

Murine Models of Acute Alcoholic Hepatitis and Their Relevance to Human Disease

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1 Title Page:

2

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

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10

11 Running head: Murine models of alcoholic liver injury (39 characters)

12

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14

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16

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20

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24

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51 **Abstract**

52

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

59

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

69

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

74

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

75 **Introduction**

76

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

92

93 Whilst there are limitations of using animal models to investigate alcoholic liver injury,
94 this approach does provide research opportunities not found in *in vitro* or clinical
95 studies. Animal models allow control over multiple pathogenetic factors such as the
96 environment, contribution of specific pathways and the amount of alcohol consumed,
97 which are difficult to replicate in human studies. Mice that are transgenic for key
98 inflammatory and metabolic disease modifying genes are widely available, and
99 confer the ability to assess the impact of regulatory processes on the induction of
100 alcoholic liver injury(7). While transgenic rats are available their use has been
101 restricted by a limited knowledge of their reproductive system and more difficult *in*
102 *vitro* embryo manipulation which is needed to develop transgenic breeds. Therefore,

103 in this review we will critically appraise the published models of acute murine alcohol-
104 induced liver injury, paying particular attention to the parameters used to define the
105 extent of liver damage, in order to highlight advantages of those models with the
106 greatest promise for new treatment options.
107

108 **Phenotype of disease**

109

110 Alcohol induces a broad spectrum of liver injury in patients ranging from steatosis, to
111 more florid inflammation and hepatocyte necrosis, and finally to fibrosis and the
112 development of hepatocellular carcinoma. The particular phenotype induced is
113 determined in part by the quantity and duration of alcohol exposure as well as patient
114 specific factors. A variety of models have been used by researchers to model this
115 spectrum(6), with each utilising a different method of alcohol administration to
116 produce a desired pattern of liver injury. In general however, whilst many of the
117 available murine models reproduce some of the early stages of liver injury, the
118 development of fibrosis and cirrhosis is harder to replicate and commonly requires an
119 injury additional to alcohol exposure. Thus, whilst steatosis has been achieved by *ad*
120 *libitum* feeding for between approximately one week to several months(7, 8), most
121 models require a second insult alongside an extended course of alcohol
122 administration in order to induce fibrosis such as either concomitant genetic
123 manipulation(9) or the addition of a second chemical insult such as carbon
124 tetrachloride (CCl₄)(10).

125 **Use of Wild Type mice to model alcohol induced liver damage**

126

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

142

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

152

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

168

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

180 around dead and fat-loaded hepatocytes. This provides a better representation of an
181 acute alcoholic hepatitis injury (Figure 1).

182

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

191

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

207 metabolic cages, single mouse housing, specialist infusion equipment and surgery to
208 be performed by the researcher.

209

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

220 **Models of alcohol induced liver damage using genetically modified mice**

221

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

230

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

248 time points after exposure to the high concentration of alcohol making the model
249 difficult to use for both logistic and ethical reasons.

250

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

263

264 Nishiyama *et al.* also investigated fat deposition(29). They used a hepatocyte specific
265 *HIF-1a* null mouse to show that HIF-1 (Hypoxia inducible factor-1) has a protective
266 role that reduces accumulation of lipids in the liver after ingestion of an alcohol/Lieber
267 deCarli liquid diet. They were also able to show that HIF-1 α suppresses Srebp-1c
268 activity and that is at least part of the reason that when HIF-1 α is removed, steatosis
269 increases. However, there are conflicting reports regarding the role of hypoxia
270 inducible factors. Nath *et al.* also used a *HIF-1a* null mouse and found a reduced
271 injury in this knockout mouse(30) while Ni *et al.* achieved similar results using a *HIF-*
272 *1b* null mouse(31). The reasons for these contrasting results are not clear, although
273 different housing conditions or development of sub-strains within the knockout
274 populations have been suggested(32).

275

276 It is interesting to note that HIF have been implicated in the tissue repair response
277 within the liver. They may be involved in regulating the angiogenic effect of hepatic
278 macrophages that induce liver sinusoidal endothelial cell proliferation and
279 migration(33). This appears to be a key step in liver repair after an acute injury.
280 Macrophages are likely to be key to fully understanding the process of tissue repair in
281 the liver. It has been shown that initially pro-inflammatory (Ly6C^{hi}) macrophages can
282 switch to a Ly6C^{low} phenotype important in tissue repair(34) after phagocytosis of
283 apoptotic hepatocytes. Further characterization of the mechanisms driving tissue
284 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.

285
286 Other pathways that have been targeted in the attempt to augment hepatic injury
287 following alcohol exposure include Ppara. Ppara is a nuclear hormone receptor and
288 transcription factor that regulates hepatic inflammation and lipid metabolism. The role
289 of this receptor is to stimulate fatty acid catabolism under fasting conditions and so
290 the authors of this study(8) anticipated that free fatty acid production associated with
291 alcohol consumption would normally activate Ppara. The *Ppara* knockout mouse was
292 given *ad libitum* Lieber deCarli liquid diet with 4% ethanol for up to six months
293 resulting in the development of both an inflammatory cell infiltrate and fibrotic
294 changes that were not seen in alcohol fed WT mice. This was confirmed by both
295 Picrosirius red and alpha smooth muscle actin staining, and demonstration of
296 induction of genes involved in fibrosis including *Thbs1*, *Col1a1* and *Col1a2*. *Ppara*
297 transgenic mice with additional genetic alterations provide further options to
298 investigate liver injury. The Glutathione S-transferase A4-4/ Peroxisome proliferator
299 activated receptor- α (*Gsta4-4/Ppara*) mouse has been described recently(35). *Gsta4-*
300 *4* is an enzyme that protects against natural and environmental toxicants through
301 glutathione conjugation which protects against harmful aldehydes, including 4-
302 Hydroxynonenal (4-Hne). Ronis *et al.* have used this double knockout in an *ad libitum*
303 Lieber deCarli/5% EtOH model to show the central role lipid peroxidation plays in

304 mediating progression of alcohol-induced necro-inflammatory liver injury, stellate cell
305 activation, matrix remodeling and fibrosis(35).

306

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

316

317 **Fibrosis**

318

319 The generation of alcohol induced fibrosis in mouse models is more challenging than
320 steatosis and inflammation and often requires a second injurious element in addition
321 to alcohol ingestion. Bataller and Gao have published a comprehensive review on
322 liver fibrosis in alcoholic liver disease and should be read for further information(38).

323 There are a variety of non-alcohol models that are utilized to induce liver fibrosis, with
324 one of the most commonly used being carbon tetrachloride(CCl₄). This involves
325 repeated intraperitoneal injections of CCl₄ over a period of weeks, although there are
326 no studies directly comparing the liver fibrosis induced by CCl₄ or alcohol. The Nagy
327 research group were able to induce liver fibrosis by administering CCl₄ and moderate
328 alcohol intake at a level not usually producing a significant liver injury. This proves
329 the additive effect of the two agents through common pathways(10). Roychowdury *et*
330 *al.* compared a high ethanol feeding regime against a moderate ethanol regime with
331 the addition of CCl₄(39). They demonstrated that steatosis, inflammation and

332 apoptosis were more prevalent in the alcohol only group as compared to the group
333 that also received CCl₄, which had more prominent fibrosis.

334

335 Chiang *et al.* exposed mice to 2% alcohol *ad libitum* for either 2 days, 2 weeks or 5
336 weeks alongside administration of CCl₄, which resulted in characteristic hepatic
337 extracellular matrix deposition and a change in sinusoidal architecture(10).

338 Genetically modified mice deficient in the HFe iron transporter, which causes
339 accumulation of hepatic iron, develop a marked steatohepatitis and fibrosis upon
340 administration of a high fat diet with ethanol(9). Versions of this dietary protocol have
341 also been used by other groups combined with other genetic backgrounds. For
342 example, Li *et al.* treated *Ppara* knockout mice with a 4% ethanol/Lieber deCarli
343 diet(8), and after 4-6 months reported fibrosis with a small amount of collagen
344 deposition in peri-venular and peri-cellular regions. Importantly, in common with other
345 models, a major drawback of this study was the length of time required for fibrosis to
346 develop, as well as the relatively modest amount of fibrosis seen. Notably, other
347 groups have demonstrated that similar or longer regimens are not able to induce
348 significant fibrosis in WT mice, necessitating further study of specific transgenic
349 animals and alternate models of alcohol delivery(8, 40).

350

351 **Mouse variables that affect experimental endpoints**

352

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

370

371 Gender is also an important factor in development of alcohol induced liver injury.
372 Female patients are more susceptible to developing more advanced alcoholic liver
373 damage both after acute and chronic administration(45), and similarly female mice
374 develop more florid liver injury than males after exposure to ethanol(46). There are
375 several different theories pertaining to this gender difference including different
376 alcohol elimination rates, different alcohol pharmacokinetics and different oestrogen
377 levels. Frezza *et al.* were the first to show that in humans, females have decreased
378 levels of gastric ADH which lessens the 'first pass effect' on alcohol and increases

379 the bioavailability of ingested alcohol when compared to males(47). Female mice
380 develop less liver fibrosis when exposed to other types of chronic liver damage, such
381 as CCl₄ injury or hepatitis C virus infection, suggesting that oestrogens may have a
382 protective effect in some disease settings(48, 49). Work still needs to be done to
383 ascertain whether this also applies to alcoholic liver injury but it does appear that
384 treatment with oestrogen in females lacking ovaries reduces hepatic steatosis(50).
385 Also, there are significant gender differences in the response to alcohol at a
386 proteomic level. Wang *et al.* found that 78 protein levels were altered by either male
387 or female mice undergoing chronic alcohol feeding and this included several
388 oxidative stress related proteins. This is consistent with studies in rats that have
389 found that oxidative stress is a possible reason for increased liver injury in females
390 after ethanol feeding(51).

391

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

406

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

407 Age is also an important variable when investigating the effects of alcohol ingestion.
408 Vogt *et al.* showed that glutathione levels take longer to recover after administration
409 of alcohol in mice aged 24 months compared to mice at 12 months(55). This would
410 appear to be replicated by other studies(56, 57). Glutathione is involved in the
411 detoxification of alcohol and this result would seem to indicate that older mice are
412 less able to metabolise repeated alcohol doses. Further work is required to establish
413 whether this results in increased toxicity and an increased liver injury. However,
414 Ramirez *et al.* found an increased liver injury in mice over 24 months when compared
415 with younger mice though this may be due to decreased rates of autophagy in the
416 older mice(58). It is not clear whether age reduces a human's ability to metabolise
417 alcohol. Wynne *et al.* showed that age did not diminish the activity of alcohol
418 dehydrogenase in the livers of male or female healthy volunteers(59). However,
419 studies suggest that both age and ethnicity influence the severity of alcoholic liver
420 disease in humans(60), and decline in mitochondrial function combined with
421 accumulated oxidative damage in older individuals may render older livers more
422 susceptible to damage from alcohol(61). Thus age is a variable that should be
423 investigated more fully in the context of alcoholic hepatitis.
424

425 **Comparison of mouse models to human AAH**

426

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

439

440 The level of neutrophil infiltration in the murine liver has been suggested as a
441 measure of how representative a model is of the picture of AAH seen in patients.
442 However, a mouse model that induces a neutrophil infiltration similar to that seen in
443 AAH has been elusive(66). Moreover, greater neutrophil infiltration is associated with
444 better survival in humans (65) and thus may not be a sensible therapeutic target.
445 Two older models that have been used in this context are the 3,5-diethoxycarbonyl-
446 1,4-dihydrocollidine (DDC) or griseofulvin (GF) models. These produce ballooning of
447 hepatocytes and accumulation of Mallory bodies but do not involve the administration
448 of alcohol to the mice.

449

450 Lamle *et al.* were able to induce inflammation within the livers of the *Nrf2*^{-/-} mice that
451 received Lieber deCarli and ethanol diet which was characterised by histological
452 finding of Kupffer cell and neutrophil infiltration of the liver(7). The chronic-binge

453 alcohol feeding method also seems to induce a liver injury that is reasonably similar
454 to human AAH and Bertola *et al.* describe raised serum ALT/AST, TNF and hepatic
455 neutrophil infiltration in this model albeit without describing the other characteristic
456 histological findings such as hepatocyte ballooning found in human AAH(17).

457

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

466

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

475

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

477

478 The literature includes a range of different read-outs and experimental endpoints that
479 are used to quantify the nature and severity of alcohol-induced liver injury. This
480 diversity can be useful for understanding pathogenesis but is challenging when trying

481 to compare the phenotype of liver damage reported across different models.
482 Moreover, there is value in tailoring the read-outs to the focus of a particular study or
483 clinical discipline, whether it is generation of steatosis, inflammation, fibrosis or
484 cancer. Certain analyses are useful in the majority of studies, such as serum ALT
485 levels, whereas other tests will be specific for the question being asked, such as the
486 amount of fibrosis as indicated by alpha-smooth muscle actin. Detail of some of the
487 more common experimental parameters is given below and summarised in Table 3.

488

489 ***Overall assessment of murine behaviour and well-being***

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

502

503 ***Biochemical assessment of liver function***

504 In the setting of severe liver injury, the most robust assessment of a model should
505 include measurement of parameters of liver synthetic function such as prothrombin
506 time, serum bilirubin, glucose and albumin levels. These provide important
507 information on the severity of injury, and can be performed on peripheral blood
508 samples whilst models are ongoing thus allowing for the rigorous assessment of

509 potential new therapies. However, as mice have approximately 50-60 ml/kg of
510 circulating blood (approximately 1.5 ml for a 25 gram mouse) (National centre for the
511 replacement raroair. Mouse : Decision tree for blood sampling. Available from:
512 <http://www.nc3rs.org.uk/mouse-decision-tree-blood-sampling>. Accessed 14/09/15),
513 there are limitations on the number of blood tests that can ethically and
514 physiologically be performed on living animals.

515

516 ***Assessment of liver damage and hepatocyte death***

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

532

533 ***Assessment of liver steatosis***

534 Although H&E staining gives a qualitative indication as to the extent of steatosis,
535 quantitative assessment can be performed using Oil Red O staining of liver sections
536 and digital imaging or morphometric analysis alongside quantification of hepatic

537 triglycerides and lipids. Liver to body weight ratio can also provide an indication of
538 the extent of steatosis although it can be confounded by concomitant liver necrosis.
539 More detailed analysis of steatosis can also include analysis of key molecules in
540 pathways contributing to its development, such as SREBP, which are involved in
541 cholesterol and fatty acid biosynthesis(71).

542

543 ***Assessment of liver inflammation***

544 Immunohistochemical staining of liver provides data on the extent and composition of
545 liver infiltrating inflammatory cells, which can be complemented by flow cytometric
546 analysis of resident immune cells from liver cell digests. For example neutrophil
547 infiltration in models of alcoholic hepatitis has been assessed using both
548 immunochemical staining(72) and cytometric detection of Ly6G positive cells in liver
549 digests(73). Cell digest analysis can provide detailed quantitative information on the
550 composition of the liver infiltrate as well as determination of the activation status of
551 any infiltrating cells. This can also be supplemented with analysis of cytokines, such
552 as tumour necrosis factor (TNF), IL-6 and IL-10, from serum and liver tissue at
553 message and protein level to provide useful information on the level of inflammation
554 and the impact of any therapeutic intervention(74). For example, in humans, IL-6, IL-
555 8, TNF and MCP-1 have all been implicated in neutrophil infiltration in patients with
556 alcoholic hepatitis(75) (76), whilst in mice IL-4 appears to promote neutrophil survival
557 and hepatitis(77).

558

559 ***Assessment of liver fibrosis***

560 Standardised assessment of liver fibrosis should include morphometric analysis of
561 fibrotic areas by picosirius red (PSR) or Van Gieson staining, qPCR for *Col1*
562 transcripts and biochemical assays of fibrosis such as hepatic hydroxyproline
563 quantification(78). Useful additional insights can be gained by studying staining for
564 activated hepatic stellate cells using alpha-smooth muscle actin (α -SMA) and

565 transcription levels of matrix metallo-proteinases (MMP) and their tissue inhibitors
566 (TIMP).

567

568 ***Additional mechanistic studies***

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

580

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

590 **Conclusion**

591

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

605

606 **Tables**

607

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.

<p>Intra-gastric infusion (21), (71), (94), (95), (96), (97)</p>	<p>Histological evidence of severe steatosis, inflammation, necrosis and hepatic stellate activation.</p>	<p>ALT up to 450 U/L.</p>	<p>Specialist surgical skill needed, extensive amount of specialist equipment and intensive monitoring needed.</p>
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608

609

610 Table 1- Established routes for administration of alcohol to mice

611

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

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.
<i>CHOP</i> 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)	<i>CHOP</i> 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 <i>CHOP</i> ^{-/-} mice) and ALT (112 U/L). <i>CHOP</i> ^{-/-} mice had no apoptosis.	As a response to ER stress, <i>CHOP</i> upregulates and is involved in causing apoptosis.
<i>Cyp2e1</i> knockout, intra gastric infusion of high fat diet + 14 g/kg/day increased to 28g/kg/day of alcohol for a	<i>Cyp2e1</i> (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)			
<i>Gsta4-4/Ppara</i> 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.
<i>Hfe</i> 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.
<i>Hif-1a</i> 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.	<i>HIF-1a</i> 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.
<i>Lipin-1</i> knockout, <i>ad libitum</i> 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 <i>Lipin-1</i> knockout mice after 4 weeks of feeding.	Suggests a role for treatments to enhance lipin-1 as a treatment for ALD.
<i>Nrf2</i> knockout, <i>ad libitum</i> 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.
<i>Ppara</i> knockout, gavage of 0.4ml/10g 52% erguotou wine for 4/52(8)	<i>Ppara</i> 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 <i>Ppara</i> agonists in treatment of ALD.

<p><i>Srebp-1c</i> 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)</p>	<p>Sterol response binding proteins (SREBP) are normally induced in the liver by alcohol. They have an essential role in hepatic triglyceride and cholesterol synthesis.</p>	<p>ALT rise up to 118 in WT mice and 80 in <i>Srebp-1c</i>^{-/-} mice with a steatosis score of 3.2 in WT and 0.9 in knockout mice.</p>	<p>Shows that hepatic triglyceride accumulation is dependent on <i>Srebp-1c</i>.</p>
<p><i>Stat3</i> knockout, ad libitum LdC + 5% alcohol for 10/7 followed by a gavage of 5 g/kg of alcohol(18)</p>	<p>Involved in the activation of IL-22- a cytokine involved in controlling bacterial infection, homeostasis and tissue repair.</p>	<p>Produces significantly higher ALT (300 U/L), AST (450 U/L) + triglycerides (50 mg/g), with microsteatosis.</p>	<p>Shows the hepatoprotective role of IL-22 is dependent on <i>Stat3</i>.</p>
<p><i>TNFR1</i> 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)</p>	<p>Tumour Necrosis Factor α(TNFα) is released by Kupffer 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.</p>	<p>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.</p>	<p>ALD has multiple complex pathways, TNFα has a modest contribution to the liver injury seen.</p>

612
613 Table 2. Summary of current transgenic models of alcohol induced liver injury. Abbreviations: ADH- Anti diuretic hormone, ALT- Alanine
614 transaminase, AST- Aspartate transaminase, CHOP- C/EBP-homologous protein of 29 kDa, EtOH- Ethanol, Stat3- signal transducer and
615 activator of transcription 3.
616 * 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.	<i>Chrebp/ Srebp, TNF-α.</i>
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.	<i>Sod1, Stat3, GRP-78, GRP-94.</i>
Fibrosis		Van Gieson or Picro sirius red staining.	α -SMA.	<i>Col1, MMP, TIMP.</i>

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

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