Review Article

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Studying non-alcoholic fatty liver disease: the ins and outs of in vivo, ex vivo and in vitro human models

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Abstract:
The prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing. Determining the pathogenesis and pathophysiology of human NAFLD will allow for evidence-based prevention strategies, and more targeted mechanistic investigations. Various in vivo, ex situ and in vitro models may be utilised to study NAFLD; but all come with their own specific caveats. Here, we review the human-based models and discuss their advantages and limitations in regards to studying the development and progression of NAFLD. Overall, in vivo whole-body human studies are advantageous in that they allow for investigation within the physiological setting, however, limited accessibility to the liver makes direct investigations challenging. Non-invasive imaging techniques are able to somewhat overcome this challenge, whilst the use of stable-isotope tracers enables mechanistic insight to be obtained. Recent technological advances (i.e. normothermic machine perfusion) have opened new opportunities to investigate whole-organ metabolism, thus ex situ livers can be investigated directly. Therefore, investigations that cannot be performed in vivo in humans have the potential to be undertaken. In vitro models offer the ability to perform investigations at a cellular level, aiding in elucidating the molecular mechanisms of NAFLD. However, a number of current models do not closely resemble the human condition and work is ongoing to optimise culturing parameters in order to recapitulate this. In summary, no single model currently provides insight into the development, pathophysiology and progression across the NAFLD spectrum, each experimental model has limitations, which need to be taken into consideration to ensure appropriate conclusions and extrapolation of findings are made.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common cause of chronic liver disease worldwide with prevalence estimates ranging from 25% to 45%, depending on the diagnostic method, age, gender and ethnicity studied [1], [2]. NAFLD parallels the prevalence of obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome, all of which increase the risk of more advanced liver disease [3]. NAFLD encompasses a spectrum of disease ranging from simple steatosis (often referred to as NAFLD), to non-alcoholic steatohepatitis (NASH), through to the development of cirrhosis and hepatocellular carcinoma [4], [5], [6], although only a relatively small proportion of NAFLD patients will progress and develop NASH [2]. NAFLD can remain asymptomatic in a significant proportion of individuals in whom diagnosis often occurs when liver function tests are abnormal. Recent evidence suggests that several genetic risk factors predispose to the development and progression of NAFLD [7]. For example, polymorphisms of patatin-like phospholipase domain-containing

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protein 3 (*PNPLA3*), transmembrane 6 superfamily 2 (*TM6SF2*), fat-mass and obesity associated (*FTO*), lipase A, lysosomal acid (*LIPA*), lysophospholipase like 1 (*LYPLAL*) and heme oxygenase 1 (*HMOX-1*) genes have been found to be associated with the development/progression of the disease [8], [9]. Lifestyle factors including increased calorie consumption and lack of physical activity/exercise have also been reported to be key mediators in the accumulation of liver fat [10], [11], [12], [13].

Definition, diagnosis and identification

NAFLD is defined by the presence of intracellular triglyceride (TG) in >5% of hepatocytes as defined by histological analysis, or the cut-off is >5.6% by a proton density fat fraction (PDFF) assessed by proton magnetic resonance spectroscopy (^{1}H-MRS) or quantitative fat/water selective magnetic resonance imaging (MRI), in the absence of other aetiologies of liver disease [2]. NAFLD diagnosis can be achieved using the following methods.

Biopsy

Liver biopsy is the current "gold standard" for diagnosing the presence and severity of NAFLD [14] and remains the only diagnostic procedure that can reliably assess histological patterns, severity of disease and associations [15]. However, it is expensive, invasive, and potentially risky making it unsuitable for screening individuals for studies, or for follow-up of patients after therapeutic intervention. Furthermore, as only a small quantity of tissue is taken (~1/50,000th relative to total liver size) [16] biopsies are subject to sampling variability. This can potentially result in misdiagnosis and staging inaccuracies because histological lesions of NASH may be unevenly distributed throughout the liver parenchyma [15].

Liver imaging methods

Due to the limitations of biopsies there is a need for accurate and non-invasive methods to diagnose and stage the severity of NAFLD. Several non-invasive liver imaging methods are routinely used in clinical practice and research settings, including ultrasonography, computed tomography (CT), MRI and MRS.

Ultrasound

This is the most common imaging modality due to its low cost, safety and relatively widespread availability [17], [18], [19]. It evaluates liver fat content indirectly based on subjective qualitative sonographic features such as echogenicity, echotexture, vessel visibility and beam attenuation [19], [20]. The diagnostic performance of ultrasound in detecting steatosis is variable and influenced by the degree of steatosis and presence of coexisting chronic liver disease (e.g. fibrosis and oedema). In patients without coexisting liver disease, ultrasound offers an accurate diagnosis of moderate-to-severe steatosis (defined as histologic degree ≥30%) [19], [20] although it is considerably less accurate when all degrees of steatosis are considered [20], [21], [22]. Ultrasound has limited sensitivity in individuals with high body mass index (BMI) (≥40 kg/m^2) [19], [23]. Further limitations of ultrasound include the substantial intra- and inter-observer variability [24] and the qualitative nature of the current four-point grading system, which is too simplistic to account for small alterations in steatosis severity on follow-up [24] and therefore may not be suitable for evaluating patients with NAFLD after therapeutic intervention.

Elastography

Ultrasound elastography and magnetic resonance (MR) elastography are emerging as promising methods for NASH diagnosis. This technology evaluates liver stiffness by measuring the velocity of the shear wave using ultrasound or MRI [25], [26]. Elastography translates the degree of fat infiltration of the hepatic parenchyma into stiffness; the higher tissue rigidity, the faster the sheer wave is propagated [25], [27], [28]. Results from ultrasound elastography demonstrate that as liver stiffness increases so does the severity of histologic fibrosis; although stiffness values did not correlate with the degree of steatosis or inflammation [29], [30], [31] suggesting ultrasound elastography can assess hepatic fibrosis without being confounded by steatosis, but provides no insight into inflammation [29], [30], [31], [32].

When studying MR elastography it has been shown that patients with steatosis and lobular inflammation had increased liver stiffness in comparison to patients with steatosis alone, and significantly lower stiffness than patients with steatosis and fibrosis [26]. Taken together, these results indicate that ultrasound or MR elastography may play a place in screening for NASH and/or advanced fibrosis.

Computed tomography

Evaluation of steatosis by CT is based on attenuation values of the liver parenchyma, measured as Hounsfield units (HUs), and is dependent on tissue composition. As the attenuation value of fat (approximately −100 HU) is lower than that of soft tissue, steatosis lowers the attenuation of parenchyma [33]. CT liver attenuation can be measured objectively and with high precision [34], [35] although the accuracy of CT in measuring liver fat content may vary but is considered precise when diagnosing moderate-to-severe steatosis [36]. The reduced accuracy in detecting low-grade steatosis suggests that CT may not be suitable for evaluating the early stages of intrahepatocellular fat accumulation [5], [37]. Several quantitative CT indices have been used to assess steatosis, and these include the absolute liver attenuation value (HU liver) and the liver-to-spleen difference in attenuation (CTL-S). Despite HU liver showing a stronger correlation with histologic measures of steatosis than CTL-S, HU liver may be subject to errors resulting from variations in attenuation values across different CT scanners [38], [39]. Threshold values of CT indices for the diagnosis of hepatic steatosis depend on the methods and populations used [20], [21], [40] which may limit the ability to generalise results across studies. Several confounding factors can influence liver attenuation on CT, including liver iron and glycogen content, fibrosis, oedema and ingestion of drugs such as amiodarone, resulting in unavoidable errors in fat quantification and low sensitivity [34]. Moreover, the potential hazard of ionising radiation makes CT unsuitable for use in paediatric studies or the longitudinal monitoring and follow-up of patients with all stages of NAFLD.

Magnetic resonance imaging and spectroscopy

MRI and MRS provide the opportunity to directly quantify liver fat content non-invasively and both measure PDFF, defined as the amount of protons bound to fat divided by the amount of all protons in the liver, including those bound to fat and water. MRI and MRS both detect the presence of liver fat greater than 5.6% (the diagnostic threshold for NAFLD) with high accuracy (nearly 100%) [41]. Comparisons have been made between the accuracy of MR techniques and other imaging modalities for the assessment of hepatic steatosis, with histologic grading as the reference standard; MRS and MRI outperform CT and ultrasound in the diagnosis and grading of hepatic steatosis [19], [20], [21]. Moreover, PDFF measurements using MRS and MRI have also been reported to be highly reproducible [42], [43], [44], [45]. Novel MRS-based imaging sequences have been developed to distinguish the composition of hepatic fat [46], [47] which has the potential to provide insight into which type of fat may be deleterious for liver fat accumulation, particularly if undertaken in conjunction with nutritional intervention and physiological studies.

Positron emission tomography

Positron emission tomography (PET) is a nuclear imaging technique that employs short-lived positron-emitting radioisotopes to label molecules of interest in order to quantify organ-specific substrate metabolism [48]. Studies investigating liver metabolism have combined PET with intravenous (i.v.) administration of radio-labelled fatty acid (FA) tracers (e.g. ^{18}F-fluoro-6-thia-heptadecanoic acid and ^{11}C-palmitate) in order to quantify hepatic FA uptake, oxidation and esterification [49], [50]. This method is non-invasive and allows for the quantification of metabolic processes in vivo. However, such methods require further validation in humans and the use of radio-labelled isotopes may prove prohibitive for longitudinal patient assessment.

Plasma markers

Circulating concentrations of liver enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and gamma glutamyl transpeptidase (γGT) are often used to screen for liver function and can be increased in individuals with NAFLD. Even though a persistently elevated level of ALT can be associated with an increased risk of disease progression, patients with advanced disease often have normal liver enzyme levels making the identification of at-risk patients challenging [51], [52]. Increased or abnormal levels of liver enzymes may also

result from alcohol excess, drug-induced liver injury, viral hepatitis, autoimmune liver disease, haemochromatosis and coeliac disease [53].

In an attempt to improve the specificity of liver enzymes to diagnose NAFLD, the "NAFLD liver fat score" was developed and this is calculated from fasting serum AST and the AST/ALT ratio (AAR), in combination with fasting serum insulin and presence of the metabolic syndrome/T2DM [54]. This algorithm has been shown to be a particularly sensitive predictor of NAFLD [54], although it is unable to distinguish between different disease stages [55].

Further plasma markers that may aid in the diagnosis of NAFLD are cytokeratin-18, which is a breakdown product resulting from caspase 3-mediated apoptosis of hepatocytes [56], [57], and pro-inflammatory cytokines including tumour necrosis factor alpha, interleukin (IL)-6 and IL-8, C-reactive protein and ferritin [58], [59], [60]. However, whilst these markers may provide information on liver damage/function and inflammation they are unable to specifically determine intrahepatic fat content.

Studying hepatic FA metabolism in vivo in humans

Human in vivo studies allow for whole-body and tissue-specific metabolism to be assessed in a physiological setting. The major challenge of studying NAFLD is the limited direct accessibility to the liver, making researchers reliant on indirect measurements. Here we will review FA metabolism relevant for NAFLD and how some of these pathways may be studied in humans by utilising stable-isotope methodologies.

Stable-isotopes are naturally occurring molecules that have been used to study human metabolism for over 80 years [61]; the most commonly utilised are carbon, hydrogen and nitrogen [62]. Stable-isotope tracers are chemically and functionally identical to their more abundant counterpart (the tracee), but differ in atomic mass, which allows for detection by mass spectrometry [63]. They allow investigation of dynamic processes [62] and multiple tracers can be used to study various aspects of human metabolism simultaneously. However, tracer costs, which can vary substantially depending on the number and position of the labels within a molecule, and access to mass spectrometers (for measurement) may limit their use.

Metabolic tracers are typically introduced into the body by (i) bolus injection, (ii) ingestion, or (iii) i.v. infusion. Blood, breath or biopsies of tissues such as adipose tissue, skeletal muscle or liver, can then be collected and the isotopic enrichment determined. The classical models which tracer data quantified are: (1) tracer dilution, whereby a known amount of tracer is intravenously infused and the rate of appearance (Ra) of the tracee is calculated from the magnitude of tracer dilution; (2) tracer incorporation, where a tracer is introduced into the precursor pool from which a more complex product is formed, and the enrichment of tracer in the end-product is used to estimate rate of synthesis; and (3) tracer conversion, which is a similar principal to tracer incorporation with the exception that the enrichment of tracer is measured in a metabolic by-product. The utility of each of these models is dependent on the question being addressed [63].

Very low-density lipoprotein-triglyceride production and secretion

Very low-density lipoprotein (VLDL) is synthesised in the liver, are a major carrier of TG and have been suggested to be the most reflective circulating lipid fraction to use for studying hepatic FA metabolism [64], [65]. The formation of VLDL-TG is a two-step process involving the fusion of a single newly-synthesised apolipoprotein B-100 molecule with cytosolic TG [66], [67]. The isolation of VLDL can be performed by density gradient ultracentrifugation, with VLDL present at a Svedberg flotation rate (S_f) 60–400. However, in the postprandial period, chylomicron remnants will also be found in this fraction. Combining density gradient ultracentrifugation with immunoaffinity chromatography against apolipoprotein B-100 (ApoB-100) results in the isolation of hepatic derived VLDL [68], [69], [70], [71], [72]. The separation of the hepatic-derived apoB-100 containing VLDL particles from those derived from the gut (i.e. apoB-48 containing chylomicron-remnants) allows for a more specific assessment of hepatic FA metabolism.

VLDL-TG kinetics can be determined through use of FA, glycerol and leucine tracers and the precursor to product method, whereby FA and glycerol tracers are used to estimate the secretion rate of VLDL-TG, and the leucine tracer used to determine VLDL-ApoB-100 synthesis [73]. Multiple tracers (e.g. glycerol and leucine) are often used in multi-compartmental models, as it is not possible to infer information regarding the whole VLDL-TG particle from a single component [73]. Using these methods, it has been shown that NAFLD patients have increased secretion of VLDL-TG when compared to those without NAFLD [74], [75]. Furthermore, the acute suppressive effect of insulin on VLDL-TG secretion has been shown to be blunted in NAFLD [76], consequently, hypertriglyceridaemia is often observed in NAFLD patients. Increased VLDL-TG secretion in NAFLD may

represent an adaptive response aimed at reducing liver fat content although ApoB-100 secretion is reported not to be increased in NAFLD patients [77], which may result in increased VLDL-TG particle size [75].

FA sources contributing to liver fat

Liver fat accumulation occurs when FA input and synthesis within the liver exceeds disposal [78]. FA sources may originate from: (1) uptake of circulating non-esterified fatty acids (NEFA) derived from subcutaneous and visceral adipose tissue (VAT) lipolysis or chylomicron-derived spillover, (2) uptake of chylomicron remnants (dietary fat) or, (3) endogenously produced through de novo lipogenesis (DNL) [79]. Within the liver FAs are broadly partitioned between esterification (to form predominantly TG, which can be stored or secreted in VLDL) and oxidation (the tricarboxylic acid (TCA) or ketogenic) pathways. The intrahepatic TG pool is dynamic and may fluctuate markedly and rapidly in response to extrinsic challenges; fasting and exercise have both been shown to acutely increase liver fat content [80], [81], whilst a single high-fat meal increases liver fat content during the postprandial period [82], [83].

Adipose tissue-derived NEFA are the major contributor to the intrahepatic FA pool [64], and hepatic NEFA uptake is elevated in obese subjects with NAFLD compared to lean, non-NAFLD controls [84]. Studies using stable isotope methodologies to assess whole-body lipolysis, have found that NAFLD patients exhibit an elevated lipolytic rate when compared to those without NAFLD, and that the relative suppression of adipose tissue lipolysis is inversely associated with liver fat content [77], [85], [86]. As the hydrolysis of adipose tissue TG releases FA and glycerol, whole-body lipolytic rate can be determined through use of both FA and glycerol tracers [87], [88], [89], with both methods resulting in reasonable levels of agreement under most circumstances [63]. Due to the rapid turnover rate of the plasma FA pool, there is no need for a priming dose of tracer as steady-state levels of enrichment are typically achieved within 30–45 min of infusion. FA tracers can also be administered by bolus injection, although the rapid FA turnover rate results in a tracer half-life of 1–4 min, drastically shortening the potential experimental period unless large quantities of tracer are used [73]. Multiple samples are also required at frequent intervals when using the bolus injection technique in order to obtain valid measures [73]. A potential advantage of using a FA tracer is the ability to then trace the fate of the FA through metabolic pathways and estimate the relative contribution of systemic NEFA to intrahepaticTG and VLDL-TG.

It has been hypothesised that increased lipolysis of VAT may play a role in the pathogenesis of NAFLD as the drainage of blood from VAT occurs via the portal vein (the "portal vein" hypothesis) [90]. However, this has yet to be demonstrated directly in humans. Studies have estimated from measurements obtained from the hepatic vein, that VAT-derived FA contribute between 5% and 10% of hepatic FA in lean adults and 30%–40% in those with increased VAT depots [91]. Using ^{11}C-palmitate in combination with PET imaging it was shown there is an increased contribution of VAT NEFA to the hepatic FA pool in obese, compared to lean individuals [49]. Together these data suggest that the contribution of VAT-derived FA to the liver increases with enlargements in adipose tissue mass, which may in part explain the association between NAFLD and obesity.

Despite NAFLD patients being reported to have elevated lipolytic rates and circulating NEFA levels [77], [85], [86] it has been found that the majority of FA incorporated into VLDL-TG are derived from non-systemic sources (i.e. lipolysis of intrahepatic TG, VAT derived-FA and DNL), and not systemic (i.e. subcutaneous) adipose tissue-derived FA [77]. This observation is in contrast with that reported by Donnelly et al., who found that the majority of FA in VLDL-TG and liver TG were derived from the circulating NEFA pool [64]. The differences between these studies may be explained through methodological differences, which include the tracers utilised ([$^2H_2$]palmitate vs. [^{13}C]palmitate), the duration of tracer infusion (12 h vs. 5 days) and the metabolic state being investigated (i.e. postabsorptive vs. postprandial). It is plausible that the extended infusion time employed by Donnelly et al. [64] resulted in increased tracer recycling which may have influenced findings.

A consideration when using labelled FA to estimate lipolytic rate relates to whether the Ra of the individual FA labelled is representative of other FA released from adipose tissue. It has previously been shown that the mobilisation of FA from adipose tissue is a selective process, in which the rate of mobilisation is decreased with increasing carbon chain length and degree of saturation [92]. Mittendorfer et al. [93] have measured the Ra of myristate, palmitate, stearate, oleate and linoleate under basal conditions and under conditions which increase (i.e. epinephrine infusion) and decrease (i.e. insulin infusion) the lipolytic rate. They found that calculating total NEFA Ra by palmitate, oleate and linoleate produces relatively similar estimates (within 10%–15%) of total FA flux, whereas stearate and myristate tracers consistently underestimated and overestimated total NEFA Ra, respectively [93]. Thus, it would be prudent to recommend that palmitate, oleate or linoleate tracers are used when estimating whole-body lipolysis. The study by Mittendorfer et al. [93] was performed in relatively lean, healthy, young males and it is unclear whether metabolic health or adipose tissue function may influence the Ra of individual FA, which may be pertinent for measuring lipolytic rate in NAFLD patients or obese individuals. The fractional uptake of individual FA by the liver has also been reported to differ, with the uptake of lauric,

myristic and palmitoleic acids being higher than palmitic, oleic, linoleic and arachidonic acids, whilst stearic acid was lower than the other FA [94]. It is plausible that the incorporation of specific FA into VLDL-TG may also be a selective process, or at least influenced by the rate of uptake into the liver of individual FA; a notion that warrants consideration when choosing a FA tracer.

When quantifying the contribution of FA sources to liver fat, it has been reported that ~15% of liver TG was derived from dietary FAs in NAFLD patients [64]; it remains unclear what the contribution is in non-NAFLD subjects and it is likely that the amount and frequency of consumption of dietary fat may influence the contribution markedly.

Tracing the fate of dietary fat requires the incorporation of FA tracers into the fat component of a test meal. On consumption, the tracer is incorporated into chylomicron-TG before entering the systemic circulation where chylomicrons are hydrolysed, and FAs are taken up into adipose tissue or skeletal muscle or they "escape" uptake and spillover into systemic circulation and may enter the liver [95]. The hydrolysis of chylomicrons also results in the generation of cholesterol-rich remnant particles which remain in circulation until removed by the liver [96], [97]. Thus, dietary FAs can enter the liver through two pathwayseither as spillover FA or chylomicron remnants. Once within the liver intrahepatic FA partitioning can be investigated as the tracer can be "followed" into esterification (appearance in VLDL-TG) or oxidation (appearance in 3-hydroxybutyrate (3-OHB) or in TCA cycle intermediates through use of ^{13}C nuclear MR methods) pathways [98], [99].

Stable isotope tracers can be given as FA, either individually in their free form, as mixed FA or TG. When given as a free FA, it has been shown that palmitate and oleate are similarly incorporated into chylomicron-TG, whereas linoleate may be differentially partitioned within the enterocyte [100], [101]. Assessment of tracer enrichment in faecal samples suggests that >98% of palmitate and oleate are absorbed [102]. Together, this suggests that labelled palmitate and oleate are ideally suited to trace fat of dietary TG. The amount of tracer incorporated into the test meal is dependent on, the pool size being traced (a big pool may require more tracer to get good enrichment), meal fat content and composition, and the sensitivity of analytical equipment. Whether the total fat and tracer content of test meal(s) should be standardised, so all participants receive the same meal, or individualised (either to bodyweight or total fat mass) between subjects is debated. Standardising fat and tracer content has the advantage of simplifying meal preparation, although this may not be appropriate in heterogeneous populations, where it is possible that some will be over- and others under-fed. Increasing the total fat content of a test meal(s) has the concomitant effect of increasing postprandial TG concentrations and this may result in increased dilution of tracer(s), resulting in lower enrichment which may make detection/measurement more challenging. Thus, it may be necessary to increase the amount of tracer used when studying hypertriglyceridaemic populations in order to achieve desired enrichment levels [103]. When using ^{13}C tracers participants should limit/avoid foods which are naturally high in ^{13}C (e.g. corn, cane sugar, etc.) in the days preceding testing in order to minimise background enrichment.

Hepatic DNL can be measured using either ^{13}C-acetate or $^{2}H_2O$ (heavy water). Anecdotally it appears that the use of $^{2}H_2O$ is becoming increasingly popular which may in part be due to its ability to be administered without i.v. infusion, along with its relative cheapness compared to other tracers [104]. Once ingested, heavy water rapidly equilibrates with the body water pool (within 1–2 h in humans) [105], producing a homogenous precursor pool for any reactions involving water (e.g. condensation/hydrolysis), thus potentially allowing for the investigation of several metabolic processes at once [106]. As the half-life of body water is approximately 10 days in humans, ^{2}H enrichment remains relatively stable for a number of days [107]. Because VLDL-TG is suggested to represent hepatic TG, the incorporation of ^{2}H atoms into VLDL-TG (either from intracellular $^{2}H_2O$ or incorporation into nicotinamide adenine dinucleotide phosphate or acetyl-CoA first) can be used to estimate hepatic DNL [71], [72], [89], [108], [109]. Heavy water is most commonly provided in bolus oral dose(s) 1–7 days prior to the assessment, and the volume of heavy water given is based on the pool size of total body water (which in humans is ~50%–70% of total body weight [110], [111], [112]), and desired level of ^{2}H enrichment. A potential side effect of heavy water administration in humans is that it may induce vertigo [113], although this is transient in the majority of participants. The use of $^{2}H_2O$ does not provide the opportunity to determine ^{2}H enrichment in the true precursor pool for FA synthesis; therefore, it is important to incorporate a correction factor into calculations when using $^{2}H_2O$ to determine hepatic DNL [114]. Alternatively, mass isotopomer distribution analysis (MIDA) can be utilised. MIDA was developed in 1992 by Hellerstein and Neese [115] and estimates precursor enrichment via a combinatorial probability model comparing measured abundances to the theoretical distribution pattern of labelling within a molecule; a comprehensive overview of MIDA is available elsewhere [116], [117].

Hepatic DNL is reported to be elevated in subjects with NAFLD (liver fat 14.9%–18.4%) compared to individuals without NAFLD (liver fat 3.1%–4.6%), when assessed using heavy water [109], [118]. Increased hepatic DNL has also been reported in hyper-insulinemic individuals, compared with their normo-insulinemic counterparts [72], and in males compared with females [71]. Despite being often implicated as the cause of NAFLD, DNL is not strongly associated with liver fat content [119]. Recently, pharmacological inhibition of the lipogenic

enzyme acetyl-CoA carboxylase in humans resulted in reductions in liver fat content, supporting a role for DNL in liver fat accumulation [120], although further evidence demonstrating that DNL plays a causative role in the development of NAFLD is required.

Hepatic DNL can also be assessed by using ^{13}C-acetate. Either 1-[^{13}C] or 2-[^{13}C] acetate is administered via i.v. infusion to enrich the acetyl-CoA pool, from which ^{13}C will be metabolised through the DNL pathway and incorporated into VLDL-TG [121]. This methodology requires a lengthy infusion period (8–24 h) prior to the assessment of hepatic DNL in order to adequately enrich the precursor pool [122], [123] and as with $^{2}H_2O$, measuring the true precursor pool for FA synthesis is challenging.

Typically, the determination of hepatic DNL is based on the isotopic enrichment of palmitic acid, however, de novo synthesis of other FA (e.g. stearic acid and oleic acid) is possible through elongation or desaturation pathways [124], [125]. The enzyme stearoyl-CoA desaturase 1 (SCD) is responsible for the desaturation of palmitic and stearic acids to palmitoleic and oleic acids, respectively [126]. Animal studies have shown that inhibition of SCD decreases liver fat content [127], [128] although whether these findings translate to humans remains unclear. It has been suggested that SCD activity can be estimated by calculating the ratio of palmitoleate to palmitate (or stearate to oleate) in VLDL-TG, termed the SCD index. Silbernagel et al. [129] found the SCD index in VLDL-TG was not associated with baseline liver fat content but showed a strong negative correlation with liver fat content in response to a 4-week high-sugar diet. Conversely, Stefan et al. [130] reported an inverse correlation between the SCD index in VLDL-TG and liver fat content. The difference in findings between these studies may be due to the fact the SCD index was calculated from different FA ratios, with Silbernagel et al., calculating the ratio of palmitoleic to palmitic acid, whereas Stefan et al., calculated SCD index from the ratio of oleic to stearic acid [129], [130]. A more direct method of estimating SCD activity is the isotopic desaturation index, which is the ratio of palmitoleate enrichment to palmitate enrichment in VLDL-TG [72], [89], [126]. Using this method we have previously shown that desaturation appears higher in normo-compared to hyper-insulinemic individuals [72]. Based on this observation it is plausible that the lower desaturation in the hyper-insulinaemic group may in part be a mechanism underpinning hepatocellular lipotoxicity [72].

FA oxidation

The use of ^{13}C labelled substrates to quantify in vivo FA oxidation rates dates back to the 1970s [131], [132], [133], whereby ^{13}C was administered either through i.v. infusion or oral ingestion and then measured in expired CO_2. When combined with indirect calorimetry, to assess CO_2 production the total (whole-body) $^{13}CO_2$ produced over time can be determined. Breath samples are easy to obtain, however a correction factor to account for the loss of ^{13}C due to fixation in the bicarbonate body pool or isotopic exchange within the TCA cycle should be included in calculations [134], [135], [136]. An alternative method of determining FA oxidation rates involves the use of a deuterium labelled palmitic acid, whereby upon oxidation the ^{2}H enriches plasma water from which FA oxidation rates can be calculated [137]. Due to minimal isotopic exchange, in comparison with carbon tracers, the use of a correction factor is not required when using deuterium tracers to determine FA oxidation [137].

Hepatic FA can be partitioned into the ketogenic pathway when acetyl-CoA concentrations are high, producing ketone bodies such as 3-OHB which is secreted into circulation for use as an energy substrate by extrahepatic tissues. The rate of ketogenesis is subject to hormonal regulation, with insulin suppressing adipose tissue lipolysis (reducing substrate availability) and promoting lipogenesis and glucose uptake and oxidation [138]. Thus, ketone body production is lower during the postprandial period under the majority of conditions [139]. Circulating levels of 3-OHB reflect the balance between 3-OHB synthesis and disposal. When a ^{13}C tracer has been included (either infused or ingested) then the incorporation of ^{13}C in 3-OHB can be traced to demonstrate that either FA from adipose tissue lipolysis (infused ^{13}C) or recently ingested FA (ingested ^{13}C) have gone through the ketogenic pathway (as a marker of hepatic FA oxidation) and differences between sex, phenotype, or the effects of diet can be studied [71], [72], [140], [141].

It has been proposed that there are differences in the oxidation rates of specific FAs, with DeLany et al., and Jones et al., both demonstrating that the appearance of $^{13}CO_2$ in expired breath is lower following the consumption of test meals containing ^{13}C-stearate in comparison to ^{13}C-oleate and ^{13}C-linoleate [142], [143]. This observation suggests consideration may be required when extrapolating the oxidation of individual FA to total FA oxidation. In addition, this data suggests that unsaturated FAs are oxidised to a greater extent than saturated FAs (SFA), which may partly explain the more lipogenic effects of diets enriched in SFA compared to polyunsaturated FAs (PUFA) [144]. However, this is an area that has received relatively little attention to date, and the studies that have been performed are of limited sample sizes (four to six subjects) [142], [143], and are therefore likely to be highly influenced by individual variations.

A computational model of hepatic energy metabolism has suggested inter-individual variation in FA oxidation has a larger impact on susceptibility for NAFLD than inter-individual variation in DNL [145]. Studies in humans have reported mixed findings regarding hepatic FA oxidation with decreased [146], similar [147]

or even increased [85], [148] rates reported in subjects with NAFLD compared to those without NALFD. The discrepancy in findings may be due to the stage of disease being examined (e.g. the findings of Sanyal et al. [148], suggest there are differences in hepatic FA oxidation in steatosis vs. NASH), and metabolic state in which hepatic FA oxidation were assessed; it is notable that whilst all these studies assessed insulin stimulated fat oxidation by way of a hyperinsulinaemic-euglycaemic clamp, the insulin infusion rates are different study to study. By using a combination of ^{2}H and ^{13}C tracers, Sunny et al. [149] showed hepatic mitochondrial oxidation to be twice as high in subjects with NAFLD compared with subjects without NAFLD; there was a strong direct association with flux through the TCA cycle and liver fat content. The increased mitochondrial oxidation was not reflected by ketone production as assessed by tracer dilution of 3-OHB, which was similar between groups [149]. This suggests that circulating 3-OHB per se does not fully reflect hepatic FA oxidation, or it does indeed remain unchanged and is regulated in a different manner to the TCA cycle. Petersen et al. [98], using ^{13}C-acetate infusion in combination with a ^{13}C-MRS methodology, reported similar rates of hepatic mitochondrial oxidation (based on a mathematical model using the appearance of ^{13}C in the TCA cycle intermediate glutamate) in subjects with high (>4%) and low (<4%) liver fat content. Discrepancies between these studies may be related to differences in mean liver fat content between the NAFLD patients examined in these studies (~9% Petersen et al. [98] and ~17% in Sunny et al. [149]).

Increased hepatic FA oxidation in NAFLD may indicate an initial compensatory response to try and normalise liver fat content. This suggestion is indirectly supported by the observation that in comparison to healthy, lean males, abdominally-obese men had a greater isotopic enrichment of ^{13}C in expired CO_2 and circulating 3-OHB, indicating increased hepatic and whole-body FA oxidation [150]. Moreover, using ^{11}C-palmitate combined with PET imaging Iozzo et al. [49] showed that hepatic FA oxidation rates were, on average, twice as high in obese (BMI 32 kg/m^2) compared with non-obese (BMI 26 kg/m^2) individuals. However, in both of these studies the liver fat content of participants was not reported. It is suggested that chronic activation of hepatic mitochondria may result in oxidative stress and cellular damage, responses which have been implicated in the development of NASH [151]. Indeed, there is evidence to suggest that NAFLD/NASH patients exhibit mitochondrial abnormalities (i.e. mitochondrial enlargement, paracristalline inclusions, and decreased expression of mitochondrial proteins) and increased FA oxidation in relation to their healthy counterparts [148], [152], [153], [154]. However, it remains unclear if FA oxidation rates are influenced across the NAFLD spectrum.

Although stable-isotope tracers allow for investigations of the dynamic processes involved in in vivo hepatic FA metabolism, the inability to directly sample the liver in the majority of instances means many tracer methodologies rely on inferring information from proxy markers (e.g. analysing VLDL-TG and 3-OHB to provide information on hepatic FA partitioning, TCA cycle intermediates, whole body measures, etc.). In addition, heterogeneity between experimental study groups make direct comparisons between studies challenging. This was evidenced in a recent study from our laboratory whereby despite similar phenotypes between individuals stratified as being normo- or hyperinsulinaemic there was overlap between the two cohorts in regards liver fat content [72]. It is plausible that the mechanisms underpinning the accumulation of liver fat differ between individuals. The ability to obtain direct measurements would greatly enhance the understanding of NAFLD pathogenesis and pathophysiology.

Ex situ models of liver fat metabolism

A novel approach to investigate hepatic lipid metabolism includes the use of a normothermic machine perfusion (NMP) device which maintains the liver in a fully functioning state ex situ by providing oxygen and nutrition at 37°C [155], [156]. The primary purpose of this technology is as a preservation method in liver transplantation, as it has been shown to be superior to standard cold storage in terms of post-transplant outcomes [156], [157]. The main constituents of the device include a blood reservoir, centrifugal pump, oxygen concentrator and heat exchanger and the liver is perfused using a red-cell suspension in a colloid. Perfusate is pumped out of the liver via the inferior vena cava before being heated and oxygenated where it is then diverted into the hepatic artery via a low-flow, high-pressure system or into a reservoir which feeds the portal vein via a high-flow, low-pressure system. Constant blood gas analysis enables control of pO_2 and pCO_2 levels, facilitating the maintenance of acid-base homeostasis. Continuous infusions enable sufficient vasodilatation, protection against coagulation and the provision of an environment that enables near-physiological metabolic and synthetic liver function [158].

Although the primary use of the NMP device is as a preservation method in liver transplantation, it offers a unique opportunity to study human liver metabolism at the organ level. By perfusing human livers that have been declined for transplantation, it is possible to explore the structural and functional effects of NMP and pharmacological adjuncts that may alter liver metabolism. The advantage of this system is that it is repro-

ducible and standardised for all perfusions. Synthetic and metabolic functional assessment can be performed through the measurement of arterial and portal flow rates, pH measurement, and evidence of bile production, lactate clearance and glucose metabolism. Frequent sampling of both liver tissue and perfusate can be performed in order to investigate the mechanisms underpinning any alterations of FA metabolism. This system is disadvantaged by the fact that it is not completely physiological; there is no differential oxygen supply between the portal and arterial systems (as is the case in vivo) and there is no peripheral metabolism which plays a key role in vivo. The perfusate does not fully represent system circulation but has the potential to be modified and indeed any inflammatory mediators are only those which are resident in the liver, which may not be reflective of whole-body in vivo metabolism. The heterogeneity of livers in the donor pool as well as the duration of cold storage before the commencement of NMP is variable. However, the potential change observed in each liver remains valid.

In the long-term, this technology has the ability to expand the diminishing potential liver donor pool which could largely be achieved by transplanting organs which may be deemed too "high-risk" and are known to have poorer post-transplant outcomes. One such group of livers are those with significant levels of macrovesicular steatosis, whose use is limited due to increased susceptibility to ischaemia/reperfusion injury, which remains the Achilles' heel of solid organ transplantation. Due to the increased prevalence of NAFLD, this markedly reduces the livers in the donor pool. It is possible that by avoiding the deleterious effects of cooling and resulting ischaemic injury, NMP may greatly enhance outcomes from these livers. Furthermore, animal models have demonstrated that NMP with or without the addition of de-fatting agents can result in a reduction of steatosis during preservation [159], [160].

In order to better understand the pathophysiology of NAFLD and key pathways involved in potential de-fatting, stable-isotope tracers can be infused into the circuit and the metabolic fate of the metabolic tracers (fats and sugars) can therefore be explored through sequential liver biopsies and perfusate sampling. Changes in lipid-droplet morphology and gene expression can also be studied over time and comparisons of various interventions made. This unique model can therefore be fully exploited to investigate liver fat metabolism in a way which would be impossible in vivo. Ultimately, having a greater insight into how a steatotic liver functions, and if it has the ability to be "de-fatted" or the function improved through use of pharmacological agents will potentially increase both the donor pool and post-transplant outcomes.

In vitro human cellular models

In vitro models offer the ability to understand disease mechanisms and investigate effect of therapies at a cellular and molecular level. However, the in vitro model used may differ depending on the scientific question being asked and it is unlikely that a single in vitro model can recapitulate the complexity of the human liver in vivo. By understanding the benefits and limitations of each model the most appropriate then can be effectively used. Here we review available human in vitro models of the liver and highlight their uses (and limitations) for understanding NAFLD and developing translational therapies.

Many in vitro liver cell models are available, with the majority focusing on hepatocytes with the "gold standard" model considered to be primary human hepatocytes (PHH) (either fresh, or immortalised). In order to overcome availability issues other hepatocyte models are often utilised including: differentiated hepatic stem cells (~1% of liver cells) [161], [162], [163], [164], differentiated human induced pluripotent stem cells (hiPSCs) and hepatocyte cell lines. Additionally, isolation and culture of primary human Kupffer and stellate cells have been reported. Precision cut liver slices (PCLs) offer the advantage of retained cellular architecture and cell-cell interactions. We have previously reviewed the pros and cons of each cell model [165] and these are summarised in Table 1.

Table 1: Overview of potential in vitro models that could be used to study NAFLD.

Model	Advantages	Disadvantages	References
Precision cut liver slices (PCLs)	– Liver cell architecture retained – Multiple cell types – Intact drug metabolism – Can be generated from biopsies – Good prediction of in vivo metabolite profiles	– Short-term (24 h) lifespan in static culture – Quality/reproducibility dependent on quality of tissue used – Cryopreservation poor – Lipid metabolism not investigated	[166], [167], [168], [169], [170], [171]

Primary human hepatocytes (PHH)	– Cryopreservation – Main liver cell type – Commercially available – 2D and 3D models established – Can be isolated from steatotic livers – Variety of *PNPLA3* genotypes – Store exogenous FA as TG – Secrete VLDL with similar TG/PL as in vivo – Capable of FA oxidation	– Dedifferentiate with time (2D) – Lose drug metabolism enzyme activity with time – Quality/reproducibility dependent on quality of tissue used – Media often un-physiological (high glucose/insulin)	[172], [173], [174], [175], [176]
Immortalised human hepatocytes (IHH)	– Proliferate – Glucose concentration dependent DNL	– Similar to hepatoma cell lines – Lose some functionality – Lipoprotein secretion mainly LDL – Storage and oxidation of exogenous FA unknown	[177], [178], [179]
Kupffer cells	– Commercially available – Can be isolated with other cell types from same liver tissue – Easily identifiable from CD68 expression – May promote VLDL secretion from hepatocytes	– Highly adherent – Isolation from steatotic livers difficult	[180], [181]
Stellate cells	– Commercially available – Can be isolated with other cell types from same liver tissue – Store exogenous FA	– Require cell-cell interactions for activation – Lipid metabolism not investigated	[182], [183], [184]
Hepatic stem cells	– Patient specific cells	– Low abundance	[161], [162], [163], [164]
Human pluripotent stem cells (hiPSCs)	– Differentiated into desired cell type – Generate cells of specific liver disease – Uptake and store exogenous FA as TG – PLIN2 expression following FA treatment	– Current differentiation protocol: fetal phenotype – Epigenetic memory may prevent differentiation – Long differentiation time – Limited investigation into lipid metabolism	[185], [186], [187]
HepG2	– Proliferate – Human serum improves phenotype – Easily cryopreserved – Uptake and store exogenous FA as TG – Uptake lipoprotein remnants – Capable of FA elongation and desaturation – Human serum improves TG secretion and lipoprotein profiles	– Fetal phenotype – *PNPLA3* mutants – Cancer origin/phenotype – High TG content on glucose media/high DNL – *PNPLA3* mutant – Mainly LDL secretion – Low FA oxidation – Limited SER	[99], [188], [189], [190], [191], [192], [193], [194], [195], [196]

Huh7/7.5	– Proliferate – Human serum improves phenotype – Easily cryopreserved – Capable of FA elongation and desaturation – Lipolytic enzyme expression – Human serum improves TG secretion and lipoprotein profiles	– Fetal phenotype – Cancer origin/phenotype – High TG content on glucose media/high DNL – *PNPLA3* mutant – Mainly LDL secretion on glucose – Low FA oxidation on glucose	[189], [197], [193], [198], [199], [200], [201]
HepaRG	– Proliferate – Uptake and store exogenous FA as TG – Secrete lipoproteins	– Retained drug metabolism enzymes – Limited investigation into lipid metabolism	[202], [203], [177], [204]

FA, Fatty acids; TG, triglyceride; VLDL, very low-density lipoprotein; PL phospholipid; LDL, low-density lipoprotein; DNL, de novo lipogenesis; SER, smooth endoplasmic reticulum.

Establishing NAFLD in vitro

Although isolation of PHH from livers with alcoholic fatty liver disease have been reported to have a low success rate (29%) [205], we have shown, PHH can be successfully isolated from steatotic livers and from small amounts of tissue [206]. However, well-characterised in vitro models of NAFLD are required. In humans, hepatic steatosis is most often macrovesicular where a large lipid droplet displaces the nucleus to the periphery and often consists of various sized lipid droplets that can come together to form larger lipid droplets [207]. In ~10% of NAFLD patients microvesicular steatosis (lipid droplets less than 1μm in diameter) can occur [207] and this phenotype is often associated with more advanced NAFLD [207]. In the majority of in vitro models only microsteatosis appears to be induced. There is currently no standard definition of steatosis in in vitro cell models so comparison between studies is often difficult and the establishment of a macrosteatoic model is challenging. However, one study has reported macrosteatosis in rodent primary hepatocytes after culturing in FA for 6 days, although the total concentration of FA used to induce this was exceptionally high at 4 mM (conjugated to 4% BSA) [208] which makes it challenging to translate to human models because it was supra-physiological and contained no saturated FA, so does not represent the human diet.

When hepatic cell lines, such as Huh7 and HepG2 cells, are grown on glucose alone, they have a high level of TG compared to PHH [188], [189] which is most likely due to an upregulation of GLUT1, which facilitates a constant uptake of glucose independently of glucose concentrations [209] leading to enhanced DNL. Therefore, the use of such conditions should be interpreted with caution in terms of what is used as non-steatotic control and whether DNL induced steatosis alone reflects steatosis in humans in vivo remains to be demonstrated. Ideally, a model of steatosis in vitro should be induced using physiologically relevant concentrations and ratios of saturated and unsaturated FAs conjugated to BSA and a mixture of glucose and/or fructose at physiological levels (5–11 mM). To best represent physiology, cells should be exposed to a mixture of FA such as oleate, palmitate and linoleate (in varying ratios) as these are the most abundant FA in humans [210] and therefore have the potential to best recapitulate the types of dietary fat that would typically be consumed and may be involved in the development of steatosis in vivo. Typically, in the literature, the most common FAs used to induce steatosis in vitro are either oleate (18:1) and/or palmitate (16:0) which are used alone or in combination [172], [188], [211], [212].

In terms of developing in vitro NAFLD models it is likely that stellate cells and Kupffer cells play important roles and therefore co-culture models are potentially required to effectively model disease progression. Additionally, a further consideration is zonation in the hepatocytes as this may play an important role in NAFLD development and progression as lipids have been shown to have distinct zonal distributions which may become dysregulated in NASH [145], [213]. Both DNL and FA oxidation have been reported to be carried out in specific hepatic zones however, the data is largely from rodent models and often contradictory [214]. Isolated PHH will represent a heterogeneous population of cells in terms of zonation and therefore will not be informative of the importance of zonation in humans; establishment of zonation in a two-dimensional (2D) single cell type culture may be a limitation that cannot be overcome. Therefore PCLs, which retain a cellular three-dimensional (3D) architecture, may be useful. Limited data is available in human in vitro models looking specifically at NAFLD and or lipid metabolism, as the majority of work has been carried out in rodent cell models.

In vitro models of genetic NAFLD

Due to natural human genetic variation PHH isolated from liver tissue offer the opportunity to study a broad range of genetic polymorphisms and their impact on NAFLD. Such a genetic variant is that of *PNPLA3* I148M. This variant has been shown to be related to liver fat content in humans [215] and alters susceptibility to development of NAFLD. Therefore, when using PHH it is advisable to genotype cells from each isolation so that wildtype, heterozygous and homozygous variant cells can be investigated. Although, the genotype of cell-lines is often not reported we have previously reported that HepG2 cells carry the *PNPLA3* I148M mutation [188] and consistent with this they have high intracellular TG levels compared to a *PNPLA3* wildtype cell line (LIV0APOLY) [188]. Although Huh7/7.5 cells have also been suggested to carry the *PNPLA3* I148M mutation [202] it has been reported that Huh7 cells do not express *PNPLA3* at the protein level [216]; it is plausible that the gene is not switched on in these cells. We have recently demonstrated that Huh7 cells have higher intracellular TG levels when grown with glucose media compared to HepG2 cells [189]. HepaRG cells, also offer the opportunity to be utilised as model of NAFLD but despite acceptance of these cells as a useful in vitro toxicological model, very little phenotyping and investigation has been carried out in relation to nutrient, specifically FA metabolism or *PNPLA3* genotype.

Hepatocyte FA partitioning

The majority of models have been shown to take up exogenous FA and store TG. Of note, PHH models have shown that a 2:1 oleic:palmitic acid ratio increases intracellular TG to levels comparable to that observed in human livers in vivo [211], [172]. Early work in HepG2 cells, utilising radio-tracers showed they were capable of taking up exogenous FA and lipoprotein remnants [190], [191], [192] and both HepG2 and Huh7 cells metabolise FAs via elongation and desaturation pathways [189], [190], [197]. HepaRG cells exposed to various polyunsaturated FA have been reported to develop microsteatosis and modulate lipogenic genes [203] whilst immortalised human hepatocyte (IHH) cells have been shown to have glucose concentration dependent DNL [177], [178]. Differentiated hiPSCs accumulate lipid following incubation with oleate and induced perilipin 2 (PLIN2) expression and peroxisome proliferator-activated receptor alpha [185]. Thus, there are a number of models that have the potential to be further developed to create an in vitro 2D cellular model of macrosteatosis.

An ideal liver cell model needs to be able to take up FA and dispose of them through oxidation and secretion pathways. However, many in vitro models do not secrete TG rich particles. HepG2 and Huh7 cells secrete lipid-poor apoB-containing lipoproteins, which resemble LDL, rather than VLDL, particles [193], [198], [199]. Although PCLs retain cell-cell interactions, to the best of our knowledge lipid metabolism has not been investigated, but work in rat and other species PCLs suggest they are able to secrete VLDL-TG [166]. Isolated PHH can secrete nascent VLDL with similar TG, cholesterol and phospholipid amounts to that seen in vivo [173]. We have previously found that treatment of PHH cells with a physiological mix of FA resulted in both storage and secretion of TG and oxidation of palmitate, which suggests FA partitioning remains intact following isolation [188]. IHH cells secrete lipoproteins but they are mainly in the LDL size range [177], [178]. The amount of FA in the culture media and the duration of culturing in FA may influence the secretion profile. VLDL secretion from hepatocytes may also be influenced by other cell types. For example, evidence from rodent cells suggests that activated Kupffer cells affect VLDL secretion of hepatocytes [180].

Normally, FA partitioning and mobilisation between intracellular pools in hepatocytes is a highly dynamic process; however, HepG2 cells exhibit levels of FA mobilisation for oxidation and VLDL secretion that are lower than those in primary hepatocytes or in vivo [193]. This is attributable to the very low expression of the carboxylesterase enzymes, which hydrolyse cholesterol esters and TG [189], [217]. Although expression levels of lipolytic enzymes are higher in Huh7 compared to HepG2 cells, they also have limited oxidation and secretion of FAs when cultured on glucose alone [189]. Additionally, HepG2 cells have limited smooth ER, demonstrating a structural deficit relevant to lipoprotein assembly [194]. However, several approaches have now been shown to overcome an insufficiency in lipoprotein secretion. It has consistently been shown that supplying exogenous FAs to culture media improves lipoprotein secretion in HepG2 and Huh7 cells [198], [218], [219], [220]. Use of human serum (HS) in the culture media has also been shown to improve TG secretion and lipoprotein profiles in HepG2 and Huh7/7.5 cells [99], [189], [200]. HS changes both gene expression of hydrolase enzymes and supplies more FAs to cells than foetal bovine serum, increasing substrate supply. Samanez et al. [177] showed that HepaRG cells are glucose and insulin-sensitive and secrete apoB-containing lipoproteins, levels of which increase with elevated glucose concentrations.

Non-parenchymal cells and NAFLD

It is likely that Kupffer cells play important roles in liver inflammation and in the progression from NAFLD to NASH. Kupffer cell isolation from steatotic livers may be difficult as fatty residue has been shown to impair the fractionation of liver cell types [181], this limits their use in understanding how Kupffer cells differ in healthy and steatotic livers, information that may be crucial in the understanding of NAFLD progression.

Stellate cells are pivotal in the initiation and progression of hepatic fibrogenesis [221] and their use in vitro allows these processes to be investigated either alone or in co-culture with PHHs. One study found that although stellate cells are able to accumulate fat, this is not involved in their activation [182] suggesting steatosis of stellate cells does not play a role in the progression of NAFLD. Immortalised stellate cells accumulate TG following exogenous FA treatments, however, they require cell-cell interactions with hepatocytes in order to be activated and stellate cell effects on extracellular matrix remodelling involve paracrine factors [182]. This latter finding highlights the importance in in vitro co-culture methods. Co-culture of PHH with non-parenchymal liver cells isolated from the same liver tissue may help to retain cell-cell interactions that would occur in vivo. This could be achieved either in direct contact or by conditioned media helps to maintain function of hepatocytes potentially through soluble factors secreted by stellate cells [222]. Human stellate cells are able to secrete adiponectin which negatively correlates with progression of NAFLD [164], however, to the best of our knowledge their lipid metabolism has not been investigated.

Potential uses of in vitro models to understand NAFLD

If tissue from healthy and diseased (e.g. NAFLD) human livers can be acquired, then PCLs can also be used to investigate pathological process and therapeutic potential of drugs. Slices from hepatic cancers [223] have been used in this way. One growing area is the use of human PCLs to identify biomarkers that can be used in vivo to monitor drug toxicity/side effects or potentially to aid in diagnosis of diseases. This could be applied to NAFLD, to identify biomarkers of, for example, various stages of NAFLD progression. Human PHH may be useful for drug discovery of NAFLD treatments as drugs that act on lipid metabolism have been shown to have different effects in rodents and humans due to enzymatic species differences [224]. Human IHH, Huh7 and HepG2 cells have been used to investigate how micro-RNAs vary between healthy and NAFLD subjects as they express three micro-RNAs thought to be induced in NAFLD [225] therefore are models to study the function of these micro-RNAs in NAFLD. The role of these micro-RNAs in causing or progressing NAFLD warrants further investigation. As micro-RNAs can be secreted from cells [226] changes in expression profiles may represent a cell-cell crosstalk mechanism between hepatic cells that could promote inflammation and progression of NAFLD. NAFLD has been modelled in hiPSCs in terms of increased TG storage, changes in metabolism gene expression and changes in hepatic microRNAs [185]. These findings were similar to that observed in liver biopsies from NAFLD patients [227].

Summary of in vitro models

In order to make findings translatable a few considerations should be made including investigating and understanding if the in vitro model(s) have: (1) the machinery and functionality to address the question of interest, and (2) the best culture conditions/time for the model to be optimal. In a number of cases it is likely that optimisation of models is required in order for it to be translational to an in vivo human situation. To validate the in vitro model, it would be most useful to study cells in parallel with in vivo human experimentation.

Expert opinion

NAFLD is a complex and heterogeneous disease that is widely prevalent in the general population and exponentially growing worldwide. Ideally, NAFLD diagnosis should be performed early to prevent the progression to NASH, and more advanced liver disease, and to adopt effective preventive strategies. Knowledge on the pathogenesis, risk factors, genetic markers and the pathophysiologic mechanisms involved in unfavourable disease progression will likely lead to develop effective treatment strategies and pharmacological treatments.

The ideal model to study NAFLD should ideally reproduce the human pathology; although no one single in vivo, ex situ or in vitro model currently encompasses the spectrum of human NAFLD progression (Figure 1). Consequently, the development of experimental models able to mimic the human condition becomes a

necessary tool and whilst undertaking human in vivo studies enables investigations into the factors that may influence the development of NAFLD to occur within the physiological setting, due to the heterogeneity between studies it is difficult to isolate and manipulate single factors. For example, in intervention studies where the intake of a dietary component (e.g. sugars) is altered, it is likely the intake of another dietary component (e.g. fat) is also altered, making it difficult to disentangle the influencing variables. Furthermore, recapitulating an "individuals" typical environment or diet is challenging, especially during standardised interventions, and it is likely that the intervention results in an artificial environment for some of the individuals under investigation, which may influence findings. The temporal resolution of studies also warrants consideration; longitudinal investigations and follow-up studies of NAFLD patients would be enlightening but are unfeasible for most. In this repsect, in vitro models, especially cultures of primary hepatocytes and hepatic cell lines, as well as ex situ models (when available) are valuable research tools, particularly when time is spent optimising the model to make it more physiologically relevant (e.g. using HS, altering sugar and FA concentrations). The main obstacle of these systems is the extrapolation of the results to the much more complex human environment.

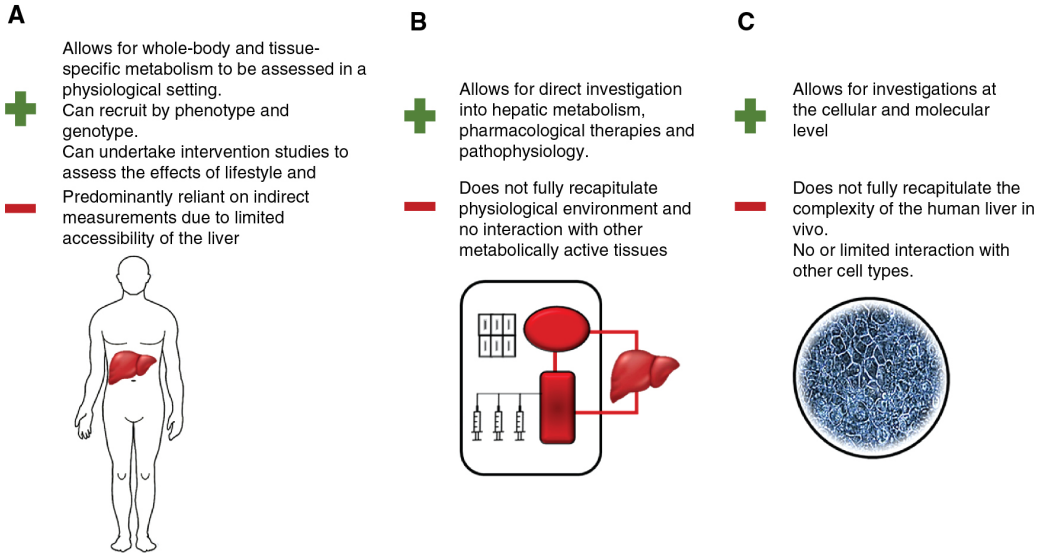

Figure 1: Summary of the advantages and limitations of in vivo, ex situ and in vitro models to study the liver. Advantages for each model are indicated by the green plus and limitations by the red minus signs. (A) in vitro human model, (B) ex situ model and (C) in vitro cell models.

Although there are many available models and methodologies to study NAFLD, researchers need to be aware of the shortcomings of the experimental models used in order to ensure scientifically sound conclusions and appropriate extrapolation to the disease situation can be made. When appropriately used, these models will continue to be indispensable tools in NAFLD research.

Outlook

In the next 5–10 years in vivo, ex situ and in vitro experimental models will evolve, and potentially more importantly, researchers will employ combinations of different models and methodologies to research NAFLD. In particular, it is likely that the development and refinement of non-invasive methods (i.e. imaging and biomarkers) to diagnose and stage NAFLD will substantially benefit the clinical and research fields, as it will allow for the periodical assessment of patients/participants in response to pharmacological, diet, or lifestyle interventions. Furthermore, the ability to identify patients in the early stages of steatosis will greatly aid in the ability to treat and manage NAFLD and characterise those at increased risk of disease development. Combining these methods with in vivo stable isotope methodologies will allow for investigation into the individual metabolic pathways influencing liver fat content and NAFLD progression. We also hypothesise that pharmacological treatments will gain approval in the relatively near future, developments of which will be accelerated as our knowledge of NAFLD increases.

Moreover, a concerted effort to establish models that fully epitomise the human condition in vitro would help elucidate the mechanisms underpinning the accumulation of liver fat and understand NAFLD pathophysiology and progression. Ideally these models will recapitulate liver cell architecture in 3D and involve physiologically relevant nutrients in a perfused, co-culture system.

A recent innovation in the field of NAFLD research is NMP. In the long-term NMP technology has the ability to expand the limited potential liver donor pool, however an additional benefit of this technology is that it allows for direct investigation of the liver, and experiments that cannot be performed in vivo. This technology has the potential to greatly enhance our understanding of liver metabolism, and the development and treatment of NAFLD.

Highlights

- Diagnosis and staging of NAFLD can be achieved using non-invasive imaging techniques.
- Stable-isotope methodology can provide mechanistic understanding of FA metabolism in health and disease in vivo and can also be applied to ex situ and in vitro models.
- NMP of non-transplantable livers allows for direct investigation into hepatic metabolism, pharmacological therapies and pathophysiology.
- Recapitulation of liver 3D architecture needs optimisation in vitro in order to provide models that better mimic human physiology.
- In vitro genetic models of NAFLD exist however, diet-induced NAFLD should be developed using more physiological components.
- No one single in vivo, ex situ or in vitro model currently encompasses the spectrum of human NAFLD progression. Researchers should consider the shortcomings of the experimental models used in order to ensure scientifically sound conclusions and appropriate extrapolation to the disease.

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