NAFLD. This was supported on analysis of phenotype scores by subgroups (Fig. 2E). While choline-deficient diets had the highest mean liver histology scores, they had few features of the metabolic syndrome.

TABLE 2. Most frequently used rodent NAFLD models

			Diet			Chemical					Phenotype Score			Ballooning		NASH		Fibrosis		HCC			
Model Name	Background	Sex	Started (Weeks)	Fat (%kcal)	Cholesterol (%weight)	Age Given	Dose	Obesity	Insulin Resistance	Dyslipidemia	Metabolic Syndrome	Liver	Overall	Yes/No	Max. Age	Yes/ No	Max. Age	Max. Stage	Max. Age	Yes/No	Max. Age	Timing of Weeks	Number Citations
Leptin deficiency (*ob/ob*)	C57BL/6J	M						Yes	Yes	Yes	3	7	10	Yes	10	Yes	48	2	21			Life	90 (e.g., PMID 24205128)
HFD	C57BL/6J	M	8	60				Yes	Yes	Yes	3	8	11	Yes	29	Yes	16	1	34	Yes	60	Diet	78 (e.g., PMID 23197411)
Zucker (*fa/fa*) rats	Zucker	M						Yes	Yes	Yes	3	8	11	Yes	23	Yes	28	2	22			Life	78 (e.g., PMID 19101115)
HFD	C57BL/6J	M	6	60				Yes	Yes	Yes	3	5	8	Yes	20	Yes	40	?	24			Diet	59 (e.g., PMID 27562717)
MCD	C57BL/6?	M	8					No	No	Yes	1	8	9	Yes	18	Yes	18	3	18			Diet	43 (e.g., PMID 28190475)
MCD	C57BL/6J	M	8					No	?	Yes	1	7	8	Yes	18	Yes	20	2	20			Diet	36 (e.g., PMID 26979540)
HFD	C57BL/6?	M	8	60				Yes	Yes	Yes	3	4	7	Yes	28	Yes	28	Yes (NR)	24			Diet	35 (e.g., PMID 28604704)
HFD	C57BL/6J	M	5	60				Yes	Yes	Yes	3	7	10	Yes	24	Yes	26	1	24			Diet	34 (e.g., PMID 23774190)
OLETF rat	Long-Evans	M						Yes	Yes	Yes	3	6	9	?	40	Yes	40	1	40			Life	33 (e.g., PMID 11106100)
Leptin deficiency (*ob/ob*)		M						Yes	Yes	Yes	3	2	5	?	9	No	12	Yes (NR)	20			Life	30 (e.g., PMID 27997977)
Leptin deficiency (*ob/ob*)	C57BL/6?	M						Yes	Yes	Yes	3	2	5			Yes	30					Life	29 (e.g., PMID 21703192)
HFD	C57BL/6J	M	4	60				Yes	Yes	Yes	3	2	5			Yes	40	No	24			Diet	28 (e.g., PMID 24704474)
LepR deficiency (*db/db*)	C57BLKS/J	M						Yes	Yes	Yes	3	6	9	No	24	Yes	24	2	20			Life	27 (e.g., PMID 24244369)
HFD	C57BL/6J	M	8	45				Yes	Yes	Yes	3	4	7	Yes	20	Yes	20	No	52			Diet	26 (e.g., PMID 23643521)
HFD	C57BL/6J	M		60				Yes	Yes	Yes	3	4	7	Yes	12	Yes	22	No	18			Diet	26 (e.g., PMID 27200103)
LepR deficiency (*db/db*)	C57BL/6J	M						Yes	Yes	Yes	3	2	5	No	12	Yes	21	No	21			Life	24 (e.g., PMID 25420998)
HFD	C57BL/6?	M	7	60				Yes	Yes	Yes	3	6	9	Yes	40	Yes	40	1	40			Diet	23 (e.g., PMID 28597936)
LepR deficiency (*db/db*)		M						Yes	Yes	Yes	3	3	6	Yes	18	Yes	18	No	18			Life	18 (e.g., PMID 27997977)
Leptin deficiency (*ob/ob*)	C57BL/6J	F						Yes	Yes	Yes	3	1	4									Life	15 (e.g., PMID 27032381)
Zucker (*fa/fa*) rats	Zucker							Yes	Yes	Yes	3	1	4			No	10					Life	15 (e.g., PMID 15349118)
HFD + STZ	C57BL/6J	M	4	57		0.29	200ug	Yes	Yes	Yes	3	9	12	Yes	13	Yes	13	3	12	Yes	20	Diet	12 (e.g., PMID 28512251)

TABLE 2. *Continued*

			Diet			Chemical			Phenotype Score				Ballooning		NASH		Fibrosis		HCC			
Model Name	Background	Sex	Started (Weeks)	Fat (%kcal)	Cholesterol (%weight)	Age Given	Dose	Obesity	Insulin Resistance	Dyslipidemia	Metabolic Syndrome	Liver	Overall	Yes/No	Max. Age	Yes/ No	Max. Age	Max. Stage	Max. Age	Yes/No	Max. Age	Timing of Weeks	Number Citations
Tunicamycin	C57BL/6?	M				8.00	1 mg/kg			Yes	1	2	3			Yes	0.4					After chemical	8 (e.g., PMID 23152128)
HFD + STZ	C57BL/6?	M	4	60		0.29	200ug	No	Yes	Yes	2	8	10	Yes	25	Yes	25	2	25	Yes	25	Diet	7 (e.g., PMID 24048504)
SHRSP5/Dmcr + HFHC	SHRSP5/-Dmcr	M	10	47	5.00			Yes	No	Yes	2	8	10	Yes	14	Yes	14	3	14			Diet	6 (e.g., PMID 22569299)

Within each of nine categories of NAFLD models, the most frequently used models (with a minimum of three citations) are reported. All ages are given in weeks. "?" is used to illustrate where there was no consensus between studies for the presence of metabolic syndrome or histological features. The PubMed identification number of one study using each model is given below the number of citations.
Abbreviations: HFHC, high-fat, high-cholesterol; LepR, leptin receptor; MCD, methionine-/choline-deficient diet; NR, (fibrosis stage) not reported; OLETF, Otsuka Long-Evans Tokushima fatty; PMID, PubMed identification number; STZ, streptozocin.

PROGRESSION TO PERIPORTAL INFLAMMATION, CIRRHOSIS, AND HCC

We also used our database of NAFLD models to identify rodent studies reporting the presence of cirrhosis in under 20 weeks (Supporting Table S5). The majority of these used choline-deficient diets, with or without methionine deficiency. Other models, including diet-induced obesity designs (e.g., 60% kcal HFD), were reported to cause advanced fibrosis (NAFLD fibrosis score > F3), but this was after a duration of 40-50 weeks.

Similarly, HCC developed in these obese rodents models typically after 1-2 years,[10] which would require a protracted study period. Therefore, we searched our database to identify models showing HCC at under 30 weeks (Supporting Table S5). This again highlighted methionine-/choline-deficient models, as well as chemically manipulated models, whereby rodents received diethylnitrosamine or streptozocin parenterally.

A further histological feature of specific interest was periportal inflammation as the presence of periportal inflammation is associated with more advanced disease in both adults and children.[24] We identified a variety of models showing portal inflammation (Supporting Table S5), including chemically induced (e.g., monosodium glutamate), dietary (e.g., high fat, high fructose and high sucrose), and genetic (e.g., galectin-3 knockout) models. We noted that portal inflammation was the least frequently described histological feature (Supporting Fig. S3) and suspect that this may be influenced by underreporting.

GENE EXPRESSION

Out of 187 studies with animal model gene expression data, eight had performed a cross-species comparison (Supporting Table S6). Most studies found statistically significant overlap between human and murine expression, using a variety of different analysis methodologies. As mentioned, Giles et al.[19] found that housing at thermoneutrality (compared to room temperature) increased the ability of gene expression profiles to predict the presence of NASH in humans from 82% to 89%. Two studies compared the expression of multiple different animal models. Firstly, Teufel et al.[25] observed that the variation between species was substantially larger than within murine models and that only a few enriched pathways overlapped, with fructose-enriched HFD models showing

TABLE 3. Models with highest overall phenotype scores

			Diet					Chemical						Phenotype Score			Ballooning		NASH		Fibrosis		HCC	
Model Name	Background	Sex	Started (Weeks)	Fat (%kcal)	Cholesterol (%Weight)	Sucrose (%kcal)	Fructose (%Weight)	Age Given	Dose	Obesity	Insulin Resistance	Dyslipidemia	Metabolic Syndrome	Liver	Overall	Yes/No	Max. Age	Yes/No	Max. Age	Max. Stage	Max. Age	Yes/No	Max. Age
HFD example studies: PMID 29092796, 22834991, 23964070)	C57BL/6?	M	8	57						Yes	Yes	Yes	3	9	12			Yes	60	3	60	Yes	60
HFD (example studies: PMID 23197411, 27997977, 27045862)	C57BL/6J	M	8	60						Yes	Yes	Yes	3	8	11	Yes	29	Yes	16	1	34	Yes	60
HFD (example studies: PMID 23395669, 26889237, 15761972)	Sprague-Dawley	M	5	22	2					Yes	Yes	Yes	3	9	12	Yes	20	Yes	20	3	20		
HFD (example studies: PMID 28539530, 29709653)	Sprague-Dawley	M	8	40	2					Yes	Yes	Yes	3	9	12	Yes	20	Yes	32	3	32		
HFD (example studies: PMID 28555525, 23200892)	Wistar	M	5	60						Yes	Yes	Yes	3	9	12	Yes	8	Yes	8	3	8		
HFHCD (example studies: 24827559, 28436319)	Sprague Dawley	M	9	51	2.5					Yes	No	Yes	2	9	11	Yes	9	Yes	9	4	9	No	9
Atherogenic + HFD (high fat, high cholesterol, low choline) (example studies: PMID 28212548, 17929294, 29599925)	C57BL/6J	M	8	60	1.25					No	Yes	Yes	2	10	12	Yes	68	Yes	68	4	68	Yes	68
Western diet (high fat, high fructose) (example studies: PMID 28939830, 29959418, 28869588)	C57BL/6J	M	8	58			2.31			Yes	Yes	Yes	3	9	12	Yes	52	Yes	52	2	52	Yes	52
HCHFD + fructose and sucrose in water (example studies: PMID 24256559, 29152605)	C57BL/6J	M	8	58			2.31			Yes	Yes	Yes	3	8	11	Yes	16	Yes	16	2	16		
High fat (trans-fat) + fructose + cholesterol (example studies: PMID 27326314, 29375204)	C57BL/6J	M	5	40	2		20			Yes	Yes	Yes	3	8	11	Yes	50	Yes	50	3	50		
High fat (trans-fat) + fructose + cholesterol (example studies: PMID 25213859, 22268099, 23886860)	C57BL/6J	M	7	40	2		22			Yes	Yes	Yes	3	8	11	Yes	30	Yes	52	2	52		
High fat, carbohydrate, cholesterol diet + fructose in drinking water (example studies: PMID 27261415, 29222421)	C57BL/6J-129/SvImJ	M	10	42	0.1		2.31			Yes	Yes	Yes	3	10	13	Yes	8	Yes	24	3	52	Yes	52

TABLE 3. *Continued*

			Diet					Chemical					Phenotype Score			Ballooning		NASH		Fibrosis		HCC	
Model Name	Background	Sex	Started (Weeks)	Fat (%kcal)	Cholesterol (%Weight)	Sucrose (%kcal)	Fructose (%Weight)	Age Given	Dose	Obesity	Insulin Resistance	Dyslipidemia	Metabolic Syndrome	Liver	Overall	Yes/No	Max. Age	Yes/No	Max. Age	Max. Stage	Max. Age	Yes/No	Max. Age
MCD (example studies: PMID 20848203, 19856102, 20520981)	Wistar	M	7							No		Yes	1	10	11	Yes	4	Yes	20	3	20	Yes	20
MCD + HFD (example studies: PMID 26820535, 23477499, 23028442)	C57BL/6?	M	8	60						No	Yes	Yes	2	9	11	Yes	2	Yes	12	4	16		
Choline-deficient diet (example studies: PMID 25843661, 22613706, 15107972)	Wistar	M	7							No		Yes	1	10	11	Yes	12	Yes	12	4	16	Yes	16
Choline-deficient, L-amino-defined diet (example studies: PMID 17914985, 23481610, 27320964)	Wistar	M	6							Yes	Yes	Yes	3	8	11	Yes	4	Yes	12	3	12		
Choline-deficient, L-amino-defined diet (example studies: PMID 24968200, 23996730, 30083132)	C57BL/6?	M	8							No	Yes	Yes	2	9	11	Yes	22	Yes	22	4	22		
HFD + STZ (example studies: PMID 28512251, 28110063, 23430399)	C57BL/6J	M	4	57				0.3	200µg	Yes	Yes	Yes	3	9	12	Yes	13	Yes	13	3	12	Yes	20
HFD + STZ (example studies: PMID 28345673, 29179453, 29216638)	C57BL/6J	M	4	60				0.3	200µg	No	Yes	Yes	2	9	11	Yes	5	Yes	20	3	20	Yes	0
ACOX1-Lampe1 (splice-site mutant) (example studies: PMID 21760938, 29563328)	C57BL/6J									Yes		Yes	2	9	11			Yes	59	4	59	Yes	59
ACOX1-Lampe1 (splice-site mutant) + HFHCD (example studies: PMID 21760938, 29563328)	C57BL/6J			58			2.31			Yes		Yes	2	9	11			Yes	52	4	52	Yes	59
ApoE*3Leiden + Western diet (example studies: PMID 29907965, 29907965)	C57BL/6J	M	9	33	1					Yes	Yes	Yes	3	8	11	Yes	16	Yes	16	3	16		
Leptin deficiency (*ob/ob*) + high fat (trans-fat), fructose, cholesterol diet (example studies: PMID 29375205, 27326314, 29375204)	B6.V-Lepob/JRj	M	5	40	2		20			Yes	Yes	Yes	3	8	11	Yes	20	Yes	20	3	30		

TABLE 3. *Continued*

			Diet					Chemical						Phenotype Score			Ballooning		NASH		Fibrosis		HCC	
Model Name	Background	Sex	Started (Weeks)	Fat (%kcal)	Cholesterol (%Weight)	Sucrose (%kcal)	Fructose (%Weight)	Age Given	Dose	Obesity	Insulin Resistance	Dyslipidemia	Metabolic Syndrome	Liver	Overall	Yes/No	Max. Age	Yes/No	Max. Age	Max. Stage	Max. Age	Yes/No	Max. Age
Leptin deficiency (*ob/ob*) + high fat (trans-fat), fructose, cholesterol diet (example studies: PMID 29107284, 29713129)	C57BL/6J	M	8	40	2		22			Yes	Yes	Yes	3	8	11	Yes	12	Yes	12	3	12		
MC4R-KO + Western diet (example studies: PMID 29202448, 29402900, 29402900)	C57BL/6J	M	8	41	0.21	34				Yes	Yes	Yes	3	9	12	Yes	52	Yes	52	3	52	Yes	52
SHRSP5/Dmcr rats + HFHCD (example studies: PMID 27037502, 29899851)	SHRSP5/Dmcr	M	6	47	5					Yes	Yes	Yes	3	9	12	Yes	16	Yes	16	4	16		
Zucker rats / *fa/fa* rats (example studies: PMID 19101115, 27636007, 16139386)	Zucker	M								Yes	Yes	Yes	3	8	11	Yes	23	Yes	28	2	22		

Rodent models with the highest overall phenotype scores (0-14), which had been used by at least two studies. The PubMed identification number of up to three studies using each model is given below the model name.
Abbreviations: ACOX1-Lampe1, acyl-CoA oxidase 1; HFHCD, high-fat high-cholesterol diet; MC4R-KO, melanocortin 4 receptor knockout; MCD, methionine-/choline-deficient diet; PMID, PubMed identification number; STZ, streptozocin.

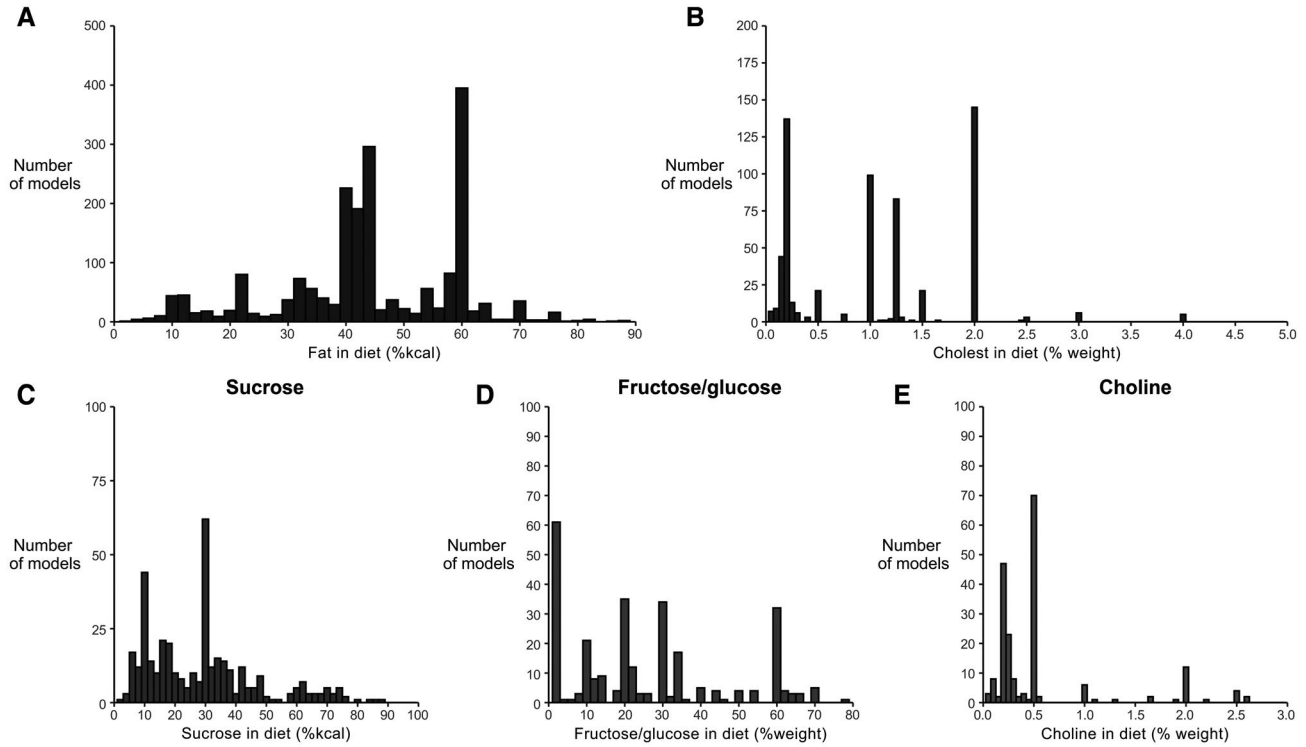

FIG. 3. Composition of HFDs. Data from 1,488 rodent HFD models. (A) Proportion of total dietary kilocalories from fat. (B) Percentage of diet as cholesterol (by weight). (C) Proportion of total dietary kilocalories from sucrose. (D) Percentage of diet as fructose/glucose (either alone or in combination). (E) Percentage of diet as choline (by weight).

the greatest similarity at the pathway level. Secondly, Tsuchida et al.[23] included data from 22 different models (or time points) and found that male C57BL/6J mice fed a Western diet (high fat, high sucrose, high cholesterol) with high fructose or glucose content in water and i.p. CCl_4 injection showed the greatest similarity to human NAFLD.

HETEROGENEITY IN NOMENCLATURE OF DIETARY MODELS

The use of specialist diets to induce hepatic steatosis, with or without obesity and insulin resistance, has been the mainstay of rodent models of NAFLD.[9] Over 75% of all models in our database included some form of dietary perturbation. However, we observed that there was inconsistent use of terminology when comparing the names of diets and their composition.

For example, 16% (237/1,488) of models described as an HFD (without specifying additional components) also contained added cholesterol (Fig. 3). Overall, 22% (334/1488) of HFDs had some added cholesterol, choline, sucrose, or fructose/glucose.

Similar results were observed for 149 "Western diet" models, which most frequently involved 40%-45% kcal from fat, plus 30% kcal from sucrose and 0.2% cholesterol. However, there was substantial variability (Supporting Fig. S4), with many models described as "Western diet" including added fructose. In addition, 18 models were described as using "Western diets" but provided no detail on diet composition.

GENETIC ANIMAL MODELS OF NAFLD ACT THROUGH ADIPOSE DYSFUNCTION AND INNATE IMMUNITY

Understanding the genetics of NAFLD in humans has provided substantial insight into the pathogenesis of fatty liver disease. Concurrently, there have been hundreds of genetically modified animal models that display features of NAFLD to varying severity. We used our database to identify 433 human orthologues

from genetically modified mice that display exacerbated severity of NAFLD. Using gene set enrichment analysis,[16] we identified that these gene targets are most highly expressed in adipose tissue and liver (Supporting Fig. S5 and Table S7). Pathways relating to insulin resistance, adipogenesis, and innate immunity, as well as genes implicated in type 2 diabetes and related metabolic syndrome traits in humans, were enriched in our gene set (Supporting Fig. S5).

RISK OF BIAS

Recommendations for reporting of preclinical studies suggest the use of blinding, randomization, a prespecified protocol, and a sample size estimate.[26] We assessed all 4,540 articles for these four risk of bias metrics and combined them into an overall score (0-4). Over half of all studies had a score of 0 or 1 (Supporting Fig. S6). Only 1.3% of all studies reported a power calculation, with blinding used in 19% and randomization in 37%.

Discussion

Preclinical animal studies of hepatic steatosis have been conducted for nearly 70 years with the aim of helping to elucidate the mechanisms of liver lipid metabolism[27] and, more recently, to find effective treatments for NAFLD. This has resulted in an increasingly complex array of animal models.[28] We performed a systematic analysis of animal models of NAFLD, providing a framework for their description, identifying the suitability of models for studying different aspects of the disease, and highlighting challenges of reproducibility.

We categorized over 3,900 unique models through detailed comparison of animal study design. This large number is due to variation in almost every aspect of design, including age of interventions, dietary composition, sex, and genetic background. Therefore, while many studies may initially appear to use the same model (e.g., high fat, high cholesterol in C57BL/6J mice), the majority have only been used by a single study. Consideration of precise details in study design is relevant in the light of recent data that suggest that even small differences in age or diet composition may affect treatment response in animal models of NAFLD.[11] Moreover, this has implications for understanding the "reproducibility crisis" of preclinical studies.[12] Our results suggest that most models have never been replicated, which adds further variability in challenging *in vivo* studies with small effect sizes. We found that genetic models were the most frequently replicated and that, in their case, the perturbation was (usually) unambiguously defined (e.g., albumin-cyclization recombination liver-specific phosphatase and tensin homolog [*PTEN*] knockout compared to "Western diet"). However, genetically modified animals are often subject to compensatory germline expression changes affecting their metabolism through mechanisms that may often be difficult to identify.

We observed marked variability in the terminology of dietary interventions. "Atherogenic diet" and "Western diet" have been used in animal studies since the 1950s and 1980s, respectively,[29,30] with their use increasing since preformulated diets became available from major laboratory feed suppliers. While there is some consensus on general composition between suppliers (e.g., atherogenic diets Harlan Teklad TD.88137 and Research Diets D12336), there does not appear to be a formal definition of terminology. From our observations, we suggest the development of consensus recommendations around the nomenclature of dietary compositions (e.g., Western diet >30% kcal from fat, >30% kcal from sucrose, no added fructose, <0.5% cholesterol) as this will support consistency and reproducibility. Similarly, an additional source of heterogeneity stems from the exact fat composition of different dietary interventions. Diets high in unsaturated fats (such as coconut oil–based diets) have been demonstrated to increase *de novo* lipogenesis more than diets high in saturated fats, including the widely used lard-based HFD, which may be an additional reason for phenotypic variation.[31]

We have used this data set to explore whether there is a single "ideal" animal model that reflects the spectrum of human NAFLD or if different study designs should be used for specific animal phenotypes.[10,32] To this end, we used a phenotype score in which we summarize the key measurable metabolic and histological features of NAFLD, which we identified following review of all included animal studies (Supporting Table S1). Although the principal aim of this score is to allow comparison across multiple papers, rather than giving an in-depth description of a single model, this phenotype score could be used as a baseline measure when developing and assessing preclinical models of NAFLD. While no model demonstrated all

features of both histological NAFLD and the metabolic syndrome, several studies using a prolonged diet of high fat and high fructose had the highest overall scores for similarity to human NAFLD.[20,22] The high-fat, high-fructose model was also identified to have the closest transcriptomic signature to human NAFLD.[25] Therefore, this core model design appears to mimic the human phenotype most closely. Fibrosis and HCC development could be expedited in these models by additional treatment with intraperitoneal CCl_4.[22] If the focus of a preclinical study were to be advanced liver disease resulting from NAFLD, a high-fat, high-fructose model with injection of CCl_4 might be preferable.

The link between a high-fat, high-fructose diet and NAFLD pathogenesis is in line with current evidence on human NAFLD. Diets high in fat might induce NAFLD through the high calorie burden and increased adiposity, leading to insulin resistance, as well as direct and differential effects of different fatty acids on lipogenesis, as discussed above.[33] Proposed mechanisms describing the causative role of fructose in the development of NAFLD include direct upregulation of *de novo* lipogenesis enzymes by fructose breakdown products in hepatocytes,[34,35] production of acetyl CoA by conversion of fructose into acetate by the intestinal microbiota, and intestinal barrier deterioration leading to increased uptake of endotoxins from the gut to the liver, driving inflammation.[36] Because few high-fat, high-fructose animal model designs have been precisely replicated, there are currently insufficient data to recommend specific dietary composition, genetic background, or timings of interventions.

The integral connection of adipose and liver metabolism is well established and supported by strong human genetic evidence, particularly from individuals with lipodystrophy.[37] We have replicated this finding through analysis of rodent models with exacerbated NAFLD but interestingly also observed a role for pathways of innate inflammation (i.e., NF-κB, IL-6, TNF-α). In humans, steatohepatitis is a state of sterile inflammation, and all common human genetic variants so far reproducibly associated with NAFLD at genome-wide significance act through perturbation of lipid metabolism, including variants in or near *PNPLA3*, transmembrane 6 superfamily member 2, glucokinase regulator, hydroxysteroid 17β-dehydrogenase 13, membrane-bound O-acyltransferase domain containing 7, and mitochondrial amidoxime reducing component 1.[7,38-40] There have been candidate studies suggesting that common variants in MER proto-oncogene tyrosine kinase[41] and interferon-lambda 4[42] are associated with severity of NAFLD in humans, though none of these variants have reached genome-wide significance, unlike for hepatitis C.[43] In addition, one recent genome-wide association study (GWAS) using electronic medical records identified a GWAS-significant variant near IL17 receptor A associated with NAFLD activity score.[44] The enrichment of inflammatory pathways in our analysis suggests that differential activity of the innate immune system might accelerate NASH across different rodent models. It will be interesting to see whether this is a finding specific to rodent models or whether further genetic evidence will implicate a causal role for innate immune activity in human NAFLD in the future.

We also examined gene expression in our data set, though only a small number of studies compared hepatic gene expression in animal models with human samples. Results were broadly congruous with our phenotype-based comparison, despite substantial heterogeneity in study design. It was not possible to perform a formal meta-analysis of these data due to lack of replication of each animal model and variation in age at sampling. In addition, selection of human samples for comparison would have been challenging given that some rodent models are focused on malignancy (with or without cirrhosis), while others principally cause steatosis without fibrosis. It should also be noted that several of these studies used the same human data set (GSE48452). Lastly, this cross-species methodology is principally limited by the substantially greater difference between human and all rodent samples than within rodent models.[25]

The scoring system used in this study facilitated comparison across a large number of models, but small numerical differences (e.g., 12 vs. 13) may hide substantially different phenotypes (e.g., choline-deficient diet vs. high-fructose diet). When considering the utility of an individual model, the presence or absence of a specific feature (e.g., rapid development of HCC) would likely be more important than reported score in this study. In addition, due to institution-specific variables (e.g., animal housing conditions), investigators may observe phenotypic differences compared to previous reports. Therefore, when selecting a model design, we encourage investigators to interpret the phenotype score in combination with the number of independent

replications, as well as specific features (e.g., periportal inflammation). Where appropriate, replicating a previously described model will help the field move toward greater reproducibility in animal studies.

The vast majority of models used only male rodents; therefore, it was not possible to make a broad statement about whether models conducted in female versus male animals more closely resemble the human phenotype. Sex differences within models can be substantial,[19] though are likely to still be smaller than between-species differences. Despite this, female animals are underrepresented in this field, particularly compared to the proportion of female participants in human NASH trials.[45]

In this study, we have concentrated on disease phenotypes, which has facilitated the inclusion of a large number of studies and allowed focus on endpoints that are directly comparable to clinical practice. In some cases, we were limited by the level of detail reported by each individual study. For example, where two studies were otherwise identical, if one described use of "C57BL/6J" mice and the other reported just "C57BL/6," these would be identified as separate designs. Although this could have led us to overestimate the number of unique models, the importance of differences in genetic background in murine NAFLD phenotypes has been demonstrated by results from the Hybrid Mouse Diversity Panel.[46-49] At the same time, we merged unique models with minimal differences in study design, such as percentage of fat in the diet varying by 1%, where the cutoffs were chosen manually based on modal values in our data, given our recent observation that most outcomes in rodent models of NAFLD can be modeled in continuous fashion.[11] At present, the significance of, for instance, a 1%-2% difference in dietary fat is not known. Therefore, the precise number of "unique models" reported in this study is a function of this methodology. Lastly, there is a wide range of other variables that can affect the phenotype that we have not included in our analysis, including gut microbiome composition.[50]

In summary, this systematic review has demonstrated substantial variability in the design of studies using rodent models of NAFLD. Due to variation in study design, most rodent models of NAFLD have only been used by a single study, highlighting the need for standardization and replication. This variation is compounded by a lack of consensus around nomenclature of diets. Genetic models that exacerbate NAFLD are enriched for genes involved in adipogenesis and innate immune pathways. Overall, high-fat, high-fructose diet models show the most phenotypic similarity to human NAFLD, though additional chemical liver injury or a prolonged study period might be required for the study of advanced stages of liver disease such as cirrhosis and HCC in these models.

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