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CONTEMPORARY REVIEW

Asking the Right Questions With Animal Models: Methionine- and Choline-Deficient Model in Predicting Adverse Drug Reactions in Human NASH

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ABSTRACT

In the past few decades, great conceptual and technological advances have been made in the field of toxicology, but animal model–based research still remains one of the most widely used and readily available tools for furthering our current knowledge. However, animal models are not perfect in predicting all systemic toxicity in humans. Extrapolating animal data to accurately predict human toxicities remains a challenge, and researchers are obligated to question the appropriateness of their chosen animal model. This paper provides an assessment of the utility of the methionine- and choline-deficient (MCD) diet fed animal model in reflecting human nonalcoholic steatohepatitis (NASH) and the potential risks of adverse drug reactions and toxicities that are associated with the disease. As a commonly used NASH model, the MCD model fails to exhibit most metabolic abnormalities in a similar manner to the human disease. The MCD model, on the other hand, closely resembles human NASH histology and reflects signatures of drug transporter alterations in humans. Due to the nature of the MCD model, it should be avoided in studies of NASH pathogenesis, metabolic parameter evaluation, and biomarker identification. But it can be used to accurately predict altered drug disposition due to NASH-associated transporter alterations.

Key words: NASH; animal models; adverse drug reactions; MCD model.

The review by Dr Choudhuri *et al.* deftly summarizes critical conceptual and technological advances that precede our understanding of toxicology and gives a comprehensive overview of the past and future development of toxicology. Toxicological research in animal models plays a critical role in expanding our current knowledge, even though there is concern as to whether animal data successfully recapitulates human toxicities. In this paper, we will share our perspective on the selection and validation of animal models to address specific research questions in the toxicological research related to nonalcoholic steatohepatitis (NASH).

Experimental animals have been extensively used in scientific research to understand particular biological phenomena and provide insight to human biological processes and human diseases. As a powerful investigative tool, utilization of animals greatly expanded the scope and complexity of biomedical research, such as generating fundamental knowledge of biological or pathological mechanisms, evaluating efficacy and safety of preclinical drugs and medical devices, and assessing the risk of industrial and consumer products as well as environmental toxicants (Rollin, 2014). The long history of using animal models in toxicology has demonstrated their utility, but also revealed that animal models are less than perfect in reflecting human diseases or in predicting the fate of drugs and chemicals and their subsequent effects in humans. A study regarding worldwide laboratory animal use estimated that about 115.3 million animals were used in 2005, which the authors considered a substantial underestimation (Taylor *et al.*, 2008). Meanwhile, a total of $14 billion is spent on animal experimentation worldwide every year and about $2.8 billion of that is spent on toxicological

studies (Hartung, 2009). Considering the tremendous investment in animal-based toxicological research, it is crucial to answer the question of how useful the current animal models are and how reliable they are. Olson *et al.* (2000) reported that only 43% of toxic effects that occurred in humans were correctly predicted in rodent models, which increased to 63% when non-rodent animals were included. Low concordance of toxicity between humans and animals demonstrates that the toxicological community faces a great challenge in animal-to-human extrapolation of experimental results.

General toxicities or adverse drug reactions (ADRs) in clinical drugs are generally categorized into 2 types: intrinsic (type A) and idiosyncratic (type B). Intrinsic ADRs are dose-dependent, and the risk of toxicity increases with increasing drug dose. Increasing dose, however, does not necessarily increase the risk of idiosyncratic ADRs, because they are generally associated with factors that are unique to individuals or certain subpopulations, such as genetic predispositions, age, sex, nutritional status, immune system, and underlying disease (Iasella *et al.*, 2017; Pirmohamed *et al.*, 1998; Ulrich, 2007). Though idiosyncratic ADRs are usually considered to be dose-independent, in some situations they result from altered pharmacokinetics but not pharmacodynamics of particular drugs. In other words, preexisting conditions in patients that lead to alterations of absorption, distribution, metabolism, and excretion (ADME) processes and effect drug metabolism and disposition can also increase the likelihood of ADRs. In this case, predictable type A ADRs may present in particular individuals or subpopulations within the normally safe therapeutic dose range. Current animal models are moderately reliable in predicting type A ADRs, and therefore most type A ADRs of drugs can be identified in preclinical animal studies or human clinical trials. Type B ADRs, however, usually are not revealed until a drug has been widely used, which suggests that current preclinical procedures of drug toxicity and ADR screening in animal models poorly reflect the heterogeneity of the human population. Additionally, identification of risk factors of type B ADRs mostly relies on awareness of potential predisposition conditions in human patients as well as knowledge of the underlying mechanism. This paper aims to present an example of utilizing an animal model in translating observations in human studies and predicting type B ADRs in a particular subpopulation and to provide a perspective of how to use current models to answer appropriate questions.

HIGHLIGHTS AND CHALLENGES IN USING METHIONINE- AND CHOLINE-DEFICIENT MODEL IN NASH RESEARCH

Nonalcoholic steatohepatitis is the progressive stage of the most prevalent chronic liver disease, nonalcoholic fatty liver disease (NAFLD), that affects about 25% of the world population (Younossi *et al.*, 2016). Nonalcoholic steatohepatitis encompasses distinct pathological features such as extensive intracellular lipid deposition, hepatocellular ballooning, and inflammation and can progress to liver cirrhosis and hepatocellular carcinoma (Chalasani *et al.*, 2012). The molecular mechanisms in the development of NASH are not fully understood, though the "multiple hit" hypothesis has been well accepted, which usually encompasses multiple sequential or parallel cytotoxic events, including insulin resistance, hormonal or nutritional perturbation, and gut microbiota alteration, along with genetic or epigenetic factors that collectively promote NASH pathology (Buzzetti *et al.*, 2016). Along with the advancement of understanding of the pathophysiology of NASH, a significant variety of targets have been identified for NASH treatment, which has been thoroughly reviewed recently (Townsend and Newsome, 2017; Wong *et al.*, 2016). Meanwhile, pharmaceutical companies have accelerated the development of specific therapeutics for NASH management, and several pipeline therapies with diverse mechanisms of action are under late-phase clinical studies (Brodosi *et al.*, 2016; Cassidy and Syed, 2016). Demonstrating the safety profiles of these potential NASH drugs will be critical to obtain U.S. Food and Drug Administration (FDA) approval and maintain long-term success of the drugs.

Even though human NASH is highly associated with obesity, one of the widely used animal models of NASH is a nutrient-deficient model, the methionine- and choline-deficient (MCD) diet fed rodent model (Table 1). The MCD diet contains a considerable amount of sucrose (40% of energy and 10% fat) but is deficient in methionine and choline (Dyets, Inc #518810). Choline deprivation in animals significantly impairs hepatic production and secretion of very-low-density lipoprotein (VLDL) (Vance, 2008; Yao and Vance, 1990). In addition, choline plays a major role in mitochondrial membrane integrity, so choline deficiency alters the mitochondrial membrane composition and leads to perturbations in mitochondrial bioenergetics and fatty acid β-oxidation (Corbin and Zeisel, 2012). In one mechanistic study, it has been shown that the MCD diet can significantly increase hepatic fatty acid uptake and reduce VLDL secretion (Rinella *et al.*, 2008). As an essential amino acid, methionine plays a critical role in protein synthesis and is also the intermediate in *S*-adenosylmethionine (SAM) and glutathione synthesis, which are 2 important endogenous antioxidants (Lu, 2000). Disruption of hepatic methyl balance in either humans or rodents can lead to changes in SAM content that affects transmethylation reactions and promotes liver injury and development of liver diseases (Mato *et al.*, 2008). In brief, in the MCD model, choline deficiency contributes more to the phenotype of steatosis, whereas methionine deficiency initially promotes oxidative stress and changes in cytokines and adipokines, which are believed to drive the progression of inflammation in the animals (Caballero *et al.*, 2010; dela Peña *et al.*, 2007; Larter *et al.*, 2008; Leclercq *et al.*, 2000). Collectively, multiple mechanisms have been proposed in the MCD model, which mostly disagree with human NASH pathogenesis mechanisms; this model does, however, lead to NASH histology, which is highly comparable with humans. Sprague Dawley rats and C57BL/6J mice on 8 weeks of MCD diet exhibit the phenotype of severe NASH (Canet *et al.*, 2014; Machado *et al.*, 2015). Excessive steatosis and cell ballooning were observed in MCD-fed rodents (Canet *et al.*, 2014). Additionally, MCD-fed animals exhibit inflammation as early as 2 weeks after the onset of diet and present pericellular and perisinusoidal fibrosis in 8–10 weeks (Leclercq *et al.*, 2000). Serum alanine aminotransferase is significantly elevated in MCD-fed rodents, indicating substantial liver injury in these animals (Canet *et al.*, 2014; Machado *et al.*, 2015). Moreover, disruptions of cellular defensive pathways, such as endoplasmic reticulum (ER) stress, oxidative stress, and autophagocytic stress, which have been shown as contributing factors to human NASH progression, are observed in MCD models as well (Machado *et al.*, 2015). In summary, the MCD model has the advantage of effectively and reproducibly introducing severe hepatic steatosis and inflammation in animals, which closely resembles human NASH histology, within a relatively shorter time than other dietary models of NASH (Figure 1).

Table 1. Metabolic Profile and Pathological Features in Human NASH and MCD Model

	Human NASH	MCD Model
Metabolic parameters		
Bodyweight	Highly associated with obesity	40% weight loss in 8–10 weeks feeding
Insulin sensitivity	Systemic insulin resistance	Improved insulin sensitivity, intrahepatic insulin resistance
Fast blood glucose	High	Low
Triglyceride	High	Low
Cholesterol	High	Low
Liver injury markers		
ALT	Increase (slightly to moderately) (Schindhelm *et al.*, 2006)	Increase
AST	Increase (slightly to moderately)	Increase
CK-18 fragments	Increase (Feldstein *et al.*, 2009)	Increase (Anstee *et al.*, 2010)
Histology		
Steatosis	Moderate and severe hepatic macrosteatosis characterized by lipid deposition as a single large vacuole within the hepatocyte cytoplasm (Brunt *et al.*, 2011)	Moderate to severe macrovesicular steatosis as human, though microvesicular lipid droplets are common in mice, which is different from typical human histology (Canet *et al.*, 2014)
Hepatocellular ballooning	Commonly but not extensively found: cellular enlargement 1.5–2 times the normal hepatocyte diameter, with rarefied cytoplasm (Brunt *et al.*, 2011; Caldwell *et al.*, 2010)	Difficulty in translating concept of cell ballooning in human patients to animal model, though cell enlargement can be found (Lackner, 2011)
Inflammation	Mild inflammation and predominantly lobular rather portal based (Brunt *et al.*, 2011)	Fast developed inflammation as early as 2-week feeding, significant hepatocellular inflammation (Leclercq *et al.*, 2007)
Fibrosis	"Pericellular" or "chicken-wire" fibrosis (Brunt *et al.*, 2011)	Fibrosis is developed after 6-week feeding, and the chicken-wire fibrosis resembles human phenotype (George *et al.*, 2003)
Inflammatory mediators		
TNF-α/TNFR1	Increase (Abiru *et al.*, 2006)	Increase (Tomita *et al.*, 2006)
IL-6	Increase (Wieckowska *et al.*, 2008)	Increase (Yamaguchi *et al.*, 2010, 2011)
Adiponectin	Decrease (Lemoine *et al.*, 2009)	Unchanged or increase (Larter and Yeh, 2008; Leclercq *et al.*, 2007; Schattenberg *et al.*, 2006)
Leptin	No significant change (Angulo *et al.*, 2004)	Decrease

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase, CK-18, cytokeratin-18; TNFR, tumor necrosis factor receptor.

The utilization of the MCD model in NASH research has been commonly criticized due to its distinct metabolic profiles from human NASH patients. Animals fed on MCD diet have dramatic weight loss, which can be up to 40% in 8–10 weeks of feeding, and along with the loss of white adipose tissue, the liver size decreases in a proportional manner (Canet *et al.*, 2014; Rinella and Green, 2004). Nonalcoholic steatohepatitis is recognized as the hepatic manifestation of metabolic syndrome; systemic insulin resistance is frequent in human NASH patients and has been considered a pivotal factor in driving NAFLD progression (Asrih and Jornayvaz, 2015). In contrast, systemic insulin resistance is absent or even improved in the MCD model (Rinella and Green, 2004), whereas impaired hepatic insulin signaling as well as intrahepatic insulin resistance were observed (Leclercq *et al.*, 2007; Schattenberg *et al.*, 2006). Schattenberg *et al.* reported JNK-associated intrahepatic insulin resistance as a driving force of steatohepatitis development in MCD mice. The role of peripheral insulin resistance versus hepatic insulin resistance in human NAFLD progression is not fully understood, and therefore the argument of hepatic insulin resistance in the MCD model reflecting human pathogenesis remains questionable. To overcome weight loss and insulin sensitivity in wildtype MCD animals, MCD diets are often fed to genetically modified mice such as the Leptin knockout (*ob/ob*) mice and the Leptin receptor knockout (*db/db*) mice to better replicate human NASH (Canet *et al.*, 2014; Clarke *et al.*, 2015; Rinella *et al.*, 2008). The advantage of *ob/ob* and *db/db* mouse models is that they attain human characteristics of metabolic syndrome. *ob/ob* mice fed with 4 weeks of MCD diet show remarkable macrosteatosis, hepatic inflammation, and fibrosis, which is usually more severe than lesions seen in wildtype MCD mice (Clarke *et al.*, 2015; Li *et al.*, 2017a). However, weight loss as well as improved insulin resistance were still observed in *ob/ob* and *db/db* mice, meanwhile genetic leptin loss or resistance in humans with obesity are very rare, which is an inevitable limitation of these models. Nevertheless, the wildtype rodents fed with the MCD diet also exhibit lower leptin levels, unchanged or increased adiponectin levels, and decreased triglyceride and cholesterol, all of which contradict the metabolic profile of human disease (Larter and Yeh, 2008; Larter *et al.*, 2008; Rinella and Green, 2004). Furthermore, a recent study showed significant dissimilarities in the metabolomics profiles of MCD rats as compared with human NASH, which further demonstrates that the metabolic context of the MCD model is distinct from human NASH

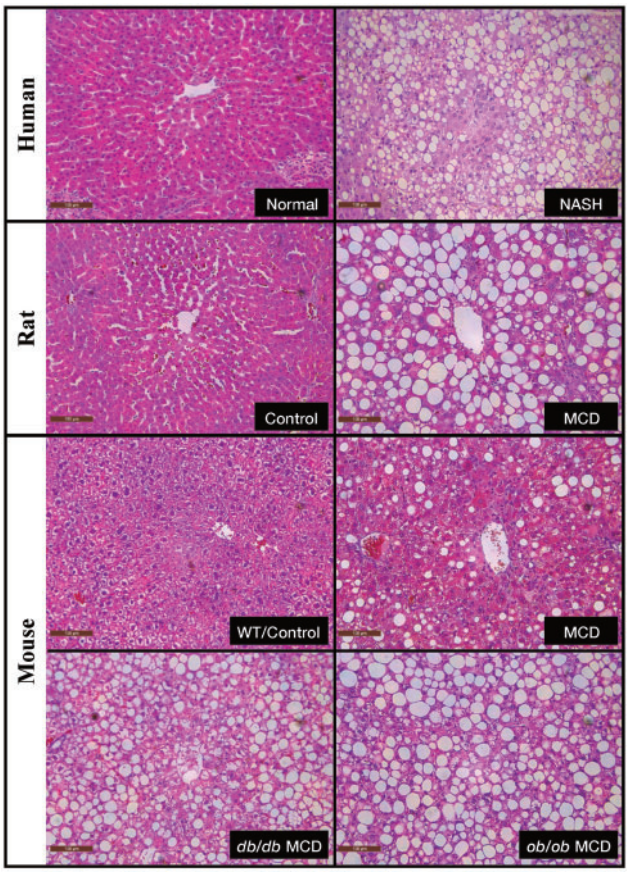

Figure 1. NASH histopathology in human and rodent models. Hematoxylin and eosin (H&E)-stained liver slides from human (normal vs NASH), rat (control vs MCD), and mouse (wild-type control, MCD, db/db MCD, and ob/ob MCD). All of the representative images are shown at × 20 magnification. NASH, nonalcoholic steatohepatitis; MCD, methionine and choline deficient; db/db, Leptin receptor knockout; ob/ob, Leptin knockout.

(Han *et al.*, 2017). Due to these limitations, the use of the MCD model to examine the metabolic parameters or study NASH pathogenesis should be discouraged.

MCD MODEL REFLECTING ADME ALTERATIONS IN HUMAN NASH

Studies have demonstrated profound hepatic ADME alterations during NASH progression, which influences multiple ADME pathways, including cytochrome P450s (CYPs), UDP-glucuronosyltransferases, sulfotransferases, glutathione-S-transferases, and hepatic drug transporters (Donato *et al.*, 2006; Fisher *et al.*, 2009b; Hardwick *et al.*, 2010, 2011, 2013; Lake *et al.*, 2011; Woolsey *et al.*, 2015) (Table 2). Several studies have reported altered pharmacokinetics in human NAFLD/NASH patients, which further implicates NASH as a risk factor of variable drug response and idiosyncratic ADRs (Canet *et al.*, 2015; Ferslew *et al.*, 2015; Woolsey *et al.*, 2015) (Table 3).

Cytochrome P450s

Cytochrome P450s belong to the largest and the most important drug metabolizing enzyme superfamily, which are involved in the metabolism of the majority of endogenous and exogenous substances. Among the known 57 putatively functional human CYPs, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are involved in metabolism of 78.7% of clinical drugs (Zanger and Schwab, 2013). Alterations in CYP activities can have substantial influence on the pharmacokinetics of substrate drugs, which, depending on drug exposure and the severity of enzyme alterations, may lead to potential compromised therapeutic effects or ADRs. Craig *et al.* performed systemic characterization of CYPs along NAFLD progression and found significant changes in expression and activity in multiple CYP isoforms, indicating a fundamental transformation of CYP-mediated phase I drug metabolism (Fisher *et al.*, 2009b). Alterations in individual CYP isoforms in response to NAFLD/NASH were also reported by other groups (Aljomah *et al.*, 2015; Aubert *et al.*, 2011; Woolsey *et al.*, 2015) (Table 2). Despite the fact that studies have demonstrated significant CYP changes in NASH, the underlying molecular mechanisms are poorly understood. Considering the complexity of physiological and pathological changes in NAFLD progression, CYPs may respond to multiple factors and cellular pathways, such as proinflammatory cytokines, oxidative stress, and nuclear receptors (Aitken *et al.*, 2006; Hardwick *et al.*, 2010; Lake *et al.*, 2016; Morgan, 2009). For instance, elevated plasma interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) have been confirmed in human NASH studies (Kugelmas *et al.*, 2003). Lipopolysaccharide (LPS) administration, which induces expression of a variety of proinflammatory cytokines, such as IL-6 and TNF-α, can significantly down-regulate hepatic mRNA expression of Cyp1a2, Cyp2c29, Cyp2e1, and Cyp3a11 in mice (Richardson and Morgan, 2005). Collectively, systemic and hepatic inflammation is not only one of the important driving forces of NAFLD progression, but may also play a role as a regulator of CYP activity. The MCD model is similar to human NASH with regard to CYP1A2/Cyp1a2 and CYP2E1/Cyp2e1, but fails to resemble human changes in CYP2C, 2D, and 3A (Leclercq *et al.*, 2000; Li *et al.*, 2017a; Rahman *et al.*, 2007; Weltman *et al.*, 1996; Yamazaki *et al.*, 2007). The disparities in CYP2C, 2D, and 3A could be a reflection of interspecies variabilities in CYP enzymes. Specific CYP isoforms in different species, even those with high sequence identity, may show diverse catalytic activity and specificity. Rat and mouse have comparable expression and activity of CYP1A and CYP2E1 when compared with human, but substantial differences in CYP2C, 2D, and 3A (Bogaards *et al.*, 2000; Martignoni *et al.*, 2006). Studies regarding CYP-mediated drug metabolism in either human NASH or the MCD model are relatively scarce. A study by Woolsey *et al.* examined CYP3A activity and expression in human NAFLD patients as well as the MCD model and cellular models. This study reported a 2.4-fold increase in plasma midazolam levels in subjects with NASH, though CYP3A4 activity, which was assessed by 4b-hydroxycholesterol, an endogenous CYP3A4/5 marker, had no significant changes (Woolsey *et al.*, 2015). This finding is consistent with a previous *ex vivo* study of CYP3A4 activity in human NASH (Fisher *et al.*, 2009b). Moreover, they also observed a significant reduction of CYP3A activity and expression in MCD mice and cell culture model (Woolsey *et al.*, 2015). Recently, our group published an *in vivo* evaluation of Cyp3a activity in the *ob/ob* MCD model, which used midazolam as a probe drug of Cyp3a. Even though increased midazolam plasma level was observed, the metabolite 1-hydroxy midazolam was also increased, leading to no significant change in the area under curve (AUC) ratio and no change in Cyp3a activity in the *ob/ob* MCD mice (Li *et al.*, 2017a). Considering that CYP3A isoforms and gene regulation mechanisms greatly differ between rodents and humans, the rodent MCD model may not accurately reflect the human response of CYP3A to NASH, which largely limits the utility of this model in predicting CYP3A-associated drug metabolism. Fisher

Table 2. Hepatic ADME Characterization in Human NASH and MCD Models

	Human	MCD Rodent Model
Phase I biotransformation		
CYP1A	• ↓ mRNA, protein, and activity in adult patients • No change of activity in pediatric patients (Fisher et al., 2009b; Li et al., 2017b)	• Male CAR$^{+/+}$ mice on 8-week MCD diet: ↓ mRNA (Yamazaki et al., 2007) • Male *ob/ob* MCD mice on 4-week MCD diet: ↓ mRNA, protein and activity (Li et al., 2017a)
CYP2B	• CYP2B6: ↑ mRNA, no change of protein and activity (Fisher et al., 2009b)	• Male C57BL/6 mice on 8-week MCD diet: ↓ mRNA and protein of Cyp2b10 (Cichocki et al., 2017) • Male Sprague Dawley rats on 8-week MCD diet: ↓ mRNA, protein and activity of Cyp2b1 (Cho et al., 2016) • Male CAR$^{+/+}$ mice on 8-week MCD diet: ↑ mRNA of Cyp2b10 (Yamazaki et al., 2007)
CYP2C	• ↑ CYP2C9 activity in adult NASH • ↓ CYP2C19 activity in both adult and adolescent (Fisher et al., 2009b; Li et al., 2017b)	• Male *ob/ob* MCD mice on 4-week MCD diet: ↓ Cyp2c29 mRNA, protein, and activity (Li et al., 2017a) • Male C57BL/6 mice on 8-week MCD diet: ↓ mRNA and protein of Cyp2c29 (Cichocki et al., 2017)
CYP2D	No change observed (Fisher et al., 2009a,b)	• Male Sprague Dawley rats on 8-week MCD diet: ↓ mRNA, protein and activity of Cyp2b1 • Male C57BL/6 mice on 8-week MCD diet (#519541, Dyets Inc): ↓ mRNA and protein of Cyp2b10. • Male *ob/ob* MCD mice on 4-week MCD diet: ↓ Cyp2d22 protein (Li et al., 2017a)
CYP2E1	• Inconsistent report of mRNA, protein, and activity of CYP2E1 (Aljomah et al., 2015; Aubert et al., 2011; Chtioui et al., 2007; Fisher et al., 2009b; Varela et al., 2008)	• Male Wistar rats on 30-day MCD diet • Male C57BL/6 mice on 8-week MCD diet and female C57BL/6 mice on 10-week MCD diet: ↑ mRNA and protein of Cyp2e1 (Leclercq et al., 2000; Rahman et al., 2007; Weltman et al., 1996)
CYP3A4/5	• ↓ Trend of protein and *ex vivo* activity (Fisher et al., 2009b) • ↓ mRNA and decreased *in vivo* activity (Woolsey et al., 2015)	• Male *ob/ob* MCD mice on 4-week MCD diet: ↓ Cyp3a11 protein (Li et al., 2017a) • Male C57BL/6 mice on 8-week MCD diet: ↓ mRNA and protein of Cyp3a11 (Cichocki et al., 2017)
NQO1	• ↑ mRNA, protein, and activity (Hardwick et al., 2010; Zhang et al., 2010)	• Male Sprague Dawley rats on 8-week MCD diet: ↑ mRNA and activity (Lickteig et al., 2007a,b)
Phase II biotransformation		
UGTs	• ↑ mRNA of UGT1A9, 2B10, 2A3, and 2B15; ↓ protein of UGT1A6 (Hardwick et al., 2013)	• Male Sprague Dawley rats on 8-week MCD diet: ↑ mRNA of Ugt2b1 (Dzierlenga et al., 2015)
SULTs	• ↑ mRNA of SULT1A1, 1C4, 2B1, and 4A1; ↑ protein of SULT1C4 (Hardwick et al., 2013)	No report
GSTs	• ↑ mRNA of GST isoform A1, A2, A4, M3, and P1 • ↑ protein of GSTA, GSTP • ↓ protein of GSTM (Hardwick et al., 2010)	• Male C57BL/6 mice on 6-week MCD diet: ↑ mRNA of Gsta1 and Gsta2; ↑ protein of Gsta1 (Sugimoto et al., 2010)
Drug transporters		
ABCs	• ↑ mRNA of ABCC1 (MRP1), ABCC4 (MRP4), ABCC5 (MRP5), ABCB1 (P-gp), and ABCG2 (BCRP) • ↑ protein of ABCC1 (MRP1), ABCC2 (MRP2), ABCC3 (MRP3), ABCC4 (MRP4), ABCC5 (MRP5), ABCC6 (MRP6), ABCB1 (P-gp), ABCG2 (BCRP), ABCC2 (MRP2) mislocalization (Canet et al., 2015; Hardwick et al., 2011; Okushin et al., 2016; Tanaka et al., 2012)	• Male Sprague Dawley rats on 8-week MCD diet: ↑mRNA of Mrp1, Mrp2, Mrp3, Mrp4, Mdr1a, Mdr1b, and Bcrp; ↑ protein of Mrp2, Mrp3, Mrp4, and Pgp • Male C57BL/6 mice on 8-week MCD diet: ↑ mRNA of Mrp2, Mrp4, and Mdr1a; ↑ protein of Mrp4 • Male *ob/ob* mice on 4-week MCD diet: ↑ mRNA of Mrp2, Mrp3, Mrp4, and Mdr1a; ↑ protein of Mrp4. • Male *db/db* mice on 4-week MCD diet: ↑ mRNA of Mrp2, Mrp3, Mrp4, and Mdr1a (Canet et al., 2014; Dzierlenga et al., 2015, 2016)
OATPs	• ↓ mRNA of OATP1B3, increased protein of OATP1B1, and ↓ protein of OATP1B3 (Clarke et al., 2014b)	• Male Sprague Dawley rats on 8-week MCD diet: ↑ mRNA of Oatp1a4 and ↓ mRNA of Oatp1b2; ↓ protein of Oatp1a4 and Oatp1b2 (Canet et al., 2014; Clarke et al., 2014a,b; Fisher et al., 2009a) • Male C57BL/6 mice on 8-week MCD diet: ↑ mRNA of Oatp1a4 and ↓ mRNA of Oatp1b2; ↑ protein of Oatp1a4, and ↓ protein of Oatp1a1, 1b2, and 2b1 • Male *ob/ob* mice on 4-week MCD diet: ↓ mRNA of Oatp1a1; ↓ protein of Oatp1b2. • Male *db/db* mice on 4-week MCD diet: ↑ mRNA of Oatp1a4 and ↓ mRNA of Oatp1a1 and 1b2; ↓ protein of Oatp1b2 (Canet et al., 2014; Clarke et al., 2014a,b)

Table 2. (continued)

	Human	MCD Rodent Model
OATs	No report	• Male Sprague Dawley rats on 8-week MCD diet: ↓ mRNA of Oat2 and Oat3 (Fisher et al., 2009a)
OCTs	No report	• Male Sprague Dawley rats on 8-week MCD diet: no change of Oct1 and Oct3 observed (Fisher et al., 2009a) • C57BL/6 mice on 8-week MCD diet and *ob/ob* mice on 4-week MCD diet: no change of Oct1 observed (Clarke et al., 2015)
NTCP	• Inconsistent report of Ntcp mRNA, ↓ protein level of NTCP (Aguilar-Olivos et al., 2015; Okushin et al., 2016)	• Male Sprague Dawley rats on 8-week MCD diet: ↓ mRNA of Ntcp (Fisher et al., 2009a) • C57BL/6NCr mice on 8-week MCD diet: decreased mRNA of Ntcp (Tanaka et al., 2012)

Abbreviations: BCRP, breast cancer resistance protein; CAR, constitutive androstane receptor; GST, glutathione S-transferases; MRD, multidrug resistance; NQO1, NAD(P)H dehydrogenase [quinone] 1; NTCP, Na^+-taurocholate co-transporting polypeptide; OAT, organic anion transporter; OCT, organic cation transporter; P-gp, P-glycoprotein; SULT, sulfotransferase; UGT-UDP, glucuronosyltransferase.

Table 3. MCD Model in Predicting Human Pharmacokinetics and ADRs

Drugs	Human	MCD Model
Morphine	• ↑ Morphine glucuronide systemic exposure (Ferslew et al., 2015) • ↑ M3G serum exposure due to reduced biliary excretion and increased basolateral efflux of M3G (Pierre et al., 2017)	• ↑ Morphine concentrations in the bile and plasma • ↑ M3G/morphine plasma AUC ratio • ↑ Systemic exposure to M3G with reduced biliary excretion and hepatic accumulation of M3G (Dzierlenga et al., 2015)
Acetaminophen	• ↑ APAP glucuronidation formation in children with NAFLD • No significant changes of the pharmacokinetics of APAP in children with NAFLD (Barshop et al., 2011) • ↑ Serum and urinary levels of APAP-glucuronide along with ↓ serum levels of APAP-sulfate in pediatric NASH patients (Canet et al., 2015)	• ↓ Biliary concentrations of APAP-sulfate, APAP-glucuronide (APAP-GLUC), and APAP-glutathione in MCD rats • ↓ Plasma APAP-sulfate in MCD rat • ↑ Plasma APAP-GLUC in MCD rat • ↑ Urinary elimination of APAP-GLUC (80% higher) in MCD rats (Lickteig et al., 2007a,b)
Midazolam	• ↑ Midazolam concentrations (2.4-fold greater) in adult NASH subjects (Woolsey et al., 2015) • No significant pharmacokinetic change in midazolam in pediatric NASH, though decrease trend in metabolism AUC ratio (Li et al., 2017b)	• ↑ Midazolam was increased in the *ob/ob* MCD mice • No significant change in the metabolism AUC ratio of midazolam in *ob/ob* and *ob/ob* MCD mice (Li et al., 2017a)
Bupropion	• No significant changes in *ex vivo* bupropion hydroxylase metabolism in human hepatic microsome (Fisher et al., 2009b)	• ↓ Enzyme-kinetic and pharmacokinetic parameters of bupropion metabolism in MCD rats (Cho et al., 2016)

Abbreviations: APAP, Acetaminophen; AUC, Area under curve; CYP, Cytochrome p450; M3G, Morphine–glucuronide; MCD, Methionine and choline deficient; NAFLD, Nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis.

et al. reported no significant changes in CYP2B6 protein expression and enzymatic activity in human NASH, when enzymatic activity was evaluated by *ex vivo* incubation of human hepatic microsomes with the CYP2B6 specific substrate bupropion hydroxylase (Fisher *et al.*, 2009b). In a recent study, Cho *et al.* investigated hepatic Cyp2b1, the rat ortholog of human CYP2B6, and *in vivo* bupropion disposition in both high-fat diet and MCD diet rats. Cyp2b1 mRNA and protein expression were significantly decreased in MCD rats, whereas the metabolism AUC ratio of bupropion was considerably reduced in MCD rats, suggesting a reduction of *in vivo* hepatic Cyp2b1-mediated metabolism of bupropion (Cho *et al.*, 2016). Additionally, a study addressed the modulation of xenobiotic disposition and metabolism and increased hepatic exposure to tetrachloethylene and trichloroacetate in MCD mice, which may be associated with a significant reduction of Cyp2b10 expression in MCD mice (Cichocki *et al.*, 2017). Based on the data produced in the MCD model, decreased Cyp2b metabolism in rodent is distinct from unchanged CYP2D6 metabolism in human NASH. In brief, the MCD diet is not an ideal model for modeling CYP alterations or predicting altered drug metabolism and disposition related to CYPs in human NASH.

Multidrug Resistance–Associated Proteins: MRP2 and MRP3

The ATP-binding cassette (ABC) family of transporters consists of a variety of proteins that use the energy of ATP hydrolysis and actively transport xenobiotics and endobiotics across cellular membranes. The ABCC subfamily of ABC transporters are also known as multidrug resistance–associated proteins (MRPs), which are all efflux transporters. In the liver, these efflux transporters reside on the sinusoidal and canalicular membranes of the hepatocyte and are responsible for substrate efflux into blood and bile (Klaassen and Aleksunes, 2010). Multidrug resistance–associated protein 2 is an efflux transporter on the

hepatic canalicular membrane that shuttles xenobiotic conjugates into the bile, whereas MRP3 is a sinusoidal efflux transporter responsible for transport into blood circulation and shares a wide range of substrate specificity with MRP2. Multidrug resistance–associated proteins 2 and 3 levels are significantly increased in human NASH, whereas evidence showed that during NASH progression, some of the MRP2 protein was mislocalized away from the canalicular membrane and led to compromised MRP2 function in human patients (Hardwick *et al.*, 2011). Similar Mrp2 and Mrp3 alterations were also found in MCD models. Increased Mrp2 and Mrp3 protein levels were universally found in all MCD-fed rodent models, whereas mislocalization of Mrp2 was also confirmed in MCD rats (Canet *et al.*, 2014; Dzierlenga *et al.*, 2015, 2016). These disease-induced alterations in MRP2/MRP3 efflux system have been associated with significant pharmacokinetic changes in clinical drugs. Barshop *et al.* (2011) defined an altered acetaminophen (APAP) glucuronidation and altered APAP-Gluc disposition in children with NAFLD, although the actual underlying mechanism was not described. Furthermore, Canet *et al.* (2015) demonstrated that a significant increase in serum and urinary levels of APAP-Gluc only presented in NASH patients, the advanced stage of NAFLD, and the study further established the mechanistic connection between the loss of MRP2 function and induced MRP3 function and altered APAP-Gluc disposition. These findings were consistent with a previous animal study, which reported significant induction of plasma and urinary APAP-Gluc and reduction of biliary APAP-Gluc in MCD rats administered APAP (Lickteig *et al.*, 2007a,b). Morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) are 2 major metabolites of morphine in humans, which mostly rely on MRP2 and MRP3 for hepatobiliary disposition. A human study showed that morphine glucuronide systemic exposure and bile acid serum concentrations were substantially higher in NASH patients. A follow-up study further established a quantitative relationship between NASH severity score and the exposure of M3G, which was attributed to reduced biliary excretion and increased basolateral efflux of M3G (Ferslew *et al.*, 2015; Pierre *et al.*, 2017). Meanwhile, a parallel morphine disposition study in MCD rats also showed increased systemic exposure to M3G, but decreased biliary excretion and hepatic accumulation of M3G (Dzierlenga *et al.*, 2015). Other than clinical drugs that were characterized in both humans and rodents, several studies reported changed disposition of MRP2/MRP3 substrate drugs, such as ezetimibe, methotrexate, and pemetrexed, in MCD-fed rodents (Dzierlenga *et al.*, 2016; Hardwick *et al.*, 2012, 2014). Collectively, current research has well documented MRP2 and MRP3 alterations in NASH and potential clinical outcomes in increased systemic toxicities and decreased hepatic drug efficacy. Furthermore, the MCD model precisely reflects molecular alterations of MRP2 and MRP3 in human NASH and successfully repeats changes in MRP2/MRP3-mediated drug dispositions in human NASH. In terms of promoting precision medicine in clinical practice, NASH should be critically considered in MRP2/MRP3 substrate drug prescriptions. Considering the wide range of these transporters, the MCD model can be a useful model in the preclinical evaluation of individual substrates to predict ADRs and mechanistic studies of variable drug responses in NASH.

Organic Anion–Transporting Polypeptides: OATP1B1, OATP1B3, and OATP2B1

Organic anion–transporting polypeptides (OATPs) belong to the superfamily of solute carriers and mediate the uptake of a broad range of compounds into cells (Kim, 2003). Organic anion–transporting polypeptide substrates include endogenous substances such as bile salts, hormones, and steroid conjugates as well as clinical drugs like the HMG-CoA-reductase inhibitors (statins), cardiac glycosides, anticancer agents like methotrexate, and antibiotics like rifampicin (König *et al.*, 2006). Among 11 human OATP transporters, OATP1B1, OATP1B3, and OATP2B1 are predominantly expressed on sinusoidal membrane of hepatocytes and play particular roles in hepatic drug pharmacokinetics (Kalliokoski and Niemi, 2009; Smith *et al.*, 2005). Additionally, OATP1B1, OATP1B3, and OATP2B1 exhibit great overlap in substrate specificity, which provides a compensatory system that maintains general uptake capacity if one transporter's function is compromised or lost (Kalliokoski and Niemi, 2009). In human NASH, OATP1B1 expression is increased 3-fold, whereas OATP1B3 expression is decreased 10-fold, though OATP2B1 is not significantly changed (Clarke *et al.*, 2014b). In MCD rats, studies reported decreased Oatp1a1, 1a4, and 1b2 protein expression levels (Canet *et al.*, 2014; Clarke *et al.*, 2014a; Fisher *et al.*, 2009a). An increase in plasma concentration of bromosulfophthalein, a common compound used in liver function test, indicated impaired function of hepatic uptake transporters in the MCD rats (Fisher *et al.*, 2009a). Furthermore, a study reported an increase in exposure to simvastatin hydroxyl and potential myopathy toxicity in MCD rats, which may be associated with an overall downregulation of OATP transporters (Clarke *et al.*, 2014a). In the MCD mice, Oatp1a4 expression is increased, whereas Oatp1a1, 1b2, and 2b1 are significantly decreased, which moderately reflects the OATP alteration pattern in human NASH to some extent (Canet *et al.*, 2014; Clarke *et al.*, 2014b). Moreover, Clarke *et al.* demonstrated a gene-by-environment effect on pravastatin disposition in MCD-fed Oatp1b2 knockout mice, which provided mechanistic insight into the occurrence of statin-induced ADRs in the general population and particularly in the subpopulation of NASH patients (Clarke *et al.*, 2014b). So far, studies regarding NASH impact on OATP substrate drugs in actual human patients are still absent, and therefore future studies into the pharmacokinetics of OATP substrates in human NASH are required to substantialize this effect as well as to evaluate the current animal models.

CONCLUSIONS

Overall, although an ideal animal model or *in vitro* model of NAFLD/NASH has yet to be discovered, especially for studies of pathogenic mechanisms and metabolic signature profiling, the current MCD model provides an auxiliary tool to study this major human disease, particularly with regard to potential drug ADRs and toxicities associated with drug transporter alterations during disease progression. Mechanistically, NASH-induced membrane transporter alterations are likely more associated with morphological changes on a cellular basis rather than metabolic pathogenic abnormalities in NASH; therefore, the MCD model, which most closely resembles human NASH histopathology, shows advantage in reflecting changes in drug transporters. In this case, alterations in rodent transporter function in response to the MCD diet closely resemble the alterations in human NASH and provide the pathological context that can reflect potential risk factors in altering pharmacokinetics or pharmacodynamics in the NASH subpopulation.

Investment in drug discovery and development in NASH are growing rapidly. It is foreseeable that medication therapy will become a crucial part of NASH management. As we come to better understand the potential for altered ADME processes in NASH and other inflammatory disease states, animal models

that accurately reflect these changes will be invaluable in identifying potential ADRs. Therefore, animal model selection and development remain a major priority to researchers in this field.

Extrapolation of animal-to-human results is always challenging, because there is no perfect animal model that predicts the pharmacokinetics and pharmacodynamics of all chemicals in humans. Although there are many reasons to reject the MCD model for various applications, the similarity of alterations in ADME processes, particularly those of drug disposition mediated by MRP2/MRP3 and OATPs, make it a useful tool in predicting NASH-associated ADRs in this sensitive population. This publication was supported by National Institutes of Health [Grants HD062489 and GM123643], and the National Institute of Environmental Health Science [Grant ES007091 and ES006694].

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