The role of hepatic lipid composition in obesity-related metabolic disease

Running title: Hepatic lipid composition and obesity

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Abbreviations: NASH, non-alcoholic steatohepatitis; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; MR, magnetic resonance; ^{1}H-MRS, proton magnetic resonance spectroscopy; DNL, *de novo* lipogenesis; DGAT, diacylglycerol transferase; DAG, diacylglycerol; LPC, lysophosphatidylcholine; ER, endoplasmic reticulum; PC, phosphatidylcholine; PKCε, protein kinase Cε; SCD1, stearoyl-CoA desaturase-1; EPA, eicosapentaenoic acid; DHA, docohexaenoic acid; AA, arachidonic acid; FADS, fatty acid desaturase; ELOVL, fatty acid elongase; NAFL, non-alcoholic fatty liver; HOMA-IR, homeostatic model assessment of insulin resistance; SI, saturation index; UI, unsaturation index; PCSK9, proprotein convertase subtilisin/kexin type 9; PPARα, peroxisome proliferator-activated receptor alpha; PUI, polyunsaturation index.

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JAK and SAW developed initial idea for this review, which was further refined with DJS, GPA, JAS, SB, and SM. SAW and JAK led the writing of this review with support from all other authors. SM and SAW drew the figures. All authors approved the final version of this manuscript and hold accountability for all aspects of the work.

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Abstract

Obesity is a primary antecedent to non-alcoholic fatty liver disease whose cardinal feature is excessive hepatic lipid accumulation. Although total hepatic lipid content closely associates with hepatic and systemic metabolic dysfunction, accumulating evidence suggests that the composition of hepatic lipids may be more discriminatory. This review summarises cross-sectional human studies using liver biopsy/lipidomics and proton magnetic resonance spectroscopy to characterise hepatic lipid composition in people with obesity and related metabolic disease. A comprehensive literature search identified 26 relevant studies published up to 31st March 2021 which were included in the review. The available evidence provides a consistent picture showing that people with hepatic steatosis possess elevated saturated and/or monounsaturated hepatic lipids and a reduced proportion of polyunsaturated hepatic lipids. This altered hepatic lipid profile associates more directly with metabolic derangements, such as insulin resistance, and may be exacerbated in non-alcoholic steatohepatitis. Further evidence from lipidomic studies suggests that these deleterious changes may be related to defects in lipid desaturation and elongation, and an augmentation of the *de novo* lipogenic pathway. These observations are consistent with mechanistic studies implicating saturated fatty acids and associated bioactive lipid intermediates (ceramides, lysophosphatidylcholines and diacylglycerol) in the development of hepatic lipotoxicity and wider metabolic dysfunction, whilst monounsaturated fatty acids and polyunsaturated fatty acids may exhibit a protective role. Future studies are needed to prospectively determine the relevance of hepatic lipid composition for hepatic and non-hepatic morbidity and mortality; and to further evaluate the impact of therapeutic interventions such as pharmacotherapy and lifestyle interventions.

Word count: 248

Key words:

Liver, fatty acid, quality, non-alcoholic fatty liver disease, ectopic fat, insulin resistance

Key points:

- Although total hepatic lipid content is often associated with obesity-related metabolic ill health, the composition of hepatic lipids may be more prognostic.
- Obesity and hepatic steatosis are associated with a higher proportion of saturated and/or monounsaturated hepatic lipids, a lower proportion of polyunsaturated hepatic lipids and an elevated *n*-6/*n*-3 polyunsaturated fatty acid ratio.
- This hepatic lipid profile may be further exacerbated in non-alcoholic steatohepatitis; however, additional larger scale studies are required.
- Future clinical studies should focus on the quality in addition to the quantity of hepatic lipids, which could represent a novel target in the management of obesity-related metabolic disease.

Introduction

The obesity pandemic has been paralleled by a rise in the prevalence of related metabolic conditions such as non-alcoholic fatty liver disease (NAFLD)[1], type 2 diabetes mellitus (T2DM)[2] and the metabolic syndrome[3]. An integral component of this obesity-related metabolic dysfunction is lipid accumulation in ectopic tissues such as the liver (hepatic steatosis), which is the defining feature of NAFLD[4]. Indeed, obesity is a primary antecedent to hepatic steatosis, as once the finite adipose tissue lipid stores are overwhelmed, the resultant adipose tissue dysfunction promotes the redistribution of lipids towards the liver for disposal[5]. Consequently, hepatic steatosis is present in ~50-75% of individuals with obesity (BMI≥30 $kg \cdot m^{-2}$)[1,6] and up to 94% of individuals with severe obesity (BMI≥40 $kg \cdot m^{-2}$)[7]. This has important consequences for cardiometabolic and liver-related morbidity and mortality[1,8,9].

The liver is an important regulator of glucose and lipid metabolism, and hepatic steatosis is linked with multiple cardiometabolic comorbidities such as insulin resistance, hyperglycaemia, dyslipidaemia and hypertension[8,10,11]. Consequently, NAFLD is often regarded as the hepatic manifestation of the metabolic syndrome[12], which augments the risk of developing T2DM and cardiovascular disease[13,14]. Additionally, the coexistence of T2DM in NAFLD is associated with an accelerated progression to non-alcoholic steatohepatitis (NASH)[15]; a more advanced form of chronic liver disease characterised by hepatocellular inflammation and injury[4]. A subset of people with NASH develop hepatic fibrosis which is a major precursor to end-stage liver diseases i.e. cirrhosis and hepatocellular carcinoma[9]. Importantly, the link between hepatic steatosis, metabolic dysfunction and liver disease progression displays significant heterogeneity; and the causal mechanisms are not completely understood.

Despite this deleterious sentiment, growing evidence suggests that hepatic lipid accumulation as triacylglycerol (TAG) may not be inherently harmful[16,17]. This notion stems from preclinical

research demonstrating that promoting hepatic TAG synthesis is protective from lipotoxicity and insulin resistance induced by the accumulation of other lipids and/or intermediates[18–21]. Instead, the composition of hepatic lipids may be a dominant factor mediating the adverse metabolic and lipotoxic consequences of hepatic steatosis[16,22]. Specifically, saturated fatty acids (SFAs) and their incorporation into other lipid species, may be more harmful compared with monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs)[22,23].

Advancements in lipidomic and magnetic resonance (MR) techniques have enabled researchers to identify marked alterations in hepatic lipid composition in individuals with obesity and obesity-related diseases. Accumulating evidence suggests that a more deleterious hepatic lipid composition profile may underpin the poorer metabolic health and more aggressive liver disease trajectory observed as NAFLD and its associated ailments progress[22]. Therefore, elucidating the role of hepatic lipid composition in obesity-related metabolic disease is of paramount importance to support therapeutic intervention and advance understanding of disease prognosis.

The purpose of this narrative review is to comprehensively evaluate existing human studies characterising hepatic lipid composition in obesity-related metabolic disease. To provide context, mechanistic aspects of hepatic lipid accumulation, and contemporary assessment techniques in humans, are discussed in the first part of this review. The review concludes by summarising the potential impact of lipid-lowering pharmacotherapies on clinical outcomes in NAFLD and hepatic lipid composition.

Dysregulated lipid metabolism in hepatic steatosis

Hepatic steatosis is characterised by an imbalance between the supply, uptake, synthesis and disposal of lipids, such that lipid supply exceeds the capacity for disposal[24,25]. Chronic energy surplus in obesity and its associated metabolic comorbidities, such as insulin resistance, are

key contributors to this dysregulation of hepatic lipid metabolism; and thus play an important role in the pathogenesis of hepatic steatosis[26].

The largest supply of hepatic lipids (~60% in NAFLD) is from circulating FFAs derived from adipose tissue lipolysis[27]. Crucially, adipose tissue dysfunction in obesity, characterised by enlarged/stressed adipocytes, chronic low-grade inflammation and insulin resistance, results in unrestricted adipose tissue lipolysis and augmented delivery of circulating FFAs to the liver[24]. Many studies report elevated rates of lipolysis in obesity and NAFLD[28–30]. The other main extra-hepatic source of hepatic lipids is from dietary fat (~15% in NAFLD)[25,27]. Notably, NAFLD is frequently associated with a Western dietary pattern in which excess dietary fat (particularly saturated and trans-fat) is prominent[31,32].

Hepatic lipids are also synthesized endogenously from non-lipid precursors such as glucose and fructose via *de novo* lipogenesis (DNL)[24]. This process involves the conversion of acetyl-CoA, to palmitoyl-CoA, the coenzyme A derivative of the SFA palmitate[33]. Compared to lean individuals, the contribution of DNL is elevated in people with obesity (19% vs. 11%), and further increased in those with coexisting hepatic steatosis (38%)[34]. These higher rates of DNL arise from both the increased intake of dietary glucose and fructose[35], and from hyperglycaemia and hyperinsulinaemia[36]. Collectively, elevations in each of these lipid sources contribute to dysregulated lipid metabolism in NAFLD.

The two lipid disposal routes in the liver are fatty acid oxidation, through β-oxidation and ketogenesis, and export into the circulation as very-low-density lipoprotein-TAG[26]. Data are conflicting about how these may be altered in hepatic steatosis, with some studies reporting increases, potentially as a buffering mechanism[28,29,37]. However, malonyl-CoA, an intermediate metabolite of DNL, can inhibit fatty acid oxidation, whilst reactive oxygen species produced from β-oxidation may also promote mitochondrial dysfunction[38]. Furthermore, rates

of very-low-density lipoprotein-TAG secretion may plateau[28] or even decrease[39] as liver fat content increases. Therefore, although no consensus exists, these lipid disposal pathways appear to be overwhelmed in people with obesity and related metabolic dysfunction[24].

Hepatic lipid composition as a mediator of lipotoxicity and metabolic dysfunction

The hepatic lipidome comprises of six main categories of lipids including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids[40,41]. The glycerolipid TAG represents the predominant form of lipid storage in the liver[42,43]. Hepatic FFAs partitioned into esterification pathways are converted to TAG via the acyltransferases glycerol 3-phosphate acyltransferase and diacylglycerol transferase (DGAT), before being compartmentalised into lipid droplets[17].

Despite hepatic TAG being used as a clinical marker for NAFLD[4], and demonstrating close associations with metabolic impairments such as insulin resistance[10,11], accumulating evidence suggests lipid deposition as TAG may not be inherently harmful[16,22,23]. Indeed, various instances exist whereby hepatic TAG dissociates from insulin resistance and lipotoxicity. For example, early rodent studies found that overexpressing DGAT2, which catalyses the conversion of diacylglycerol (DAG) to TAG, promotes hepatic steatosis without affecting insulin resistance or hepatic inflammation[19]. Conversely, inhibiting hepatic DGAT2 reduces hepatic TAG synthesis but exacerbates oxidative stress, inflammation and fibrosis in mice with obesity and NASH[20]. Therefore, hepatic TAG stored in lipid droplets may be relatively inert and could actually represent a protective mechanism to combat the accumulation of more harmful lipid species[16,17]. Nevertheless, excessive hepatic lipid flux leads to the accumulation of SFAs and other associated bioactive lipid intermediates such as ceramides, lysophosphatidylcholines (LPCs) and DAG[22]. Importantly, these lipid species have been directly implicated in the development of hepatic lipotoxicity and/or insulin resistance[44–47].

178 Therefore, hepatic lipid composition, rather than absolute quantity, may underpin the hepatic
179 and systemic metabolic dysfunction associated with hepatic steatosis.

180 *Saturated fatty acids (SFAs)*

181 Palmitate (C16:0) and stearate (C18:0) are the two most abundant hepatic SFAs; characterised
182 by the absence of double bonds within their hydrocarbon chains[42,43]. SFAs, particularly
183 palmitate, are suggested to be more metabolically harmful than MUFAs and PUFAs, as high
184 saturated fat diets in people with overweight or obesity lead to greater increases in hepatic TAG
185 content and insulin resistance when compared to energy-matched unsaturated and
186 polyunsaturated fat diets[48,49]. Notably, exposure of cultured hepatocytes to palmitate induces
187 cellular apoptosis but does not stimulate TAG synthesis[18]. This suggests SFAs may be less
188 preferentially directed towards TAG synthesis in favour of more lipotoxic fates. Indeed,
189 multiple studies report inverse associations between SFA-induced lipotoxicity and TAG
190 synthesis[21,50,51].

191 The mechanisms of SFA-induced lipotoxicity and insulin resistance in hepatocytes are depicted
192 in Figure 1. Preclinical research demonstrates that SFAs promote both endoplasmic reticulum
193 (ER) stress[52] and oxidative stress[46] in cultured hepatocytes. Notably, an important contributor
194 to ER stress is the incorporation of SFAs into phospholipid species which are integral structural
195 components of the ER membrane[53]. Subsequently, these factors initiate the c-Jun N-terminal
196 kinase pathway which promotes apoptosis and disrupts insulin signal transduction[54]. In non-
197 parenchymal cells, SFAs activate hepatic stellate cells and Kupffer cells leading to the initiation
198 of pro-inflammatory and pro-fibrogenic responses[55,56].

199 These deleterious effects may be partly mediated by their preferential conversion to bioactive
200 lipid intermediates such as ceramides, LPCs and DAG[22]. Ceramides are sphingolipids which
201 can promote inflammation, mitochondrial dysfunction, insulin resistance and apoptosis[57,58].

Notably, palmitate is the preferred substrate for *de novo* ceramide synthesis[47], and inhibiting this process alleviates hepatic inflammation, fibrosis and insulin resistance in murine models of obesity and NAFLD[59,60]. In humans, plasma and hepatic concentrations of ceramides are elevated in patients with NAFLD and NASH, and are positively associated with markers of insulin resistance[61,62]. Furthermore, in the aforementioned overfeeding study by Luukkonen and colleagues[48], high saturated fat overfeeding also increased plasma ceramide concentrations and other intermediates of *de novo* ceramide synthesis. Consequently, ceramides are recognised as important lipids in both the pathogenesis of NAFLD and NASH, and the associated metabolic dysfunction.

LPCs are glycerophospholipids derived from phosphatidylcholines (PCs) via the enzyme phospholipase A2 and share similar lipotoxic effects to SFAs including ER stress, mitochondrial dysfunction, inflammation and apoptosis[45,63]. These lipids may be a downstream mediator of SFA-induced lipotoxicity as pharmacological inhibition of phospholipase A2 in isolated hepatocytes impairs the conversion of palmitate to LPC, leading to a reduction in palmitate-induced apoptosis[22,45]. DAG are formed during the penultimate step of TAG synthesis and have been strongly implicated in the development of hepatic insulin resistance through activation of protein kinase Cε (PKCε)[44,64]. Indeed, palmitate treatment in HepG2 cells resulted in the accumulation of DAG rather than TAG which was associated with greater insulin resistance and PKCε activation[65]. Therefore, SFAs and their associated bioactive lipid intermediates are important contributors to hepatic lipotoxicity and insulin resistance resulting from excessive lipid flux in the liver.

Insert Figure 1

Monounsaturated fatty acids (MUFAs)

MUFAs such as palmitoleate (C16:1) and oleate (C18:1) contain a single double bond and can be converted from palmitate and stearate, respectively, by the rate-limiting enzyme stearoyl-CoA desaturase-1 (SCD1)[66]. Compared to SFAs, MUFAs are less lipotoxic and appear to be preferentially incorporated into TAG species[22]. Indeed, oleate supplementation in cultured hepatocytes results in marked TAG accumulation with minimal impact on apoptosis, whilst co-supplementing oleate with palmitate sufficiently prevents palmitate-induced lipotoxicity[18]. Furthermore, SCD1 knockout in mice with diet-induced NASH reduces hepatic steatosis but exacerbates hepatic inflammation and injury compared to mice with intact SCD1 activity[50]. Together, these data suggest that the conversion of SFAs to MUFAs and their preferential incorporation into hepatic TAG may be a key mechanism by which hepatic TAG accumulation protects the liver from SFA-induced lipotoxicity.

Polyunsaturated fatty acids (PUFAs)

PUFAs contain multiple double bonds in their hydrocarbon chain and form two classes based on the position of the first double bond in relation to the methyl end of the fatty acid chain: *n*-3 and *n*-6 PUFAs[22,42]. Eicosapentaenoic acid (C20:5*n*-3; EPA) and docohexaenoic acid (C22:6*n*-3; DHA) are important long-chain *n*-3 PUFAs, whilst arachidonic acid (C20:4*n*-6; AA) is an important long-chain *n*-6 PUFA[67]. These PUFAs are formed from a series of desaturation and elongation steps via several fatty acid desaturase (FADS) and fatty acid elongase (ELOVL) enzymes, respectively[68]. Notably, only a minor portion (5-10%) are derived from 18-carbon precursors such as linoleic acid (C18:2*n*-6) and α-linolenic acid (C18:3*n*-3); thus, intake from the diet represents an essential source[68].

PUFAs, particularly of the *n*-3 series, have been shown to exert protective metabolic effects as *n*-3 PUFA supplementation reduces hepatic steatosis, improve markers of liver injury and

enhance insulin sensitivity in humans and rodents with NAFLD[69,70]. Mechanistically, PUFAs interact with transcription factors to upregulate oxidative pathways and downregulate pathways relating to lipogenesis, inflammation and fibrogenesis[71,72]. Additionally, they are readily incorporated into phospholipid species to maintain cell membrane fluidity and permeabilization[73]. Therefore, PUFAs appear to play an active role in maintaining hepatic lipid homeostasis and alleviating hepatic lipotoxicity and insulin resistance resulting from excessive lipid accumulation.

The aforementioned long-chain *n*-3 and *n*-6 PUFAs are also synthesised into specialised pro-resolving mediators and eicosanoids, respectively[22]. Specialised pro-resolving mediators are a class of signalling molecules which exhibit profound anti-inflammatory and anti-fibrogenic properties[74,75]. In contrast, eicosanoids such as prostaglandins, thromboxane and leukotrienes are signalling molecules which are primarily considered to play a proinflammatory role in the liver[76]. Consequently, given that *n*-6 PUFAs are the major precursors for proinflammatory eicosanoids, an increased flux through the *n*-6 PUFA pathway and a concomitant increase in the *n*-6/*n*-3 PUFA ratio may also contribute to the pathogenesis of NASH and its associated metabolic dysfunction[22,43,77].

Assessment of hepatic lipid composition

Liver biopsy/lipidomics

Liver biopsy is the gold-standard technique for the clinical diagnosis of hepatic steatosis and is currently the only technique which can reliably detect other features of NASH such as hepatocyte ballooning, lobular inflammation and fibrosis (although non-invasive markers are increasingly used to distinguish advanced stages of fibrosis)[4,78]. This permits the distinction between stages of chronic liver disease. Lipidomic analysis techniques such as chromatography and mass spectrometry can be paired with liver biopsies to quantify the relative amounts of

different lipid species in liver tissue samples and assess their fatty acid compositions[79]. Gas chromatography and liquid chromatography (particularly high-performance liquid chromatography) have traditionally been used to assess the composition of multiple hepatic lipid species, including TAG, DAG, FFAs and phospholipids, in populations with obesity and obesity-related metabolic disease[80–83]. A major recent advancement is the coupling of these techniques with mass spectrometry, enabling a more detailed characterisation of the hepatic lipidome[84,85]. Recent studies using this approach have examined differences in the composition of sphingolipids, PCs, LPCs, cholesterol esters, and free cholesterol, in addition to the other lipid species mentioned previously[61,62,77,86]. A key limitation, however, is that its invasiveness has often limited its application to those with severe metabolic phenotypes[80,81,86,87]. The heterogenous nature of hepatocytes is another consideration with small biopsy samples[88].

Proton magnetic resonance spectroscopy (^{1}H-MRS)

Recent advances in precision imaging have enabled scientists to non-invasively monitor important metabolic pathways and outcomes in obesity-related metabolic disease[89]. Specifically, advanced MR techniques, such as ^{1}H-MRS and chemical-shift-encoded MR imaging, now permit the non-invasive assessment of hepatic lipid composition *in vivo*[90]. ^{1}H-MRS has emerged as the gold-standard non-invasive technique for assessing liver fat content and strongly correlates with histologically-derived measurements (r=0.93)[91]. The theoretical basis for ^{1}H-MRS is underpinned by the 'chemical shift' effect whereby, when placed in a strong magnetic field, hydrogen protons within water molecules and hydrocarbon fatty acid chains resonate at different frequencies based on their surrounding chemical milieu (Figure 2A)[90]. By irradiating these molecules with a radio frequency field, protons are excited and subsequently emit a signal at specific frequencies. Consequently, in a typical liver MR spectrum, water forms a large spectral peak, whilst six smaller lipid peaks are formed owing to different functional groups within a fatty acid chain (Figure 2B)[92]. Liver fat fraction is then

299 expressed as the total fat signal as a percentage of the combined water and fat signal, with
300 ≥5.56% being considered clinically elevated[93].

301

302 ***Insert Figure 2***

303

304 Of the six visible lipid peaks, two (methene and allylic) relate to functional groups associated
305 with double bonds in a fatty acid chain i.e. unsaturated fatty acids, whilst one (diallylic) relates
306 to a functional group exclusively associated with consecutive double bonds i.e. PUFAs[94]. With
307 this knowledge, multiple research groups have developed indices to represent the ratio of
308 saturated, (mono)unsaturated and polyunsaturated lipids within the liver[92,94,95]. Due to the
309 ethical considerations of liver biopsies, the ^{1}H-MRS technique has currently only been
310 validated against gas chromatography in human adipose tissue samples. Nevertheless, these
311 studies report strong correlations between techniques[95,96], supporting ^{1}H-MRS as a non-
312 invasive alternative to liver biopsy. Given the potential for repeat measurements, the technique
313 is ideally suited for use in therapeutic trials[89].

314 Unlike lipidomic approaches, ^{1}H-MRS only provides a semi-quantitative assessment of hepatic
315 lipid saturation measured through ratios of fat groups, rather than a quantitative assessment of
316 the relative abundances of different lipid species and their fatty acid constituents[90].
317 Additionally, ^{1}H-MRS is currently unsuitable for determining hepatic lipid composition in
318 individuals with low liver fat fractions owing to insufficient fat signal and therefore spectral
319 resolution at clinical field strengths[97]. Other limitations include high costs, low spatial coverage
320 from single voxel spectroscopy, and the specialist expertise required.

321 **Hepatic lipid composition in obesity-related metabolic disease**

Liver biopsy/lipidomic studies

We identified 18 cross-sectional studies using liver biopsy and lipidomic analyses to examine the hepatic lipid composition of people with obesity and related metabolic disease (Table 1).

Insert Table 1

Takahashi and Tanaka[98] first noted that posthumous liver samples of individuals with hepatic steatosis comprised of a greater percentage of MUFAs and a lower percentage of PUFAs in multiple lipid fractions compared to individuals without hepatic steatosis. More recently, Araya et al.[80] observed a relative depletion of *n*-3 and *n*-6 long-chain PUFAs and an increase in the *n*-6/*n*-3 PUFA ratio in the livers of patients with NAFLD compared to healthy controls; however, no differences were observed between NAFLD stages i.e. non-alcoholic fatty liver (NAFL; elevated steatosis without hepatic inflammation) and NASH. Similar findings have been reported by others[83,99–101]; however, these studies observed greater long-chain PUFA depletion in individuals with NASH compared to NAFL. Notably, the NAFLD populations recruited by Araya et al.[80] were undergoing bariatric surgery and the extreme metabolic phenotype may have negated potential differences. Nevertheless, these studies indicate that a depletion of hepatic long-chain PUFAs, particularly of the *n*-3 series, and a concomitant increase in the *n*-6/*n*-3 PUFA ratio may contribute to the pathogenesis and progression of NAFLD. This may be through favouring lipid synthesis over lipid export and oxidation[71], and promoting a proinflammatory state[76].

Hepatic long-chain PUFA depletion may be related to deficiencies in dietary intake, desaturase activity and/or greater lipid peroxidation[80,83,99,100]. Importantly, no differences in dietary intake

were reported in any of these studies; however, the product/precursor ratios for *n*-6 (AA/C18:2*n*-6) and *n*-3 PUFAs (EPA+DHA/C18:3*n*-3), which indirectly reflect desaturase enzyme activity (FADS1 and FADS2, respectively), were decreased in NAFLD[80,100] and NASH[99]. Thus, defective desaturation and synthesis from their precursors may contribute to hepatic long-chain PUFA depletion. PUFAs are also highly susceptible to lipid peroxidation induced by oxidative stress and inflammation[68]; however, data are inconsistent regarding the contribution of lipid peroxidation to hepatic PUFA depletion in humans.

Puri et al.[43] comprehensively characterised the human liver lipidome in patients with NAFL and NASH, and compared their compositions to controls with obesity. Alongside a stepwise depletion of long-chain PUFAs in hepatic TAG, DAG and FFA fractions, it was found that SFAs and MUFAs were augmented in hepatic TAG and DAG in the NAFL and NASH groups, despite similar BMIs and metabolic profiles to controls[43]. In agreement, Peng et al.[86] recently observed greater SFAs and MUFAs in hepatic TAG and DAG, respectively, in NAFL and NASH along with lower long-chain PUFAs. Therefore, this enrichment of hepatic TAG and DAG with SFAs and MUFAs represents a more lipotoxic hepatic lipid profile in individuals with NAFLD. Interestingly, the greater MUFA concentrations in these studies were primarily driven by an increase in oleate (C18:1), whilst reductions were seen in the SFA stearate (C18:0), suggesting that an accelerated conversion of SFAs to MUFAs may occur to limit SFA-induced lipotoxicity[22].

The fatty acid composition of phospholipids was also altered such that patients with NAFL and NASH exhibited progressively lower long-chain PUFAs in hepatic PCs[43,86]. Furthermore, total hepatic PCs were lower and hepatic LPCs were higher in these populations[43]. Elizondo et al.[81] specifically compared the fatty acid composition of phospholipids in the liver and erythrocytes of lean individuals and people with severe obesity/NAFLD. The NAFLD group, who had markedly higher insulin resistance and hyperglycaemia, displayed 34% higher total SFAs and

63% lower total *n*-3 long-chain PUFAs in hepatic phospholipids, resulting in a 2.4-fold greater *n*-6/*n*-3 PUFA ratio[81]. Given that phospholipids are integral to membrane bilayers, a reduction in total hepatic phospholipids and enrichment with SFAs may disrupt membrane integrity of structures such as the ER and mitochondria, thereby promoting ER stress and mitochondrial dysfunction[53,102]. In support, Peng et al.[86] noted elevated concentrations of the mitochondrial lipids cardiolipin, ubiquinone and acylcarnitine in NASH, suggestive of mitochondrial dysfunction.

To investigate the relationship between hepatic lipid composition and metabolic dysfunction, Luukkonen et al.[61] recruited 125 bariatric surgery patients and divided them into two groups based on their median homeostatic model assessment of insulin resistance (HOMA-IR). In addition to 2-fold higher liver fat content, the high HOMA-IR group exhibited a greater proportion of SFAs and MUFAs in hepatic TAG and FFA fractions compared to the low HOMA-IR group[61]. These associations between insulin resistance, augmented hepatic SFAs and MUFAs, and depleted PUFAs have also been replicated recently in patients with less severe metabolic dysfunction and obesity[103]. Whilst observational, these findings concur with preclinical evidence implicating SFAs in the development of insulin resistance[104].

In a further analysis, Luukkonen et al.[61] investigated the apparent dissociation between insulin resistance and hepatic steatosis by additionally dividing their cohort based on *PNPLA3* genotype (rs738409), given that carriers of the I148M variant display hepatic steatosis without metabolic dysfunction[105]. As expected, these individuals exhibited a 3-fold greater liver fat content compared to non-carriers, whilst metabolic parameters were similar between groups[61]. Interestingly, the greater hepatic lipid accumulation in I148M variant carriers was driven by elevated PUFAs in hepatic TAG[61]. These findings are corroborated by Peter et al.[82] who reported 44% higher concentrations of the *n*-3 PUFA α-linolenic acid and reductions in multiple *n*-6 PUFAs in hepatic TAG in I148M variant carriers vs. non-carriers. *PNPLA3*

functions as a transacylase, transferring PUFAs from hepatic TAG and DAG to phospholipid species[106]; however, recent *in vivo* and *in vitro* experiments demonstrate that the I148M variant promotes the retention of PUFAs in hepatic TAG[107]. Collectively, these studies demonstrate that 'metabolic NAFLD' associated with insulin resistance is characterised by an enrichment of SFAs in hepatic TAG; whilst PUFA enrichment in 'genetic NAFLD' may underpin a more favourable metabolic profile.

Alterations in multiple ceramide species and their derivatives have also been observed in obesity and NAFLD[61,62,86]. Specifically, Luukkonen et al.[61] reported higher hepatic concentrations of almost all ceramide species in metabolic NAFLD but minimal differences in genetic NAFLD; whilst Apostolopoulou et al.[62] observed elevated total hepatic ceramides in patients with NASH compared to NAFL and controls with obesity. These differences were primarily accompanied by higher dihydroceramide species, indicating an upregulation of the *de novo* ceramide synthetic pathway in which palmitate is a key substrate[61,62]. However, the potential contribution of DAG to insulin resistance could not be discounted in the study by Luukkonen et al.[61] as multiple DAG species were also elevated. In support, Kumashiro et al.[64] found that hepatic DAG species containing C16:0 and C18:1 were the most abundant in individuals with severe obesity, whilst total DAG content in lipid droplets strongly correlated with greater insulin resistance (r=0.80) and hepatic PKCε activation (r=0.67). Therefore, these studies support a role for both ceramides and DAG in the development of insulin resistance in people with metabolic-associated NAFLD.

Mechanistically, multiple studies have identified marked changes in the expression of numerous genes involved in hepatic lipid metabolism which could underpin the altered hepatic lipid composition in obesity-related metabolic disease[77,83,108–110]. Indeed, these studies consistently show an upregulation of genes related to lipogenesis (e.g. sterol regulatory element-binding protein 1, acetyl-CoA carboxylase, fatty acid synthase) and a downregulation

of genes related to lipid oxidation (e.g. peroxisome proliferator-activated receptor-α; PPAR α)[77,108,110], which is consistent with higher rates of DNL in obesity and NAFLD[34,111]. Hepatic DNL may be a key driver of the more saturated hepatic lipid profile in obesity-related metabolic disease[61] given that DNL exclusively produces palmitate and is directly stimulated by hyperglycaemia and hyperinsulinaemia[33,34,36]. Additionally, the expression of hepatic SCD1, FADS2 and ELOVL5 were also elevated in NASH[77,109,110], consistent with an enhanced conversion of SFAs to MUFAs in these individuals. Conversely, Chiappini et al.[77] reported lower hepatic FADS1 and ELOVL6 expression and activity in humans and rodents with NASH which the authors suggested was responsible for a bottleneck upstream of long-chain PUFA synthesis, leading to long-chain PUFA depletion and the accumulation of <20-carbon SFAs and MUFAs. Earlier studies, however, noted elevated FADS1[109] and ELOVL6[110] expression in patients with NASH; thus further research is required to clarify this discrepancy.

Proton magnetic resonance spectroscopy (^{1}H-MRS) studies

Eight cross-sectional studies were identified using ^{1}H-MRS to assess hepatic lipid composition in individuals with obesity-related metabolic disease (Table 2).

Insert Table 2

Pollesello et al.[112] first used ^{1}H-MRS to determine the composition of lipid extracts from liver biopsy samples of patients with vs. without hepatic steatosis by calculating the average fatty acid chain length and a lipid unsaturation ratio. Each outcome was lower in patients with hepatic steatosis, indicating the presence of shorter, more saturated fatty acids in hepatic lipids in NAFLD. However, the first *in vivo* assessment of hepatic lipid composition was performed

443 by Johnson et al.[94] who developed and validated indices to assess the degree of hepatic lipid
444 saturation. In this study, the hepatic lipid saturation index (SI), unsaturation index (UI) and
445 polyunsaturation index (PUI) were compared between people with obesity, NAFLD and
446 healthy controls. It was found that the hepatic SI was higher and the hepatic PUI was lower in
447 the groups with NAFLD and obesity compared to controls, whilst the hepatic PUI was depleted
448 to a greater extent in the NAFLD group[94].

449 Using the same indices, Erickson et al.[113] recently reported that patients with NAFLD exhibited
450 a higher hepatic SI and lower hepatic UI when compared to controls with overweight/obesity.
451 Notably, the greater hepatic lipid saturation in NAFLD was accompanied by greater
452 dyslipidaemia, lower exercise capacity and poorer peripheral insulin sensitivity[113]. The
453 relationship between hepatic steatosis and a more saturated hepatic lipid profile was further
454 highlighted by Hamilton et al.[97] who found inverse associations between liver proton density
455 fat fraction and the number of double bonds and methylene-interrupted double bonds in
456 patients with suspected or diagnosed NAFLD, reflecting unsaturated and polyunsaturated
457 hepatic lipids, respectively. Therefore, in line with the liver biopsy/lipidomic literature, these
458 findings demonstrate that NAFLD is characterised by a greater proportion of saturated and a
459 lower proportion of unsaturated/polyunsaturated hepatic lipids, and this lipid profile is
460 accompanied by greater metabolic dysfunction.

461 Roumans et al.[95] most recently introduced novel indices to specifically quantify hepatic SFA,
462 MUFA and PUFA fractions, and compared these fractions in individuals with NAFL, T2DM
463 and overweight/obesity. The hepatic SFA fraction was elevated in both the NAFL and T2DM
464 groups compared to controls with overweight/obesity; however, the hepatic MUFA and PUFA
465 fractions were similar between groups[95]. Furthermore, hepatic insulin sensitivity was inversely
466 associated with the hepatic SFA fraction (r=-0.55) and positively associated with the hepatic
467 MUFA fraction (r=0.39). Only one other study has examined hepatic lipid composition in

T2DM which reported greater total hepatic unsaturated fatty acids in NAFLD patients with vs. without T2DM[114]. Notably, however, these findings are not directly comparable as the unsaturation ratio used by van Werven et al.[114] was calculated by expressing the methene (unsaturated) resonance as a proportion of the total water rather than total lipid signal. Therefore, this elevated unsaturation ratio suggests greater absolute amounts of unsaturated hepatic lipids but does not necessarily represent differences in the relative proportion of lipid fractions, and could merely reflect greater total liver fat in the T2DM group (data not reported)[114].

Given the proposed role of hepatic DNL as a key contributor to the saturated hepatic lipid pool, Roumans et al.[95] determined rates of hepatic DNL in a sub-group of their study volunteers. Hepatic DNL was positively associated with the hepatic SFA fraction (r=0.52) and negatively associated with the hepatic MUFA fraction (r=-0.71)[95]. To further scrutinise this relationship, the authors recruited an additional group of participants with glycogen storage disease 1a[95], a condition characterised by a genetic deficiency in glucose-6-phosphatase activity, leading to elevated rates of hepatic DNL[115]. These individuals displayed a greater hepatic SFA fraction compared to the NAFL and control groups; whilst the hepatic MUFA fraction was lower compared to the control group[95]. These data provide further support that hepatic DNL may play a causal role in the accumulation of saturated hepatic lipids.

Only one ^{1}H-MRS study has compared hepatic lipid composition between patients with NAFL and NASH, and no differences in hepatic SI, UI or PUI were observed[116]. In contrast, a greater hepatic SFA fraction has been reported in NASH compared to NAFL when assessed using MR imaging as opposed to ^{1}H-MRS[117]. In addition to the differing assessment techniques, these discrepant findings may be related to the fact that Leporq et al.[117] restricted their analyses to participants with a liver fat fraction >15%; whilst Trausnigg et al.[116] included a large number of participants with more mild steatosis. Consequently, further studies using ^{1}H-MRS are

493 required to compare the hepatic lipid composition of patients with NAFL and NASH, and
494 confirm the differences reported in studies using a lipidomic approach.

495 Trausnigg et al.[116] also examined the influence of *PNPLA3* on hepatic lipid composition by
496 sub-dividing their participants into carriers and non-carriers of the I148M variant. In agreement
497 with lipidomic studies[61,82], homozygous carriers had a greater hepatic PUI and a lower HOMA-
498 IR compared to non-carriers despite all participants possessing hepatic steatosis[116]. From the
499 opposing perspective, Fellinger et al.[118] recently compared hepatic lipid composition between
500 people with acromegaly and healthy controls. Notably, acromegaly is an endocrine disorder
501 resulting from excessive growth hormone production and is characterised by insulin resistance
502 despite having reduced liver fat content[119]. Accordingly, Fellinger et al.[118] reported greater
503 insulin resistance and lower hepatic lipids in people with acromegaly compared to healthy
504 controls and this was accompanied by a lower proportion of unsaturated hepatic lipids.
505 Therefore, these studies provide further human evidence of the dissociation between hepatic
506 steatosis and insulin resistance, and support the notion that elevated saturated hepatic lipids,
507 rather than total hepatic lipids, may be a more important determinant of the metabolic
508 consequences of hepatic lipid accumulation.

509 **Lipid-lowering agents as a potential pharmacotherapy**

510 To date, no approved pharmacotherapies exist for the treatment of NAFLD/NASH; however,
511 multiple pharmacological approaches are currently under investigation. These include existing
512 antidiabetic medications with insulin-sensitising/glucose-lowering properties and experimental
513 agents currently in phase II and III clinical trials which target multiple aspects of hepatic lipid
514 metabolism; both have been reviewed recently[41]. Lipid-lowering agents are another category
515 of pharmaceuticals demonstrating potential efficacy in NAFLD treatment. These broadly act
516 through reducing circulating concentrations of various lipids and are currently used for the

treatment of dyslipidaemia and cardiovascular disease[12]. Given the potential of these lipid-lowering effects in NAFLD treatment, the following section will summarise the available evidence regarding their potential impact on hepatic lipid composition.

Statins are a class of lipid-lowering agents which inhibit cholesterol biosynthesis and may exert anti-inflammatory and antioxidative effects[120]. Multiple (albeit mainly uncontrolled) studies have demonstrated some efficacy for statins in improving liver biochemistry, steatosis grade and risk of cardiovascular events in patients with NAFLD[121–123]; however, their impact on histological endpoints is more equivocal[124–126]. Nevertheless, preclinical research suggests statins may also alter lipid composition in multiple cell lines including human liver cells[127]. *In vivo*, data are currently restricted to plasma fatty acid compositions (which may not reflect hepatic lipid composition); however, these studies found that statins increase the proportion of long-chain PUFAs and decrease the proportion of SFAs and MUFAs[128,129]. Furthermore, statin treatment also led to elevations in the *n*-6/*n*-3 PUFA ratio[130]; however, the physiological role this plays in their mode of action remains unclear. Additional studies are required to determine the impact of statins on hepatic lipid composition.

Ezetimibe is another cholesterol-lowering agent which acts through inhibiting the intestinal reabsorption of cholesterol[120]. No definitive consensus exists on the effectiveness of ezetimibe in NAFLD treatment as a meta-analysis of the available literature found that ezetimibe treatment was effective at improving liver biochemistry, steatosis severity and hepatocellular ballooning but had no effect on hepatic inflammation and fibrosis[131]. However, when the analyses were restricted to randomised controlled trials (n=2), only the positive effect on hepatocellular ballooning remained[131]. Only one previous study has examined the impact of ezetimibe on hepatic lipid composition, and it was found that six months of ezetimibe treatment increased multiple hepatic SFAs and MUFAs in patients with NAFLD[132]. Notably, these changes were accompanied by impairments to glycaemic control and insulin sensitivity which

could be related to the shift in hepatic lipid composition[132]. Thus, combination therapy with insulin-sensitising agents may be useful to combat this adverse off-target effect and has shown potentially greater benefit to liver-related outcomes[120].

Fibrates such as fenofibrate are PPARα agonists used clinically to lower circulating TAG and increase circulating high-density lipoprotein[12]. Whilst some studies show that fenofibrate lowers circulating liver enzymes and indirect markers of hepatic fibrosis, stiffness and inflammation[133,134], others have failed to report any histological benefits[133,135]. Conversely, pemafibrate is a novel selective PPARα modulator in phase III trials which produces more potent lipid-lowering effects and has recently been shown to improve markers of hepatic inflammation, function and fibrosis in patients with NAFLD[136,137]. Regarding hepatic lipid composition, fenofibrate treatment increases the SFA and MUFA content and decreases the PUFA content of hepatic lipids in C57BL/6J mice[138]. These changes were ascribed to the dual role of PPARα in promoting hepatic lipogenesis and esterification in addition to β-oxidation following fenofibrate administration[138]. Similar observations have been made in multiple plasma lipid fractions in patients with hypercholesterolaemia[139]; however, the effect of fibrates on hepatic lipid composition in humans remains to be elucidated.

Long-chain *n*-3 PUFAs, namely EPA and DHA, are used pharmacologically in the treatment of hypertriglyceridaemia[12]. Given the established anti-inflammatory properties of *n*-3 PUFAs and their modulatory role in hepatic lipid metabolism[71,72,74,75], research has focused on their potential in NAFLD/NASH treatment. Recent meta-analyses have found *n*-3 PUFA supplementation to be effective in reducing hepatic steatosis and liver enzyme concentrations concomitantly with improvements in circulating lipid profiles and insulin sensitivity[140,141]. Conversely, no effects of *n*-3 PUFA supplementation on histological features of NASH were reported however[140,141]. Interestingly, *n*-3 PUFA supplementation in a diet-induced murine model of NASH has been shown to increase hepatic *n*-3 PUFAs and decrease hepatic SFAs,

MUFAs and the *n*-6/*n*-3 PUFA ratio[142]. In humans, Stephenson et al.[143] examined the effect of three months of *n*-3 PUFA supplementation on ^{1}H-MRS-assessed hepatic lipid composition in patients with NAFLD but reported no changes in hepatic lipid composition. Notably, validated hepatic lipid composition indices were not used by the authors, thus further clarification is required to establish the influence of *n*-3 PUFA supplementation on hepatic lipid composition in NAFLD.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors are an emerging pharmacotherapy which reduce circulating low-density lipoproteins and cardiovascular risk by inhibiting the PCSK9-mediated lysosomal degradation of the low-density lipoprotein receptor[120]. Most[144–146], but not all[147], studies demonstrate positive associations between circulating concentrations and hepatic expression of PSCK9 and steatosis severity, whilst it was recently found that the PCSK9 R46L loss-of-function genetic variant was protective from hepatic steatosis and the histological features of NASH[146]. Experimentally, preliminary evidence has shown that two different PCSK9 inhibitors were successful in ameliorating hepatic steatosis and resolving NASH in 40 patients with heterozygous familial hyperlipidaemia[148]. No studies have examined the relationship between PCSK9 inhibition and hepatic lipid composition, although multiple studies report positive associations between PCSK9 and hepatic expression of DNL-related genes[144–146]. Therefore, PCSK9 inhibition may theoretically promote a less saturated hepatic lipid profile through a reduction in DNL; however, this hypothesis needs to be tested experimentally.

Conclusions

Hepatic lipid accumulation is a central feature of obesity-related metabolic dysfunction and is associated with a greater risk of developing T2DM, cardiovascular disease and advanced liver disease[13–15]. It is increasingly recognised, however, that the composition rather than the

591 quantity of hepatic lipids may be the primary factor impacting liver disease progression and
592 related metabolic consequences[16,22,23]. This paper reviewed studies using liver
593 biopsy/lipidomic approaches and ^{1}H-MRS to characterise the hepatic lipid composition in
594 people with obesity and related metabolic disease. The available data provide a consistent
595 picture demonstrating that people with hepatic steatosis exhibit an elevated proportion of
596 saturated and/or monounsaturated hepatic lipids and a reduced proportion of polyunsaturated
597 hepatic lipids (Figure 3). This more lipotoxic hepatic lipid profile is associated with metabolic
598 derangements such as insulin resistance and may be further exacerbated in NASH. Data
599 published very recently by Ooi et al.[149] challenge this notion as the authors failed to observe
600 differences in the composition of multiple liver lipid species in patients with NASH compared
601 to NAFL. Definitive conclusions remain elusive, however, owing to a far smaller sample size
602 in their NASH group; therefore, additional larger scale studies are required. Nevertheless, the
603 observations from these studies are consistent with mechanistic studies implicating SFAs in
604 the development of hepatic lipotoxicity and wider metabolic dysfunction[18,23,46,52–56,104], whilst
605 MUFAs and PUFAs may exhibit a protective role[18,50,68–72]. However, it must be appreciated
606 that the studies included in this review are cross-sectional, limiting judgements about causal
607 inference.

608

609 ***Insert Figure 3***

610

611 Studies employing liver biopsy/lipidomic approaches have provided the most detailed
612 characterisation of hepatic lipid composition and related metabolic pathways within this
613 review. In NAFLD, these studies detail an enrichment of SFAs/MUFAs and a depletion of
614 long-chain PUFAs in hepatic TAG, DAG, FFA and phospholipid species, and an accumulation

615 of toxic lipid intermediates such as ceramides and LPCs. These differences are related to
616 dysregulated hepatic lipid metabolism, specifically defective lipid desaturation and elongation,
617 and an upregulation of hepatic DNL[77,83,108,110].

618 ^{1}H-MRS offers a non-invasive alternative to assess hepatic lipid composition, and whilst
619 currently only providing a semi-quantitative measurement, the technique demonstrates the
620 same general trends observed with liver biopsy/lipidomic techniques. These studies
621 demonstrate higher indices of hepatic lipid saturation and lower indices of hepatic lipid
622 unsaturation/polyunsaturation in obesity-related metabolic disease which is associated with
623 greater hepatic and peripheral insulin resistance. However, this technique is yet to be validated
624 against the gold-standard gas chromatography measurement in human liver tissue. Other
625 limitations include a lack of consistency with post-processing techniques and lipid composition
626 indices used[90], and measurement difficulty at low liver fat fractions[97]. Nevertheless, ^{1}H-MRS
627 enables repeat assessments of hepatic lipid composition in response to therapeutic interventions
628 (previously unviable in many instances). Preliminary (mainly preclinical) evidence suggests
629 that pharmacotherapies such as lipid-lowering agents may be able to alter hepatic lipid
630 composition[41,67], whilst lifestyle interventions have also shown promising results in patients
631 with NAFLD[150]. Further experimental studies are needed to extend this evidence base and to
632 prospectively determine whether changes in hepatic lipid composition impact metabolic and
633 liver-related health.

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Table 1. Summary of the findings from 18 liver biopsy/lipidomic studies examining hepatic lipid composition in individuals with obesity-related metabolic diseases.

Ref.	Study Year	Population	Analysis Method	Lipid Species	Main differences in hepatic lipid fatty acid composition
[98]	1961	Healthy liver vs. Steatosis	GC	Total lipids Neutral lipids Phospholipids CEs	*Total lipids:* ↑ MUFAs (C16:1, C18:1) and ↓ SFAs (C18:0) and PUFAs (C18:2) in steatosis vs. healthy liver. *Neutral lipids:* ↑ C14:0 and MUFAs (C16:1) and ↓ C18:0 and PUFAs (C18:2) in steatosis vs. healthy liver. *Phospholipids:* ↑ C14:0 and MUFAs (C16:1, C18:1) and ↓ C18:0 and PUFAs (C18:2) in steatosis vs. healthy liver. *CEs:* ↑ MUFAs (C16:1) and ↓ PUFAs (C18:2) in steatosis vs. healthy liver.
[80]	2004	Control (obesity) vs. NAFL vs. NASH	GC	Total lipids TAG Phospholipids	*Total lipids*: ↑ C16:0, MUFAs (C16:0, C14:1, C16:1, C18:1) and *n*-6/*n*-3 PUFA ratio, and ↓ *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NAFLD vs. control. *TAG:* ↓ C18:0, *n*-3 PUFAs (C18:3, EPA, DHA) and *n*-6 PUFAs (AA) in NAFLD vs. control. *Phospholipids:* ↑ *n*-6 PUFAs (AA) and *n*-6/*n*-3 PUFA ratio and ↓ *n*-3 PUFAs (EPA, DHA) in NAFLD vs. control.
[43]	2007	Control (obesity) vs. NAFL vs. NASH	TLC / GC	Total lipids TAG DAG FFAs Phospholipids	*Total lipids:* ↑ proportion of MUFAs and *n*-6/*n*-3 PUFA ratio, and ↓ proportion of PUFAs in NAFLD vs. control. *TAG:* ↑ SFAs (C16:0), MUFAs (C18:1) and *n*-6/*n*-3 PUFA ratio, and ↓ in *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NASH vs. NAFL vs. control (trend).

				CEs Cholesterol	*DAG:* ↑ SFAs (C16:0), MUFAs (C18:1) and *n*-6/*n*-3 PUFA ratio, and ↓ in *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NASH vs. NAFL vs. control (trend). *FFAs:* ↓ *n*-3 PUFAs (EPA, DHA) and ↓ *n*-6 PUFAs (AA, C18:3) in NASH vs. NAFL vs. control (trend). ↑ *n*-6/*n*-3 PUFA ratio in NASH vs. control. *Phospholipids:* ↑ *n*-6/*n*-3 PUFA ratio and ↓ in *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in phosphatidylcholines in NASH vs. NAFL vs. control (trend). *CEs:* ↓ SFAs and ↑ *n*-3 PUFAs (trend) and *n*-6 PUFAs in NAFLD vs. control.
[81]	2007	Control (lean) vs. NAFLD	GC	Phospholipids	↑ total SFAs (C18:0), C22:5*n*-6 and *n*-6/*n*-3 PUFA ratio in NAFLD vs. control. ↓ total *n*-3 PUFAs (EPA, DHA, C22:5*n*-3) and *n*-6 PUFAs (AA, C18:2) in NAFLD vs. control.
[99]	2008	Healthy liver vs. NAFL vs. NASH	GC	Total lipids	↑ MUFAs (C16:1, C18:1) in NASH vs. healthy liver. ↓ *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NASH vs. healthy liver. ↓ total *n*-6 PUFAs in NASH vs. NAFL.
[108]	2009	Control (lean) vs. NAFLD	GC	Total lipids	↓ *n*-3 PUFAs (DHA) in NAFLD vs. control.
[100]	2011	Control (healthy) vs. NAFLD	GC	Total lipids	↓ *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NAFLD vs. control.
[64]	2011	Obesity	LC-MS / MS	DAG	C18:1-C16:0, C18:1-C18:1, C18:1-C18:2 and C16:0-C18:2 were most abundant and positively correlated with HOMA-IR.

					C20:4-C20:5 inversely correlated with HOMA-IR.
[82]	2014	*PNPLA3*^{148II} vs. *PNPLA3*^{I148M}	TLC	TAG DAG FFAs Phospholipids CEs	*TAG:* ↓ SFAs (C18:0, C20:0, C22:0) and *n*-6 PUFAs (AA, 20:3, 22:4, 22:5) and ↑ *n*-3 PUFAs (C18:3) in *PNPLA3*^{I148M} vs. *PNPLA3*^{148II}. *FFAs:* ↓ *n*-6 PUFAs (AA, 20:3) and ↑ *n*-3 PUFAs (18:3) in *PNPLA3*^{I148M} vs. *PNPLA3*^{148II}.
[109]	2014	Control (healthy) vs. NASH	GC	Total lipids	↑ *n*-6/*n*-3 PUFA ratio and ↓ *n*-3 PUFAs (EPA, DHA) in NASH vs. control.
[83]	2015	NAFL vs. NASH	GC	Total lipids	↓ *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA, C20:3) in NASH vs. NAFL.
[110]	2015	Control (lean) vs. NAFL vs. NASH	GC	Total lipids	↑ proportion of SFAs (C14:0, C16:0), MUFAs (C16:1, C18:1) and *n*-6/*n*-3 PUFA ratio in NASH vs. NAFL vs. control. ↓ proportion of C18:0, *n*-3 PUFAs (DHA) and *n*-6 PUFAs (C18:2, C22:2) in NASH vs. NAFL vs. control.
[61]	2016	NAFLD (high HOMA-IR vs. low HOMA-IR / *PNPLA3*^{148II} vs. *PNPLA3*$^{148MM/MI}$)	GC-MS / UHPLC-MS	TAG DAG FFAs Ceramides	*TAG:* ↑ saturated and monounsaturated TAG in high HOMA-IR vs. low HOMA-IR. ↑ polyunsaturated TAG in *PNPLA3*$^{148MM/MI}$ vs. *PNPLA3*^{148MII}. *FFA:* ↑ SFAs (C16:0, C18:0) and MUFAs (C18:1) in high HOMA-IR vs. low HOMA-IR. *Ceramides:* ↑ ceramide (almost all species) in high HOMA-IR vs. low HOMA-IR. *DAG:* ↑ (4 species) in high HOMA-IR vs. low HOMA-IR and ↑ polyunsaturated DAG in *PNPLA3*$^{148MM/MI}$ vs. *PNPLA3*^{148MII}.
[77]	2017	Control (lean) vs.	GC /	Neutral lipids	↑ SFAs (C14:0, C16:0), MUFAs (C16:1, C18:1*n*-7, C18:1*n*-9), C18:2*n*-6 and *n*-6/*n*-3 PUFA ratio in NASH vs. NAFL and control.

		NAFL vs. NASH	LC-MS		↓ *n*-3 PUFAs (EPA, DHA) and *n*-6 PUFAs (AA) in NASH vs. NAFL and control.
[86]	2018	Control (obesity) vs. NAFL vs. NASH	LC-MS	TAG DAG Phospholipids Sphingolipids CEs	*TAG:* ↑ SFAs (C14:0, C17:0, C18:0) in NASH vs. control and ↓ *n*-6 PUFAs (AA) in NASH vs. NAFL vs. control. *DAG:* ↑ MUFAs (C16:1, C18:1) and ↓ SFAs (C18:0), *n*-3 PUFAs (DHA, C22:5*n*-3) and *n*-6 PUFAs (AA, C18:2) in NAFLD vs. control. *CEs:* ↑ C16:1, C16:2, C17:1, C18:2, C18:3, C22:6 and ↓ C16:0, C18:1, C20:1, C20:2, C22:4 in NASH vs. control. ↑ C16:2 and ↓ C16:0 in NAFL vs. control. ↓ C20:2 in NASH vs. NAFL. *Phospholipids:* ↓ *n*-3 PUFAs (DHA) in NAFLD vs. control. *Sphingolipids*: ↑ Cer(d18:0/18:0) in NAFLD vs. control and ↑ Hex2Cer(d18:1/18:0) and Hex2Cer(d18:1/24:1) in NASH vs. NAFL and control.
[62]	2018	Control (healthy) vs. Obesity vs. NAFL vs. NASH	LC-MS / MS	Sphingolipids	*Ceramides:* ↑ total ceramides and C24:0 in NASH vs. other groups. ↑ C16:0 in obesity vs. control. *Dihydroceramides:* ↑ total dihydroceramides and C16:0, C22:0 and C24:1 in NASH vs. control. *Lactosylceramides:* ↑ total lactosylceramides and C24:1 in NASH vs. control. *Hexosylceramides:* ↑ C22:0 and C24:0 in NASH vs. other groups.
[101]	2019	NAFL vs. NASH	GC	Total lipids	↑ proportion of C16:0 and MUFAs (C16:1, C18:1) in NASH vs. NAFL. ↓ proportion of C18:0, MUFAs (C22:1), *n*-3 PUFAs (DHA) and *n*-6 PUFAs (C18:2, C22:2) in NASH vs. NAFL.

[103]	2020	Healthy liver vs. Steatosis / High HOMA-IR vs. Low HOMA-IR	GC	FFAs	↑ proportion of SFAs (C14:0, C16:0) and MUFAs (C16:1, C18:1) and ↓ proportion of C18:0 and PUFAs (AA, C18:2) in steatosis vs. healthy liver. ↑ proportion of SFAs (C14:0, C16:0) and MUFAs (C16:1, C18:1) and ↓ proportion of C18:0 and PUFAs (AA, C18:2) in high HOMA-IR vs. low HOMA-IR.

AA, arachidonic acid; CE, cholesterol ester; DAG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acids; GC, gas chromatography; HOMA-IR, homeostatic model assessment of insulin resistance; LC, liquid chromatography; LPC, lysophosphatidylcholine; MS, mass spectrometry; MUFA, monounsaturated fatty acid; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PC, phosphatidylcholine; *PNPLA3*, patatin-like phospholipase domain-containing protein 3; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TLC, thin-layer chromatography; UHPLC, ultra-high-performance liquid chromatography.

Table 2. Summary of the findings from eight proton magnetic resonance spectroscopy studies examining hepatic lipid composition in individuals with obesity-related metabolic diseases.

Ref.	Study Year	Population	Main differences in hepatic lipid composition indices
[112]	1993	Healthy liver vs. NAFLD	↓ unsaturation ratio and chain length in NAFLD vs. healthy liver.
[94]	2008	Lean vs. Obesity vs. NAFLD	↑ SI in obesity and NAFLD vs. lean. ↓ PUI in NAFLD vs. obesity vs. lean.
[114]	2010	Suspected NAFLD (With T2DM vs. Without T2DM)	↑ proportion of unsaturated fatty acids in group with T2DM vs. without T2DM.
[116]	2017	NAFL vs. NASH	No differences in SI, UI or PUI in NAFL vs. NASH. ↓ PUI/UI ratio in participants with vs. without obesity. ↑ PUI in *PNPLA3* I148M homozygous carriers vs. non-carriers.
[113]	2019	Healthy liver vs. NAFLD	↑ SI and ↓ UI in NAFLD vs. healthy liver. No differences in PUI in NAFLD vs. healthy liver.
[97]	2020	NAFLD (or suspected)	↓ number of double bonds and number of methylene-interrupted double bonds with ↑ liver proton density fat fraction.
[95]	2020	Overweight/Obesity vs. NAFL vs. T2DM vs.	↑ SFA fraction in NAFL and T2DM vs. overweight/obesity. ↑ SFA fraction in GSD1a vs. NAFL and overweight/obesity. ↓ MUFA fraction in GSD1a vs. overweight/obesity.

		GSD1a	
[118]	2020	Control (healthy) vs. Acromegaly	↓ UI in acromegaly vs. control.

[1]H-MRS, proton magnetic resonance spectroscopy; GSD1a, glycogen storage disease type 1a; HOMA-IR, homeostatic model assessment of insulin resistance; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; *PNPLA3*, patatin-like phospholipase domain-containing protein 3; PUI, polyunsaturation index; SI, saturation index; T2DM, type 2 diabetes mellitus; UI, unsaturation index.

Figure legends

Figure 1. The mechanistic pathways underpinning SFA-induced lipotoxicity and insulin resistance in hepatocytes. SFAs induce ER stress through activation of the unfolded protein response pathway, and promote oxidative stress via mitochondrial dysfunction and the accumulation of reactive oxygen species. Both factors indirectly trigger apoptosis through activation of the JNK stress signalling pathway. Additionally, SFAs can directly promote apoptosis through binding to various death receptors located on the cell membrane. JNK activation also impairs insulin signalling via phosphorylation of the insulin receptor substrate-1, leading to the development of insulin resistance. SFAs also promote lipogenesis which may be mediated by their conversion to MUFAs which are preferentially incorporated into lipid droplets as triacylglycerol. Furthermore, the activation of TLR4 signalling by SFAs leads to the production of proinflammatory cytokines through the upregulation of the transcription factor NF-kB. Notably, some of these effects of SFAs on lipotoxicity and insulin resistance within hepatocytes may be mediated through their conversion to bioactive lipid intermediates such as ceramides, LPCs and DAG. DAG, diacylglycerol; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; LPC, lysophosphatidylcholine; MUFA, monounsaturated fatty acid; NF-kB, nuclear factor kappa B; SFA, saturated fatty acid; TLR4, toll-like receptor 4.

Figure 2. (A) Example chemical structures of a saturated (palmitic), monounsaturated (oleic) and polyunsaturated (linoleic) fatty acid chain with the hydrogen protons associated with different functional fatty acid groups highlighted. (B) Peak assignments and chemical shifts of the functional fatty acid groups in a typical lipid proton magnetic resonance spectrum.

Figure 3. Summary of the relative alterations in the fatty acid composition of hepatic lipids across the progression of NAFLD. The percentage of SFAs and MUFAs in hepatic lipids are elevated in NAFL compared to individuals with a healthy liver; whilst the percentage of PUFAs

are reduced, leading to an elevation in the *n*-6/*n*-3 PUFA ratio. The percentage of SFAs and MUFAs and the *n*-6/*n*-3 PUFA ratio may be further increased in NASH compared to NAFL; whilst there is a further depletion in the percentage of PUFAs. These alterations in hepatic lipid composition are associated with greater obesity-related metabolic dysfunction and hepatic lipotoxicity as NAFLD progresses. MUFA, monounsaturated fatty acid; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Figure 1

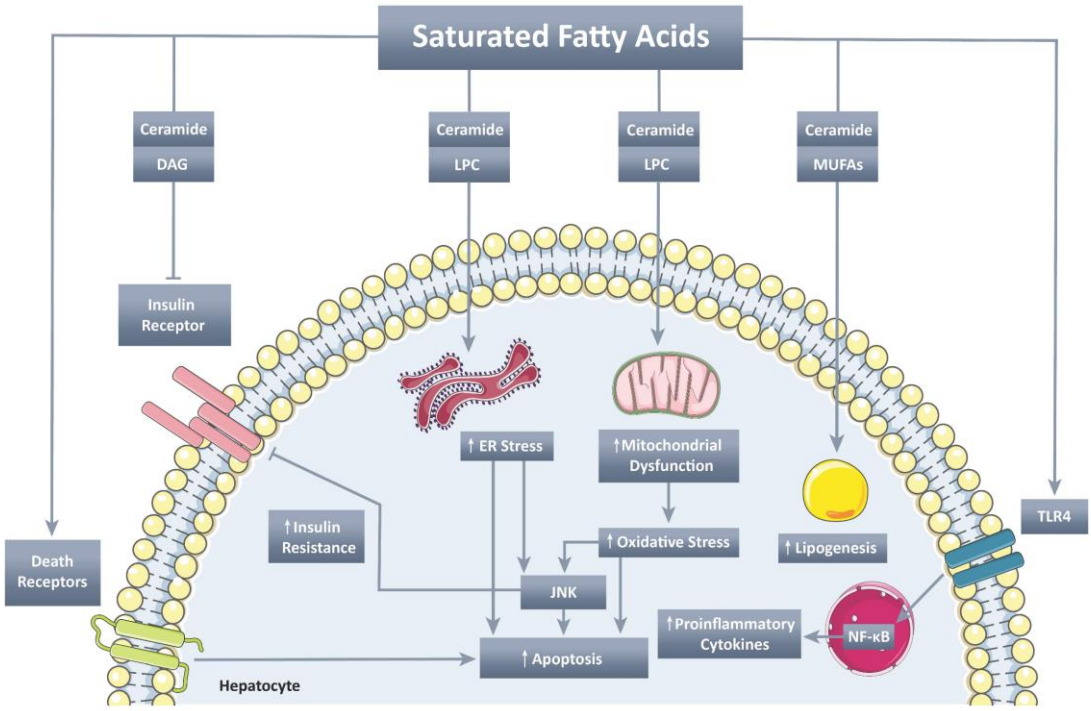
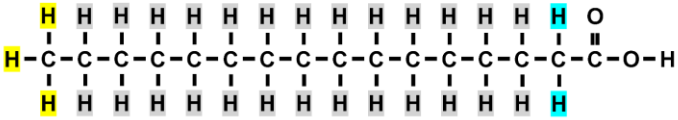

Saturated Fatty Acids
Ceramide
DAG
Ceramide
LPC
Ceramide
LPC
Ceramide
MUFAs
Insulin Receptor
↑ ER Stress
↑Mitochondrial Dysfunction
↑Insulin Resistance
↑ Oxidative Stress
↑ Lipogenesis
TLR4
Death Receptors
JNK
↑Proinflammatory Cytokines
NF-κB
↑ Apoptosis
Hepatocyte

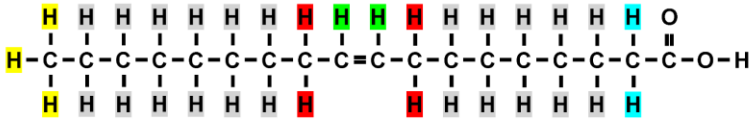
Figure 2

A

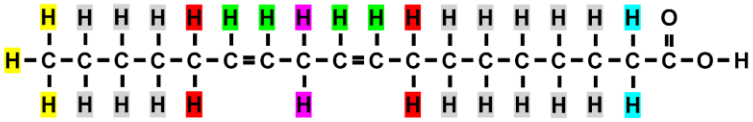
Palmitic acid - C16:0 - $CH_3(CH_2)_{14}COOH$



Oleic acid - C18:1 - $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$



Linoleic acid - C18:2 - $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$



B

Peak #	Name	Chemical formula	Chemical shift (ppm)
1	Methene	$-CH{=}CH-$	5.2-5.4
2	Diallylic	$-CH{=}CH-CH_2-CH{=}CH-$	2.8-3.1
3	α-Methylene to carboxyl	$-CH_2-COOH$	2.2-2.4
4	Allylic	$-CH_2-CH{=}CH-CH_2-$	2.0-2.1
5	Methylene	$-CH_2-$	1.2-1.4
6	Methyl	CH_3-	0.8-1.0

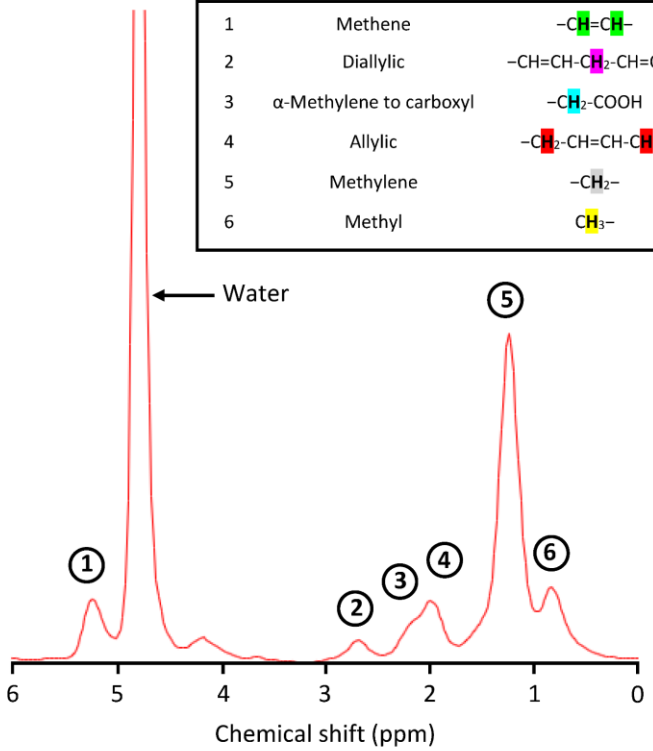
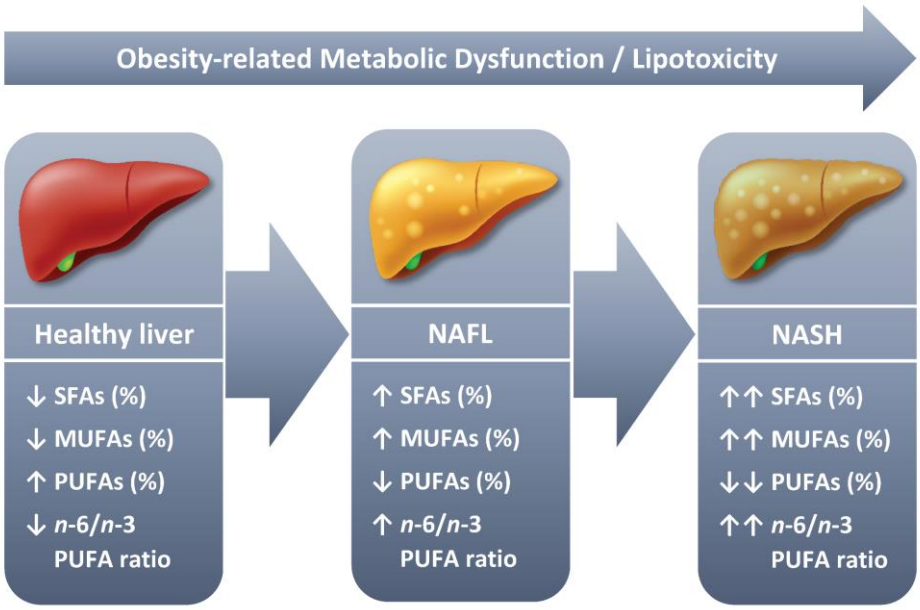


Figure 3


Obesity-related Metabolic Dysfunction / Lipotoxicity
Healthy liver
↓ SFAs (%)
↓ MUFAs (%)
↑ PUFAs (%)
↓ n-6/n-3 PUFA ratio
NAFL
↑ SFAs (%)
↑ MUFAs (%)
↓ PUFAs (%)
↑ n-6/n-3 PUFA ratio
NASH
↑↑ SFAs (%)
↑↑ MUFAs (%)
↓↓ PUFAs (%)
↑↑ n-6/n-3 PUFA ratio