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Title Page:

Murine models of acute alcoholic hepatitis and their relevance to human disease

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Abstract

Alcohol induced liver damage is a major burden for most societies, and murine studies can provide a means to better understand its pathogenesis and test new therapies. However, there are many models reported with widely differing phenotypes, not all of which fully recreate the spectrum of human disease. Thus understanding the implications of these variations is key for clinicians/clinician scientists who wish to model human disease.

This review critically appraises key papers in the field, detailing the spectrum of liver damage seen in different models, and how they relate to the phenotype of disease seen in patients. A range of different methods of alcohol administration have been studied ranging from *ad libitum* consumption of alcohol and water to modified diets e.g. Lieber deCarli liquid diet. Other feeding regimens have taken more invasive routes using intra-gastric feeding tubes to infuse alcohol directly into the stomach. Notably, models utilising wild-type (WT) mice generally produce a milder phenotype of liver damage than those using genetically modified mice, with the exception of the chronic binge feeding model.

The review also recommends panels of tests that should be considered so as to standardise end-points for the evaluation of the severity of liver damage. This is key for comparison of models of injury, testing of new therapies, and for subsequent translation of findings into clinical practice.

Comment [RW1]: Abstract cut down to 220 words.

Introduction

The burden of alcohol and related liver disease is significant, in terms of both human and financial costs. In 2010, 7.2 deaths per 100 000 people globally were caused by alcohol related cirrhosis equating to 0.9% of deaths from all causes(1). The economic burden is much more difficult to calculate, and the World Health Organisation estimated that in 2003, the total tangible cost of alcohol to EU society was 125 billion euros, with non-tangible costs (value placed on pain, suffering and lost life due to social and health harms caused by alcohol) amounting to 150-760 billion euros(2). Whilst alcohol excess is a major cause of cirrhosis, as many as 60% of patients presenting with alcohol induced liver damage also have evidence of concomitant acute alcoholic hepatitis (AAH)(3). As the most dramatic presentation of alcohol-induced liver injury, AAH has a much higher short and long-term mortality approaching 20% and 50% respectively, despite current medical therapy(4, 5). The understanding of its pathogenesis and hence development of novel therapies has been in part hampered by the lack of relevant, reproducible animal models of AAH(6).

Whilst there are limitations of using animal models to investigate alcoholic liver injury, this approach does provide research opportunities not found in *in vitro* or clinical studies. Animal models allow control over multiple pathogenetic factors such as the environment, contribution of specific pathways and the amount of alcohol consumed, which are difficult to replicate in human studies. Mice that are transgenic for key inflammatory and metabolic disease modifying genes are widely available, and confer the ability to assess the impact of regulatory processes on the induction of alcoholic liver injury(7). While transgenic rats are available their use has been restricted by a limited knowledge of their reproductive system and more difficult *in vitro* embryo manipulation which is needed to develop transgenic breeds. Therefore, in this review we will critically appraise the published models of acute murine alcohol-induced liver injury, paying particular attention to the parameters used to define the extent of liver damage, in order to highlight advantages of those models with the greatest promise for new treatment options.

Phenotype of disease

Alcohol induces a broad spectrum of liver injury in patients ranging from steatosis, to more florid inflammation and hepatocyte necrosis, and finally to fibrosis and the development of hepatocellular carcinoma. The particular phenotype induced is determined in part by the quantity and duration of alcohol exposure as well as patient specific factors. A variety of models have been used by researchers to model this spectrum(6), with each utilising a different method of alcohol administration to produce a desired pattern of liver injury. In general however, whilst many of the available murine models reproduce some of the early stages of liver injury, the development of fibrosis and cirrhosis is harder to replicate and commonly requires an injury additional to alcohol exposure. Thus, whilst steatosis has been achieved by *ad libitum* feeding for between approximately one week to several months(7, 8), most models require a second insult alongside an extended course of alcohol administration in order to induce fibrosis such as either concomitant genetic manipulation(9) or the addition of a second chemical insult such as carbon tetrachloride (CCl_4)(10).

Use of Wild Type mice to model alcohol induced liver damage

Alcohol has been administered to mice by a variety of different routes/regimens, each having their respective advantages and disadvantages (Table 1). Choice of model is often governed by the features of liver injury that are required and the skills/resources available. The simplest method of administering alcohol, known as *ad libitum*, is to mix it into the drinking water and allow the mouse free access to this alongside their normal chow. However, due to a natural aversion to alcohol, the mice generally only develop low blood alcohol levels (BAL) and mild liver injury(11). This model can be useful in some circumstances as it replicates human patterns of alcohol exposure and dietary intake. The other *ad libitum* option involves the addition of alcohol to a Lieber deCarli (LdC) diet, in which normal mouse chow is replaced by a high fat, nutritionally complete liquid diet. This partially overcomes the murine dislike of alcohol and tends to produce a more significant liver injury than the conventional water/alcohol mix(12). There is conflicting evidence as to whether the increased liver injury is a reflection of higher blood alcohol levels or the additive effect of combining a high fat diet with alcohol exposure(12, 13).

Another *ad libitum* method is to provide the ethanol in an agar gel(14). This has been used much less commonly than a liquid diet although there is some evidence that the alcohol evaporation from a gel is low. The model was developed to try and simplify the administration of alcohol. The gel diet does appear to induce a liver injury- the alcohol fed mice developed a significantly higher steatosis score, triglyceride level and ALT level than control mice not fed alcohol. The drawback for this method is the complicated gel preparation and custom made feeding tubes required. In contrast the Lieber deCarli liquid diet is easier to make and Richter tubes are a simple delivery method.

Another approach consists of administering alcohol via gavage directly into the animal's stomach, which is a relatively straightforward procedure that can be easily taught(15). However, the procedure needs to be repeated on a daily basis, thus inducing stress in the mouse, and again only produces mild liver injury with a 25% increase in serum alanine aminotransferase (ALT) levels in ethanol fed animals(16). The gavage model can be used in combination with *ad libitum* delivery of alcohol, such as in the chronic-binge model(17), where mice have access to a Lieber-deCarli/ethanol mix and also receive a single gavage of ethanol on the day the experiment is terminated. This produces a more significant liver injury than just gavage or *ad libitum* delivery alone, with peak levels of ALT and aspartate aminotransferase (AST) 9 hours post-gavage of 250IU and 420 IU respectively(18). Notably, there is also evidence of greater triglyceride deposition in the liver and increased hepatic inflammation in the chronic binge group. The ability of this relatively simple model to induce a moderately severe alcoholic liver injury has led to its adoption by many groups(19).

Recently, a hybrid model of a solid chow high in cholesterol and saturated fat along with intra-gastric feeding of a liquid high fat/ethanol diet has been developed by the Tsukamoto group(20). The intra-gastric feeding model was first described by Tsukamoto and French in 1985(21), and involves complex surgery to place a tube through the skin into the rodent's stomach. This tube is then used to administer feed and alcohol to the mouse. It has been shown to produce higher BALs (between 100 to 500mg/dL in rats)(6) and a more severe liver injury than *ad libitum* feeding methods(22). The hybrid model produces a liver injury consistent with chronic alcoholic steatohepatitis- with a marked transaminase rise, and significant steatosis with inflammation and occasional neutrophil infiltration present. The addition of weekly alcohol binges induces an increased neutrophil infiltration with clustering seen around dead and fat-loaded hepatocytes. This provides a better representation of an acute alcoholic hepatitis injury (Figure 1).

The length of high fat diet administration has been investigated by Chang *et al*. who fed mice for either three days or three months of high fat diet with a single gavage of alcohol on the final day of feeding(23). This model produced raised ALT/AST in the 3 day model, with higher levels in the 3 month model. Increases in infiltrated neutrophils and serum free fatty acids were also seen, however, the activation markers of macrophages was only slightly increased by the alcohol binge compared to the model without the alcohol. This seems to partially correlate with the human picture of alcoholic hepatitis (see below).

The diet composition is also very important. Lieber and deCarli developed their eponymously named diet to accentuate the liver injury that could be induced by alcohol administration and it has since been shown that a diet that is high in saturated fats can reduce hepatic lipid accumulation, whilst a diet containing polyunsaturated fats promotes liver injury. You *et al.* found that adiponectin mediated the protective effect of saturated fats, which may provide therapeutic options that should be explored(24). However, recently Chen *et al*. showed that while saturated fats can reduce hepatic fat deposition, they increased fibrotic changes within the liver(25). Importantly, the majority of murine studies follow a pair fed diet protocol. This involves matching the amount of diet without alcohol that is provided to control mice to the amount of diet and alcohol that the main study mice consumed in the previous 24 hours. This provides a control group to show that the liver injury is due to the alcohol and not the high fat diet. Ultimately, logistical issues may determine choice of regimen; *ad libitum* models require considerably less expertise and specialist equipment, whilst the more involved intra-gastric feeding model requires metabolic cages, single mouse housing, specialist infusion equipment and surgery to be performed by the researcher.

In WT mice the severity of liver damage is closely linked to the duration and quantity of alcohol consumption, both of which are strongly influenced by the method of alcohol delivery. The *ad libitum* methods are limited by the mouse's appetite whereas the intra-gastric feeding method is limited by the length of time the mouse can tolerate a feeding cannula in its stomach. Consequently, the duration of each model is determined both by the tolerability of the model and the level of liver injury that is required. Thus, whilst there are advantages to using WT mice in such studies, the extended duration of alcohol exposure needed to generate more severe liver injury may be challenging, highlighting the potential advantages of using transgenic mice that have an increased susceptibility to the injurious effects of alcohol.

Models of alcohol induced liver damage using genetically modified mice

To date, multiple different regulatory and metabolic genes have been knocked out to assess their impact on the process of liver injury (see Table 2). Some of these affect normal pathways of ethanol metabolism or metabolism of harmful by-products of ethanol, such as the *Nrf2* knockout mouse that is susceptible to oxidative stress caused by alcohol breakdown products(7). Others, such as the *Hfe* knockout mouse, which results in hepatic iron overload, augment the injurious effect of alcohol(11). Some of the more commonly used models with profound phenotypes are described in greater detail below, with a more comprehensive summary of models in Table 2.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) protects cells against xenobiotic and oxidative stress, such that mice with this gene knocked out incur a severe, acute form of acute liver injury after they ingest alcohol(7). Mice are typically given three days of Lieber deCarli diet for adaptation purposes, and then alcohol is added at increasing concentrations of 2.1%, 4.2% and 6.4% v/v for three-day blocks respectively. This gives a total of nine days of alcohol administration during which time significant amounts of hepatocellular damage were reported, as demonstrated by marked rises in ALT and development of clinical signs (7). The *Nrf2*$^{-/-}$ mouse thus provides a good model to study severe acute liver injury as seen in the setting of AAH where oxidative stress is an important factor (26, 27), although the high level of mortality reported necessitates close monitoring of mice. No evidence of liver fibrosis was presented in this model which potentially limits its utility given most patients with AAH have concomitant fibrosis, although its absence may be explained by the short duration of alcohol administration. However, it is possible that modification and extension of the regimen could potentially induce development of fibrosis. The acute onset of injury in this model presents a challenge as the cohort of mice with severe liver injury are identified by their moribund appearance and this occurs at varying time points after exposure to the high concentration of alcohol making the model difficult to use for both logistic and ethical reasons.

Other groups have targeted hepatic lipid homeostasis to exacerbate alcohol-induced liver injury. Lipin-1 is a vital regulator of lipid metabolism, acting as an enzyme in the triglyceride synthesis pathway and a transcriptional co-regulatory protein that is highly upregulated in alcoholic fatty liver disease. Hu *et al.* demonstrated that administering alcohol to mice with deletion of lipin-1 led to the rapid onset of severe liver injury, as indicated by levels of serum ALT and inflammatory cytokines, and progression to alcoholic steatohepatitis(28). In this study mice were fed low fat Lieber deCarli diet, with and without ethanol for four weeks. Wild type mice typically developed only mild liver injury while the lipin-1 knockout mice showed increased serum levels of ALT, AST, and free-fatty acids, as well as micro and macrovesicular steatosis suggesting that lipin-1 may exert a protective role by limiting inflammation and promoting efficient lipid storage and metabolism.

Nishiyama *et al.* also investigated fat deposition(29). They used a hepatocyte specific *HIF-1a* null mouse to show that HIF-1 (Hypoxia inducible factor-1) has a protective role that reduces accumulation of lipids in the liver after ingestion of an alcohol/Lieber deCarli liquid diet. They were also able to show that HIF-1α suppresses Srebp-1c activity and that is at least part of the reason that when HIF-1α is removed, steatosis increases. However, there are conflicting reports regarding the role of hypoxia inducible factors. Nath *et al*. also used a *HIF-1a* null mouse and found a reduced injury in this knockout mouse(30) while Ni *et al*. achieved similar results using a *HIF-1b* null mouse(31). The reasons for these contrasting results are not clear, although different housing conditions or development of sub-strains within the knockout populations have been suggested(32).

It is interesting to note that HIF have been implicated in the tissue repair response within the liver. They may be involved in regulating the angiogenic effect of hepatic macrophages that induce liver sinusoidal endothelial cell proliferation and migration(33). This appears to be a key step in liver repair after an acute injury. Macrophages are likely to be key to fully understanding the process of tissue repair in the liver. It has been shown that initially pro-inflammatory ($Ly6C^{hi}$) macrophages can switch to a $Ly6C^{low}$ phenotype important in tissue repair(34) after phagocytosis of apoptotic hepatocytes. Further characterization of the mechanisms driving tissue repair in alcoholic liver injury are needed to identify targets for potential therapies.

Comment [RW2]: New text added to discuss the papers investigating HIF and the tissue repair response.

Other pathways that have been targeted in the attempt to augment hepatic injury following alcohol exposure include Pparα. Pparα is a nuclear hormone receptor and transcription factor that regulates hepatic inflammation and lipid metabolism. The role of this receptor is to stimulate fatty acid catabolism under fasting conditions and so the authors of this study(8) anticipated that free fatty acid production associated with alcohol consumption would normally activate Pparα. The *Ppara* knockout mouse was given *ad libitum* Lieber deCarli liquid diet with 4% ethanol for up to six months resulting in the development of both an inflammatory cell infiltrate and fibrotic changes that were not seen in alcohol fed WT mice. This was confirmed by both Picrosirius red and alpha smooth muscle actin staining, and demonstration of induction of genes involved in fibrosis including *Thbs1*, *Col1a1* and *Col1a2*. *Ppara* transgenic mice with additional genetic alterations provide further options to investigate liver injury. The Glutathione S-transferase A4-4/ Peroxisome proliferator activated receptor-α (Gsta4-4/Pparα) mouse has been described recently(35). Gsta4-4 is an enzyme that protects against natural and environmental toxicants through glutathione conjugation which protects against harmful aldehydes, including 4-Hydroxynonenal (4-Hne). Ronis *et al.* have used this double knockout in an *ad libitum* Lieber deCarli/5% EtOH model to show the central role lipid peroxidation plays in mediating progression of alcohol-induced necro-inflammatory liver injury, stellate cell activation, matrix remodeling and fibrosis(35).

Other alternatives to transgenic knockout mice include transfecting mice with adenoviruses to silence the expression of a specific gene, This reduces but does not completely turn off gene expression. The Postic group used this method to show that silencing the Carbohydrate Responsive Element Binding Protein (ChREBP) prevents alcohol induced steatosis in an acute model of injury(36). Another strategy is to genetically alter mice to over express a certain gene. Butura *et al.* used this method to investigate the role of the *Cyp2e1* gene(37). They inserted approximately 20 extra copies of the gene into mice. They found that overexpression of this gene aggravates the liver injury with increased levels of oxidative stress.

Fibrosis

The generation of alcohol induced fibrosis in mouse models is more challenging than steatosis and inflammation and often requires a second injurious element in addition to alcohol ingestion. Bataller and Gao have published a comprehensive review on liver fibrosis in alcoholic liver disease and should be read for further information(38). There are a variety of non-alcohol models that are utilized to induce liver fibrosis, with one of the most commonly used being carbon tetrachloride(CCl_4). This involves repeated intraperitoneal injections of CCl_4 over a period of weeks, although there are no studies directly comparing the liver fibrosis induced by CCl_4 or alcohol. The Nagy research group were able to induce liver fibrosis by administering CCl_4 and moderate alcohol intake at a level not usually producing a significant liver injury. This proves the additive effect of the two agents through common pathways(10). Roychowdury *et al.* compared a high ethanol feeding regime against a moderate ethanol regime with the addition of CCl_4(39). They demonstrated that steatosis, inflammation and apoptosis were more prevalent in the alcohol only group as compared to the group that also received CCl_4, which had more prominent fibrosis.

Chiang *et al.* exposed mice to 2% alcohol *ad libitum* for either 2 days, 2 weeks or 5 weeks alongside administration of CCl_4, which resulted in characteristic hepatic extracellular matrix deposition and a change in sinusoidal architecture(10). Genetically modified mice deficient in the HFe iron transporter, which causes accumulation of hepatic iron, develop a marked steatohepatitis and fibrosis upon administration of a high fat diet with ethanol(9). Versions of this dietary protocol have also been used by other groups combined with other genetic backgrounds. For example, Li *et al.* treated *Ppara* knockout mice with a 4% ethanol/Lieber deCarli diet(8), and after 4-6 months reported fibrosis with a small amount of collagen deposition in peri-venular and peri-cellular regions. Importantly, in common with other models, a major drawback of this study was the length of time required for fibrosis to develop, as well as the relatively modest amount of fibrosis seen. Notably, other groups have demonstrated that similar or longer regimens are not able to induce significant fibrosis in WT mice, necessitating further study of specific transgenic animals and alternate models of alcohol delivery(8, 40).

Mouse variables that affect experimental endpoints

There are practical benefits in using a model where mice freely consume alcohol in large quantities. However as noted above, most mouse strains are not inclined to voluntarily ingest alcohol and this means that modified liquid diets, gavage or intra-gastric infusion are often required. There are marked strain differences in murine attraction to alcohol, and one of the more comprehensive studies compared the consumption of unsweetened alcohol, sweetened alcohol and sweetened water in 22 in-bred strains of mouse(41). C57Bl/6J strain of mice freely consumed the most alcohol, drinking more than 10g/kg/day compared to less than 2g/kg/day consumed by DBA/2J mice. Moreover, it has been shown that C57Bl/6 mice would consume diet containing a higher concentration of alcohol than other strains of mice(42). Patterns of alcohol consumption over time were also explored, and notably, mice with restricted daily access to alcohol consumed similar quantities to mice that had unlimited 24 hour access(43), with both groups having similar blood alcohol levels(42). It is not clear why the C57Bl/6 mice are able to consume higher concentrations of alcohol but there are parallels with consumption in humans where there is a marked difference in susceptibility to alcohol induced liver damage across ethnic groups (44).

Gender is also an important factor in development of alcohol induced liver injury. Female patients are more susceptible to developing more advanced alcoholic liver damage both after acute and chronic administration(45), and similarly female mice develop more florid liver injury than males after exposure to ethanol(46). There are several different theories pertaining to this gender difference including different alcohol elimination rates, different alcohol pharmacokinetics and different oestrogen levels. Frezza *et al*. were the first to show that in humans, females have decreased levels of gastric ADH which lessens the ‘first pass effect’ on alcohol and increases the bioavailability of ingested alcohol when compared to males(47). Female mice develop less liver fibrosis when exposed to other types of chronic liver damage, such as CCl_4 injury or hepatitis C virus infection, suggesting that oestrogens may have a protective effect in some disease settings(48, 49). Work still needs to be done to ascertain whether this also applies to alcoholic liver injury but it does appear that treatment with oestrogen in females lacking ovaries reduces hepatic steatosis(50). Also, there are significant gender differences in the response to alcohol at a proteomic level. Wang *et al*. found that 78 protein levels were altered by either male or female mice undergoing chronic alcohol feeding and this included several oxidative stress related proteins. This is consistent with studies in rats that have found that oxidative stress is a possible reason for increased liver injury in females after ethanol feeding(51).

Alcohol consumption is different from alcohol metabolism, but female mice seem to have an equal or increased consumption compared to males. Female mice will ingest more alcohol than their male counterparts if given free access to alcohol, although when access is restricted to a defined time period, their intake is similar(52). The females will also achieve higher blood alcohol levels after ingesting an equal amount of alcohol as male mice(52). This would also seem to mirror the human setting in which women need a lower alcohol intake to achieve equal blood levels to men (National Institute on Alcohol Abuse and Alcoholism. Women and alcohol 2015. Available from: http://pubs.niaaa.nih.gov/publications/womensfact/womensfact.htm. Accessed 14/09/15). Also, women that drank a moderate amount of alcohol were at higher risk of developing alcoholic liver disease than men that drank a similar amount(53, 54). All of the above underlines the importance of gender in induction of an alcoholic liver injury and reinforces the need to use mice of a single gender in murine models to achieve consistent results.

Comment [RW3]: Text added to expand and clarify the importance of gender as a variable affecting liver injury.

Age is also an important variable when investigating the effects of alcohol ingestion. Vogt *et al*. showed that glutathione levels take longer to recover after administration of alcohol in mice aged 24 months compared to mice at 12 months(55). This would appear to be replicated by other studies(56, 57). Glutathione is involved in the detoxification of alcohol and this result would seem to indicate that older mice are less able to metabolise repeated alcohol doses. Further work is required to establish whether this results in increased toxicity and an increased liver injury. However, Ramires *et al.* found an increased liver injury in mice over 24 months when compared with younger mice though this may be due to decreased rates of autophagy in the older mice(58). It is not clear whether age reduces a human's ability to metabolise alcohol. Wynne *et al*. showed that age did not diminish the activity of alcohol dehydrogenase in the livers of male or female healthy volunteers(59). However, studies suggest that both age and ethnicity influence the severity of alcoholic liver disease in humans(60), and decline in mitochondrial function combined with accumulated oxidative damage in older individuals may render older livers more susceptible to damage from alcohol(61). Thus age is a variable that should be investigated more fully in the context of alcoholic hepatitis.

Comparison of mouse models to human AAH

Inflammation of the liver caused by excess alcohol intake occurs after sustained excessive intake and consists of a combination of signs, symptoms and histological findings(62). Clinically, it causes a rapid onset of jaundice with fever, ascites and proximal muscle loss that may be accompanied by an enlarged and tender liver. Unfortunately, none of these parameters can be used to demonstrate the relevance of a mouse model to human disease. In patients, serum ALT/AST, bilirubin and INR are commonly raised and liver histology will reveal the presence of hepatocyte ballooning which represent amorphous eosinophilic inclusion bodies, called Mallory-Denk bodies(63), and a high number of infiltrating neutrophils. Bilirubinostasis is common and associated with susceptibility to infection (64) and poor survival (65). Due to the long history of alcohol excess, steatosis and fibrosis are also commonly seen in human livers.

The level of neutrophil infiltration in the murine liver has been suggested as a measure of how representative a model is of the picture of AAH seen in patients. However, a mouse model that induces a neutrophil infiltration similar to that seen in AAH has been elusive(66). Moreover, greater neutrophil infiltration is associated with better survival in humans (65) and thus may not be a sensible therapeutic target. Two older models that have been used in this context are the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) or griseofulvin (GF) models. These produce ballooning of hepatocytes and accumulation of Mallory bodies but do not involve the administration of alcohol to the mice.

Lamle *et al.* were able to induce inflammation within the livers of the *Nrf2*$^{-/-}$ mice that received Lieber deCarli and ethanol diet which was characterised by histological finding of Kupffer cell and neutrophil infiltration of the liver(7). The chronic-binge alcohol feeding method also seems to induce a liver injury that is reasonably similar to human AAH and Bertola *et al.* describe raised serum ALT/AST, TNF and hepatic neutrophil infiltration in this model albeit without describing the other characteristic histological findings such as hepatocyte ballooning found in human AAH(17).

Human alcoholic hepatitis(AH) commonly occurs after repeated, long-term alcohol ingestion with an acute flare up producing the inflammation. It may be that our mouse models do not reflect this longer term ingestion and thus do not produce the same phenotype of disease. This is supported by the findings of cirrhosis in human biopsies which is not normally reflected in the mouse models. An elevated bilirubin is not reproduced by any of the mouse models which may indicate that this feature is linked to the more chronic features of the disease, although how this occurs still needs further clarification.

In the search for murine model/human disease crossover, Xu *et al*. identified murine hepatic *Fsp27* and the human homolog *Cidec*(67). Both genes are elevated in correlation within a setting of AAH and *Fsp27* is thought to be upregulated by ChREBP and Ppar-γ. Interestingly, *Cidec* up-regulation was found to correlate with the degree of hepatic steatosis, severity of disease and the mortality of the AH patients. Xu *et al*. were able to show that knocking out *Fsp27* in the mouse, ameliorated the liver injury seen. This suggests that *Cidec* may be a therapeutic target that could reduce the level of liver injury sustained by patients with AH.

Standardisation of endpoints for use in models of alcohol-induced liver injury

The literature includes a range of different read-outs and experimental endpoints that are used to quantify the nature and severity of alcohol-induced liver injury. This diversity can be useful for understanding pathogenesis but is challenging when trying to compare the phenotype of liver damage reported across different models. Moreover, there is value in tailoring the read-outs to the focus of a particular study or clinical discipline, whether it is generation of steatosis, inflammation, fibrosis or cancer. Certain analyses are useful in the majority of studies, such as serum ALT levels, whereas other tests will be specific for the question being asked, such as the amount of fibrosis as indicated by alpha-smooth muscle actin. Detail of some of the more common experimental parameters is given below and summarised in Table 3.

Overall assessment of murine behaviour and well-being

Murine behavioural patterns are often monitored with a view to animal welfare, although their assessment with standardised scoring systems can provide important information on the effect of alcohol on the mouse. Done reliably, such scoring systems have the potential to provide objective information on the severity of illness in mice thus providing a censorable end-point for experiments, whether they be induction of injury or response to treatment (Supplemental Table S1). This bears comparison with clinical scoring systems such as the Glasgow alcoholic hepatitis score (GAHS), which increasingly focus on clinical features of function rather than static measures of liver damage. Given the reported individual variation in level of liver damage following some murine models of ethanol exposure, the added advantage of a clinical assessment is that it ensures mice are more likely to have developed a similar level of liver damage.

Biochemical assessment of liver function

In the setting of severe liver injury, the most robust assessment of a model should include measurement of parameters of liver synthetic function such as prothrombin time, serum bilirubin, glucose and albumin levels. These provide important information on the severity of injury, and can be performed on peripheral blood samples whilst models are ongoing thus allowing for the rigorous assessment of potential new therapies. However, as mice have approximately 50-60 ml/kg of circulating blood (approximately 1.5 ml for a 25 gram mouse) (National centre for the replacement raroair. Mouse : Decision tree for blood sampling. Available from: http://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling. Accessed 14/09/15), there are limitations on the number of blood tests that can ethically and physiologically be performed on living animals.

Assessment of liver damage and hepatocyte death

Liver damage, as opposed to function, can be assessed in a variety of ways ranging from measurement of serum ALT/AST through to scoring of liver histology. Serum ALT/AST are commonly measured in studies and provide a standardised measurement of liver damage. This is generally used to compare the extent of liver damage across studies using different models and different strains of mice, although there is strain-dependent difference in susceptibility to injury. For example, Mizuhara *et al.* have shown that ALT levels vary significantly between C57Bl/6 and BALB/c mice following induction of liver injury with concanavalin A(68). Haematoxylin & Eosin (H&E) staining of liver sections provides valuable information on the extent of tissue necrosis, inflammation and steatosis, and TUNEL staining can allow quantification of the amount of apoptosis. Histological analysis for the presence of hepatocyte ballooning and presence of Mallory bodies by ubiquitin staining(69) is of particular relevance in the setting of AAH, whilst analyses of superoxide dismutase 1 (SOD1) and malondialdehyde (MDA) may provide useful insights into the level of oxidative stress during acute liver injury(70).

Assessment of liver steatosis

Although H&E staining gives a qualitative indication as to the extent of steatosis, quantitative assessment can be performed using Oil Red O staining of liver sections and digital imaging or morphometric analysis alongside quantification of hepatic triglycerides and lipids. Liver to body weight ratio can also provide an indication of the extent of steatosis although it can be confounded by concomitant liver necrosis. More detailed analysis of steatosis can also include analysis of key molecules in pathways contributing to its development, such as SREBP, which are involved in cholesterol and fatty acid biosynthesis(71).

Assessment of liver inflammation

Immunohistochemical staining of liver provides data on the extent and composition of liver infiltrating inflammatory cells, which can be complemented by flow cytometric analysis of resident immune cells from liver cell digests. For example neutrophil infiltration in models of alcoholic hepatitis has been assessed using both immunochemical staining(72) and cytometric detection of Ly6G positive cells in liver digests(73). Cell digest analysis can provide detailed quantitative information on the composition of the liver infiltrate as well as determination of the activation status of any infiltrating cells. This can also be supplemented with analysis of cytokines, such as tumour necrosis factor (TNF), IL-6 and IL-10, from serum and liver tissue at message and protein level to provide useful information on the level of inflammation and the impact of any therapeutic intervention(74). For example, in humans, IL-6, IL-8, TNF and MCP-1 have all been implicated in neutrophil infiltration in patients with alcoholic hepatitis(75) (76), whilst in mice IL-4 appears to promote neutrophil survival and hepatitis(77).

Assessment of liver fibrosis

Standardised assessment of liver fibrosis should include morphometric analysis of fibrotic areas by picrosirius red (PSR) or Van Gieson staining, qPCR for *Col1* transcripts and biochemical assays of fibrosis such as hepatic hydroxyproline quantification(78). Useful additional insights can be gained by studying staining for activated hepatic stellate cells using alpha-smooth muscle actin (α-SMA) and transcription levels of matrix metallo-proteinases (MMP) and their tissue inhibitors (TIMP).

Additional mechanistic studies

Existing mouse models are useful in replicating human disease but, as discussed above, they have limitations. One interesting area that could be expanded upon in the future is the use of genome wide association studies (GWAS) to identify human pathways/molecules involved in alcoholic liver injury. Current results from these studies have helped identify an allele that has an association with alcoholic liver injury(79). Other studies have identified specific genes that have a role in the pathogenesis of alcoholic liver injury, such as osteopontin(80). There is potential to expand on this work to identify further genes that put individuals at risk of developing severe alcoholic liver injury. This clinical information could be used to create new transgenic mice to investigate pathways involved in alcohol metabolism, help future refining of animal models and discover new treatments for alcoholic liver disease.

Thus, future mechanistic studies may consider useful biomarkers to identify individuals at risk of experiencing alcoholic liver injury(81). Manna *et al*. used metabolomics to show that indole-3-lactic acid and phenyl lactic acid are potential biomarker candidates(82), while microarray data has identified that serum insulin-like growth factor binding protein 1 could provide an easily measured biomarker for early detection of alcohol-induced liver injury(83). The Szabo group reported that microRNAs may serve as biomarkers that can differentiate between hepatocyte inflammation and injury. They found that different miRNAs can be elevated by either alcoholic, drug-induced or inflammatory liver disease(84).

Conclusion

In conclusion, murine models of alcoholic liver disease are an invaluable tool that can be used to investigate the whole spectrum of alcohol-induced liver damage encountered in the human population. Murine models have several advantages which allow researchers to investigate the full time-course and specific mechanisms of the disease in more depth than is possible from human studies. It is clear that before commencing any mouse model work, the human liver injury feature to be replicated must be identified. When this is known, a specific mouse model can be chosen by selecting a transgenic mouse, the alcohol administration method and the duration/amount of alcohol required to replicate that clinical picture. However, researchers should exert caution and ensure that factors such as gender, age and strain of mice are carefully considered. This is vital to ensure the mouse liver injury mirrors that seen in patients and thus provides a robust means in which to test new pathophysiological mechanisms or therapeutic agents.

Tables

Mode of Delivery	Liver histology findings	Change in serum ALT	Practical/resource issues
Ad Libitum- Water + Ethanol (11),(85) (86), (87)	Histologically normal liver or mild steatosis only.	Minimal or no rise in ALT up to 160 U/L.	Easy to deliver.
Ad libitum- Lieber-DeCarli diet + Ethanol (8), (10), (17), (28), (88), (89), (90), (91), (92)	Histological evidence of mild to moderate micro and macrosteatosis only.	Variable rise in ALT from a minimal increase up to 350 U/L with long term feeding.	Easy to deliver, special diet needed.
Acute gavage (16), (93)	Histological evidence of mild steatosis and inflammatory injury only.	A rise of between 30 to 50 U/L.	Skill needed for gavage technique.
Ad libitum + gavage (18), (19), (22)	Histological evidence of neutrophil infiltration into the liver. Steatosis with occasional areas of necrosis, but no fibrosis.	Increase of up to 270 U/L.	Skill needed for gavage technique.

Intra-gastric infusion (21), (71), (94), (95), (96), (97)	Histological evidence of severe steatosis, inflammation, necrosis and hepatic stellate activation.	ALT up to 450 U/L.	Specialist surgical skill needed, extensive amount of specialist equipment and intensive monitoring needed.

Table 1- Established routes for administration of alcohol to mice

Genetic manipulation	Function of key gene	Liver injury indices	Conclusions
Hepatic *ADH* knockout, *ad libitum* LdC + 1, 2 or 3.5% EtOH (98), (99)	ADH catalyses the oxidation of ethanol - the main pathway by which ethanol is metabolized during chronic alcohol abuse.	No significant oxidative stress levels or inflammatory response. Produced pan lobar vacuolization in response to 3.5% EtOH diet.	Dose of ethanol and ADH deficiency are key factors in initiation and progression of alcoholic fatty liver disease. The *ADH* KO mice produced higher BALs(99) and consequently increased hepatic lipid vacuolization. Deer mice and this model can be used to study chronic alcoholic liver injury.
BiP (heavy chain immunoglobulin binding protein/ Grp78) knockout, *ad libitum* high fat diet + 4 g	*BiP* mediates the unfolded protein response which reduces protein translation, enhances protein folding and increases degradation	Raised ALT to approximately 320 U/L in *BiP* KO mice compared to approximately 45 U/L in WT mice. Also showed increased lipid	HCCs were only found in the knockout mice, suggesting that more than one ‘insult’ needs to be present to induce

alcohol/kg body weight(89)	of unfolded proteins. This serves as a model of ER stress with alcohol added to study the development of hepatocellular carcinoma (HCC).	accumulation and increased rate of HCC.	carcinogenesis. Alcohol induced stress was age related, with younger animals more resistant to stress.
CHOP knockout, intra gastric infusion of high fat diet + 18 g/kg/day increased to 29 g/kg/day of alcohol for a total of 4 weeks(100)	CHOP is a transcriptional regulator involved in apoptosis caused by endoplasmic reticulum stress.	WT & transgenic mice had significant changes in steatosis score, liver triglyceride levels (fivefold increase in WT but 50% decrease in *CHOP* -/- mice) and ALT (112 U/L). *CHOP* -/- mice had no apoptosis.	As a response to ER stress, *CHOP* upregulates and is involved in causing apoptosis.
Cyp2e1 knockout, intra gastric infusion of high fat diet + 14 g/kg/day increased to 28g/kg/day of alcohol for a	Cyp2e1 (cytochrome P450) is induced in the hepatocyte by ethanol and appears to correlate with the level of liver injury.	Mild steatosis, slight inflammation and necrosis as shown by pathology scores.	Shows that CYP2E1 has a minimal role in early alcohol induced liver injury.

total of 4 weeks(97)			
Gsta4-4/Ppara double knockout, ad libitum 5% EtOH/ LdC for 40 days(35)	Gsta4-4 (Glutathione S-transferase A4-4) is a detoxification enzyme that eliminates toxins via glutathione conjugation. Ppar-α is a hormone receptor that regulates hepatic inflammation and lipid metabolism.	Produces increased hepatic injury with significantly increased inflammatory response, necrosis and fibrosis.	Shows the importance of lipid peroxidation products mediating the early progression of ALD.
Hfe knockout, High fat diet and ad libitum water + alcohol at 20% v/v for 8 weeks(9)	Model of iron overload consistent with haemochromatosis.	Produces profound steatohepatitis, significant fibrosis and increased apoptosis.	Highlights a combined effect of iron overload, alcohol and a high fat diet cause significant steatosis, inflammation, oxidative stress and apoptosis.
Hif-1a knockout mice, *ad libitum* 6% ethanol/LdC diet	HIF (hypoxia inducible factor) is a master controller adapting to	Increased steatosis, serum and liver cholesterol and triglycerides.	*HIF-1a* induction provides protection against alcohol

for 4 weeks(29)	hypoxia by controlling expression of hundreds of genes.		induced fatty liver disease and modulating its activity may provide therapeutic potential.
Lipin-1 knockout, *ad libitum* low fat LdC + alcohol for 4 weeks*(28)	Lipin-1 is a vital regulator of lipid metabolism.	Produces an ALT of 90 U/L with fibrosis in *Lipin-1* knockout mice after 4 weeks of feeding.	Suggests a role for treatments to enhance lipin-1 as a treatment for ALD.
Nrf2 knockout, ad libitum LdC + 2.1% v/v alcohol for 3/7, 4.2% for 3/7 followed by 6.3% alcohol until the mice became moribund(7)	Nuclear factor-erythroid 2-related factor 2 (NrF2) is a transcription factor that protects against oxidative stress.	An ALT of 3000 U/L and severe steatosis with increased number of Kupffer cells.	Central role for Nrf2 in the protection against alcohol induced liver injury.
Ppara knockout, gavage of 0.4ml/10g 52% erguotou wine for 4/52(8)	Pparα stimulates fatty acid catabolism under fasting conditions (similar to chronic alcohol ingestion).	Fibrosis in knockout mice fed ethanol for 4-6 months, with severe steatosis and inflammatory cell infiltration.	Suggests a pathway for alcohol metabolism. Possible role for Pparα agonists in treatment of ALD.

Srebp-1c knockout, intra-gastric infusion of high fat diet + 18 g/kg/day of alcohol increased to 29 g/kg/day for a total of 4 weeks(71)	Sterol response binding proteins (SREBP) are normally induced in the liver by alcohol. They have an essential role in hepatic triglyceride and cholesterol synthesis.	ALT rise up to 118 in WT mice and 80 in *Srebp-1c*$^{-/-}$ mice with a steatosis score of 3.2 in WT and 0.9 in knockout mice.	Shows that hepatic triglyceride accumulation is dependent on *Srebp-1c*.
Stat3 knockout, ad libitum LdC + 5% alcohol for 10/7 followed by a gavage of 5 g/kg of alcohol(18)	Involved in the activation of IL-22- a cytokine involved in controlling bacterial infection, homeostasis and tissue repair.	Produces significantly higher ALT (300 U/L), AST (450 U/L) + triglycerides (50 mg/g), with microsteatosis.	Shows the hepatoprotective role of IL-22 is dependent on *Stat3*.
TNFR1 knockout, intra-gastric infusion of high fat diet + 18 g/kg/day of alcohol increased to 29 g/kg/day for a total of 4 weeks(95)	Tumour Necrosis Factor α(TNFα) is released by Kuppfer cells primed by gut endotoxins and plays a major role in early alcoholic liver injury- It's effect is stopped if its receptor (TNFR1) is knocked out.	Knock-out mice have smaller increases in ALT (45 vs 115 U/L), liver triglycerides (0.27 vs 0.34 mg/mg), inflammatory foci and apoptotic cells than WT mice.	ALD has multiple complex pathways, TNFα has a modest contribution to the liver injury seen.

Table 2. Summary of current transgenic models of alcohol induced liver injury. Abbreviations: ADH- Anti diuretic hormone, ALT- Alanine transaminase, AST- Aspartate transaminase, CHOP- C/EBP-homologous protein of 29 kDa, EtOH- Ethanol, Stat3- signal transducer and activator of transcription 3.

* Ethanol level calculated according to percentage of calories in the liquid diet. Mice given 29% of the daily calories as ethanol.

Phenotype of liver injury	**Blood analyses**	**Histological assessment**	**Flow cytometry**	**PCR**
Steatosis	Serum AST/ALT, Triglycerides, free fatty acids, cholesterol.	H&E staining, Oil Red O staining.	Fatty Acid Synthase.	*Chrebp/ Srebp, TNF-α*.
Acute alcoholic hepatitis	Serum AST/ALT, markers of synthetic function (PT or bilirubin) and TNF, IL-6, IL-10.	CD45, CD68, CD11b, MPO staining.	Identification of inflammatory cells i.e. CD3, CD4, CD8, CD19 & CD45.	*Sod1, Stat3, GRP-78, GRP-94*.
Fibrosis		Van Gieson or Picro sirius red staining.	α-SMA.	*Col1, MMP, TIMP*.

Table 3. Summary of suggested tests according to phenotype of liver damage being established.

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