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Human *in vitro* models of nonalcoholic fatty liver disease

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Abstract

Systems-oriented approaches involving integrated *in vitro* and *in silico* models are emerging as a basis for future risk assessment strategies to address the current challenge in chemical risk assessment of increased numbers of registered chemicals with no toxicological data. Liver toxicity poses a major concern in risk assessment as the liver is exposed to most xenobiotic compounds and their bioactive metabolites, making the liver susceptible to chemically induced liver diseases. Nonalcoholic fatty liver disease (NAFLD) is a rising global burden with an estimated prevalence of about 25% in the general population. Risk factors for NAFLD include metabolic disorders, genetics, drugs, and environmental exposures. To integrate *in vitro* data into computational models and use *in vitro* to *in vivo* extrapolation to systematically assess the risk of chemicals to induce NAFLD, sophisticated human cell models are needed. Reviewed here are characteristics and limitations of 2D and 3D monoculture and coculture human liver cell models, including microphysiological systems, in which NAFLD-relevant end points were addressed together with their results. The aim of this review is to inform selection of experimental test systems depending on the toxicological question to be addressed and to promote the further development of *in vitro* approaches that address existing technological gaps.

Addresses

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Keywords

Nonalcoholic fatty liver disease, Hepatocytes, *In vitro* models, Hepatotoxicity, Steatosis.

Abbreviations

NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; FFA, free fatty acid; PHH, primary human hepatocyte; HLC, hepatocyte-like cell; iPSC, induced pluripotent stem cell; HSC, human stellate cell.

Introduction

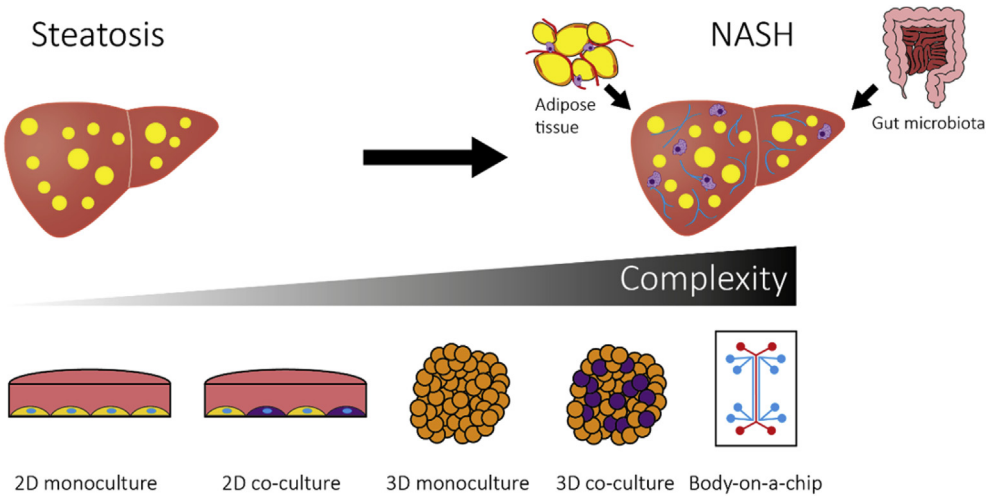
Current chemical risk assessment faces a huge challenge as there are increasing numbers of chemicals registered at federal agencies, in the United States over 80000 [1] and in the European Union over 90′000 [2]. Most have limited or no toxicological data suitable for informing risk assessment. Together with the widely acknowledged ethical and financial requirements that limit animal use in toxicological studies, systems-oriented approaches involving integrated *in vitro* and *in silico* models are an emerging future risk assessment strategy [3,4].

Liver toxicity poses a major concern in risk assessment as the liver is exposed to xenobiotic compounds and their metabolites, making the liver susceptible to chemically induced liver diseases. Nonalcoholic fatty liver disease (NAFLD) is a rising global burden with an estimated prevalence of about 25% in the general population, suggesting it will probably be the leading cause of end-stage liver disease in the coming decades [5,6]. Risk factors for NAFLD include metabolic disorders, genetics, drugs, and environmental exposures [7–9]. A number of drugs, such as amiodarone, valproic acid, and some cancer chemotherapeutics, are known to cause NAFLD in some patients, particularly after chronic treatment [10–13]. Exposure to environmental chemicals, such as solvents, persistent organic pollutants, and pesticides, has been implicated as a risk factor [8,14]. Epidemiological studies suggest a link between exposure to heavy metals (lead and mercury) and polychlorinated biphenyls and NAFLD prevalence [15,16]. To integrate *in vitro* data into computational models and use *in vitro* to *in vivo* extrapolation to assess systematically the risk of chemicals to induce NAFLD, sophisticated human cell models are needed that reflect molecular processes underlying the etiology of NAFLD (Figure 1, Table 1).

Pathology of NAFLD

NAFLD, often referred to as the hepatic manifestation of metabolic syndrome, encompasses a wide spectrum of liver diseases starting from simple fat accumulation (steatosis) to nonalcoholic steatohepatitis (NASH) with or without fibrosis, cirrhosis, and eventually hepatocellular carcinoma (Figure 1). The underlying molecular mechanisms are complex, involving multiple parallel

Figure 1



From a relatively simple disease phenotype (steatosis) to a more complex one (NASH), multiple tissues are involved and more complex cells models are required. These start from 2D monoculture to 2D coculture over to 3D monoculture and coculture up to body-on-a-chip models. NASH, nonalcoholic steatohepatitis.

factors acting synergistically on the liver and leading to the development or progression of the disease (Figure 2). One of the key events in the development of NAFLD is insulin resistance, which leads to an increase in hepatic *de novo* lipogenesis and impaired inhibition of adipose tissue lipolysis. Together with elevated free fatty acid (FFA) levels in serum, the accumulation of FFA within the liver increases and results in steatosis, a key cellular event in NAFLD. Ongoing accumulation of FFA leads to endoplasmic reticulum stress and mitochondrial dysfunction where the latter will induce an overproduction of reactive oxygen species. Genetic variants in the form of single-nucleotide polymorphisms in genes mostly involved in lipid metabolism influence hepatic FFA flux, oxidative stress, response to endotoxins, and cytokine production and activity, increasing the risk of NAFLD [17,18]. Thus, all these molecular and cellular events result in inflammation and apoptosis and eventually in liver disease.

Human *in vitro* models of NAFLD: current approaches and challenges

2D monoculture cell models

Primary human hepatocytes (PHHs, Table 1) are considered the gold standard short-term human *in vitro* liver model because of their high functionality relative to the human organ *in vivo*. They can be obtained either from isolation of liver resections or liver tissues improper for transplantation or cryopreserved from commercial suppliers. Therefore, they are considered to be highly reliable for predictive results in toxicological and pharmacological research; however, limited culture duration of only a couple of days and donor variability are two major drawbacks. The donor variability is a double-edged sword; as on one hand, the relevance of interindividual variations in genetic polymorphisms can be investigated using samples from different donors, while on the other hand, donor difference and cell alteration after isolation can lead to variance in experimental results and poor reproducibility [19–21]. Nevertheless, FFA-exposed PHHs showed a dose-dependent increase in lipid accumulation, expression of C/EBP homologous protein (key protein leading to endoplasmic reticulum (ER) stress), induction of the profibronetic gene transforming growth factor beta (TGFβ), fibronectic activation of hepatic stellate cells via conditioned media from lipid-loaded PHH, and expression of the chemokine regulated upon activation, normal T-cell expressed and secreted [22–25]. Thus, despite limitations, PHHs are used effectively to study various key events of NAFLD.

A promising alternative to PHHs is hepatocyte-like cells (HLCs) derived from induced pluripotent stem cells (iPSCs). These iPSCs are pluripotent stem cells reprogrammed from somatic cells by the ectopic coexpression of transcription factors. They can be used to generate patient-derived HLCs to screen for drugs tailored to individual patients to address hepatotoxicity and interindividual variation in response as major reasons for drug failure in preclinical trials [20,26]. Graffmann et al. [27] used HLCs differentiated from iPSCs to create an NAFLD model, whereupon exposure to FFA lipid accumulation, upregulation of perilipin 2, and genes involved in the peroxisome proliferator–activated

Table 1 Overview of human *in vitro* cell models of NALFD.

	Cell type	Exposure	Duration	NAFLD-relevant end points analyzed	Validation	Reference
2D monoculture	PHH	Oleic acid Palmitic acid Elaidic acid	24 h	Steatosis ER stress apoptosis	A[c]	[22]
		Either oleic acid or palmitic acid	24 h	Steatosis Secretion of TGFβ		[23]
		Palmitic acid	24 h	Expression of RANTES		[24]
		Palmitic acid	24 h	Steatosis Fibrosis		[25]
		Mixture of oleic acid and palmitic acid	14 h, 24 h, 36 h	Steatosis Apoptosis		[31]
	HepG2	Bisphenol A	48 h	Steatosis	B[d]	[28]
		Valproate	24 h & 48 h	Steatosis	B, C[e]	[29]
		Oleic acid	24 h	Steatosis Apoptosis	A	[30]
		Mixture of oleic acid and palmitic acid	14 h, 24 h, 36 h	Steatosis Apoptosis,		[31]
		Oleic acid	24 h	Steatosis		[27]
	HuH 7	Palmitic acid	24 h	Steatosis		[35]
		Oleic acid and palmitic acid	24 h	Steatosis, ER stress Apoptosis		[36]
	HepaRG	16 previously known steatosis inducer 12 previously known as non-steatosis inducer	24 h	Steatosis MMP Oxidative stress Cell viability	B, C	[40]
		Either stearic acid or oleic acid to induce lipid accumulation. APAP to study cytotoxicity under steatotic conditions	FFA: 1 week APAP: Last 6 h, 24 h or 48 h of the 1 week FFA exposure	Steatosis CYP2E1 and CYP3A4 expression and activity Oxidative stress	A	[39]
		Tetracycline Amiodarone	24 h or 14 days	Steatosis	C	[41]
		Cyproconazole	24 h or 72 h	Steatosis Nuclear receptor activation Mitochondrial respiration	B	[42]
	iPSC	Oleic acid	iPSC: 48 h	Steatosis	A	[27]
2D coculture	Huh 7 and LX2 (human stellate cell line)	Mixture of oleic acid and palmitic in a molar ratio of 2:1 (oleic acid:palmitic acid)	24 h	Steatosis in hepatocyte and stellate cells, activation of stellate cells	A	[43]
3D monoculture	PHH	Mixture of oleic acid and palmitic acid (1:1 M ratio) High fructose	7, 14 and 21 days	Steatosis Insulin resistance CYP3A4 expression	A	[46]
		2:1 mixture of oleic acid and palmitic acid under constant flow	7 and 14 days	Steatosis Metabolic capacity Expression of inflammatory and fibrotic markers		[47]
3D coculture	HepaRG and primary HSC	APAP Ally alcohol Methotrexate	24 h 13 days[a]	Activation of HSC and expression of profibrotic markers Mitochondrial dysfunction	B	[49]
	PHH and primary Kupffer cells PHH and primary HSCs PHH and primary biliary cells	Cyclosporine A[b]	48 h	Steatosis	B	[50]

(*continued on next page*)

Table 1 (*continued*)

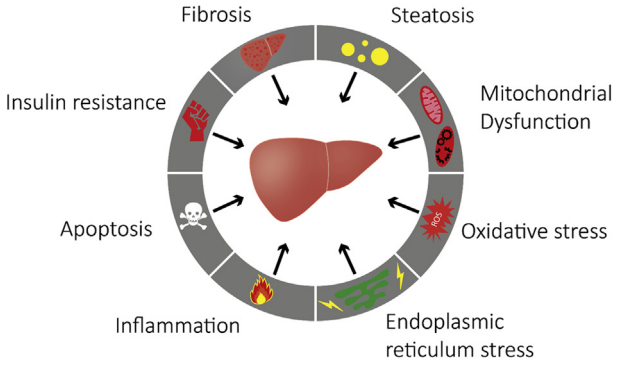
	Cell type	Exposure	Duration	NAFLD-relevant end points analyzed	Validation	Reference
	PHH and primary human HSCs and primary human macrophages	Lipotoxic environment: High glucose Oleic acid Palmitic acid	10 days	Steatosis Dysregulation of insulin signaling Oxidative stress Apoptosis Inflammation Expression of profibrotic markers	D[f]	[51]

NAFLD, nonalcoholic fatty liver disease; iPSC, induced pluripotent stem cell; PHH, primary human hepatocyte; FFA, free fatty acid; HSC, human stellate cell; ER, endoplasmic reticulum stress; TGFβ, transforming growth factor beta; RANTES, regulated on activation normal T cell expressed and secreted; MMP, mitochondrial membrane potential. APAP, N-acetyl-para-aminophenol, acetaminophen.
[a] Repeated exposure.
[b] Monospheroids only.
[c] A: selected FFAs are found the be elevated in obese patients with fatty liver.
[d] B: confirmed in animal studies.
[e] C: confirmed in human studies.
[f] D: *in vitro* data compared to clinical data.

receptor pathway was observed. Despite the advantage of studying patient-specific disease models, a major limitation is difficulties in producing large volumes of these cells and the risk of teratoma formation.

Another alternative to PHH or HLC are human hepatoma cell lines (HepG2, Huh 7, and HepaRG). HepG2 cells have been widely used to model NAFLD *in vitro*. Bisphenol A—treated cells showed a dose-dependent increase in lipid accumulation, expression of sterol regulatory element—binding transcription factor 1, and other genes involved in *de novo* lipogenesis. Valproate treatment induced lipid accumulation via upregulation of cluster of differentiation 36, a fatty acid transporter, and diacylglycerol transferase 2. FFA-exposed cells showed a dose-dependent increase in lipid accumulation with an elevated production of tumor necrosis factor alpha, a proinflammatory cytokine [28—31]. In addition, a high-content imaging assay has been developed to simultaneously investigate lipid accumulation, oxidative stress, mitochondrial membrane potential, and cell viability of 16 previously reported steatosis-inducing drugs where all were positive for lipid accumulation in this assay [32]. A major drawback in using HepG2, however, is the lack or low expression of drug metabolizing enzymes, which can be partially overcome by transfecting with vectors containing relevant enzymes [20,33].

Figure 2



Hallmarks of NAFLD. Key cellular events important in the induction and progression of the disease. NAFLD, nonalcoholic fatty liver disease.

To further address limitations in important metabolic enzyme functions, there exist cell lines with metabolic enzyme expression patterns similar to PHH. HuH 7 cells exhibit effective CYP3A4 activity when grown to confluence [34] and have been used to investigate the mechanism of steatosis-ameliorating chemicals as well as unveiling new mechanisms in the pathogenesis of NAFLD [35,36]. In addition, HepaRG cells are emerging as a popular cell line with expression patterns of drug-metabolizing enzymes and drug transporters similar to PHH. Derived from a human hepatocellular carcinoma, differentiated HepaRG cell cultures typically contain hepatocyte- and biliary-like cells and were shown to express various drug-metabolizing enzymes including CYPs and transporters of the phase II metabolism [37,38]. Therefore, it has been used in toxicological and drug metabolism studies [37,38]. Recently, HepaRG cells exposed to FFAs showed an increase in triglycerides levels and a decrease in CYP2E1 activity, which resulted in a higher decrease in cell viability upon acetaminophen treatment, compared with nonsteatotic cells [39]. In addition, a high-content analysis of HepaRG cells exposed to 16 compounds previously known to induce steatosis flagged them as positive for lipid accumulation in this assay [40]. Moreover, the HepaRG cells have been used in elucidating the

mechanism of the induction of steatosis by amiodarone and tetracycline after short-term and repeated exposure [41]. Lately, they also have been used to investigate key events from the adverse outcome pathway of chemically induced liver steatosis, by exposing HepaRG cells to cyproconazole. They confirmed activation of retinoic acid receptor alpha and pregnane X receptor as molecular initiating events and triglyceride accumulation and mitochondrial dysfunction as key events. However, they also observed different molecular changes than those described in the adverse outcome pathway [42]. Thus, HepaRG and other 2D cell models are suitable for the study of several key events in NAFLD, but most studies have addressed steatosis and oxidative stress, and underlying mechanisms individually, rather than tracking multiple parallel cellular events and how this networked interaction impacts NAFLD risk.

2D coculture cell models

The relatively easy handling of 2D monoculture systems and possibility of scaling up to high-throughput experiments make them attractive models, but they lack dimensionality and do not have medium flow or other liver cell types such as Kupffer or Stellate cells. Introducing a second cell type into *in vitro* NAFLD models increases their complexity, but some drawbacks from monoculture systems can be effectively overcome. A recent study demonstrated an *in vitro* model of NASH involving cocultured HuH 7 and human stellate cell line (LX2), which is an immortalized cell line derived from a normal liver and retains key features of cytokine signaling and fibrogenesis. After exposure to FFA for 24 h, lipid accumulation in both cell types and activation of human stellate cells (HSC) were observed [43,44]. A clear advantage in such a model is that the same methodologies as in monoculture can be used and they are also scalable to high-throughput experiments. However, 2D coculture systems with human hepatocytes and another human cell type (Kupffer cell, HSC) where NAFLD is reflected are scarce, potentially due to the challenge of finding optimal culture condition and sources for both cell types.

3D monoculture cell models

In the past decades, major advances in 3D cell culture system have been made, especially in the field of liver research. One of the most common techniques used is the 2D sandwich culture where hepatocytes are put between layers composed of extracellular matrix components [45]. Other techniques involve hydrogels, synthetic scaffolds, and spheroids. 3D cell culture has the advantage of more closely mimicking the *in vivo* situation than 2D, suggesting more physiological or pathophysiological relevance. A recently published study introduced a 3D spheroid model with PHH to investigate steatosis and insulin resistance. They treated the spheroids at physiologically and pathophysiologically relevant concentrations of FFA and insulin to induce lipid accumulation and insulin resistance over a timeframe of 21 days. Transcriptional analysis also revealed the upregulation of lipogenic genes and they could also reverse the steatotic phenotype with antisteatotic chemicals [46].

Microfluidic devices as a basis of perfused microphysiological systems are of great interest to model *in vivo* blood flow. Thus, another 3D NAFLD-relevant *in vitro* model involved a perfused platform using PHH *I*cells. The system was exposed to FFA over 7 and 14 days, and metabolic, transcriptome, and phenotypic changes were then measured [47]. Gori et al. also developed a microfluidic perfused liver model with HepG2 cells to create liver-on-a-chip devices and exposed those to FFA to induce steatosis. They showed that accumulation in the chip model is much slower compared with the traditional 2D cell culture and the viability is higher as well [48]. These three models emphasize the advantages of 3D as PHHs can be cultivated up to 3 weeks, allowing for experiments with repeated exposure and thus lowering the concentration of NAFLD-inducing agents to more relevant levels. Moreover, 3D liver spheroids are scalable for high throughput screening (HTS) experiments. Besides using only one cell type, however, the development of such 3D models is time consuming and requires significantly more validation work as it is still in early stages of use. The future full potential of 3D models is anticipated to be exploited with the advent of coculture systems and capacity for sustaining long-term exposure scenarios.

3D coculture cell models

3D coculture models can be used to study the more complex phenotype of NASH, where inflammation and liver fibrosis occur. For that, human liver cells need to be cocultured with either human Kupffer cells or HSC or both together. Recently, Leite et al. published a model where they created 3D spheroids consisting of HepaRG cells and primary HSC and that maintained their metabolic activity over 21 days, allowing for repeated exposure. They also showed that the hepatic organoids showed fibrotic features after treating them with profibrotic chemicals such as allyl alcohol and methotrexate. In addition, they also showed that acetaminophen can activate HSC *in vitro* and confirmed it *in vivo*, which was previously unknown [49].

Besides fibrosis, inflammation is another important key event in NAFLD. Bell et al. demonstrated that PHH spheroids can be cocultured with either Kupffer cells, HSC, or biliary cells, and they showed that stimulating PHH spheroids cocultured with Kupffer cells elicit an increase in interleukin-6 secretion, modeling an inflammatory response. They also investigated steatosis in pure PHH spheroids by treating them with cyclosporine A. However, other key cellular events of NAFLD or

other NAFLD inducing chemicals were not further analyzed as the main focus lied on characterization of PHH spheroids [50].

A different study by Feaver et al. presented an *in vitro* NAFLD model where PHH were cocultured with human macrophages and HSCs under hemodynamic and transport condition. They exposed the model to a lipotoxic environment, consisting of elevated glucose, insulin, and FFA levels to induce a NASH phenotype. With that, they showed that the model exhibited lipid accumulation, mitochondrial dysfunction, an increase in oxidative stress, secretion of inflammatory markers, as well as fibrotic markers. They also challenged the disease state with obeticholic acid, a phase III drug and showed an amelioration in the phenotype [51]. Besides directly coculturing hepatocytes with nonparenchymal cells, microfluidic devices are capable of coculturing different tissues on a chip, mimicking closer the *in vivo* situation. A recent published study presented for the first time a gut-liver-on-a-chip model, where they included gut absorption of FFA to successfully induce steatosis in hepatocytes [52].

These 3D coculture models of NAFLD are the closest to mimicking the *in vivo* phenotype. They can recapitulate multicellularity in 3D and maintain important metabolic function up to 3 weeks, and phenotypes can be induced under pathophysiologically relevant conditions. In addition, microphysiological systems allow for coculturing multiple tissues on a chip, which can address the multitissue etiology of NAFLD. Nevertheless, owing to its complexity, these models are time consuming and costly to establish, limiting scalability and allowing for testing a small number of drugs or chemicals.

Conclusion

Developing *in vitro* models of NAFLD poses a highly relevant challenge to the research community with several recent advances (see Table 1). The majority of models used are 2D involving either PHH or a human cell lines. With these relatively simple systems, steatosis as a key event has been most investigated and these models were mainly exposed to FFAs and chemicals known to induce steatosis. A few studies have advanced to the level of testing hundreds of chemicals for their steatotic potential with a high-content assay, while there are also emerging complex 3D and microfluidic models with the potential to address synergistic processes contributing to the complex pathology of NAFLD.

Emerging new techniques such as the liver on a chip or body on a chip will allow future studies to expand the NAFLD scope beyond liver cells to also include adipose tissue or the gut microbiome as important contributors to the pathogenesis of the disease, together with critical computational models such as SteatoNET, which is a multipathway and multitissue model that identified pathway branches that drive a healthy liver toward steatosis [53]. NAFLD disease mechanisms are anticipated to be better understood. This mechanistic knowledge and *in vitro* tools will contribute to novel therapies and improve the capacity to predict the contributions of drugs and chemicals to NAFLD risk.

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Conflict of interest statement

Nothing declared.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. US EPA, About the TSCA chemical substance inventory. https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory (accessed February 14, 2019).
2. ECHA, Registration statistics infograph - ECHA. https://echa.europa.eu/registration-statistics-infograph# (accessed February 14, 2019).
3. Sturla S, Boobis AR, FitzGerald RE, Hoeng J, Kavlock RJ, Schirmer K, Whelan M, Wilks MF, Peitsch MC: **Systems toxicology: from basic research to risk assessment**. *Chem Res Toxicol* 2014, **27**:314–329. https://doi.org/10.1021/tx400410s.
4. Hartung T, FitzGerald RE, Jennings P, Mirams GR, Peitsch MC, Rostami-Hodjegan A, Shah I, Wilks MF, Sturla SJ: **Systems toxicology: real world applications and opportunities**. *Chem Res Toxicol* 2017, **30**:870–882. https://doi.org/10.1021/acs.chemrestox.7b00003.
5. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, George J, Bugianesi E: **Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention**. *Nat Publ Gr* 2017, **14**. https://doi.org/10.1038/nrgastro.2017.109.
6. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M: **Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes**. *Hepatology* 2016, **64**:73–84. https://doi.org/10.1002/hep.28431.
7. Rabinowich L, Shibolet O: **Drug induced steatohepatitis: an uncommon culprit of a common disease**. *BioMed Res Int* 2015, **2015**:1–14. https://doi.org/10.1155/2015/168905.
8. Wahlang B, Beier JI, Clair HB, Bellis-Jones HJ, Falkner KC, McClain CJ, Cave MC: **Toxicant-associated steatohepatitis**. *Toxicol Pathol* 2013, **41**:343–360. https://doi.org/10.1177/0192623312468517.
9. Arciello M, Gori M, Maggio R, Barbaro B, Tarocchi M, Galli A, Balsano C: **Environmental pollution: a tangible risk for NAFLD pathogenesis**. *Int J Mol Sci* 2013, **14**:22052–22066. https://doi.org/10.3390/ijms141122052.
10. Begriche K, Massart J, Robin M-A, Borgne-Sanchez A, Fromenty B: **Drug-induced toxicity on mitochondria and lipid metabolism: mechanistic diversity and deleterious consequences for the liver**. *J Hepatol* 2011, **54**:773–794. https://doi.org/10.1016/J.JHEP.2010.11.006.

11. Jennings P, Schwarz M, Landesmann B, Maggioni S, Goumenou M, Bower D, Leonard MO, Wiseman JS: **SEURAT-1 liver gold reference compounds: a mechanism-based review**. *Arch Toxicol* 2014, **88**:2099–2133. https://doi.org/10.1007/s00204-014-1410-8.

12. Schumacher JD, Guo GL: **Mechanistic review of drug-induced steatohepatitis**. *Toxicol Appl Pharmacol* 2015, **289**:40–47. https://doi.org/10.1016/j.taap.2015.08.022.

13. Willebrords J, Pereira IVA, Maes M, Crespo Yanguas S, Colle I, Van Den Bossche B, Da Silva TC, de Oliveira CPMS, Andraus W, Alves VA, Cogliati B, Vinken M: **Strategies, models and biomarkers in experimental non-alcoholic fatty liver disease research**. *Prog Lipid Res* 2015, **59**:106–125. https://doi.org/10.1016/j.plipres.2015.05.002.

14. Kaiser JP, Lipscomb JC, Wesselkamper SC: **Putative mechanisms of environmental Chemical?Induced steatosis**. *Int J Toxicol* 2012, **31**:551–563. https://doi.org/10.1177/1091581812466418.

15. Cave M, Falkner KC, Ray M, Joshi-Barve S, Brock G, Khan R, Bon Homme M, McClain CJ: **Toxicant-associated steatohepatitis in vinyl chloride workers**. *Hepatology* 2010, **51**:474–481. https://doi.org/10.1002/hep.23321.

16. Cave M, Appana S, Patel M, Falkner KC, McClain CJ, Brock G: **Polychlorinated biphenyls, lead, and mercury are associated with liver disease in American adults: NHANES 2003–2004**. *Environ Health Perspect* 2010, **118**:1735–1742. https://doi.org/10.1289/ehp.1002720.

17. Buzzetti E, Pinzani M, Tsochatzis EA: *The multiple-hit pathogenesis of non-alcoholic fatty liver disease*. NAFLD); 2016. https://doi.org/10.1016/j.metabol.2015.12.012.

18. Anstee Q, Daly A, Day C: **Genetics of alcoholic and nonalcoholic fatty liver disease**. *Semin Liver Dis* 2011, **31**:128–146. https://doi.org/10.1055/s-0031-1276643.

19. Bell CC, Dankers ACA, Lauschke VM, Sison-Young R, Jenkins R, Rowe C, Goldring CE, Park K, Regan SL, Walker T, Schofield C, Baze A, Foster AJ, Williams DP, van de Ven AWM, Jacobs F, van Houdt J, Lähteenmäki T, Snoeys J, Juhila S, Richert L, Ingelman-Sundberg M: **Comparison of hepatic 2D sandwich cultures and 3D spheroids for long-term toxicity applications: a multicenter study**. *Toxicol Sci* 2018, **162**:655–666. https://doi.org/10.1093/toxsci/kfx289.

20. Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Böttger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho C-S, Choi Y-J, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CEP, Gómez-Lechón MJ, Groothuis GMM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Häussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhütter H-G, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EHK, Stieger B, Stöber R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM, Hengstler JG: **Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME**. *Arch Toxicol* 2013, **87**:1315–1530. https://doi.org/10.1007/s00204-013-1078-5.

21. Zeilinger K, Freyer N, Damm G, Seehofer D, Knöspel F: **Cell sources for *in vitro* human liver cell culture models**. *Exp Biol Med* 2016, **241**:1684–1698. https://doi.org/10.1177/1535370216657448.

22. Sharma S, Mells JE, Fu PP, Saxena NK, Anania FA: **GLP-1 analogs reduce hepatocyte steatosis and improve survival by enhancing the unfolded protein response and promoting macroautophagy**. *PLoS One* 2011, **6**:e25269. https://doi.org/10.1371/journal.pone.0025269.

23. Wanninger J, Neumeier M, Hellerbrand C, Schacherer D, Bauer S, Weiss TS, Huber H, Schäffler A, Aslanidis C, Schölmerich J, Buechler C: **Lipid accumulation impairs adiponectin-mediated induction of activin A by increasing TGFbeta in primary human hepatocytes**. *Biochim Biophys Acta* 2011, **1811**:626–633. https://doi.org/10.1016/j.bbalip.2010.11.001.

24. Kirovski G, Gäbele E, Dorn C, Moleda L, Niessen C, Weiss TS, Wobser H, Schacherer D, Buechler C, Wasmuth HE, Hellerbrand C: **Hepatic steatosis causes induction of the chemokine RANTES in the absence of significant hepatic inflammation**. *Int J Clin Exp Pathol* 2010, **3**:675–680. http://www.ncbi.nlm.nih.gov/pubmed/20830238. Accessed 8 January 2019.

25. Wobser H, Dorn C, Weiss TS, Amann T, Bollheimer C, Büttner R, Schölmerich J, Hellerbrand C: **Lipid accumulation in hepatocytes induces fibrogenic activation of hepatic stellate cells**. *Cell Res* 2009, **19**:996–1005. https://doi.org/10.1038/cr.2009.73.

26. Robinton DA, Daley GQ: **The promise of induced pluripotent stem cells in research and therapy**. *Nature* 2012, **481**:295–305. https://doi.org/10.1038/nature10761.

27. Graffmann N, Ring S, Kawala M-A, Wruck W, Ncube A, Trompeter H-I, Adjaye J: **Modeling nonalcoholic fatty liver disease with human pluripotent stem cell-derived immature hepatocyte-like cells reveals activation of PLIN2 and confirms regulatory functions of peroxisome proliferator-activated receptor alpha**. *Stem Cell Dev* 2016, **25**:1119–1133. https://doi.org/10.1089/scd.2015.0383.

28. Lin Y, Ding D, Huang Q, Liu Q, Lu H, Lu Y, Chi Y, Sun X, Ye G, Zhu H, Wei J, Dong S: **Downregulation of miR-192 causes hepatic steatosis and lipid accumulation by inducing SREBF1: novel mechanism for bisphenol A-triggered non-alcoholic fatty liver disease**. *Biochim Biophys Acta Mol Cell Biol Lipids* 2017, **1862**:869–882. https://doi.org/10.1016/j.bbalip.2017.05.001.

29. Bai X, Hong W, Cai P, Chen Y, Xu C, Cao D, Yu W, Zhao Z, Huang M, Jin J: **Valproate induced hepatic steatosis by enhanced fatty acid uptake and triglyceride synthesis**. *Toxicol Appl Pharmacol* 2017, **324**:12–25. https://doi.org/10.1016/j.taap.2017.03.022.

30. Cui W, Chen SL, Hu K-Q: **Quantification and mechanisms of oleic acid-induced steatosis in HepG2 cells**. *Am J Transl Res* 2010, **2**:95–104. http://www.ncbi.nlm.nih.gov/pubmed/20182586. Accessed 27 September 2016.

31. Gómez-Lechón MJ, Donato MT, Martínez-Romero A, Jiménez N, Castell JV, O'Connor J-E: **A human hepatocellular in vitro model to investigate steatosis**. *Chem Biol Interact* 2007, **165**:106–116. https://doi.org/10.1016/j.cbi.2006.11.004.

32. Donato MT, Tolosa L, Jiménez N, Castell JV, Gómez-Lechón MJ: **High-content imaging technology for the evaluation of drug-induced steatosis using a multiparametric cell-based assay**. *J Biomol Screen* 2012, **17**:394–400. https://doi.org/10.1177/1087057111427586.

33. Gerets HHJ, Tilmant K, Gerin B, Chanteux H, Depelchin BO, Dhalluin S, Atienzar FA, Chanteux H: **Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins**. *Cell Biol Toxicol* 2012, **28**:69–87. https://doi.org/10.1007/s10565-011-9208-4.

34. Sivertsson L, Ek M, Darnell M, Edebert I, Ingelman-Sundberg M, Neve EPA: **CYP3A4 catalytic activity is induced in confluent Huh 7 hepatoma cells**. *Drug Metab Dispos* 2010, **38**:995–1002. https://doi.org/10.1124/dmd.110.032367.

35. Khamphaya T, Chukijrungroat N, Saengsirisuwan V, Mitchell-Richards KA, Robert ME, Mennone A, Ananthanarayanan M, Nathanson MH, Weerachayaphorn J: **Nonalcoholic fatty liver disease impairs expression of the type II inositol 1,4,5-trisphosphate receptor**. *Hepatology* 2018, **67**:560–574. https://doi.org/10.1002/hep.29588.

36. Takahara I, Akazawa Y, Tabuchi M, Matsuda K, Miyaaki H, Kido Y, Kanda Y, Taura N, Ohnita K, Takeshima F, Sakai Y, Eguchi S, Nakashima M, Nakao K: **Toyocamycin attenuates**

free fatty acid-induced hepatic steatosis and apoptosis in cultured hepatocytes and ameliorates nonalcoholic fatty liver disease in mice. *PLoS One* 2017, **12**:e0170591. https://doi.org/10.1371/journal.pone.0170591.

37. Lübberstedt M, Müller-Vieira U, Mayer M, Biemel KM, Knöspel F, Knobeloch D, Nüssler AK, Gerlach JC, Zeilinger K: **HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro**. *J Pharmacol Toxicol Methods* 2011, **63**:59–68. https://doi.org/10.1016/j.vascn.2010.04.013.

38. Andersson TB, Kanebratt KP, Kenna JG: **The HepaRG cell line: a unique *in vitro* tool for understanding drug metabolism and toxicology in human**. *Expert Opin Drug Metabol Toxicol* 2012, **8**:909–920. https://doi.org/10.1517/17425255.2012.685159.

39. Michaut A, Le Guillou D, Moreau C, Bucher S, McGill MR, Martinais S, Gicquel T, Morel I, Robin MA, Jaeschke H, Fromenty B: **A cellular model to study drug-induced liver injury in nonalcoholic fatty liver disease: application to acetaminophen**. *Toxicol Appl Pharmacol* 2016, **292**:40–55. https://doi.org/10.1016/j.taap.2015.12.020.

40. Tolosa L, Gómez-Lechón MJ, Jiménez N, Hervás D, Jover R, Donato MT: **Advantageous use of HepaRG cells for the screening and mechanistic study of drug-induced steatosis**. *Toxicol Appl Pharmacol* 2016, **302**:1–9. https://doi.org/10.1016/j.taap.2016.04.007.

41. Anthérieu S, Rogue A, Fromenty B, Guillouzo A, Robin MA: **Induction of vesicular steatosis by amiodarone and tetracycline is associated with up-regulation of lipogenic genes in heparg cells**. *Hepatology* 2011, **53**:1895–1905. https://doi.org/10.1002/hep.24290.

42. * Luckert C, Braeuning A, de Sousa G, Durinck S, Katsanou ES, Konstantinidou P, Machera K, Milani ES, Peijnenburg AACM, Rahmani R, Rajkovic A, Rijkers D, Spyropoulou A, Stamou M, Stoopen G, Sturla S, Wollscheid B, Zucchini-Pascal N, Lampen A: **Adverse outcome pathway-driven analysis of liver steatosis *in Vitro*: a case study with Cyproconazole**. *Chem Res Toxicol* 2018, **31**:784–798. https://doi.org/10.1021/acs.chemrestox.8b00112.

An AOP driven study investigating the effect of Cyproconazole on HepaRG cells. They observed activation of RARα and PXR, together with lipid accumulation and mitochondrial dysfunction upon chemical treatment.

43. * Barbero-Becerra VJ, Giraudi PJ, Chávez-Tapia NC, Uribe M, Tiribelli C, Rosso N: **The interplay between hepatic stellate cells and hepatocytes in an in vitro model of NASH**. *Toxicol Vitro* 2015, **29**:1753–1758. https://doi.org/10.1016/J.TIV.2015.07.010.

A 2D co-culture model consisting of HuH7 cells and hepatic stellate cells to study NASH *in vitro*

44. Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ: **Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis**. *Gut* 2005, **54**:142–151. https://doi.org/10.1136/gut.2004.042127.

45. Dunn JCY, Tompkins RG, Yarmush ML: **Long-term in vitro function of adult hepatocytes in a collagen sandwich configuration**. *Biotechnol Prog* 1991, **7**:237–245. https://doi.org/10.1021/bp00009a007.

46. * Kozyra M, Johansson I, Nordling Å, Ullah S, Lauschke VM, Ingelman-Sundberg M: **Human hepatic 3D spheroids as a model for steatosis and insulin resistance**. *Sci Rep* 2018, **8**:14297. https://doi.org/10.1038/s41598-018-32722-6.

Induction of steatosis over a timecourse of 3 weeks in 3D liver spheroids using pathophysiological concentrations of FFA, carbohydrates and insulin

47. Kostrzewski T, Cornforth T, Snow SA, Ouro-Gnao L, Rowe C, Large EM, Hughes DJ: **Three-dimensional perfused human in vitro model of non-alcoholic fatty liver disease**. *World J Gastroenterol* 2017, **23**:204–215. https://doi.org/10.3748/wjg.v23.i2.204.

48. Gori M, Simonelli MC, Giannitelli SM, Businaro L, Trombetta M, Rainer A: **Investigating nonalcoholic fatty liver disease in a liver-on-a-chip microfluidic device**. *PLoS One* 2016, **11**:e0159729. https://doi.org/10.1371/journal.pone.0159729.

49. Leite SB, Roosens T, El Taghdouini A, Mannaerts I, Smout AJ, Najimi M, Sokal E, Noor F, Chesne C, van Grunsven LA: **Novel human hepatic organoid model enables testing of drug-induced liver fibrosis in vitro**. *Biomaterials* 2016, **78**:1–10. https://doi.org/10.1016/J.BIOMATERIALS.2015.11.026.

50. Bell CC, Hendriks DFG, Moro SML, Ellis E, Walsh J, Renblom A, Fredriksson Puigvert L, Dankers ACA, Jacobs F, Snoeys J, Sison-Young RL, Jenkins RE, Nordling Å, Mkrtchian S, Park BK, Kitteringham NR, Goldring CEP, Lauschke VM, Ingelman-Sundberg M: **Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease**. *Sci Rep* 2016, **6**:25187. https://doi.org/10.1038/srep25187.

51. ** Feaver RE, Cole BK, Lawson MJ, Hoang SA, Marukian S, Blackman BR, Figler RA, Sanyal AJ, Wamhoff BR, Dash A: **Development of an in vitro human liver system for interrogating nonalcoholic steatohepatitis**. *JCI Insight* 2016, **1**:e90954. https://doi.org/10.1172/jci.insight.90954.

in vitro NAFLD model incorporating sinusoidal flow with PHH, HSC and human macrophages. Upon posure to lipotoxic environment, insulin resistance, lipid accumulation, secretion of inflammatory proteins as well as fibronetic activation was observed.

52. * Lee SY, Sung JH: **Gut–liver on a chip toward an in vitro model of hepatic steatosis**. *Biotechnol Bioeng* 2018, **115**:2817–2827. https://doi.org/10.1002/bit.26793.

Body-on-a-chip incorporating a gut and liver model to study absorpotion of FFA and their impact on the liver. Upon treating the gut layer with FFA, lipid acumulation in liver cells was observed.

53. Naik A, Rozman D, Belič A: **SteatoNet: the first integrated human metabolic model with multi-layered regulation to investigate liver-associated pathologies**. *PLoS Comput Biol* 2014, **10**:e1003993. https://doi.org/10.1371/journal.pcbi.1003993.