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Animal models of NASH: Getting both pathology and metabolic context right

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of referral to liver clinics, and its progressive form, non-alcoholic steatohepatitis (NASH), can lead to cirrhosis and end-stage liver disease. The main risk factors for NAFLD/NASH are the metabolic abnormalities commonly observed in metabolic syndrome: insulin resistance, visceral obesity, dyslipidemia and altered adipokine profile. At present, the causes of progression from NAFLD to NASH remain poorly defined, and research in this area has been limited by the availability of suitable animal models of this disease. In the past, the main models used to investigate the pathogenesis of steatohepatitis have either failed to reproduce the full spectrum of liver pathology that characterizes human NASH, or the liver pathology has developed in a metabolic context that is not representative of the human condition. In the last few years, a number of models have been described in which the full spectrum of liver pathology develops in an appropriate metabolic context. In general, the underlying cause of metabolic defects in these models is chronic caloric overconsumption, also known as overnutrition. Overnutrition has been achieved in a number of different ways, including forced feeding, administration of high-fat diets, the use of genetically hyperphagic animals, or a combination of these approaches. The purpose of the present review is to critique the liver pathology and metabolic abnormalities present in currently available animal models of NASH, with particular focus on models described in approximately the last 5 years.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is increasingly prevalent and strongly associated with obesity, diabetes and the metabolic syndrome. In 1980, the term non-alcoholic steatohepatitis (NASH) was first used by Ludwig *et al.*[1] to describe, among non-drinkers, the liver pathology (steatohepatitis) previously thought to be uniquely associated with alcoholic liver disease. NAFLD is the preferred term to NASH when the pathology has not been defined, because the same metabolic determinants are associated with a spectrum of pathology from bland steatosis through steatohepatitis to cirrhosis and, possibly, hepatocellular carcinoma. NAFLD/NASH was initially thought to be a disease of the Western world, but it is now clear that the prevalence is very high in many regions, from the USA to South America, the Middle East, Europe, Asia and Australia.[2,3] The major risk factors are overnutrition and its resultant disorders: obesity, insulin resistance, glucose intolerance and dyslipidemia.[4]

Clinical research into mechanisms of steatohepatitis development and progression are constrained by ethical considerations, particularly with respect to obtaining liver and other tissue, and by limited ability to delineate cause and effect from complex, interactive disease pathogenic pathways. It is therefore attractive experimentally to use animal models. However, for the information derived from these models to have clear relevance to human liver disease, the models need to accurately reflect not only the liver pathology of NASH, but also the context within which it develops. There are a variety of genetic and dietary models of NAFLD, but few show progression to the inflammatory condition of steatohepatitis. Conversely, the methionine- and choline-deficient (MCD) model does show steatohepatitis and has been widely used, but there is understandable controversy over its validity.[5] It is fair to say that the popularity of the MCD model stemmed from a lack of alternative models which recapitulate the liver pathology of human NASH. It is now reasonable considered that the weight loss and whole-body insulin sensitivity associated with MCD feeding (despite demonstrated impairment of hepatic insulin receptor signaling)[6] limits the extrapolation of data from this model to NASH.[7,8] In recent years, several new animal models have been described. These appear promising for researchers investigating one of the key issues in NASH; not so much why steatosis occurs, but what causes the transition from bland steatosis to the inflammatory and progressive fibrosing condition of steatohepatitis.

NASH: What we know and what we don't know

Histopathology

While diagnostic criteria for pathological description of human NASH continue to evolve, current concepts center on steatosis, inflammation with liver cell injury, and the distinctive pattern of pericellular fibrosis.[9] By using these criteria, steatohepatitis can be separated from steatosis conceptually, but in practice, however, NASH may be difficult to distinguish from simple steatosis with minor inflammation if one uses oversimplified criteria.[10] In humans, ballooning of hepatocytes is a morphological manifestation of liver cell injury. It is thought to result from accumulation of intracellular fluid and/or other forms of toxic cell injury. Ballooning (or cellular) degeneration is characterized by swelling of hepatocytes which show rarefied cytoplasm (Fig. 1c, inset).[11] A frequently quoted clinicopathological study conducted by Matteoni *et al.* suggested that hepatocytic ballooning is a key feature that distinguished progressive NASH from the less progressive forms of NAFLD.[12] It has also been shown that patients whose livers show ballooning degeneration with fat accumulation and Mallory hyaline or fibrosis have a higher rate of developing cirrhosis and liver-related death, compared with patients whose livers show fat accumulation alone or with lobular inflammation only.[4] Furthermore, a clinicopathological study based on a blinded review of entry biopsy specimens for a treatment trial showed that hepatocytic ballooning was associated with higher serum cholesterol levels.[13] There was also a trend toward the presence of ballooning in biopsy specimens from patients with abnormal glycemic control, greater insulin resistance, and increased serum markers of necroinflammation.[13]

Using multiple regression analysis, it has been shown that the diagnosis of NASH is not dependent on a single histological feature. Instead, it involves assessment of multiple independent features.[14] Using multivariate analysis, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-sponsored NASH Clinical Research Network study identified several features that are significantly associated with a diagnosis of NASH; these include lobular inflammation, ballooning degeneration, fibrosis, and steatosis.[15] The aforementioned histological changes are therefore now considered to be the most helpful features to establish a diagnosis of NASH, and they comprise a common set of minimal criteria for this diagnosis.[16] It is noted that minor differences in the predominance of each of these features may occur under some circumstances. For instance, ballooning is less prominent in children, in whom periportal fibrosis may be more conspicuous than pericentral fibrosis,[15,17] whereas livers showing cirrhosis may exhibit little or no steatosis.[16] The extent to which these criteria can be used to define animal models of NASH remains unclear, but in the present review we will comment on both similarities and substantial differences.

Pathogenesis

The most reproducible risk factors for NASH are abdominal (central or visceral) obesity, insulin resistance, fasting hyperglycemia and hypertriglyceridemia.[4] The severity of NAFLD correlates with the number of criteria met for metabolic syndrome; in two large series, >85% of patients with NASH had established metabolic syndrome.[18,19] More recently, it has become clear that fatty liver may precede the onset of metabolic syndrome and its complications.[20,21] In light of these observations, it is appropriate that NAFLD, and particularly NASH, is increasingly referred to as the hepatic manifestation of metabolic syndrome.[22]

A key feature of metabolic syndrome is the profile of changes in serum levels of adipokines (increased leptin and tumor necrosis factor-alpha [TNF-α], decreased adiponectin). It is therefore not surprising that the same alterations of serum adipokine profile are prevalent in NAFLD. Importantly, there have been several independent reports that serum adiponectin levels correlate inversely with NASH severity.[23–25] Insulin resistance likely plays a pathogenic role in the development and progression of NAFLD, but the complex relationship between hepatic steatosis and insulin sensitivity makes it difficult to assign cause and effect.[26] Obesity, but more particularly, increased intra-abdominal (visceral) adipose tissue (VAT) mass is almost universal in NASH.[27] Similar to insulin resistance, this likely contributes to the pathogenesis of fatty liver disease.[26] For example, enlarged visceral adipose stores are associated with an increased flux of free fatty acids from VAT to liver via the portal circulation, impaired insulin sensitivity, increased levels of pro-inflammatory cytokines such as TNF-α and interleukin (IL)-6, and decreased levels of adiponectin.[28–31]

The potential causes of steatosis in NAFLD are generally understood and have been reviewed elsewhere.[26,32,33] In contrast, the cellular mechanisms involved in inflammatory cell recruitment, hepatocyte injury and fibrogenesis are not well understood, and have been the subject of conjecture and controversy. Roles for oxidant stress, mitochondrial injury, pro-inflammatory cytokines and lipotoxicity have all been suggested.[34] Clearly, more research is required to determine the contribution of these factors, and their potential interaction, to steatohepatitis pathogenesis. Such mechanistic understanding of hepatocellular injury and inflammatory recruitment in metabolic liver disease is important for the development of targeted therapeutic interventions. In order to progress this understanding, animal models that accurately reflect not only the liver pathology of human NASH, but also the metabolic milieu in which it develops are essential.

Animal models of NASH: What are we looking for?

The importance of animal models and knowledge derived from earlier studies have been reviewed by others over the last few years.[35–37] The purpose of the present review is to focus more particularly on those models with appropriate pathology for NASH, and with the same metabolic setting of overnutrition rather than nutritional depletion or genetic manipulation. As mentioned, the two key criteria that animal models of NASH should fulfil are: (i) that the pathological pattern of liver injury reflects that which defines human steatohepatitis; and (ii) the model recapitulates the context within which human NASH develops. Shortcomings in either the liver pathology or metabolic context of experimental NASH make it difficult to translate research from the laboratory bench into the clinical setting. Explicitly, an animal model of NASH must have liver pathology that features steatosis, inflammation, liver cell injury (ballooning of hepatocytes) and progression to fibrosis, particularly the specific perivenular/ pericellular (chicken wire) pattern of fibrosis

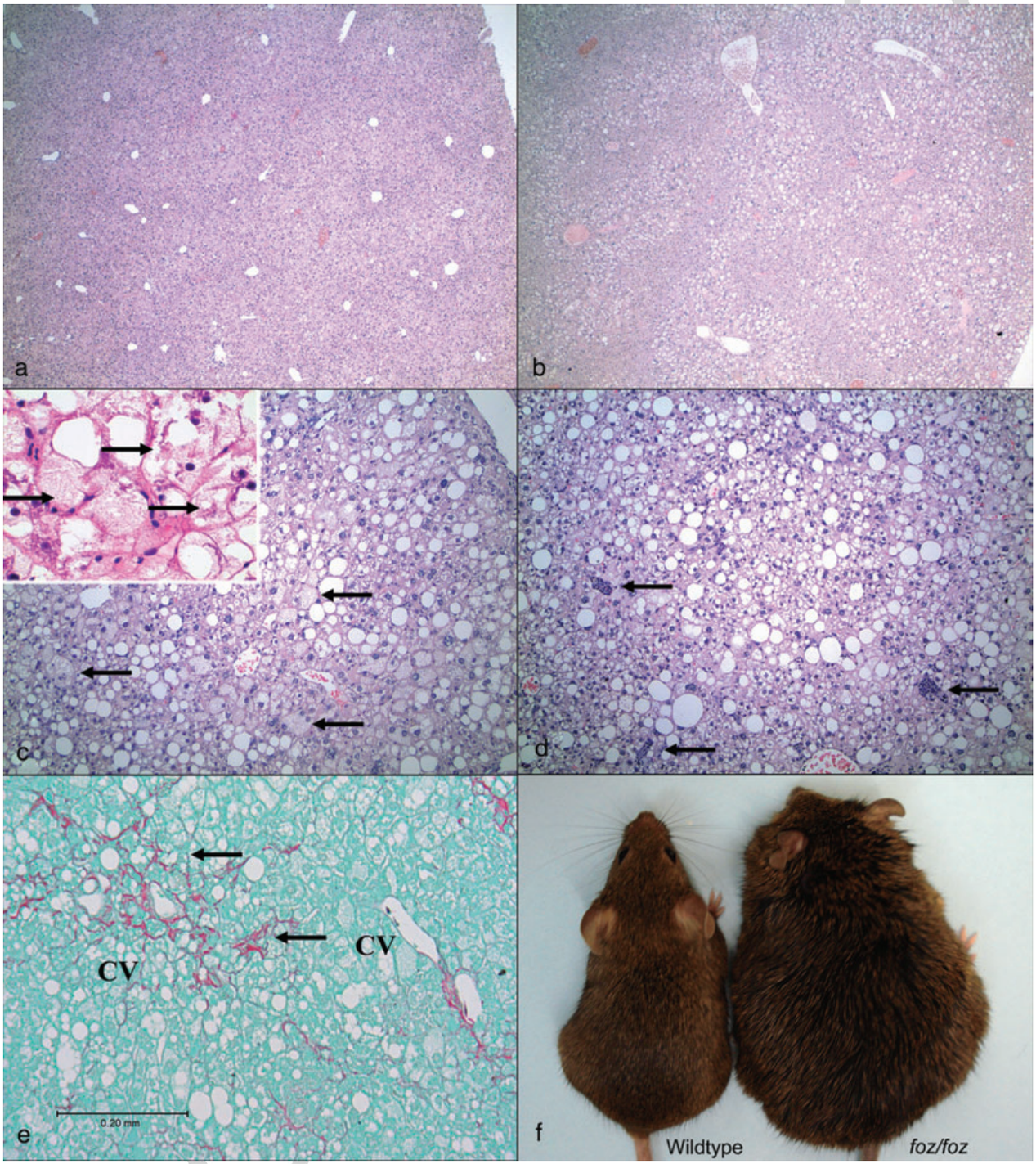

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Figure 1 Liver histology of obese high-fat-fed *foz/foz* mice shows fibrosing steatohepatitis. (a) Liver of the wild-type mice fed with a high-fat diet for 300 days showed no significant histological changes including no significant steatosis or inflammation (H&E stain, 40× magnification). (b) *foz/foz* Mice fed with a normal diet for 300 days showed mild mixed micro- and macrovesicular steatosis, predominantly macrovesicular, in a zone 3 distribution pattern (H&E stain, 40× magnification). (c) *foz/foz* Mice fed with a high-fat diet for 300 days showed more extensive steatosis, there were ballooned hepatocytes (arrows, H&E stain, 100× magnification). Inset: Ballooned hepatocytes in a background of steatosis in human non-alcoholic steatohepatitis (arrows, H&E stain, 600× magnification). (d) There were also many foci of inflammatory cells, composed predominantly of neutrophils and mononuclear cells, in the hepatic lobules in *foz/foz* mice fed with a high-fat diet for 300 days (arrows, H&E stain, 100× magnification). (e) Sirius red stain shows perivenular and pericellular fibrosis in *foz/foz* mice fed with a high-fat diet for 300 days (arrows, Sirius red stain, 100× magnification). CV, central vein. (f) *foz/foz* Mice are severely obese in comparison to wild-type controls (mice depicted are 6 months of age).

commonly seen in adult NASH. Also, the model should exhibit metabolic abnormalities such as obesity, insulin resistance, fasting hyperglycemia, dyslipidemia and altered adipokine profile. Preferably, animals will be both obese and insulin resistant. Furthermore, the more of the above-mentioned metabolic criteria that are met, the more intrinsically useful the model becomes as it will enable examination of the separate and interactive impacts of individual metabolic abnormalities on liver pathology. Given the complexities of human behavior and biology, it is also critical that steatohepatitis researchers do not study the liver in isolation: appetite regulation, physical activity, food choices, genetics and humoral determinants of body composition, metabolic regulation, and inflammation in extrahepatic tissues may each play a role in NASH pathogenesis.

Animal models of steatohepatitis

Genetic models

Genetic models of obesity-related liver injury can be broadly classified into two groups: (i) those in which steatohepatitis develops with no or minimal features of metabolic syndrome; and (ii) those in which steatosis develops in the context of obesity and features of metabolic syndrome, but where there is only minor and non-progressive liver injury. Many of these models have been reviewed previously,[35,36] so only a brief overview will be provided here. The first category includes mice nullizygous for acyl-CoA oxidase (ACOX), methionine adenosyltransferase (MAT)-1A (MATO mice), and those with liver-specific *pten* deletion. ACOX is the first and rate-limiting step of peroxisomal β-oxidation of long chain fatty acids. Deletion of this gene leads to severe steatosis and inflammatory infiltration of the liver with hepatocyte apoptosis, but animals are growth retarded and do not exhibit features of metabolic syndrome.[38] Further, at 6–8 months of age, ACOX null mice become resistant to steatosis following a process of liver regeneration associated with peroxisome proliferator-activated receptor (PPAR)α induction. This regeneration limits the utility of this model for studying the pathogenesis of steatohepatitis, as disease progression thereafter is different from human NASH.

Steatohepatitis develops in chow-fed MATO mice at approximately 8 months of age.[39] MAT-1A is involved in the synthesis of phosphatidylcholine, a phospholipid required for hepatic triglyceride export as very low-density lipoprotein (VLDL). Steatohepatitis in MATO mice is associated with marked oxidant stress,[40] a candidate mediator of inflammatory recruitment, and hepatocarcinogenesis, a later stage in the evolution of resultant liver disease. Although MATO mice are hyperglycemic, they have normal insulin levels and appear to not develop other features of metabolic syndrome.[39] *pten* is a multifunctional phosphatase and tumor suppressor. Steatohepatitis develops in mice lacking hepatocyte *pten* expression, but mice actually have improved insulin sensitivity and low serum insulin levels.[41] Like MATO mice, liver-specific *pten* deletion is also associated with hepatocarcinogenesis; the timeframe of development is similar to steatohepatitis. It is therefore difficult to delineate effects of steatohepatitis from tumorigenesis in this model.

In contrast to the above models, mice lacking either leptin (*ob/ob*) or the long form of the leptin receptor (*db/db*) are obese, hyperphagic, insulin resistant and develop hepatic steatosis and type 2 diabetes.[42] Although the metabolic abnormalities resemble NAFLD, spontaneous development of steatohepatitis is not a feature of these strains, although older males do show some mononuclear cell infiltration of liver and elevation of serum alanine aminotransferase (ALT). Another feature of *ob/ob* mice is that they are protected against fibrosis,[43] a phenomenon which led to the characterization of leptin as an essential mediator of hepatic fibrogenesis.[43,44] Two methods are commonly used to induce steatohepatitis in these obese mice, either a second insult, such as low-dose lipopolysaccharide,[45] or administration of the methionine- and choline-deficient diet (see following section).

Another genetic model of steatosis in association with insulin resistance is the sterol regulator element binding protein (SREBP)-1c transgenic mouse. In these animals, SREBP1c, a lipogenic transcription factor, is overexpressed in adipose tissue. This creates a model of congenital lipodystrophy in which severe insulin resistance and diabetes develop secondary to impaired adipose differentiation.[46] The restriction in adipose mass causes hepatic lipid accumulation, with marked steatosis present from as young as 8 days of age. When fed standard rodent chow, steatosis, lobular inflammation, and perivenular and pericellular fibrosis were observed in SREBP-1c transgenic mice by 20 weeks (Table 1).[47] Ballooned hepatocytes and Mallory bodies were described, which are important histological features for the diagnosis of steatohepatitis. However, it is unclear whether the morphology depicted is representative. At 20 weeks of age, serum leptin and adiponectin levels were decreased, whereas serum triglyceride and cholesterol levels were increased. ALT levels were not increased, although serum aspartate aminotransferase (AST) levels were increased. Overall, this model appears to be well suited for the study of lipodystrophy-associated steatohepatitis, in which low serum leptin levels are also found, but note that serum leptin levels are usually increased with obesity and in NAFLD/NASH. However, the fact that these mice exhibit decreased adipose mass limits their applicability to the broader field of NAFLD/NASH research, because in obesity-related NASH, the adipose tissue is a storage compartment which is likely to contribute to perturbations of whole-body lipid homeostasis.

Methionine- and choline-deficient model

Methionine and choline deficiency rapidly induces steatohepatitis in rodents. In contrast to choline-only deficiency, MCD-fed mice exhibit a liver phenotype that is strikingly similar to the MATO mouse, in which intrahepatic methionine deficiency likewise occurs.[39] Triglycerides and lipoperoxides accumulate, and while these changes are variable, levels usually become significantly increased compared to appropriate dietary controls after 2–5 days of MCD feeding. After 5 days of MCD feeding, serum ALT levels are consistently increased, although generally not significantly so until day 10.[48] After 3 weeks of MCD feeding, steatohepatitis is well developed, and by 8–10 weeks pericellular and perisinusoidal fibrosis are present.[7,49] After 10 weeks of dietary feeding, there is extensive macrovesicular steatosis in all zones except the periportal region, together with multiple foci of necroinflammation in the liver. The hepatic inflammatory infiltrate includes lymphocytes

Table 1 Animal models of steatohepatitis showing appropriate pathology, but inappropriate metabolic context

Model	Type of model	Metabolic phenotype				Liver histology			
		Obesity	Insulin resistance	Abnormal adipokines	Dyslipidemia	Steatosis	Inflammation	Hepatocyte injury	Fibrosis
MCD diet[7,8,48–55]	Dietary Blocks hepatic lipid export	No ↓Weight ↓Adiposity	No ↓Insulin ↓Glucose	No ↑Adiponectin (or no change) ↓Leptin	No ↓Cholesterol ↓Triglyceride	Yes Macrovesicular	Yes Lymphocytes & neutrophils	Yes Ballooning	Yes Perivenular and pericellular
MCD-fed *ob/ob* mice[56]	Dietary and genetic	Variable ↓Weight in some	Not described	Not described	No Similar to ob/ob controls	Yes Macrovesicular	Yes similar to wild-type	Yes Ballooning	No Protected against fibrosis
MCD-fed *db/db* mice[56,57,58]	Dietary and genetic	No ↓Age-related weight gain ↓Adiposity	No ↓Insulin ↓Glucose compared to db/db controls	↑Leptin (but non-functional long leptin receptor)	No ↓Cholesterol ↓Triglyceride ↓FFA	Yes Macrovesicular	Yes Severe	Not described	Yes Pericellular
SREBP-1c transgenic mice[47]	Genetic Adipocyte overexpression of SREBP1c	No ↓Adiposity	Yes	Mixed ↓Leptin ↓Adiponectin	Yes	Yes	Yes	Yes Ballooning	Yes Perivenular and pericellular
Atherogenic diet-fed mice[63]	Dietary	No ↓Weight after 24-week feeding	Yes	Yes ↑Hepatic TNF-α mRNA	Yes ↑Cholesterol ↓Triglyceride	Yes	Yes	Yes Ballooning	Yes Perivenular and pericellular

FFA, free fatty acids; MCD, methionine- and choline-deficient; SREBP, sterol regulator element binding protein; TNF, tumor necrosis factor.

and neutrophils. Pervivenular and pericellular fibrosis, the so-called chicken-wire fibrosis typically seen in human NASH, also readily develops. One recent article reported severe fibrosis at 4 weeks,[50] which differs from the extensive experience in the authors' laboratory and elsewhere, which finds no fibrosis at week 3 and minimal increase in collagen at week 5 of MCD dietary feeding. Furthermore, the depicted fibrosis did not show the typical chicken-wire pattern observed in MCD-induced steatohepatitis. Administration of the MCD diet for 3 weeks leads to steatosis, inflammation and ballooning degeneration of hepatocytes, culminating in NAFLD activity scores significantly higher than in control-fed mice.

The major disadvantage of the MCD model is that it is associated with significant weight loss, often >20% of bodyweight after 3 weeks, low serum leptin and peripheral insulin sensitivity (Table 1).[8] The severe atrophy of adipose tissue in MCD-fed mice suggests that steatohepatitis in this model may reflect that associated with lipodystrophy rather than metabolic syndrome.[51] Further, the metabolic profile of MCD-fed mice is generally the converse of human NASH; serum insulin and leptin levels are decreased, fasting blood glucose levels are low, animals are peripherally insulin sensitive, and serum adiponectin levels are unchanged or increased.[52–55]

Attempts to overcome these metabolic obstacles include giving the MCD diet to genetically obese mice. MCD-fed *ob/ob* mice develop steatohepatitis, but exhibit marked weight loss and liver disease does not progress to fibrosis, presumably due to leptin deficiency.[56] Fibrosis does develop in *db/db* mice,[56] but despite some reports of persistent obesity and insulin resistance,[56] other studies clearly demonstrate significant reductions in bodyweight[57] and also marked decreases in serum insulin and glucose levels.[58] As only modest weight loss is required to improve the metabolic phenotype,[59] it is evident that use of *db/db* mice for administration of the MCD diet does not completely overcome the limitations of this model of nutritional depletion. The concurrent weight loss and improvements in insulin sensitivity and glucose metabolism would be expected to obscure any effect of these important metabolic perturbations on steatohepatitis development.

Steatohepatitis in atherogenic models

Non-alcoholic steatohepatitis is associated with increased risk of cardiovascular disease, even independently of its association with metabolic syndrome,[20] and alterations in blood cholesterol as well as triglyceride levels are common. Previously, it has been observed that genetic susceptibility to atherosclerosis or feeding an atherogenic diet, typically containing cholate and increased cholesterol and/or fat content, leads to hepatic steatosis and liver injury. Recent studies have specifically addressed whether giving an atherogenic diet to genetically susceptible or wild-type mice induces steatohepatitis. Apolipoprotein E (ApoE) is involved in hepatic lipoprotein clearance from blood. Both ApoE2 knock-in and ApoE null mice develop marked hyperlipoproteinemia when given an atherogenic diet (Table 2).[60,61] ApoE2 knock-in mice express a mutant human form of ApoE which has reduced binding affinity for lipoprotein lipase, thereby leading to decreased lipoprotein clearance. When fed a 'Western diet' with 21% fat and 0.2% cholesterol by weight for 3 weeks, ApoE2 knock-in mice exhibited marked elevations in serum cholesterol and triglyceride levels, as well as hepatic abnormalities.[60] Although steatosis developed early, the extent was very mild, and hepatic triglyceride content was not reported. A striking observation was the early appearance of macrophages in the liver, even preceding the emergence of steatosis. Kupffer cell numbers increased over time, together with other inflammatory cells, to form larger aggregates. Conversely, fibrate treatment (to stimulate PPARα) reduced the number of macrophages. Together, these data indicate that an increase in hepatic macrophage numbers is an early event during the development of steatohepatitis in this model, and that this can be mitigated by PPAR-α activation. Development of fibrosis was described in this model, but the only hard data were upregulation of collagen synthesis genes; the morphological pattern of fibrosis was not depicted.[60]

In a similar study, ApoE null mice were fed a 20% palm or olive oil diet with modest cholesterol (0.1%) for up to 24 weeks. Consequently, animals developed macrovesicular steatosis and inflammatory foci consisting of mononuclear cells and neutrophils.[61] Notably, there were again abnormally high numbers of macrophages in the hepatic parenchyma. While the former two findings are common observations in non-alcoholic models of steatohepatitis, prominent macrophages are not typical.[61] Few data were provided as to the metabolic phenotype of high-fat-fed ApoE null mice, although the expected increases in plasma cholesterol were observed. Other reports of high-fat-fed ApoE null mice indicate that they are actually protected against diet-induced obesity and maintain peripheral glucose tolerance. A likely explanation is decreased lipid delivery to tissues such as muscle and adipose.[62] Thus, whereas published data are insufficient to fully assess the validity of this model for the study of NAFLD pathogenesis, it appears likely that the similarities with human NASH are limited to the association with dyslipidemia and some (but not all) common histopathological features.

In a similar approach using wild-type mice, giving a high-fat atherogenic (Ath+HF; 60% fat, 1.25% cholesterol and 0.5% cholate by weight) diet for up to 24 weeks appeared to induce steatohepatitis (Table 1).[63] Pathological features included steatosis, inflammation, fibrosis and, in addition, ballooned hepatocytes; the latter is a critical histological component for the diagnosis of human NASH and has been noted in very few animal models. The addition of a high-fat component to the Ath diet accelerated the development of steatosis, inflammation, fibrosis, and ballooned hepatocytes. It appears the pattern of fibrosis is perivenular and pericellular, which is similar to human NASH. However, there are a number of features in this model that raise concern over the validity of its use to study the pathogenesis of steatohepatitis. First, hepatic triglyceride content decreased with time, as did serum ALT levels in Ath+HF-fed mice. Second, mice fed the Ath+HF diet were smaller than control mice and had smaller epididymal adipose pads. Further, insulin sensitivity and glucose tolerance were similar between Ath+HF and control-fed mice, albeit there was some evidence of hepatic insulin resistance. Last, and of most concern, was the formulation of the diets used. Instead of increasing dietary fat content at the expense of carbohydrate content, additional fat, cholesterol and cholate were added to a normal rodent diet. This approach resulted in a significant reduction in dietary protein content (from 22% to 8.6% by weight), which is below the maintenance requirements of mice.[64] There was also a

Table 2 Animal models of steatohepatitis showing appropriate metabolic context, but limited characterization pathology

Model	Type of model	Metabolic phenotype				Liver histology			
		Obesity	Insulin resistance	Abnormal adipokines	Dyslipidemia	Steatosis	Inflammation	Hepatocyte injury	Fibrosis
ob/ob mice reviewed in[32,33]	*Genetic leptin deficient*	Yes ↑ Weight ↑Adiposity	Yes ↑Insulin ↑Glucose	Yes ↑TNFα ↓Adiponectin	Yes ↑Triglyceride ↑Cholesterol ↑FFA	Yes	Age-related inflammation in males	Not described, but reports of ALT elevation	No Resistant to fibrosis
db/db mice reviewed in[32,33]	Genetic Leptin receptor deficient	Yes ↑Weight ↑Adiposity	Yes ↑Insulin ↑Glucose	Yes ↑TNF-α ↓Adiponectin	Yes ↑Triglyceride ↑Cholesterol ↑FFA	Yes	Not described	Not described	No Does not develop spontaneously
High-fat-fed ApoE2 knock-in mice[60]	Dietary and genetic	Not described	No Normal glucose	Not described	Yes ↑Triglyceride ↑Cholesterol	Yes Mild	Yes High number of macrophages	Not described	Upregulation of collagen synthesis genes
High fat-fed ApoE null mice[61]	Dietary and genetic	Not described	Not described Other reports indicate glucose tolerance	Not described	Yes ↑Cholesterol	Yes Macrovesicular	Yes High number of macrophages	Not described	Not described
Lieber-deCarli diet-fed Sprague–Dawley rats[67]	Dietary Liquid high-fat diet	No Not obese	Yes ↑Insulin	Not described	Not described	Yes	Yes	Mitochondrial injury Normal ALT	Not described

Apo, apolipoprotein; FFA, free fatty acids; TNF, tumor necrosis factor.

concomitant reduction in dietary vitamin and mineral content to a level below that consumed by control animals. Therefore, the effects of protein, vitamin and mineral restriction need to be considered in the interpretation of this model. A tentative conclusion is that this model, like the MCD dietary model, has striking histological similarities to human NASH. However, the failure of the model to recapitulate the metabolic milieu (obesity, insulin resistance, glucose intolerance, metabolic syndrome) could limit its applicability for steatohepatitis research directed at disease pathogenesis and therapeutic intervention.

Overnutrition as a model of steatohepatitis

Overnutrition, which is chronic energy intake surfeit to the daily energy requirements of the individual, is a central feature of the 'modern lifestyle' that predisposes to overweight and obesity, insulin resistance and fatty liver disease. It follows that animal models which use caloric overload as the main abnormality to drive liver injury are conceptually desirable for their similarity to the human condition. High-fat feeding has been used to induce overnutrition. However, a common problem is that rodents may adapt to high-fat feeding and become resistant to the development of obesity, and/or other metabolic abnormalities.[65] Researchers have attempted to overcome this self-correcting mechanism in two ways; first, through the use of forced feeding via gavage, implanted gastrostomy tube or total enteral nutrition and, second, by using animals which exhibit hyperphagia by virtue of appetite dysregulation.

Overnutrition induced by high-fat diet

The effects of giving a high-fat diet to rodents can be highly variable. Some studies show clear induction of steatosis and steatohepatitis, whereas others show very few, if any, liver abnormalities. Some of this variability could be explained by the influence of rodent strain which is known to be important in the susceptibility to several forms of liver disease. In a recent longitudinal study, chronic administration of a high-fat diet (60% of calories from fat) led to the development of steatohepatitis in male C57Bl/6J mice.[66] After 10 weeks of feeding, high-fat-fed animals were heavier, with raised plasma insulin, total cholesterol and hepatic triglyceride levels, and mice showed impaired glucose tolerance. Hepatic triglyceride content was further increased at 19 weeks, and remained elevated throughout the study. Serum ALT and AST levels were increased after 34 weeks of high-fat feeding, but histological data were only provided for mice fed for 50 weeks. At this time, high-fat feeding had induced marked steatosis accompanied by inflammatory infiltrate. Azan stain showed mild fibrosis but with a perivenular and pericellular pattern. In summary, NASH develops in high-fat-fed C57Bl/6J mice, and is linked to similar pathogenic factors as in humans, with steatosis and metabolic syndrome preceding the transition to steatohepatitis (Table 3). However, if a 50-week feeding period is required for the development of histological steatohepatitis, the practicality of this model is limited by the length of feeding required.

In rats, Sprague–Dawley animals appear susceptible to steatohepatitis development when fed a high-fat diet, and this is likely associated with their susceptibility to diet-induced obesity. A highly cited study by Lieber and colleagues described the effects of feeding a liquid high-fat diet, the Lieber-DeCarli diet, to Sprague–Dawley rats.[67] In this study, 3-weeks *ad libitum* high-fat feeding induced steatosis and inflammation. The pathology was attenuated by restricting dietary intake. By ultrastructural analyses, high-fat-fed rats also showed more extensive mitochondrial abnormalities, including rarefied matrix, loss of cristae, and herniation. While mitochondrial dysfunction produces reactive oxygen species that can provoke an array of responses to result in hepatocyte injury and cell death, inflammation, and fibrosis, it is not certain whether the abnormal mitochondrial morphology viewed by electron microscopy represents the cause or result of the observed histopathology. It is also unclear whether significant fibrosis developed in this model (Table 2). Interestingly, despite clear histomorphological abnormalities, there were no biochemical markers of liver injury, and, in particular, serum ALT and AST levels remained similar to controls. During *ad libitum* feeding, a similar caloric intake was observed between high-fat and control-fed rats, and there was no difference in final bodyweights. However, there was an approximately twofold increase in serum insulin levels in high-fat-fed rats, suggesting the presence of insulin resistance in this model. Overall, this model does not convincingly recapitulate the full spectrum of liver pathology, or the metabolic context of human NAFLD/NASH.

In another study examining the effects of high-fat-feeding on Sprague–Dawley rats, animals were fed a solid high-fat diet for up to 6 months.[68] In this study, chronic caloric overconsumption was achieved in high-fat-fed rats and this was accompanied by increased weight gain and visceral adiposity. In comparison to low-fat-fed controls, serum glucose and insulin levels were elevated after 1 month of high-fat feeding and remained elevated throughout the 6-month study period; hyperglycemia became more exaggerated with time. A time-dependent increase in portal serum free fatty acids (FFA) was also observed, as were increased serum TNF-α and decreased serum adiponectin levels. Analyses of insulin receptor substrate, (IRS)1, demonstrated increased serine phosphorylation, which inhibits the pathway of tyrosine phosphorylation. This signaling abnormality provided further evidence of hepatic insulin resistance in this model. Histologically, the rats fed with a solid high-fat diet developed predominantly macrovesicular steatosis in the liver. The distribution of steatosis was zone 3 and zone 1 by the first month, but became more generalized by 3 and 6 months. Scattered lobular inflammatory infiltrates were evident in zone 3 after 1 month of dietary feeding, and became more extensive by 3 and 6 months. These inflammatory infiltrates contained not only polymorphs, but also foci of mixed inflammatory cells, together with hepatocytic necrosis and apoptosis throughout the hepatic lobule. These observations correlated with the increased levels of hepatic TNF-α, expressed at both the mRNA and protein levels. In addition, zone 3 pericellular and perisinusoidal fibrosis was observed (Table 3).

Giving *n*-3 polyunsaturated fatty acids, which are natural PPARα ligands, to these animals reduced both hepatic steatosis and necroinflammation scores, and also the number of apoptotic bodies. These findings were accompanied by a decline in both hepatic TNF-α mRNA and serum ALT levels. In summary, this high-fat-feeding model appears to recapitulate many of the metabolic features of human NASH, as well as developing liver

Table 3 Towards more optimal animal models of NASH: Liver pathology develops in an appropriate metabolic context

Model	Type of model	Metabolic phenotype				Liver histology			
		Obesity	Insulin Resistance	Abnormal Adipokine	Dyslipidemia	Steatosis	Inflammation	Hepatocyte Injury	Fibrosis
High-fat-fed C57Bl mice[66]	Dietary Mice fed for up to 50 weeks	Yes	Yes ↑Insulin ↓Glucose tolerance	Not described	Yes ↑Cholesterol	Yes	Yes	Not described	Yes Mild—perivenular and pericellular
High-fat-fed Sprague–Dawley rats[68]	Dietary Solid high-fat diet fed for up to 6 months	Yes ↑Adiposity	Yes ↑Insulin ↓Insulin signal ↑Glucose	Yes ↑TNF-α ↓Adiponectin	Yes ↑FFA	Yes Generalized distribution	Yes Lobular	Yes Hepatocytic necrosis and apoptosis	Yes Zone 3 pericellular and perisinusoidal
Intragastric overnutrition in mice[69]	Dietary	Yes Severe	Yes ↑ Insulin ↓Insulin sens. & glucose tol. ↑glucose	Yes ↑TNF-α ↑Leptin ↓Adiponectin	Not described	Yes	Yes Mainly neutrophils	Not described	Yes Pericellular and perisinusoidal
Total enteral overnutrition in rats[70]	Dietary	Yes ↑Adiposity	Yes ↑ Glucose	Yes ↑Leptin ↓Adiponectin	Yes ↑Triglyceride ↑FFA	Yes	Yes Lobular	Yes Mild ballooning	Yes Portal/periportal and lobular
Caloric overload (by gavage) in rats[71]	Dietary Chow-fed with gavage of lipid-rich emulsion	Yes	Yes ↑Insulin ↑Glucose	Yes ↑TNF-α	Yes ↑Triglyceride ↑Cholesterol ↓HDL ↑FFA	Yes Macrovesicular	Yes	Yes Mitochondrial abnormalities	Not described
High-fat-fed *fa/fa* rats[75]	Dietary and genetic	Yes	Yes ↑Glucose Strain is insulin resistant	Yes ↑TNF-α Others not described	Not described	Yes Periportal, extending to central region	Yes Scattered foci	Yes Ballooning	Yes Accentuated in portal region
High-fat-fed *foz/foz* mice[77]	Dietary and genetic	Yes Severe ↑Adiposity	Yes ↑Insulin ↑Glucose	Yes ↓Adiponectin	Yes ↑Cholesterol	Yes Macrovesicular	Yes Multiple foci	Yes Ballooning	Yes Pericellular and perivenular

FFA, free fatty acids; HDL, high-density lipoprotein; sens., sensitivity; TNF, tumor necrosis factor; tol., tolerance.

histology that appears to mimic human NASH pathology, including steatosis, inflammation, and liver cell injury.

Despite the above findings, chronic administration of a high-fat diet to rodents does not always ensure development of steatohepatitis. In a study of Wistar rats, feeding high saturated-fat diets for up to 14 weeks caused no abnormalities in liver histology, and few aberrations in the metabolic health of these rodents.[65] High-fat feeding was associated with increased caloric intake, but minimal or no increase in bodyweight. Adipose mass and adipocyte size increased in high-fat-fed rats, but this apparent partitioning of triglyceride into adipose prevented hepatic triglyceride accumulation. Another adaptation to high-fat feeding was observed in the brown adipose tissue, which showed a modest expansion and increase in expression of uncoupling protein. The latter indicates increased thermogenesis, which may counter the effects of increased caloric intake in high-fat-fed Wistar rats. Thus, although some high-fat-feeding models appear well suited for NASH research, the challenge to reproduce them reliably between different laboratories may limit their utility and popularity.

Intragastric overnutrition

A number of models have now been described in which caloric overload is deliberately given to rodents. The pioneering study was in 2005 by Deng and colleagues, who described a gastric overnutrition model that delivered a caloric intake up to 185% that of normal mice through an implanted gastrostomy tube.[69] The feeding emulsion consisted of 37% of calories from fat (corn oil) and 39% as dextrose. In control mice fitted with an intragastric feeding device, but fed equivalent to normal caloric intake, weight gain was similar to that reported with *ad libitum* feeding. In contrast, over-fed mice developed severe obesity that was associated with increases in fasting serum glucose and insulin levels, and impaired insulin sensitivity. In adipose tissue, leptin mRNA increased in association with serum leptin levels, whereas adiponectin mRNA levels fell. Adipocyte TNF-α mRNA levels were increased, but overfeeding in TNF-α receptor-1 null mice demonstrated TNF-α signaling was not required for steatohepatitis development. All mice in the overfeeding group developed hepatomegaly associated with steatosis and increased serum ALT. Approximately half (six of 13 mice) went on to develop steatohepatitis. Steatosis was induced in most hepatocytes. The hepatic parenchyma was notable for aggregates of inflammatory cells, predominantly neutrophils and surrounding hepatocytes that showed microvesicular steatosis. Whereas human NASH histology is typically characterized by macrovesicular steatosis, and neutrophilic aggregates are not common (instead they are more often seen in alcoholic hepatitis), the histological injury in this model correlated with elevated serum ALT levels. As demonstrated by reticulin stain, there was also perisinusoidal and pericellular fibrosis, resembling the typical fibrosis pattern seen in human NASH. The gastric overnutrition model has many advantages (Table 3). First, transition to steatohepatitis appears to be spontaneous. Second, it occurs in the context of obesity, insulin resistance and metabolic syndrome. Third, the liver histology mimics human NASH. However, a 10–15% mortality rate and the requirement for technical expertise (or expensive purchasing from an experimental facility) may prevent this model from becoming a readily available tool for studying steatohepatitis development.

A similar model has recently been described in rats. Modest caloric overload is given via an intragastric cannula. In this study, rats given a 5% fat (as energy) diet that was 17% in excess of caloric requirement for 21 days became obese; there was increased adiposity as well as increased serum leptin and blood glucose levels.[70] Comparable caloric overload, but with dietary fat content increased to 70% fat (as energy), caused similar metabolic perturbations, but was also associated with decreased serum adiponectin levels, increased serum triglyceride, FFA and ALT levels, as well as histological evidence of steatohepatitis. Whereas overfeeding with regular diet alone was not associated with significant liver pathology, overfeeding with increased dietary fat content caused a dose-dependent increase in macro- and microvesicular steatosis, and lobular inflammation. These findings were accompanied by elevated serum ALT levels. When overfed rats were studied to 65 days, there was a further increase in serum ALT and evidence of hepatic fibrosis in the portal/periportal and lobular regions, suggesting progressive liver injury. In this model, ballooning degeneration of the hepatocytes was described, but the score was not significantly different between high- and low-dietary fat groups, and photomicrographs of ballooned hepatocytes were not presented in the article. Another feature of this model is that rats are sedentary. Previous studies indicated that activity levels were reduced by approximately 50% as rats do not expend energy to eat. Although this model successfully recapitulates the metabolic context of human NASH, at 21 days the extent of liver injury and inflammation and hepatic triglyceride content is only modest (Table 3). Fibrosis is evident at the later time-point (65 days), but the omission of histological scores for inflammation and ballooning degeneration at this time-point prevent critical assessment of this model at this stage of development.

Another approach to overnutrition was described by Zou and colleagues in 2006.[71] In their study, rats were allowed *ad libitum* access to food, water and an 18% saccharose solution. To induce caloric overload, rats were then given daily, by gavage, 10 mL/kg of a high-fat, high-cholesterol emulsion, whereas controls were given saline. After 6 weeks of overfeeding, rats given the high-fat emulsion had gained more weight, had increased blood glucose, insulin, triglyceride, total cholesterol, LDL–cholesterol, FFA and TNF-α levels, whereas HDL–cholesterol levels were decreased. Serum ALT levels were elevated and liver histology demonstrated significant steatosis, predominantly macrovesicular, and an inflammatory cell infiltrate. However, biochemical measures of lipid accumulation demonstrated only modest triglyceride accumulation (~1.5-fold), as well as increased total cholesterol (~fivefold) and free fatty acids (~threefold). Ultrastructurally, the hepatocytes showed aberrant mitochondria, including mitochondrial swelling, rarefied matrix, and loss of cristae. Similar ultrastructural changes in mitochondria have been described in human NASH, and may reflect injury or adaptive changes.[72,73] Although not specifically described, under light microscopy, these changes are suggested to reflect the megamitochondria seen in human NASH; megamitochondria are round or needle-shaped intracytoplasmic inclusions.[11,15,16] Pericentral hepatocytic necrosis was also observed in this study. However, typical liver cell injury and sequelae seen in human NASH, such as hepatocytic ballooning and fibrosis, were not described. A more thorough description of the liver pathology is required before the utility of this model can be fully assessed (Table 3). However, as the model requires daily

gavage of the lipid emulsion for 6 weeks (possibly longer to induce fibrosis), it is clearly labor intensive and the practicality of such a model will likely limit its use in steatohepatitis research.

Hyperphagia-driven overnutrition

Caloric overconsumption is thought to be one of the main underlying causes of the obesity epidemic.[74] Therefore, animal models of steatohepatitis in which hyperphagia is a central feature are attractive for their similarity to humans. Genetically obese animals such as *ob/ob* and *db/db* do not readily develop steatohepatitis, as discussed earlier. However, the Zucker fatty rat (*fa/fa*), which has a defect in the leptin receptor, does develop steatohepatitis when given a high-saturated-fat diet. After 8 weeks of high-fat feeding, *fa/fa* rats were obese with fasting hyperglycemia. They exhibited elevated serum ALT levels, which correlated with histological liver injury.[75] In contrast, high-fat-fed controls developed only mild steatosis, as did low-fat-fed *fa/fa* rats which were also obese but showed only mild fasting hyperglycemia and minor ALT elevation.

In high-fat-fed *fa/fa* rats with steatohepatitis, the distribution of steatosis began in the periportal region and extended to the lobules and central region. Fibrosis was also accentuated in the portal region. Thus, the lobular pattern of steatosis, liver injury and deposition of collagen in this model seem different from that seen in human adults with NASH, which show injury and fibrosis initially in zone 3. Ballooning degeneration of the hepatocytes was also described in this brief report, but it was not shown in the figures. In summary, high-fat-fed *fa/fa* rats appear to exhibit most metabolic and histological features similar to human NASH (Table 3). However, a more thorough description of the metabolic phenotype, for example, serum adipokine levels, as well as more extensive illustration of hepatic pathology, including images of ballooned hepatocytes, would enable the utility of this model to be established.

Another model of hyperphagic obesity is the *foz/foz* mouse. These animals have a spontaneous, truncating mutation in *Alms1*, the gene responsible for Alström syndrome in humans. Chow-fed *foz/foz* mice consume approximately 30% more calories than their wild-type littermates, are obese with increased adiposity, hypercholesterolemia, insulin resistance and glucose intolerance; diabetes develops by approximately 4 months of age.[76] High-fat feeding exacerbates this phenotype, and a striking reduction in serum adiponectin levels occurs. This appears to be associated with the spontaneous transition from the simple steatosis as observed in chow-fed *foz/foz* mice (and wild-type high-fat-fed mice) to severe steatohepatitis (Fig. 1).[77] Histologically, *foz/foz* mice fed a high-fat diet for approximately 300 days show mixed microvesicular and macrovesicular (predominantly macrovesicular) steatosis, and also ballooning degeneration of hepatocytes (Fig. 1c) with multiple foci of inflammatory cells (neutrophils and mononuclear cells) in hepatic lobules (Fig. 1d). Perivenular and pericellular (chicken-wire) fibrosis is also present in *foz/foz* mice with steatohepatitis (Fig. 1e). Of note, severely affected animals showed architectural distortion of the liver due to dense fibrosis. We have recently presented data showing that high fat-fed *foz/foz* mice develop liver histology representing steatohepatitis (steatosis, lobular inflammation, ballooned hepatocytes and fibrosis) as early as 6 months.[78] In fact, metabolic abnormalities and serum ALT elevation have been observed from as early as 6 weeks of high-fat feeding (C Larter & G Farrell, unpubl. data, 2008). Thus, the phenotype of high-fat-fed *foz/foz* mice appears to recapitulate the liver pathology that defines human NASH, in the context of multiple features of the metabolic milieu in which steatohepatitis develops (Table 3). Specifically, mice are obese with increased adiposity, insulin resistance, hyperglycemia, hyperinsulinemia, high serum leptin levels and low serum adiponectin levels.

Summary and conclusion

A number of rodent models of steatohepatitis have recently been described. Some recapitulate the liver injury observed in human NASH, but in a different metabolic context (nutritional deficiencies, or insulin sensitivity) (Table 1), whereas others develop in the metabolic context of NAFLD but without full development of steatohepatitis (Table 2). More recently, models of overnutrition have been achieved through high-fat feeding, forced caloric overload or studying genetically hyperphagic mice fed a high-fat diet (Table 3). These models appear most suited to studying the complex biological interactions that are involved in the development of fatty liver disease. In particular, these models enable steatohepatitis researchers to assess the contribution of the metabolic milieu of insulin resistance, obesity, dyslipidemia, altered adipokine and cytokine profile, as well as oxidant stress to the development of liver injury. The mechanisms of hepatocellular injury, inflammatory recruitment, fibrosis and carcinogenesis can appropriately be studied in such models.

We trust that the need for a thorough description of new animal models is evident from this review. It must be emphasized that comprehensive information on both liver pathology and metabolic phenotype should be included when new models are described. From the metabolic viewpoint, markers of obesity (weight gain, adipose mass), body fat distribution, insulin resistance (blood glucose and insulin levels, and or dynamic measures of insulin sensitivity), serum lipids and adipokine profile (serum adiponectin, leptin, TNF-α and IL-6 levels), provide valuable information for judging the value of models in terms of their metabolic similarities to human NASH.

Comparative studies of liver pathology between models, as well as within models at different stages of their evolution, would be a valuable contribution to knowledge in this area. Agreement on what comprises steatohepatitis in experimental models is urgently required if we are to reliably interpret studies that use established models to investigate pathogenesis or therapeutic pathways. For example, the sum of scores for steatosis, lobular inflammation, and hepatocellular ballooning was used to generate a NAFLD activity score (NAS) proposed by the NIDDK sponsored NASH Clinical Research Network (CRN). NAS has been widely used for clinical trials in patients since its validation. We have also adopted this scoring system in experimental animals such as MCD mice and the *foz/foz* model (C Larter *et al*., unpubl. data, 2008). In both models, we have found good correlation between the NAS and serum ALT levels.[79,80] However, lack of agreement on the validity of this or other approaches currently hampers laboratory-based NASH research.

In conclusion, in order to determine pathogenic mechanisms of liver injury and, particularly, what causes the transition from steatosis to steatohepatitis, appropriate animal models are required. As NASH appears to be a slowly evolving form of liver pathology

in humans, models of chronic overnutrition with spontaneous progression of steatosis to steatohepatitis may be the most valid and practical means to study the complex interplay between metabolic abnormalities and liver injury. The 'two-hit hypothesis', which usefully emphasized that mechanisms of liver injury and inflammatory recruitment may not be directly related to those for lipid accumulation (steatosis),[81] may be an oversimplification of steatohepatitis pathogenesis. A more realistic conceptual basis for NASH is that cumulative small pathogenic steps ('hits'), such as overnutrition, underactivity and insulin resistance on the background of genetic susceptibility initiate the transition of steatosis to NASH. It is critical that as more animal models become available these reflect this pattern of disease development. In the meantime, we believe that developments since 2005 place the field where preference should be given to 'metabolic models' that show appropriate pathology over models with parallel pathology, but created by non-physiological metabolic pathways or nutrient deficiency. In 2009, it is time for NASH researchers to look beyond the MCD model.

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