

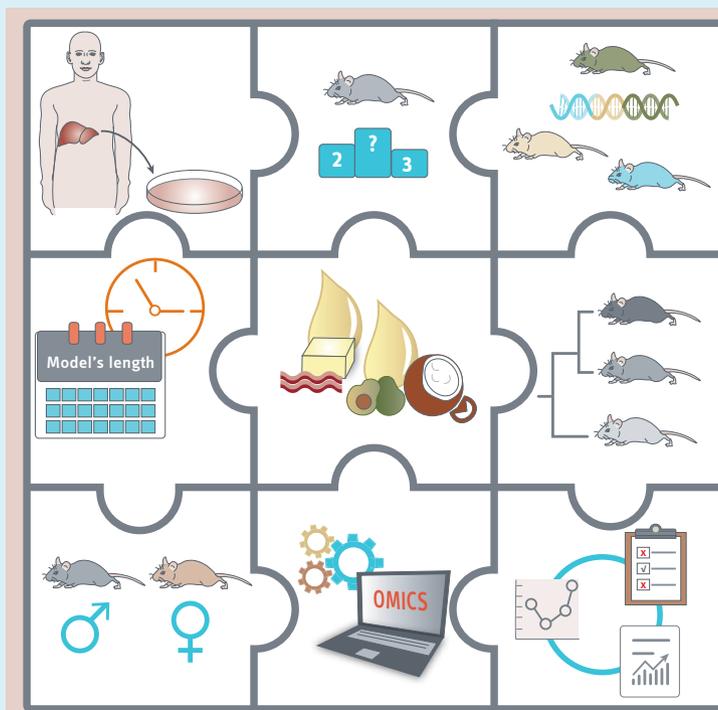
Mistakes in mouse models of nonalcoholic steatophepatitis and how to avoid them

Rui E. Castro and Anna M. Diehl

Nonalcoholic fatty liver disease (NAFLD) is a growing cause of chronic liver disease worldwide that can manifest as nonalcoholic fatty liver (NAFL) or nonalcoholic steatohepatitis (NASH). Compared with NAFL, NASH poses a substantially higher risk of progression to advanced liver disease, cirrhosis and hepatocellular carcinoma (HCC). Given the lack of directed pharmacological therapies and the complex, multifactorial disease aetiology and pathology, NAFLD is expected to become the leading cause of end-stage liver disease in the coming decades.

Preclinical research aimed at elucidating the molecular mechanisms driving disease and identifying reliable biomarkers and potential treatments is critical and has gained significant attention in recent years. Several animal models attempt to mirror the histopathology and pathophysiology of each stage of human NAFLD, including the development of NASH and fibrosis, up to HCC development. Most *in vivo* studies use mouse models owing to their relatively low cost, short lifespan and ease of genetic manipulation, which allow for a level of experimental control that is not possible with human studies. Independent of each model's inherent advantages and disadvantages, making a mistake when choosing, performing, or even analyzing results for a particular animal NASH model may jeopardize our ability to obtain accurate results or draw firm conclusions.

Here, we discuss some mistakes commonly made in NASH preclinical research. We also consider the challenges and opportunities when selecting animal models for the study of NAFLD.



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Mistake 1 Thinking that an animal model is absolutely necessary

Despite the undeniable value of animal models for studying NASH, ethical concerns have been pushing experimentation towards the increased use of *in vitro* cell systems. Furthermore, while animal models will always have translational limitations due to species differences, human *in vitro* systems are increasingly gaining physiological relevance and may provide a more faithful representation of disease biology. Human *in vitro* systems therefore allow for a clear and independent focus on specific mechanistic aspects of the disease, without the need for animal models.

Using human hepatocytes incubated with free fatty acids (FFAs) allows for basic studies of liver steatosis in the context of NAFLD. Using hepatocytes and nonparenchymal cells in co-culture further allows for the study of stellate cell and profibrogenic gene activation.

Human liver cells can also be structurally organized into sandwich or spheroid cultures, in which cell-cell and cell-extracellular matrix interactions reduce functional decline and allow experimental approaches to be extended. Similar interactions are also maintained in precision-cut liver slices.¹⁻³ These organotypic liver *in vitro* systems more closely resemble the complexities of the native human liver, including a three-dimensional (3D) multicellular architecture and a dynamic microenvironment.⁴

Organotypic liver *in vitro* systems thus embody viable alternatives for select animal experiments, including preliminary evaluation of drug safety and hepatotoxicity. Upon evidence of clinical translation, promising drugs can be thoroughly evaluated *in vivo*. For example, a microfluidic *in vitro* system (comprised of primary human hepatocytes, stellate cells and Kupffer cells) exposed to circulating FFAs, glucose, insulin and

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inflammatory cytokines was shown to reproduce select transcriptomic, cell-signalling and pathophysiological changes observed in NASH (e.g. increased *de novo* lipogenesis [DNL], gluconeogenesis and oxidative stress, cytokine production and stellate cell activation).^{5,6} Furthermore, obeticholic acid, which is currently undergoing clinical trials as a potential treatment for NAFLD, has been evaluated in this system, eliciting strong anti-steatotic, anti-inflammatory and antifibrotic effects,^{5,7} further highlighting the usefulness of *in vitro* systems for anti-NASH drug testing.

Mistake 2 Expecting a single model to recapitulate all features of human NASH and focusing solely on the liver

At present, no single dietary or genetic animal model recapitulates all pathological features of human NASH. As such, researchers should focus on particular aspects of the disease and, accordingly, choose the most appropriate model. Regardless, models reflecting not only hepatic histopathology but also the global metabolic disarrangement of human NASH are more meaningful. This means that the animal model should obviously encompass liver steatosis, intralobular inflammation, hepatocellular ballooning and perisinusoidal fibrosis, but that metabolic abnormalities, such as obesity (weight gain and adipose mass), body fat distribution, insulin resistance (blood glucose and insulin levels), fasting hyperglycaemia, dyslipidaemia and an altered adipokine profile, should ideally also be present.^{8,9}

Going deeper into the complexities of human behaviour and biology, it should be noted that appetite and food choices, physical activity, genetics and humoral determinants of body composition, as well as metabolic regulation and inflammation in extrahepatic tissues, particularly the adipose tissue, all have a role in NASH pathogenesis. It is important that, whenever possible, these features are investigated and reported. In addition, particularly for preclinical studies of potential anti-NASH drugs, it is suggested that at least two individual, complementary NASH models are used, with at least one consistently reproducing obesity and histology-proven liver fibrosis.¹⁰

Mistake 3 Setting aside genetic animal models

Dietary animal models rank among those of highest relevance to human NAFLD. However, it should be noted that genetic animal models

can be extremely useful in elucidating the significance of particular pathways during NASH development. For instance, T-cell knockout mouse lines were used to prove that adaptive immunity has a critical role in NASH and its progression to HCC.¹¹ Furthermore, transgenic animal models are also useful for clarifying the effect of genetic background on NASH; it is well known that distinct single nucleotide polymorphisms (SNPs) associate with NASH, most notably variants of *PNPLA3* and *TM6SF2*, while specific monogenic conditions lead to the development of severe NAFLD.^{12,13}

Nonetheless, most genetic NASH mouse models comprise gene mutations that are not commonly altered in patients (e.g. *ob/ob*, *db/db*, *foz/foz* mice and others). In this case, the value of these models lies in the ability to study isolated pathways that are involved in metabolic homeostasis, as well as the consequences of their dysregulation. It is also possible to model advanced NASH using genetic models through the application of additional stimuli, usually in the form of a modified diet, leading to development of inflammation and fibrosis.^{13,14} In comparison with traditional dietary models, these “mixed” models generally exhibit a more severe disease phenotype within a shorter time period, thus increasing their attractiveness from a practical and/or economic perspective.

Mistake 4 Expecting an animal model to work in a shortened timeframe

Most NASH animal models need a long period of time to achieve a certain phenotype. For instance, depending on the model, it can take up to 4 months to achieve different degrees of steatosis, with or without significant necro-inflammatory changes. Development of fibrosis usually requires additional time and is often mild, if present. Finally, most models trying to reproduce the natural disease course, up to the development of HCC, require an experimental period of 12 months, on average.

In practice, temporal resources are often limited and animal models requiring a long experimental duration can be extremely costly, particularly when a preclinical lead is being tested. For this reason, it may be appealing to reduce the duration of the model. Unfortunately, this almost never is a good choice—the extreme diversity of the NAFLD disease spectrum means that animal models of NASH are also inherently variable, and the histopathological features are not always consistent. For instance, in most animal models of NASH progressing to HCC, neoplastic nodule numbers, size and degree of

malignancy vary from animal to animal and are often unpredictable.

Trying to reduce the length of time required for an animal model to display a given phenotype only serves to increase phenotypic variability and can even prevent the desired phenotype from being obtained. Of course, although it is possible to add a carcinogen or use certain modified diets to shorten the time needed for disease development and/or neoplastic nodules to appear, there will be an extra layer of complexity that must be appreciated and dealt with when interpreting the data.

Mistake 5 Assuming that all fat is created equal

Diet composition for animal models of NASH varies markedly in the published literature, with the fat source being either lard, butter or coconut, olive, corn and soybean oil, among others.¹⁵ These distinct fat sources have different compositions in terms of fatty acids (polyunsaturated [PUFA], monounsaturated [MUFA], saturated [SFA], and trans [TFA]), which undergo distinct metabolic processing and, as such, lead to variable amounts of lipid accumulation in the liver.¹⁶

Generally speaking, dietary SFAs and TFAs negatively impact liver function,^{15,16} although different SFA species have distinct effects. One study showed that replacing dietary lard with coconut oil, in order to elevate the ratio of medium-chain fatty acids to long-chain fatty acids, mitigates high-fat diet (HFD)-induced NASH in mice.¹⁷ Insulin resistance is also influenced by the dietary lipid content and is more likely to occur with diets rich in SFA and MUFA. By contrast, insulin resistance can be minimized by the consumption of PUFAs.¹⁵

Last, but not least, the amount of fat included in the diet (regardless of the fat type), is also not standardized, generally ranging from 30–60% of energy content. This variation can also significantly impact experimental outcomes.

An overview of the differential effects of distinct fat-source diets on rodent liver bioenergetics and oxidative imbalance was published by Kakimoto and Kowaltowski in 2016.¹⁵ Overall, for any type and amount of fat in a NASH diet, and to increase future reproducibility in this area, the composition of the HFD and control diet should ideally be paired, with the only notable change being the fat content itself. It is also recommended that the content of the diet should be clearly specified in publications, for both the control and HFD groups, particularly with regard to the source and type of dietary fat.

Mistake 6 Failing to consider the mouse strain

In parallel with the macronutrient and fat composition of a diet, as well as the duration of feeding, the genetic background of the mouse strain used also determines disease severity. Although most models rely on C57BL/6 mice, it is important to recognize that other strains or recombinant inbred strains could be more or less susceptible to NASH development. Even the mouse substrain should be carefully chosen prior to any experiment, as key differences may exist.

C57BL/6j mice are more insulin resistant compared with C57BL/6N mice.¹³ Intriguingly, it has been reported that C57BL/6j mice from The Jackson Laboratory may carry a spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene (*NNT*) that could affect mitochondrial function and hence NASH development, but not C57BL/6j mice from other suppliers, nor C57BL/6N mice.¹⁵ This calls for awareness when selecting the supplier of any given mouse strain.

As another example, both Alstrom syndrome 1 (*ALMS1*)-deficient *foz/foz* C57BL/6j and *foz/foz* BALB/c mice have been shown to gain weight when on an HFD, although NAFLD-associated liver fibrosis is more severe in the C57BL/6j strain.^{13,18,19} More recently, Asgharpour and colleagues created a novel isogenic B6/129 mouse strain derived from the C57BL/6j and 129S1/SvImj backgrounds. When on an HFD containing 0.1% cholesterol plus fructose/sucrose-enriched drinking water, the B6/129 mice developed NASH with fibrosis, and formation of liver tumours was observed from week 32 onwards. Of note, NAFLD activity and liver fibrosis in these mice was more pronounced when compared with either parental strain, of which only 129S1/SvImj mice developed liver tumours.²⁰

Mistake 7 Not appreciating gender differences

Men and women exhibit major differences in NAFLD susceptibility and severity and, similar to the situation in humans, male rodents appear to be more susceptible to the development of NASH than female rodents. Largely for this reason, most published *in vivo* studies use only male animals.

In different dietary models of NASH, male rodents have been shown to exhibit more pronounced steatosis and have higher levels of serum alanine aminotransferase, cholesterol, TGs and leptin than their female counterparts.^{21,22} Similarly, Fujii et al. found that only male STAM mice developed sequential

steatohepatitis, fibrosis and carcinoma,²³ suggesting the protective role of oestrogen or other as-yet-unknown factors. Indeed, another study has shown that myeloid IKK β deficiency prevents Western-diet-induced obesity and visceral adiposity in females only.²⁴

Oestrogen does appear to be a key factor responsible for the gender disparities in NASH susceptibility and severity. The prevalence of NAFLD is higher in women aged 55 years or older,²⁵ and disease severity is decreased in female patients prior to menopause.²⁶ In support of the role of oestrogen, post-menopausal women are more prone to develop extrahepatic complications of NAFLD, such as visceral obesity, insulin resistance and type 2 diabetes,²⁷ with oestrogen treatment attenuating these complications.²⁸

The opportunity to study particular risk factors and pathophysiological molecular and cellular circuits in women that account for this differential susceptibility to disease development should not be missed. For this reason, more female-only mouse models of NASH are eagerly anticipated. Furthermore, when accompanied by male mouse studies, they might aid the development of novel and more precise directed therapies for NASH.

Mistake 8 Not taking advantage of omics technologies

The definition of what comprises NASH in animal models remains unclear. In addition to the limited applicability of numerous NASH animal models to model such a complex multifactorial human disease, the lack of a detailed definition of NASH in animal models further fuels the difficulty predicting accurate translation of effective treatment strategies. To narrow this gap, many researchers are now taking advantage of omics data from human patients and animal models, where the clinical phenotype, genomic heterogeneity, transcriptomics, and metabolomic changes are combined to identify the ideal NAFLD animal model for a specific scientific question or to test a particular drug.^{29,30}

Evidently, different animal models will show different degrees of overlap in their gene expression profiles when compared with human NAFLD. But overall, and thus far, the gene expression patterns in the livers of HFD-fed mice appear to more closely resemble human NAFLD when compared with other models.³¹ In the dietary isogenic B6/129 mouse model, hepatic gene expression at 52 weeks had a similar signature to human liver cirrhosis and, later on, HCC was concordant with gene expression observed in specific human molecular subclasses.²⁰

More recently, Tsuchida et al. described a NASH mouse model with rapid progression of extensive fibrosis and HCC.³² They performed global transcriptome profiling of the liver and HCC tumour tissues from their mouse model and also of two human NASH cohorts and several previously published diet, chemical, and/or genetic NASH mouse models. Their animals were shown to have dysregulated molecular signatures similar to those of early/mild human NASH. Animals developing tumours at later time points also had a transcriptomic pathway similar to human HCC molecular subclasses.^{32,33} Such work highlights the power of omics in elucidating more meaningful animal models that parallel human disease progression.

Mistake 9 Failing to report critical issues or not publishing “bad” results

That the results of animal research should be published only when they conform to agreed international standards, namely the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines, is undeniable.³⁴ While fundamental animal experimentation rules should be followed, including humane and healthy animal husbandry, as well as following ‘the three Rs’ (replacement, reduction and refinement) policies, ARRIVE recommendations also include reporting extended details of the animals used, such as strain/genetic fidelity, use of littermates and the specifics of diet/nutrients. As stated previously, these represent crucial factors in NASH animal models. However, it should not be interpreted that unexpected/negative results should not be reported or published, as this may contribute to suboptimal interpretation of animal data, particularly when describing a new *in vivo* NASH model.

Given the complex aetiology and pathology of human NASH, and the absence of a single animal model featuring all of its components (and with each existing model having their inherent strengths and weaknesses), it is likely that false positives, false negatives and/or inconclusive data will be obtained. A typical example is failing to achieve the reported phenotype of a particular model and deciding not to publish those findings. Provided the ARRIVE recommendations were followed, making the results available should be encouraged, either via specialized journals or through an online dataset, as this information is vital for the research community. These data are particularly relevant for drug development studies—without them preclinical leads could advance to clinical trials based on incomplete, critical information.

Mistake 10 Neglecting outliers when interpreting study data

Given the oratory character of preclinical animal studies, outliers are often neglected when interpreting study data, although they should ideally always be reported. To circumvent potential bias, eligibility and exclusion criteria should be defined a priori and experiments performed in a blinded and randomized fashion. Failing to do so has been shown to increase the odds of reaching statistically significant results more than threefold when compared with appropriately designed studies³⁵ Even for correctly designed studies, outliers are to be expected, particularly for normally distributed data and large sample sizes—roughly 1 in 22 observations will differ by twice or more the standard deviation from the mean. Whatever the case, outliers should always be carefully examined to establish whether they actually reflect end spectrums of NAFLD pathology (or treatment) or are the result of experimental artefacts.³⁶ Furthermore, excluding outliers in a targeted fashion (that is, considering whether or not it supports the expected results), may have extreme consequences with regard to false positives and skewed interpretation.

Last but not least, animals dropped from any study should also always be reported. In clinical research, reporting standards such as the Consolidated Standards of Reporting Trials (CONSORT) and the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines, require reporting of all dropouts in a given clinical trial. By contrast, many animal studies fail to report this number. Add targeted outlier exclusion, and results may be fourfold more likely to be significant, with the effectiveness of a given treatment overstated by up to almost 200%.³⁷

In summary, given the many different NASH animal models used by researchers, outliers should not be neglected. Outliers may provide crucial information about the intrinsic characteristics of the model or, in drug development, the intrinsic characteristics of the compound being studied.

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Your NASH briefing

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- Further relevant articles can be found by navigating to the ‘hepatobiliary’ category in the UEG ‘Standards & Guidelines’ repository [<https://www.ueg.eu/guidelines/>] and via the EASL Clinical Practice Guidelines webpage [<http://www.easl.eu/research/our-contributions/clinical-practice-guidelines>].