The regulation of hepatic fatty acid synthesis and partitioning: the effect of nutritional state

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is an increasing global public health burden. NAFLD is strongly associated with type 2 diabetes mellitus, obesity and cardiovascular disease and begins with intrahepatic triacylglycerol accumulation. Under healthy conditions, the liver regulates lipid metabolism to meet systemic energy needs in the fed and fasted states. The processes of fatty acid uptake, fatty acid synthesis and the intracellular partitioning of fatty acids into storage, oxidation and secretion pathways are tightly regulated. When one or more of these processes becomes dysregulated, excess lipid accumulation can occur. Although genetic and environmental factors have been implicated in the development of NAFLD, it remains unclear why an imbalance in these pathways begins. The regulation of fatty acid partitioning occurs at several points, including during triacylglycerol synthesis, lipid droplet formation and lipolysis. These processes are influenced by enzyme function, intake of dietary fats and sugars and whole-body metabolism, and further affected by the presence of obesity or insulin resistance. Insight into how the liver controls fatty acid metabolism in health and how these processes might be affected in disease offers the potential for new therapeutic treatments for NAFLD to be developed.

[H1] Introduction

The liver is a key regulator of systemic lipid metabolism. It is connected to the gut by the hepatic portal vein, which provides the majority of the liver's blood supply, with the hepatic artery delivering blood from the systemic circulation. As the main parenchymal cells of the liver, hepatocytes make up approximately 80% of liver tissue and are the primary site of hepatic

nutrient metabolism [1]. Hepatocyte distribution is defined as periportal or pericentral, depending on the proximity to the portal vein and hepatic artery or central veins, respectively, with an intermediate zone in between [2,3]. Hepatocytes in the periportal zone are exposed to the highest supply of nutrients, with the concentrations decreasing progressively for subsequent hepatocytes depending on the uptake rates of periportal hepatocytes [3].

Within the human body, there is a constant flux of fatty acids to the liver from a variety of sources, including those liberated by adipose tissue triacylglycerol (TAG) lipolysis and dietary fat (as chylomicron remnants), along with a continual recycling of fatty acids secreted as VLDL–TAG and taken up in the form of VLDL remnant particles. Once within the hepatocyte, exogenous fatty acids mix with endogenously synthesised fatty acids (which can be derived from non-lipid precursors), where they can act as signalling molecules and transcription factor ligands. The majority of fatty acids are partitioned between two pathways: either esterification to form glycerolipids (predominantly, but not exclusively, TAG and phospholipids) or oxidation. Which pathway fatty acids are partitioned toward is dependent on physiological and/or nutritional state [4]. In this Review, we will present what current research shows about how diet, especially those with altered macronutrient composition (i.e. high-sugar/high-fat), and metabolic diseases might have on these processes and how diets and disease interact to alter hepatic fatty acid synthesis and partitioning. …

[H1] Liver fat turnover and accumulation

In health, a balance exists between fatty acids entering the liver and those being synthesised within the liver and fatty acid disposal from the liver. Historical data clearly demonstrate that after an 18 h fast, of the fatty acids entering the liver in healthy individuals who are normolipidaemic, approximately two-fold more enter oxidation pathways than esterification pathways, whilst in individuals who are hyperlipidaemic, similar proportions of fatty acids enter the oxidation and esterification pathways [5]. In the transition to the postprandial state, the hormonal effect of insulin shifts cellular metabolism away from oxidation toward esterification of fatty acids at the endoplasmic reticulum (ER), predominantly producing TAG [6], which can then be secreted as VLDL–TAG or stored within lipid droplets (**Figure 1A**). It has long been proposed that the liver stores TAG to accommodate fatty acids that have accumulated in excess of the body's requirements for oxidation and/or secretion as VLDL–TAG [7-9]. A net retention of intrahepatic TAG (IHTAG) is a prerequisite for the development of non-alcoholic fatty liver disease (NAFLD), which encompasses a spectrum of diseases, starting with simple steatosis

(often referred to as NAFLD), through to the development of cirrhosis and hepatocellular carcinoma [10-12]. Importantly, IHTAG is strongly associated with obesity, insulin resistance, and type 2 diabetes mellitus (T2DM) [13] (**Figure 1B**).

Steatosis is defined by the presence of intracellular TAG in >5% of hepatocytes as determined by histological analysis, or >5.6% by proton density fat fraction assessed by proton magnetic resonance imaging or spectroscopy [14]. The causes of steatosis are complex and multifactorial; a combination of factors are probably involved. These include lifestyle factors (such as over-nutrition and lack of physical activity or exercise) [15], systemic changes (including insulin resistance or low-grade inflammation) [16] and molecular perturbations, which are characterised by increased reactive oxygen species (ROS) generation and ER stress [17]. Additionally, inherited factors, such as common variants in patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), transmembrane 6 superfamily 2 (*TM6SF2*) and glucokinase regulator (*GCKR*), have been demonstrated to predispose individuals to the development and progression of NAFLD [18].

[H1] Hepatic fatty acid uptake and activation

[H2] Plasma non-esterified fatty acids. The liver is supplied with non-esterified fatty acids (NEFA) from two sources; the largest contribution is from the intracellular lipolysis of TAG in the adipose tissue (endogenous NEFA), while lipolysis of chylomicron-derived dietary TAG (exogenous NEFA) represent a smaller contribution [19]. Adipose tissue lipolysis is under the control of insulin, which inhibits the activity of the two major lipolysis enzymes, adipose TAG lipase (ATGL) and hormone sensitive lipase (HSL) [20]. As a result, plasma concentrations of NEFA are highest in the fasting state and in the transition to the postprandial state levels decrease after consumption of a mixed meal [21,22]. By contrast, chylomicron-TAG concentrations increase in the systemic circulation over the course of the postprandial period until they peak around 2–4 h after consumption of a meal [21,23,24]. As chylomicron-TAG is hydrolysed by lipoprotein lipase, the majority of liberated NEFAs are taken up by adipose tissue; however, some escape uptake and appear in the systemic plasma NEFA pool: these fatty acids are often referred to as spillover NEFA [19]. NEFA turnover is a key determinant of VLDL–TAG production and stable isotope tracer studies have demonstrated that adipose-derived NEFA contribute the largest proportion of fatty acids that are esterified to form intracellular TAG [23,25-28] and for secretion as VLDL–TAG [29].

Although plasma concentrations of NEFA are often elevated in obesity, NAFLD and T2DM [30,31], the mechanism by which this occurs is unclear. It is now accepted that elevated plasma concentrations of NEFA are not due to increased adipose tissue fat mass: NEFA release per kilogram fat mass is reduced in obesity and associated with a downregulation of ATGL and HSL in adipose tissue [31]. Furthermore, the inability of adipose tissue to carry out sufficient uptake of dietary fat spillover, evidence suggests that this spillover is not increased in obesity [19,21]. An inverse relationship has been found between HOMA-IR and NEFA spillover [19], which suggests that lipoprotein lipase action is reduced in response to poor insulin sensitivity; expression of lipoprotein lipase in adipose tissue is significantly reduced in individuals with obesity compared with those who are lean [21]. A reduction in insulin-mediated inhibition of lipolysis could also explain the elevated postprandial NEFA concentrations [21]. Further evidence indicates that the relationship between insulin resistance and lipolysis is more complex, since in obesity, insulin sensitivity and NEFA levels are dissociated [30,31], an effect that might be mediated by changes in levels of adipokines during fat mass expansion, namely reduced adiponectin and increased TNF, which inhibit and stimulate lipolysis, respectively [32,33].

Dietary composition has also been reported to effect subcutaneous adipose tissue lipolysis. For example, a study that utilised stable isotope tracer methodology demonstrated that a 3-week diet enriched in saturated fat, compared with an unsaturated fat or free sugar-enriched diet, was associated with higher adipose tissue lipolysis during a hyperinsulinaemic clamp after the diet intervention, which would lead to a potentially greater flux of fatty acids (adipose tissue and dietary) to the liver [34]. This finding is in agreement with a previous dietary study that found that when men who were overweight or obese consumed a high-fat diet for 2 weeks, the postprandial suppression of adipose tissue lipolysis was reduced compared with when a moderate-fat diet had been consumed [35]. The effect of a high-fat diet on adipose tissue lipolysis might be due to a reduction in insulin sensitivity noted in the studies; however, an increase in inflammation in the adipose tissue might also contribute [34]. Taken together, the type and amount of dietary fat consumed might affect adipose tissue function, leading to a greater and more lengthened flux of fatty acids to the liver.

Once at the hepatic vein, NEFAs are transported across the plasma membrane, mainly via transporter-mediated mechanisms, whilst passive diffusion has a minor role. To date, plasma membrane fatty acid-binding protein (FABPpm), caveolins and fatty acid translocase (FAT, also known as cluster of differentiation 36 (CD36) have been identified as proteins that

facilitate and regulate the entry of NEFAs into hepatocytes [36] (Box 1). By using positron emission tomography or computed tomography in combination with labelled palmitate (^{11}C) or the palmitate analogue fluoro-6-thia-heptadecanoic acid (^{18}F-FTHA), hepatic fatty acid uptake in participants with morbid obesity, obesity or overweight has been assessed [37,38]. Although not significantly different, hepatic fatty acid uptake tended to be higher in participants with obesity than in those who were overweight[38]. By contrast, in participants with morbid obesity, hepatic fatty acid uptake was significantly higher before and 6 months after bariatric surgery than in lean controls, despite IHTAG content and insulin sensitivity being normalised after the surgery [37]. A negative correlation was noted between portal venous blood flow and hepatic fatty acid uptake, suggesting an adaptive upregulation of fatty acid transport that persisted after weight loss [37]. It remains unclear if this response was maintained beyond 6 months and if this is a specific adaption that occurs with weight loss induced by bariatric surgery, rather than lifestyle (that is, diet and exercise).

[H2] Dietary chylomicrons. In the postprandial state, chylomicrons are produced by the enterocytes and enter the blood stream. Once in systemic circulation, the estimated half-life of the TAG content in chylomicrons is approximately 5 mins [39]. Work in a rat model has estimated that around only half of the chylomicron TAG content is lost in the process of chylomicron remnant formation [40]. The liver is the major site of removal of chylomicron remnants, either via the LDL receptor (LDLR) or LDLR-related protein 1 (LRP1) [7,41,42] and once in the liver, remnants are hydrolysed by hepatic lysosomes to release fatty acids [43,44]. In both obesity and NAFLD, hepatic expression of LDLR and LRP1 are either unchanged or downregulated [45-47]. This effect might result in chylomicron remnants staying in the systemic circulation for longer periods of time than in people without obesity or NAFLD and could partly explain the higher plasma concentrations of TAG observed in individuals with obesity and/or insulin resistance compared with lean and/or insulin-sensitive individuals [21,24]. Work in the *Ldlr*$^{-/-}$ mouse found that when fed a high-fat, high-cholesterol diet, IHTAG accumulation occurred in association with hepatic inflammation and liver damage compared with the *Ldlr*$^{+/+}$ mouse [48]. Moreover, the type of dietary fat consumed also effects hepatic LDLR activity and expression, with a diet high in saturated fat decreasing LDLR activity [49] and expression [50] compared with diets containing polyunsaturated fat or that are low in fat. Thus, LDLR activity and expression might have a key role in modulating the dyslipidaemia that is often associated with metabolic disease and in protecting the liver from oxidised LDL-mediated injury.

[H1] Fatty acid and triacylglycerol synthesis

[H2] *De novo* lipogenesis. Non-lipid precursors (such as sugars and proteins) can be used as substrates for fatty acid synthesis through *de novo* lipogenesis (DNL). For example, during glycolysis, the production of acetyl-CoA from pyruvate by pyruvate dehydrogenase provides the substrate required for DNL. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) then perform the subsequent steps to produce malonyl-CoA and a fatty acyl-CoA, respectively [51]; specifically, palmitoyl-CoA is often considered the fatty acyl-CoA end product of the DNL pathway. In addition to contributing newly synthesised fatty acids to the intrahepatic pool, increased DNL might have indirect effects on IHTAG accumulation. include suppression of hepatic fatty acid oxidation via malonyl-CoA inhibiting the activity of carnitine palmitoyl transferases 1 (CPT1) [52,53], increased ceramide synthesis from palmitoyl-CoA [54,55], which might cause mitochondrial dysfunction, oxidative stress and cell death [56], proinflammatory pathways due to accumulation of DNL-derived saturated fatty acids [57-59]. All three of these effects might lead to IHTAG accumulation.

Regulation of DNL occurs via transcriptional regulation of ACC and FAS, primarily by sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate-responsive element-binding protein (ChREBP). For SREBP1c to activate target gene transcription, which includes *ACACA* (the gene encoding the major hepatic isoform of ACC, ACC1) and *FASN* (which encodes FAS [60]), it must be translocated to the cell nucleus. ChREBP also requires nuclear translocation, which is facilitated by glycolytic by-products and results in increased transcription of genes with the carbohydrate response element, including *ACACA* and *FASN* [61]. Both transcription factors are stimulated via activation of liver X receptor (LXR), which upregulates transcription. LXR can be activated by oxysterols and cholesterol intermediates [62], as well as insulin [63]. Activation by insulin occurs through both increased transcription of LXR (*NR1H3*) and potentially indirectly through production of ligands (that is, oxysterols) that increase activity [64]. SREBP1c is also directly stimulated by insulin through nuclear SREBP1c translocation. (**Figure 2**) [51] SREBP-1c exists in a membrane-bound, inactive form at the ER in association with SREBP cleavage-activating protein (SCAP) and insulin-induced gene (INSIG). Insulin signalling via the PI3K/PKB pathway causes the SREBP1-c-SCAP complex to dissociate from INSIG, which enables the complex to move to the Golgi apparatus. Cleavage of SREBP-1c produces a mature form that translocates to the nucleus and increases transcription of its target genes.

Very few studies directly measure the contribution of DNL-derived fatty acid to IHTAG. Instead, the contribution is measured by determining levels of VLDL–TAG, which has been suggested to be a good surrogate of IHTAG [25]. Hepatic DNL is considerably higher in individuals with NAFLD than in those without NAFLD: stable isotope tracer studies suggest that DNL-derived fatty acids contribute between 14% and 25% to VLDL–TAG in these individuals [25,65-67], compared with around 10% or less in those with the metabolic syndrome but low IHTAG content [65,67]. Moreover, impaired insulin signalling seems to have a direct effect, as DNL is associated with hyperinsulinaemia [68], even when participants are matched for BMI [69]. These functional changes are associated with increased expression levels of the DNL master regulators, LXR, SREBP1c and ChREBP [70-72]. Although DNL seems to be induced as a result of insulin resistance and/or NAFLD development, it remains unclear whether an increase in DNL might precede, and thus contribute to, the development of steatosis and insulin resistance.

Given the effect of sugars as substrates and regulators of DNL, dietary intake has a strong influence on this pathway: hepatic DNL increases in response to high-carbohydrate, low-fat feeding. For example, the contribution of DNL-derived fatty acid to VLDL–TAG was 41% after consumption of a high carbohydrate (75% total energy) diet for 2 weeks, compared with 10% after consumption of a moderate carbohydrate (55% total energy) diet [73]. Similarly, DNL-derived fatty acids in VLDL–TAG increased by 35% (to ~20% of total fatty acids in VLDL–TAG) after a low fat diet for 3 days (~23% total energy fat, 59% total energy carbohydrate) compared with a high-fat diet (~37% total energy fat, 48% total energy carbohydrate) in healthy participants [74]. , the effect of dietary sugars, and specifically excess fructose, on DNL and IHTAG accumulation has gained attention. Fructose upregulates lipogenic gene expression and enzyme activity [75] and can also be used as a substrate for DNL; however, tracer studies have shown that the amount of fructose used for this pathway is minimal (<1%) [76,77]. Upregulation of DNL reduces β-oxidation and energy expenditure [78] and unlike the metabolism of glucose by glucokinase, the metabolism of fructose by fructokinase is an unregulated pathway that depletes intracellular ATP and generates uric acid, which might contribute to inflammation and oxidative stress in the development and progression of NAFLD [79,80].

Experimental evidence consistently shows that acute consumption of hypercaloric, fructose-enriched diets leads to IHTAG accumulation in human intervention studies [81-83]. However, systematic reviews and meta-analyses have concluded that there is insufficient evidence to

implicate fructose as causative in IHTAG accumulation when fed isocalorically [84-86]. In agreement with the latter finding, when a high fructose diet was compared to a sucrose and starch diet in rats (all 60% of energy intake), no differences were found in IHTAG accumulation, despite increased nuclear presence of SREBP1c and ChREBP in fructose and sucrose-fed animals [75].

In a whole dietary context, the effect of free sugars on IHTAG accumulation is unclear. Although meta-analyses that included only observational studies have concluded that there is a notable effect of fructose on IHTAG accumulation [87,88], it was sucrose intake in the form of sugar sweetened beverages that drove this association. However, in a Finnish cohort, fructose consumption was inversely associated with NAFLD risk, probably because fructose tended to be consumed in the form of fruits rather than sugar sweetened beverages [89]. In addition, compared with other macronutrients, the 33% increase in IHTAG content after consumption of a sugar-enriched diet was lower than that reported after a diet enriched with saturated fat, which increased IHTAG by 55% [34].

[H2] Triacylglycerol synthesis. Hepatocytes can esterify fatty acyl-CoAs to form TAG via the glycerol-3-phosphate acyltransferase (GPAT) or monoacylglycerol pathway. In the canonical GPAT pathway, a fatty acyl-CoA is joined to glycerol-3-phosphate to form lysophosphatidic acid and with a further addition of a fatty acyl-CoA, catalysed by sn-1-acyl-glycerol-3-phosphate acyltransferase, phosphatidic acid is formed. The phosphate group is then removed by phosphatidic acid phosphatase (also known as lipin) to form DAG [90]. The GPAT pathway is stimulated in conditions of energy excess postprandially and inhibited in conditions of energy depletion through phosphorylation and dephosphorylation of GPAT [91]. Although TAG synthesis predominantly occurs at the ER, it can also occur at lipid droplets, mitochondria and the nuclear envelope and several organelle-specific isoforms of each enzyme in the GPAT pathway exist. Their relevance to normophysiology and pathophysiology has been reviewed elsewhere[92]. Of note, expression of the mitochondrial-resident isoform of GPAT, GPAT1, is positively correlated with steatosis occurrence in mice [92]. This enzyme specifically utilises palmitoyl-CoA, including that derived from DNL, as a substrate [93]; altered function of this enzyme might therefore affect TAG accumulation from DNL. In line with this idea, GPAT is also a target of SREBP1c, which allows coordination of fatty acid synthesis through DNL and TAG synthesis [91]. The monoacylglycerol pathway of TAG synthesis is typically used during hydrolysis and re-esterification of TAG, where a preformed monoacylglycerol molecule has a

fatty acyl-CoA added by monoacylglycerol acyltransferase to form DAG, with both pathways having the final fatty acyl-CoA added to DAG by DAG acyltransferase (DGAT) [94].

[H1] Partitioning of hepatic fatty acids

[H2] Hepatic lipid droplets. Within the hepatocyte, TAG is stored primarily in lipid droplets. Although lipid droplets can be found in the ER lumen, as primordial VLDL particles and within the nucleus [95], cytosolic lipid droplets are the most studied. In individuals with NAFLD, steatosis is histologically defined as either macrovesicular steatosis or microvesicular steatosis the cytosolic lipid droplet pattern. Macrovesicular steatosis describes large lipid droplets that displace the nucleus to the periphery of the cell, causing structural disruption [96]; however, macrovesicular steatosis can be present with both large and small droplets that might be seen to coalesce [97]. Moreover, macrovesicular steatosis can be further sub-divided into large droplet macrovesicular steatosis (a single lipid droplet, larger than half of the cell, displacing the nucleus) or small droplet macrovesicular steatosis (lipid droplet is smaller than half of the cell and does not displace the nucleus) [98]. By contrast, microvesicular steatosis is characterised by multiple small lipid droplets that create a foamy appearance and uniformly occupy the whole cell with a centrally-located nucleus. Microvesicular steatosis is usually present in acute fatty liver onset in association with severe impairment of mitochondrial β-oxidation, including that caused by certain drugs, pregnancy, Reye syndrome, and hepatitis C infection [99,100]. However, microvesicular steatosis accounts for around 10% of steatosis in NAFLD, where it is associated with more advanced histology markers and progression to NASH [97,101].

The factors regulating the size and location of lipid droplets in the development and progression of steatosis are yet to be completely elucidated. Lipid droplet formation is hypothesised to occur when neutral lipids accumulate between the membranes of the ER, initially forming a lens, before a budding lipid droplet is formed, which eventually buds off into the cytoplasm [102,103]. The size of the lipid droplet formed at the ER, fusion and coalescence of cytoplasmic lipid droplets and *in situ* TAG synthesis have all been proposed to contribute to lipid droplet growth [102,104]. Of particular relevance to lipid droplet size and pattern (that is, micro or macrovesicular steatosis) are the perilipin family of lipid droplet surface proteins. The expression of different perilipin proteins has been tracked across several disease states and during lipid accumulation, showing that perilipin 3 (PLIN3) and PLIN5 were more common on smaller lipid droplets, and PLIN1 and PLIN2 were more common on the largest lipid droplets [105]. However, levels of all perilipin proteins, especially PLIN1, which is not usually

expressed in hepatocytes, are increased in NAFLD [106,107]. Furthermore, both PLIN1 and PLIN2 have previously been associated with NASH [108,109], suggesting that a large lipid droplet pattern might determine fatty acid partitioning and contribute to NAFLD progression.

In line with a regulatory role of perilipins in fatty acid partitioning, perilipin proteins have been demonstrated to regulate ATGL activity by inhibiting its action in the fed state in multiple tissues [110], which downregulates fatty acid lipolysis and disposal. Although known to contribute to TAG lipolysis, a growing body of evidence has focused on the role of ATGL in lipophagy. Lipophagy, the autophagic process that specifically contributes to lipid droplet degradation in hepatocytes [111], occurs via both macro-based and micro-based mechanisms, involves a number of proteins and liberates fatty acids primarily for oxidation [112]. The current model of lipid droplet catabolism is that ATGL and lipophagy directly contribute to lipid droplet degradation, with ATGL not only being necessary and sufficient to promote the expression of genes with proteins products involved in autophagy, but also promoting lipid droplet turnover [112,113]. It has been suggested that in some patients with NAFLD, a decreased expression of the enzyme glycine N-methyltransferase might result in increased serum levels of methionine and S-adenosylmethionine, leading to impairment in lipophagy [114]. Alternatively, a slight elevation of the autophagy-inhibiting protein, Rubicon, in liver samples taken from patients with NAFLD has been reported [115]. The liberation of fatty acids from TAG in lipid droplets is still an area of investigation [113]. However, given that PLIN1 is not usually expressed in the liver, how expression of this protein in steatosis might interact with ATGL activity and affect fatty acid liberation from lipid droplets remains to be elucidated.

[H2] Intrahepatic mitochondrial β-oxidation: complete oxidation and ketogenesis. The predominant oxidative pathway for energy production in the liver is β-oxidation in the mitochondria; however, β-oxidation can also occur in peroxisomes and oxidation can also occur via the alternative pathway of The use of microsomal oxidation, either to shorten long-chain fatty acids, or when mitochondrial overload occurs, can lead to the production of ROS [116]. Entry of fatty acyl-CoAs into the mitochondria occurs via CPT1; β-oxidation then consists of a cycling process involving dehydrogenation, hydration, dehydrogenation and acylation that produces acetyl-CoA [117,118]. A branch point in fatty acid oxidation pathways is the partitioning of intra-mitochondrial acetyl-CoA between complete oxidation via the TCA cycle or ketogenesis. Which of these two pathways acetyl-CoA is partitioned is dependent on supply of oxaloacetate (derived from pyruvate during glycolysis). hen levels are sufficient, oxaloacetate

317 condenses with acetyl-CoA and enters the TCA cycle; however, in situations when glucose
318 levels become low (such as fasting), oxaloacetate is preferentially utilised in the process of
319 gluconeogenesis and acetyl-CoA is diverted to ketogenesis [119].

320 Ketogenesis produces acetoacetate, acetone and 3-hydroxybutyrate (3-OHB); plasma levels of
321 3-OHB are commonly used as a surrogate marker of hepatic fatty acid oxidation [6] (**Figure 2**).
322 In healthy individuals, a major regulator of ketogenesis is the rate of supply of fatty acids from
323 adipose tissue; in the postprandial period ketogenesis is suppressed compared with in the
324 fasting state due to the effect of insulin on suppressing adipose tissue lipolysis. The primary
325 regulator of β-oxidation is the transcription factor peroxisome proliferator-activated receptor α
326 (PPARα), the action of which is upregulated by fatty acids and glucagon and suppressed via
327 insulin [120]. Direct shuttling of fatty acyl-CoAs to oxidative organelles upon entry to the
328 hepatocyte might occur[121], otherwise, ATGL is the predominant lipase that directs mobilised
329 fatty acid toward oxidation, with PLIN5 facilitating oxidation by promoting lipid droplet–
330 mitochondria interactions and PLIN2 blocking ATGL access to the lipid droplet surface [103,122].
331 As lipid droplets become larger (that is, steatosis progresses), the increased expression of
332 PLIN2 and lower expression of PLIN5 [105] could therefore be speculated to downregulate fatty
333 acid oxidation.

334 Indeed, a number of studies have investigated hepatic fatty acid oxidation *in vivo* in humans.
335 By using a combination of stable isotope labelled tracers (^{2}H and ^{13}C), it was found that fasting
336 mitochondrial oxidation was twice as high in patients with NAFLD (17% IHTAG) than in
337 those without NAFLD (3% IHTAG). In addition, a strong direct association between oxidative
338 flux and IHTAG was found, although no difference in ketone body production was observed
339 [123]. By contrast, by using ^{13}C-acetate infusion in combination with a ^{13}C-MRS methodology,
340 similar rates of fasting hepatic mitochondrial oxidation (based on mathematical modelling)
341 were found in participants with high (~9%) and low (~2%) IHTAG content [124]. Studies
342 measuring plasma concentrations of 3-OHB in the fasting state as a marker of hepatic fatty acid
343 oxidation have reported mixed findings, with concentrations being decreased [125], similar
344 [23,24,27,126] or increased [127,128] in individuals with insulin resistance and/or NAFLD. Despite
345 inconsistent associations between oxidation measures and IHTAG content, increased markers
346 of oxidative stress and redox imbalances have been noted in people with steatosis [129-131]. An
347 alternative hypothesis to the role of perilipins in downregulation of fatty acid oxidation (as
348 discussed previously) is that in the initial stages of IHTAG accumulation there is an increase

in mitochondrial activity to dispose of fatty acids, before the mitochondria become overloaded, microsomal oxidation and ROS increase and mitochondrial activity decreases [132,133].

It is often speculated that dietary polyunsaturated fatty acids (PUFA) preferentially enter oxidation pathways compared with monounsaturated and saturated fatty acids, suggesting that a diet enriched with PUFAs would result in reduced IHTAG accumulation. This theory is supported by limited evidence using stable isotope tracers in humans. By measuring the appearance of ^{13}C from recently ingested fatty acids in breath CO_2 (a marker of whole-body fatty acid oxidation), a greater recovery of unsaturated (both monounsaturated and polyunsaturated fatty acids) compared with saturated fatty acids has been reported [134-136], suggesting that unsaturated fatty acids enter oxidation pathways to a greater extent than saturated fatty acids. The mechanism underpinning this observation remains to be elucidated but it could be speculated that unsaturated fatty acids stimulate fat oxidation by activating transcription factors such as PPARα [137]. In support of these observations, two studies have reported that IHTAG accumulation is lower on a PUFA-enriched diet compared with a saturated fat enriched diet; as there was no notable change in fasting plasma levels of 3-OHB this finding might be attributable to a concomitant increase in complete fatty acid oxidation and a reduction in DNL [34,138].

[H2] Secretion of hepatic fatty acids. The liver has a role in the regulation of systemic lipid metabolism as it assembles and secretes TAG-rich VLDL particles into systemic circulation for distribution of fatty acids to peripheral tissues. The formation of VLDL begins with a nascent apoB100 particle passing from the rough to the smooth ER, where the addition of TAG via microsomal TAG transfer protein (MTP) forms a primordial $VLDL_2$ particle [139,140]. A second lipidation step is required for mature $VLDL_1$ particle secretion, but the exact mechanism underlying this step remains unclear. However, it has been suggested that luminal lipid droplets are utilised as a substrate pool either through a lipolysis–re-esterification cycle or fusion with the primordial $VLDL_2$ particle [121,141] (**Figure 2**). However, convincing evidence for the fusion hypothesis in VLDL assembly is still lacking [140]. If insufficient lipid is transferred to a primordial $VLDL_2$ particle in this second lipidation process, apoB will undergo degradation [142]. Otherwise, mature $VLDL_1$ particles undergo vesicle-mediated transfer to the Golgi apparatus, before migration to the sinusoidal membrane for release into the circulation [143,144]. Molecular regulators of VLDL assembly are discussed in **Box 2**.

Patients with T2DM and those with NAFLD have been reported to have an overproduction of VLDL particles, particularly $VLDL_1$ [145]. Evidence suggests that apoB100 secretion is not increased in patients with NAFLD [146]. Instead, the particles secreted are more TAG-rich with increased particle size compared with those from people without NAFLD [147]. These changes might be partly mediated by insulin resistance, as insulin suppresses $VLDL_1$ production in individuals who are insulin sensitive but not those who are insulin resistant [148,149]. Although secretion of $VLDL_1$ increases with increasing amounts of IHTAG [150], a limit to VLDL–TAG production seems to exist. Indeed, a plateau in secretion has been reported beyond 10% IHTAG [146]. At a gene expression level, when steatosis accounted for >30% of liver volume, the genes encoding MTP and apoB were downregulated compared with people who had lower levels of steatosis [151]. In line with this finding, a mutation in *TM6SF2*, which encodes an ER-resident protein, is strongly associated with NAFLD. This protein is involved in determining the partitioning of lipid towards intracellular lipid droplets and VLDL particles [152] and in humans the mutation causes a reduction in VLDL secretion [153], confirming a role for impaired TAG secretion in steatosis development.

Dietary influences on VLDL secretion have been reported in a limited number of studies. A study has compared the influence of isocaloric diets high (26% total energy) and low (6% total energy) in free sugars, consumed for 12 weeks, on VLDL–TAG kinetics in individuals with and without NAFLD [154]. While the $VLDL_1$–TAG production rate increased in individuals with and without NAFLD after the high sugar diet, the $VLDL_2$ production rate only increased after the high sugar diet in the individuals with NAFLD [154]. In participants who were healthy or had hypertriglyceridaemia, a 5-week high carbohydrate (68% total energy) diet resulted in elevated VLDL–TAG concentrations and a reduction in VLDL–TAG uptake compared with a 1-week control diet (carbohydrate 50% total energy), but responses did not differ between the groups [155]. The effect of different dietary fats on VLDL–TAG production and secretion are less clear; however, low (7.8% total energy), medium (10.3% total energy) and high (13.7% total energy) levels of monounsaturated fatty acids in the diet did not affect production of $VLDL_1$ and $VLDL_2$ in people with mild hypercholesterolaemia [156]. This limited evidence suggests a larger effect of sugar than fat on VLDL–TAG secretion and uptake, which might be supportive of DNL-derived TAG being partitioned directly towards a secretory pool.

[H1] Therapeutic targets

Decreasing or increasing the synthesis and/or partitioning of intrahepatic fatty acids into specific pathways has been suggested to result in an attenuated IHTAG content or risk of developing NAFLD. As individuals with T2DM typically have IHTAG accumulation, several studies have investigated the therapeutic effects of pharmacological agents and their effect on IHTAG content [157]. Briefly, these studies report that insulin-sensitising agents, including metformin and sulphonylureas, do not seem to decrease IHTAG content in humans, in contrast to the effects of metformin on reducing IHTAG levels in rodents [158]. Thiazolidinediones, which are selective ligands of the PPARs (of which there are α, β/δ and γ forms), seems to decrease IHTAG content [157]. Proposed mechanisms include increasing fatty acid uptake and re-esterification in adipose tissue, thus lowering the flux of fatty acids to the liver (via PPARγ), and influencing β-oxidation (via PPARα) [157]. Glucagon-like peptide 1 (GLP1) has the potential to decrease IHTAG levels, but requires further exploration. Uncertainty surrounds dipeptidyl peptidase 4 (DPP4) and sodium glucose cotransporter 2 (SGLT2) inhibitors in their ability to alter IHATG content [157].

Supplementation with marine-derived *n*-3 PUFA, namely eicosapentaenoic acid and docosahexaenoic acid when given as ethyl esters, at a dose of 4 g per day for 8 weeks has been reported to decrease IHTAG content in women with polycystic ovary syndrome [159]. Furthermore, two independent reviews in patients with NAFLD concluded that *n*-3 PUFA reduces IHTAG content [160,161]. The proposed mechanisms by which *n*-3 PUFA lower IHTAG are through hepatic transcription factors downregulating lipogenic pathways and upregulating β-oxidation pathways [160,162]. We have previously reported pilot data showing that *n*-3 PUFA supplementation resulted in decreased IHTAG content and fasting hepatic DNL and increased postprandial hepatic β-oxidation [163]. More human studies replicating these observations are required.

As increased hepatic DNL has been suggested to be a cause of IHTAG accumulation, a number of studies have been undertaken in which specific enzymes or genes in the DNL pathway were inhibited (Table 1). Taken together, it appears that inhibition of either ACC or DGAT2 results in a reduction in DNL, thus lowering substrates for IHTAG synthesis, as well as reducing levels of DNL intermediates such as malonyl-CoA, which can have an inhibitory effect on fatty acid β-oxidation, allowing increased IHTAG disposal (**Table 1**). Although these inhibitors seem to be a potential treatment for IHTAG accumulation, what remains unclear is what effect inhibiting DNL has on other intrahepatic and extrahepatic metabolic pathways. For example, a

study reported a significant decrease in IHTAG content when DNL was inhibited, but there was a concomitant significant increase in plasma TAG concentrations [164]. Whether this is a transient or long-term effect, or is due to increased TAG production or decreased clearance is unclear. As a result, the utility of DNL inhibitors remains unclear.

[H1] Conclusion

Although many factors are involved in the regulation of intrahepatic fatty acid metabolism and partitioning, current evidence suggests that both dietary intake and disease state are likely to have molecular implications that might cause an imbalance of hepatic fatty acid uptake and utilisation. In disease, whether these disturbances in input and output are a cause or consequence of fat accumulation in diseases including obesity, T2DM and NAFLD is unclear. These diseases are linked by impaired insulin signalling, which is traditionally thought to manifest a 'selective' profile of hepatic insulin resistance, where both DNL and gluconeogenesis remain upregulated. However, the nuances of hepatic insulin resistance, and how this might cause, or result from, fat accumulation remain to be elucidated. As dietary intake can also influence insulin levels as well as tissue nutrient exposure, the interaction between these pathways requires optimisation of physiologically relevant models of hepatic fat and carbohydrate metabolism. The development of systems that enable the interaction of multiple pathways to be studied will allow the processes involved in IHTAG accumulation and its effects on intracellular fatty acid partitioning to be more fully understood.

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Competing interests

The authors declare no competing interests.

Publisher's note

Key points

- Intrahepatic triacylglycerol (IHTAG) accumulation occurs through an imbalance between fatty acid uptake and synthesis and fatty acid disposal; however, the exact mechanisms by which this occurs in humans are poorly understood.
- Insulin signalling seems to be an important factor that links intrahepatic and extrahepatic fatty acid metabolism; hepatic insulin signalling regulates pathways linked to fatty acid uptake, synthesis and storage.
- Both non-esterified fatty acid (NEFA) delivery and fatty acid synthesis through DNL seem to be upregulated during IHTAG accumulation, which might be worsened by high saturated fat and high free sugar intake, respectively.
- Secretion of IHTAG as VLDL–TAG and partitioning into oxidation pathways might have a dynamic response, depending on disease state; the regulation of the pathways requires further investigation.
- Dietary intake influences insulin levels as well as tissue nutrient exposure; the interaction between these pathways requires optimisation of physiologically relevant models of hepatic fat and carbohydrate metabolism.

Box 1. Regulation of hepatic fatty acid uptake and activation

The mechanisms of fatty acid uptake and activation to fatty acyl-CoAs are an area of continuing investigation. Although expressed at low levels in the liver, mRNA and protein levels of the most well-characterised fatty acid membrane transporter, CD36, are positively correlated with liver levels of fat [165,166]. Moreover, in a mouse model, this transporter was regulated by lipogenic transcription factors, including LXR, which suggests an important functional role for CD36 in steatosis development [167]. CD36 dysfunction has been implicated in lipophagy reduction and NAFLD development [168]. In addition, while more commonly associated with skeletal muscle fatty acid uptake, expression of the gene that encodes FABPpm was upregulated in adolescents with NASH [165]. The same study noted upregulation of FATP2 and FATP5, the most common liver isoforms of the FATP family of proteins, which function both as mediators of fatty acid uptake and activators of very long chain fatty acids [165]. Caveolin 1 has diverse functions in addition to fatty acid uptake, including on liver function, the cell cycle and accumulation of deleterious lipid species [169]. As such, its associations with IHTAG accumulation are complex; in humans, genetic mutations resulting in reduced caveolin 1 levels are associated with congenital generalized lipodystrophy [170], making isolating adipocyte and liver-specific effects of the resulting hepatic steatosis difficult. Similarly, the most abundant hepatic FABP (FABP1) has functions beyond shuttling fatty acids to different cellular compartments, including in mitosis and as an antioxidant [171]. However, expression of FABP1 [172], as well as FABP4 and FABP5 [173], is associated with fat infiltration in patients NAFLD.

Box 2. Regulation of VLDL–TAG substrate supply and assembly

A major determinant of VLDL–TAG production is substrate supply from hepatic lipid droplets. As well as the canonical lipolysis pathway by ATGL and HSL, there might be additional secretion-specific pathways present in hepatocytes. In humans, carboxylesterase (CES) enzymes are the most well-defined lipases associated with VLDL assembly: CES1 and CES2 are present at the ER where they are hypothesised to hydrolyse TAG from luminal lipid droplets for second-step $VLDL_1$ lipidation [174]. Specifically, CES2 acts as a TAG and DAG hydrolase and activity of this enzyme is downregulated in human obesity [175]. Through

its activation of ATGL, alpha-beta hydrolase domain containing 5 has historically been proposed to have a role in liberating fatty acids towards VLDL assembly; however, the evidence is inconclusive and a mechanism controlling partitioning is lacking [176]. By contrast, PLIN2, which is upregulated in NAFLD [105], seems to have an inhibitory role on VLDL synthesis, probably by blocking lipase action [177]. Through inter-organelle lipid transfer, CIDEB and the Arf1–COPI complex might promote transfer of pre-formed TAG contained within cytosolic lipid droplets to the ER lumen for lipoprotein assembly. Finally, although still under investigation, DGAT2 has been proposed to use DNL-derived fatty acids for TAG synthesis, which might then be partitioned towards a VLDL assembly pool, [141] potentially in association with fat storage-inducing transmembrane protein 2 (FITM2 or FIT2) [178,179].

Figure Legends

Figure 1. Hepatic and whole-body pathways of fatty acid metabolism. (**A**) In the fasting state (solid lines), when insulin levels are low, lipolysis of subcutaneous and visceral adipose tissue liberates non-esterified fatty acids, which enter the liver via the hepatic artery and mix with fatty acids from the cytosolic triacylglycerol (TAG) pool. Fatty acids in the liver can be used to synthesise TAG, which is incorporated into VLDL particles for delivery of fat to peripheral tissues. Alternatively, fatty acids can be oxidised, primarily via β-oxidation, for energy production in the liver. Fatty acids partitioned into storage in the liver are esterified to predominantly produce TAG and stored within lipid droplets. After eating (dashed lines), dietary fat is incorporated into chylomicrons in the gut as TAG before entering the circulation to deliver fatty acids to tissues, where they are liberated by lipoprotein lipase, before being taken up by the liver as chylomicron remnants. Dietary sugars absorbed into the circulation at the small intestine can be used to form fatty acids by *de novo* lipogenesis (DNL). The postprandial increase in plasma concentrations of insulin suppresses adipose tissue lipolysis and upregulates the DNL pathway, which would shift the cellular metabolism of fatty acids away from oxidative pathways towards esterification. (**B**) In individuals with an 'unhealthy' phenotype (for instance, insulin resistance, obesity or NAFLD) these pathways become dysregulated (blue arrows). In the fasting state, peripheral insulin resistance reduces lipolysis inhibition, which might cause increased non-esterified fatty acid concentrations, while in both the fasting and the postprandial state the DNL pathway will be constitutively upregulated. Chylomicron and VLDL–TAG concentrations are increased, either through elevated production, reduced clearance or both. Findings on measurements of fatty acid oxidation levels are mixed, with both increased and decreased levels reported [123,125].

Figure 2. Overview of hepatocellular partitioning of fatty acids. A fatty acid entering the hepatocyte is rapidly 'activated' by acyl-CoA synthetases to form fatty acyl-CoA. Alternatively, fatty acids might originate from lipoprotein uptake and catabolism within lysosomes or be synthesised from non-lipid precursors by *de novo* lipogenesis (DNL), which is catalysed by acetyl-CoA carboxylase 1 (ACC1; encoded by *ACACA*) and fatty acid synthase (FAS; encoded by *FASN*). The transcription of these enzymes is increased by nuclear translocation of carbohydrate-responsive element bind protein (ChREBP) and sterol-regulatory element binding protein 1c (SREBP1c), which is stimulated by glycolytic by-products and

insulin, respectively, and inhibited by fatty acids. Transcription of the genes encoding SREBP1c (*SREBF1*) and ChREBP (*MLXIPL*) is stimulated by insulin via liver X receptor (LXR) and inhibited by fatty acids. A 'pool' of fatty acyl-CoAs might either enter the mitochondrion for oxidation via carnitine palmitoyl transferase 1 (CPT1), or enter the cytosolic esterification pathway for glycerolipid synthesis, the final step of which is TAG synthesis by diacylglycerol acyltransferase (DGAT) enzymes. This primarily occurs at the endoplasmic reticulum (ER; pictured), but might also occur on lipid droplets, at the mitochondrial membrane and at the nuclear envelope. Malonyl-CoA, an intermediate in DNL, inhibits CPT1 action and downregulates fatty acid oxidation. At the ER, TAG might be partitioned towards an apoB-associated lipid droplet, which requires microsomal triglyceride transfer protein (MTP), for maturation and secretion as a VLDL particle via the Golgi apparatus, or form a budding lipid droplet for storage in the cytosol; transmembrane 6 superfamily 2 (TM6SF2) has a role in determining the partitioning of TAG between these pools. Once within the cytosol, TAG might undergo lipolysis and enter back into the fatty acid pool by the sequential actions of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoglyceride lipase (MGL), or by lipophagy.

Table 1. Overview of inhibitors used to lower hepatic *de novo* lipogenesis

Model	Target	Compound and dose	Summary of findings
HepG2 [180]	ACC	Soraphen A (100nM) for 6 h	↓↓ intracellular malonyl-CoA, newly synthesised 16:0 and elongation of fatty acids
Rodent [181]	ACC	ND-630 1. Single dose of 0, 0.3, 3 or 30 mg/kg 2. DIO for 4 weeks followed by treatment with 0, 0.3, 3 or 30 mg/kg for 28 d	1. Dose dependent ↓↓ in malonyl-CoA (nmol/g liver), DNL and RQ. **[Au: Please define RQ.]** 2. ↓↓ body weight in highest dose and ↓↓ IHTAG (3 and 30mg/kg doses).
Rodent [182]	ACC	ND-645 Single dose of 0.3, 3 or 30 mg/kg	↓↓ Dose-dependent intrahepatic malonyl-CoA (nmol/g tissue)
Rodent [164]	ACC	MK-4074 Single dose: 3-30 mg/kg Daily dose: 10 or 30 mg/kg for 4 weeks	Single dose: dose-dependent ↓↓ intrahepatic DNL and ↑↑ plasma ketones over 12 h Daily: ↓↓ with 10 or 30 mg/kg for 4 weeks in IHTAG (mg/g tissue)
Human [164]	ACC	MK-4074 1. Healthy: 1x140 mg or 2x70mg daily for 7 days 2. Healthy: 200mg single dose 3. Patients with NAFLD: 2x200mg per day for 4 weeks	1. ↑↑ fructose-stimulated DNL (%) 2. ↑↑ fasted and fed concentrations of ACAC and B-OHB (μM) **[Au: Please define ACAC and B-OHB.]** 3. ↓↓ IHTAG and ↑↑ increase in plasma levels of TAG
Human [183]	ACC	Patients with clinical diagnosis of NAFLD: 1. GS-0976 20mg per day for 2 weeks 2. GS-0976 5mg per day for 12 weeks 3. placebo for 12 weeks	1. ↓↓ IHTAG, ↑↑ plasma concentrations of TAG 2. ↓ IHTAG, ↑ plasma concentrations of TAG 3. ↓ IHTAG, ↓ plasma concentrations of TAG
Human [184]	ACC	NDI-010976: cross-over study in patients with overweight or obesity 1. 20 mg single dose 2. 50 mg single dose 3. 200 mg single dose	In 1-3. ↓↓ fructose-stimulated DNL (%) appeared to be dose-dependent with increasing dose of NDI-010976
Murine primary hepatocytes [178]	DGAT2	Example 109B: 5μM for 4 h	↓↓ mean area in individual lipid droplets, abundance of Acaca, Fasn, Scd1 and Srebf1c, and DNL-derived fatty acids and secreted TAG
Rodents and non-human primates [185]	DGAT2	Compound 2 and compound 16 1. Acute (rodent) 30 mg/kg 2. Chronic (rodent) compound 2 only (100mg/kg per day) for 19 days 3.Acute (non-human primate) (4.5mg/kg/h infused for 4 h)	1. ↓↓ newly synthesised TAG and VLDL–TAG 2. ↓↓ newly synthesised TAG and liver-TAG 3. ↓↓ production rate of TAG

↓↓, significant (P<0.05) decrease, ↑↑, significant (P<0.05) increase, ↓, non-significant decrease.

Abbreviations: ACAC, acetoacetate; B-OHB, beta-hydroxybutyrate; DIO, diet induced obesity; DNL, *de novo* lipogenesis; FA, fatty acids; TAG, triacylglycerol; RQ, respiratory quotient; Acaca, acetyl-

CoA carboxylase; Fasn, fatty acid synthase; Scd1, stearoyl Co-A desaturase 1; Srebf1c, sterol-regulatory element binding protein 1c.