

EASL position paper on preclinical models of steatotic liver disease

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Summary

Steatotic liver disease (SLD) comprises a heterogeneous group of liver diseases defined by pathological hepatic lipid accumulation with varying degrees of steatohepatitis and progressive fibrosis, which may culminate in cirrhosis and/or hepatocellular carcinoma. SLD encompasses metabolic dysfunction-associated steatotic liver disease (MASLD), formerly called non-alcoholic fatty liver disease (NAFLD); MetALD, describing patients with MASLD consuming moderately high amounts of alcohol; and alcohol-associated/related liver disease (ALD). In addition, SLD encompasses less common aetiologies, including drug-induced and monogenic causes, as well as cryptogenic disease, which lacks metabolic risk factors or an identifiable cause. As the leading cause of chronic liver disease worldwide, MASLD, including metabolic dysfunction-associated steatohepatitis (MASH), is a complex multisystem disorder associated with extrahepatic organ dysfunction. Consequently, MASLD has become a major focus of multidisciplinary research, driving innovation across hepatology, immunology, cardiometabolism, addiction medicine, nutrition, prevention, and beyond. Over the past two decades, a broad array of *in vivo*, *in vitro*, and *ex vivo* experimental models have been developed to recapitulate diverse aspects of SLD pathophysiology, genetics, inflammation, treatment response, and multi-organ crosstalk, substantially advancing mechanistic understanding and therapeutic development. However, the rapid expansion and heterogeneity of available models have also highlighted the need for improved categorisation, standardisation, and harmonisation in line with evolving disease definitions and clinical concepts. In this EASL position paper, we critically review and classify currently available experimental SLD models and propose key criteria required for their appropriate use across distinct SLD subtypes. These criteria encompass systemic and hepatic metabolism, cardiometabolic comorbidities, histopathology, immunopathology, and molecular features relevant to disease stage and aetiology. This position paper aims to guide informed model selection, promote consistent nomenclature, and enhance rigour and translational relevance in preclinical and experimental SLD research.

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Introduction

Clinical setting – a perspective on SLD

Steatotic liver disease (SLD) embodies a broad spectrum of disorders characterised by hepatic steatosis, detected by imaging or biopsy, and includes metabolic dysfunction-associated steatotic liver disease (MASLD), alcohol-associated/related liver disease (ALD), and metabolic and alcohol-associated/related liver disease (MetALD).¹ This revised nomenclature, following a multi-stakeholder Delphi process, has been endorsed by major international societies including the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases, as well as various patient advocacy groups. The shift from the term non-alcoholic fatty liver disease (NAFLD) to

MASLD, and the introduction of the new MetALD disease category, better reflects the underlying metabolic drivers of the disease while also avoiding potentially stigmatising language.¹

MASLD, as defined by the international consensus,¹ requires hepatic steatosis in the presence of at least one cardiometabolic risk factor, including obesity/overweight, impaired glucose regulation, hypertension, or dyslipidaemia with low alcohol consumption. This reconceptualisation better frames MASLD, including metabolic dysfunction-associated steatohepatitis (MASH), as the hepatic manifestation of metabolic syndrome, a multi-organ/systemic disorder with profound implications for pathogenesis and therapeutic response.^{2,3} Importantly, the updated nomenclature reframes and raises questions about the validity of widely used pre-clinical models, many of which were established under the

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previously defined “NAFLD” paradigm and may not fully align with current clinical definitions.⁴

ALD, in parallel, remains a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma (HCC) worldwide.^{5,6} The introduction of MetALD highlights the biological and clinical overlap between metabolic dysfunction and alcohol exposure, emphasising the need to consider both drivers in disease classification and modelling.¹ While the original SLD consensus paper defined MetALD as a steatotic liver disease with at least one MASLD criterion and high alcohol consumption,^{1,6} recent expert opinions argue for at least two MASLD risk factors in the diagnosis of MetALD.^{7,8}

Disease progression across MASLD, MetALD and ALD is highly variable and influenced by numerous modifiers, including age (adult vs. paediatric), sex, comorbidities, gut microbiota, genetic polymorphisms (e.g. *PNPLA3*, *TM6SF2*), and lifestyle factors such as diet, physical activity, smoking, alcohol consumption, and medications.^{3,5,9,10} These variables highlight the heterogeneity of SLD and the need for appropriate preclinical models that capture both the metabolic and environmental determinants of disease.

SLD is a spectrum of diseases that are highly heterogeneous

Across the SLD spectrum (MASLD, MetALD, and ALD), disease progression is characterised by hepatic steatosis, inflammation, hepatocyte injury and fibrogenesis, though the initiating drivers differ. In ALD, ethanol metabolism promotes hepatocellular injury through acetaldehyde toxicity, mitochondrial dysfunction, and perturbations in adipose tissue, gut-liver, and gut-brain axes. In MASLD, insulin resistance, adipose tissue dysfunction, and dyslipidaemia dominate, whereas MetALD reflects the additive or synergistic impact of moderate alcohol exposure on a background of metabolic stress.^{6,9}

MASLD has now emerged as the most prevalent chronic liver disease worldwide, affecting approximately 38% of the global adult population.¹⁰ MASLD is strongly associated with obesity, type 2 diabetes (T2D) and other features of metabolic syndrome. It encompasses a spectrum of diseases ranging from simple steatosis (MASL) to steatohepatitis (MASH) and can present with or without fibrosis.¹ MASH can progress to cirrhosis and HCC, which is among the leading causes of cancer and cancer-related mortality.⁹ Notably, degree of fibrosis remains the primary determinant of adverse liver-related outcomes in MASH, which includes cirrhosis with portal hypertension, hepatic decompensation and carcinogenesis.^{11,12} Nevertheless, most patients with MASLD succumb to extrahepatic complications, including cardiovascular disease (CVD) and chronic kidney disease (CKD), underscoring its systemic nature.^{2,3}

The natural history of MASLD and MetALD reflects a dynamic interplay of metabolic stress, lipotoxicity, inflammation, and regeneration. Patients may transition from early MASL or MetALD to advanced MASH/MetALD or fibrotic stages over years, with comorbidities and duration of exposure strongly influencing disease progression. Importantly, disease trajectories in MASLD and MetALD are not unidirectional. Spontaneous improvement or regression of steatohepatitis and fibrosis has been consistently reported in placebo arms of

clinical trials,^{13,14} highlighting the inherent plasticity of metabolic liver disease and the influence of lifestyle and metabolic factors. Hepatic steatosis is the most rapidly reversible component, whereas inflammation and hepatocellular injury resolve more gradually. Fibrosis is the most persistent feature, requiring prolonged intervention and often showing only partial regression. In addition, distinct molecular and phenotypic subsets of MASLD are increasingly recognised. Recent work has categorised patients into liver-predominant and cardiometabolic clusters,^{15,16} with further subclassification into molecular subtypes based on transcriptomic and metabolic profiles.¹⁷ These insights emphasise that MASLD is not a single entity but rather a heterogeneous disease with diverse metabolic, immunologic, and pathologic pathways.

ALD similarly displays marked heterogeneity, with differences in susceptibility, progression, and outcomes shaped by sex, genetic polymorphisms, drinking patterns (e.g. binge drinking compared to chronic exposure), and the presence of metabolic risk factors.^{5,6} Together, these variables determine whether patients progress from steatosis to alcohol-associated/related steatohepatitis, fibrosis, or cirrhosis.

The objective of this position paper

In the past 20 years, a wealth of *in vivo*, *in vitro* and *ex vivo* models have been successfully established that aimed to mimic all aspects of SLD from molecular and cellular pathology to genetics, treatment response and multi-organ cross-talk.^{18–20} This has greatly improved understanding of the pathogenesis of SLD and has helped stratify patients, tailor novel therapies, and predict disease outcomes. However, it has also become clear that improving the understanding and modelling of SLD requires better categorisation, standardisation, and harmonisation of the vast array of *in vivo*, *in vitro*, and *ex vivo* models to ensure they align with current criteria and knowledge of SLD. Thus, in this EASL position paper, the authors have attempted to categorise, standardise and harmonise the most appropriate *in vivo*, *in vitro* and *ex vivo* SLD models that are currently available.

To help researchers both within and beyond the SLD field select the most suitable models to investigate specific SLD subtypes, we sought to define the key features that the most appropriate SLD models should embody. These included aspects of systemic and hepatic metabolism, links to cardiometabolic disease and other organ disorders, immunology, and molecular and tissue pathology, with the aim of integrating general requirements that a particular model needs to fulfil in order to be correctly recognised as an appropriate model for a specific SLD type. This EASL position paper does not seek to prescribe the use of specific models for distinct SLD types; rather, it aims to guide researchers toward the models that, according to the current literature, are best suited to the specific aetiology of interest. These recommendations harmonise nomenclature to benefit basic research and ultimately facilitate efficient translation of findings to clinical practice. Moreover, this EASL position paper is intended to motivate researchers to develop new and optimise existing models so they more accurately recapitulate the various types of SLD.

A new therapeutic landscape in MASLD

With expanding insight into the molecular underpinnings of SLD, the therapeutic landscape for MASH is shifting from exclusively lifestyle-based interventions toward pharmacological therapies.^{21,22} Nevertheless, clinical practice guidelines from EASL, European Association for the Study of Diabetes (EASD), and European Association for the Study of Obesity (EASO) still underscore that weight loss, via caloric restriction, dietary optimisation, and regular exercise, remains the indispensable first pillar of therapy.²³ In severe obesity, bariatric surgery delivers profound and durable histological improvements in steatohepatitis,²⁴ underscoring the potent impact of substantial weight reduction. Equally critical is rigorous management of cardiometabolic comorbidities, ideally benefiting both cardiovascular and hepatic outcomes.²³

As these interventions primarily target extrahepatic drivers of MASLD, such as nutrition, adiposity, and the gut-liver axis, not all cell-based or rodent models are suited to reflect the potency of these interventions for liver and cardiometabolic health. Among pharmacological approaches, glucagon-like peptide-1 receptor agonists (GLP-1RAs) such as semaglutide, already mainstays in diabetes and obesity management, induce substantial weight loss and histological improvement in non-cirrhotic MASH.²⁵ Semaglutide demonstrated a significant benefit on steatohepatitis resolution and fibrosis improvement (placebo-adjusted histological response rate of 29% and 14%, respectively) after 72 weeks of therapy in a phase III randomised controlled trial (RCT) in MASH, and was recently granted accelerated approval by the FDA.²⁶ Dual agonists (GLP-1/GIP, e.g. tirzepatide,²⁷ or GLP-1/glucagon, e.g. survodutide²⁸) and triple agonists (GLP-1/GIP/glucagon, e.g. retatrutide²⁹) are entering phase III RCTs, with early data suggesting enhanced weight loss and metabolic control, albeit limited by poor gastrointestinal tolerability. In addition, a recent meta-analysis reported that weight regain following cessation of weight management drugs was significantly higher than in patients who lost weight through lifestyle interventions.³⁰ Similarly, sodium-glucose transport protein 2 (SGLT2) inhibitors, which are approved for T2D, heart failure and kidney disease, may offer benefits for MASH.³¹

With a liver-directed mode of action, resmetirom became the first therapy granted accelerated approval for MASH. By selectively activating thyroid hormone receptor (THR)- β in the liver, resmetirom enhances hepatic lipid clearance and glucose metabolism. In the phase III RCT, non-cirrhotic patients with stage ≥ 2 fibrosis experienced significant reductions in steatohepatitis activity histology scores and improvement in fibrosis after 52 weeks, without the off-target cardiac effects associated with THR α activation.³² Gastrointestinal complaints (diarrhoea, nausea) were the most common adverse events, and early signals of cardiovascular benefit such as lower low-density lipoprotein (LDL) and non-high-density lipoprotein (HDL) cholesterol further reinforce its appeal.³² However, the placebo-adjusted response rate for resmetirom reached only about 10–20% for histological endpoints, highlighting the remaining unmet need and fuelling interest in tailored combinations.

Direct reprogramming of hepatic energy balance via fibroblast growth factor 21 (FGF21) analogues is another emerging strategy for MASH.³³ Long-acting FGF21 analogues such as

efruxifermin, pegozafermin or efimosfermin enable weekly or monthly dosing, with efruxifermin demonstrating fibrosis regression even in a population with MASH-related cirrhosis in a phase II trial over 96 weeks.³⁴ These agents are now being tested in phase III RCTs for both their efficacy and long-term safety.

Additional metabolic targets include peroxisome proliferator-activated receptors (PPARs), given their central roles in fatty acid oxidation, lipid storage, and insulin sensitivity.³⁵ While lanifibranor, an $\alpha/\beta/\gamma$ PPAR agonist, demonstrated encouraging phase II results, improving steatohepatitis, glucose metabolism, and fibrosis, single or dual PPAR agonists (e.g. elafibranor) have not met primary endpoints in pivotal trials.³⁶ Farnesoid X receptor (FXR) agonists likewise showed promise by modulating bile acid signalling, but the clinical development of obeticholic acid was curtailed because it did not meet the primary endpoint of fibrosis improvement of ≥ 1 stage without worsening of steatohepatitis in the phase III RCT.^{37,38}

Targeting hepatic *de novo* lipogenesis (DNL) with inhibitors of acetyl-CoA carboxylase, fatty acid synthase, and diacylglycerol-O-acyltransferase 2 yielded proof-of-concept reductions in hepatic triglyceride content, but safety concerns (hypertriglyceridaemia, platelet decline) and modest antifibrotic effects have slowed progress.³⁹ Similarly, anti-inflammatory and/or anti-fibrotic strategies including CCR2/CCR5 antagonists or inhibiting inflammasome activation did not meet the primary endpoints of fibrosis improvement of ≥ 1 stage without worsening of steatohepatitis in the AURORA phase III RCT.^{40–42} Emerging cell-based therapies ranging from restorative macrophage infusions to CAR T cells targeting senescence markers or fibrogenic integrins are under early investigation but face hurdles of cell manufacturing complexity and off-target risks.^{43,44} Moreover, hepatic stellate cells, a key player in fibrosis, have proven elusive pharmacologic targets; while integrin inhibitors like PLN-74809 aim to prevent local TGF- β activation, robust human data are still pending.⁴⁵

It is increasingly clear that targeting a single pathway will not suffice to resolve MASH and improve fibrosis. Instead, modular regimens melding lifestyle interventions with agents that correct lipid dysregulation, reduce inflammation, and reverse fibrotic scarring are poised to define the next era of care. In addition, therapeutic efficacy will likely depend on appropriate matching of interventions to disease subtypes and underlying drivers. For instance, allele-specific approaches, such as *PNPLA3*-targeting antisense oligonucleotides, are expected to be most effective in individuals homozygous for the I148M variant.⁴⁶ This highlights the need for stratified and precision-based treatment strategies, as additional subtype-therapy relationships continue to emerge. In addition, cell-based and animal models will remain critical to identify and validate new drug targets.

With the emergence of effective interventions from clinical trials, current model systems should consider including positive control interventions if the model is technically capable of responding to these clinically validated treatments. Additionally, such models should ideally account for the heterogeneity of MASLD, as well as the impact of cardiometabolic comorbidities and environmental risk factors on disease progression, to enable meaningful translation.

Recommendations

- Based on the available positive data from phase IIb and III clinical trials in humans (primarily in MASH or MASH-fibrosis), experimental cell-based or rodent models of SLD evaluating novel therapeutic strategies should include a pharmacological positive control (e.g. resmetirom or semaglutide) whenever feasible. Controls should be selected to reflect dynamic changes in the key endpoint(s) studied in the model, e.g. obesity, metabolic syndrome, steatosis, metabolic injury, intrahepatic and systemic metabolite alterations, inflammation, hepatocyte injury and/or fibrosis.
- Mechanistic studies elucidating disease mechanisms or signalling pathways should prioritise robust functional validation of target or pathway engagement, supported by appropriate baseline or vehicle controls.

Alignment and standardisation of preclinical SLD models

The now outdated belief that pathobiology and pathology are the same across SLD of different aetiology has led to the development and wide use of “phenocopy” preclinical animal models of liver disease with little consideration of mechanism.^{18–20,47} Dozens of models have been characterised, using various genetic, dietary, and chemical components (alone or combined) to induce widely varying degrees of hepatic steatosis, inflammation, hepatocyte injury and fibrosis. However, the lack of clear guidelines, methodological standardisation, and alignment with clinical features of the human disease has undoubtedly skewed our mechanistic understanding of SLDs and slowed therapeutic development. It has also likely contributed to the lack of translatability, resulting in a steep decline in the use of preclinical models for disease-relevant research. There is a growing misconception that animal models, particularly rodents, are a poor model of human metabolic disease, based on commonly propagated assumptions such as “mice do not develop MASH”, “female mice do not develop MASLD”, or “the histopathology of rodent MASLD is not the same as that of humans”. While no single animal model is likely to fully recapitulate MASLD diversity, specific models can closely mirror distinct disease subtypes at the metabolic, histopathological and transcriptomic levels.^{48,49} Notably, a thorough histopathological comparison between humans and mice is presented later in this paper, underscoring the importance of carefully aligning the research question with the most appropriate model. In addition, preclinical models of SLD should be aligned with current clinical guidelines.

Given the diverse causes and divergent developmental paths of these diseases, accurate modelling will help to develop targeted and effective therapies for each SLD type. Better alignment with clinical definitions will also allow researchers to not only understand the causes of each SLD and identify treatments, but to predict long-term health risks of each disease and uncover how they are linked to other disorders. This is of utmost importance, as MASLD is a chronic disease, causing liver damage but also significantly increasing the risk of T2D and death from CVD.⁵⁰

Consensus statement

Alignment and standardisation of preclinical SLD models will:

- Increase trust in preclinical animal modelling.
- Increase chances of reproducible discovery.
- Provide a higher chance for translation to the clinic.
- Be relevant to the most prevalent form of the disease.

Mouse models of SLD – experimental factors influencing disease pathogenesis

This section aims to cover some of the most important experimental factors influencing SLD pathogenesis from the microbiome to diet, housing conditions and confounders.

Microbiome – human vs. mouse comparability

The gastrointestinal tract of mice is significantly different from that of humans in anatomy, genetics, and microbiome composition. In particular, very few gut microbial genes are shared between humans and mice (about 4%),^{51,52} thus it is inappropriate to directly compare gut microbiome findings between these two species. With this caveat, reduction in some gut microbes, such as *Akkermansia muciniphila*, is observed in alcohol-related liver disease and MASH in both humans and mouse models.^{53,54}

Human gnotobiotic mouse models (transplantation of human stools into microbiota-depleted germ-free mice) have been widely used, as they allow for studies of causality with high-fidelity replicates of the human gut microbiome.⁵⁵ However, these humanised models do not necessarily recapitulate the actual features in humans, as the complex crosstalk between host and gut microbes is host specific. For example, mice transplanted with a human microbiome exhibit compromised immune maturation and expansion compared to mice transplanted with a mouse microbiome.⁵¹ Moreover, the marked difference in diets and environmental exposures between humans and mice, which are the main contributors to microbiome variation,⁵⁶ can further reduce their comparability. Thus, a humanised diet will also be required to facilitate the colonisation of human gut microbiota in human gnotobiotic mouse models. Of note, in contrast to microbiome composition, numerous studies have shown that up to 95% of gut microbial functions may overlap between humans and mice.^{57,58} To this end, different catalogues that functionally map the mouse microbiome have been established, representing robust tools for studying host-specific microbial functions and enabling translation between the mouse and human gut microbiome.^{57,59} In addition, the wildling mouse model, laboratory mice rederived with a natural, pet store-derived microbiota, recapitulates features of human immune-microbiota interactions and could therefore provide a relevant platform to study microbiota-driven immune modulation in the future.⁶⁰ Nevertheless, one caveat is that a wild-derived microbiome was shown to prevent high-fat diet (HFD)-induced weight gain, metabolic syndrome and advanced MASLD; thus, further studies are required to determine how well the wildling model truly captures diet-induced obesity and metabolic syndrome in humans.⁶¹ Collectively, instead of modelling

human microbiome composition and alterations, mouse models can be utilised to validate microbial functions observed in humans, whilst providing mechanistic insights in health and disease.

Heterogeneity of diet and alcohol

Supplementation of a high-caloric diet combining fats and sugars is the most common method to induce spontaneous pathogenesis of MASL and MASH in mice. These diets, including variations of HFDs, Western diets (WDs), and choline-deficient HFDs, contain approximately 40–60% fat. In humans, a diet with total fats exceeding 35% of total daily calories is considered to have high fat content (2015–2020 Dietary Guidelines for Americans). Hence, the dietary fat content given to mice to induce MASLD is comparable to humans. Given the widespread consumption of sugar-rich foods and beverages in human diets, mouse models of MASLD often employ diets high in both fat and sugar, particularly fructose and sucrose.¹⁸ An alternative approach is to combine a WD with toxins (e.g. low-dose weekly carbon tetrachloride [CCl₄])⁶² or transgenic mice (e.g. *Foz/Foz* mice harbouring a mutation in the *Alms1* gene)⁶³ to induce MASLD more rapidly. It is noteworthy that HFD supplementation alone in mice cannot mimic MASL and MASH in normal-weight humans, a phenotype that is more prevalent among South and East Asian populations.⁶⁴ Instead, a diet deficient in choline and restricted in methionine (0.1%) is needed to induce steatohepatitis with advanced fibrosis in lean mice.^{65,66} However, these diets merely recapitulate pathological characteristics of steatohepatitis in humans, as they do not induce systemic insulin resistance and they cause weight loss with reduced triglyceride and cholesterol levels.^{67,68} Compared with dietary manipulations, modelling alcohol consumption in mice is more challenging, as commonly used approaches fail to accurately recapitulate the complexity of human drinking behaviours. Moreover, as the amount of alcohol consumed varies greatly among individuals, current mouse models, such as co-supplementation of a WD with 5–10% alcohol,⁶⁹ focus on disease pathogenesis and mimic chronic exposure rather than recapitulating the actual alcohol consumption of humans.

The need for high cholesterol levels in diet models

C57BL/6 mice are known to absorb dietary cholesterol poorly, so it is necessary to use a high-cholesterol (1–2%) diet to obtain the level of liver cholesterol seen in human MASH livers. Although such dietary concentrations may initially appear supraphysiological, this is required to match the hepatic cholesterol exposure of humans due to inherent differences in intestinal cholesterol absorption between the two species. For instance, the cholesterol component of the commonly used fructose-palmitate-cholesterol (FPC) diet increases liver cholesterol to a level similar to that in human MASH liver.⁷⁰ Liver cholesterol, which is important in human MASH,^{70,71} activates human-relevant MASH pathways in hepatocytes in FPC-fed mice without causing cytotoxicity.⁷⁰ Similarly, a high-fat, high-sucrose, and 0.2% cholesterol (w/w) diet was also shown to effectively induce MASH and MASH-induced HCC by modulating the gut microbiome.⁷² Importantly, the LITMUS (Liver Investigation: Testing Marker Utility in Steatohepatitis) consortium analysed over 40 distinct mouse models and found

that 2% cholesterol is a key disease accelerant required to potentiate liver damage and induce advanced (>F2) fibrosis.⁴⁹

Thermoneutrality

The impact of housing temperature on mouse models is increasingly acknowledged, as standard housing (i.e. around 21°C–22 °C) can induce cold stress in mice.^{73,74} In particular, cold stress stimulates brown adipose tissue-mediated thermogenesis and alters immune responsiveness, thereby blunting MASLD development in mice.^{48,75} As opposed to standard housing, thermoneutral housing has been proposed, showing that MASLD pathogenesis is markedly accelerated in HFD-fed mice housed under thermoneutral conditions at 30 °C.⁴⁸ Of note, these observations appear to be dependent on the duration of high-calorie diet feeding. While increased hepatic steatosis, inflammation, ballooning, and fibrosis were observed in HFD-fed mice housed at 30 °C for 24 weeks,^{48,76} these effects were not significant in mice housed under thermoneutral conditions for 16 weeks.⁷⁷ In general, current evidence suggests that long-term (24 weeks) thermoneutral housing is advantageous in boosting diet-induced MASLD pathogenesis in different mouse models, yet its necessity for disease modelling remains debatable. Housing temperature may also be advantageous when modelling MASLD in female mice, as thermoneutrality appears to reduce the sex differences seen in male vs. female mice.⁴⁸ However, the reproducibility and robustness of this observation across strains is still being established.⁷⁷ Laboratories may also face technical challenges in simultaneously housing mice at different temperatures, and the impact of housing temperature may be negligible if the desired MASLD phenotype is achieved and all mouse groups are maintained under the same conditions.

Confounders

The mouse genetic background is a major determinant of the physiological responses to metabolic challenges, and different mouse strains present with different susceptibility to MASH.⁷⁸ When assessing the development of steatosis across a panel of >100 inbred mouse strains fed a high-fat, high-sucrose diet for 8 weeks, a broad spectrum of hepatic triglyceride content was observed, with more than a 30-fold difference between high and low responders.⁷⁹ Similarly, upon transgene expression of human apolipoprotein E*3-Leiden and cholesteryl ester transfer protein, and feeding with a high-fat and -cholesterol diet, these strains exhibited striking variability in liver injury, with divergent degrees of inflammation and fibrosis, reaching up to a 500-fold difference in fibrotic burden as assessed by Picrosirius Red staining.⁸⁰ Consistent with this variability, comparative analyses across seven inbred strains revealed marked differences in MASH susceptibility, with PWK/PhJ mice uniquely progressing to steatohepatitis with advanced fibrosis and exhibiting a human-like transcriptional signature linked to mitochondrial dysfunction, whereas strains such as CAST/EiJ remained largely resistant.⁷⁸ Likewise, C57BL/6 mice are highly susceptible to diet-induced obesity and hepatic steatosis, whereas BALB/c mice are associated with milder liver disease and a lower propensity to develop obesity.^{81,82}

The selection of appropriate mouse strains based on study requirements is therefore critical. In fact, even the substrain can impact on disease modelling. For instance, C57BL/6J mice, the

most widely used inbred strain of mice, are more glucose intolerant than C57BL/6N mice due to a mutation in the *Nnt* gene encoding a mitochondrial enzyme.^{83,84} In addition, it was reported that a subset (5%-25%) of C57BL/6 mice from different commercial breeders have significantly higher serum bile acids and spontaneously develop cholaemia.⁸⁵ This subset of mice develops exacerbated MASH with advanced fibrosis and accelerated liver cancer upon feeding of a WD for 32 weeks.⁸⁵ While the genetic basis for the existence of this subset remains unclear, future mouse studies may consider measuring serum bile acids prior to the start of experiments and using these values for randomisation to reduce potential variation. If this is not feasible, cholaemic mice should be excluded retrospectively when assessing serum liver damage markers or other parameters at later time points.⁸⁵ In practice, researchers should ensure the use of littermate controls. The use of non-littermate controls (even from the same supplier) is strongly discouraged due to possible strain divergence as well as adaptation to local environments.^{86,87} On this note, controlling for homogeneous litter sizes is also recommended, as it directly influences early-life nutritional status and developmental programming of critical metabolic tissues.

There is wide variability in the literature regarding the age at which a MASLD dietary intervention is initiated, with most models ranging from 4 weeks (weaning) to 8 weeks ("young adulthood"),^{49,88} in order to save time and reduce costs. This may introduce developmental programming effects and not necessarily mirror adult-onset human MASLD. In fact, when challenged with the same MASLD-inducing diet, aged mice appear to develop more severe MASH and fibrosis more rapidly than young mice.^{89,90} At the same time, MASLD is a sexually dimorphic condition, and female C57BL/6 mice are more resistant to diet-induced obesity compared to male mice,^{91,92} possibly due to the protective effects of hormones, but only at reproductive age. Interestingly, the magnitude of protection seems to depend on diet composition.⁹³ Still, the influence of sex as a biological variable is rarely integrated into preclinical MASLD studies.⁹⁴⁻⁹⁶ Hopefully this will soon change, with major research funding schemes (e.g. Horizon Europe) requiring that the gender dimension is integrated into the research context, alongside a more structured approach to addressing sex bias in preclinical research proposals.⁹⁷ Finally, one should also be aware of the impact of circadian rhythm on SLD,⁹⁸ as well as on liver metabolism and size.⁹⁹

Recommendations

The following need to be considered when conducting pre-clinical mouse studies:

- Altogether, the most well characterised and validated models (Tables 1-3) should be used, thereby preventing dilution of evidence across multiple animal systems and improving effective translation to humans.
- When possible, both male and female mice of at least 8-12 weeks of age should be used for modelling human adult MASLD.
- Avoid extrapolating the compositional changes in human gastrointestinal tract to mice but rather validate functional

mechanistic outcomes of specific gut-derived metabolites and bacterial species.

- Mice should be randomised prior to treatment based on body weight and, where applicable, metabolic parameters such as fasted glucose, triglycerides and/or cholesterol, as well as serum markers of hepatic damage, such as alanine aminotransferase (ALT).
- Spontaneous cholaemia in 5-25% of C57BL/6 mice is a confounder in MASLD studies and therefore these mice should be excluded, at least retrospectively.
- The use of littermate controls is strongly encouraged. Using control mice ordered from a supplier, bred in a different colony, or housed separately is strongly discouraged (due to possible strain divergence, cage effects and adaptation to local environments).

Standardisation in reporting

Ensuring rigorous and standardised reporting of preclinical models is critical for reproducibility and improving translation to clinical practice. The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines, originally developed in 2010 and updated in 2020 (ARRIVE 2.0),^{100,101} provide a structured list of the minimum criteria to report in manuscripts documenting animal research. Similar guidance for *in vitro* models is being developed and should be adopted to increase reproducibility. Despite these recommendations, many studies still fail to report critical details related to study design, sample size, experimental animals, and experimental procedures, among others, hampering translational relevance and limiting comparability across studies. Hence, we recommend adherence to the ARRIVE Essential 10 guidelines when reporting SLD animal experiments, including species-appropriate details, housing and husbandry conditions, and low-stress handling practices, all of which can profoundly influence experimental outcomes.

In parallel with standardisation of experimental design and procedures, the use of different animal models to mimic distinct features of human SLD (e.g. metabolic relevance and/or ability to induce MASH-fibrosis;⁴⁹ or cardiometabolic MASLD and liver-specific MASLD¹⁵) warrants the consistent measurement and reporting of a core set of endpoints. Herein, we outline a set of experimental and reporting standards that complement the ARRIVE 2.0 guidelines and should be provided in manuscripts describing preclinical SLD models (Fig. 1). In addition, we provide an overview of the most important metabolic, histopathological and biochemical endpoints that should be provided in an *in vivo* study on SLD. Adopting a standardised reporting framework will allow for objective comparisons across models using precise parameters, reduce discrepancies related to underreported variables, and align preclinical studies with the new SLD nomenclature, which is key for clinical translation. The technologies and endpoints used to characterise mouse disease should evolve in parallel with human studies. The field should strive for "maximal" characterisation, integrating multi-omics, advanced imaging, digital pathology, and metabolic phenotyping while

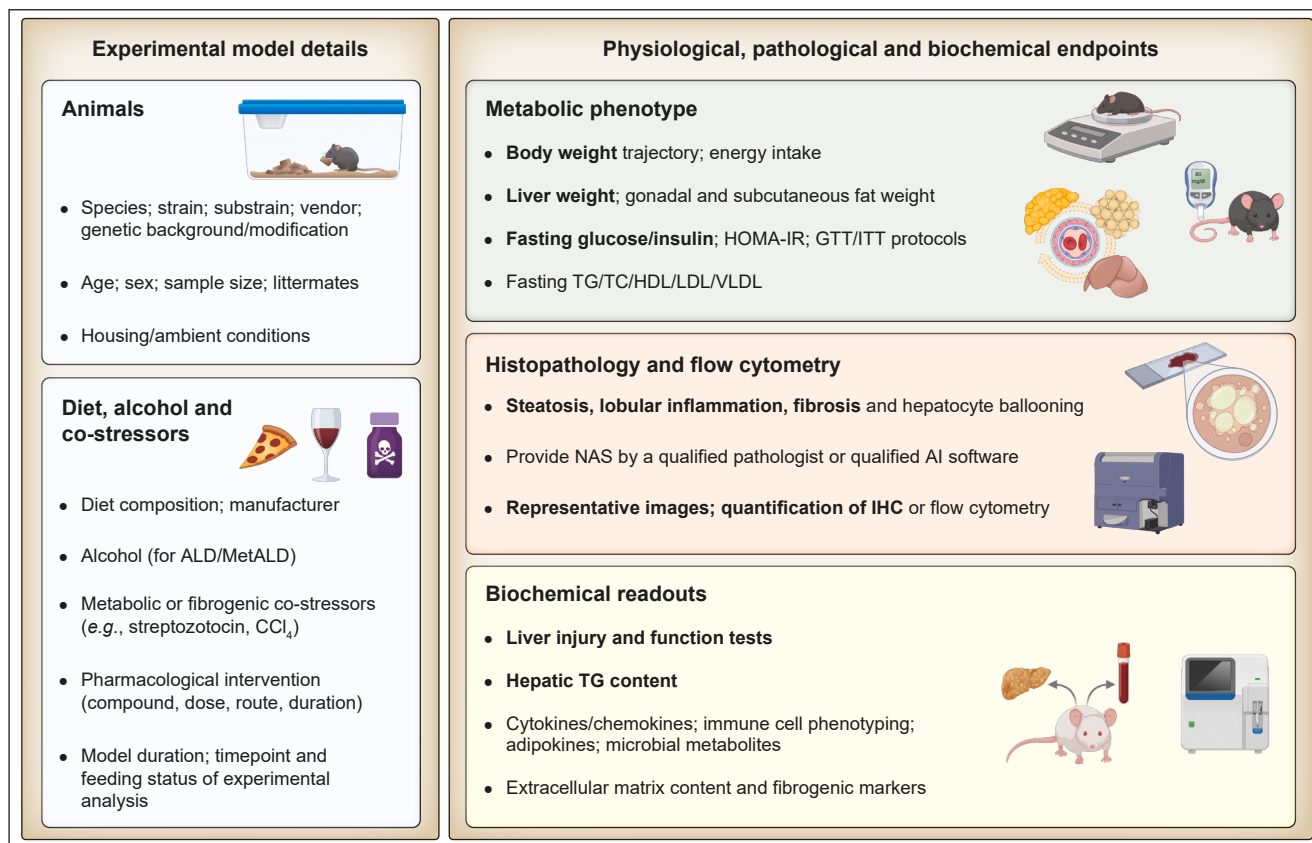


Fig. 1. Criteria for standardised reporting in the evaluation of animal models of SLD. Left Top and Bottom: SLD animal models should provide a sufficient level of detailed information enabling one to reproduce the experiment in a robust and reproducible manner. We recommend adherence to the Essential 10 minimum reporting criteria by the ARRIVE guidelines. Right: Animal models of SLD can be systematically characterised using a combination of metabolic, histopathological, and biochemical readouts. Top: Metabolic assessment should include longitudinal monitoring of body weight, food/water intake when applicable, macroscopic endpoints, including liver weight (grams and % of body weight), size (hepatomegaly), adipose tissue distribution (e.g. gonadal and subcutaneous fat depots), as well as evaluation of glucose homeostasis, insulin sensitivity and systemic lipid status to capture features of obesity and metabolic dysfunction. Middle: Histopathological assessment should include standardised grading of steatosis and necroinflammatory activity and staging of fibrosis by an experienced histopathologist or qualified digital pathology software and quantitative assessment of hepatic steatosis (Oil Red O or Sudan Red), inflammation, fibrosis (Sirius Red or Masson's trichrome) and hepatocyte ballooning using immunohistochemistry and/or flow cytometry-based approaches. Bottom: Biochemical characterisation should encompass quantitative measures of liver injury and function tests (e.g. serum ALT, AST, ALP, TBIL and total bile acids), circulating inflammatory and metabolic parameters (e.g. IL-1 β , TNF α , IL-6, MCP-1, adiponectin and leptin), immune cell phenotyping (e.g. macrophages, neutrophils, t-cells etc), microbial metabolites (e.g. endotoxin/lipopolysaccharide) and hepatic triglyceride content. Consequences of MASH on extracellular matrix content and fibrogenesis should also be evaluated using biochemical approaches such as hepatic hydroxyproline content. **The minimum recommended readouts for SLD studies in disease phenotyping are highlighted in bold.** AI, artificial intelligence; ALD, alcohol-associated/related liver disease; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCl₄, carbon tetrachloride; GTT, glucose tolerance test; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; IHC, immunohistochemistry; ITT, insulin tolerance test; LDL, low-density lipoprotein; MASH, metabolic dysfunction-associated steatohepatitis; MetALD, metabolic and alcohol-associated/related liver disease; NAS, NAFLD activity score; SLD, steatotic liver disease; TBIL, total bilirubin; TC, total cholesterol; TG, triglyceride; VLDL, very-low-density lipoprotein.

also defining “minimal” standards that guarantee comparability across laboratories.

Animals

- Species (e.g. mouse or rat), strain (e.g. C57BL/6 or BALB/c), substrain (C57BL/6J or C57BL/6N), vendor (e.g. Jackson, Charles River, Janvier, Taconic), and genetic background/modification (e.g. *Ldlr*^{-/-}, *ob/ob*, *db/db*), with rationale relative to human phenotype (obesity, insulin resistance, dyslipidaemia).
- Age at induction and at endpoint, sex (both male and female animals are strongly recommended), litter effects, and sample size per group.
- Fed/fasted state at time of dissection (e.g. were animals in a fed state or precise time of fasting prior to dissection).
- Timepoint of experimental analysis (because of the potential impact of circadian rhythm).

- Housing/ambient conditions: temperature (thermoneutral vs. standard), light–dark cycle, cage density/enrichment, activity (e.g. running wheels), and microbiological status (e.g. conventional, specific-pathogen-free, or germ-free).

Diet, alcohol and co-stressors

- Diet composition: energy density (kcal/g), carbohydrates (%kcal or %weight; refined carbohydrates – fructose/sucrose in diet/water), proteins (%kcal), methionine and choline (% or g/kg), fat (%kcal; main source of fat, type of fat including fully or partial hydrogenation), cholesterol (%), and fibre. Manufacturer (including catalogue number). For healthy control mice and to promote reproducibility, avoid ‘standard chow’ and use a low-sucrose/fructose, lower fat standardised diet.
- Alcohol (for ALD/MetALD): liquid diet vs. vapour, g/kg/day or g/day, blood ethanol concentrations at standardised timepoints

(concentrations in mice rapidly decrease and measurements for chronic consumption are best performed a few hours into the dark cycle when the mice are actively consuming), binge vs. chronic vs. chronic-binge design, pair-feeding controls, withdrawal timing, and nutritional compensation. Align dose ranges/patterns to human SLD subclasses where feasible (e.g. MetALD vs. ALD thresholds). Report any signs of ethanol intoxication (mortality rate), weight loss, or behavioural changes.

- Metabolic or fibrogenic co-stressors: e.g. streptozotocin (STZ dose, timing), CCl₄ (dose, route, schedule), cigarette smoke/particulates, lipopolysaccharide (LPS), and viral vectors. Include justification relative to human disease stage.
- Duration: timepoints targeting steatosis, MASH features and ≥F2 fibrosis, as appropriate.

Metabolic phenotype

- Adiposity & body composition: body weight trajectory (weekly). Energy intake (g food/week) and body mass composition (EchoMRI), when available.
- Method of humane sacrifice (e.g. cervical dislocation, exsanguination, CO₂).
- After humane sacrifice: liver weight, gonadal and subcutaneous fat weight (grams and % of body weight), and whether the lymph nodes are removed from the inguinal fat pad.
- Glucose homeostasis and insulin sensitivity: fasting (4–6 hours) glucose and/or insulin, HOMA-IR (with caveats). If necessary for the research question, glucose tolerance tests/insulin tolerance tests (provide timing, fasting duration) and/or hyperinsulinaemic-euglycaemic clamps (when available) can be performed with the caveat that these induce significant stress to the mice and can impact weight curves in the following few weeks. Please see here^{102–104} for more information on important confounders when performing glucose tolerance tests/insulin tolerance tests, such as the need to normalise for lean mass when assessing mice of substantially different weights or the need to use 2 g/kg of glucose to robustly induce insulin secretion.
- Lipid phenotype: post-prandial fasting (4–6 hours) triglycerides (TGs) and total cholesterol. If applicable use fast protein liquid chromatography to define HDL, LDL, and very-low-density lipoprotein (VLDL) fractions, with the major component being cholesterol and TG as a second component.
- Blood pressure and cardiovascular readouts (when available/ if relevant).

Histopathology

- Steatosis (macro- vs. microvesicular steatosis), lobular inflammation and hepatocyte ballooning (not to be confused with microvesicular steatosis in mouse models, with most mouse models usually reaching a score of only 1 and very rarely 2 under the NAS/SAF criteria). Zonal topography of the lesions (zone 1 vs. zone 3 accentuation). H&E and fibrosis stains (Sirius Red or Masson's trichrome).
- Use validated systems with central, blinded expert assessment to reduce subjective bias (where possible) and quantification across multiple replicates. Provide representative images and region/zone descriptors (pericellular/perivenular vs. periportal) if applicable. Provide histological grading of steatosis and necroinflammatory activity and staging of fibrosis by a certified pathologist or a qualified digital pathology software.

- Report any hepatic and/or extrahepatic pathological features relevant to MASLD/ALD (e.g. liver tumours or alcohol-induced intestinal lesions).
- Please see a thorough histopathological comparison between humans and mice later in this paper.

Biochemical readouts

- Liver function and injury: ALT, aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and (when available) total bile acids and K18 fragments.
- Hepatic lipid content/composition: TG levels. Mass spectrometry-based lipidomics when available (including total cholesterol, cholesteryl ester, diacylglycerol/ceramide species). Oxidised LDL can be measured as a possible metabolic co-stressor for MASLD.
- Bile acid pools/profiles (when available).
- Cytokines/chemokines (IL-1β/inflammasome, TNFα, IL-6, MCP-1), adipokines (adiponectin, leptin), endotoxin/LPS (for gut-liver axis contributions), and oxidative/endoplasmic reticulum stress markers (4-HNE, MDA; CHOP/GRP78), when available.
- Fibrogenic and ductular markers: hepatic hydroxyproline content. When available: α-SMA, COL1A1/3A1, K19, osteopontin, TIMP1.

Omics/transcriptomic alignment to human disease (when describing new models)

- Transcriptomic benchmarking: When RNA-sequencing/arrays are used, report pipelines and human benchmarking approaches (e.g. concordance of differentially expressed pathways/genes with human MASH datasets; use of cross-species “human proximity” metrics). Summarise whether the model clusters with human MASH that incorporates signatures for steatohepatitis and cardiometabolic factors at the transcriptome level and whether fibrosis-related signatures are also captured. State specifically which cell populations are analysed by single-cell or single-nucleus RNA-sequencing approaches. In general, single-nucleus RNA sequencing is preferred for hepatocyte analysis whereas single-cell RNA is better suited for immune cell profiling, since many immune activation markers constitute cytoplasmic transcripts and are therefore missed in nucleus-based sequencing approaches. On this note, the use of CITE-seq could potentially be attractive, since it enables simultaneous quantification of surface protein markers alongside transcriptomic profiles at the single-cell level, thereby enhancing the resolution and accuracy of immune cell phenotyping beyond RNA-based readouts alone.¹⁰⁵

Recommendations

- Reporting of results from SLD mouse models should follow the Essential 10 minimum reporting criteria from the ARRIVE guidelines.
- To enable disease phenotyping, the minimum recommended readouts for SLD studies should include: body weight (g), liver weight (g and %), serum liver damage markers (e.g. ALT, AST), fasted (4–6 h) glucose/insulin, histopathology (hepatic steatosis, inflammation and fibrosis [Sirius Red or Masson's trichrome]), and hepatic TG content.

Animal models of MASLD

Rodents have been extensively and effectively used as a model organism for metabolic diseases for decades, and in general, closely reproduce the metabolism and pathogenesis of human diseases, including MASLD.^{49,106} Arguably, rodents are the most common preclinical model, and their use has led to numerous translatable discoveries in our field, such as GLP-1RAs and SGLT2 inhibitors.^{107–109} Of course, as with all models, mice or rats have limitations. In relation to MASLD, many mouse strains readily develop obesity and insulin resistance, but most also have an impressive ability to protect their functional beta cell (β -cell) mass and produce sufficient insulin to maintain blood glucose levels under most metabolically challenging conditions.^{110–112} In contrast, mice rarely develop cardiovascular disease (hypertension or atherosclerosis) by diet alone, and rodent serum lipid profiles differ greatly from humans.^{113–115} In this section, we summarise the most appropriate mouse models of MASLD subdivided into whether they are purely dietary; or whether diet is combined with a genetic mutation or hepatotoxin (Table 1).

Considering the clinical definition of MASLD (hepatic steatosis plus either obesity, impaired glucose regulation, hypertension, and/or dyslipidaemia) with these limitations of mice, we propose the following prioritisation of physiological characteristics when considering whether an animal model is appropriate for MASLD:

- Hepatic steatosis is a required precondition.
- Secondary to this, obesity and insulin resistance are the most prevalent cardiometabolic risk factors in MASLD, and we strongly recommend that any model chosen has, at a minimum, these three features.
- If hypertension and dyslipidaemia are noted in the absence of obesity and insulin resistance, one should reflect on whether the aetiology of the model appropriately aligns with the human condition.
- Arterial hypertension with MASLD can be elicited by a combination of HFD and inhibition of constitutive nitric oxide synthase using L-NAME (N ω -nitro-L-arginine methyl ester), a mouse model originally designed to recapitulate features of heart failure with preserved ejection fraction.¹¹⁶
- Liver histopathology at the time of experimentation should align with what is observed in humans. Hepatic steatosis in the absence of inflammation or fibrosis should be referred to as MASL. It is notable that most short-term (8–16 weeks), diet-induced mouse models only have MASL or early stages of MASH.^{18,47,49}

Recommendations

- The selection of MASLD mouse models that display not only hepatic steatosis but also obesity and systemic insulin resistance (skeletal muscle and adipose tissue) is recommended, given that these are two of the most common cardiometabolic risk factors found in humans. However, if the aim is to phenocopy MASLD in normal-weight patients, hepatic steatosis and systemic insulin resistance and/or dyslipidaemia may suffice.

- Liver pathology of MASLD models should align with the human hepatic pathology including steatosis, lobular inflammation, and ballooning (albeit to a lesser degree than observed in humans).

Diet-induced models of MASLD

Diet-only mouse models remain the backbone of MASLD research because they replicate the human context of nutritional excess as a primary driver of disease. Even when accounting for various confounders such as age and sex, diet-induced obesity remains one of the most important risk factors driving MASLD.¹¹⁷ However, the composition of diet-only models varies substantially, and the inclusion of specific nutrients such as fructose, cholesterol, or saturated fats profoundly influences the severity and nature of liver injury.^{18,49} Notably, a systematic review of over 4,000 articles concluded that WDs rich in fats and fructose most closely mimic human MASLD.¹¹⁸ These findings were corroborated by the recent European LITMUS consortium analysis of more than 40 MASLD models.⁴⁹ They identified that WDs high in fats, fructose, and 2% cholesterol most closely mimic MASH with fibrosis at the histological, transcriptional, and metabolic levels, termed the human proximity score.⁴⁹

WDs better phenocopy human MASLD than simple HFDs as it is the combination of high fats together with sugars that potentially induces hepatic steatosis, inflammation and fibrosis. However, the composition of a WD can also differ vastly, in particular the type of sugars that are utilised. To this end, fructose is a critical dietary component in rodent models of MASLD, as it closely mirrors the pathogenic impact of sugar-sweetened beverage (e.g. high-fructose corn syrup) consumption in humans.^{18,119} Unlike glucose, fructose is metabolised almost exclusively in the liver, where it rapidly fuels DNL, promotes oxidative stress, and alters gut microbiota composition, thereby disrupting the gut–liver axis.^{119,120} These effects collectively drive steatosis, inflammation, and fibrosis.^{119,120} The form and route of administration markedly influence disease severity: liquid fructose accelerates steatosis and fibrosis, whereas solid fructose tends to provoke stronger inflammatory responses.¹²¹ Consequently, combining fructose with other sugars such as sucrose, whether in food or drinking water, enhances total caloric intake and better reproduces the metabolic and hepatic features of human MASLD.^{18,121}

WD – GAN and NTF formulations

Although the term WD is widely used, it carries different connotations across research fields, including atherosclerosis and MASLD, because its exact composition varies substantially between models. Therefore, careful consideration of the specific WD formulation is essential, with the Gubra Amylin NASH (GAN) and the non-trans fat (NTF) diets representing two of the most commonly used and relevant WD variants in MASLD research.^{49,85,122–126} Both consist of ~40% kcal from fat (GAN: palm oil; NTF: non-trans fat Primex Z), supplemented with 22% fructose by weight, 10.6% sucrose by weight, and 2% cholesterol. When administered for 32 weeks or longer in C57BL/6 mice, these diets robustly induce the spectrum of

Table 1. Widely used MASLD and 'other SLD'* mouse models.

Model	Strain & sex	Diet	Duration of diet feeding (wk)	Primary components known to induce damage	MASH ± fibrosis		Similarity to human MASLD	Extrahepatic complications	Key references (PMID)
				i. Source of fat ii. Source of sugar iii. Other critical components	Cardiometabolic risk factors	Liver pathology			
MASLD – Diet									
WD - GAN or NTF	C57BL/6J ♂	GAN - D09100310 NTF - D16022301 (Research Diets)	32	i. GAN: 40% kcal - palm oil; NTF: 40% kcal - non-trans fat Primex Z ii. Fructose and sucrose iii. 2% cholesterol	Obesity (++) Dysglycaemia (++) Insulin resistance (++) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (+)	Transcriptome and histology	NA	38867022; 32631250; 38718791; 34883280; 31293441
FPC	C57BL/6J ♂	TD.160785.PWD (Inotiv)	28	i. 53% kcal – trans and saturated fat (Primex); anhydrous milk fat, palmitic acid ii. Sucrose in the diet and fructose (45%) and glucose (55%) in the water (g/L) iii. 1.25% cholesterol	Obesity (+) Dysglycaemia (+) Insulin resistance (++) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (++)	Histology	NA	28068223; 32259482; 39911943
HFHS + cholesterol	C57BL/6J ♂	SF11-078 (Specialty Feeds)	32	i. 44% kcal - lard ii. Sucrose iii. 0.2% cholesterol	Obesity (++) Dysglycaemia (++) Insulin resistance (++) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (+)	Histology	NA	32694178; 30367044
CDHFD	C57BL/6J ♂	D05010402 (Research Diets)	32	i. 45% kcal - lard ii. Sucrose iii. Choline deficient diet	Obesity (++) Dysglycaemia (++) Insulin resistance (++) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (+) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (-/+)	Histology	NA	25314080; 30936549; 37224925
MASLD – Diet + genetics									
WD + Foz/Foz mice	C57BL/6J ♂	AIN-76A (Test Diet)	12-24	i. 40% kcal - milk fat ii. Sucrose iii. 0.2% cholesterol; mutation in the <i>Alms1</i> gene	Obesity (++) Dysglycaemia (++) Insulin resistance (NA) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (++)	Transcriptome	CKD at 12 wk/CVD at 24 wk	34062281; 32629454
MASLD – Diet + toxin									
WD + sugar water + low dose CCl ₄	C57BL/6J ♂	TD120528 (Inotiv)	12-24	i. 42% kcal - milk fat ii. Sucrose in the diet and 18.9 g/L glucose & 23.1 g/L fructose in the water iii. 1.25% cholesterol; CCl ₄ (0.32 µg/q weekly)	Obesity (-) Dysglycaemia (-/+) Insulin resistance (-/+) Dyslipidaemia (+)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (++)	Transcriptome and histology at 24 wk	CKD at 12 and 24 wk	29572095; 38159678

(continued on next page)

Table 1. (continued)

Model	Strain & sex	Diet	Duration of diet feeding (wk)	Primary components known to induce damage		MASH ± fibrosis	Liver pathology	Similarity to human MASLD	Extrahepatic complications	Key references (PMID)
				i. Source of fat	ii. Source of sugar					
				iii. Other critical components		Cardiometabolic risk factors				
Other SLD* - Diet										
CDAHFD	C57BL/6J ♂	A06071302 (Research Diets)	12	i. 60% kcal - lard ii. Sucrose iii. Choline-deficient & methionine-restricted (0.1%) diet		Obesity (-) Dysglycaemia (-) Insulin resistance (-) Dyslipidaemia (-)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Hepatocyte ballooning (+) Lobular inflammation (++) Fibrosis (++)	NA	NA	23305254

CCl₄, carbon tetrachloride; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; CDHFD, choline-deficient high-fat diet; CKD, chronic kidney disease; CVD, cardiovascular disease; FPC, fructose-palmitate-cholesterol; GAN, Gubra Amylin NASH; HFHS, high-fat, high-sucrose; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NA, not available; NTF, non-trans fat; SLD, steatotic liver disease; WD, Western diet.

Dysglycaemia: glucose tolerance test, fasting glucose; insulin resistance: insulin tolerance test, fasting insulin, HOMA-IR; dyslipidaemia: elevated fasting (5 h) serum triglycerides or fed serum cholesterol; serum markers: ALT, AST, ALP; hepatomegaly: liver weight (g or % of body weight); other SLD*: steatotic liver disease not meeting MASLD criteria.

-/+ trend observed but not statistically significant.

+ statistically significant alteration.

++ statistically significant with profound alterations.

human MASLD and metabolic syndrome including obesity, glucose intolerance, insulin resistance (e.g. elevated HOMA-IR and plasma insulin), dyslipidaemia, hepatomegaly, and histopathological features of MASH, including hepatic steatosis, lobular inflammation, and hepatocyte ballooning with fibrosis.^{49,85,122–124} However, extended feeding durations (over 40 weeks) are required to induce significant fibrosis (F2 or higher),⁴⁹ while feeding for 8–16 weeks will induce a state of MASL or early stages of MASH. Transcriptomic and histological analyses demonstrate a high degree of overlap with human MASLD, making them strong candidates for translational research.¹²⁴ Notably, mice fed either the GAN or NTF WD subjected to either semaglutide or an intermittent 5:2 fasting regimen display weight loss and MASH amelioration, faithfully phenocopying the human situation.^{123,125,126} Overall, the GAN and NTF WDs are not only some of the most widely used diets in the field but are consistently ranked among the best models to phenocopy human MASLD.^{49,118}

FPC diet

The fructose-palmitate-cholesterol (FPC) diet combines 53% kcal from fully hydrogenated, anhydrous milk fat, palmitic acid, 1.25% cholesterol and high sucrose, paired with *ad libitum* access to sugar water (55% glucose and 45% fructose w/w g/L).^{70,127} After 28 weeks, C57BL/6 mice develop dyslipidaemia, insulin resistance, and advanced steatohepatitis with fibrosis.^{70,127} Mice fed the FPC diet also develop obesity and mildly impaired glucose homeostasis but to a lesser extent than mice fed the GAN or NTF WD formulations.^{70,127} Nevertheless, the FPC diet may induce a more rapid fibrotic response (F1 after only 16 weeks and F2–F3 after 28 weeks), but further studies should compare the two models directly to ascertain such differences. Similar to the GAN/NTF WD formulations, the FPC diet given for 8–16 weeks will induce a state of MASL or early stages of MASH.^{70,127} Of note, trans fats utilised in the first iteration of the FPC diet are now banned for human use in the EU and USA due to their health implications, but the new iteration with fully hydrogenated fats works very well.^{128,129} In addition to the GAN and NTF WDs, the FPC diet is another MASLD model that is frequently used in the field, particularly for studying advanced fibrosis over a shorter time frame while retaining key cardiometabolic characteristics.

HFHS + cholesterol diet

Similar to the WD and FPC diets, the high-fat, high-sucrose (HFHS) + cholesterol diet (44% kcal from lard, rich in sucrose, and 0.2% cholesterol) also induces obesity and significant hepatic injury over extended feeding periods (~32 weeks), closely mirroring human pathophysiology.^{72,130} Importantly, C57BL/6 mice fed the HFHS + cholesterol diet displayed hepatic steatosis, inflammation and fibrosis at 32 weeks of feeding.^{72,130} HFHS + cholesterol-fed mice also displayed glucose intolerance, elevated fasting insulin, and dyslipidaemia.^{72,130} However, a proper pathological comparison between human and mouse liver pathology with the HFHS + cholesterol diet needs to be carried out. In addition, the HFHS + cholesterol diet given for 8–16 weeks will induce a state of MASL or early stages of MASH.^{72,130}

CDAHFD vs. CDHFD

In addition to variations of WDs, different formulations of choline-deficient diets have been extensively used in MASLD research.¹⁸ However, the 'devil lies in the detail' since most forms of choline-deficient diets are also deficient or significantly restricted for methionine, an essential amino acid. Therefore, these low choline/methionine diets, despite containing high fats/sugars, almost always induce either rapid weight loss or prevent weight gain, thereby limiting their ability to model cardiometabolic risk factors.

One of the most widely used of these, particularly in earlier 'non-alcoholic steatohepatitis (NASH)' research, is the choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD/CDAA-HFD/HF-CDAA – 60% kcal lard, 9.6% sucrose by weight), which is choline-deficient and methionine-restricted (0.1%). The CDAHFD rapidly induces severe steatohepatitis and advanced fibrosis within 12 weeks of feeding.^{65,131–133} As the CDAHFD is not only choline deficient but is also methionine restricted (0.1%), mice remain lean and are metabolically healthy, retaining insulin sensitivity and normal glucose homeostasis.^{132,133} It is therefore important to emphasise that the CDAHFD represents a model of 'other SLD' not meeting MASLD criteria. Although the absence of obesity and cardiometabolic risk factors limits its translational relevance for MASLD, the rapid development of advanced fibrosis within a predominantly myeloid-driven inflammatory milieu makes this model highly valuable for fibrosis-focused mechanistic studies.

In contrast, the choline-deficient high-fat diet (CDHFD) that lacks only choline but is methionine sufficient has been extensively used in the MASLD field over the past decade.^{134–136} Specifically, the CDHFD comprises 45% of kcal from lard, 9% sucrose (by weight), and is choline-deficient.^{134–136} Notably, C57BL/6 mice fed a CDHFD for ~32 weeks develop obesity and features of metabolic syndrome, including glucose intolerance, insulin resistance and dyslipidaemia.^{134–136} In addition, CDHFD-fed mice display significant steatosis and inflammation but present with milder fibrosis compared to GAN/NTF-fed mice.^{134–136} Furthermore, CDHFD induces a distinct immune profile compared to WD with a higher tendency towards adaptive immunity; thus, the CDHFD functions as an alternative model to confirm findings or potential novel treatments in addition to a typical WD. The CDHFD also induce a state of MASL or early MASH when given for 8–16 weeks.^{134–136}

Taken together, while both CDAHFD and CDHFD are widely used choline-deficient dietary models, they differ substantially in their metabolic phenotype and relevance to MASLD. This distinction has become particularly important with the transition from NAFLD to MASLD nomenclature, which requires the presence of at least one cardiometabolic risk factor, and highlights the need for careful consideration of dietary composition when selecting experimental models. The broader implications of nomenclature changes for the interpretation and use of prior MASLD models have also been discussed recently.⁴

Diet + genetic models of MASLD

Diet-induced models are powerful but can take months to recapitulate advanced MASLD features.^{18,49} Combining a metabolic diet with a susceptible genetic background

accelerates MASLD progression, including systemic complications, thereby further recapitulating key features of the human disease.

WD + *Foz/Foz* mice (*Alms1* mutation)

Foz/Foz mice carry a loss-of-function mutation in *Alms1*, a gene linked to the human ciliopathy Alström syndrome, characterised by obesity, insulin resistance, and cardiometabolic disease. When placed on a WD (40% kcal milk fat, sucrose and 0.2% cholesterol), the *Foz/Foz* hyperphagic mice develop obesity, hyperinsulinaemia, dyslipidaemia, and severe steatohepatitis within 12 weeks, which is much faster than diet-only models.^{63,137} Importantly, this combination leads to extrahepatic complications including CKD at 12 weeks and CVD at 24 weeks, mimicking the systemic burden of MASLD in humans.^{63,137} Notably, transcriptomic profiling confirmed strong alignment with human MASH signatures.^{63,137} The major strengths of this model lie in its ability to recapitulate both metabolic syndrome and extrahepatic manifestations, which are central to MASLD. Nevertheless, while phenocopying many aspects of human MASLD, *ALMS1* mutations in the general population are rare, making it more a model of accelerated disease rather than a direct genetic parallel to common MASLD where polymorphisms in *PNPLA3* or *TM6SF2* are more frequent.

Other genetic models

Some of the most frequent single nucleotide polymorphisms in human MASLD are in *PNPLA3*, *TM6SF2* and *MBOAT7*.¹³⁸ Therefore, multiple genetic models have been developed to investigate the impact of human MASLD-associated genetic variants, including the *PNPLA3* rs738409 (I148M), *TM6SF2* rs58542926 (E167K), and *MBOAT7* rs641738/rs8736 C>T polymorphisms.

The most common single nucleotide polymorphism in *PNPLA3* is rs738409, which induces a substitution at position 148 from isoleucine to methionine (I148M).¹³⁸ This mutation not only reduces the triglyceride hydrolase activity of *PNPLA3*, but causes the mutant *PNPLA3*-I148M protein to accumulate on lipid droplets and sequester the lipase co-activator ABHD5/CGI-58 to limit the activity of adipose triglyceride lipase (ATGL/*PNPLA2*).^{139–142} By altering retinyl-palmitate lipase activity in stellate cells, the I148M variant has been linked to fibrosis and liver-related complications, such as decompensation and HCC, pointing to its potential as a therapeutic target.^{143,144} Notably, knock-in mice carrying the *Pnpla3* 148M/M variant displayed increased hepatic triglycerides when fed a high-sucrose diet or a WD containing 40% kcal from trans fats, 22% fructose by weight and 2% cholesterol.^{140,145,146}

The *TM6SF2* variant rs58543926, encoding an E167K substitution, results in increased hepatic lipid accumulation and fibrosis by reducing APOB stability and VLDL secretion.^{147,148} The E167K substitution represents a loss-of-function effect and mice with hepatocyte-specific loss of *TM6SF2* displayed worsened hepatic steatosis and fibrosis without body weight alterations when fed an FPC diet or a high trans fat, high-fructose diet for 26 or 20 weeks, respectively.^{147,149} Nevertheless, although it increases susceptibility to MASLD, the E167K variant is paradoxically associated with

lower levels of circulating cholesterol and triglycerides, thereby reducing cardiovascular risk.¹⁵⁰

Patients with the *MBOAT7* rs641738 C>T polymorphism display lower protein levels of MBOAT7, consistent with a loss-of-function mutation. Therefore, mice with hepatocyte-specific deletion of *Mboat7* were utilised to mimic this phenotype.^{128,151} These mice not only displayed a similar lipidomic profile to human carriers, but also showed enhanced hepatic fibrosis without changes in steatosis with a high-fat, methionine-low, choline-deficient diet.^{128,151}

Furthermore, the DIAMOND model consists of a stable isogenic cross between C57BL/6J and 129S1/SvImJ mice fed a WD containing 0.1% cholesterol with sugar water (18.9 g/L D-glucose and 23.1 g/L fructose) for 24 weeks.¹⁵² These mice developed obesity, insulin resistance and histopathological features of MASH with a transcriptome resembling human MASH.¹⁵² A recent manuscript demonstrated that feeding *Ldlr*^{-/-} mice Western or CDHF diets containing 0.2% cholesterol induces both CVD and MASH in a sex-dependent manner.¹⁵³

These transgenic models provide valuable tools for dissecting the molecular mechanisms by which genetic variants contribute to MASLD pathophysiology.

Diet + toxin models of MASLD

While diet-only and diet + genetic models are more physiological, they require extended feeding to produce advanced fibrosis. To overcome this, hepatotoxins are sometimes combined with dietary excess to accelerate disease progression. Two models that have come to prominence are combining WDs with either low dose CCl₄ or streptozotocin (STZ – when given in adults) termed the “FAT-MASH” and STZ+HFD models, respectively. These models have been shown to be particularly useful for studying MASH with accelerated fibrosis and to recapitulate transcriptional signatures in human MASH. However, it is important to note that there are several caveats, as they do not often display metabolic syndrome or phenocopy the natural pathophysiology of the human disease to the same extent as pure dietary models.

WD + sugar water + low-dose CCl₄ (FAT-MASH)

This model combines a WD (42% kcal milk fat, 1.25% cholesterol) with sugar water (18.9 g/L D-glucose and 23.1 g/L fructose) and weekly low-dose CCl₄ (0.32 µg/g) injections.^{62,154} C57BL/6 mice developed severe steatohepatitis, with ballooning, inflammation, and fibrosis, accompanied by CKD as early as 12 weeks.^{62,154} By 24 weeks, these mice displayed histopathological and transcriptomic changes very similar to steatohepatitis in humans.⁶² Mechanistically, CCl₄ generates free radical-mediated hepatocellular necrosis, which synergises with lipid-induced injury to accelerate fibrosis. However, this model has metabolic limitations, as mice in the WD+CCl₄ group displayed only marginal body weight gain compared to chow-fed controls at 24 weeks and remain weight-matched at 12 weeks.⁶² Furthermore, blood glucose and plasma insulin levels were not significantly altered at either 12 or 24 weeks compared to chow-fed mice, but the WD+CCl₄ group displayed elevated serum cholesterol levels.⁶² Overall, this model is highly effective for studying late-stage MASH and fibrosis

but has limited utility for studying natural disease initiation, since toxin-mediated injury is not a feature of human MASLD.

HFD + low-dose STZ in adult mice

In this model, adult C57BL/6 mice (7-week-old) are treated with low-dose STZ (40 mg/kg for 5 days) and fed a HFD (60% kcal lard) from 8 weeks of age.¹⁵⁵ STZ selectively destroys pancreatic β-cells, leading to insulin deficiency and impaired glucose homeostasis. When performed in adult mice, as done here, this resembles more of a T2D phenotype. When combined with a HFD, mice rapidly develop obesity, insulin resistance, dyslipidaemia, and steatohepatitis by 12 weeks of feeding (20 weeks of age).¹⁵⁵ Fibrosis developed from 12 weeks of feeding and progressed to advanced fibrosis by 24 weeks of feeding (32 weeks of age).¹⁵⁵ The hepatic transcriptome profile of these mice was similar to patients with MASL (NAS 2-6 and fibrosis stage 0-1) at 12 weeks of feeding and to patients with MASH (NAS 4-7 and fibrosis stage 4) at 24 weeks of feeding.¹⁵⁵ The strength of this model is its relevance to patients with MASLD and co-existing T2D, where β-cell dysfunction amplifies hepatic lipotoxicity. Its limitation is that toxin-induced β-cell death is mechanistically distinct from the hyperinsulinaemia and progressive β-cell failure seen in humans. In addition, it should be noted that this adult model of HFD+STZ has yet to be reproduced by multiple other groups, thus further studies are still needed to evaluate the robustness of the model.

MASLD in normal-weight mice

MASLD is typically associated with obesity; however, 5–35% of patients are lean (BMI <25 kg/m² [<23 in Asia]), with a higher prevalence reported in South and East Asia.⁶⁴ MASLD in lean individuals is heterogeneous in origin and may be driven by genetic factors, including *PNPLA3* variants, as well as conditions such as lipodystrophy.⁶⁴ Although these individuals generally exhibit a lower metabolic burden, insulin resistance, elevated triglyceride levels and increased visceral adiposity are still commonly observed.⁶⁴ Compared with obese MASLD, lean patients often present with more favourable histological features and a lower cardiovascular risk at diagnosis.^{64,156} Nevertheless, longitudinal data suggest that both Western (<25 kg/m²) and Asian (<23 kg/m²) patients who are lean with MASLD are at significantly increased risk of all-cause mortality and liver-related events compared to those with non-lean MASLD, but a similar risk of HCC and a lower risk of CVD.^{156,157} Nevertheless, limited attention has been given to modelling this phenomenon in mice, and the optimal mouse model for this subgroup is so far unclear. To this end, there are a few models that phenocopy at least some aspects of MASLD in normal-weight patients. Approaches utilised include a modified atherogenic diet to induce dyslipidaemia and MASH with a microbiome reflecting humans,¹⁵⁸ or the STAM model where neonatal (2-day-old) STZ is combined with HFD feeding from 4 weeks of age to induce hyperglycaemia and MASH by 8 weeks of age, but with the caveat that it reflects a type-1 diabetic phenotype.⁶⁶

The modified atherogenic diet (9.5% cocoa butter and soya oil, 33% sucrose, 2% cholesterol, and 0.5% cholate) induces dyslipidaemia (hypercholesterolaemia), alterations in bile acid metabolism, and histological steatohepatitis after 16 weeks.¹⁵⁸

Cholate is supplemented to aid in intestinal cholesterol absorption, but with the caveat that it is also a hepatotoxin. Notably, the gut microbiome and bile acid profile in these mice resembled MASLD in normal-weight patients.¹⁵⁸ However, mice fed atherogenic diets do not develop obesity, impaired glucose tolerance, or insulin resistance.¹⁵⁸ Thus, while the model is valuable for dissecting the roles of cholesterol and bile acids in liver injury as mice displayed dyslipidaemia, it lacks other metabolic syndrome features such as insulin resistance.

Recommendations

- The use of diet-only models, in particular a variation of the WD (e.g. GAN/NTF) or the FPC diet, is recommended. While the GAN/NTF WD induces a greater degree of obesity and glucose intolerance, the FPC diet induces advanced fibrosis.
- Alternative models can be utilised to confirm findings. These include the HFHS + cholesterol diet and the CDHFD as diet-only models, as well as accelerated models such as combining a WD with *Foz/Foz* transgenic mice or with hepatotoxic agents such as low-dose CCl₄, sugar water, or STZ, which can be used to model MASH with advanced fibrosis over a shorter timeframe (12–24 weeks).
- Long-term experiments (28–32 weeks of diet) are needed to investigate advanced MASH with moderate hepatic fibrosis using diet-only models (e.g. WD, FPC, HFHS, and CDHFD) in mice.
- Ultimately, model selection should align with the research question. As human MASLD stratification advances, specific models may map to specific metabolic and molecular (sub)types, enhancing mechanistic insights and precision therapeutic strategies.

Animal MetALD models

MetALD can be modelled by combining a MASLD-inducing diet with ethanol administration to recapitulate the coexistence of MASLD and alcohol-associated/related hepatitis. Current mouse models predominantly mimic either chronic heavy drinking or acute binge-drinking patterns observed in humans, with only a limited number of models integrating both approaches¹⁵⁹ (Table 2). For chronic drinking, mice fed a MASLD-inducing diet are constantly exposed to 10–20% alcohol in drinking water or liquid diet. The induction time of steatohepatitis in these chronic models is dependent on the type of diet, length of diet feeding and the dose of ethanol.^{160,161} In contrast, to mimic binge drinking patterns, ethanol is administered to mice through intragastric delivery or oral gavage typically once or twice per week.^{162–164} By feeding mice a HFD plus multiple binges of ethanol (dosage varies across studies), hepatic steatosis, hepatocyte injury, inflammation, triglyceride accumulation, and fibrosis can develop in 12 weeks.^{162–164} A single binge of ethanol following 12 weeks of HFD feeding was sufficient to induce hepatic steatosis in

mice but had milder effects than multiple binges.¹⁶⁴ Finally, some have attempted to combine chronic ethanol with weekly gavages to accelerate the process.¹⁶⁵ Importantly, in many of the chronic models of binge drinking, mice are humanely killed 9 hours after the last ethanol binge. Therefore, one is studying the effects of both chronic and acute ethanol intoxication. Overall, over the past decade, several MetALD models have been developed that more closely phenocopy the human condition, as summarised below.

WD with chronic ethanol supplementation in drinking water – DUAL model and alternating weekly ethanol concentrations

Recently a novel MetALD model termed DUAL was developed, combining a widely used WD rich in trans fats (40% kcal Primex, 22% fructose by weight, 10.6% sucrose by weight and 2% cholesterol) with chronic 10% v/v ethanol in sweetened (6.75% D-glucose) drinking water and a gradual increase in ethanol concentration from 1% to 10% v/v ethanol.¹⁶¹ 10% v/v ethanol was the optimum concentration as C57BL/6 mice consumed significantly less ethanol when increasing the ethanol concentration to 20%.¹⁶¹ Mice on the DUAL protocol also consumed more calories compared to either WD or ethanol groups supplemented with sugar solution.¹⁶¹ Mice on the DUAL protocol displayed increased body weight, hyperglycaemia, hypercholesterolemia, elevated liver damage as well as hepatomegaly, hepatic steatosis, ballooning, inflammation and advanced fibrosis after 23 weeks.¹⁶¹ Notably, these mice also developed bridging fibrosis and liver nodules after 52 weeks.¹⁶¹ The phenotype observed with the DUAL model was more pronounced than with either WD or ethanol alone.¹⁶¹ This is perhaps one of the best currently available models of MetALD to study chronic liver pathology and even tumorigenesis, as it is well tolerated and not associated with ethanol-induced mortality.¹⁶¹

Another study combined a milder form of WD (40% kcal fat, 0.2% cholesterol with sucrose) with alternating ethanol concentrations in drinking water over a weekly cycle for 16 weeks, consisting of 20% ethanol for 4 consecutive days followed by 10% ethanol for 3 consecutive days.¹⁶⁰ In the initial phase (~2 weeks), ethanol concentration was progressively increased from 1%, 3%, 10%, 15% to 20% with each interval lasting for 3 days.¹⁶⁰ The authors found that male mice, in particular, consumed less liquid when given chronic 20% ethanol; however, the alternating protocol resolved this issue, allowing chronic 20% ethanol to be administered in the final 2 weeks.¹⁶⁰ Mice fed this WD with alternating ethanol concentrations not only exhibited body weight gain comparable to WD alone, but also showed more pronounced serum liver injury markers, hepatic steatosis, inflammation (particularly within the myeloid compartment), and fibrosis after 16 weeks.¹⁶⁰

HFD with multiple binges of ethanol

To mimic binge drinking and overnutrition in humans, several studies aimed to combine HFD or WD feeding with weekly ethanol gavages. One study utilised a WD diet (55% kcal fat, 1% cholesterol with sucrose and fructose) in combination with weekly ethanol (5 g/kg) gavages for 12 weeks in C57BL/6 mice.¹⁶² Importantly, the survival rate of these mice was ~75%.¹⁶² Over the span of 12 weeks, C57BL/6 mice exhibited

Table 2. Widely used MetALD mouse models.

Model	Strain & sex	Diet	Diet feeding (wk)	Primary components known to induce damage	Ethanol consumption		MetALD ± fibrosis		Key references (PMID)	Remarks
				i. Source of fat ii. Source of sugar iii. Other critical components	Duration (wk)	Dose and administration	Cardiometabolic risk factors	Liver pathology		
Diet + chronic drinking										
DUAL	C57BL/6J ♀ (♂ - data not shown)	D09100301 (Research Diets)	23	i. 40% kcal - Primex (transaturated fats) ii. Fructose and sucrose iii. 2% cholesterol	23	10% v/v ethanol in sweetened (6.75% D-glucose) water given <i>ad libitum</i> . Gradual increase in ethanol concentration from 1% to 10% v/v.	Body weight (++) Dyslipidaemia (NA)	Serum markers (++) Hepatomegaly (++) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	34523155	Hyperglycaemia, glucose intolerant. Male mice showed a more pronounced phenotype (data was not shown)
WD + alternating ethanol	C57BL/6J ♀ and ♂	D12079B (Research Diets)	16	i. 40% kcal - milk fat, corn oil ii. Sucrose iii. 0.2% cholesterol	16	20% v/v ethanol for 4 days/wk (Thu-Sun) and 10% v/v ethanol for 3 days/wk (Mon-Wed) in drinking water. 20% v/v ethanol was given in the final 2 wk. Initial phase - 3 days each of 1%, 3%, 10%, 15% and 20% ethanol.	Body weight (++) Dyslipidaemia (++)	Serum markers (+) Hepatomegaly (+) Steatosis (++) Lobular inflammation (+) Fibrosis (++)	34523155	Differential effect on pathology in males and females depending on the outcome.
Diet + binge drinking										
WD + multiple binges	C57BL/6J ♂	D09061704 (Research Diets)	12	i. 55% kcal - coconut oil, hydrogenated 101, soy-bean oil ii. Fructose and sucrose iii. 1% cholesterol	12	5 g/kg binge drinking once per wk (oral gavage). Mice sacrificed 9 hours after the last oral gavage.	Body weight (+) Dyslipidaemia (+)	Serum markers (++) Hepatomegaly (NA) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	38777573	Elevated blood glucose. Survival rate of mice was ~75%.
HFD + multiple binges	C57BL/6J ♂	D12451 (Research Diets)	12	i. 45% kcal - lard, soy-bean oil ii. Sucrose iii. 0.25% cholesterol	12	Ethanol binges twice a wk at 2 g/kg in 30% saline solution (oral gavage). 48-96 h recovery between administrations.	Body weight (+) Dyslipidaemia (+)	Serum markers (-) Hepatomegaly (+) Steatosis (+) Lobular inflammation (+) Fibrosis (+)	25915743	Elevated serum insulin. No effect on kidney or heart weights. No observed mortality.
HFD + multiple binges	C57BL/6J ♂	D12492 (Research Diets)	12	i. 60% kcal - lard, soy-bean oil ii. Sucrose iii. None	12 + 4	12 wk after HFD, mice were given ethanol (5 g/kg as a 53% ethanol solution in water) binges twice a wk for 4 wk (oral gavages).	Body weight (++) Dyslipidaemia (NA)	Serum markers (++) Hepatomegaly (NA) Steatosis (++) Lobular inflammation (+) Fibrosis (++)	29552626	High mortality rate: ~80% in C57BL/6N mice and ~30% in C57BL/6J mice.

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Table 2. (continued)

Model	Strain & sex	Diet	Diet feeding (wk)	Primary components known to induce damage		Ethanol consumption		MetALD ± fibrosis		Key references (PMID)	Remarks
				i. Source of fat	ii. Source of sugar	iii. Other critical components	Duration (wk)	Dose and administration	Cardiometabolic risk factors	Liver pathology	
Diet + chronic + binge drinking											
WD + ethanol + multiple binges	C57BL/6J ♂	D09061704 (Research Diets)	12	i. 55% kcal - coconut oil, hydrogenated 101, soybean oil		ii. Fructose and sucrose iii. 1% cholesterol	12	10% v/v ethanol in drinking water given <i>ad libitum</i> + ethanol (5 g/kg) binges by oral gavage once per wk on the weekends. Mice sacrificed 9 hours after the last oral gavage.	Body weight (++) Dyslipidaemia (++)	Serum markers (++) Hepatomegaly (NA) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	38896082 10-15% of mice died. Elevated fasted glucose.

HFD, high-fat diet; MetALD, metabolic and alcohol-associated/related liver disease; WD, Western diet.

Dyslipidaemia: elevated fasting (5 hour) serum triglycerides or fed serum cholesterol; serum markers: ALT, AST, ALP.

- not altered.

-/+ trend observed but not statistically significant.

+ statistically significant alteration.

++ statistically significant with profound alterations.

body weight gain, albeit to a lesser degree than with WD alone, as well as hypercholesterolaemia, elevated serum liver injury markers, hepatic steatosis, inflammation, and more pronounced fibrosis compared to WD feeding alone.¹⁶² There was an increase in Ly6G⁺ neutrophil infiltration and neutrophil extracellular trap formation with ethanol gavages either alone or together with WD feeding.¹⁶² In a similar study, mice were fed a HFD (45% kcal fat and 0.25% cholesterol) and administered ethanol twice weekly (2 g/kg in 30% solution in saline) for 12 weeks.¹⁶³ One key difference is that the dose of ethanol was lower (2 g/kg) compared to the aforementioned study (5 g/kg).^{162,163} Mice on this protocol showed no mortality despite receiving twice weekly ethanol binges, thus demonstrating that repeated low doses can overcome ethanol-induced toxicity.¹⁶³ Mice on the HFD and ethanol binges displayed body weight gain, dyslipidaemia, and hepatomegaly but there were no overt changes in serum liver damage markers.¹⁶³ These mice also showed histopathological features of hepatic steatosis, myeloid-driven inflammation and fibrosis.¹⁶³ In contrast to chronic binges, another study utilised a protocol where mice were first fed an HFD (60% kcal fat) for 12 weeks and then subjected to twice weekly ethanol (5 g/kg) binges, as a 53% ethanol solution in water by oral gavage.¹⁶⁴ Notably, ~30% of C57BL/6J and ~80% of C57BL/6N mice died in this model.¹⁶⁴ Nevertheless, mice fed the HFD followed by ethanol binges displayed obesity as well as elevated liver injury markers, hepatic steatosis and extensive fibrosis with neutrophil infiltration.¹⁶⁴

Combining WD with both chronic ethanol supplementation and weekly binges

One recent study developed a novel MetALD model combining chronic ethanol (10%) in the drinking water given *ad libitum* with once per week ethanol (5 g/kg) binges on the weekend for 12 weeks to mimic human behaviour.¹⁶⁵ In addition to the ethanol supplementation, these mice were also fed a WD (55% kcal fat, 1% cholesterol with sucrose and fructose) to resemble a MetALD environment.¹⁶⁵ One caveat, however, is that roughly 10-15% of the mice died due to complications.¹⁶⁵ Nevertheless, combining a WD with both chronic ethanol and weekly binges induced body weight gain, elevated fasting glucose, hepatic steatosis, inflammation (particularly within the myeloid compartment) and fibrosis within 12 weeks.¹⁶⁵ Notably, the hepatic phenotype at the 12-week time point was more pronounced in the WD plus ethanol group compared to either the WD or ethanol alone groups.¹⁶⁵ The WD used can be modified to incorporate the GAN or NTF formulations, which are well characterised in a MASH setting. In addition to the DUAL approach, this represents another robust MetALD model that reproduces key features of metabolic syndrome and steatohepatitis with advanced fibrosis, albeit with the caveat of ethanol-induced mortality in a subset of mice.

Combining genetic models of obesity with ethanol supplementation

Finally, genetic mouse models of MASLD (e.g. *db/db* mice, *ob/ob* mice) have been utilised in combination with ethanol protocols to mimic MetALD, but these approaches were not as successful as the diet-only models. For example, chronic ethanol supplementation in drinking water leads to hepatic

steatosis in *ob/ob* mice within 6 weeks; however, surprisingly, these mice show a milder phenotype compared with *ob/ob* mice without ethanol.¹⁶⁶ Alternatively, a single binge of ethanol in an acute setting (9 hours) was adequate to increase hepatic injury, oxidative stress, and neutrophil infiltration in 10-week-old *ob/ob* mice.¹⁶⁷ Nevertheless, it would be intriguing to elucidate the effects of either chronic ethanol or ethanol binges in widely used transgenic MASH models, such as in *Foz/Foz* mice, in future studies.

Recommendations

- The use of a WD (e.g. GAN/NTF) combined with 10% v/v ethanol in drinking water is recommended as the most appropriate model to study chronic MetALD.
- Disease pathogenesis can be accelerated with weekly or twice weekly ethanol gavages. The dose (2-5 g/kg) and frequency (once weekly or every 2 weeks) of the oral gavages should be optimised to reduce, and preferably eliminate, ethanol-associated mortality.

Animal ALD models

Alcohol in drinking water is the simplest approach to model ALD in mice, of which simple steatosis and minor inflammatory infiltrates are initiated after alcohol drinking (5%-20%) for 8-10 weeks (Table 3). However, alcohol drinking cannot induce pathological changes beyond steatosis in the absence of a secondary stress, and it is difficult to control the nutritional effects of alcohol drinking.¹⁶⁸ To this end, incorporating ethanol into a liquid diet formula has been widely used (*i.e.*, the Lieber-DeCarli [LDC] model) to model chronic ALD, which forces the mice to have nothing to eat or drink except the ethanol-containing liquid diet.¹⁶⁹ A 4-week supplementation of LDC diet up to a maximum of 4% (v/v) ethanol or a modified version up to a maximum of 6% (v/v) induced mild steatosis, elevation of serum ALT, and minor inflammation, phenocopying the initial stages of ALD.^{170,171} On the other hand, prolonged supplementation of LDC diet (8-12 weeks) induces more pronounced steatosis and liver damage.¹⁷² The LDC model, however, is greatly divergent from human drinking behaviours. A common modification of the LDC is to combine chronic liquid diet with a single binge of ethanol through oral gavage (*i.e.*, the NIAAA model).¹⁷³ This NIAAA model can be extended up to 8 weeks by giving mice multiple ethanol binges, causing moderate steatosis and marked elevation of serum ALT. Nevertheless, the NIAAA model cannot induce significant fibrosis, and long-term modelling is associated with body weight loss and high mortality.¹⁷³ It is also noteworthy that the NIAAA model combines chronic LDC diet feeding with acute high-dose binge drinking, thus it primarily models acute-on-chronic alcohol-associated hepatitis, rather than chronic ALD. Given the differences in pathogenesis and molecular phenotypes between chronic ALD and acute hepatitis, it is recommended not to use the NIAAA model for studying chronic ALD but rather for alcohol-associated hepatitis. Moreover, cautions are needed when comparing findings from

the NIAAA model, as its protocol has been greatly varied across studies, including endpoints, gavage frequency, and exposure duration.

Besides binge drinking, hepatotoxins such as diethylnitrosamine¹⁷⁴ and CCl₄^{171,175} can be added to the chronic LDC liquid ethanol diet as a “second hit” to increase liver damage, leading to more severe liver injuries, cirrhosis, and HCC. The Tsukamoto-French model is another robust modification of the LDC model, as it can accurately resemble ALD pathology in humans, including severe hepatic steatosis, inflammation and mild fibrosis.^{176,177} This model also allows for complete control of ethanol intake and diet. However, this approach is greatly limited by its technical complexity, as the LDC liquid diet is directly infused into the mouse stomach via an implanted catheter connected to an infusion pump. Hence, the Tsukamoto-French model is highly invasive, requires skilled surgical implantation and expensive equipment. It should be noted that the LDC liquid diet used in chronic alcohol feeding has a relatively high fat (35%) content compared to standard chow diets (10-20% fat) used in mice.

Recommendations

- To study chronic ALD, the LDC ethanol-containing liquid diet model is recommended. Short-term feeding with the LDC diet (4 weeks) can phenocopy the initial stages of ALD with mild steatosis, while prolonged supplementation with the LDC diet (8-12 weeks) induces more pronounced steatosis with moderate inflammation and liver damage.
- The LDC model cannot develop histologically relevant fibrosis. Therefore, hepatotoxins (diethylnitrosamine or CCl₄) are needed to accelerate disease pathogenesis in the LDC model. The Tsukamoto-French model (intragastric infusion of ethanol) is a modification of the LDC model that can trigger perisinusoidal and pericellular fibrosis but with severe technical caveats.
- For studies of alcohol-associated hepatitis, we recommend the use of the NIAAA model, as the NIAAA model combines chronic LDC diet feeding with acute high-dose binge drinking.

Recommendations for *in vivo* models of SLD

Our final recommendations for *in vivo* models of SLD are summarised in Fig. 2. These recommendations are based on currently available models that are best suited to address distinct disease characteristics while remaining broadly accessible and experimentally feasible. The models highlighted represent those that, in the authors' view, most closely align with contemporary clinical definitions and pathophysiological features of human SLD.

This overview is not intended to be exhaustive, nor to exclude alternative or emerging approaches. Rather, it reflects a pragmatic selection of widely used and well-characterised models that collectively capture key aspects of metabolic dysfunction, alcohol exposure, fibrosis severity, and translational relevance. Fig. 2 is intended to serve as a decision-

Table 3. Widely used ALD mouse models.

Model	Strain	Ethanol consumption		ALD ± fibrosis		Key references (PMID)	Remarks
		Duration (wk)	Dose and administration	Body weight	Liver pathology		
Chronic drinking							
Alcohol in drinking water	Mouse	24-48 wk	5% and 10% in drinking water each for 3 days, then 20% until experiment ends	-	Serum markers (+) Hepatomegaly (-) Steatosis (+) Lobular inflammation (-/+) Fibrosis (-)	17681030; 22017344; 22960051	
Liquid-only diet							
Lieber-DeCarli model	C57BL/6	4 wk	1% and 2% (v/v) diet each for 2 days, 4% and 5% (v/v) diet each for 1 wk, 6% (v/v) diet for 1 wk until sacrifice	-	Serum markers (+) Hepatomegaly (+) Steatosis (+) Lobular inflammation (-/+) Fibrosis (-)	2673971; 22273278	Rats preferred; mice are more resistant to the diet
Lieber-DeCarli model	C57BL/6	8-12 wk	Ethanol concentrations were raised gradually from 2% to 4% over the first wk and maintained at 4% for the residual administration period	-	Serum markers (++) Hepatomegaly (++) Steatosis (++) Lobular inflammation (-/+) Fibrosis (-)	29072704	Rats preferred; mice are more resistant to the diet
Liquid-only diet + binge drinking							
NIAAA model	C57BL/6	10 days	5% liquid diet for 10 days + 5 g/kg single binge then sacrifice after 9 hours	-	Serum markers (++) Hepatomegaly (++) Steatosis (++) Lobular inflammation (-/+) Fibrosis (-)	20842630; 23449255	
NIAAA model	C57BL/6	4-6 wk	5% liquid diet for 4-6 wk + 5 g/kg binges during feeding, twice per wk	-	Serum markers (+) Hepatomegaly (++) Steatosis (++) Lobular inflammation (-/+) Fibrosis (-)	23449255	High mortality rate
Chronic drinking + hepatotoxin							
Alcohol in drinking water + DEN	B6C3F1	24-48 wk	1 mg/kg DEN, 1 dose, i.p., then 5% and 10% in drinking water each for 3 days, 20% until end of experiment	+	Serum markers (+) Hepatomegaly (+) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	22017344	HCC development
Liquid-only diet + hepatotoxin							
Lieber-DeCarli model + DEN	C57BL/6	15 wk	40 mg/kg DEN, 10 doses, every 4 days, i.p., then 5% liquid diet for 10 wk	++	Serum markers (++) Hepatomegaly (++) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	28566719	HCC development
Lieber-DeCarli model + CCl4	C57BL/6	7 days	1% and 2% diet each for 2 days, then 1 µl/g CCl4, 1 dose, i.p., 2% diet for 3 days	N/A	Serum markers (NA) Hepatomegaly (NA) Steatosis (NA) Lobular inflammation (NA) Fibrosis (++)	22273278	This model was established only for studying fibrosis

(continued on next page)

Table 3. (continued)

Model	Strain	Ethanol consumption		ALD ± fibrosis		Key references (PMID)	Remarks
		Duration (wk)	Dose and administration	Body weight	Liver pathology		
Liquid-only diet + intragastric infusion Tsukamoto-French model	C57BL/6	4 wk - 3 months	5% liquid diet (intragastric infusion)	-	Serum markers (++) Hepatomegaly (++) Steatosis (++) Lobular inflammation (++) Fibrosis (++)	22461066	

ALD, alcohol-associated/related liver disease; CCl₄, carbon tetrachloride; DEN, diethylnitrosamine; HCC, hepatocellular carcinoma.

Serum liver damage markers - ALT, AST, ALP

- not altered.

-/+ trend observed but not statistically significant.

+ statistically significant alteration.

++ statistically significant with profound alterations.

making aid to guide investigators in selecting appropriate *in vivo* models based on the specific SLD subtype and experimental question being addressed.

Ex vivo and in vitro MASLD and MetALD models

With the advent of precision medicine over the last decade, demand has increased for additional MASLD models to improve understanding and treatment of this multifactorial disease. Commonly used MASLD animal models are useful tools and provide translational insights,¹⁷⁸ but require corroboration in other experimental setups due to their limitations. Therefore, there is a desire to find non-animal alternatives that recapitulate human MASLD as either complementary or replacement models. Furthermore, the FDA announcement that animal testing is no longer required before human drug trials¹⁷⁹ has paved the way for *ex vivo* models to be embedded into regulatory drug testing pipelines.¹⁸⁰ Consequently, there has been a surge in the development of *ex vivo* single- and multicellular models, including 2D cultures using primary hepatocytes, human induced pluripotent stem cells (iPSCs), or cell lines, as well as 3D models such as precision-cut liver slices, organoids/spheroids, bioprinted liver tissues, and organ-on-a-chip systems^{181–187} (Table 4). The advancement of these *ex vivo* models also aligns with the '3Rs' principles (Replacement, Reduction, and Refinement), significantly reducing reliance on *in vivo* experiments. The successful development of *ex vivo* models hinges on their capacity to accurately recapitulate the full spectrum of MASLD (*i.e.* steatosis, inflammation, insulin resistance, and fibrosis). The most widespread approach to generate *in vitro* models of MASLD is the addition of non-esterified fatty acids (NEFAs) to cell culture medium. In particular, a NEFA mixture containing oleic and palmitic acids (OA and PA, at a 2:1 ratio) has been shown to induce lipid accumulation, cell-intrinsic insulin resistance, lipotoxicity (*e.g.* oxidative/ER stress, mitochondrial dysfunction), cell death (*e.g.* apoptosis/necroptosis), the release of damage-associated molecular patterns, and inflammatory and immune responses. Validation of *ex vivo* MASLD models requires demonstration of their ability to replicate these critical pathways (Fig. 3).

2D cell cultures: primary hepatocytes or cell lines

2D MASLD cell cultures are the most established *in vitro* models.^{184,187–189} These cultures involve plating hepatic cells as a single layer either directly on plastic or an extracellular matrix (ECM) substrate such as type I collagen (rat-tailed) or Matrigel, and offer advantages such as simplicity, cost-effectiveness, and suitability for high-throughput studies like pharmaceutical screening. As mentioned, generating *in vitro* MASLD models in 2D cultures involves the addition of NEFAs to the cell culture medium. OA and PA in a 2:1 ratio have been shown to induce key features of MASLD, including triglyceride accumulation, ER stress, cell death, and inflammation. Various cell sources are utilised in these 2D MASLD models, contributing to the versatility of this *in vitro* approach.

- **Hepatic cell lines** such as HepG2, HuH-7, AML12 and HepaRG, are frequently used in research due to their ease of culture and expandability, as they are neoplastic in origin.^{189–193} These characteristics make them well-suited for large-scale studies

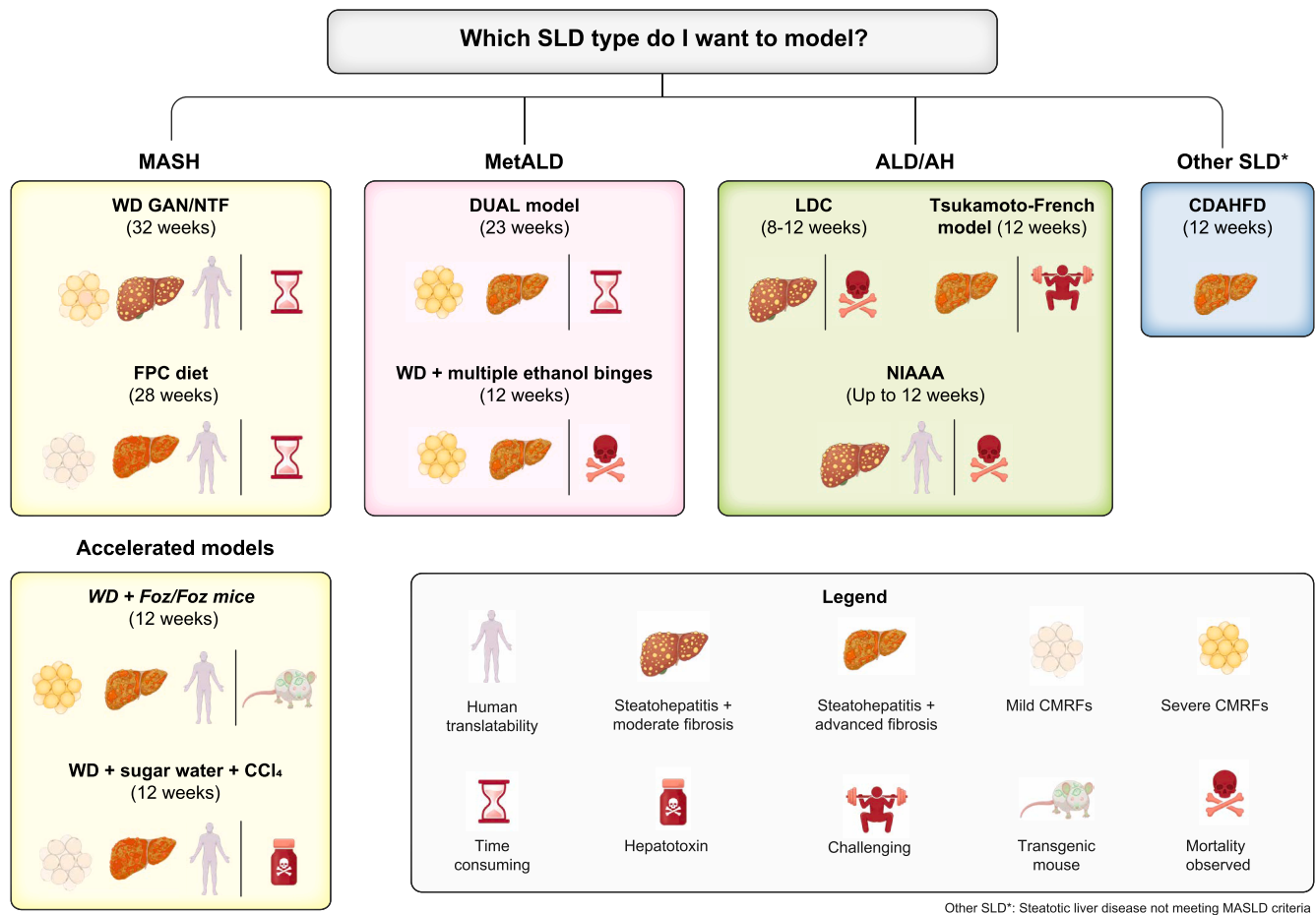


Fig. 2. Flowchart for the selection of mouse models for MASLD, MetALD, ALD and Other SLD*. Schematic overview of representative and widely used *in vivo* mouse models recommended for studying MASLD, MetALD, ALD, and SLD not meeting MASLD criteria (Other SLD*). Models are organised according to the primary disease entity to be modelled and include information on typical study duration, degree of cardiometabolic risk, fibrosis severity, and translational relevance. Accelerated models, incorporating genetic susceptibility or hepatotoxic injury, are shown separately and highlight trade-offs between experimental duration, disease severity, and physiological fidelity. Icons indicate key features including human translatability, time requirements, cardiometabolic risk factor burden, fibrosis stage, use of hepatotoxins or transgenic approaches, experimental challenge, and observed mortality. The figure is intended as a practical guide to support informed model selection rather than an exhaustive catalogue of available SLD models. ALD, alcohol-associated/related liver disease; CCl₄, carbon tetrachloride; CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; CMRF, cardiometabolic risk factors; FPC, fructose-palmitate-cholesterol; LDC, Lieber-DeCarli model; MASLD, metabolic dysfunction-associated steatotic liver disease; MetALD, metabolic and alcohol-associated/related liver disease; SLD, steatotic liver disease; WD GAN/NTF, Western diet Gubra Amylin NASH/non-trans fat.

and enable longer maintenance compared to other *ex vivo* MASLD models. However, a significant limitation of many hepatic cell lines, particularly HepG2 and HuH-7, is their atypical liver-specific profile. This includes impaired drug metabolism, a lack of insulin responsiveness, deficient VLDL secretion, and the presence of genetic abnormalities. In addition, HepG2 and HuH-7 cells have limited capacity to metabolise ethanol due to low expression of alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1). In contrast, HepaRG cells are recognised for their superior expression of various drug-metabolising enzymes and better overall liver function compared to other hepatoma cell lines.^{194,195} Nevertheless, the effective use of these models depends on carefully optimising their differentiation. Research using the HepaRG system has revealed that hepatocytes treated with fatty acids form two distinct metabolic subpopulations, one of which exhibits a steatotic state characterised by excessive lipid accumulation. Furthermore, IL-17A-induced inflammation can disrupt the balance of these metabolic states, a process regulated by NF-κB signalling.¹⁹²

○ **Primary human hepatocytes (PHHs)** are widely recognised as the gold standard for *in vitro* liver research.^{189,196} PHHs are obtained from various sources (e.g. resected liver tissue, commercial cryopreserved options) and typically coated on type I collagen (rat-tailed) coated plates. Their utility is somewhat restricted by a short culture duration (3-5 days). Donor variability is another notable challenge. While this variability can be beneficial for investigating interindividual genetic differences, it can also lead to inconsistent experimental results and poor reproducibility across studies. Even with these limitations, PHHs are effectively utilised to study key aspects of MASLD or ALD. Notably, primary hepatocytes metabolise ethanol and are suitable for modelling ALD, while stem cell-derived organoids and dHepaRG are suitable alternatives. It is also important to note that PHHs tend to rapidly dedifferentiate when cultured in traditional 2D monolayer systems. However, researchers can employ strategies such as higher density culturing or co-culturing with ECM to help maintain their hepatic phenotype and functions (e.g. glucose and lipid homeostasis).^{187,197,198}

Table 4. Widely used *in vitro/ex vivo* models of SLD.

Cells or tissue	Primary components known to induce damage	MASH ± fibrosis	Relevance to human	Tested drug response	Key references (PMID)	Pros	Cons
	i. Lipid ± sugar ± insulin ii. Inflammatory stimuli iii. Fibrotic stimuli iv. Ethanol stimuli						
2D human models - MASLD - monoculture							
dHepaRG cell line	i. Oleic acid (66 μM) & palmitic acid (33 μM) ii. 50 ng/ml interleukin 17-α	Steatosis (++) Inflammation (+) Apoptosis (-) Fibrosis (-)	Metabolomic	-	34226721	Cheap; simple; scalable; can be CRISPR edited; relatively high throughput and human validated.	Not patient derived; no matrix; lacks NPC; 2D culture; not highly reproducible due to differentiation process.
PHHs	i. BSA-conjugated palmitic acid & oleic acid (1:5, final conc 0.15, 0.3, & 0.5 mM) 10 nM insulin and/or glucagon ii. 10 μM LPS	Steatosis (++) Inflammation (+) Apoptosis (+) Fibrosis (+)	RNA	+	38977136	Cheap; simple; relatively high throughput.	No matrix, lacks NPC; 2D culture; can only culture for a relatively short period.
2D human models - MASLD - co-culture							
PHHs or hESC-derived hepatocytes + PSC differentiated HSC-like cells & macrophages	i. 800 μM oleic acid iii. 25 ng/ml TGFβ	Steatosis (+) Inflammation (+) Apoptosis (-) Fibrosis (+)	-	+	34304139	Complex model; cheap; simple; can be CRISPR edited; medium-high throughput.	Not patient derived; no matrix; 2D culture.
2D human models - SLD/MetALD - monoculture							
dHepaRG	i. Stearic & oleic acids (150 μM of each) 5 μg/ml insulin iv. 25 mM ethanol	Steatosis (++) Inflammation (-) Apoptosis (+) Fibrosis (-)	-	-	30147834	Complex model; cheap; simple; can be CRISPR edited; medium-high throughput.	Not patient derived; lacks NPC; no matrix.
3D human models MASLD - spheroid							
PHHs	i. 320 μM FFA (1:1 mix palmitic & oleic acids) 5.5 mM glucose + 5 mM or 10 mM fructose 0.1 nM insulin	Steatosis (++) Inflammation (-) Apoptosis (-) Fibrosis (-)	Lipidomic	+	30250238	3D culture model, technically easy, medium-high throughput, human validated and therapy response.	Not patient derived; random organisation, no matrix; NPC varies by donor. Donor to donor variability.
PHHs ± liver NPCs	i. 480 μM FFA (1:1 mix palmitic & oleic acids) 10 μg/ml insulin ii. 1 μg/ml LPS iii. 5 ng/ml TGFβ	Steatosis (+) Inflammation (-) Apoptosis (-) Fibrosis (++)	-	+	32295224	Complex 3D culture model, genotyped donors (commercial), technically easy, medium-high throughput, human validated and therapy response.	Not patient derived or validated against human disease. Random organization; donor to donor variability
PHHs ± HSCs and KCs/LECs	i. FFAs - 167 μM, supplements; CP-02-302, CP-02-303, InSphero AG Glucose & fructose (22.5 mM total) ii. 5 μg/ml LPS	Steatosis (++) Inflammation (+) Apoptosis (+) Fibrosis (++)	-	+	34815444; 30972180	Complex model; commercially available (simple); drug response.	Random organization. Donor to donor variability. No matrix.
PHHs ± liver NPCs	i. Palmitic & oleic acids (1:1 ratio, 160 μM each), 5.5 mM Glucose + 10 mM fructose ii. 1 μg/ml LPS iii. 1 ng/ml TGF-β1 iv. 100 mM ethanol	Steatosis (+) Inflammation (+) Apoptosis (-) Fibrosis (+)	RNA	+	38833372; 39522884	Complex model; drug response; medium-high throughput.	Access to human tissue to generate own cells (technical skills required) or cost to buy cryopreserved lines. Random organization, Donor to donor variability in response to stimuli.
3D human models MASLD - organoid							

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Table 4. (continued)

Cells or tissue	Primary components known to induce damage	MASH ± fibrosis	Relevance to human	Tested drug response	Key references (PMID)	Pros	Cons
	i. Lipid ± sugar ± insulin ii. Inflammatory stimuli iii. Fibrotic stimuli iv. Ethanol stimuli						
Human inducible PSC/human HLO	i. 300 µM sodium oleate 5 µg/ml insulin	Steatosis (++) Inflammation (+) Apoptosis (-) Fibrosis (-)	scRNA-seq & metabolomic	+	36240780	Complex model; multiple lines genotyped for MASLD relevant SNPs, drug response; medium-high throughput.	Technical skills required, long differentiation period, matrix free (pro/con), lacks NPC, cost media & supplements. Random organization. Donor to donor variability
H1 hESCs (WA-01) & PSC-derived hepatic organoids	i. 200 µmol/L or 500 µmol/L oleic and palmitic acids	Steatosis (++) Inflammation (+) (ROS) Apoptosis (+) Fibrosis (-)	RNA	-	32553762	Complex model; multiple organoid lines characterised, drug response; medium-high throughput once HLO generated.	Technical skills required, differentiation period, matrix free (pro/con), lacks NPC, cost media & supplements. Random organization. Generating genotyped clonal HLO lab intensive.
3D human models - ALD - spheroid							
dHepaRG	iv. 100 mM ethanol, up to 72 h	Steatosis (+) Inflammation (+) (ROS) Apoptosis (+) Fibrosis (-)	CYP1B1 induction	-	32738395	Complex model; cheap; simple; can be CRISPR edited; medium-high throughput.	Lacks NPC; no matrix.
3D human models ALD - organoid							
H9 hESC & hFLMC-derived hepatic organoid	iv. 100 mM ethanol, 7 days	Steatosis (+) Inflammation (+) (ROS) Apoptosis (+) Fibrosis (++)	ADH1A/B induction	-	31628434	Complex model; can be CRISPR edited; medium-high throughput.	Technical skills required, long differentiation period. Random organization. hepatocyte and fibroblasts only; no matrix.
Human adipose stromal/stem cell-derived multicellular liver organoids (hALOs)	iv. 100 mM ethanol, for 72 h	Steatosis (+) Inflammation (+) Apoptosis (+) Fibrosis (+)	ADH1A/B induction	-	38749443	Complex model; can be CRISPR edited; medium-high throughput.	Technical skills required, long differentiation period, hepatocytes only, lacks NPC; no matrix.

ALD, alcohol-associated/related liver disease; hESC, human embryonic stem cell; HLO, hepatocyte liver organoid; HSCs, hepatic stellate cells; MASLD, metabolic dysfunction-associated steatotic liver disease; MetALD, metabolic and alcohol-associated/related liver disease; NPCs, non-parenchymal cells; PHH, primary human hepatocytes; PSC, pluripotent stem cells; SLD, steatotic liver disease.

- not altered or not tested

+ steatosis analysed by BODIPY.

++ steatosis analysed by BODIPY and triglycerides.

+ fibrosis analysed by a single output.

++ fibrosis analysed by multiple outputs.

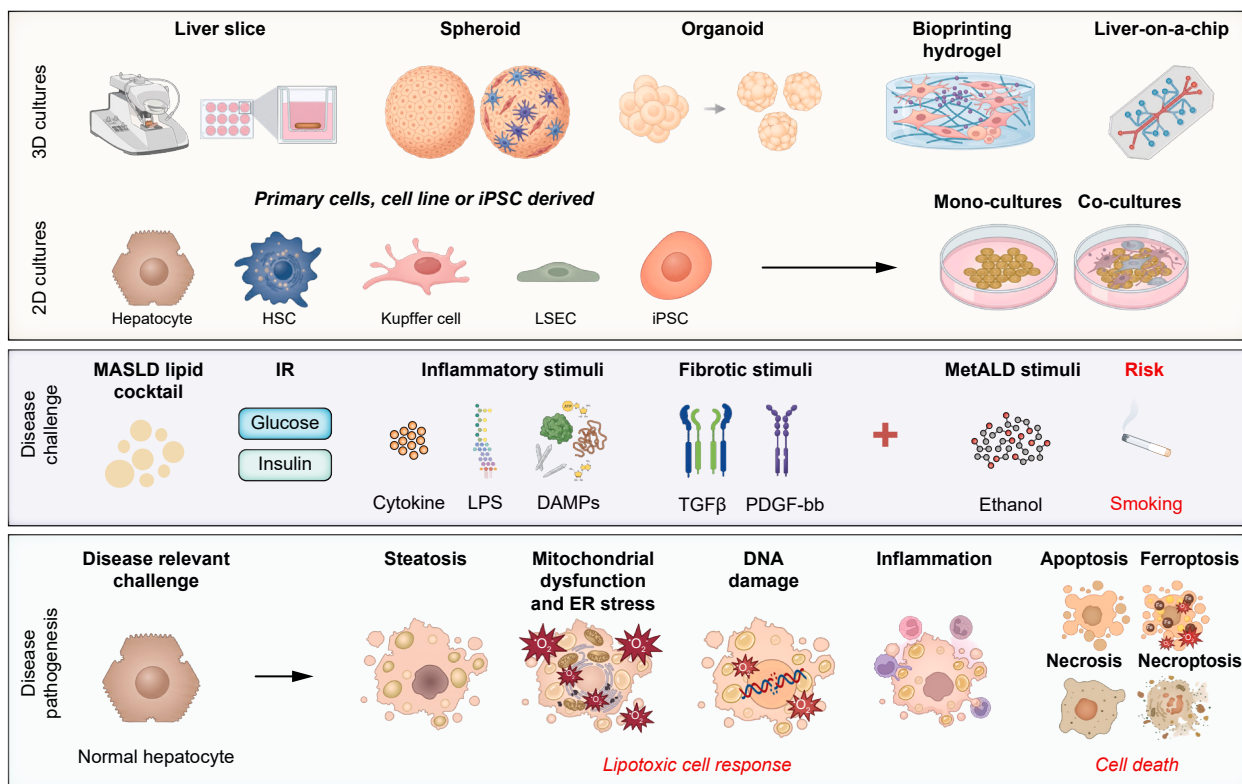


Fig. 3. *In vitro* and *ex vivo* experimental models to study SLD. *In vitro* and *ex vivo* systems enable controlled interrogation of disease-relevant pathways across increasing levels of cellular complexity. Upper panel illustrates commonly used 2D and 3D culture platforms, including precision-cut liver slices, spheroids, organoids, bioprinted hydrogel systems, and liver-on-a-chip devices. The middle panel summarises disease-relevant stimuli used to model distinct SLD subtypes, including MASLD-associated lipid overload and insulin resistance (IR), inflammatory signals (e.g. cytokines, LPS, DAMPs), fibrotic mediators (e.g. TGF β , PDGF-bb), and MetALD-related challenges such as ethanol exposure, as well as additional risk modifiers. The lower panel depicts key cellular and molecular disease phenotypes that can be assessed in these systems, including steatosis, mitochondrial dysfunction and endoplasmic reticulum (ER) stress, DNA damage, inflammation, and regulated cell death pathways (apoptosis, necrosis, necroptosis, and ferroptosis). Collectively, these platforms provide complementary approaches to dissect SLD pathogenesis and to evaluate therapeutic interventions in a human-relevant context. DAMPs, damage-associated molecular patterns; LPS, lipopolysaccharide; MASLD, metabolic dysfunction-associated steatotic liver disease; MetALD, metabolic and alcohol-associated/related liver disease; SLD, steatotic liver disease.

Zeigerer and collaborators recently introduced a robust 3D human steatotic hepatocyte *in vitro* system (collagen sandwich culture system) that successfully replicates key features of MASLD, including insulin resistance, mitochondrial dysfunction, and inflammation (TNF- α /TGF- β secretion into the medium). This model's high phenotypic resolution and clinical relevance make it an excellent pre-screening platform for identifying and validating therapeutic targets for MASLD.¹⁹⁹

- Human iPSCs** are generated by directly reprogramming somatic cells and can be used to produce a large number of liver cells (e.g. hepatocytes, cholangiocytes, liver sinusoidal endothelial cells [LSECs], Kupffer cells [KCs] and hepatic stellate cells [HSCs]) and a potentially unlimited cell source to model MASLD.^{187,200–202} Human iPSCs can be generated from individual patients with different genetic backgrounds, disease severity, and comorbidities (e.g. obesity, T2D, dyslipidaemia). Notably, this allows us to study the impact of specific genetic variants (e.g. *PNPLA3* I148M).²⁰³ However, high associated culturing costs, difficulty reproducing phenotypes across lines, and a lack of consistency in differentiation are limitations of iPSCs that have prevented widespread use to date.

While 2D monoculture MASLD models offer simplicity and are valuable for initial mechanistic studies, their primary

drawback is a lack of crucial cell-cell and cell-ECM interactions, leading to altered cellular functions. To address this, co-culture systems incorporating other liver cell types (e.g. HSCs, KCs, LSECs) are being developed to create a more representative *in vitro* environment.^{184,189,200} Despite these limitations, 2D models remain a fundamental basic tool for early-stage MASLD research.

3D cell cultures

While 2D cultures have been fundamental in MASLD research, 3D spheroid and organoid models offer significant advantages for studying the complex pathophysiology of MASLD by providing a more physiologically relevant and predictive platform that better mimics the human liver environment and disease progression (Table 5).^{184,204–208}

- Spheroids** are spherical 3D constructs, the simplest of which consist of aggregated hepatocytes, often derived from immortalised hepatic cell lines (HepG2/HepaRG), differentiated embryonic stem cells, PSCs, or PHHs obtained from healthy or diseased livers (e.g. MASL or MASH livers).^{209–211} More comprehensive models of MASL-MASH progression are frequently engineered by incorporating non-parenchymal cells (NPCs), such as HSCs, KCs/macrophages, and endothelial

Table 5. Complex 3D human *in vitro/ex vivo* models of SLD.

Model type	Cell(s) or tissue	Commercially available	Culture duration	Primary components known to induce damage	MASH ± fibrosis	Relevance to human	Tested drug response	Key references (PMID)
				i. Lipid ± sugar ± insulin ii. Inflammatory stimuli iii. Fibrotic stimuli iv. Ethanol stimuli				
Bioprinting, microfluidics & Liver-on-a-chip (LOC) devices - High translational relevance but typically require specialist equipment and/or access to fresh human tissue								
MASLD - bioprinted primary cells	PHH ± liver NPCs; KC & HSC ± LSEC, from healthy and/or NASH-diseased donors	Yes	Up to 21 days	i. 3 mM palmitic acid & oleic acid 11 mM glucose + fructose (ratio not stated) ii. 10 µg/ml LPS	Steatosis (++) Inflammation (-) Apoptosis (-) Fibrosis (++)	RNA	+	38158078; 39775546
MASLD - microfluidic devices	NASH-on-a-chip (Custom fabricated device containing PHH, KC, LSEC and HSC)	Yes	Up to 14 days	i. 0.66 mM oleic and 0.33 mM linoleic acid ii. 10 µg/ml LPS	Steatosis (+) Inflammation (+) Apoptosis (+) Fibrosis (++)	-	+	33553970
MASLD - LOC model	PHH ± NPC (KC and/or HSC)	Yes	Up to 15 days	i. 600 µmol/L of oleate acid and palmitate acid (2:1 mix) or HEP-FAT medium with physiologically relevant quantities of sugar & insulin (proprietary to CN Bio Innovations) ii. 0.5 ng/ml LPS	Steatosis (++) Inflammation (+) Apoptosis (-) Fibrosis (++)	-	+	31909357; 32694734
Precision cut liver slices - High translational relevance but typically requires specialist equipment and/or access to fresh human tissue								
MASLD - Human PCLS	Fresh human liver tissue	No	Up to 4 days	i. Palmitic acid (240 µM) & Oleic acid (480 µM) 61 mM glucose + 5 mM fructose 1 nM insulin	Steatosis (++) Inflammation (+) Apoptosis (-) Fibrosis (+)	RNA	-	38474754
MASLD/ALD/MetALD - Human PCLS	Fresh human liver tissue	No	Up to 5 days	i. 0.1 mM oleic and 0.1 mM linoleic acid 10 µg/ml insulin ii. 10 µg/ml LPS iv. 250 mM ethanol (or ethanol only for ALD)	Steatosis (++) Inflammation (+) Apoptosis (+) Fibrosis (++)	-	-	38203321
ALD	Fresh human liver tissue	No	Up to 72 hours	iv. 100-250 mM ethanol	Steatosis (-) Inflammation (+) Apoptosis (+) Fibrosis (-)	RNA	-	23300006 30515676
3D genetic models - Allow the study of disease and drug screens to be performed in the context of genetically relevant drivers of MASLD								
SLD - primary cells	PHHs (Wild type or heterozygous TM6SF2 E167K variant donors)	Yes	Up to 10 days	i. Up to 320 µM FFA 1:1 mix palmitic acid and oleic acid 5.5 mM glucose 100 pM insulin	Steatosis (++) Inflammation (-) Apoptosis (-) Fibrosis (-)	-	-	31406127
MASLD - hepatic cell lines	HepG2 and LX-2 cells (homozygotes PNPLA3 I148M variant)	Yes	48 hours	i. Palmitic acid and oleic acid 500 µM (1:2) conjugated to BSA ii. 10 ng/ml TGF-β or 10 ng/ml PDGF	Steatosis (-) Inflammation (-) Apoptosis (-) Fibrosis (+)	-	+	30986904; 33036387

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Table 5. (continued)

Model type	Cell(s) or tissue	Commercially available	Culture duration	Primary components known to induce damage			Key references (PMID)
				i. Lipid ± sugar ± insulin	ii. Inflammatory stimuli	iii. Fibrotic stimuli	
MASLD/MetALD - Human iPSCs	hiPSC differentiated Hepatocytes (CRISPR generated PNPLA3 gene knockout or PNPLA3 I148M variant)	Yes/ collaboration	Up to 7 days	i. Oleic acid and/or palmitic acid and/or stearic acid (250 µM of each)			34288010

ALD, alcohol-associated/related liver disease; hESC, human embryonic stem cell; HSCs, hepatic stellate cells; LOC, liver-on-a-chip; MASLD, metabolic dysfunction-associated steatotic liver disease; MetALD, metabolic and alcohol-associated/related liver disease; NPCs, non-parenchymal cells; PHH, primary human hepatocytes; PCLS, precision cut liver slice; PSC, pluripotent stem cells; SLD, steatotic liver disease.

- not altered or not tested

+ steatosis analysed by BODIPY.

++ steatosis analysed by BODIPY & triglycerides.

+ fibrosis analysed by a single output.

++ fibrosis analysed by multiple outputs.

cells. These advanced spheroids exhibit enhanced steatogenesis, collagen production indicative of fibrogenesis, and upregulation of genes involved in fibrosis progression.^{205,211–214} Spheroid formation most commonly utilises low-attachment plates coated with non-adhesive materials like agarose or specialised polymers, or hanging droplets, which compel cells to aggregate. The resulting spheroids are then incubated in a MASH-inducing cocktail, typically containing OA, PA, LPS, and TGFβ1, usually between 7–14 days post-spheroid formation.^{204,215–219} Cell viability, assessed by ATP content, demonstrates the stability of these spheroids for over 21 days. Further analyses of these spheroids include measuring lipid accumulation, inflammatory chemokines, and fibrogenic markers. The function of specific genes within defined cell populations in these human liver spheroids can be investigated using siRNA knockdown approaches. Consequently, these models have been instrumental in elucidating the roles of key MASLD hallmarks (e.g. lipid accumulation, DNL, insulin resistance, ER stress, inflammation, and cell death).^{211,213,215,216,218,219} Several studies have demonstrated the relevance of these 3D spheroids for investigating the mechanism of action of drug candidates targeting DNL, apoptosis, senescence, and fibrogenesis.^{213,215,216,220–223} Spheroid models have also enhanced our understanding of genetic variants and proven promising for investigating clinically relevant associations with MASLD.^{128,224–226} Recently, Brenner *et al.* made a major advance by developing 3D MASH human liver spheroids containing all the liver cell types at physiological concentrations observed in human MASH livers.²⁰⁷ Challenging these liver spheroids with a MASH cocktail induced steatosis, inflammation and fibrogenesis, but these changes could be reversed upon withdrawal of the MASH cocktail from the culture media. Using this model, they identified a novel therapeutic target for MASLD (RUNX1/2-SERPENTINE axis).²²⁷ To model MetALD, the human liver spheroids were challenged with a MetALD cocktail (MASH cocktail + ethanol) to promote alcohol-induced hepatocellular injury, lipid accumulation and fibrogenesis.^{207,228} To model ALD, a 72-hour ethanol challenge in dHepaRG cell organoid/spheroids promoted alcohol-dependent changes comparable to those observed *in vivo*, including induction of CYP2E1 expression, mitochondrial dysfunction, reactive oxygen species production, mitophagy and steatosis.²²⁹ While spheroid models are cost-effective, they possess inherent drawbacks; specifically, size variability and a tendency to aggregate into large clumps can impede nutrient and oxygen diffusion, complicating scalability.

- **Bioprinting** is an emerging technology being developed by researchers in the field to generate spatially controlled, multi-cellular models of MASLD for studying disease biology and testing drugs. Recently, Brenner *et al.* described two approaches to create 3D bioprinted liver tissue models of NASH/MASH.^{181,185} In both models, bioprinted hydrogels contained human hepatocytes with one or more type of NPC (HSCs, LSECs or KCs) in physiological ratios. In the inducible human 'MASH in a dish' model, bioprinted healthy human hepatocytes, LSECs, HSCs, and KCs were challenged with PA and OA, LPS, glucose, and fructose for 21 days to induce molecular and histological features of MASH, including steatosis, inflammation, and fibrosis.¹⁸¹ In the second model, human hepatocytes were bioprinted with NPCs from either healthy

donors or NASH/MASH donors, with the latter creating a chimeric model that spontaneously develops a NASH phenotype.¹⁸⁵ Advantages of bioprinting include reproducible cultures and the potential to modify the bioink and create pre-defined tissue structures to regulate the spatial organisation of the different cell types.

- **Human liver organoids (HLOs)** emerge by embedding liver-derived stem cells, progenitor cells, or tissue-resident cells within a hydrogel ECM. Their development protocols enable precise control over the multicellular aggregate's spatial organisation, more closely mimicking the heterogeneity and organotypic architecture found *in vivo*.^{19,184,208,230} Their ability to be maintained in culture for extended periods allows for the study of chronic MASLD progression.^{19,184,206,231} Human PSC-derived foetal liver-like organoids with hepato-haematopoietic co-development enable the coordinated development of hepatic and immune lineages, providing a physiologically relevant platform to interrogate immune-metabolic interactions central to MASLD pathogenesis.²³²

Liver organoid applications include: (1) dissecting the diverse phenotypes arising from varying modelling conditions, such as exposure to different lipids, glucose levels, and other metabolites;^{233–235} (2) pinpointing the intracellular reactions that drive key pathological features of MASLD, including steatosis, inflammation, and fibrosis;^{200,236–238} (3) elucidating the complex intercellular interactions occurring between crucial liver cell types like hepatocytes, macrophages, LSECs, and HSCs;^{200,235,239} (4) delineating the genetic underpinnings of metabolic mechanisms;^{233,234,240–242} and (5) evaluating drugs targeting DNL, fibrogenesis, inflammation, and DNA damage in MASLD.^{233,235,236,242,243}

Notably, "en masse" phenotyping of organoids has recently emerged as a powerful approach for advancing precision medicine, particularly in addressing the complexity and heterogeneity of MASLD. Hendriks *et al.* developed FatTracer, a CRISPR-based screening platform using human foetal liver organoids, to pinpoint genes influencing hepatic steatosis and reveal potential drug targets.²³³ This platform effectively identified key genes, including FADS2, critical in the development of steatosis. Similarly, Kimura *et al.* established a system to generate and analyse a large number of liver organoids derived from multiple human donors.²⁴² This "en masse" approach allowed them to link genetic variations to specific organoid phenotypes relevant to MASH.

ALD can be modelled in hepatic organoid cultures by supplementing the culture media with ethanol. Organoids comprised of human embryonic stem cell-derived hepatocyte and human foetal liver mesenchymal cells, showed increased CYP2E1 activity and reactive oxygen species production, developed hepatocellular injury, and elevated expression of inflammatory and fibrogenic markers in response to alcohol challenge.²⁴⁴ Similarly, hepatic steatosis, inflammation and induction of ethanol metabolising enzymes could be induced in human adipose-derived hepatocellular organoids challenged with ethanol.²⁴⁵

Despite their promise, incomplete cell differentiation and random organisation remain key limitations that hinder the reproducibility of organoid models. The use of Matrigel for organoid formation is a further limitation due to the batch-to-batch variation in protein composition and mechanical

properties, e.g. matrix stiffness, and the mouse sarcoma origin which presents ethical and immunogenicity considerations.²⁴⁶ Achieving complete differentiation and standardised architecture necessitates further refinement of current methods. Recently, Arino *et al.* described a new method for generating hepatocyte and biliary organoids from liver biopsies obtained from patients with early- and late-stage ALD.²⁴⁷ These organoids retain the molecular features of ALD in culture and were responsive to disease stimuli and prednisolone therapy.²⁴⁷ In the future, similar approaches could be applied to create organoids from SLD, MASLD and MetALD.

Liver-on-a-chip models/microphysiologic system

MASLD organoid and spheroid models face challenges in fully replicating the liver's complex immune environment, lack interactions with the ECM, do not perfectly mimic human liver physiology, and show variability depending on the cell donor. Fibrosis is a key driver of liver disease pathology, and with the emerging concept of 'hot' and 'cold' fibrosis, ECM composition appears to play a central role in shaping the hepatic immune environment, regulating scar matrix resolution, and potentially predicting therapeutic response.²⁴⁸ Therefore, the quantity and quality of ECM composition and stiffness for *in vitro* models is an important factor to consider. *In vitro* liver disease models that combine natural scaffolds or decellularised tissue²⁴⁹ with liver cells offer one approach to model aspects of liver disease biology in the context of a physiologically relevant ECM.^{143,250,251} Recent studies show that background liver disease (steatosis) and donor age – ECM density seems to increase with age – can influence the phenotype and function of exogenously added cells, for example the differentiation of iPSC-derived hepatocytes.²⁵⁰ ECM derived from steatotic livers has been shown to increase the expression of lipid metabolism genes in engrafted iPSC-derived hepatocytes and PHHs. Interactions between ECM disease status and donor genetics can also modulate cell phenotype, with mitochondrial dysfunction and decreased antioxidant capacity observed in PNPLA3 I148M variant HSCs being accentuated when cultured on cirrhotic ECM.¹⁴³

Liver-on-a-chip (LoC) models are advanced *in vitro* systems designed to mimic the complex microenvironment and functions of the human liver.^{252–258} These models can also be referred to as a microphysiologic system.²⁵⁹ These microfluidic devices integrate liver cells with controlled fluid flow allowing for the modelling of blood or other fluid content (e.g. nutrients; oxygen, cytokines, therapeutic agents). The dynamic culture conditions in LoC systems lead to improved hepatocyte differentiation, maintenance of liver-specific functions (e.g. albumin production, cytochrome P450 activity) and extended cell viability. These models have emerged as promising platforms for replicating the complex hepatic microenvironment to study MASLD pathophysiology.^{187,231,260–262} These microfluidic systems integrate human hepatocytes (hiPSCs, HepG2, HepaRG, PHH) and NPCs. Freag *et al.* created a "NASH-on-a-chip" using the four main human liver cell types (hepatocytes, KCs, LSECs, and HSCs) which maintained liver function for at least 2 weeks. Exposure to a fatty environment on the chip mimicked the development of NASH/MASH features like intracellular lipid accumulation, hepatocellular ballooning, HSC activation, and elevation of inflammatory and profibrotic

markers.²⁶³ An alternative LoC platform, incorporating four primary mouse liver cell populations and primary mouse blood cells, provides a sophisticated microfluidic environment that effectively recapitulates the complex cellular interactions between metabolic injury and immune-mediated therapeutic responses (e.g. resmetirom vs. lanifibranor) in MASLD.²⁶⁴

Using a LoC “3D NASH microtissue” model, comprised of cryopreserved human hepatocytes, KCs, and HSCs, cultured in a Hep-Fat media, Vacca *et al.* identified BMP8b as a novel driver of MASH progression.²⁶⁵ This highlights how LoC models can be employed to elucidate new disease mechanisms. Interestingly, culturing liver cells with the *PNPLA3 I148M* mutant on a LoC device showed increased inflammation under fatty acid treatment, highlighting the device’s usefulness in predicting how genetic changes affect MASLD liver disease progression.²⁶² The potential to integrate patient-derived cells into LoC devices opens avenues for studying individual responses to therapeutic interventions.^{261,263,266} Importantly, advancements have been made to develop multi-organ-on-a-chip systems that connect liver tissue units with other relevant organs like adipose tissue or the intestine, providing a more holistic view of the systemic metabolic dysregulation in MASLD.^{267,268} In addition, ALD was modelled using a LoC model comprised of PHH, LSEC and KC in a sandwich culture.²⁶⁹

However, designing and fabricating sophisticated LoC devices can be complex and expensive. Scaling these platforms for high-throughput drug screening presents a significant challenge, primarily because the enclosed flow system inherently limits the number of drugs that can be independently tested. Sophisticated LoC systems can effectively mimic aspects of human liver disease and validate findings from circulating protein analysis, strengthening the potential of such *in vitro* models for translational research.

Furthermore, an urgent need remains for sophisticated multi-organ systems capable of capturing the systemic nature of SLD and its associated comorbidities. Despite the inherent technical challenges, including the harmonisation of disparate culture media and the mechanical (and tissue) engineering complexity of multi-organ integration, addressing these gaps is essential to improve the clinical translatability of *in vitro* models.

Precision-cut liver slices

Precision-cut liver slices (PCLSs) offer a significant advantage for studying MASLD by providing an *ex vivo* model that closely mimics the liver parenchyma. Unlike simpler cell culture systems, PCLSs retain the intricate cellular architecture, diverse cell populations, ECM and crucial cell-cell interactions of the native liver. This allows researchers to investigate the complex interplay of lipid metabolism, inflammatory responses, and fibrotic processes that drive MASLD pathogenesis in a more physiologically relevant context.^{187,270–273} These slices offer the advantage of multiple readouts from a single sample, analysing both the tissue and culture medium. The tissue used for human PCLS preparation is usually obtained from partial hepatectomies, surgical liver specimens (excess liver tissue not required for diagnosis), explanted livers, or non-transplantable tissue. To prepare PCLSs, liver samples can be perfused with a cold solution to minimise ischaemic

damage before being embedded in agarose and precisely sliced (150–250 μm)²⁷⁰ or cored and processed using a tissue slicer.²⁷⁴ These slices are then cultured in William’s E medium plus supplements, using static, dynamic, or bioreactor systems. Static cultures exhibit a limited lifespan (48–72 hours) due to hypoxia and cell death, which can be alleviated by employing oxygen carriers, rocking, shaking, or perfusion bioreactors to enhance oxygen and nutrient supply or culturing static or agitated PCLS under a carbogen (95% O₂, 5% CO₂) atmosphere.^{270,275,276} Of note, ATP levels alone or combined with albumin or urea production, serve as an indicator of PCLS functionality, remaining stable for approximately 5 days before decreasing in parallel with the degradation of the cultures’ metabolism. Healthy PCLS cultured in medium enriched with sugars (glucose and/or fructose), insulin, fatty acids $\pm$ an inflammatory stimulus such as LPS, can develop key features of MASLD (e.g. steatosis/inflammation, and even fibrosis).^{273,275,277–279} Short-term alcohol exposure in the absence of lipid upregulates the expression of inflammatory cytokines, chemokine receptors and adhesion molecules, and enhances lymphocyte infiltration in human PCLS.²⁸⁰ To model ALD or MetALD, Palma *et al.* cultured normal human PCLSs with ethanol alone or in combination with fatty acids (oleic & linoleic), LPS and ethanol for up to 5 days to induce hepatotoxicity, inflammation and fibrosis.²⁸¹ In these models, the combination of free fatty acids and LPS with ethanol challenge accentuated the hepatocellular injury and inflammatory response.²⁸¹

The acute effects of alcohol have been tested in human PCLSs, with short-term ethanol challenge increasing expression of alcohol metabolising enzymes, adhesion molecules, inflammatory cytokines and chemokines.²⁸⁰ Whilst a separate study reported hepatocyte mitochondrial enlargement after 72-hour ethanol challenge.²⁷³ In addition, CYP2E1, ADH1A, and ADH3 expression, as well as hepatocellular injury, were induced in organoid and PCLS models, as expected during alcohol metabolism. Alcohol has been shown to be actively metabolised in rat liver slices and the addition of 4-methylpyrazole blocked ethanol metabolism and the induction and activity of CYP2E1.²⁸²

Alternatively, PCLSs can be produced from MASLD livers.^{273,283,284} In these different settings, PCLSs have been used to explore multicellular mechanisms of MASLD, notably the role of FXR and PPAR α signalling.^{285,286} Researchers have also used this system to assess the potential of drug candidates targeting metabolic disturbances and fibrogenesis.^{276,284,287–294} Recently, Mabire *et al.* demonstrated that mucosal-associated invariant T cell inhibition promotes liver fibrosis regression via macrophage phenotype reprogramming.²⁸⁴ Interestingly, the use of PCLSs has underlined clear differences in the fibrotic process and the efficacy of antifibrotic compounds between species, explaining why several drugs with proven antifibrotic effects in animal studies are not effective in humans.²⁷⁸

However, research using PCLSs faces limitations, including variability in tissue source and challenges in maintaining long-term viability and function beyond 48–96 hours.^{187,231} Moreover, the mechanical slicing has been shown to induce damage potentially confounding MASLD-specific observations.²⁷⁶ In fact, this injury triggers a rapid wound-healing and regenerative response, leading to the activation of HSCs.²⁷¹

Advances in culture techniques have extended PCLS viability and mitigated some issues.²⁹⁵ These limitations necessitate careful experimental design and interpretation to maximise the utility of PCLSs in MASLD research.

Additional considerations for experimental design of *in vitro* models

Sex, gender, and sex hormones can influence liver physiology, hepatic zonation, and metabolism, and may contribute to increased susceptibility to different liver disease aetiologies.⁹⁶ Sex-specific differences are observed in MASLD, with higher prevalence in males than females, largely due to the protective role of oestrogens in suppressing hepatic lipid accumulation, inflammation, and fibrosis. However, MASLD prevalence and the risk of accelerated fibrosis significantly increases in post-menopausal women.^{94,296,297} For *in vitro* studies, the implications for hepatic sexual dimorphism should be considered in the experimental study design and, where possible, primary liver cells, human iPSCs and PCLSs from both male and female donors should be used to increase translatability.

Recommendations

- While current *in vitro* models of SLD are increasingly well characterised and validated against clinical datasets, a critical gap persists: unlike established *in vivo* frameworks, a direct comparison between *in vitro* platforms to establish standardised benchmarks for fidelity has not been performed. However, prioritising the adoption of 3D models, such as organoids and PCLSs, which more accurately recapitulate the complex *in vivo* microenvironment, is recommended.
- As alternatives, 2D primary hepatocytes or similar entities, such as dHepaRG cells or AML12 cells, can be used.

What is needed to faithfully phenocopy the human condition

Humanised liver models – the next generation

Increasing efforts are being made to integrate human hepatocytes and NPCs into a mouse liver environment to enable more faithful modelling of human liver disease. In recent years, “humanised” mouse liver models have been established to investigate liver function and disease, mainly, but not solely, applying the MISTRG6 mouse model system.²⁹⁸ MISTRG6 mice harbour knock-in alleles encoding human M-CSF (CSF1), IL-3, SIRPα, thrombopoietin, GM-CSF (CSF2), and IL-6, each inserted into their respective mouse loci, on an immunodeficient *Rag2^{-/-}Il2rg^{-/-}* background.²⁹⁸ To permit efficient hepatocyte engraftment, these mice are additionally rendered fumarylacetoacetate hydrolase-deficient (*Fah^{-/-}*).²⁹⁸

Using this system, Kaffe *et al.* transplanted human CD34⁺ foetal liver cells together with human hepatocytes into 2-day old MISTRG6; *Fah^{-/-}* mice, resulting in the generation of chimeric livers with human hepatocytes alongside human immune cells, LSECs, and HSCs.²⁹⁸ This approach enables the

coordinated reconstitution of human parenchymal and non-parenchymal compartments within the liver, providing a more physiologically relevant context for studying human-specific liver biology.²⁹⁸ Notably, this model supports key aspects of human hepatic metabolism and immune interactions and has been shown to recapitulate features of human SLD.²⁹⁸ An alternative and simpler approach is to use chimeric mice engrafted exclusively with human hepatocytes.^{299–301}

For ALD research, humanised CYP2E1 mouse models have been widely employed and consistently demonstrate that expression of human CYP2E1 exacerbates ethanol-induced liver injury, characterised by increased oxidative stress, inflammation, and progression towards fibrosis.^{302–305} These models provide strong *in vivo* evidence that CYP2E1 is a key mechanistic driver of ALD pathogenesis and enhance the translational relevance of preclinical findings.^{302–305}

Ultimately, humanised mouse models represent a significant advance in liver research, enabling more accurate studies of human SLD and therapeutic interventions.

Testing current golden-standard models for systemic effects

With humanised and non-humanised models in mind, it will be of utmost importance to further test the systemic effects of different diets (e.g. WD GAN/NTF or FPC), including their impact on the integrity of other organs such as the heart, kidney, brain, adipose tissue, pancreas and the vasculature. These experiments may open new avenues for identifying novel therapeutic targets.

Models mimicking MASLD in normal-weight patients

As mentioned, the development of MASLD in individuals without systemic obesity is not a novel phenomenon but is observed both in Asian countries, where it is more prevalent, and in Europe and the USA.⁶⁴ Thus, it is necessary to understand the metabolic vulnerability of patients with MASLD presenting with a ‘normal’ body mass index and how they would respond to distinct therapies. Unlike classical obesity or MASLD mouse models, current MASLD models in which mice remain of normal weight are based on largely metabolically healthy animals, such as those fed a modified atherogenic diet, where mice develop dyslipidaemia but show no signs of systemic insulin resistance or glucose intolerance.^{65,132,158} Appropriate genetic and lifestyle-related MASLD models in normal-weight mice are needed in the near future to study disease pathogenesis, as well as to identify prognostic markers and preventive strategies.

Mechanisms and models of MASH-fibrosis regression

MASH-induced fibrosis contributes to liver dysfunction as well as the transition to MASH-HCC. It has become evident that advanced stages of fibrosis are in part reversible;^{306–308} however, the exact mechanisms and the distinct liver environment that would favour such a development have remained largely unknown. Thus, it will necessitate distinct models of MASH-induced fibrosis that enable the study of fibrosis regression. In addition to conventional dietary models that are time-consuming, several dietary setups have been published that enable rapid and efficient development of MASH-related

fibrosis and have been used to study fibrosis regression.^{9,309} Nevertheless, models of “MASH cirrhosis with portal hypertension” are currently lacking and therefore future studies are needed to understand the progression to decompensated cirrhosis and acute-on-chronic liver failure.

Relevance to human pathology

The histological spectrum of human SLD comprises simple steatosis, which refers to accumulation of triglycerides within >5% of hepatocytes, or steatosis with lobular inflammation that may progress to steatohepatitis with or without fibrosis, and finally cirrhosis. Steatosis is usually macrovesicular (Fig. 4A,B), consisting of one large intracytoplasmic fat droplet displacing the nucleus or many smaller distinct droplets with a centrally located nucleus (small droplet macrovesicular steatosis). Microvesicular steatosis, when numerous tiny fat droplets give an indistinct foamy appearance in the cytoplasm, may co-exist in many cases (mixed micro- and macrovesicular steatosis). Pure microvesicular steatosis is not a feature of human MASLD but may rarely occur in ALD (alcohol-related foamy degeneration). In human MASLD, lobular inflammation is usually mild and characterised by a chronic or mixed inflammatory infiltrate, composed of lymphocytes, macrophages, plasma cells, occasional eosinophils and/or neutrophils (Fig. 4A,B). The minimal diagnostic histological criteria of steatohepatitis are steatosis,

lobular inflammation and hepatocyte ballooning indicative of cell injury. Classical ballooned hepatocytes are enlarged ($\geq 2\times$ the size of normal hepatocytes) with a round shape and clear or flocculent/reticulated cytoplasm (Fig. 4A–C). Chronic portal inflammation may be seen in human MASLD and is usually mild, increasing in severity in MASH and advanced fibrosis stages. In addition, changes in intrahepatic immune cells are observed even before measurable histological inflammatory foci are present, including a shift in Kupffer cells towards a proinflammatory phenotype and platelet secretion of α - and dense granules.^{136,310} Consensus definitions of histological criteria for human MASLD/MASH, along with an atlas of agreed histological features and mimickers, have recently been published.³¹¹

The diagnostic features of pre-cirrhotic adult MASLD and ALD show zone 3 (centrilobular) predominance, whereas in paediatric MASLD, zone 1 (periportal) accentuation of lesions is common. Fibrosis is not required for the diagnosis of MASH or alcohol-associated/related steatohepatitis (ASH) but when present in adults, it starts in zone 3 and is characteristically perisinusoidal/pericellular (Fig. 4D,F). With disease progression, portal/periportal fibrosis additionally develops (Fig. 4D), followed by bridging fibrosis (Fig. 4E), and finally cirrhosis. In rare cases of patients with MASH in the context of obesity and in paediatric MASH, isolated portal/periportal fibrosis without zone 3 perisinusoidal fibrosis may occur.³¹² Grading of

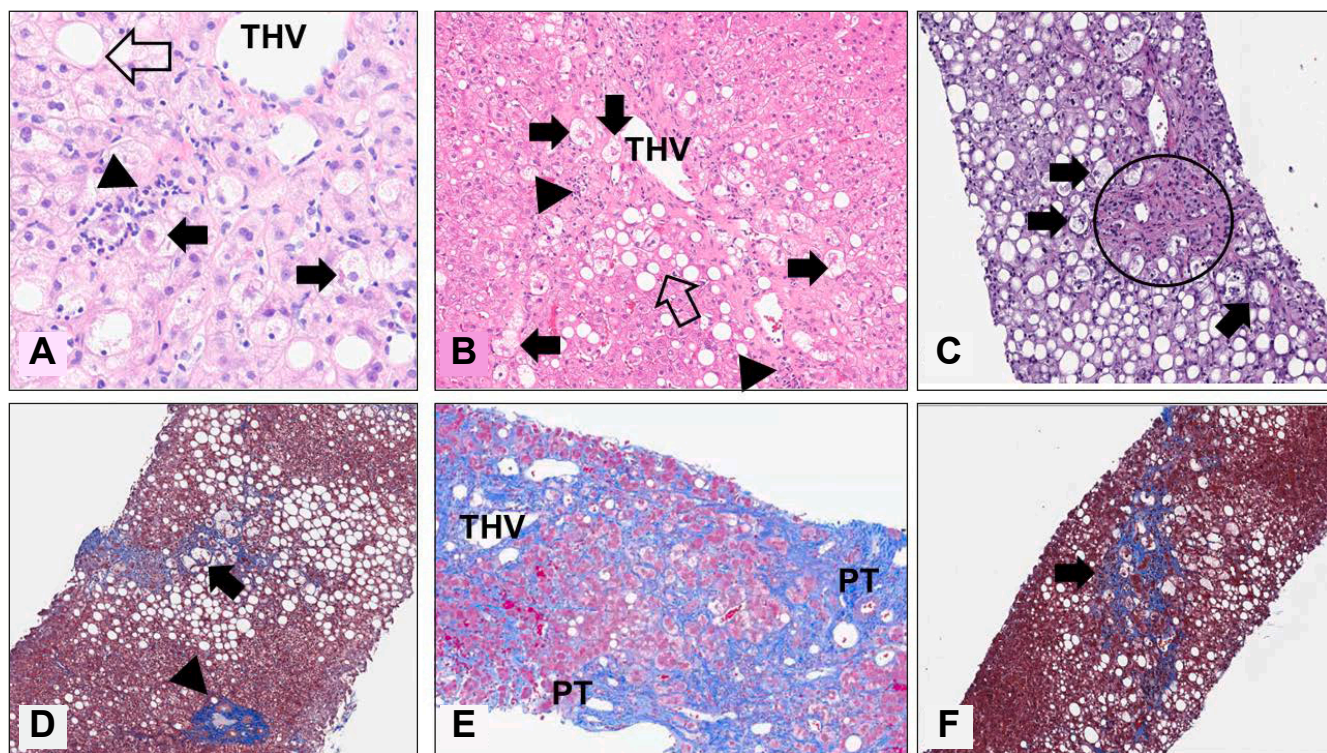


Fig. 4. Human adult MASLD pathology from needle liver biopsy material. (A) MASH: Zone 3 ballooned hepatocytes with Mallory-Denk bodies (black arrows), lobular inflammation with satellitosis (arrowhead) and macrovesicular steatosis (empty arrow) in MASH, H&E 200 $\times$; (B) ASH: Numerous ballooned hepatocytes (black arrows) in zone 3, lobular inflammation (arrowheads) and mild microvesicular steatosis (empty arrow), H&E, 100 $\times$; (C) MetALD: Veno-occlusive lesion (circle), ballooned hepatocytes (black arrows) and severe steatosis., H&E, 100; (D) MASH: Zone 3 sinusoidal fibrosis (black arrow), mild periportal fibrosis (arrowhead) and moderate steatosis with zone 3 and 2 accentuation, stage 2 NASH CRN/SAF, Masson's trichrome, 40 $\times$; (E) ASH: Dense and extensive pericellular/sinusoidal fibrosis and advanced bridging fibrosis, Stage 4Cp SALVE, Masson's trichrome, 40 $\times$; (F) MetALD: Dense pericellular/perisinusoidal fibrosis and steatosis with Zone 3 accentuation (black arrow), Masson's trichrome, 40 $\times$. ASH, alcohol-associated/related steatohepatitis; MASH, metabolic dysfunction-associated steatohepatitis; MetALD, metabolic and alcohol-associated/related liver disease; PT, portal tract; THV, terminal hepatic venule.

necroinflammatory activity and staging of fibrosis in human MASLD, but also in animal MASLD, is performed according to the NASH Clinical Research Network³¹³ or the SAF grading and staging system,³¹⁴ while for human ALD, the SALVE histological grading and staging system has recently been published.³¹⁵ The latter has not yet been applied to ALD animal models. Recently, EPoS, a seven-tiered fibrosis staging system has been proposed for human MASLD that could also be applied in MASLD animal models.³¹⁶ Fibrosis is often measured by a combination of histopathological stains, such as Sirius Red or Masson's trichrome, especially in preclinical models. Importantly, the vessel regions should be excluded in fibrosis quantifications.

Similarities vs. differences in MASH, ASH and MetALD

In humans, alcohol-related liver injury has specific histological features that, when present, can help in identifying the leading driver of SLD and should be reported to aid SLD subtyping. These features include sclerosing hyaline necrosis, characterised by dense perivenular fibrosis, presence of Mallory-Denk bodies (MDB) and hepatocyte necrosis in zone 3, resulting in the formation of large perivenular scars, veno-occlusive lesions (Fig. 4C), acute or chronic cholestasis, cholangiolitis and extensive microvesicular steatosis. In the absence of these changes, alcohol-related injury cannot be excluded based on histology, as histopathological studies on human MetALD features are currently lacking. However, it is generally accepted that the overall histopathological appearance of human MASH is usually milder than that of ASH. Non-specific features that may raise the suspicion of concurrent

alcohol-related injury in human MASH (MetALD), are severe neutrophilic lobular inflammation, extensive "satellitosis" (comprising of neutrophils surrounding ballooned hepatocytes), abundant MDB, and extensive dense pericellular/perisinusoidal fibrosis (Fig. 4F).³¹⁷

Differences in quality of fibrosis between MASH and ASH can be assessed using reticulin staining, with solid fibrosis composed of collagen I fibres being more common in ASH and lattice fibrosis rich in collagen III fibres characterising MASH.³¹⁸ Recently, fibrotic pattern analysis by digital pathology corroborated these results, showing heterogeneous distribution of fibrosis with thicker and longer collagen fibres in ALD compared to MASLD. In MetALD, fibre distribution resembled that of MASLD.³¹⁹

Comparative pathology between mice and humans

In mouse models of MASLD, severe steatosis (Fig. 5A,B), microvesicular steatosis, and milder lobular inflammation are more common compared with human MASLD. Features of steatosis and lobular inflammation are similar to human SLD, but ballooned hepatocytes are less frequent and more difficult to identify, mainly because changes in cytoplasmic quality and size are more subtle. Ballooned hepatocytes in mouse MASH are rounded with clear cytoplasm but are usually not markedly enlarged, and intracytoplasmic inclusions such as MDBs are less frequent than in human MASH (Fig. 5A,B). Portal inflammation is less frequent in mouse SLD models than in human SLD. Fibrosis develops and progresses in a similar manner to human SLD, with perisinusoidal/pericellular fibrosis followed by portal/peripoportal (Fig. 5C,D) and bridging

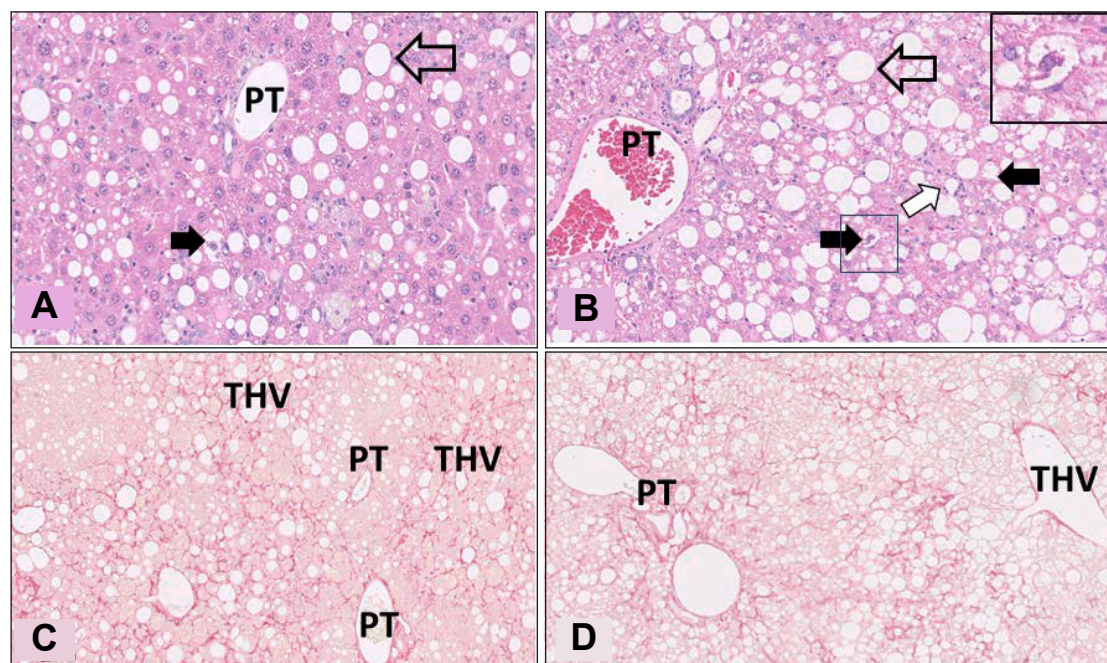


Fig. 5. Mouse MASLD pathology. (A) CDAHFD 12 weeks: Steatohepatitis with severe steatosis (empty arrow) and a rare, ballooned hepatocyte (black arrow), H&E 100 $\times$; (B) WD GAN 42 weeks: Steatohepatitis. Severe macrovesicular steatosis, hepatocyte ballooning (black arrows) and a lipogranuloma (white arrow), H&E 100 $\times$. Inset shows higher magnification of the ballooned hepatocyte within the quadrangle, containing eosinophilic Mallory-Denk body-like inclusions; (C) CDAHFD 12 weeks: Periportal and perisinusoidal fibrosis, stage 2 NASH CRN/SAF, Sirius Red, 50 $\times$; (D) WD GAN 42 weeks: Periportal and perisinusoidal fibrosis highlighted in red, stage 2 NASH CRN/SAF, Sirius Red, 50 $\times$. CDAHFD, choline-deficient, L-amino acid-defined, high-fat diet; MASLD, metabolic dysfunction-associated steatotic liver disease; WD GAN, Western diet Gubra Amylin NASH.

fibrosis leading to cirrhosis in some models. Recent data from the LITMUS comparative animal MASLD study had shown that some WD (GAN/NTF or FPC) models may show early zone 3 accentuation of cardinal histological features resembling human adult MASLD, while rare models may replicate zone 1 topography of paediatric MASLD, like *ob/ob* on 8-week chow diet.⁴⁹

The most important points to consider when choosing an animal model of adult SLD, independent of type, is that it recapitulates all the main histological features of human steatohepatitis (steatosis, lobular inflammation, hepatocyte ballooning, fibrosis), with zone 3 accentuation in early disease. To avoid overstaging SLD, fibrosis should be evaluated 3 mm away from the liver capsule.

***In vivo* vs. *in vitro* pathological differences**

From a histological perspective, *in vivo* models of SLD have the advantage of retaining liver tissue architecture, enabling evaluation of zonal topography of individual histological features and facilitating comparison with human MASLD and ALD histology.⁴⁷ The best *in vitro* alternative are PCLSs, which enable assessment of the liver parenchyma, architectural changes and identification of spatial relationships of SLD features.^{47,273,275,320}

Conclusion

In this EASL position paper, we summarise the most recent and relevant advances in clinical and basic research in the field of SLD, providing a framework to guide the selection and use of appropriate experimental SLD models. The introduction of a new nomenclature and emerging subtyping schemes has fundamentally redefined the diversity of SLD in humans. In parallel, clinical, molecular, and histopathological assessments of liver disease have been refined, supported by advances such as AI-assisted pathology, while increasing emphasis has been placed on cardiometabolic risk factors and multiorgan crosstalk in the definition and progression of SLD. These developments necessitate a corresponding re-evaluation of experimental SLD models, whether derived from *in vivo*, *ex vivo*, or *in vitro* systems. Accordingly, this position paper

describes and contextualises currently available SLD models in light of updated clinical definitions and guidelines. Particular emphasis is placed on models that best recapitulate key features of human disease, including histopathology, metabolic and immunologic signatures, and inter-organ interactions, and for which molecular and cellular markers support alignment with specific SLD subtypes.

Both the clinical disease spectrum and the experimental model landscape are highly dynamic and continue to evolve as understanding of SLD pathogenesis deepens. Experimental models will inevitably be adapted over time to better mirror human disease complexity, progression, and reversibility. Such evolution is essential to improve mechanistic understanding and to enable the development of more effective, subtype-specific therapeutic strategies.

Importantly, this EASL position paper is not intended to prescribe or restrict the use of specific SLD models, nor to discourage the development of novel approaches or the modification of existing models to better phenocopy human SLD. Rather, it reflects the current 'status quo' in the field, highlighting models that are best suited to address distinct disease characteristics while remaining accessible and broadly applicable. The goal is to support both established investigators and new entrants to the field in selecting appropriate experimental systems that align with contemporary standards and terminology. By offering recommendations, outlining key considerations for model selection, and promoting the use of more accurate disease classification, this position paper aims to promote greater standardisation, improve harmonisation of nomenclature, and enhance reproducibility in basic and translational SLD research; areas in which inconsistency remains a challenge.

Looking ahead, continued advances in biomedical research, including the expanding use of artificial intelligence, spatial profiling technologies, and integrative multi-omics approaches, will further refine human SLD subtypes. These developments will, in turn, drive the evolution of more sophisticated and clinically relevant experimental models. This EASL position paper is intended to serve as a foundation for that ongoing process.

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Abbreviations

ADH, alcohol dehydrogenase; ALD, alcohol-associated/related liver disease; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ASH, alcohol-associated/related steatohepatitis; AST, aspartate aminotransferase; CCl₄, carbon tetrachloride; CDAHFD, choline-deficient, L-amino acid-defined, high-fat

diet; CDHFD, choline-deficient high-fat diet; CVD, cardiovascular disease; CYP2E1, cytochrome P450 2E1; EASL, European Association for the Study of the Liver; FPC, fructose-palmitate-cholesterol; FXR, farnesoid X receptor; GAN, Gubra Amylin NASH; GGT, gamma-glutamyltransferase; GLP-1, glucagon-like peptide 1; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HFD, high-fat diet; HFHS, high-fat, high-sucrose; HLO, human liver organoid; HSCs,

hepatic stellate cells; iPSCs, induced pluripotent stem cells; KCs, Kupffer cells; LDC, Lieber-DeCarli; LDL, low-density lipoprotein; LoC, liver-on-a-chip; LPS, lipopolysaccharide; LSECs, liver sinusoidal endothelial cells; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; MDB, Mallory-Denk bodies; MetALD, metabolic and alcohol-associated/related liver disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NPCs, non-parenchymal cells; NTF, non-trans fat; OA, oleic acid; PA, palmitic acid; PCLSs, precision-cut liver slices; PHHs, primary human hepatocytes; PPAR, peroxisome proliferator-activated receptor; RCT, randomised controlled trial; SLD, steatotic liver disease; STZ, streptozotocin; T2D, type 2 diabetes; TG, triglyceride; VLDL, very-low-density lipoprotein; WD, Western diet.

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Conflicts of interest

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Authors' contributions

SG, REC, and MH conceptualized the manuscript with feedback from JE, IT, QMA, DGT, GZ, JY, CD, FT and FO. SG, REC, DGT, CD, FO and MH conceptualized and designed the figures with feedback from all authors. All authors contributed to writing and revising the manuscript, including the Tables, and approved the final version.

Declaration of generative AI and AI-assisted technologies in the writing process

Prior to publication of this work, the author(s) used ChatGPT 5.2 to check for grammar and clarity of the text. After using this tool, the author(s) reviewed and

edited the content as needed and take(s) full responsibility for the content of the published article.

Supplementary data

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