Non-alcoholic fatty liver disease (NAFLD) consists of a complex combination of liver maladies, ranging from benign hepatic steatosis (fatty liver) to its more aggressive inflammatory manifestation, non-alcoholic steatohepatitis (NASH). In parallel to soaring rates of obesity and type 2 diabetes (T2D), the prevalence of NAFLD is rising rapidly. An estimated 25% of adults worldwide currently have NASH and ~30-59% of these patients will develop NASH (see Figure 1). NASH is a dynamic condition that can regress back to isolated steatosis, or cause progressive fibrosis that leads to irreversible cirrhosis (stage F4 fibrosis) and/or hepatocellular carcinoma (HCC). Approximately 9% of patients with NASH will progress to these end-stage liver diseases. Some experts believe the crisis is so significant in the United States that NASH will become the leading cause for liver transplantation by 2020.

The looming global health crisis of NASH represents a substantial opportunity for pharmaceutical companies and market analysts estimate the peak drug market size for NASH therapeutics could be as high as $40 billion. There have already been more than 750 trials relating to NAFLD to date, yet despite the intense race to develop therapeutics, no approved treatments are available. Most clinical Phase III trial results have been disappointing, and even the most promising preclinical drugs have not performed as expected once tested in humans. In addition to lack of efficacy, significant safety concerns have been raised for some new drug candidates. These disappointing results are not only aggravating for companies funding trials, but also frustrating for patients waiting for a cure.

Why so many setbacks in this ‘golden age’ of medicine?
The R&D delays for efficacious therapies for NASH can be largely attributed to a lack of physiologically relevant and predictive preclinical models that translate to humans. Selecting and applying relevant disease models for drug discovery requires an understanding of clinical etiology, both in terms of the causes of the disease and its pathogenesis. Part of a systemic metabolic syndrome, NASH involves many complex mechanisms and there is not yet consensus in the field about disease initiation and progression. Some researchers propose a ‘dual-hit’ hypothesis (with steatosis from increased lipogenesis in hepatocytes as the first hit, proinflammatory mediators from macrophages the second); others favour a ‘multi-hit’ hypothesis (where free fatty acids (FFA) and their metabolites promote NASH through multiple toxic pathways), but most involve some form of FFA-mediated lipotoxicity. Hepatic stellate cells, activated by sustained lipotoxic stress, promote fibrogenesis, whereas macrophages are thought to contribute to inflammation and fibrosis.

Evolving models for NASH drug discovery: tools for stemming a silent epidemic

By Dr Sue Grepper, Dr Radina Kostadinova, Dr Eva Thoma and Professor Armin Wolf
inflammation, are responsible for excessive extracellular matrix deposition, leading to fibrosis and eventually cirrhosis. Signalling interactions between hepatocytes, Kupffer cells and stellate cells are critical for NASH pathogenesis. Successfully modelling all the key physiological events (from steatosis to inflammation and fibrosis) evident in clinical NASH has been extremely challenging—in both in vivo animal models and in vitro cell-based ones. Meanwhile, researchers are realising it is unlikely that a single, ‘one-size-fits-all’ wonder drug will be discovered. Many pharmaceutical companies are shifting their R&D efforts toward combination therapies, making an extremely expensive bet that their preclinical models will accurately predict the best combinations to test in the clinic.

In vivo animal models—predictive or just poorly-treated mice?

To date, most NASH therapeutics have been tested for efficacy and toxicity in mouse models prior to being advanced to clinical trials. Why mice? Their short lifespan, relatively low cost (compared to other laboratory animals) and ease of genetic manipulation. More than 20 different mouse models have been used to study NASH and it is clear that these models have come a long way over the past 50 years (see Figure 2).

One of the first animal models used for NAFLD drug discovery was the carbon tetrachloride (CCL4) model, introduced in the 1970s. CCL4 treatment in mice causes significant fibrosis within a relatively short treatment period, and it is still occasionally utilised for testing drugs that target the fibrotic aspect of NASH. Understandably, this highly-toxic chemical model does not truly reflect the manifestation of NASH in humans.

More appropriate in vivo mouse models have since been developed for NASH drug discovery and development, each with advantages and limitations. These models range from chemical-induced, high fat diet-induced, Methionine Choline Deficient (MCD) diet, genetically-altered, or some combination/adaptation thereof. To better mimic the major human risk factors associated with NASH, most of these mouse models are at least partially diet-based and the animals typically become obese. However, genetically identical mice do not have identical eating habits, and a confirmatory surgery and liver biopsy is typically required prior to the initiation of drug treatment (at ~week 20-30, depending on the model), to prove that they truly developed NASH. For this reason, a significant percentage of diet-induced NASH mice are subsequently excluded from the remainder of the experiment.

Many dietary-only mouse models progress only as far as steatosis, with or without mild inflammation, and require additional stimulation to progress...
to advanced stages of NASH, such as inflammation and fibrosis. For example, the HDF-CDA A model combines a high fat diet and a modified version of the MCD diet (as methionine deficiency leads to hepatic injury, inflammation and fibrosis, and choline deficiency leads to macrovesicular steatosis). The STAM™ model first induces diabetes with streptozotocin treatment in mice at birth, followed by feeding a high fat diet for several months. The DIAMOND™ model7 takes a different approach. Rather than induce NASH by chemical intervention, this model relies on genetics and diet. It is a stable isogenic cross between C57BL/6J (B6) and 129S1/SvImJ (S129) mice, fed a high fat diet with ad libitum consumption of glucose and fructose. Impressively, this model is thought to mimic all the physiological, metabolic, histological and clinical endpoints of human NASH and it is now considered the most physiologically-relevant and predictive of all the available in vivo NASH animal models.

Even though in vivo mouse models serve a valuable purpose in the quest for NASH therapeutics, scalability and time continue to be major limitations with animal models in general. Most mouse models employed for NASH research typically require ~20 weeks to show the first hallmarks of NASH disease progression, while the appearance of severe fibrosis can take 30 weeks or longer. Considering that combination therapies are now the approach for most companies, and every new combination of drugs tested in mice requires this length of time, it might be years before the right combination makes it to the clinic.

**Engineering better NASH models: human in vitro models pave the way for new therapies**

Due to high costs and time required for animal testing, there is growing interest in in vitro models of NASH, particularly for early preclinical screening of single/combinatorial therapies. Historically, the predominant approach for modelling diseases in vitro has been limited to single cell types, such as cell lines (eg HepG2), hepatocytes, or stellate cells, cultured in a two-dimensional (2D) monolayer on a flat surface (see Figure 2). While these simple cell-based systems have advanced our understanding of the disease, cells placed in artificial 2D micro-environments do not reflect human biology. The lack of interactions between relevant cell types

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**Figure 2**

The evolution of NASH models. Numerous in vivo mouse and in vitro human cell-based models have been used in NASH drug discovery. Although not an exhaustive list, this timeline highlights models that have influenced the field over the past 50 years. Since 2010, researchers have benefited from significant advances in both in vivo and in vitro models appropriate for NASH research.
and the extracellular environment results in significant changes in cell morphology, polarity and function. For example, primary hepatocytes are known to become dedifferentiated and quickly lose their metabolic functionality (within 2-3 days) in 2D culture, whereas HepG2 simply lack these metabolic capabilities. As hepatocyte metabolism plays a crucial in NASH pathophysiology, the time window for disease induction and drug efficacy testing is therefore quite limited when working in 2D. While 2D cultured HepG2 cells and primary hepatocytes cultured are still quite useful for investigating lipid loading and de novo lipogenesis (DNL) aspects of NASH, the primary limitation of these models is the lack of the downstream non-parenchymal cell contributions to the progression of NASH.

Similarly, stellate cell lines (eg LX-1 and LX-2) and primary hepatic stellate cells have been valuable for studying the fibrotic aspect of NASH, because stellate cells play a pivotal role in the initiation, progression and regression of liver fibrosis. However, as with 2D hepatocyte monoculture, 2D stellate cell monoculture models ignore the contribution and interplay of other liver cell types essential for NASH initiation and progression. In addition, 2D stellate cell models harbour another serious liability: stellate cells become spontaneously activated upon attaching to plastic surfaces in culture. These artificially-activated stellate cells should not be used to evaluate the efficacy of drug candidates that target slowly progressing activation processes, as observed in NASH.

The shortcomings of 2D models are particularly problematic for investigators working on new therapies for complex diseases, such as NASH. Recent advances in cell culture techniques are now enabling more complex and robust disease models. For example, it is now possible to incorporate multiple cell types, along with biochemical and biomechanical micro-environments, to better mimic in vivo pathophysiology. These ‘next gen’ models are driving greater understanding of the underlying mechanisms and progression of disease and should, in turn, help identify the most promising drug candidates to advance to clinical trials. It is also important to consider that NASH develops in humans over the course of years, if not decades. Researchers focusing on NASH model development must tackle the monumental task of condensing what occurs in humans over 10 or more years… in a dish, within an extremely accelerated timeframe.

In summary, to meet the needs of today’s pharma drug discovery efforts, in vitro NASH models must:

- Recreate the pathophysiology of the progression of NAFLD and NASH in humans, from lipid accumulation in hepatocytes (steatosis), inflammatory response (hepatitis) and fibrotic scarring (fibrosis).
- Mimic chronic drug exposure of clinical treatment programmes by maintaining longevity in culture for longitudinal, repeat-dose treatments.
- Reflect the human response to disease inhibitors/inducers, enable monitoring of pathways perturbed in the disease state and predict patient response to treatment dosing in the clinic.
- Deliver robust functionality to ensure reproducible and comparable results over multiple experimental assays.
- Enable scalable, screening-compatible, clinically-

Figure 3: Inducible in vitro NASH model. In humans, the progression of NASH starts with lipid accumulation in the hepatocytes (steatosis). High content imaging of this 3D multicellular human liver model shows the changes in model phenotype under healthy control conditions (A) and after NASH induction with a specialised media that contains higher sugar levels and free fatty acids (lipids). Treatment with low (C) and high (D) concentrations of an anti-steatotic clinical drug candidate leads to a decrease in intracellular lipids. Nile Red staining (magenta) captures a normal amount of lipids (green) in the control and after treatment with high drug concentrations of the drug, whereas steatotic hepatocytes are abnormally enlarged and filled with lipid vacuoles. Hoechst staining for nuclei (blue) further highlights macrovesicular steatosis (engorgement of hepatocytes by lipids that displace nuclei), mimicking the fatty liver disease state in humans. Photo courtesy of InSphero AG, imaged on a Yokogawa high-content screening system.
relevant endpoint readouts that capture pathological aspects of NASH.

**A new era of game-changing NASH models**

Over the past few years, enormous strides have been made in cell-based NASH model development. Several research groups have engineered advanced models for studying NASH (see Figure 2). For example, in 2016 scientists at Hemospher establish an *in vitro* NASH model that combined flow technology with primary cell culture in a transwell format. In their model, primary hepatocytes cultured on a collagen-matrix are separated from stellate cells and Kupffer cells by a polycarbonate membrane. Upon exposure to lipotoxic stimuli, key hallmarks of NASH, including lipid loading, changes in hepatocyte metabolism and stellate cell activation, can be recapitulated.

Researchers at AstraZeneca and the University of Gothenburg have furthered the field by collaborating on 3D spheroid co-cultures of HepG2 with LX-2 cell lines. FFA treatment in this co-culture model leads to significant lipid loading and stellate cell activation. Compared to the Hemospher model, this approach achieves much greater throughput, as it utilises a fraction of the number of cells, in a 96-well format. The team also used a monoculture of individual donor primary hepatocytes in a similar spheroid format to demonstrate important differences in lipid metabolism dependent on the genetic background of individual donors.

Aiming to combine the advantages of primary liver cells within a scalable 3D culture system, InSphero developed a 3D human liver NASH model that includes all liver cell types thought to be involved in the development of NASH: hepatocytes, hepatic stellate cells, liver endothelial cells and Kupffer cells. This model is disease tunable in that treatment with FFA, sugars and inflammatory stimuli induce lipid accumulation in hepatocytes (steatosis), release pro-inflammatory cytokines and chemokines from Kupffer cells (inflammation) and deposit fibril collagens (fibrosis) by stellate cells (see Figure 3). As in humans, this model progresses over multiple stages upon induction with NASH stimuli, but the process is condensed down to two weeks. With this compact induction protocol, this model offers a significant advantage over mouse models, which typically require several months to achieve experimental maturity. Furthermore, since combination therapies may be needed for effectively fighting NASH, this approach provides a promising tool for high throughput screening with various mechanisms of action.

Each of these advanced human cell-based approaches can be used to complement findings from mouse models and optimise use of animal testing in discovery programmes. Aside from issues such as relevance, scalability and predictivity, rodent models are most useful for single ‘snapshot’ endpoints, typically collected at the completion of the study (eg sacrifice of the animals and histopathological assessment). For progressive complex diseases like NASH, it is especially crucial to gain more insight in the dynamics and kinetics of the underlying mechanisms. The latest generation of advanced *in vitro* models retain longevity, and when combined with newer sophisticated methods such as live cell imaging, allow deconstruction of the sequential steps in pathophysiology of NASH. From another perspective, they can also be used to generate a large amount of relevant data (eg, combination therapies, multiple timepoints, numerous endpoints) in a much shorter timeframe than possible with an animal study. Theoretically, this should allow for a much more rapid prioritisation of promising drug combinations.

**A drug developer’s wish list for in vitro human NASH models**

Of course, there are still major hurdles to be overcome with cell-based models. Even the most advanced *in vitro* human NASH models currently lack extra-hepatic contributions to the progression of NASH, such as circulating blood macrophages (important for inflammation and further activation of stellate cells), intestinal cells (important for FXR-stimulated release of FGF-19), or gut microbiota. Furthermore, the increasing complexity of cellular models makes robust experimental readouts more challenging to implement. The amount of data increases remarkably in complex cellular assays, strengthening the need for appropriate tools for data management, analysis and interpretation. The rapid progress in cutting-edge technologies, such as high-content imaging (see Figure 3) or single cell sequencing, as well as the increasing availability of sophisticated bioinformatic tools is encouraging, however, fitting these technologies to the needs of complex *in vitro* models remains a major challenge.

The interpretation of results – putting them in context with existing data from *in vivo* and *in vitro* studies – is also a challenge with 3D models. Ironically, the difficulty here is due to the complexity of model. As with animal studies, it is not easy to determine pathways leading to an unexpected effect of treatment.
A vision for the future of NASH drug discovery

The pharmaceutical research community has long been under intense pressure to improve R&D productivity, reduce animal use and reduce drug attrition rates. The most promising drug candidates need to be accurately validated with physiologically-relevant preclinical models prior to being advanced to expensive and lengthy clinical trials. This is especially true in the hunt for therapeutics to treat NASH – a complex disease with a growing prevalence and costly impact on society.

Multicellular human disease models, such as those for NASH, are now able to more faithfully recapitulate complex in vivo conditions. They are better suited for mechanistic studies of disease progression and high-throughput drug efficacy screening. These robust, validated models are readily integrated into R&D workflows and can improve discovery and preclinical development efforts. Aware of the current limitations of available models, biotech companies working on the next generation of human in vitro models must maintain an open dialog with pharmaceutical researchers to identify desired enhancements, such as the addition of systemic components (eg, infiltrating blood immune cells or microbiome components). In the near future, scientists will be able to apply emerging ‘organ-on-a-chip’ technology to create organ networks of liver and pancreatic tissues with immune cells in a single system that effectively recreate metabolic syndrome at the bench.

As the prevalence of NAFLD and NASH continues to increase, the clinical and economic burden will become even more staggering. Ground-breaking research tools such as these will lead to more efficient development of truly-effective NASH therapies and better address this growing pandemic. DDW

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