The influence of dietary fatty acids on liver fat content and metabolism

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Abstract

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of conditions from hepatic steatosis through to cirrhosis; obesity is a known risk factor. The liver plays a major role in regulating fatty acid metabolism and perturbations in intrahepatic processes have the potential to impact on metabolic health. It remains unclear why intra-hepatocellular fat starts to accumulate, but it likely involves an imbalance between fatty acid delivery to the liver, fatty acid synthesis and oxidation within the liver and triglyceride export from the liver. As humans spend the majority of the day in a postprandial rather than postabsorptive state, dietary fatty acid intake should be taken into consideration when investigating why intra-hepatic fat starts to accumulate. This review will discuss the impact of the quantity and quality of dietary fatty acids on liver fat accumulation and metabolism, along with some of the potential mechanisms involved. Studies investigating the role of dietary fat on liver fat accumulation, although surprisingly limited, have clearly demonstrated that it is total calorie intake, rather than fat intake *per se*, that is a key mediator of liver fat content; hypercaloric diets increase liver fat whilst hypocaloric diets decrease liver fat content irrespective of total fat content. Moreover, there is now, albeit limited evidence emerging to suggest the composition of dietary fat may also play a role in liver fat accumulation, with diets enriched in saturated fat appearing to increase liver fat content to a greater extent when compared to diets enriched in unsaturated fats.

Introduction

The worldwide prevalence of individuals defined as overweight and obese has risen by over 25% since 1980 [1]. Whilst the development of overweight and obesity likely involves a combination of genetic, environmental and psycho-social factors, in its simplest form it can be attributed to an imbalance between energy intake (*i.e.* diet) and energy expenditure (*i.e.* physical and metabolic activity) [2]. Diet is a modifiable risk factor for overweight and obesity, and due to the strong associations between total body fatness and risk of metabolic diseases such as type 2 diabetes (T2DM) and non-alcoholic fatty liver disease (NAFLD), the increase in the number of individuals defined as overweight and obese represents a major global health challenge [3]. Here we review the evidence regarding the influence of dietary fats on liver fat content.

Non-alcoholic fatty liver disease: an overview

NAFLD, which has been termed the hepatic manifestation of the metabolic syndrome, encompasses a spectrum of liver disease ranging from steatosis to hepatocellular carcinoma, the causes of which are not attributable to alcohol or substance abuse [4]. NAFLD is currently recognised as the most prevalent form of liver disease worldwide, estimated to affect ~25% of the global population [5], although due to the limited availability of valid and reliable, non-invasive diagnostic tests, it has been suggested that the true figure may be even greater [6]. Hepatic steatosis (which is often referred to as NAFLD, and is the first stage in the NAFLD spectrum) is the net retention of intracellular triacylglycerol (TG), and is defined histologically as the presence of intracellular TG in >5% of hepatocytes, or when the proton density fat fraction is greater than 5.6% when measured by magnetic resonance imaging/spectroscopy [7].

NAFLD risks factors

A number of modifiable and non-modifiable risk factors have been identified for NAFLD. Non-modifiable risk factors include 1) sex, with Caucasian males being more susceptible to NAFLD than Caucasian females [8,9]; 2) ethnicity, Asian and Hispanic populations have a greater prevalence of NAFLD compared to Caucasian and Black populations [10,9,5]; and 3) carrying specific genetic variants of patatin-like phospholipase domain containing protein three (PNPLA3)), or transmembrane 6 superfamily member 2 (TM6SF2) amongst others[11].

The major modifiable risk factors include: 1) increased body fatness [12]; 2) a sedentary lifestyle or low physical activity levels [13]; and 3) dietary intake [14]. However, it is challenging to define the contribution that specific dietary components may play in the aetiology of NAFLD from those of total macronutrient intake, as total body fatness (which is typically a product of excess energy consumption) is strongly associated with increased liver fat [15]. This inability to disentangle the effects of total calorie and macronutrient intakes likely explains, in part, the observation that both high-fat diets enriched in saturated fatty acids (SFA), and high-carbohydrate diets enriched with free sugars are both associated with increased liver fat [16,17]. In addition to total macronutrient intakes a further area of interest relates to how consumption of SFA, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) may differentially affect liver fat content independently of calorie intake. However, at this point in time, only a few studies have been undertaken comparing the influence of isocaloric diets with different fat compositions on liver fat content *in vivo* in humans, making it challenging to draw robust conclusions. Thus, the precise role of specific dietary fats on liver fat content, and the mechanisms through which they have their effects, are yet to be fully clarified. In this review we summarise the available evidence from human studies regarding dietary fat consumption (and where available composition) and the effect on liver fat content, and highlight areas for potential future investigation.

Overview of hepatic dietary fatty acid metabolism

The accumulation of fat within the liver represents an imbalance between the amount of fatty acids entering the liver (input), fatty acid synthesis within the liver, and fatty acid disposal from the liver (output) [18]. Investigation of these pathways will provide an understanding of the effect of diet and dietary components on the pathogenesis, and potentially progression, of NAFLD; we have recently reviewed methodologies for investigating hepatic fatty acid metabolism in humans [19].

Briefly, in the fasted state the input of fatty acids to the liver are derived predominantly from adipose tissue lipolysis (known as systemic non-esterified fatty acids (NEFA)) [20-23], and in the postprandial period dietary fatty acids enter, as chylomicron-derived spillover fatty acid [24,22,25] and chylomicron remnants [26,22] (Figure 1). Fatty acids synthesised within the liver from non-lipid precursors (*e.g* dietary sugars), via the process of *de novo* lipogenesis (DNL) [27,28] also contribute to the intrahepatic fatty acid pool (Figure 1**)**. Within the liver, fatty acids are broadly partitioned into either esterification pathways to form predominantly TG which

may then be incorporated into very low density lipoprotein (VLDL) and secreted into systemic circulation or stored in the cytosolic TG (as lipid droplets) or, an alternative fat for intrahepatic fatty acids is that they may be partitioned into oxidation pathways (either the tricarboxylic acid (TCA)/Krebs cycle or ketogenesis) [18].

To elucidate the contribution of dietary fatty acids to intrahepatic TG content ideally, the contribution of dietary fatty acids to liver TG would be measured directly. To date, only one study has assessed the contribution of different fatty acid sources to intrahepatic TG (IHTG) in subjects with NAFLD. By using stable-isotope tracer methodology, they found after 5 days of labelling that the relative contribution of systemic NEFA, DNL fatty acids and dietary fatty acids to IHTG was 59%, 26% and 15%, respectively [20]. The authors reported that the contribution from the respective fatty acid sources was similar in VLDL-TG [20], suggesting that VLDL-TG could be used as a proxy marker of IHTG. Further support for the notion that VLDL-TG may be a proxy for IHTG comes from Peter *et al*., [29] who, when comparing the fatty acid composition of liver tissue with various blood lipid fractions, found that the 16:1/16:0 ratio in IHTG strongly correlated with the 16:1/16:0-ratio in VLDL-TG. Studies that have used stable-isotope methodology and measured the contribution of different fatty acid sources to VLDL-TG have found that 75-84% of fatty acids are derived from the circulating NEFA pool, whereas DNL fatty acids are estimated to contribute 10-22%; although both these pathways may be influenced by obesity and insulin resistance [30]. The data for the contribution of dietary fatty acids is somewhat variable, ranging between 12 – 39% [20,21,31,22,23], as this will be influenced by meal composition (i.e. the amount of fat consumed), size of the meal, and timing of when samples are collected after the meal(s). Studies utilising ^{13}C / ^{31}P magnetic resonance spectroscopy (MRS) have reported that liver fat content is rapidly increased (within 360 min) in response to a single high-fat load in healthy lean individuals, and remains elevated for up to 5 hours [32,33]. Ravikumar *et al*., [34] reported a significantly higher increment in liver fat content in individuals with T2DM after consumption of a mixed test meal, compared to lean controls; baseline liver fat content was also significantly higher in T2DM compared to controls. Thus, it is plausible that consumption of high fat foods at regular intervals over the course of a day long-term, would lead to an increased delivery of dietary fatty acids to the liver that exceeds the livers disposal capacity, leading to an accumulation of IHTG.

The limited availability of liver tissue also makes it challenging to investigate intrahepatic fatty acid composition. It has been reported that when compared to age and BMI matched non-NAFLD controls, NAFLD patients have lower levels of intrahepatic PUFA [35], although it is unclear whether this is attributable to differences in specific lipid fractions. By analysing the fatty acid composition of erythrocytes and liver phospholipids from individuals with and without NAFLD, Elizondo *et al.,* [36] demonstrated that liver phospholipids from obese patients with NAFLD had a lower abundance of 20:4n-6, 22:5n-3, and 22:6n-3 when compared to controls. They also found that levels of DHA within the liver were positively correlated with those in erythrocytes [36], suggesting that erythrocyte fatty acid composition could be a useful indicator of liver phospholipid fatty acid composition in obese, NAFLD patients. Petit *et al.,* [37] measured erythrocyte fatty acid composition in T2DM patients with and without NAFLD, and found higher proportions of total SFAs, and lower proportions of PUFAs in erythrocytes in those who had NAFLD (n=109) compared to those without (n=53).

Measuring the fatty acid composition of blood lipid fractions is an objective and useful marker of dietary fatty acid intake [38]. To date a limited number of studies have measured plasma fatty acid composition and liver fat content and investigated associations. For example, Rosqvist et al [39] observed that the abundance of linoleate in plasma phospholipids and cholesterol esters were inversely associated with liver fat content in a population-based sample of 78 elderly men and women. Although observational, these data suggest that individuals that have a higher abundance of plasma lineolate, presumably due to a higher dietary intake, have a lower IHTG than individuals with a lower intake.

Dietary fatty acids and liver fat

The findings from cross-sectional / observational studies investigating the associations between dietary fat intake and liver fat content are variable and inconsistent in demonstrating whether the total amount and the type of fat consumed is associated with liver fat content [40-43,17,44]. The variability and discrepancies in results between studies is likely to be in part explained by how and when liver fat content was assessed, in combination of how dietary intake was assessed and over what time period. To fully understand the impact (and potentially causal nature) of dietary fat on liver fat accumulation evidence from intervention studies provides some insight.

Dietary fatty acids and liver fat content: evidence from intervention studies

Total fat: Whether the proportions of dietary carbohydrate and fat play a key role in liver fat accumulation is highly debated. During isocaloric conditions, in interventions lasting ≤4 weeks diets higher in carbohydrate (~60% total energy (TE) carbohydrate, ~20%TE fat) appear to reduce liver fat to a greater extent when compared to diets that are higher in fat (~50%TE fat, ~30%TE carbohydrate) [45-48]. In contrast, in interventions lasting 6-12 weeks diets higher in fat (~45% TE fat, ~35% TE carbohydrate) have been reported to decrease liver fat to a greater extent than diets higher in carbohydrate (~50%TE carbohydrate, ~25%TE fat) [49-51]. Notably, the higher-fat diets in the latter interventions (6-12 weeks) were primarily enriched in unsaturated fats, in contrast to the shorter term studies where the higher-fat diets were primarily enriched in SFAs [45-48]. Furthermore, subjects in the longer-term studies had prediabetes, T2DM and/or NAFLD, whilst subjects in the shorter-term studies were considered metabolically healthy, which may have contributed to the divergence in findings between high- (~40% to 56% TE as fat) and low-fat diets (~16% to 30% TE as fat). A recent meta-analysis reported that due to the large heterogeneity between studies, there is no difference in liver fat reductions between low-carbohydrate (~24% TE as carbohydrate) and low-fat diets (~20% TE as fat) [52].

In a recent short-term (2 weeks) study without a control group, Mardinoglu *et al.,* [53] investigated the effects of an extreme low-carbohydrate (4% TE), high-fat (72% TE, fat quality not specified) diet in 10 subjects with NAFLD (IHTG content ~12%) and demonstrated large reductions (>40%) in IHTG content. The large decrease in liver fat is likely to be due to a combination of factors including: i) modest (2 kg) weight loss, ii) decreased hepatic DNL, which is likely to be explained by a lack of non-lipid precursors, and iii) increased fatty acid oxidation. Although results are impressive, the longer term effects (including compliance) to such an extreme low-carbohydrate, high fat diet remains to be determined. Considering the large effect of caloric restriction (*i.e.* weight loss) on liver fat content, it is unsurprising that dietary composition appears to not be a key mediator of liver fat content during hypocaloric conditions [54,55]. Although it has been suggested that extreme carbohydrate restriction (8% TE) may aid in decreasing IHTG content further than fat restriction, despite hypocaloric conditions [56], further well-controlled investigations are required to clearly demonstrate this.

In a long term (18-month) randomised trial in 278 subjects (89% male), Gepner *et al*., [57] compared a hypocaloric low-fat diet with a hypocaloric Mediterranean diet and found that

although overall weight loss (~3 kg) was not different between diets, the Mediterranean compared to the low-fat diet resulted in a greater loss of IHTG after statistical adjustment. However, although the macronutrient compositions of the diets are unclear, when calculated, it appears that the dietary intakes of carbohydrate and fat were quite similar between the two interventions (the low-fat diet containing ~45% TE carbohydrate, and ~34% TE fat, and the Mediterranean diet ~39% TE carbohydrate, and ~41% TE fat) suggesting other dietary factors may underpin the observed reductions in IHTG content.

Few studies have compared the proportions of fat and carbohydrate during hypercaloric conditions [58,59]. Sobrecases *et al*., observed a 16% increase in liver fat after 7 days of fructose overfeeding (+35% TE) and a 86% increase after 4 days of SFA overfeeding (+30% TE) in young, lean and healthy men, suggesting SFA increases liver fat to a greater extent than fructose during hypercaloric conditions [58]. The combination of fructose and SFA overfeeding for 4 days resulted in a 133% increase in IHTG content however as surplus energy intake also increased (+65% TE) it is unclear how much is due to nutrient synergy compared to energy excess. As weight gain is likely a key driver of increased IHTG it is difficult to disentangle nutrient-specific effects during concurrent weight gain. In agreement with these findings, Lecoultre *et al*., [59] reported a greater relative increase (~90% increase) in IHTG after a SFA (30% energy excess) enriched diet for 6-7 days compared to overfeeding 3.0 g/kg bodyweight glucose (~60% increase in IHTG), although they found no significant difference in IHTG after feeding 1.5, 3.0, or 4.0 g/kg bodyweight fructose in young, healthy, males. The differences in energy intake and durations of overfeeding between diets within and between studies make it difficult to compare and interpret the findings. For example, the increased IHTG content after carbohydrate/sugar overfeeding may in part be explained due to increased hepatic DNL, although as this is an energy inefficient method of accumulating liver fat compared to storing pre-formed fat, it is plausible that the increase in liver fat is smaller when overfeeding carbohydrate/sugar compared to isocaloric fat overfeeding [60].

<u>*Saturated fat:*</u> Marina *et al*., [48] reported that increasing SFA intake from ~12% TE to ~24% TE (total fat from 35% TE to 55% TE) over a four week period failed to increase IHTG during isocaloric conditions in 10 healthy subjects with elevated liver fat (8-9%), whereas a slight decrease in SFA intake (from ~12% TE to ~8% TE) within a low-fat/high-carbohydrate diet (20% TE fat, 62% TE carbohydrate) decreased IHTG in a within-group

analysis however, diets were not significantly different in a between-group comparison. In a larger study (n=61), Bjermo *et al.*, [61] found that an isocaloric increase in SFA (from ~15% TE to ~20% TE) over 10 weeks increased IHTG compared to a diet enriched in n-6 PUFA in obese, middle-aged subjects. In line with this, Rosqvist *et al.*, [62] demonstrated that overfeeding SFA for 7 weeks increased IHTG to a greater extent compared with similar overfeeding of n-6 PUFA in young and lean subjects. Luukkonen *et al.*, [63] also recently reported a 55% increase in IHTG after 3 weeks of SFA overfeeding (+1000 kcal/day) compared to a 33% increase and a 15% increase in IHTG after 3 weeks of simple sugar or unsaturated fat (mixture of MUFA and PUFA) overfeeding (+1000 kcal/day) in obese, middle-aged men and women. By using stable-isotope tracer methodology, Luukkonen *et al.*, [63] demonstrated that the SFA diet was associated with an increase in adipose tissue lipolysis which would lead to a greater fatty acid flux (adipose tissue and dietary) to the liver, whilst the simple sugar diet was associated with an increase hepatic DNL. Although the unsaturated fat diet was associated with a decrease in lipolysis and no change in DNL, it is likely that the excess calories from the fat resulted in an increased flux of dietary fatty acids to the liver. What remains unclear from these studies is how the specific fatty acids alter intrahepatic fatty acid partitioning.

Overall, it appears that increased consumption of a SFA-enriched diet increases IHTG, although it remains unclear if different sources of SFA (e.g dairy vs meat) may potentially have different effects on liver fat content. For example, Kratz *et al.*, [64] observed that circulating biomarkers of dairy fat intake (the fatty acids 15:0, 17:0 and trans-16:1) were inversely associated with liver fat content in 17 subjects with NAFLD (assessed by computer tomography-derived liver-spleen ratio) and 15 age and BMI-matched subjects without NAFLD. Although based on a small sample size, this finding is in-line with the general finding of reduced risk of T2DM with higher circulating abundance of dairy fat biomarkers [65]. Different dairy products have distinctly different effects on cholesterolemia, even when total intake of SFA from these food items are matched [66,67], highlighting the importance of other food components. A differential effect of various SFA sources on IHTG has yet to be demonstrated in humans but would be a logical and interesting direction for future studies.

Monounsaturated fat: Bozzetto *et al.*, [49] reported that consumption of an isocaloric high-MUFA diet (27% TE) for 8 weeks decreased IHTG (~30% relative reduction) compared to a normal-MUFA diet (16% TE). Similarly, Errazuriz *et al.*, [51] reported IHTG to decrease

during 12 weeks of a high- (22% TE) compared with a low- (7% TE) MUFA diet, during isocaloric conditions. In a randomized cross-over study where the majority of foods were provided, Ryan *et al.*, [50] compared a high-MUFA Mediterranean diet (44% TE fat, 23% TE MUFA) with a low-fat diet (21% TE fat, 8% TE MUFA) over 6 weeks and found reductions in IHTG tended to occur to a greater extent after the high- compared to low-MUFA diet. In contrast, Properzi *et al.*, [68] showed similar reductions in IHTG when a high-MUFA Mediterranean diet (45% TE fat, 24% TE MUFA, 37% TE carbohydrate) was compared to a lower-fat diet (31% TE fat, 12% TE MUFA, 48% TE carbohydrate) consumed *ad libitum* for 12 weeks. The similar decrease in IHTG may, in part, be due to the slightly hypocaloric nature of the diet as there were reductions in body weight of between 1.6-2.1 kg; there was no difference in SFA intake between groups. As a Mediterranean diet consists of a relatively high intake of nutrient dense foods such as fruits and vegetables [69], it is challenging to isolate the effects of MUFA as it is plausible that other components of a Mediterranean diet are beneficial for reductions in IHTG [70]; elucidating the influence of specific components on IHTG, although interesting would be an extremely challenging endeavour. Moreover, this would not be specific to the Mediterranean diet but to all diets/dietary patterns based on minimally processed food items. For example, Otten *et al* [71] demonstrated superior reductions in IHTG by a higher-fat (43%TE fat, 30%TE carbohydrate) paleolithic-type diet (based on lean meat, fish, eggs, vegetables, fruits, berries and nuts) compared to a lower-fat (32%TE fat, 44%TE carbohydrate) conventional diet. However, as both diets were hypocaloric and also differed in fat quality it is difficult to disentangle which component(s) may contribute the greatest to the change in IHTG.

The potential importance of phytochemicals (for example β-carotene, vitamin E, folate and flavonoids) , which are high in the Mediterranean diet [70], in the prevention and management of NAFLD is gaining interest, with studies in different animal models showing beneficial effects on both steatosis and various biomarkers (*e.g.* liver enzymes) [72,73]. Studies in humans are thus far limited but of those available, results generally show beneficial effects on liver enzymes [72,73], despite having very reductionist designs unlikely to represent a complex diet. Taken together, the reductions in IHTG when a a plant-based diet rich in minimally processed food items (*e.g.* the Mediterranean diet) is consumed is likely to be explained by both changes in the classical components such as fat- and carbohydrate quality but also by the content of micro- and non-nutrient compounds. More studies in humans

investigating this concept are needed, given that lifestyle modifications are still the first and most effective line of treatment of NAFLD.

n-6 and n-3 polyunsaturated fat: The specific effects of dietary n-3 and n-6 PUFA intakes are of interest due to their potential influence on metabolic health [74,75].

n-3 fatty acids: The two most recognised n-3 PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are predominantly found in fatty fish and fish oils. Another n-3 PUFA, α-linolenic acid is present in certain seed and plant based foods (*e.g.* flaxseed oil, walnuts, soybeans and soybean oil, pumpkin seeds, rapeseed oil, and olive oil [76]). Mammalian cells have the capacity to further metabolise α-linolenic acid to EPA and DHA through elongation and desaturation pathways [75].

Animal models have suggested that increasing n-3 PUFA intakes leads to a reduction in IHTG [77,78] and evidence from observational studies suggests that when compared NAFLD compared to non-NAFLD patients consume consumes less n-3 PUFA [79]. Evidence from human intervention studies demonstrates that, when compared to placebo, supplementation with n-3 PUFA decreased IHTG across a spectrum of phenotypes, including patients with T2DM [80], NAFLD [81-84], and polycystic ovarian syndrome [85], with the response appearing to occur independent of changes in body weight; although not all studies have observed a reduction in IHTG with n-3 PUFA supplementation [86]. Moreover there is some suggestion that n-3 PUFA supplementation may ameliorate the clinical symptoms of NASH [87,88], although this is not observed by all [89].

The majority of studies investigating the effect of n-3 PUFA on IHTG have been supplementation studies with EPA and/or DHA in combination or alone with concentrations ranging from 250mg up to 4 g/day; the upper intake exceeds both the WHO recommendations for EPA/DHA intakes (250-2000mg/day [90]), and the estimated intakes of all n-3 PUFA for UK adults (~2.2g/day [91]), although the recommended clinical dose for lowering TG levels is 2–4 g/day of EPA and DHA [92]. To put this in context, a typical portion of Salmon contains ~500mg of EPA and 1.3g of DHA per 100g consumed[75], thus supplementation, rather than diet, is the only plausible way to achieve the intakes reported to lower IHTG. As the duration of the studies investigating n-3 PUFA supplementation and IHTG ranges from 8 weeks to 24 months, in combination with the wide range of n-3 PUFA

doses used, it is challenging to determine what (if any) optimal n-3 PUFA intakes could be for reducing IHTG and whether they could be achieved by diet alone. Furthermore, it is unclear if the ratio between EPA and DHA is a significant mediator of IHTG, as studies comparing EPA vs. DHA yet to be undertaken.

To date, the majority of the focus has been on EPA and DHA and their effects on IHTG, with relatively little attention being given to α-linolenic acid. Of the limited work been undertaken, Nogueira *et al*., [93] investigated differences in histopathological features in NASH patients before and after supplementation with 945mg/day of a α-linolenic acid, EPA and DHA mix (~64, 16 and 21% of total fatty acids, respectively) for 6 months. The authors reported that n-3 PUFA supplementation did not influence hepatocellular ballooning, steatosis or fibrosis when compared to a mineral oil control [93], although a substantial confounder to the study results was the fact that plasma n-3 PUFA levels also increased in the control group, suggesting an increased n-3 PUFA intake (by fish or supplements) [93] making conclusions difficult to draw. By comparing the whole cohort, the authors observed a significant positive correlation between the rate of increase in plasma α-linolenic acid and EPA levels and improvements in steatosis, lobular inflammation and ballooning, suggesting a beneficial role for n-3 supplementation in NASH patients [93].

n-6 fatty acids: Whilst the evidence would suggest that n-3 PUFA intake, specifically EPA and DHA, may play a role in modulating liver fat content, n-6 PUFA consumption is notably higher than n-3 PUFA consumption in a typical Western style diet, with the majority of dietary PUFA being from linoleic acid. Despite linoleic acid being the major PUFA consumed, only a few studies have investigated the effects of this fatty acid on IHTG. Bjermo *et al.*, [61] demonstrated that a high intake of n-6 PUFA (10-15% TE from linoleic acid) in abdominally-obese men and women for 10 weeks reduced IHTG compared to a higher intakes of SFA, in the context of an isocaloric diet. In a double-blind follow-up study, Rosqvist *et al.*, [62] observed that a similarly high intake of n-6 PUFA (10-15% TE) during hypercaloric conditions for seven weeks did not lead to accumulation of IHTG, which was in contrast to the group consuming SFA. As the subjects in the study by Rosqvist *et al* [62] were healthy, young, lean adults, with very low levels of IHTG, it would be of interest to undertake a similar study in other populations. To date, no study has directly compared the effects of n-3 and n-6 PUFA on IHTG, which would be interesting, although challenging to do due to the imbalanced dietary intake of n-3 vs n-6 PUFA.

Potential mechanisms of fatty acids; specific effects on liver fat metabolism

Although it appears that different fatty acids may have differential effects on IHTG accumulation, the mechanistic basis remains to be elucidated, although a number of proposed mechanism have been suggested, work is required to provide a clear demonstration.

There have been a number of mechanisms proposed for the effect of SFA on IHTG accumulation and these include: increased inflammation in adipose tissue leading to increased lipolysis [63], thereby increasing the fatty acid flux to the liver, and increased ceramides [63]; the fatty acid 16:0 is a precursor for ceramide synthesis and ceramides have been suggested to cause insulin resistance (which may lead to IHTG accumulation). The latter hypothesis is supported by animal work which has demonstrated a role for ceramides in IHTG accumulation [94,95]. Moreover, evidence from animal and cellular studies has suggested that increased intracellular accumulation of SFAs caused cell dysfunction and up-regulate pro-inflammatory pathways [96-98] which may, eventually lead to IHTG accumulation.

There is a commonly held, but largely unsubstantiated view that dietary PUFAs are more prone to oxidation compared with both MUFA and SFA, and hence the latter (SFA) are more likely to be to be stored promoting liver fat accumulation. Although, human evidence is limited, this view is generally supported by animal work. From the available evidence in humans, Jones *et al.*, [99] fed, in random order, meals labelled with either ^{13}C-labeled stearic, oleic and linoleic acid to six young (mean age 27.3 years) and lean (mean BMI 20.9 kg/m^2) males and analysed the recovery of ^{13}C in breath CO_2 as a measure of whole-body dietary fatty acid oxidation. They found significantly greater recovery of ^{13}C from oleate, compared to ^{13}C from linoeate in breath (15.1% vs 10.2% cumulative recovery after 9 hours, respectively), and this occurred to a greater extent than the appearance of ^{13}C from stearate (2.9% cumulative recovery after 9 hours) [99]. In a cross-over study, Schmidt *et al.*, [100] fed ^{13}C-labeled oleate and palmitate in frequent (every 20 minutes) small meals over 7 hours to ten young (mean age 24.7 years) and lean (mean BMI 22.9) men and women and found a 21% (95% CI 10-32%) higher oxidation of oleate compared with palmitate. Finally, DeLany *et al.*, [101] fed ^{13}C-labeled laurate, palmitate, stearate, oleate, elaidate, linoleate and linolenate in random order as part of a single meal to 4 normal-weight men. It was found that oxidation of SFA decreased with increasing carbon number and oxidation of 18-carbon fatty acids was

positively correlated with the number of double-bonds; the overall order of oxidation was laurate > linolenate > elaidate > oleate > linoleate > palmitate > stearate [101]. However, palmitate, stearate, oleate and linoleate were not statistically different from each other, with cumulative recoveries of ^{13}C in breath CO_2 ranging between 11% and 17% 9 hours after the meal [101]. On the basis of the limited, available evidence it appears that there may be differential oxidation of unsaturated and SFA however it would be important to determine if these responses diverge between males and females, as we have previously reported sexual dimorphism in dietary fatty acid oxidation [102]. Regardless, if different fatty acids are differentially oxidised or not, some (unsaturated) fatty acids may potentially stimulate general fat oxidation by activating transcription factors such as PPARα [103]. Whether this translates to meaningful effects *in vivo* in humans has, to our knowledge, not yet been demonstrated and evidence would in fact suggest a contrasting response as, somewhat counterintuitively, a synthetic PPARα agonist was recently observed to increase IHTG content in subjects with NAFLD [86].

The results from studies using indirect calorimetry to assess fasting and postprandial energy expenditure and fat oxidation are conflicting and challenging to interpret. For instance there are reports of increased postprandial fat oxidation after meals enriched in unsaturated fatty acids when compared to meals enriched in SFA [104-106], and there is some evidence to suggest that consuming diets enriched in oleic acid results in a greater postprandial fat oxidation compared to diets enriched in SFA. However, not all studies are in agreement with these findings, as they have found no difference in postprandial fat oxidation following meals that are high in SFA or unsaturated fatty acids [107-110], or following diets enriched in fatty acids of different saturation status [111,112]. Indeed, it was found that consuming a diet enriched in MUFA for 28 days, resulted in a reduced postprandial fat oxidation compared to diets enriched in SFA or trans-fatty acids [113]. Plausible explanations for the discrepancies in findings between studies include differences in study design (*e.g.* single meal crossover vs. interventions lasting several weeks), study populations (*e.g.* sex and BMI) and different methodology used (*e.g.* metabolic chamber vs. ventilated hood) and the total macronutrient composition and content of test meals given to participants.

In regards the effects of n-3 PUFA on IHTG, work from animal and cellular models have demonstrated that EPA and DHA affect the metabolic nuclear receptors, liver X receptor (LXR), hepatocyte nuclear factor-4α (HNF4-α), farnesol X receptor (FXR) and peroxisome

proliferator-activated receptors (PPARs); all of which play a role in modulating plasma TG concentrations [114] and presumably IHTG content. Therefore, a proposed model for the effects of n-3 PUFA (namely EPA and DHA) on IHTG accumulation is at the level of gene transcription by co-ordinately suppressing hepatic lipogenesis through SREBP-1c inhibition and up-regulating hepatic fatty acid oxidation through PPAR activation [114]. Taken together the coordination of these effects would have the net result of an intrahepatic effect of repartitioning fatty acids away from esterification and toward oxidation pathways. In support of this, in a pilot study we observed that in individuals who achieved a change in erythrocyte DHA enrichment ≥2%, after 15-18 months supplementation with EPA+DHA (4g /day) there was a significant reduction in fasting hepatic DNL, along with a more notable (although non-significant) decrease in IHTG content compared to individuals who had a <2% change in DHA abundance (placebo group) [115]. As this was only a pilot study, further work is required to confirm these observations.

The findings that n-6 PUFA could decrease IHTG and/or prevent IHTG accumulation (compared to SFA) is generally supported by animal studies, but the primary mechanism remains unclear. Animal studies have suggested differential effects on hepatic DNL but this is not supported by findings in humans. Konrad *et al.,* fed isocaloric diets high or low in palmitate and linoleate for 21 days to healthy males and females and found no difference in isotopically assessed hepatic DNL [116]. Similarly, Luukkonen *et al.,* found no difference in isotopically assessed hepatic DNL after overweight/obese adults consumed hypercaloric diets rich in SFA or unsaturated fats for three weeks [63] despite IHTG increasing to a notably greater extent on the SFA diet. Together these data imply that hepatic DNL is not the primary mechanism behind the differential effects of SFA and n-6 PUFA on liver fat content in humans.

In addition to oxidation and storage pathways, palmitate derived from both hepatic DNL and dietary sources may be further metabolised within the liver, *e.g.* desaturated by stearoyl-CoA desaturase (SCD) to generate palmitoleate and/or elongated to generate stearate, vaccinate, and oleate. Whether partitioning through these pathways is an important mediator for liver fat accumulation in response to dietary SFA (or PUFA) and although animal studies suggest a potential role for SCD [117], this has not been investigated in humans. The overall intracellular partitioning of DNL-derived lipids is likely to be of importance and may, in part, explain inter-individual outcomes in response to fatty acid overload [118]. It could be

speculated that linoleate may modify IHTG accumulation by affecting the efficiency of partitioning through these pathways as linoleate is known to repress SCD [117]. More studies are needed on the potential role of fatty acid desaturation and elongation and the effects on IHTG in response to different diets. However, disentangling what is causally-related compared to what is an adaptive mechanism to reduce further harm, or responses that just parallel phenomena is challenging to decipher in human studies.

Conclusion

Taking all the available evidence to date, clearly demonstrated that total calorie intake is a key mediator of IHTG accumulation, with hypercaloric diets increasing, and hypocaloric diet decreasing IHTG content, irrespective of dietary composition (Figure 2). In addition to this, there is now emerging evidence to suggest that dietary fatty acid composition may also play a role in regulating of IHTG, with diets enriched in SFA being associated with greater IHTG accumulation than diets enriched in unsaturated fatty acids; further work is needed to understand the mechanistic basis for this divergence in the effect on IHTG accumulation. Supplementation with n-3 PUFA (namely EPA and DHA) appears to decrease IHTG, although more studies are required to clearly demonstrate this and whether the same effect can be achieved through dietary intake (rather than supplements) alone, remains to be elucidated. Finally, more work is required to determine the mechanisms by which specific fatty acids elucidate their effects on IHTG content, and although challenging, taking time to develop *in vitro* models that better replicate the physiological conditions (i.e. mixture of fatty acids and sugars) and the human of disease of interest (i.e. NAFLD) would aid in bridging this knowledge gap.

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Figure legends

Figure 1. Overview of hepatic fatty acid (FA) input, synthesis and disposal in the postprandial state. FA input to the liver derives from 1) the lipolysis of adipose (subcutaneous and visceral) tissue triacylglycerol, and 2) dietary fat, which enter the liver as either chylomicron remnants or chylomicron-derived spillover fatty acids. FA synthesis occurs within the liver, via *de novo* lipogenesis (DNL) which involves the synthesis of FA from acetyl-CoA derived from non-lipid precursors, such as glucose. These FA enter a common pool and can then be broadly partitioning between two pathways for disposal. One is the esterification pathway, where predominantly triacylglycerol (TG) is produced which can then be either stored in the cytosol (as a lipid droplet) or can lipidate very-low density lipoprotein (VLDL) in the endoplasmic reticulum (ER) to form VLDL-TG and then secreted into the systemic circulation. The other possible fate for fatty acid disposal is oxidation either via the tricarboxylic acid (TCA) cycle to form CO_2, or the ketogenic pathway where β-hydroxybutyrate (3OHB) is produced and enters the systemic circulation.

Figure 2. Overview of dietary hyper- and hypo-calorie dietary intervention studies and the observed relative liver fat content. The time of the intervention, along with the caloric (or body weight) increase or decrease is shown, along with the type of fat (where known) that was increased.