REVIEW

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Liver fat storage pathways: methodologies and dietary effects

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Purpose of review
Nonalcoholic fatty liver is the result of an imbalance between lipid storage [from meal, *de novo* lipogenesis (DNL) and fatty acid (FA) uptake] and disposal (oxidation and VLDL output). Knowledge on the contribution of each of these pathways to liver fat content in humans is essential to develop tailored strategies to prevent and treat nonalcoholic fatty liver. Here, we review the techniques available to study the different storage pathways and review dietary modulation of these pathways.

Recent findings
The type of carbohydrate and fat could be of importance in modulating DNL, as complex carbohydrates and omega-3 FAs have been shown to reduce DNL. No effects were found on the other pathways, however studies investigating this are scarce.

Summary
Techniques used to assess storage pathways are predominantly stable isotope techniques, which require specific expertise and are costly. Validated biomarkers are often lacking. These methodological limitations also translate into a limited number of studies investigating to what extent storage pathways can be modulated by diet. Further research is needed to elucidate in more detail the impact that fat and carbohydrate type can have on liver fat storage pathways and content.

Keywords
dietary modulation, fatty acid storage, nonalcoholic fatty liver, stable isotope techniques

INTRODUCTION

A dramatic rise in the prevalence of nonalcoholic fatty liver (NAFL) has been observed over the last few decades and it is now considered to be the most common liver disorder worldwide [1]. NAFL is characterized by excessive fat accumulation in the liver that is not associated with high alcohol consumption and NAFL can progress to more severe stages of liver disease. Importantly, even if no further progression of liver disease occurs, NAFL *per se* is also very strongly associated with metabolic diseases such as cardiovascular disease and type II diabetes [2–4]. Excessive fat accumulation in the liver is thought to be the result of an imbalance between lipid storage (due to increased delivery and synthesis), and disposal (Fig. 1). It is now well established that fat that is stored in the liver (in hepatocytes) originates from three main sources: first, direct fat storage from a meal; second, *de novo* synthesis of fatty acids (FAs) from glucose, fructose or amino acids (*de novo* lipogenesis; DNL); third, from uptake of plasma non-esterified FAs (NEFA) mainly derived from adipose tissue lipolysis (Fig. 1). Knowledge on the contribution of each of these pathways to liver fat content in humans is sparse [5,6], in part because appropriate techniques are lacking. Gaining a better understanding of the mechanisms, which contribute to hepatic fat accumulation is crucial to the development of effective treatment strategies for NAFL and its associated metabolic disturbances. Here, we discuss the techniques available to study

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KEY POINTS

- Stable isotope techniques can be used to accurately determine liver fat storage pathways.
- High-carbohydrate diets increase liver fat mainly by strongly stimulating DNL.
- Dietary fat retention in liver seems to be robust and not strongly affected by diet composition.
- Non-esterified FAs uptake is the main contributor to liver fat, however only limited number of studies examined if it is affected by dietary fat and carbohydrate composition, and therefore needs further study.

storage pathways contributing to NAFL and review recent nutritional studies using these techniques to investigate whether these pathways can be modulated by diet.

TECHNIQUES DETERMINING DIETARY FAT UPTAKE

Following a meal, dietary fat is taken up in the enterocytes where chylomicrons are formed that will enter the systemic circulation. As the particles deposit triglyceride in muscle and adipose tissue, chylomicron remnants are formed. The liver is the major site for uptake of these remnant particles.

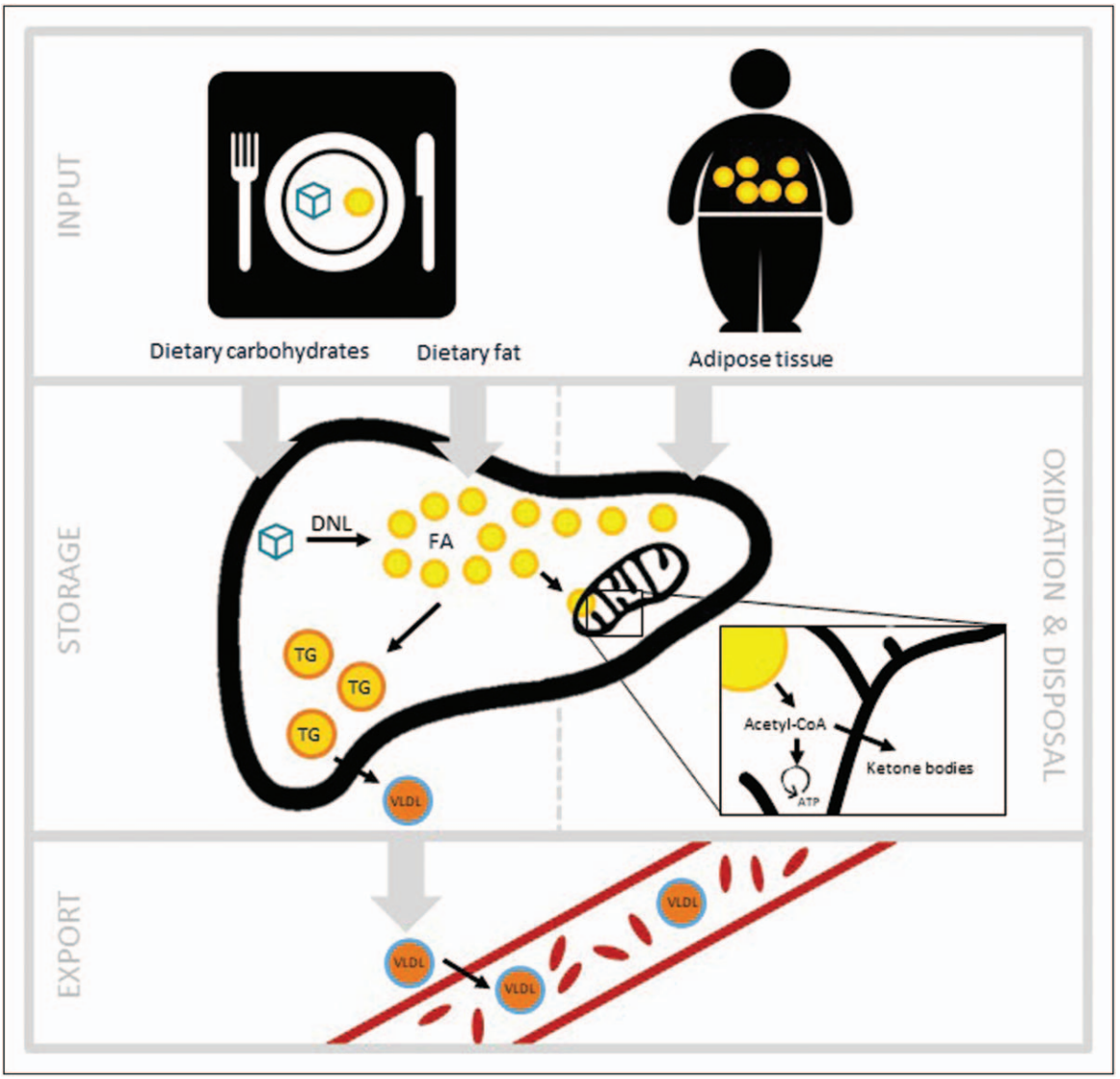

FIGURE 1. Overview of liver fat storage and disposal pathways. Storage pathways include direct fat storage from a meal, *de novo* lipogenesis from carbohydrates and adipose tissue derived non-esterified fatty acid uptake. Liver lipid disposal pathways are mitochondrial fatty acid oxidation and ketogenesis after initial ß-oxidation (acetyl-CoA disposal), and triglyceride incorporation into VLDL-particles to be secreted into the circulation.

Dietary fat uptake is commonly measured by using FA tracers enriched with the stable carbon isotope ^{13}C [7,8,9▪▪,10▪▪] or deuterium (^{2}H) [5,6,11,12], due to their low natural abundance (1.1 and 0.015%, respectively). Usually, a meal with ^{13}C-labeled palmitate, deuterated tri-palmitate or [$^{2}H_{35}$] stearate is given to trace incorporation of meal fat in VLDL triglyceride (VLDL-TG) [5–8,9▪▪,10▪▪,11,12]. The FA composition and the tracer enrichment were shown to be similar in VLDL-TG and liver triglyceride (determined from liver biopsies), therefore the tracer enrichment of plasma VLDL-TGs can be used as a surrogate for liver fat enrichment and can be used to determine hepatic storage of meal fat [5,13]. Isotopic enrichments in VLDL-TG are generally determined by gas chromatography–mass spectrometry.

To investigate tracer enrichments directly in the liver, liver biopsies have been used [5]. An alternative approach is through magnetic resonance spectroscopy (MRS) or PET methodology, assessing which proportion of the lipids in a meal is ending up in the liver. MRS techniques can be used to measure ^{13}C enrichment directly in the liver after consumption of ^{13}C–labeled FAs [14,15]. With so called ^{13}C-edited methods, the superior sensitivity and localization of ^{1}H-MRS can be used to quantify the signal of ^{1}H nuclei directly linked to ^{13}C and therefore, the ^{1}H-MRS signal becomes proportional of ^{13}C enrichment ('indirect' ^{13}C spectroscopy or ^{13}C-edited ^{1}H-MRS). Indeed, it was shown that such indirect ^{13}C spectroscopy can be used to 'track' the ^{13}C-FAs originating from a meal [15,16▪]. Since the ^{13}C signal is followed over time in the liver, the measured ^{13}C signal in the liver reflects net storage of dietary fat (uptake minus disposal), also referred to as dietary fat retention.

PET has been used in combination with oral intake of 14(R,S)-[(18)F]fluoro-6-thia-heptadecanoic acid (^{18}FTHA) tracer, a long chain FA analog containing ^{18}fluor [17]. The radioactive signal of this tracer can be measured in time and in different target organs, including the liver. ^{18}FTHA cannot be metabolized after entering the organs, but can be esterified and incorporated in protein complexes and therefore can leave the liver when secreted in VLDL. Therefore, it reflects the balance between uptake and export, where oxidation is not considered [17].

TECHNIQUES DETERMINING *DE NOVO* LIPOGENESIS

DNL is another pathway contributing to liver fat accumulation. Acetyl-CoA, derived from catabolic pathways of carbohydrates or amino acids, serves as the main substrate for this process. Most frequently, ^{13}C-acetate [5,6,11,12,18–25] and deuterium oxide [8,9▪▪,26–31,32▪▪] have been used in studies to determine DNL contribution to liver fat, and have already been described elaborately in earlier reviews [33,34]. In short, ^{13}C-acetate is intravenously infused and will be converted into acetyl-CoA in hepatocytes, thereby labeling the intrahepatic acetyl-CoA pool and becoming a substrate for DNL to ultimately end up in newly formed palmitate. Based on tracer enrichments in the intrahepatic acetyl-CoA precursor pool and the product pool of VLDL-palmitate, fractional synthesis of FAs can be determined from the precursor to product ratio using mass isotopomer distribution analysis [35,36]. Less demanding is the use of deuterium oxide, which is administrated orally and enriches the body water pool in deuterium. Consequently, deuterium will also be incorporated in NADPH, a metabolite that is used in the last step of the DNL pathway for the *de novo* synthesis of palmitate, thus labeling the palmitate formed in DNL [33].

In addition to the use of stable isotope tracers, plasma FA levels/ratios are often used to infer hepatic DNL, as reviewed before [33]. In large-scale studies, where more costly and time-consuming techniques would not be feasible, these indices can be used as an alternative marker for tracer-based methods. The most widely used plasma (VLDL-TG) marker is the lipogenic index (16 : 0/18 : 2*n*6) [7,24,37–39], which has been shown to be in agreement with ^{13}C labeled acetate measurements following a high-carbohydrate diet [24]. Furthermore, the percentagewise increase in palmitate (new palmitate) upon fructose (and glucose) feeding has been suggested as marker for DNL [40]. Important to note is that these markers should be used within the defined feeding conditions they are designed for, namely high simple carbohydrate and fructose feeding, as recently it has been shown that the lipogenic index poorly reflects DNL in habitual diet conditions [41▪▪]. This is likely due to the significant effect that dietary fat intake can have on the lipid composition, and thereby also palmitate content, of VLDL-TG. The Stearoyl-CoA desaturase index of 16 : 1*n* – 7 to 16 : 0 ($SCD1_{(16)}$) has also been linked to DNL [7,42], however also here its use under habitual diet conditions has been questioned [41▪▪].

TECHNIQUES DETERMINING NON-ESTERIFIED FATTY ACID UPTAKE

The largest contributor to hepatic fat originates from uptake of plasma NEFA, mainly originating from adipose tissue lipolysis, while spillover FAs can also contribute. Contribution of NEFA to liver fat can be assessed using intravenous infusion of palmitate tracer, to label the plasma NEFA pool, and subsequent determination of tracer enrichment in

VLDL-TG. The assumption is made that palmitate is representative for all plasma free FAs with respect to turnover and incorporation in VLDL [43]. Basically, the method is similar to the method used to measure dietary fat uptake by labeling dietary FAs. However, by infusing the labeled palmitate instead of providing the tracer orally, the plasma NEFA pool is labeled and the palmitate that will be taken up by the liver will represent NEFA contribution to liver fat. Tracing back the labeled palmitate in VLDL-TG thus provides information on the contribution of plasma NEFA to liver fat. The most frequently used palmitate tracer is ^{13}C-labeled palmitate [5,6,8,11,20], but also intravenous deuterium palmitate tracers have been used to assess NEFA contribution to VLDL-TG [12,44]. The preferred tracer depends on whether the measurement is combined with other tracers and which isotopes these contain.

In addition to tracing the labeled FAs in VLDL-TG, FA radiotracers have also been used in combination with PET imaging [45–47]. In this respect, the earlier mentioned ^{18}FTHA tracer can be used to trace NEFA uptake by the liver. Upon intravenous injection, FTHA dilutes in the NEFA pool and can be taken up by the liver. The amount of FTHA trapped in the liver determined with PET imaging provides information on the balance between hepatic NEFA uptake and export, as FTHA cannot be oxidized. Another FA tracer that has been combined with PET imaging is ^{11}C-labeled palmitate [48,49]. In contrast to FTHA, ^{11}C-labeled palmitate can be oxidized completely and therefore fat oxidative rates can be determined by using compartmental modeling. Also the uptake of FA can be determined with ^{11}C palmitate.

NUTRITIONAL EFFECTS ON DIETARY FAT UPTAKE

Nutritional effects on dietary fat contribution to fattening of the liver are hardly studied, likely due to the fact that this source is the smallest contributor with reported values of around 10–20% of the total liver fat pool [5,6,15]. In 2008, a study performed by Chong *et al.* [7] showed by using oral administration of [U-^{13}C]palmitate that dietary fat contribution to VLDL-TG was similar upon a 3-day high-fat and 3-day high-carbohydrate diet in eight healthy volunteers (around 15% 6 h after a mixed meal). Recently, several nutritional intervention studies have been performed focusing on the type of fat and carbohydrate. Parry *et al.* [10▪▪] showed that, compared with a 4-week diet enriched with free sugar, a 4-week diet enriched with saturated fat (SFA) increased liver fat content and exaggerated postprandial plasma glucose and insulin responses in 16 overweight males. There was, however, no difference in dietary fat contribution to VLDL-TG, with values around 5–10% 6 h after a meal as determined by using [U-^{13}C]palmitate [10▪▪]. Another study, by Green *et al.* [9▪▪], investigated the effect of omega-3 FA supplementation [4 g/day eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) as ethyl esters] for 8 weeks in 38 healthy men and did not find differences in dietary fat contribution either, as measured 6 h after a meal and compared with baseline. Also here, liver fat content did change upon the nutritional intervention [9▪▪]. These results suggest that the effect of type of FA on liver fat content is not specifically due to changes in dietary fat contribution. However, as these studies lack a proper control arm, it remains not unequivocally determined what the exact potential impact of fat type on dietary fat contribution is. The type of carbohydrates in a meal might also influence dietary fat contribution to liver fat. A randomized cross-over study investigating the effects of a high-fructose/low-glucose meal compared with a low-fructose/high-glucose meal on DNL, FA partitioning and dietary FA oxidation, showed by using [U-^{13}C]palmitate that in 16 healthy volunteers the relative contribution of dietary FAs to VLDL-TG 6 h after meal consumption is lower after a high-fructose/low-glucose compared with a low-fructose/high-glucose meal [32▪▪]. The absolute amount of dietary fat contribution was however not significantly different between the two meals. Together with above mentioned results this suggests that the relative contribution of meal-derived fat storage is rather robust. To date, spectroscopy and PET methods have not been used to investigate the impact of nutrition on dietary fat contribution.

NUTRITIONAL EFFECTS ON *DE NOVO* LIPOGENESIS

DNL can be a significant contributor to liver fat accumulation, as is shown by increased fasting DNL contribution to VLDL-TG in people with NAFL (20–25% vs. 5–10% in healthy individuals) [5,6,8]. Postprandially, DNL contribution is expected to be higher, and indeed, contribution of 20–25% to VLDL-TG after two meals were reported in healthy individuals determined by ^{13}C-acetate experiments to measure DNL contribution up to 11 h after the first meal in six individuals [6]. Effects of dietary interventions on DNL have been studied frequently. Specifically, the effect of dietary carbohydrate and fat on DNL has been a topic of great interest. Using the before mentioned tracer methodologies, several studies indicate that high-carbohydrate diets increase fasting and postprandial fractional DNL in both lean and obese volunteers when compared with diets high in fat and similar in protein

[20,24,30,39,50,51]. Mardinoglu *et al.* [52] found that replacing carbohydrates (4 vs. 40% energy) by both fat (72 vs. 42% energy) and protein (24 vs. 18% energy) for 14 days in 10 overweight/obese volunteers with NAFL rapidly reduced fasting DNL, as determined by deuterium oxide. Importantly, the reduction in DNL was associated with other favorable metabolic changes as increased ß-hydroxybutyrate, reflecting increased liver fat oxidation, probably underlying the drastic reduction of 44% in liver fat content over the 14-day study period. Furthermore, overfeeding with simple carbohydrates for 3–4 weeks has been shown to increase DNL, as measured by deuterated water and lipogenic index, parallel to an increase in liver fat [37,38,53]. The effect of carbohydrate intake on DNL may be dependent on the type of carbohydrate consumed, as DNL rates have been reported to be higher upon meals/diets high in fructose than meals/diets high in glucose or complex carbohydrates [19,21,32▪▪] and it has been shown in a small study population of three healthy volunteers that an increase in palmitate-rich and lineolate-poor VLDL-TG mediated by a 10-day high-sugar diet can be reduced by 7–10 day substitution of dietary starch for sugar [54]. Dietary fat composition might also influence DNL, as Green *et al.* [9▪▪] recently showed by using deuterated water that 8-week supplementation with the omega-3 FAs EPA and DHA at a dose of 4 g/day decreased both fasting and postprandial DNL compared with baseline in 38 healthy men. Protein content could also be of interest in modulating hepatic DNL, as a randomized crossover study in nine healthy males comparing the effects of a control meal (15% protein) and an isoenergetic high-protein meal (lower in fat and carbohydrate, 32% protein) showed that the lipogenic index (C16 : 0/C18 : 2) was increased 4 h after the high-protein meal compared to the control meal [55▪▪].

NUTRITIONAL EFFECTS ON NON-ESTERIFIED FATTY ACID UPTAKE

The largest contributor to hepatic fat, at least in the fasted state, is NEFA uptake with a contribution of around 60–65% [5,6]. Nevertheless, nutritional studies focusing on dietary impact on hepatic NEFA contribution are limited. Parks *et al.* [20] determined NEFA contribution to VLDL-TG using intravenous infusion of ^{13}C-palmitate tracer upon a 1-week control diet (35% fat) and upon a following 5-week low-fat/high-carbohydrate diet (15% fat) in six healthy volunteers and five hypertriglyceridemic volunteers, and showed that the contribution in the fasting state was lower upon the low-fat diet in hypertriglyceridemic volunteers, but not different in healthy volunteers. NEFA contribution to VLDL-TG has also been compared between a 3-day high-carbohydrate/low-fat diet and 3-day high-fat/low-carbohydrate diet in a randomized crossover study, showing no differences in NEFA contribution 6 h post meal in eight healthy volunteers by using intravenous infusion of $^{2}H_2$-palmitic acid [7]. Recently, NEFA contribution was compared between a 4-week relatively high-fat diet enriched in SFA and a 4-week relatively high-carbohydrate diet enriched in free sugars under eucaloric conditions, using $^{2}H_2$-palmitate in sixteen overweight males [10▪▪]. Previously, it was found that under conditions of excess calorie intake, overconsumption of SFA increases liver fat content to a larger extent (55% relative increase) as compared with overconsumption of free sugars (33% relative increase), independent of body weight changes [53]. The increase upon excess SFA intake was found to be mediated by increased lipolysis rates, suggesting larger NEFA contribution [53]. Under eucaloric conditions however, NEFA contribution 6 h after meal consumption was not increased upon 4-week high-SFA intake when compared with 4-week high simple carbohydrate intake, consistent with similar effect of these diets on liver fat content [10▪▪]. Similar as mentioned for the studies on dietary fat retention, PET techniques have not been used to investigate the dietary effects on NEFA contribution.

FUTURE DIRECTIONS

Of the three different storage pathways, DNL has been studied most extensively, with diets high in carbohydrates (and especially fructose and simple sugars) leading to the strongest stimulation of DNL. The other pathways have been studied less intensively and need further investigation as both have been shown to significantly contribute to liver fat [5,6,15]. Important to take into consideration is the composition of the different macronutrients, as some recent studies have shown that specific types of carbohydrate and fat could have distinct effects, mainly on DNL. To investigate such nutritional effects, MRS and PET imaging methodologies, which have hardly been applied, can be of great value.

CONCLUSION

Despite the availability of a wide range in techniques to measure liver fat storage pathways, knowledge on the effect of nutrition on the contribution of each pathway to liver fattening in humans is still very limited. This is most likely due to the specialized expertise and facilities needed to perform isotope tracer studies and the high costs of such studies.

Future research on the modulation of storage pathways is however crucial to the development of effective treatment strategies for NAFL and its associated metabolic disturbances.

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Conflicts of interest

There are no conflicts of interest.

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The article shows that a high-protein meal can induce *de novo* lipogenesis as indicated by an increased lipogenic index. These results indicate that high-protein feeding to tackle metabolic disease requires greater consideration.