Liver substrate metabolism in non-alcoholic fatty liver

Liver substrate metabolism in non-alcoholic fatty liver:

role of hepatic lipid composition and hepatic glycogen measured by MR-techniques

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CHAPTER 1

General introduction and outline

Non-alcoholic fatty liver

Obesity and overweight are a major worldwide problem. Worldwide obesity rates have tripled in the last 45 years (1). In 2016, 13% of the adults were obese and 40% of the adults were overweight (1). Obesity and overweight are characterized by excessive fat storage in adipose tissue. However, fat is also stored in non-adipose tissue, such as in liver, muscle and heart. Such fat storage is referred to as ectopic fat storage. In the liver, fat can be stored in hepatocytes and when intrahepatic lipids exceed 5% of liver weight, in absence of excessive alcohol consumption, this is defined as non-alcoholic fatty liver (NAFL). Currently, NAFL is the leading cause of chronic liver disease and it is estimated that 25% of the worldwide population has NAFL (2). Moreover, NAFL is even more prevalent in obesity, with rates estimated as high as 55-75% (3, 4). NAFL can progress to more severe stages of liver disease, such as non-alcoholic steatohepatitis (NASH) and liver cirrhosis, conditions in which liver function is severely hampered and the risk for hepatocellular carcinoma is increased. Importantly, NAFL *per se* is already very strongly associated with metabolic diseases such as cardiovascular disease (CVD) and type II diabetes (T2D) (5-7). As obesity is still on the rise and NAFL prevalence, together with its metabolic consequences, is expected to increase even further, it will have a tremendous clinical and economic impact (2). Knowledge on how NAFL contributes to cardiometabolic disease is limited, particularly because of the limitations in techniques available to study this relationship non-invasively. Thus, advanced methodologies are needed to investigate metabolic processes in NAFL.

Advanced MR methodologies to assess metabolic processes in NAFL

Non-invasive imaging techniques, such as magnetic resonance spectroscopy (MRS) can be applied to acquire metabolic information. MRS yields chemical information of tissues, thereby making it a useful technique to study substrate stores and dynamics in different organs. As such, MRS has been shown to be of great value in determining ectopic lipid accumulation. In the liver, ^{1}H-MRS is the gold standard to determine fat content. Using ^{1}H-MRS, a spectrum is generated from which the lipid and water resonances can be quantified and from these resonances, intrahepatic lipid (IHL) content can be calculated. This technique has been used frequently in human studies, showing that IHL stores relate to hepatic insulin resistance (8, 9) and are increased in patients with type 2 diabetes (10) and furthermore, change upon exercise (11-13) and dietary (14-17) interventions. Apart from determining the total amount of fat stored in the liver, ^{1}H-MRS could also be used to get more qualitative information of stored IHL (i.e. type of fatty acids; saturated (SFA), mono-unsaturated (MUFA), poly-unsaturated fatty acids (PUFA)). Determining the hepatic fatty acid fractions is however challenging as the resonance of the unsaturated fatty acids are

small, resonances are overlapping and imperfect suppression of the large water resonance can lead to contamination of other resonances. So far, parameters linked to the degree of unsaturation have been measured in some studies (18-21). These parameters indicate the relative abundance of double bonds in the fatty acid signal, however they do not specifically and robustly differentiate between hepatic SFA, MUFA and PUFA fractions. Such separation in SFA, MUFA and PUFA can yield interesting metabolic information as it is suggested that specifically SFA may have detrimental effects on metabolic health and the nature of fatty acid can also be indicative of the source of the hepatic fat. Other nuclei that can be investigated using MRS include ^{13}C. Direct ^{13}C-MRS has been applied on the liver in the past and can be used to determine hepatic glycogen stores. Studies have applied this method showing reductions in hepatic glycogen during the fasting period (22-24), postprandial increases in hepatic glycogen (23, 25) and defects in hepatic glycogen metabolism in patients with type 2 diabetes (23). Furthermore, hepatic glycogen has been shown to change upon dietary changes in glycaemic index (16). Presently, very little is known about glycogen dynamics in NAFL, even though it may be highly relevant in hepatic substrate partitioning. These advanced MRS techniques can be applied to investigate hepatic substrate metabolism and to better understand the metabolic changes that take place during the development of NAFL.

Thesis outline

The aim of this thesis is to investigate liver substrate metabolism in NAFL using advanced MR methodology. In **chapter 2** the techniques available to study the different pathways leading to IHL accumulation are reviewed. Furthermore, studies using these techniques to estimate the relative contribution of the different pathways to IHL accumulation or to study dietary modulation of these pathways are discussed. *De novo* lipogenesis (DNL) is one of these pathways and represents the synthesis of fatty acids from carbohydrates. DNL is found to be strongly increased in NAFL and may be one of the underlying pathways leading to NAFL. The end product of DNL is SFA, and therefore high DNL rates might contribute to an increased hepatic SFA fraction. It is suggested that specifically these SFA negatively influence metabolic health. Therefore, in **chapter 3** a novel magnetic resonance technique is developed, validated and applied, that enabled non-invasively quantification of the fractions of hepatic SFA, MUFA and PUFA separately, in healthy and metabolically compromised human volunteers. Using this methodology, the hypothesis that higher rates of DNL are associated with an increased fraction of SFA in human liver is tested. Additionally, it is investigated if populations at higher risk to develop metabolic complications are

characterized by altered hepatic fatty acid composition, and whether hepatic fatty acid composition is related to hepatic insulin sensitivity.

Hepatic lipid- and carbohydrate metabolism are strongly intertwined and a key factor in determining substrate metabolism and partitioning towards oxidation or storage may be hepatic glycogen. Glycogen represents a dynamic local carbohydrate store and is usually thought to change dynamically over 24 hours; decreasing during fasting and being replenished upon refeeding. The fasting-induced glycogen depletion is generally thought of being a healthy reflection of metabolic flexibility and it is suggested that the regular depletion of hepatic glycogen stores may be underlying the beneficial effects seen with time restricting eating regimes. To study the importance of hepatic glycogen stores in NAFL, prolonged overnight fasting is used as a tool to modulate hepatic glycogen in volunteers with NAFL in **chapter 4**. In this chapter it is investigated whether prolonged fasting would acutely lower hepatic glycogen and thereby, improve substrate oxidation during the night and postprandially in volunteers with NAFL. Additionally, effects of 5 days prolonged fasting on liver fat content and composition are studied.

In **chapter 5** it is investigated whether changing the degree of fat saturation and the glycemic index (GI) in the diet could influence IHL content. Human studies investigating the effect of dietary fat and carbohydrate type on IHL are limited to proof of principle studies with exaggerated differences in diet composition. Therefore, two two-week diets differing in GI and SFA content are designed to study the effect of changes in dietary type of carbohydrate and fat, while still matching dietary patterns of the general population. Furthermore, it is studied whether a low vs. high GI/SFA diet increased whole-body fat oxidation, decreased glycemic response and reduced hepatic glycogen levels.

Chapter 6 focusses on the importance of different factors that could influence IHL quantification. Specifically, it is evaluated how variations in T2 relaxation times affect the calculations of absolute IHL% (w/w) and to what extend the bias occurs while using different IHL quantification formulas. In **chapter 7** advanced techniques are applied to assess the metabolic effect of six-week nicotamine riboside (NR) supplementation. Results from animal studies suggest that NR, as an NAD+ precursor, can have metabolically beneficial effects, counteracting the negative effects of overweight and aging, however, human data is largely lacking. Therefore, insulin sensitivity and IHL content are investigated by hyperinsulinemic-euglycemic clamp technique and MRS respectively after NR and placebo. Finally, in **chapter 8** the main results and conclusions of the previous chapters in this thesis are discussed in a broader perspective. Furthermore, future directions in the field of liver substrate metabolism are discussed.

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CHAPTER 2

Liver fat storage and disposal pathways: methodologies, contributions to liver fat accumulation and dietary effects

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ABSTRACT

Non-alcoholic fatty liver (NAFL) is the most common liver disorder in western society and is strongly associated with metabolic diseases such as cardiovascular disease and type II diabetes. This condition of excessive liver fat storage is the result of an imbalance between lipid storage (direct storage of fat from a meal, *de novo* lipogenesis and uptake of non-esterified fatty acids mainly derived from adipose tissue lipolysis) and disposal (oxidation and secretion). Knowledge on the contribution of each of these pathways to liver fat content in humans is essential in order to develop strategies to prevent and treat NAFL. Techniques used to assess these individual pathways are predominantly stable isotope and radioisotope techniques, which require specific expertise and are costly. Validated indirect markers that are easier to apply, are often lacking. These methodological limitations also translate into a limited amount of studies that have been performed to investigate to what extent the different accumulation and disposal routes can be modulated by diet. Here, we review the techniques available to study the different pathways leading to liver fat accumulation. Furthermore, we will review studies using these techniques to estimate the relative contribution of the different pathways to liver fat accumulation or to study dietary modulation of these pathways.

INTRODUCTION

A dramatic rise in the prevalence of non-alcoholic 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. NAFL can progress to more severe stages of liver disease, such as non-alcoholic steatohepatitis (NASH) and liver cirrhosis, conditions in which liver function is severely hampered and the risk for hepatocellular carcinoma is increased. 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 (CVD) and type II diabetes (T2D) (2-4). Strikingly, the prevalence of NAFL in obese people is estimated to be as high as 55–75% (5, 6). Therefore, reducing liver fat content is a promising target to decrease the risk of these metabolic diseases.

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. It is now well established that fat that is stored in the liver (in hepatocytes) originates from three main sources: 1) direct fat storage from a meal, 2) *de novo* synthesis of fatty acids from glucose, fructose or amino acids (*de novo* lipogenesis; DNL) 3) from uptake of plasma non-esterified fatty acids (NEFA) mainly derived from adipose tissue lipolysis (figure 1). In healthy individuals, these processes are balanced by the activation of metabolic pathways that contribute to the mobilization and clearance of hepatic fat. These pathways include: 1) mitochondrial fatty acid oxidation for the production of ATP and fatty acid shuttling towards ketogenesis after initial ß-oxidation (acetyl-CoA disposal), 2) fatty acid incorporation into VLDL-particles to be secreted into the circulation (figure 1). While the pathways leading to hepatic fat accumulation have long been identified, knowledge on the contribution of each of these pathways to liver fat content in humans is sparse, 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.

In this review, an overview of the techniques available to study the different pathways leading to liver fat accumulation will be provided. Furthermore, we will review studies using these techniques to estimate the relative contribution of the different pathways to liver fat accumulation or to study dietary modulation of these pathways.

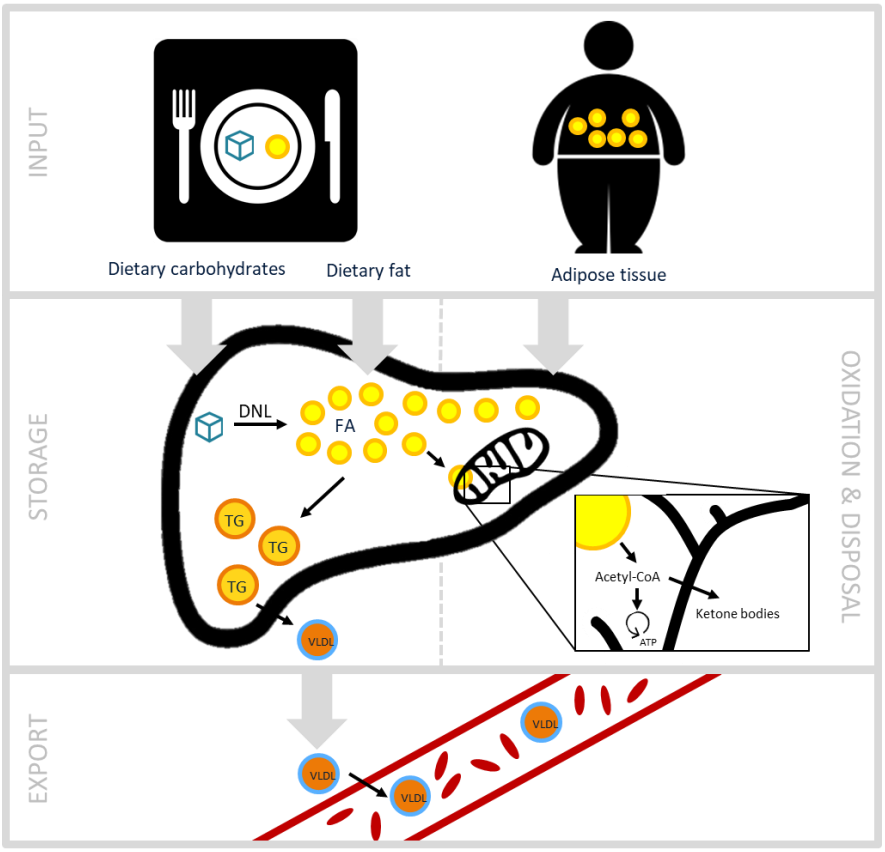

Figure 1: Overview of liver fat storage and disposal pathways. Storage pathways include direct fat storage from a meal, *de novo* lipogenesis (DNL) from carbohydrates and adipose-tissue derived non-esterified fatty acids (NEFA) uptake. Liver lipid disposal pathways are mitochondrial fatty acid (FA) oxidation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

1. DIETARY FAT STORAGE

1.1 Pathway description

Following a meal, dietary fat is taken up in the intestines (7). In the enterocytes chylomicrons (CM) are formed. These large complexes of triglycerides (TG), phospholipids, cholesterol and proteins enter the circulation via the lymphatic system (7). The CM particles are usually too big to pass the fenestrations of hepatic sinusoids and will not reach the surface of the hepatocyte (8). Therefore, the particles first need to be 'cleared' by lipoprotein lipase (LPL)-mediated hydrolysis in muscle and adipose tissue. As a result, CM

lose TG content and remnant particles are formed. These remnant particles, in contrast to CM itself, can penetrate the fenestrations, reach the surface of hepatocytes and can be taken up in the hepatocytes through receptor-mediated uptake where they are cleared of their remaining TG (8). Another route by which dietary fat reaches the liver is by so-called fatty acid spill-over: the fatty acids that are released by LPL-mediated lipolysis at the endothelium in adipose tissue or muscle will not all be taken up by the peripheral tissues, but in part remain in the circulation and thereby feed into the plasma NEFA pool (9). Subsequently, the liver can also take up these 'spill-over fatty acids' mediated by fatty acid transport proteins (10).

1.2 Techniques to measure this pathway

1.2.1 Stable isotope measurements in plasma

Dietary fat uptake is commonly measured by using fatty acid tracers, for example, fatty acids that are enriched with the stable carbon isotope ^{13}C (11-14) or deuterium (^{2}H) (15-18). Carbon-13 and deuterium have a low natural abundance (1.1% and 0.015%, respectively), making them good candidates for tracer studies focusing on dietary fat uptake. 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 very low-density lipoprotein TG (VLDL-TG) (11-18). The fatty acid composition and the tracer enrichment were shown to be similar in VLDL-TG and liver TG (determined from liver biopsies), therefore the ^{13}C and ^{2}H 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 (15, 19). Isotopic enrichments in VLDL-TG are generally determined by GC/MS.

1.2.2 Spectroscopy measurements in the liver

To investigate tracer enrichments directly in the liver, liver biopsies have been used (15). An alternative approach is through spectroscopy or PET methodology, assessing which proportion of the lipids in a meal is ending up in the liver. Magnetic resonance spectroscopy (MRS) techniques can be used to measure ^{13}C enrichment directly in the liver after consumption of ^{13}C–labeled fatty acids (20, 21). The difficulty with standard ^{13}C-MRS is the low sensitivity of this method and the high contamination of the ^{13}C lipid spectra of the liver by ^{13}C signal from adipose tissue, which is due to suboptimal localization of the measured signal to the liver. To overcome these problems, so called ^{13}C-edited methods can be used. With this method, the superior sensitivity and localization of ^{1}H-MRS can be used by applying ^{1}H-MRS techniques that specifically quantify the signal of ^{1}H nuclei that are 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 quantify the ^{13}C lipid signal in the liver and thereby 'track' the ^{13}C-fatty acids originating from a meal (21). 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.

1.2.3 Radioisotopic measurements in the liver

Another technique that has been used to determine dietary fat uptake in the liver is positron emission tomography (PET) in combination with oral intake of 14(R,S)-[(18)F]fluoro-6-thia-heptadecanoic acid (^{18}FTHA) tracer, a long chain fatty acid analog containing ^{18}fluor, which can be detected by PET (22). 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. Therefore, in tissues like skeletal muscle or the heart ^{18}FTHA is trapped in the tissue and reflects fat uptake only, while in the liver, this tracer can also leave the organ when secreted in VLDL, similar to the recycling of dietary fatty acids in VLDL. Therefore, it reflects the balance between uptake and export, where oxidation is not considered (22).

1.3 Contribution to liver fat accumulation and influence of diet

Generally, dietary fat storage in the liver is thought to be small. Using deuterated tri-palmitate as a tracer in whole-food meals, the percentage of VLDL-TG contributed by dietary fat over four days in NAFL patients was estimated to be 15% (15). Less physiologically relevant, oral and duodenal administration of liquid food has been used to determine contribution in healthy subjects, finding values of around 15-20% and 10-15%, respectively five hours after a meal (16). Using indirect ^{13}C spectroscopy, postprandial retention of orally administered ^{13}C-labeled fatty acids in the liver after 6 hours has been found to be 1.5%, for both lean and obese subjects (21). This equals dietary fat making up around 10% of the total liver fat pool per day for an individual with a relatively low liver fat percentage (21). Also between glucose tolerant and glucose intolerant subjects no differences in dietary fat uptake have been reported upon a standardized mixed meal, when measuring between 90 and 120 minutes after oral administration of ^{18}FTHA tracer (22).

A study performed by Chong et al. showed by using oral administration of [U-^{13}C]palmitate that dietary fat contribution to VLDL-TG was similar upon a three-day high-fat and high-carbohydrate diet in eight healthy volunteers (around 15% six hours after a mixed meal)

(11). Using the same tracer, Parry et al. showed that there was no difference in dietary fat contribution to VLDL-TG upon a 4-week diet enriched with saturated fat compared to a diet enriched with free sugars in sixteen overweight males, with values around 5-10% six hours after a meal (14). In addition, supplementation with omega-3 fatty acids (4 g/day eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) as ethyl esters) for eight weeks in thirty-eight healthy men did also not change dietary fat retention six hours after a meal compared to baseline (13). With respect to the type of carbohydrates in a meal, [U-^{13}C]palmitate experiments have shown in sixteen healthy volunteers that there is lower relative contribution of dietary fatty acids to VLDL-TG after consumption of a high fructose/low glucose compared to a low fructose/high glucose meal (23). To date, spectroscopy and PET methods have not been used to investigate the impact of different diet compositions on dietary fat retention.

Altogether, data on the importance of dietary fat retention in the liver is limited and measured in different populations and different diets. Based on a few studies, it seems that the relative contribution of meal-derived fat storage is rather robust. However, the potential impact of carbohydrate type and fat type is interesting to investigate in more detail.

2. *DE NOVO* LIPOGENESIS

2.1 Pathway description

De novo lipogenesis (DNL) is another pathway contributing to the accumulation of fat in the liver. This pathway comprises reactions in the mitochondrial matrix and the cytosol of hepatocytes that lead to the formation of fatty acids (24). Acetyl-CoA, derived from catabolic pathways of carbohydrates or amino acids, serves as the main substrate for this process (24). The cytosolic acetyl-CoA will be converted to the saturated fatty acid palmitate through several enzymatic reactions involving the enzymes Acetyl-CoA carboxylase (ACC), which forms malonyl-CoA from acetyl-CoA, and fatty acid synthase (FAS), which in turn synthesizes palmitate from malonyl-CoA (10).

2.2 Techniques to measure this pathway

2.2.1 ^{13}C-acetate infusion

^{13}C-acetate has frequently been used in studies to determine DNL contribution to liver fat. Upon intravenous infusion, this labeled acetate will be taken up by the liver. In hepatocytes, the acetate is converted to acetyl-CoA, thereby labeling the intrahepatic acetyl-CoA pool.

This ^{13}C labeled acetyl-CoA is then converted to palmitate by the enzymes ACC and FAS. Based on tracer enrichments in the intrahepatic acetyl-CoA precursor pool and the product pool of VLDL-palmitate, fractional synthesis of fatty acids can be determined from the precursor to product ratio, however this requires taking hepatic biopsies for determination of acetyl-CoA precursor pool enrichment (25). To overcome this, mass isotopomer distribution analysis (MIDA) has been introduced by Hellerstein and Neese (26). Through this technique, the acetyl-CoA precursor enrichment can be determined from the relative distribution pattern of labeled isotopomers in the product (VLDL-palmitate).

Most studies using this tracer have used short infusion protocols ranging from several hours to two days (16-18, 27-34). However, infusion protocols of more than two days have also been used (15), improving the assessment of DNL contribution to liver fat in people with larger hepatic lipid pools: since the intrahepatic lipid pool in NAFL takes days or even weeks to turnover, short-labeling protocols may underestimate the true DNL contribution (35). A disadvantage of longer protocols, however, is that with stable isotope approaches there will be recycling of isotope labeled fatty acids that can interfere with the determination of DNL.

2.2.2 Deuterium oxide measurements

Another frequently used stable isotope tracer for DNL measurement is deuterium oxide (12, 13, 23, 36-41). Upon oral administration, the body water pool will become enriched 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 (42). Compared to the intravenous infusion of ^{13}C-acetate, the use of deuterium oxide is less demanding as this tracer can be easily consumed orally as enriched water.

Similar to ^{13}C infusion protocols, deuterium tracer protocols largely differ in duration, varying from several hours to up to 10 days (12, 36). Most frequently, a short protocol is used, consisting of a loading dose of deuterium labeled water provided in two servings in the evening, followed by *ad libitum* intake of low-dose deuterium labeled water to maintain body water enrichment levels (13, 36-38, 41). In case of applying a deuterium protocol over up to 10 days, with oral intake of deuterium oxide at home, it should be noted that standardization of participant's lifestyle over these days is very important to limit unwanted lifestyle influences.

2.2.3 Plasma markers of DNL

Besides the use of stable isotope tracers, plasma FA levels/ratios are often used to infer hepatic DNL. The main plasma markers used are the lipogenic index, the percentage increase in palmitate ('new palmitate'), and the Stearoyl-CoA desaturase (SCD) index. 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 lipogenic index (11, 33, 43-45) is expressed as the ratio of palmitate (16:0) to linoleate (18:2n6) in VLDL-TG, and has been shown to be in agreement with ^{13}C labeled acetate measurements following a high carbohydrate diet (33). As the primary end product of DNL is saturated fatty acids in humans, increased DNL rates will lead to a higher relative contribution of palmitate to VLDL-TG mainly at the expense of PUFAs such as linoleate (32). The body cannot synthesize linoleate and therefore its appearance in plasma can only be from diet and adipose tissue. By matching the proportion of linoleate in the diet to the proportion in adipose tissue or assuming that linoleate in adipose tissue and diet are similar, linoleate contribution to VLDL-TG reflects the contribution of fatty acids not synthesized by the body and its dilution reflects DNL (33).

Instead of expressing palmitate relatively to linoleate, determining the percentage increase in only palmitate has also been used to measure DNL (46). The relative increase in palmitate contribution to VLDL-TG in response to fructose (and glucose) feeding has been shown to highly correlate with the change in DNL as determined by ^{13}C labeled acetate infusion combined with MIDA (46).

As isotope tracer studies are more costly and more challenging to perform, using these two surrogate markers of DNL could be a good alternative for large-scale studies. 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 (47). 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.

Next to the lipogenic index and increase in palmitate, the $SCD1_{(16)}$ index has been proposed as a marker for DNL (11, 48). SCD1 is a key enzyme involved in lipogenesis as it converts SFAs (mainly palmitic and stearic acid) to MUFAs. The ratio of 16:1n-7 to 16:0 [$SCD1_{(16)}$] is often used to estimate SCD1 activity (11, 43, 49). The validity of $SCD1_{(16)}$ as a marker of DNL has been tested against the isotope tracer deuterium oxide with some conflicting findings. A study by Lee et al. reported a strong positive association between $SCD1_{(16)}$ and DNL

assessed through 10 days of D_2O enrichment (48). In contrast, a more recent study by Rosqvist et al. suggests that there is no association between $SCD1_{(16)}$ and DNL (12h D_2O enrichment) when subjects consume their habitual diet (47). It is yet unclear what is at the basis of these discrepancies. Possibly, the differences in tracer enrichment duration play a role.

2.3 Contribution to liver fat accumulation and influence of diet

Both iv infusion of ^{13}C-acetate tracer and oral intake of deuterated water have been used to assess the relative contribution of DNL to VLDL-TG, showing that DNL contributed to around 5-10% of VLDL-TG in healthy individuals in the fasted state, whereas contribution increased to around 20-25% in people with NAFL (12, 15, 16). In the postprandial state, the proportion of DNL derived fatty acids in VLDL-TG increased from 5-10% in the fasted state up to 15-20% after a first meal and to 20–25% after a second meal in healthy individuals (16). In people with NAFL this increase seemed to be absent, with DNL contributions similar during fasting and postprandial periods (around 20% of TG-rich lipoproteins (tTRL-TG) after 3 days of ^{13}C-acetate labeling) (15).

Effects of dietary interventions on DNL have also 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 subjects when compared to diets high in fat and similar in protein (29, 33, 40, 45, 50, 51) and that replacement of carbohydrates by both fat and protein leads to a reduction in fasting DNL (52). In addition, 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 (43, 44, 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 (23, 28, 30). In line with these isotope tracer studies, carbohydrate type effects have also been shown using the lipogenic index. 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). Regarding the effect of fatty acids on DNL, Green et al. recently showed by using deuterated water that 8-week supplementation with the omega-3 fatty acids EPA and DHA at a dose of 4g/day decreased both fasting and postprandial DNL compared to baseline in thirty-eight healthy men (13).

Summarizing, DNL seems to be a significant contributor to liver fat, making it an interesting target for intervention studies aiming at decreasing liver fat storage. In this respect, diets high in carbohydrates stimulate DNL most, with fructose and simple sugars as most potent stimulators. Also, the lipogenic index has been shown to change upon dietary interventions stimulating DNL and therefore can render some valuable information on DNL in a less invasive and less costly way when studying dietary effects in large-scale studies.

3. NEFA UPTAKE

3.1 Pathway description

The largest contributor to hepatic fat originates from uptake of plasma non-esterified fatty acids (NEFA) (15, 16). The main source of plasma NEFAs is fatty acids originating from adipose tissue lipolysis, while spillover fatty acids can also contribute (15). These plasma NEFAs can be taken up by the liver through protein mediated transport (10). Uptake of NEFAs by the liver is strongly dependent on the level of NEFAs in the plasma, with lower plasma NEFA levels resulting in reduced uptake of NEFA by the liver (55).

3.2 Techniques to measure this pathway

3.2.1 Stable isotope measurements in plasma

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 fatty acids with respect to turnover (56). Additionally, palmitate tracer is most commonly used as this fatty acid is highly abundant in plasma and is the cheapest fatty acid tracer. Basically, the method is similar to the method used to measure dietary fat uptake by labeling dietary fatty acids. 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 (12, 15-17, 29), but also intravenous deuterium palmitate tracers have been used to assess NEFA contribution to VLDL-TG (18, 57). The preferred tracer depends on the combination with other tracers (e.g. when for example ^{13}C-palmitate is used to track dietary fat contribution, a deuterium labeled palmitate tracer is preferred to study hepatic NEFA uptake).

3.2.2 Radioisotope measurements in the liver

Besides tracing the labeled fatty acids in VLDL-TG, fatty acid tracer has also been used in combination with PET imaging (58-60). In this respect, ^{18}FTHA tracer, the earlier mentioned long chain fatty acid analog containing ^{18}fluorine, 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. As mentioned before, this tracer cannot be oxidized and therefore the amount of FTHA trapped in the liver determined with PET imaging provides information on the balance between hepatic NEFA uptake and export. Another fatty acid tracer that has been combined with PET imaging is ^{11}C-labeled palmitate (55, 61). In contrast to FTHA, ^{11}C-labeled palmitate can be oxidized completely. If this is taken into account by using compartmental modeling, also the uptake of FA can be determined.

3.3 Contribution to liver fat accumulation and influence of diet

The relative contribution of NEFA to VLDL-TG has been determined using ^{13}C-palmitate in subjects with NAFL and healthy subjects. Under fasting conditions the proportion from NEFA in VLDL-TG was around 65% and 60% in healthy volunteers and volunteers with NAFL, respectively (15, 16), showing that NEFA uptake is the largest contributor to hepatic fat, at least in the fasted state. Of the NEFA pool approximately 80% was originating from adipose tissue in volunteers with NAFL in the fasting state, showing that adipose tissue is the main contributor to plasma NEFA. Postprandially, plasma insulin concentrations will rise, leading to suppression of adipose tissue lipolysis. In NAFL, adipose tissue contribution to NEFA decreases to around 60% in the fed state. The decrease in adipose tissue NEFA release into the circulation translates into a decreased uptake of plasma NEFA by the liver. NEFA contribution to VLDL-TG in the postprandial state decreased to around 40% in healthy participants (16). In participants with NAFL postprandial contribution was determined in tTRL-TG and decreased from 55% fasted to 32% postprandially (15). In addition, using PET methodology, an ^{18}FTHA tracer study showed that fasting uptake of NEFA by the liver was impaired in people with impaired glucose tolerance (IGT) compared to healthy control subjects (59), whereas no differences in hepatic fat uptake have been shown between obese and lean subjects using infusion of ^{11}C-palmitate in combination with PET (61).

Studies focusing on the impact of diet on hepatic NEFA contribution are limited. Comparing high carbohydrate/low fat diets to high fat/low carbohydrate diets, using either intravenous infusion of ^{13}C-palmitate or $^{2}H_2$-palmitic acid tracer showed that fasting and 6-hour post-meal contributions of NEFA to VLDL-TG were similar in healthy volunteers (11, 29). 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, using $^{2}H_{2}$-palmitate in sixteen overweight males (14). NEFA contribution was not increased upon high saturated fat intake when compared to high simple carbohydrate intake 6h after meal consumption.

In summary, several studies have shown that NEFA uptake is the largest contributor to hepatic fat in the fasted state. Nevertheless, dietary studies are however limited and specifically the effects of different types of fat and carbohydrates on hepatic NEFA uptake using before mentioned tracer methodologies still awaits further study.

4. FAT OXIDATION AND ACETYL-COA DISPOSAL

4.1 Pathway description

So far, methods that focused on lipid storage were discussed. Here and in the next paragraph we discuss methods that focus on lipid disposal. Oxidation of hepatic fat is one of the pathways contributing to liver fat removal. Hepatic FA will be broken down to acetyl-CoA in mitochondrial β-oxidation. The formed acetyl-CoA can subsequently be used in the TCA cycle to be oxidized further, thereby producing NADH, which serves as an electron donor needed in the oxidative phosphorylation to produce ATP. Alternatively, acetyl-CoA can be used for the formation of ketone bodies (62). Ketone body synthesis mainly takes place in the hepatic mitochondria via the formation of acetoacyl-CoA and β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) and produces the ketone bodies acetoacetate, β-hydroxybutyrate (BHB) and acetone. Ketogenesis takes place when acetyl-CoA formation exceeds its use by the TCA cycle, in such situation, acetyl-CoA accumulates and levels increase (63). As an example, ketone body formation is stimulated under fasted conditions, when fatty acid degradation to acetyl-CoA is increased (63).

4.2 Techniques to measure this pathway

4.2.1 Direct measurements of hepatic fat oxidation

A method that is used to measure complete oxidation of hepatic fatty acids is the combined use of intravenously infused ^{11}C-palmitate tracer and PET imaging (61, 64). As mentioned before, by making use of modeling approaches several liver fat fluxes including hepatic palmitate oxidation can be determined (61). This is the only approach used to measure oxidation of hepatic fat directly. There are techniques that can determine hepatic TCA cycle flux using stable isotope tracers in combination with MRS (65, 66) or plasma analyses (67), and techniques determining complete hepatic oxidation by a ^{13}C-methionine breath test

(68-71), however such techniques do not specifically determine hepatic fat oxidation and therefore are outside the scope of this review.

4.2.2 Indirect markers of hepatic fat oxidation and acetyl-CoA disposal

Next to stable isotope measurements, indirect markers have been used to provide an indication of liver specific fat oxidation (57, 61, 72, 73). Most often, circulating beta-hydroxybutyrate (BHB) is used, which is widely accepted as marker for hepatic fat oxidation. Although plasma BHB levels are an indirect measure, it is seen as an organ-specific marker for liver fat oxidation as it is mainly produced by hepatic mitochondria. BHB is exported from the liver and can be used by other tissues. Therefore, circulating levels of BHB not only depend on the export rate from the liver, but also on extra-hepatic use. Nevertheless, Iozzo and colleagues compared hepatic fat oxidation determined by the ^{11}C-PET technique to circulating BHB levels and found a strong positive association between the two measurements (61).

4.3 Contribution to liver fat accumulation and influence of diet

To date, the importance of hepatic fat oxidation in relation to liver fat accumulation has only been studied by comparing BHB levels in people with and without NAFL and these studies show decreased (74, 75), increased (76, 77), and similar (78) BHB levels in NAFL compared to people without NAFL. These discrepancies might be explained by differences in population characteristics, sample size and fasting time. Thus, these studies so far have not been able to provide a clear answer on the role of liver fat oxidation in the accumulation of liver fat. To answer this question the earlier mentioned PET methodology could be applied, providing direct instead of indirect information on liver fat oxidation. This methodology has already been used to compare liver fat oxidation in lean and obese subjects (61), and could in a similar way be used to compare people with and without NAFL.

Dietary effects have been studied extensively, mostly using blood BHB levels as a marker for hepatic fat oxidation. Plasma BHB levels increase upon consumption of a high fat meal, whereas BHB levels do not increase upon a carbohydrate rich meal (79). In line with this, a 3-day high fat/low carbohydrate diet and a 2-week high fat/low carbohydrate diet resulted in higher BHB levels compared to isoenergetic low fat/high carbohydrate diets (57, 72). Similar, upon a two-week low carbohydrate diet, higher in both fat and protein, BHB has been shown to increase compared to baseline (52). Furthermore, an 8-week high MUFA-diet has been shown to decrease fasting plasma BHB (when compared to baseline) in patients with type 2 diabetes (80). No changes in plasma BHB where found after an 8-week

high carbohydrate/high fibre/high GI diet in patients with type 2 diabetes (80). However, no definitive conclusions regarding the effects of the type of fat and carbohydrate on liver fat oxidation can be drawn from these studies as effects were only compared to baseline.

Overall, direct hepatic fat oxidation methodology is not often applied, as this requires advanced stable isotope and imaging techniques and exposure to ionizing radiation. Such methodology would however be interesting to apply in studies investigating the role of liver fat oxidation in NAFL. In nutritional studies BHB has frequently been used as marker for hepatic fat oxidation, as it is a widely accepted and easy to determine blood marker. These nutritional studies have shown increased hepatic fat oxidation upon diets high in dietary fat and low in carbohydrates, but effects may differ depending on the type of carbohydrates or fatty acids. The latter has hardly been studied. While theoretically increased hepatic fat oxidation seems a good way to reduce liver fat accumulation, the physiological relevance of fat oxidation rates in determining hepatic fat is not well investigated. Future studies should measure hepatic fat oxidation directly in people with NAFL and in interventions combined with measurements of hepatic steatosis, in order to investigate whether decreased fat oxidation may underlie NAFL etiology.

5. VLDL SECRETION

5.1 Pathway description

Another pathway contributing to hepatic fat clearance is the secretion of VLDL. Liver TGs are used as component for VLDL assembly, together with cholesterol and apolipoproteins. Assembled TG rich VLDL particles can be secreted immediately or can be stored in the cytosol for delayed secretion (18). Upon secretion, liver TGs are transported within these VLDL particles through the circulation to peripheral tissues, where LPL ensures the release of the packed TGs.

5.2 Techniques to measure this pathway

5.2.1 Tracers to determine VLDL particle production

VLDL particle production has been assessed by studying the kinetics of apo B100, an important lipoprotein on VLDL particles. For its biosynthesis, amino acids are needed and therefore an amino acid tracer can be used to label apoB100 in order to study VLDL production. Different leucine tracers, such as trideuterated leucine and ^{13}C-leucine, have been used to intravenously label apoB100 (81-85). The enrichment of leucine tracer in VLDL apoB100 can be determined with GC/MS and is used for the calculation of VLDL production

based on compartment modeling (81, 83). However, changes in the amount of VLDL particles produced by the liver do not necessarily have to translate into changes in secreted VLDL-TG as the degree of TG loading also plays a role. For example, increased production of VLDL particles can take place without changes in VLDL-TG secretion, reflecting the formation of smaller particles (84).

5.2.2 Tracers to determine VLDL-TG secretion

To study the amount of TGs secreted from the liver in VLDL, ^{13}C-glycerol and ^{13}C-palmitate tracers can be used. Upon intravenous infusion, the labeled glycerol or palmitate will be incorporated into TG formed in the liver, which will subsequently be assembled in VLDL particles. Based on the incorporation curves of ^{13}C-glycerol or ^{13}C-palmitate in VLDL, the synthesis of VLDL-TG can then be determined (18, 29, 84, 86). Another palmitate tracer method that can provide information on the amount of TG released from the liver is ^{11}C-palmitate PET imaging, as mentioned earlier (61).

In addition to glycerol and palmitate tracers, VLDL-TG tracers have been used (64, 72, 87, 88). The VLDL-TG tracer technique is based on the tracer dilution principle. Endogenous produced VLDL-TG by the liver, will dilute the infused, labeled VLDL-TG enrichment and therefore, absolute VLDL-TG secretion rates can be determined based on the tracer infusion rate and arterial tracer enrichment levels. The main advantage of this technique is that it is based on fewer assumptions than modeling based methods. VLDL-TG tracers however, have to be induced *in vivo* or *ex vivo*, as there are no VLDL-tracers commercially available.

5.3 Contribution to liver fat accumulation and influence of diet

To which extent VLDL secretion contributes to the development of hepatic steatosis is yet unclear, as studies investigating this are lacking. It is however known that people with mutations in ApoB genes develop NAFL, as is the case in familial hypobetalipoproteinemia (FHBL) (89). Liver fat content in FHBL individuals has been reported to be three times higher as compared to healthy individuals (89). Liver fat may accumulate in these people because of impaired VLDL assembly. The combined PET/^{11}C tracer technique has been used to show that of the liver fat disposal pathways, VLDL-TG secretion seems of less importance to hepatic fat disposal compared to fat oxidation (61). Furthermore, VLDL-TG secretion has been studied in different populations indicating similar TG secretion between lean and obese people (61), and increased TG secretion in people with hypertriglyceridemia (18) and type 2 diabetes (88) compared to healthy controls. In NAFL, VLDL-TG export is increased (86, 90) and therefore, changes in VLDL-TG export do not explain NAFL. Thus, the increased

VLDL-TG export seems to be a consequence of NAFL rather than its cause. Nevertheless, changes in VLDL-TG export will always have consequences for liver fat storage.

Some dietary intervention studies have been performed in which VLDL-TG secretion was assessed. Conflicting results have been reported on the comparison of high carbohydrate/low fat and low carbohydrate/high fat diets on VLDL-TG secretion. In healthy lean subjects VLDL-TG secretion was increased upon a two-week high carbohydrate diet (75% of energy as CHO vs 30% energy as CHO in HF diet) as determined with VLDL tracer (72), whereas Parks et al. did not find differences between a 5 week high carbohydrate/low fat diet and a 1 week control diet (consisting of 68% vs 50% energy as CHO) in normolipidemic lean and hypertriglyceridemic overweight subjects when using ^{13}C-palmitate tracer (29). Whether these conflicting results are due to the different study populations or the differences in carbohydrate and fat content between the 2 studies is unknown.

The effects of fish oils, linoleic acid and medium chain fatty acids (MCFA) have been studied on VLDL ApoB secretion. Three-week supplementation of 60 mg/d linoleic acid compared to MCFA did not result in differences in ApoB secretion (81). Six-week fish oil supplementation on the other hand showed reductions in VLDL ApoB secretion compared to placebo (corn oil) (83, 91). No differences in VLDL ApoB secretion have been shown upon 10-week ingestion of EPA, DHA or corn oil (85). In these studies VLDL-TG secretion was not determined.

Summarizing, little is known about the importance of TG secretion from the liver in determining liver fat accumulation. VLDL-TG export is increased in NAFL, indicating that changes in VLDL-TG are not causing NAFL. However, changes in VLDL-TG will have consequences for NAFL. A limited number of studies have investigated dietary effects on VLDL-TG secretion and similar to nutritional studies on hepatic fat oxidation, the physiological relevance of these nutritional findings remain unknown.

CONCLUSIONS

Hepatic fat accumulation is the result of an imbalance between lipid storage on the one hand and disposal on the other hand. The different pathways involved in fattening of the liver can accurately be measured by stable isotope techniques in combination with VLDL-TG analysis or imaging techniques as MRS and by PET methodologies. As an alternative, a few validated plasma markers and indexes can be used as a more feasible alternative in studies with large sample sizes. Despite the availability of a wide range in techniques to measure these pathways, knowledge on the contribution of each pathway to liver fattening

in humans in health and disease is still 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. Furthermore, relative contributions depend on factors as study population and nutritional state (fed vs. fasted), factors which require more detailed investigation as well. While some specific questions with respect to dietary influence on pathway contributions have been answered, much remains unclear. Future research on the role of liver fat pathways in liver fat accumulation will improve our understanding of the mechanisms contributing to liver fat accumulation, which is crucial to the development of effective treatment strategies for NAFL and its associated metabolic disturbances.

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CHAPTER 3

Hepatic saturated fatty acid fraction is associated with *de novo* lipogenesis and hepatic insulin resistance

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ABSTRACT

Hepatic steatosis is associated with poor cardiometabolic health, with *de novo* lipogenesis (DNL) contributing to hepatic steatosis and subsequent insulin resistance. Hepatic saturated fatty acids (SFA) may be a marker of DNL and are suggested to be most detrimental in contributing to insulin resistance. Here we show in a cross-sectional study design (ClinicalTrials.gov ID: NCT03211299) that we are able to distinguish the fractions of hepatic SFA, mono- and polyunsaturated fatty acids in healthy and metabolically compromised volunteers using proton magnetic resonance spectroscopy (^{1}H-MRS). DNL is positively associated with SFA fraction and is elevated in patients with non-alcoholic fatty liver and type 2 diabetes. Intriguingly, SFA fraction shows a strong, negative correlation with hepatic insulin sensitivity. Our results show that the hepatic lipid composition, as determined by our ^{1}H-MRS methodology, is a measure of DNL and suggest that specifically the SFA fraction may hamper hepatic insulin sensitivity.

INTRODUCTION

Non-alcoholic fatty liver (NAFL) is the most common cause of chronic liver disease, with estimated prevalence rates of 20-35% in Western countries (1). In obese people NAFL prevalence rates as high as 50-70% have been reported (2). NAFL can progress to steatohepatitis, fibrosis, and cirrhosis, which can lead to liver failure, and hepatocellular carcinoma (3). Moreover, ectopic fat accumulation in the liver is associated with impairments in cardiometabolic health (4, 5). In this respect, NAFL has been shown to be associated with insulin resistance on hepatic and whole-body level (6-9).

However, not all individuals with NAFL will develop insulin resistance, steatohepatitis or other liver disease, and it is of utmost clinical importance to understand which factors contribute to a pathologic fatty liver. It has been suggested that the pathway by which fat accumulation in the liver occurs may impact the clinical outcome. It has been shown that high rates of *de novo* lipogenesis (DNL) are associated with metabolic risk (10-12). In addition, animal experiments have suggested that the degree of saturation of the accumulating fatty acids in the liver may impact the metabolic consequences, with more saturated fatty acids (SFA) leading to worsened metabolic outcome (13). Interestingly, the end product of *de novo* synthesis of fatty acids is mainly SFA and therefore, high rates of DNL may result in a higher proportion of hepatic SFA, possibly explaining why DNL is associated with poor metabolic health. These findings stress the importance for a more detailed characterisation of hepatic lipid composition in humans, to ultimately understand the risk factors for the development of hepatic insulin resistance and disease.

However, in humans such data is very scarce, mainly due to the invasiveness of liver biopsy procedures that are needed. Therefore, only very few studies have determined hepatic fat composition, and these studies have been performed in patients in which liver biopsies were justified due to their risk for liver disease. Generally, these studies showed higher mono-unsaturated fatty acids (MUFA) fraction at the expense of the poly-unsaturated fatty acids (PUFA) fraction in people with NAFL compared to people without NAFL (14, 15). Earlier MR-based studies already investigated some parameters that are linked to degree of unsaturation(16-19), however these do not specifically and robustly quantify hepatic SFA, MUFA and PUFA fraction separately.

Here, we develop, validate and apply a magnetic resonance (MR) post-processing tool that enables to non-invasively quantify the fractions of hepatic SFA, MUFA and PUFA separately, in healthy and metabolically compromised human volunteers. Using this methodology, we test the hypothesis that higher rates of DNL are associated with an increased fraction of SFA in human liver. We also investigate if populations at higher risk to develop metabolic

complications are characterized by altered hepatic fatty acid composition, and whether hepatic fatty acid composition is related to hepatic insulin sensitivity. We show that our ^{1}H-MRS postprocessing methodology can be used to measure hepatic fatty acid composition in humans, that hepatic SFA content is strongly related to rates of DNL and that specifically the hepatic SFA fraction is related to hepatic insulin resistance.

METHODS

Clinical Study Design

The study was conducted at Maastricht University Medical Center, the Netherlands, between August 2017 and June 2018, and was approved by the Medical Ethical Committee of Maastricht University Medical Centre. Research was performed in accordance with relevant ethical regulations regarding human research participants. The study was registered at clinicaltrials.gov with identifier NCT03211299.

Participants

All participants recruited for this study provided written informed consent. Twenty-two healthy overweight/obese participants (BMI 27-35 kg/m^2), aged 45-70 years with a large range in liver fat content (0.9-38.4%) were recruited for this study. Female study participants were postmenopausal. Exclusion criteria were engagement in exercise for more than 2 hours per week, unstable body weight (weight loss or gain more than 3 kg in 3 months preceding enrollment), alcohol consumption more than 2 units per day, smoking more than 5 cigarettes per day, contra-indication for MRI, use of anti-coagulants, use of other medication known to interfere with the outcome parameters, diabetes or other active disease. Participants participated in an MRS measurement, deuterated water measurement and two-step hyperinsulinemic-euglycemic clamp, including baseline subcutaneous adipose tissue biopsy. All measurements took place within a time window of six weeks. Two days before each of the measurements, participants were instructed to refrain from physical exercise and alcohol consumption. The evening before the measurements, participants consumed a standardised high carbohydrate diner and fasted overnight. For group comparisons, two patient groups were included in the study: nine patients with type 2 diabetes and seven patients with glycogen storage disease type 1a. Patients with type 2 diabetes were aged 40-75 years, had a BMI between 25-38 kg/m^2 and relatively well controlled type 2 diabetes: HbA1c < 9.5%. Patients had stable dietary habits, were on treatment with oral medication only (Metformin, Tolbutamide or Gliclazide) and did not use other medication known to interfere with the outcome parameters. Female patients were

postmenopausal. Exclusion criteria were engagement in exercise for more than 3 hours per week, uncontrolled hypertension, anemia, unstable body weight (weight loss or gain more than 5 kg in 3 months preceding enrollment), alcohol or drug abuse, being vegetarian or vegan, having significant food allergies, contra-indication for MRI, use of anti-coagulants and clinically relevant active disease. GSD1a patients were recruited aged 18 years and older, clinically diagnosed with GSD1a and without contra-indications for MRI. *In vivo* validation in adipose tissue was performed in a subgroup of the healthy overweight/obese participants (n=8). Reproducibility measurements in the liver were performed in a separate group of individuals aged between 25-71 years and BMI between 25.7-38.5 kg/m^2 (n=7).

Overview of specified outcomes

The primary outcome was lipid composition (SFA fraction) as measured by Magnetic Resonance Spectroscopy and rates of *de novo* lipogenesis as measured by incorporation of deuterated water. Secondary outcome was hepatic insulin sensitivity as determined by suppression of hepatic glucose output during the low insulin phase in a 2-step hyperinsulinemic euglycemic clamp.

Measurement of lipid content and lipid composition

In this study, the lipid content and lipid composition (fraction of hepatic SFA, MUFA and PUFA) were determined by proton magnetic resonance spectroscopy (^{1}H-MRS) (healthy overweight/obese controls without NAFL: n=7 for total liver fat content, n=6 for liver fat composition, healthy overweight/obese with NAFL: n=15, patients with type 2 diabetes: n=9 and patients with GSD type 1a: n=7). All ^{1}H-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 32-channel sense cardiac/torso coil (Philips Healthcare, Best, Netherlands). All spectra were obtained by using a STEAM sequence (20) with the following parameters; repetition time (TR) 4500 ms/echo time (TE) 20 ms/ mixing time (TM) 16 ms, spectral bandwidth 2000 Hz and data points 2048. For the *in vivo* hepatic lipid spectra VAPOR water suppression (21) was applied and an additional water reference scan was obtained. The number of averages was 16 for the phantom experiments and in adipose tissue and 128 for the *in vivo* hepatic spectra. We used a voxel size of 30 x 30 x 30 mm for the hepatic and 15 x 15 x 15 mm for the adipose tissue measurements.

All obtained lipid spectra were post-processed in a home-written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States.) script in which, prior to fitting, all spectra are individually corrected for phase and frequency shift. Additionally, we

performed eddy current correction for the individual lipid spectra. Phasing, frequency alignment and eddy current correction were all performed on individual spectra before signal averaging. Bad quality spectra from *in vivo* (e.g. due to motion) were removed automatically. For this, the linewidth, amplitude and frequency offset of the peaks in each individual spectrum were compared to the average values of all spectra.

For the setup of the post-processing routine we used a four-step approach. First, we acquired both ^{1}H and ^{13}C high-resolution (HR) NMR spectra from five different oils (olive, arachis, sunflower, safflower and rice oil), by using a pulse-acquire sequence. The lipid composition of the different oils was determined by integration of the peaks in the methyl region in the ^{13}C HR spectra. In the ^{1}H HR spectra four different regions were integrated, corresponding to the methyl protons (around 0.90 ppm), the allylic protons (around 2.02 ppm), the alpha carbonyl group (around 2.20 ppm) and the diallylic protons (around 2.75 ppm) (supplementary figure 1). The ^{1}H HR spectra were used to develop a basis set used by the developed fitting algorithm. The lipid signal was described by 16 individual resonances (2 for the methyl group, 5 for the methylene group, 1 for the beta methyle-group, 2 for the allylic group, 2 for the alpha-carbonyl group and 1 for the diallylic group). In this basis set, the relative frequency shifts, the splitting patterns and the initial linewidths were described.

In a second setup step, we acquired spectra from the different oils on our clinical 3T scanner. Eddy current correction was applied based on an additional water reference scan with an identical experimental setup. The oil spectra were fitted with the developed Matlab algorithm. To this end, using the basis set from the HR spectra, the time domain signal was simulated. The amplitudes and relative frequency shifts of the individual peaks were updated iteratively. In each step the difference between the simulated spectrum and the acquired spectrum was minimized in the frequency domain. Next to these individual parameters, also the Gaussian line broadening, the Lorentzian line broadening, the zero order phase, an overall frequency shift and the baseline offset were automatically updated, affecting all the peaks in an identical fashion. Essential for our approach, we furthermore fixed the ratio of the methyl group and the alpha-carbonyl group (which overlaps with the allylic group at 3T) to 0.6. Theoretically this factor should be 0.67, as the methyl group contains 3 protons and the alpha-carbonyl group contains 2 protons and both groups are present only once in every fatty acid. However, due to TE-induced losses this factor will be affected. Therefore, this factor was determined empirically. In a final fine-tuning step of the fitting routine, the zero-order phase was accurately fitted by minimizing the residual in the spectrum in the methyl and allylic + alpha-carboxyl region specifically.

The signal amplitude ratio of the diallylic over the methyl peaks and the ratio of the alpha-carbonyl + allylic over the methyl peaks was determined in both the phantom and the HR experiment, to empirically determine correction factors for TE-induced signal in our STEAM recordings. These empirically determined correction factors were then used for the measurements in adipose tissue and in the liver to calculate the lipid composition using the following formulas:

$$\%PUFA = CA * \left(\frac{2}{3} * \frac{Sdiallylic}{Smethyl}\right) * 100$$

$$\%MUFA = CB * \left[\frac{3}{4} * \left(\frac{Salphacarb + Sallylic}{Smethyl} - \frac{2}{3}\right) * 100\right] - \%PUFA$$

$$\%SFA = 100 - \%PUFA - \%MUFA$$

with CA and CB the empirically determined correction factor for the PUFA and MUFA calculation respectively (CA = 0.83 and CB = 0.99; see also supplementary table 1).

In a third step, the lipid composition in subcutaneous adipose tissue was determined *in vivo* with ^{1}H-MRS using the developed fitting routine and this was compared to the *ex vivo* lipid composition as determined by mass spectrometry analysis in adipose tissue biopsies, in eight participants. For the adipose tissue ^{1}H-MRS measurement, we applied a gradient cycled STEAM sequence, to correct for eddy currents, as previously described (22).

In a final validation step to determine reproducibility, we applied the developed methodology in the liver of seven healthy individuals (BMI 30.6 ± 3.7 kg/m^2; age 49 ± 17.2 year; 3 women), with a wide range of intrahepatic fat (2.1-19.6%). To this end, we performed the ^{1}H-MRS acquisition and repeated this after replacement of the subject on the table. The ICC was calculated for the lipid composition and total lipid content as a measure to determine the reproducibility between two consecutive measurements. Lipid content was calculated after T_2 correction as ratio of the CH_3 peak relative to the unsuppressed water resonance, expressed as percentage weight/weight.

Deuterated water measurement of DNL

A background blood sample was drawn in the afternoon before intake of the deuterated water in 18 healthy overweight/obese participants. Together with the evening meal, participants were given 2.86g/kg body weight deuterated water (70% 2H2O, Cambridge Isotope laboratories) in two servings. A blood sample for DNL analysis was drawn at fasting in the morning, 16 hours after the first serving of deuterated water. The DNL was analyzed

from enrichment in VLDL-TG of deuterated water (10, 23). Two participants were excluded from the analysis of DNL because of TG levels higher than 4 mmol/L, influencing the reliability of the deuterated water measurement.

Body composition

Body mass and body volume were assessed using air-displacement plethysmography (ADP) using the Bod Pod device (Cosmed, Italy, Rome) according to the manufacturer's instructions on the day of the hyperinsulinemic-euglycemic clamp (healthy overweight/obese volunteers: n=21, patients with type 2 diabetes: n=9) (24). Thoracic gas volume was predicted based on equations included in the Bod Pod software (version 4.2.0). From these data, body fat percentage was calculated as described by Siri (25).

Subcutaneous adipose tissue biopsy

In a subgroup of eight healthy overweight/obese participants, a subcutaneous adipose tissue biopsy (~1 g) was collected 6 – 8 cm lateral from the umbilicus, under local anesthesia (1% lidocaine) by needle biopsy before the start of the hyperinsulinemic-euglycemic clamp. Upon tissue collection, the tissue was rinsed with sterile saline and visible blood vessels were removed. Thereafter, the biopsy was snap-frozen in liquid nitrogen and stored at 80°C for later lipid composition analyses.

Analysis of VLDL and adipose tissue triglycerides

Plasma samples used for VLDL-TG analyses were taken around 16 pm before the intake of the deuterated water. The VLDL fractions (20-50µl) and adipose tissues (10-20mg) were extracted using the BUME method(26, 27). The total lipid extracts were evaporated under a stream of nitrogen and reconstituted in 250µL chloroform/methanol [2:1]. Triglycerides were detected by direct infusion (shotgun) analysis on a QTRAP 5500 mass spectrometer (Sciex, Concord, Canada) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion BioSciences, Ithaca, NJ) according to previous work (28). More specifically, a fraction of the total lipid extract was diluted 1:100 (for VLDL) and 1:100 000 (for adipose tissue) in chloroform:methanol [1:2] with 5mM ammonium acetate and infused at 250nl/min for 15 minutes. The NanoMate was run with a voltage of 1.2kV and a gas pressure of 0.8 psi. The analysis was performed in positive ion mode by neutral loss detection of 11 common acyl fragments formed during collision induced dissociation of the ammoniated triglycerides. The nano-interface of the mass spectrometer was heated to 60 degrees and the measurements was made using a scan speed of 200 dalton/sec. The neutral loss scans

were cycled and in total 50 cycles were acquired for each neutral loss scan (one neutral loss for each fatty acid). The data was processed using the LipidView 1.2 software (Sciex, Concord, Canada) and the most abundant signal intensities from the most commonly found triglyceride species were used to calculate the abundance and composition of the different triglycerides (supplementary table 2 and 3). Quantification was made using a one point calibration against glyceryl-d_5-hexadecanoate (CDN isotopes, Quebec, Canada), which was added to the infusion solvent.

Hyperinsulinemic-euglycemic clamp

A two-step hyperinsulinemic-euglycemic clamp was performed to assess hepatic insulin sensitivity using the low-dose phase (10 mU/m^2/min) (healthy overweight/obese participants: n=21). A primed continuous infusion of D-[6,6-2 H2]glucose (0.04 mg/kg/min) was started to determine rates of endogenous glucose production (EGP), glucose appearance (Ra), and glucose disposal (Rd). After 180 min, participants were given infusion of low insulin (10 mU/m2 /min) for 3h. During the last 30 min of the insulin infusion step, blood samples were collected. Steele's single pool non- steady state equations were used to calculate glucose Ra and Rd (29). Volume of distribution was assumed to be 0.160 L/kg for glucose. Hepatic insulin sensitivity was calculated as the percentage of EGP suppression during the low-dose phase. In addition, hepatic insulin sensitivity was determined according to this methodology in patients with type 2 diabetes (n=9). Clamp parameters are shown in supplementary table 4.

Statistical analysis

Results are expressed as mean ± SEM for group comparisons. Population characteristics are expressed as mean ± SD. Continuous variables were tested for normality. Two-sided pearson correlation was performed to identify correlations between variables. For non-normally distributed data two-sided spearman correlation was performed. Group comparisons were assessed by one-way ANOVA. For non-normally distributed data Kruskal-Wallis analyses was performed to compare groups. In case of significant group differences in the group comparisons, post-hoc analyses were performed using Bonferroni correction to test which groups were significantly different. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 23.0 for Mac OS.

RESULTS

Development and validation of hepatic ^{1}H-MRS method

To allow determination of hepatic fatty acid composition, we developed a ^{1}H-MRS analysis protocol. To this end, we used information from ^{1}H high-resolution (HR) NMR spectra from five different vegetable oils to improve our analysis routine and used ^{13}C HR NMR to determine the true lipid composition of these oils. We acquired spectra from these different oils with proton magnetic resonance spectroscopy (^{1}H-MRS) on our clinical 3T scanner and calculated average corrections factors to correct for TE-induced losses in the ^{1}H-MRS spectra. When using the average corrections factors, the lipid composition as determined by our clinical protocol showed excellent agreement with the true lipid composition determined by high-resolution NMR: the intraclass correlation coefficient (ICC) for SFA, MUFA and PUFA fraction was 0.982, 0.970, 0.987 and the CV was 6%, 9% and 9%, respectively (figure 1).

As it is known that *in vivo* several factors can influence MR spectra, the next step was to validate our method *in vivo*. To this end, we aimed to validate our ^{1}H-MRS lipid composition measurement with analysis performed in biopsy material. As it is ethically difficult to take liver biopsies for this purpose, we rather performed subcutaneous adipose tissue biopsies, which is far less invasive. Therefore, we validated our MRS method *in vivo* by comparing lipid composition in subcutaneous adipose tissue acquired by the ^{1}H-MRS technique and by mass spectrometry analysis in subcutaneous adipose tissue biopsies in eight participants (figure 2). As can be seen in figure 2, there was reasonably good agreement between the two methods. The ICC for SFA, MUFA and PUFA fraction was 0.333, 0.146, 0.306 and the CV was 8%, 7%; 12%, respectively. Thus, lipid composition determined by ^{1}H-MRS *in vivo* is in close agreement with lipid composition measured *ex vivo* in adipose tissue biopsies. The final validation step was to apply our protocol in the liver *in vivo*. We tested reproducibility in seven healthy individuals (separate group) with intrahepatic lipid content ranging from 2-18% (figure 3). As can be seen in figure 3, the ICC for total IHL content, SFA, MUFA and PUFA fraction was found to be 0.997, 0.562, 0.756, 0.557 and the CV was 4%, 4%, 3%, and 12%, respectively. These results indicate high reproducibility for determining *in vivo* hepatic lipid composition using our developed approach.

In addition, we compared hepatic lipid composition by our MRS method to plasma VLDL-triglyceride (VLDL-TG) composition acquired by mass spectrometry analysis in seventeen participants (figure 4). As can be seen in figure 4, especially the SFA fraction showed a strong correlation with hepatic SFA% (Pearson $r=0.80$, $p<0.001$).

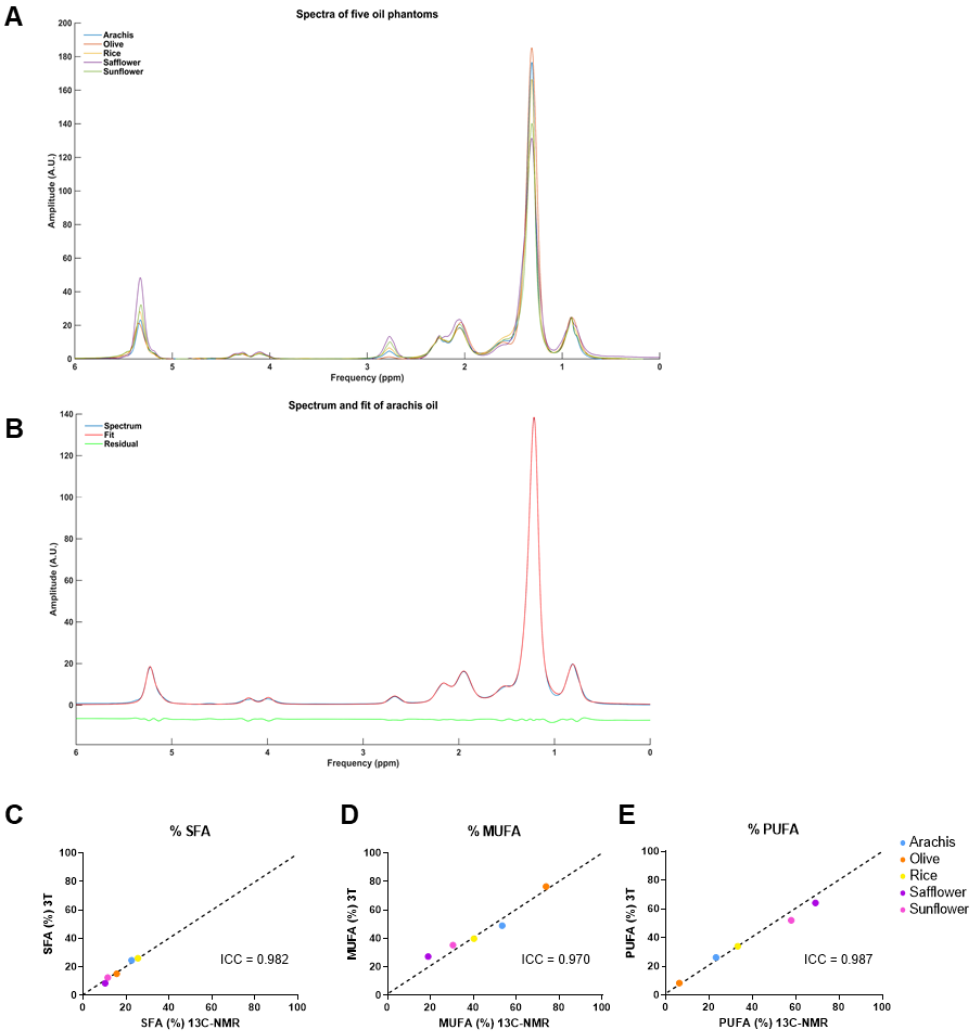

Figure 1: Validation of ^{1}H-MRS method in oil phantoms. A Lipid spectra acquired from five different oil phantoms (arachis, olive, rice, safflower and sunflower oil) showing the different lipid proton peaks and their position. **B** Lipid spectrum and fit of arachis oil. Correlations between **C** SFA, **D** MUFA, **E** PUFA measured at 3T with ^{1}H-MRS and measured with high-resolution ^{13}C-NMR spectroscopy. The intraclass correlation coefficient (ICC) is shown in the respective plots (Intraclass correlation).

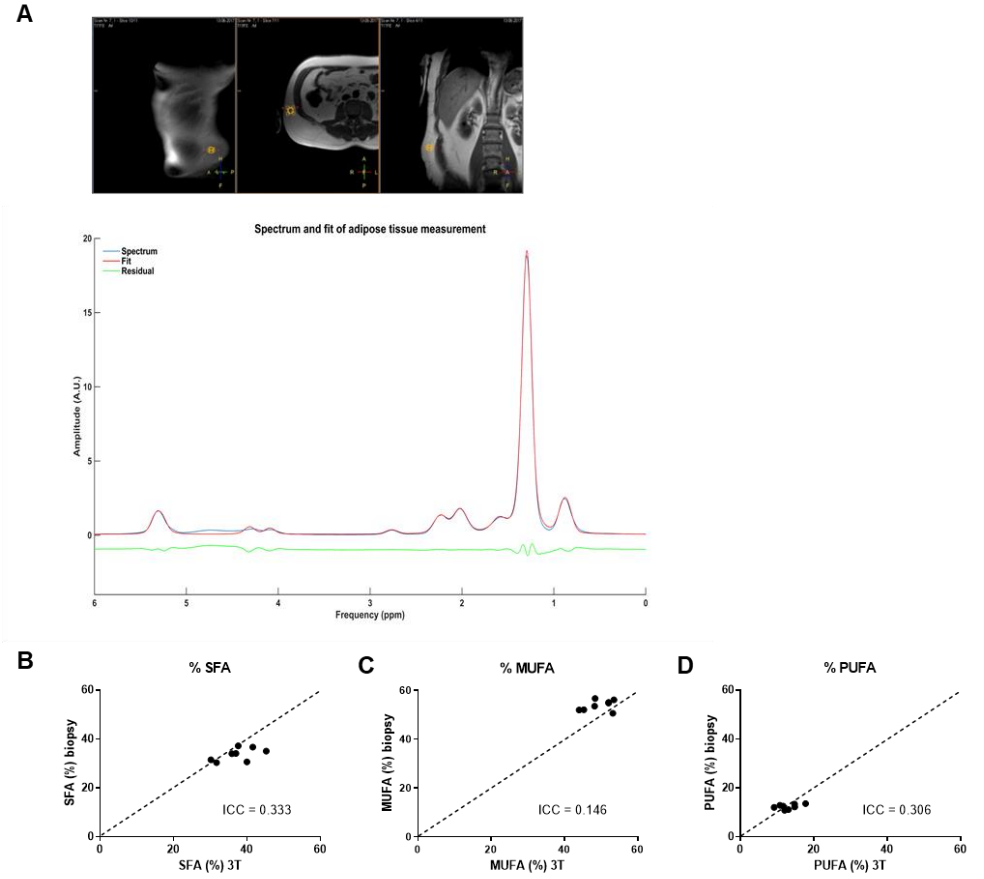

Figure 2: Validation of ^{1}H-MRS method in subcutaneous adipose tissue. A T2 weighted Turbo spin echo MR image showing the voxel position located on adipose tissue and its corresponding lipid spectrum together with the fitted spectrum. The relationships between subcutaneous adipose tissue measured at 3 T and adipose lipid composition determined through biopsy for the different lipid fractions: **B** SFA, **C** MUFA and **D** PUFA (n=8). The intraclass correlation coefficient (ICC) is shown in the respective plots (Intraclass correlation).

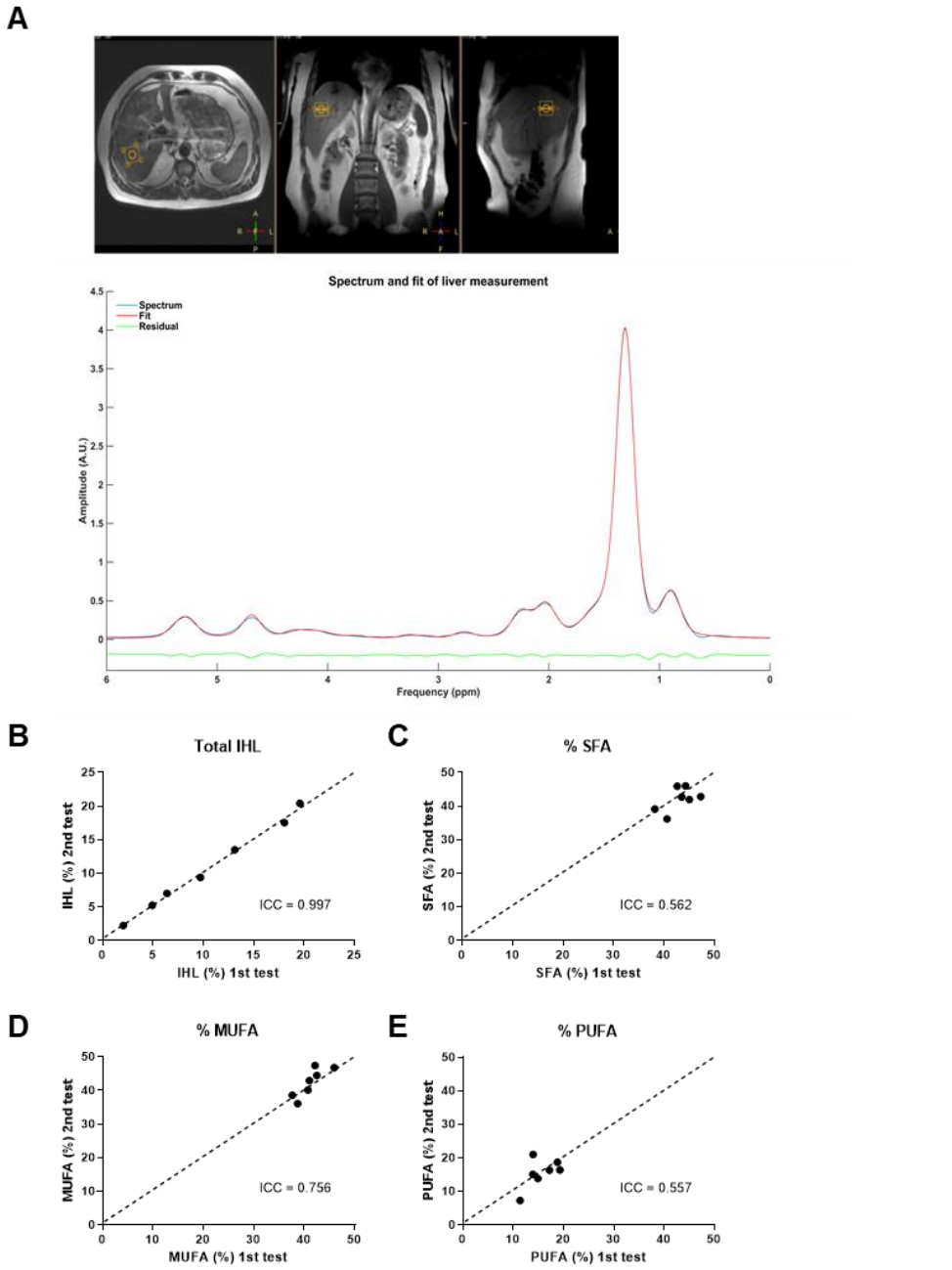

Figure 3: Validation of ^{1}H-MRS method by testing reproducibility *in vivo*. A T2 weighted Turbo spin echo MR image showing the voxel position located on liver and its corresponding lipid spectrum together with the fitted spectrum. Scatter plots showing the reproducibility of **B** total liver fat content and **C** SFA fraction, **D** MUFA fraction and **E** PUFA fraction. (n=7). Reproducibility was tested by performing two repeated measurements. The intraclass correlation coefficient (ICC) is shown in the respective plots (Intraclass correlation).

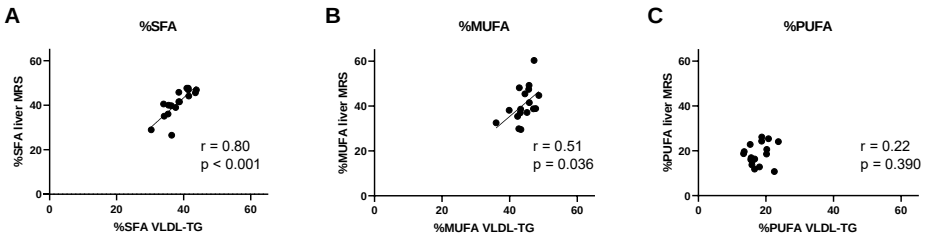

Figure 4: The relationship between hepatic lipid composition and plasma VLDL-TG composition. Relationships are shown for the different lipid fractions: **A** SFA, **B** MUFA and **C** PUFA (n=17). Hepatic %SFA determined with MRS and %SFA in VLDL-TG correlated significantly ($p=1.38x10^{-4}$). The correlation coefficient is shown in the respective plots (two-sided Pearson correlation).

Higher hepatic SFA is associated with increased DNL

DNL is an important factor in the development of fatty liver (30). We hypothesized that DNL would specifically lead to the accumulation of saturated fatty acids, as palmitate is the main product of DNL. Therefore, we determined DNL by deuterated water in overweight and obese participants with a wide range of liver fat content (n=16, 0.9-38.4%) and related it to the hepatic fatty acid composition determined by our MRS protocol in the same volunteers. DNL was not associated with total liver fat content (figure 5A). Interestingly, however, DNL correlated positively with the hepatic SFA fraction (Pearson r=0.52, p=0.047; figure 5B). Furthermore, we found a strong negative correlation between DNL and hepatic MUFA fraction (Pearson r=-0.71, p=0.003; figure 5C). In addition, DNL was negatively correlated with MUFA/SFA ratio (Pearson r=-0.64 p=0.010; figure 5E). No association was found between DNL and PUFA fraction (figure 5D). Of note, SFA fraction in plasma VLDL-TG did not correlate with DNL (Pearson r=0.27, p=0.308). These results suggest that 1) higher rates of DNL lead to altered saturation of hepatic lipids, and 2) that our non-invasive method can be used as a measure of the degree of DNL, directly in the liver.

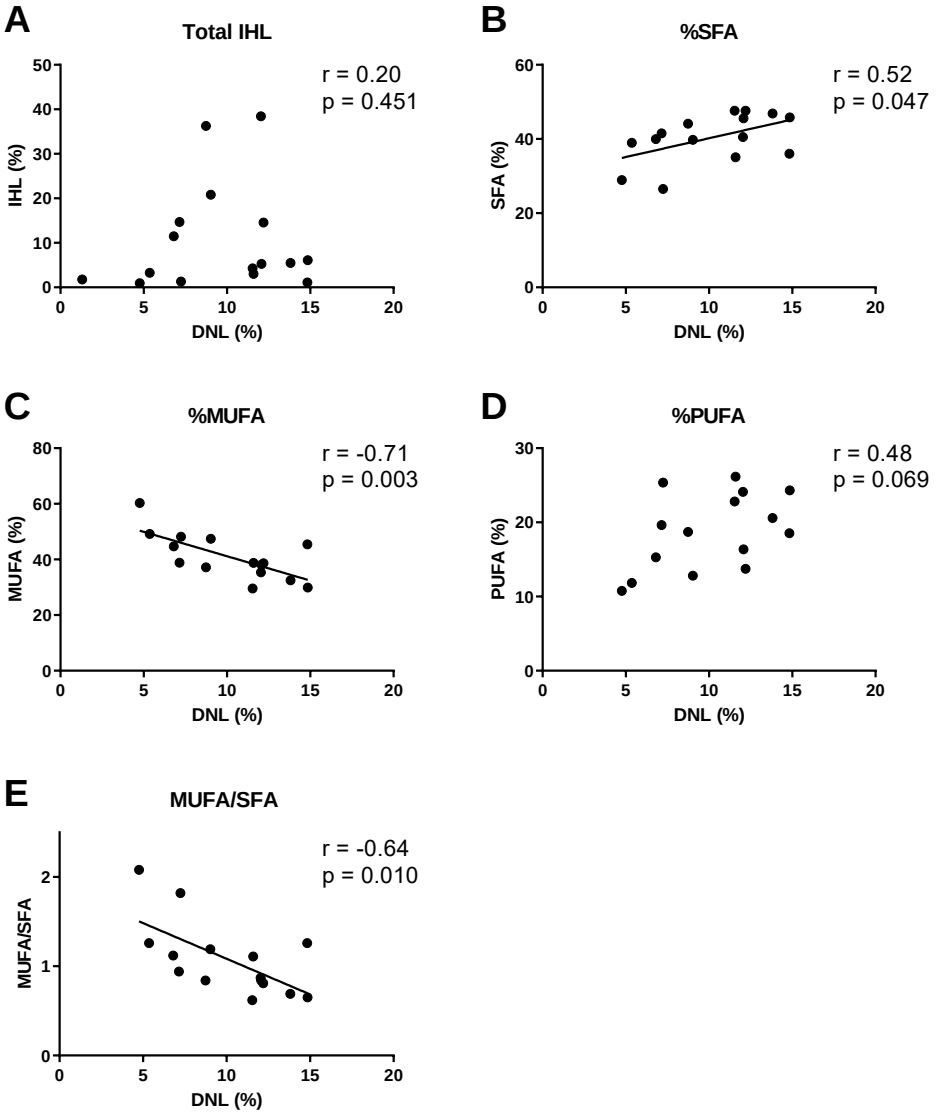

Figure 5: Relationship between DNL and liver fat composition. The relationships between DNL and **A** total liver fat content, **B** SFA fraction, **C** MUFA fraction, **D** PUFA fraction and **E** MUFA/SFA ratio in healthy overweight/obese participants (with and without NAFL, n=16 for total liver fat content and n=15 for the fatty acid fractions and MUFA/SFA ratio). The correlation coefficient is shown in the respective plots (A; two-sided Spearman correlation, B-E; two-sided Pearson correlation).

Hepatic SFA fraction is of clinical relevance in humans

To investigate if hepatic lipid composition may also have clinical relevance, we compared hepatic lipid composition in volunteers with a range in metabolic complications. We subdivided the volunteers in whom we demonstrated the relationship between DNL and hepatic SFA, into participants with NAFL (liver fat content > 5%, n=15) and control participants (liver fat content < 5%, n=7); volunteers with NAFL are known to be at increased risk to develop type 2 diabetes and other cardiometabolic disorders (6-8). In addition, we measured hepatic lipid fractions and hepatic insulin sensitivity (IS) in a group of patients with type 2 diabetes (n=9) and furthermore, determined hepatic lipid fractions in glycogen storage disease type 1a (GSD1a) patients (n=7), known to have elevated rates of DNL. This inborn error of metabolism is caused by a defect in glucose-6-phosphatase, which hampers the final step in gluconeogenesis and glycogenolysis in liver and kidney, and, as a consequence, favours the shift of glucose-6-phosphate towards DNL and hepatic steatosis (31). Control and NAFL participants and patients with type 2 diabetes were comparable in age and BMI. All subject characteristics are summarized in table 1.

Table 1: Subject characteristics of control and NAFL participants, patients with T2D and GSD1a.

	Control (n=7)	NAFL (n=15)	T2D (n=9)	GSD1a (n=7)
Age (years)	59 ± 6.8	58 ± 7.1	65 ± 4.5	37 ± 12.3 $^{A, B, C}$
BMI (kg/m^2)	29.1 ± 2.3	30.7 ± 3.1	29.4 ± 4.2	27.5 ± 3.2
Sex (f/m)	6/1	7/8	2/7	5/2
Body fat (%)	42.1 ± 8.1	42.1 ± 7.8	34.1 ± 5.8	-
Plasma glucose (mmol/L)	5.2 ± 0.4	5.6 ± 0.5	7.5 ± 1.1 $^{A, B}$	3.9 ± 0.8 $^{A, B, C}$
Plasma insulin (pmol/L)	35.2 ± 8.1	89.8 ± 40.4 ^{A}	72.8 ± 58.3	13.8 ± 5.0 $^{B, C}$
Plasma NEFA (mmol/L)	667 ± 58	623 ± 141	566 ± 211	876 ± 421
Plasma TG (mmol/L)	1.5 ± 0.8	2.6 ± 1.2	1.6 ± 0.5	5.0 ± 1.5 $^{A, B, C}$
ALT (U/L)	22 ± 4.1	35 ± 14.4	27 ±11.4	18 ± 4.9 ^{B}
AST (U/L)	22 ± 3.7	28 ± 6.3	23 ± 5.9	34 ± 9.3 $^{A, C}$
Intrahepatic fat content (% weight/weight)	2.2 ± 1.3	14.4 ± 10.4 ^{A}	6.9 ± 5.2	16.5 ± 18.9 ^{A}

Data are presented as mean ± SD. Overweight/obese controls without NAFL (controls, n=7), overweight/obese with NAFL (NAFL, n=15), patients with type 2 diabetes (T2D, n=9) and GSD type 1a (GSD1a, n=7). Age was significantly lower in GSD1a compared to control ($p=2.5x10^{-5}$), NAFL ($p=3.0x10^{-6}$) and T2D ($p=1.47x10^{-7}$). Plasma glucose was significantly lower in GSD1a compared to control (p=0.011), NAFL ($p=5.6x10^{-5}$) and T2D ($p=1.44x10^{-10}$), and was significantly higher in T2D compared to control ($p=4.0x10^{-6}$) and NAFL ($p=6.0x10^{-6}$). Plasma insulin was significantly lower in GSD1a compared to NAFL ($p=3.4x10^{-5}$) and T2D (p=0.006), and was significantly higher in NAFL compared to control (p=0.048). Plasma TG was significantly higher in GSD1a compared to control ($p=3.0x10^{-6}$), NAFL ($p=1.33x10^{-4}$) and T2D ($p=2.0x10^{-6}$). ALT was significantly higher in NAFL compared to GSD1a (p=0.011). AST was significantly higher in GSD1a compared to control (p=0.006) and T2D (p=0.017). Intrahepatic fat content was significantly higher in NAFL compared to control (p=0.002) and GSD1a compared to control (p=0.027). ^{A} Significantly different from control, ^{B} significantly different from NAFL, ^{C} significantly different from T2D (Kruskal-Wallis, p<0.05 for plasma insulin, plasma NEFA, ALT and intrahepatic fat content, and one-way ANOVA, p<0.05 for all other parameters). Bonferroni correction was used for post-hoc analyses.

Hepatic SFA fraction was higher in NAFL individuals (42.9% ± 0.8%, ANOVA, p=0.022, mean ± SEM; figure 6B) and in type 2 diabetes patients (43.9% ± 1.7%, ANOVA, p=0.016; figure 6B) compared to controls (35.5% ± 3.1%). Hepatic MUFA and PUFA fractions did not significantly differ between these groups (figure 6C and 6D). No significant associations were found between hepatic fat content and any of the FA fractions in healthy participants with and without NAFL. Therefore, these data suggest that specifically the SFA fraction is elevated in metabolically compromised volunteers.

To further confirm that DNL and SFA fraction may be causally related, and to further investigate the clinical relevance of SFA, we determined SFA fraction in a specific group of patients, known to have elevated rates of DNL, i.e. patients with glycogen storage disease type 1a (GSD1a) (n=7). In these patients, SFA fraction (49.5% ± 2.0%) was markedly higher compared to NAFL participants (42.9% ± 0.8 %, ANOVA, p=0.034; figure 6B) and controls (35.5% ± 3.1%, ANOVA, p<0.001; figure 6B). The MUFA fraction in these patients was reduced compared to controls (33.2% ± 1.1% vs. 45.2% ± 4.2%, ANOVA, p=0.006; figure 6C). These results further confirm that the fraction of hepatic SFA and MUFA reflect the rate of DNL and can be used as a non-invasive measure to characterise hepatic metabolism in clinical patients in more detail.

Higher hepatic SFA is associated with reduced hepatic IS

Our results so far suggest that specifically the saturated fat fraction is elevated in patients and individuals with an enhanced metabolic risk. These data are consistent with findings in animal studies, where it was found that specifically hepatic SFA is of importance in relation to insulin resistance (13). We therefore investigated the relationship between hepatic lipid composition and hepatic IS using the golden-standard two-step hyperinsulinemic euglycemic clamp. Hepatic IS was found to be 40% lower in patients with type 2 diabetes compared to controls (EGP suppression controls: 74.2% ± 7.0% and T2D: 41.6% ± 4.1%, ANOVA, p=0.002; figure 7F), with intermediate values in NAFL (EGP suppression 56.9% ± 4.9%; figure 7F). The hepatic SFA fraction was strongly and negatively associated with hepatic IS (Spearman r=-0.55, p=0.002; figure 7B), whereas correlation with hepatic MUFA fraction was weaker (Spearman r=0.39, p=0.034; figure 7C) and PUFA fraction and total hepatic fat content did not correlate with hepatic IS (figure 7A, D). Furthermore, the MUFA/SFA ratio was positively associated with hepatic IS (Spearman r=0.51, p=0.005; figure 7E). These results suggest that specifically the SFA fraction in the liver negatively contributes to hepatic IS, rather than the total amount of hepatic fat.

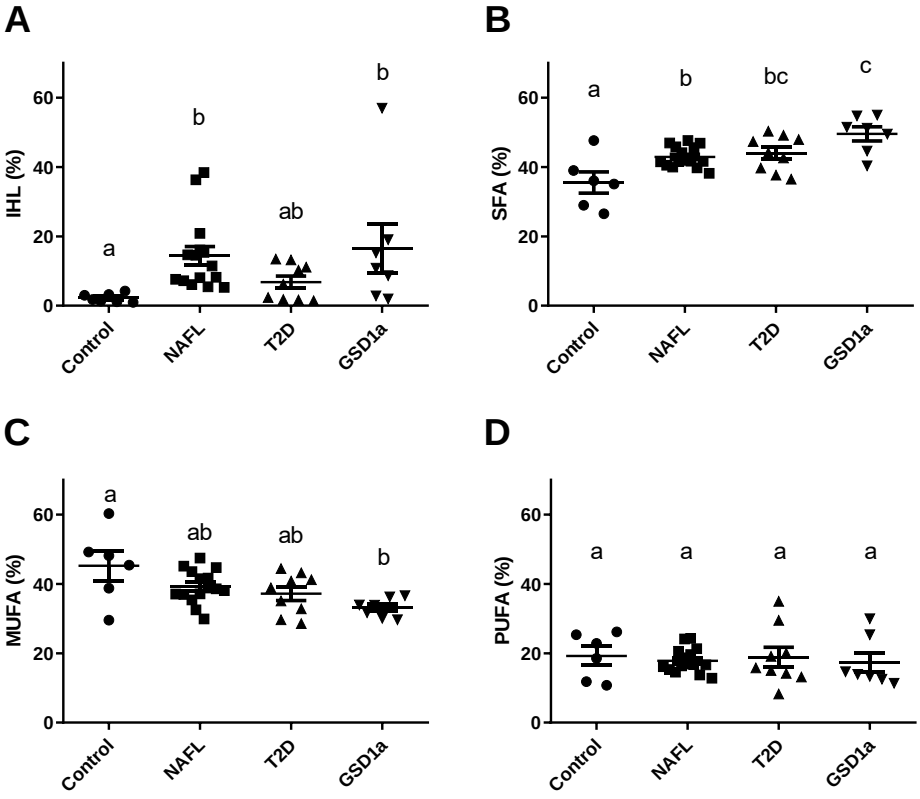

Figure 6: Liver fat content and composition in groups with different metabolic disorders. Comparisons between overweight/obese controls without NAFL (controls, n=7 for total liver fat content, n=6 for liver fat composition), overweight/obese with NAFL (NAFL, n=15), patients with type 2 diabetes (T2D, n=9) and GSD type 1a (GSD1a, n=7). **A** Total liver fat content in control, NAFL, T2D and GSD1a. Total liver fat content was significantly higher in the NAFL group compared to the control group (p=0.002) and in the GSD1a group compared to the control group (p=0.027). **B** SFA fraction in control, NAFL, T2D and GSD1a. SFA fraction was significantly higher in the GSD1a group compared to the control group ($p=7.3x10^{-5}$) and NAFL group (p=0.034), significantly higher in the T2D group compared to the control group (p=0.016), and significantly higher in the NAFL group compared to the control group (p=0.022). **C** MUFA fraction in control, NAFL, T2D and GSD1a. MUFA fraction was significantly lower in the GSD1a group compared to the control group (p=0.006). **D** PUFA fraction in control, NAFL, T2D and GSD1a. Data are presented as mean with error bars showing the SEM. Different letters indicate significant differences between groups (Kruskal-Wallis, p<0.05 for IHL and PUFA, and one-way ANOVA, p<0.05 for SFA and MUFA). Bonferroni correction was used for post-hoc analyses.

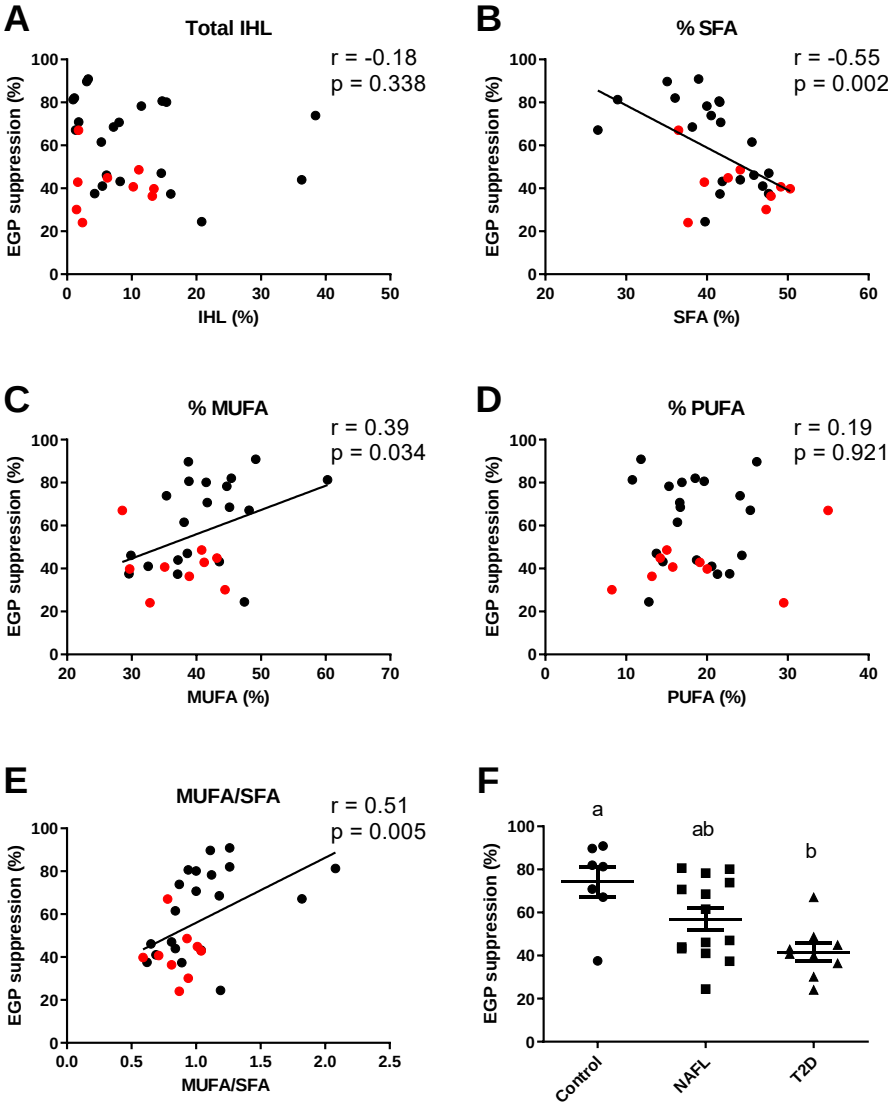

Figure 7: The relationship between liver fat composition and hepatic insulin sensitivity. The relationship between hepatic IS (EGP suppression) and **A** total liver fat content, **B** SFA fraction, **C** MUFA fraction and **D** PUFA fraction and **E** MUFA/SFA ratio in overweight and obese individuals (healthy with and without NAFL in black and patients with T2D in red, n=30 for total liver fat content and n=29 for the fatty acid fractions and MUFA/SFA ratio). The correlation coefficient is shown in the respective plots (Two-sided Spearman correlation). **F** EGP suppression in healthy overweight/obese without NAFL (controls, n=7), overweight/obese with NAFL (n=14) and patients with T2D (n=9). EGP suppression in patients with T2D was significantly lower compared to controls (p=0.002). Data are presented as mean with error bars showing the SEM. Different letters indicate significant differences between groups (one-way ANOVA, p<0.05). Bonferroni correction was used for post-hoc analyses.

DISCUSSION

The relation of hepatic lipid composition to cardiometabolic health and specifically insulin resistance has not been investigated in humans. Due to the invasive procedures necessary and due to challenges accompanying the use of non-invasive methods such as ^{1}H-MRS, determination of hepatic lipid composition is hampered. Here, we developed, validated and applied ^{1}H-MRS methodology that enabled us to non-invasively quantify the fractions of hepatic SFA, MUFA and PUFA separately. We showed that DNL was positively related to the hepatic SFA fraction and negatively to the MUFA fraction in overweight/obese participants, suggesting that high rates of DNL modify fatty acid composition. We confirmed this association between DNL and hepatic SFA fraction by showing increased hepatic SFA fraction in GSD1a patients, a genetic model of increased DNL. Furthermore, we showed that the hepatic SFA fraction is elevated in NAFL and type 2 diabetes patients and related to hepatic insulin resistance. These results show that our ^{1}H-MRS protocol can be used to determine hepatic fat composition in humans and that particularly the SFA fraction can be seen as a non-invasive measure of DNL, directly measured in the liver. Furthermore, our results indicate that hepatic lipid composition may be a clinically important feature of hepatic fat accumulation.

Previously, a few studies have used similar approaches using ^{1}H-MRS for hepatic lipid profiling, but none of these studies have reported SFA, MUFA and PUFA fractions separately (16-19). Even more so, in case of changes in MUFA/PUFA ratios, the calculation of the saturation index by using the methine resonance can lead to a misinterpretation of the lipid composition. We here show that by acquiring high quality liver spectra and by applying a sophisticated post-processing method for these spectra it is possible to differentiate between the hepatic SFA, MUFA and PUFA fractions *in vivo* in subjects with and without NAFL. Implementation of this methodology in future studies makes it possible to further explore the importance of hepatic fat composition in metabolic health and the factors that could modulate fatty acid composition.

High rates of DNL may change hepatic lipid composition. The end product of the lipogenic pathway is palmitate (C16:0), which in turn can undergo elongation to stearate (C18:0) and desaturation by Stearoyl-CoA desaturase-1 (SCD1) to the MUFAs palmitoleate (C16:1 n-7) and oleate (C18:1 n-9). Previously, it has been shown that changes in DNL as determined by stable isotope techniques correlate with changes in the fraction of palmitate in VLDL-TG (32). In line with this, DNL was positively correlated with the hepatic SFA fraction in the current study. The hepatic MUFA fraction was negatively correlated with DNL, whereas the hepatic PUFA fraction did not correlate with DNL. From these results, it follows that upon

increased DNL, the production of SFA is not necessarily accompanied by subsequent desaturation to MUFAs in humans. This is in contrast to animal studies that indicate that desaturation is upregulated in parallel to upregulation of lipogenic enzymes (13). In line, Peter et al. showed that SCD1 mRNA expression and C18:1/C18:0 ratio in TG and phospholipid fractions in human liver were not increased in individuals with NAFL (33). Thus, handling of fatty acids originating from DNL by the liver may be different in humans and animals. Increased desaturation following lipogenesis is believed to be a rescue mechanism to reduce the negative effects exerted by saturated fatty acids (13). Further, we find higher hepatic SFA fraction in patients with NAFL and T2DM compared to controls, which is in line with earlier results, showing that the relative contribution of DNL derived FA to VLDL-TG was around 5-10% in healthy individuals in the fasted state, whereas their contribution increased to around 20-25% in people with NAFL (11, 30, 34, 35). These results suggest that the negative health effects that have been attributed to increased rates of DNL may also be due to the high fraction of SFA that result from DNL. Therefore, drug development targeting DNL is promising (36-38) although it is important to note that the evidence in the present study is only associative and future research will have to show whether decreasing the amount of SFA has beneficial effects on metabolic health. Interestingly however, animal studies support this notion. In mice overexpressing carbohydrate-responsive element-binding protein (ChREBP), resulting in increased activity of SCD1 and higher MUFA fraction, the increased MUFA fraction at the expense of SFA fraction has been associated with increased insulin sensitivity, despite high amounts of total hepatic fat content (13). Furthermore, it has been shown in vitro that specifically SFA negatively affect insulin signaling and that desaturation of fatty acids can rescue these negative effects (13). In line with these animal and cell data, we here show that the hepatic SFA fraction was negatively correlated and the MUFA/SFA ratio positively correlated with hepatic insulin sensitivity in overweight and obese participants. Plasma (VLDL-)TG is often used as a surrogate for hepatic TG. In addition, with sophisticated centrifugation methods, saturation of plasma VLDL-TG can be determined. Here we showed that saturation of hepatic lipids correlated with the saturation in plasma VLDL-TG. Interestingly however, unlike the MRS determined SFA fraction, the SFA fraction in VLDL-TG did not correlate with DNL. These results indicate, that the SFA fraction determined by MRS is a better measure of DNL compared to the SFA fraction in VLDL-TG, and thus the use of our 1H-MRS approach provides extra valuable information that could not be determined from plasma VLDL-TG.

In summary, we developed an MR protocol that enabled us to non-invasively quantify the fractions of hepatic SFA, MUFA and PUFA. Applying this ^{1}H-MRS methodology, we here show that the hepatic SFA fraction is positively associated with DNL, is elevated in patients with NAFL and T2D, and negatively associated with hepatic insulin sensitivity. These results

indicate that hepatic lipid composition, as determined by our ^{1}H-MRS methodology, might be used as measure of DNL and furthermore suggest that specifically the hepatic SFA fraction may determine metabolic health.

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These authors contributed equally: Kay Roumans, Lucas Lindeboom

K.R. performed the experiments, analysed data, and wrote the manuscript. L.L. was involved in design of experiments, has set up the post-processing pipeline and contributed to writing the manuscript. P.V. was involved in setting up the post-processing pipeline, performed experiments, analysed data and reviewed and edited the manuscript. C.R., E.P., B.H., Y.B., M.S. performed experiments and reviewed and edited the manuscript. M.B., M.A., H.P., R.M., B.S., M-R.T., J.B., P.S., V.S. were involved in the design of the study, interpretation of the data, and reviewed and edited the manuscript.

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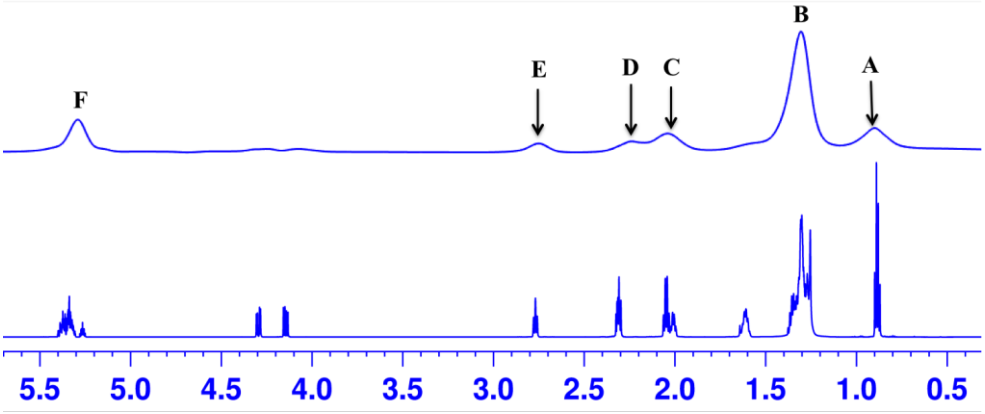
HP and MA were employees of Unilever at the time this research was designed and conducted and have since changed their professional affiliations. HP: Superfoods, Landsmeer. MA: Health Counsel, NL.

The authors declare no competing interests.

SUPPLEMENTARY MATERIAL FOR CHAPTER 3



Supplementary figure 1: Comparison of lipid spectrum acquired at 3T and high-resolution ^{1}H-NMR. Lipid spectrum acquired from the sunflower oil at 3T (top) and high-resolution ^{1}H-NMR spectrum (bottom) showing the variation in spectra resolution. A-methyl (-CH_3); B-methylene (-CH_2); C-allylic (CH_3-CH=CH-CH_2); D-alpha carbonyl (CH_2-COO); E-diallylic (=CH-CH_2-CH=); F-methine (CH=CH).

Supplementary table 1: Correction factors for PUFA and MUFA calculation

	CA	CB
Olive oil	0,759	0,997
Arachis oil	0,735	0,987
Sunflower oil	0,907	1,003
Safflower oil	0,889	0,982
Rice oil	0,849	0,990

Supplementary table 2: List of triglyceride species determined by mass spectrometry

Triglyceride species
TAG 48:0
TAG 48:1
TAG 48:2
TAG 48:3
TAG 50:0
TAG 50:1
TAG 50:2
TAG 50:3
TAG 50:4
TAG 50:5
TAG 52:0
TAG 52:1
TAG 52:2
TAG 52:3
TAG 52:4
TAG 52:5
TAG 52:6
TAG 54:1
TAG 54:2
TAG 54:3
TAG 54:4
TAG 54:5
TAG 54:6
TAG 54:7
TAG 56:2
TAG 56:3
TAG 56:4
TAG 56:5
TAG 56:6
TAG 56:7
TAG 56:8
TAG 58:7
TAG 58:8
TAG 58:9

Supplementary table 3: List of fatty acid species determined by mass spectrometry

FA species	Saturation
FA 14:0	SFA
FA 16:0	SFA
FA 18:0	SFA
FA 16:1	MUFA
FA 18:1	MUFA
FA 18:2	PUFA
FA 20:3	PUFA
FA 20:4	PUFA
FA 20:5	PUFA
FA 22:5	PUFA
FA 22:6	PUFA

Supplementary table 4: Clamp parameters in control participants, and patients with NAFL and T2D

	Control (n=7)	NAFL (n=14)	T2D (n=9)
Ra (µmol/kg/min)			
Baseline	9.74 ± 2.40	7.64 ± 1.50	12.84 ± 2.03 [A,B]
Low insulin	11.88 ± 1.34	9.08 ± 1.69[A]	9.83 ± 2.44
High insulin	35.16 ± 11.29	26.58 ± 7.74	23.02 ± 10.76
Rd (µmol/kg/min)			
Baseline	8.87 ± 2.06	7.18 ± 1.64	13.28 ± 3.10 [A,B]
Low insulin	12.68 ± 1.30	9.04 ± 1.58 [A]	10.28 ± 2.47 [A]
High insulin	35.41 ± 10.80	26.16 ± 7.51	23.55 ± 9.93 [A]
EGP (µmol/kg/min)			
Baseline	9.74 ± 2.40	7.64 ± 1.50	12.84 ± 2.03 [A, B]
Low insulin	2.68 ± 2.55	3.15 ± 1.22	7.54 ± 2.09 [A, B]
High insulin	0.98 ± 1.47	1.07 ± 0.81	2.73 ±0.51 [A, B]
% suppression low insulin	74.20 ± 18.40	56.92 ± 18.50	41.61 ± 12.15 [A]
% suppression high insulin	89.33 ± 15.82	85.24 ± 12.06	78.44 ± 4.03

Data are presented as mean ± SD. Overweight/obese controls without NAFL (controls, n=7), overweight/obese with NAFL (NAFL, n=14), patients with type 2 diabetes (T2D, n=9). Ra baseline was significantly higher in T2D compared to control (p=0.010) and NAFL ($p=2.0x10^{-6}$). Ra low insulin was significantly lower in NAFL compared to control (p=0.010). Rd baseline was significantly higher in T2D compared to control (p=0.002) and NAFL ($p=3.0x10^{-6}$). Rd low insulin was significantly higher in control compared to NAFL (p=0.001) and T2D (p=0.045). Rd high insulin was significantly lower in T2D compared to control (p=0.045). EGP baseline was significantly higher in T2D compared to control (p=0.010) and NAFL ($p=2.0x10^{-6}$). EGP low insulin was significantly higher in T2D compared to control ($p=2.35x10^{-4}$) and NAFL (p=0.001). EGP high insulin was significantly higher in T2D compared to control (p=0.003) and NAFL (p=0.001). EGP suppression low insulin was significantly lower in T2D compared to control (p=0.002). [A] Significantly different from control, [B] significantly different from NAFL (Kruskal-Wallis $p<0.05$ for EGP Low Insulin and EGP suppression high insulin, and one-way ANOVA $p<0.05$ for all other parameters). Bonferroni correction was used for post-hoc analyses.

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CHAPTER 4

Hepatic glycogen is not modulated by one night of prolonged fasting in people with non-alcoholic fatty liver

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Submitted

ABSTRACT

Time-restricted eating has been shown to improve metabolic health, even in energy balance. These beneficial effects may at least partially be due to creating a more pronounced fasting state, leading to larger fluctuations in hepatic glycogen. Here, we investigated whether prolonging an overnight fast from 9.5 hours to 16 hours reduces hepatic glycogen, improves substrate metabolism and, when repeating this fasting for 5 days improves hepatic lipid content and composition in individuals with non-alcoholic fatty liver (NAFL). Using carbon-13 magnetic resonance spectroscopy (^{13}C-MRS), we determined overnight changes in hepatic glycogen after one day of fasting for 9.5 versus 16 hours. In addition, we determined substrate oxidation overnight as well as in the morning upon a meal using whole-body respirometry. Hepatic lipid content and composition after prolonged overnight fasting for 5 days were determined by ^{1}H-MRS. We found no effect of one night of prolonged fasting on hepatic glycogen content. Interestingly, a prolonged fast of 16h did also not result in higher fat oxidation rates during the night, suggesting that these volunteers with NAFL did not fully reach the fasting state. Postprandial substrate oxidation was also unchanged after one prolonged overnight fast. Furthermore, 5 days of 16 versus 9.5 hours of fasting did not improve hepatic lipid content and composition. These results suggest that hepatic substrate metabolism is not sensitive to extending acute fasting periods in individuals with NAFL.

INTRODUCTION

Non-alcoholic fatty liver (NAFL) has become one of the most common causes of liver disease worldwide (1). The main characteristic of this condition is the build-up of excessive fat in the liver. In case hepatic fat storage exceeds 5%, in absence of excessive alcohol consumption, non-alcoholic fatty liver (NAFL) is diagnosed. Over time, excessive hepatic fat storage can progress to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and even hepatocellular carcinoma. Moreover, consequences of NAFL are not limited to the liver itself, as NAFL is also associated with an increased risk for other metabolic diseases such as type II diabetes and cardiovascular disease (2-4).

Our 24-hour lifestyle, in which food is no longer restricted to daytime, may be at the basis of the increasing prevalence of metabolic diseases such as NAFL. Food intake is usually spread over about 15h a day by the majority of people (5), which means that the overnight fasting time is limited to around 9h a day. However, a long overnight fasting period may be of great importance in maintaining metabolic health. In the post-absorptive state the body relies on hepatic glycogen stores to maintain blood glucose levels, and hepatic glycogen is reduced in the fasted state (6-8). However, when overnight fasting time is limited, as is the case for the majority of people nowadays, the need for glycogen as energy source is less evident and glycogen stores may not be depleted overnight. As a consequence, dietary carbohydrates originating from breakfast may not be used to replenish hepatic glycogen stores, but may lead to elevated plasma glucose levels and may be converted to fatty acids in the liver by *de novo* lipogenesis (DNL) instead (9). Furthermore, when the body does not reach a truly fasted state, there is less need to switch from carbohydrate to fatty acid oxidation, further favouring lipid storage (10) .

Recently, time-restricted eating (TRE) has gained a lot of attention, as such a regime may improve metabolic health through increasing fasting time (11). Here, we hypothesize that prolonging overnight fasting, such as in TRE, leads to lower hepatic glycogen levels and may explain the beneficial metabolic changes observed upon TRE. Furthermore, we hypothesize that modulating hepatic glycogen storage by prolonging overnight fasting time may be an interesting strategy to improve substrate metabolism and reduce liver fat content in people with NAFL.

Therefore, we here tested the hypothesis that prolonging overnight fasting time will reduce hepatic glycogen levels in volunteers with NAFL, resulting in increased whole-body fat oxidation during the night and upon a breakfast meal, decreased liver fat saturation (reflecting lower DNL rates) and reduced liver fat content. Using carbon-13 magnetic resonance spectroscopy (^{13}C-MRS), we determined overnight changes in hepatic glycogen

after one day of fasting for 9.5 versus 16 hours. In addition, we determined substrate oxidation overnight as well as in the morning upon a meal using whole-body respirometry. Finally, we investigated the effect of 5 days of extended overnight fasting on liver fat content and hepatic lipid composition. Contrary to our hypothesis, we show that prolonging the overnight fast by 6.5 hours does not influence hepatic glycogen in volunteers with NAFL. Similarly, we did not find changes in whole-body fat oxidation, liver fat content or composition.

METHODS

This study was conducted at Maastricht University Medical Center, the Netherlands, between January 2019 and January 2020, and was approved by the Medical Ethical Committee of Maastricht University Medical Centre. Research was performed in accordance with all relevant ethical regulations regarding human research participants.

Participants

All participants provided written informed consent. Ten overweight/obese (BMI 27-38 kg/m^2) volunteers with NAFL (liver fat content ≥5% determined by ^{1}H-MRS), aged 45-75 years were recruited for this study. Subject characteristics are shown in table 1. NAFL was verified in all participants by ^{1}H-MRS prior to inclusion. Female study participants were postmenopausal. Exclusion criteria were engagement in exercise for more than 2 hours per week, unstable body weight (weight loss or gain more than 3 kg in 3 months preceding enrollment), alcohol consumption more than 2 units per day, smoking more than 5 cigarettes per day, contra-indication for MRI, use of medication known to interfere with the outcome parameters, diabetes or other active disease.

Table 1: Subject characteristics.

	Participants (n=10)
Age (years)	62 ± 8.7
BMI (kg/m^2)	30.1 ± 2.2
Sex (f/m)	2/8
Body fat (%)	37.1 ± 5.1
Plasma glucose (mmol/L)	5.8 ± 0.5
Plasma TG (mmol/L)	1.8 ± 0.7
ALT (U/L)	38 ± 13
AST (U/L)	30 ± 7
Intrahepatic fat content (% weight/weight)	13.3 ± 7.7

Data are presented as mean ± SD, n=10.

Clinical Study Design

Volunteers participated in a randomized cross-over trial. One time, participants stayed fasted overnight for 9.5h during 6 consecutive nights, another time participants stayed fasted overnight for 16h during 6 consecutive nights (figure 1). Both conditions started with an overnight stay at the research facility, to study acute effects on hepatic glycogen, and were followed by adherence to the overnight fasting protocol at home, to study the effect on hepatic liver fat content and composition. During their stay at the research facility participants received all meals: breakfast 20 En%, lunch 30 En%, snack 10 En% and dinner 40 En% (2550 kcal diet). In the 9.5h fasting protocol, the snack was provided at 4.30 pm and the evening meal was provided at 11 pm, whereas in the 16h fasting protocol both the evening meal and snack was provided at 4.30 pm (figure 1). Participants were instructed to continue the fasting protocol at home. During this part of the study, participants were allowed to follow their own diet, as long as their diet would be similar between the two periods, so that only timing of the meals and therefore fasting time was different. To monitor at home compliance, 5-day food diaries were used and volunteers wore a continuous glucose monitoring device. Two days before day 1 and day 7, participants were instructed to refrain from physical exercise and alcohol consumption. Between the two fasting protocols a wash-out period of 2-5 weeks was applied. During the entire study period no changes in lifestyle were allowed, except for the change in fasting time as part of the study design.

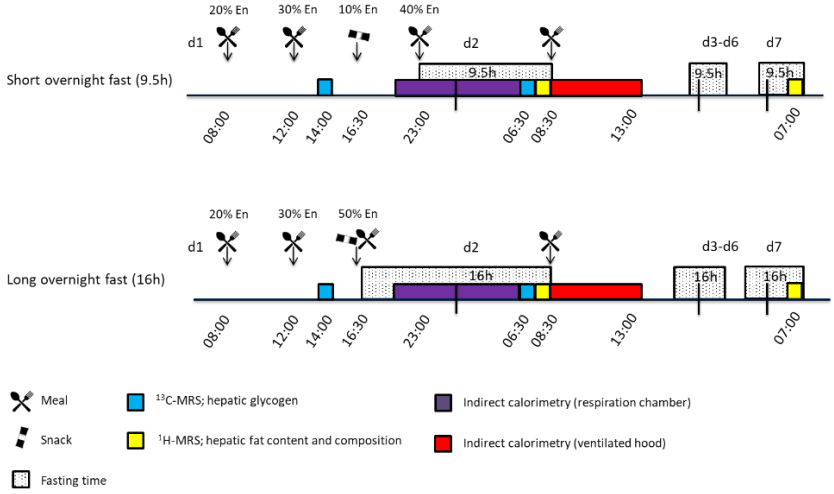

Figure 1: Study protocol. Participants participated in two overnight fasting protocols (9.5h fast and 16h fast). In the 9.5h fasting protocol, the snack was provided at 4.30 pm and the evening meal was provided at 11 pm, whereas in the 16h fasting protocol both the evening meal and snack was provided at 4.30 pm. Each protocol consisted of a 1.5 day visit to the research facilities, followed by adherence to the protocol at home and finished with an ^{1}H-MRS scan at day 7.

Measurement of hepatic glycogen

At day 1 at 2.00 pm and at day 2 after the overnight fast at 6.30 am, hepatic glycogen was determined by ^{13}C-MRS. All ^{13}C-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 21x24cm ^{13}C quadrature detection surface coil (RAPID Biomedical GmbH, Germany). Hepatic glycogen levels were measured by acquiring non-localized ^{13}C MR spectra (FID; TR: 280ms; NSA: 4096). Data analysis was performed with an in-house developed Matlab script. Phase-correction and baseline correction was applied in the morning and afternoon spectra simultaneously with the aim to minimize differences between spectra and therefore ensure identical pre-processing of both spectra. The AUC of glycogen signal was determined by integration of +-5 PPM around the C1 of glycogen at 100.5 PPM. Relative changes in glycogen AUC between the afternoon and morning measurement ((hepatic glycogen $_{6.30\ am}$ - hepatic glycogen $_{2\ pm}$)/ hepatic glycogen $_{2\ pm}$ * 100) were calculated. Reproducibility was tested by performing two repeated measurements in two young lean and two middle-aged overweight individuals. Additionally, postprandial measurements and 12h fasting measurements were performed in three young lean volunteers to show differences in hepatic glycogen were measurable.

Liver volume measurement

Liver volume was measured directly after the hepatic glycogen measurements in the afternoon and fasted in the morning by MRI. Analyses were performed manually on cross-sectional MRI images in MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States). Due to practical problems liver volume MRI images could not be assessed at all timepoints for every participants, therefore, changes in liver volume upon overnight fasting could only be calculated for 6 participants.

Measurement of hepatic lipid content and composition

At day 2 and at day 7, at 7.15 am after an overnight fast, hepatic lipid content and lipid composition (fraction of hepatic SFA, MUFA and PUFA) were determined by proton magnetic resonance spectroscopy (^{1}H-MRS). All ^{1}H-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 32-channel sense cardiac/torso coil (Philips Healthcare, Best, Netherlands). All spectra were obtained by using a STEAM sequence (12) with the following parameters; repetition time (TR) 4500 ms/echo time (TE) 20 ms/ mixing time (TM) 16 ms, spectral bandwidth 2000 Hz and data points 2048. For the hepatic lipid spectra VAPOR water suppression (13) was applied and an additional water reference scan was obtained. The number of averages was 128 for the

hepatic lipid spectra. We used a voxel size of 30 x 30 x 30 mm. All spectra were post-processed in a home-written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States.) script as previously described (14). Lipid content was calculated after T_2 correction as ratio of the CH_2 peak relative to the sum of the unsuppressed water resonance and CH_2 peak, and converted to weight/weight percentage. Relative fatty acid fractions were calculated as described previously (14).

Respiration chamber measurement

Participants stayed in a respiration chamber from day 1 9.00 pm until the next morning 6.00 am. The metabolic chamber is a small room with a bed, toilet, television, computer, and access to water, in which oxygen consumption and carbon dioxide production were measured continuously in sampled room air by whole-room indirect calorimetry (Omnical, Maastricht Instruments, Maastricht, The Netherlands) (15). During the overnight stay in the respiration chamber, sleeping metabolic rate (SMR), respiratory exchange ratio (RER), fat oxidation and carbohydrate oxidation were assessed. Energy expenditure was calculated based on the measured averaged oxygen and carbon dioxide concentrations in the inspired and expired gases with the assumption that protein oxidation was negligible, using the Weir equation (16). SMR was calculated as the lowest average 3-h energy expenditure during the sleeping period. During this 3h period also RER, carbohydrate and fat oxidation were determined. RER was also determined for the early (12.00 am - 3.00 am) and late (3.00 am - 5.30 am) phase of the night. Carbohydrate oxidation and fat oxidation rates were calculated according to Péronnet and Massicotte (17). Participants were instructed to go to sleep at 11.30 pm in both protocols. At 6.00 am the next morning participants were woken up and left the chamber in an overnight fasted state.

Meal test

After the MRS measurements in the morning of day 2, a meal test was performed. To this end, a high-energy breakfast was provided to the participants at 8:30 AM (t=0) (28% energy from fat, 57% energy of carbohydrates, and 15% energy form proteins, total energy content of 800 kcal). Before the meal (t=-15), and at t=45, t=75, t=135, t=195, t=255 minutes, indirect calorimetry measurements using a ventilated hood system (Omnical, Maastricht Instruments, Maastricht University) were performed. VO_2 and VCO_2 were used at these timepoints to assess energy expenditure (16), respiratory exchange ratio, fat and carbohydrate oxidation (17).

Continuous glucose monitoring

Before the start of the meal test, a continuous glucose monitoring sensor (FreeStyle Libre, Abbott) was placed at the back of the upper arm. Interstitial fluid glucose was measured every 15 minutes by the sensor. Measurements took place up to day 7, when the sensor was removed in the morning before the ^{1}H-MRS measurement.

Body composition

Body mass and body volume were assessed on day 1 of the first fasting protocol before breakfast using air-displacement plethysmography (ADP) using the Bod Pod device (Cosmed, Italy, Rome) according to the manufacturer's instructions (18). Thoracic gas volume was predicted based on equations included in the Bod Pod software (version 4.2.0). From these data, body fat percentage was calculated as described by Siri (19).

Statistical analyses

Results were expressed as mean ± SEM. Population characteristics were expressed as mean ± SD. Continuous variables were tested for normality. Comparisons between periods and comparisons between days within one period were assessed by paired-sample t-test.

Changes across the time course during the meal test after a 9.5h and 16h overnight fast were assessed using two-way repeated-measures ANOVA to evaluate any significant main effect of fasting time (9.5h vs. 16h) and timepoint during the meal test and any interaction between fasting time and timepoint. All significant main effects were followed up by pairwise comparisons using a paired t-test and Bonferroni adjustment was applied for multiple comparisons.

A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 23.0 for Mac OS.

RESULTS

Validation of the hepatic ^{13}C-MRS method

To validate the hepatic glycogen quantification methodology, we tested reproducibility in 2 healthy young lean and 2 middle-aged overweight individuals (figure 2A). The difference in AUC between two repeated measurements averaged 5.8% in young healthy individuals and averaged 2.4% in overweight middle-aged individuals and the intraclass correlation coefficient based on these reproducibility measurements was 0.990. These results indicate

good reproducibility for the hepatic glycogen quantification method that was used. To further test whether we were able to show differences in hepatic glycogen as they occur after one overnight fast, we measured hepatic glycogen in the evening 4 hours after an evening meal and the following morning upon an overnight fast of around 12 hours in three healthy young volunteers. In all three young volunteers hepatic glycogen was decreased in the morning compared to the evening (-19.4 ± 1.9%; figure 2B), showing that the used glycogen quantification approach can be used to measure differences in hepatic glycogen as they occur overnight.

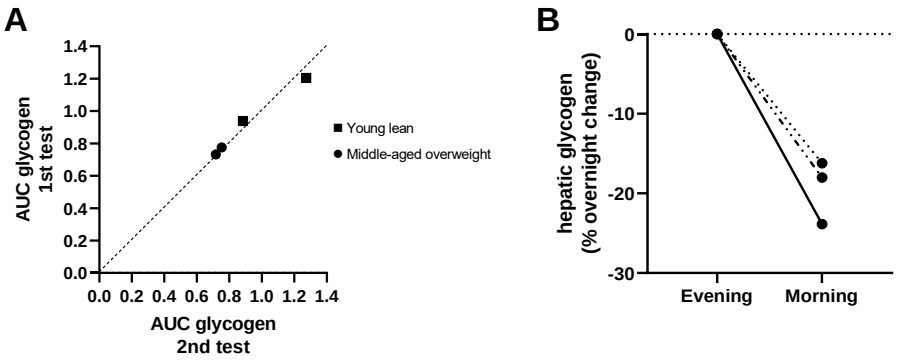

Figure 2: Validation of the hepatic glycogen quantification by ^{13}C-MRS. A Reproducibility of hepatic glycogen tested by two repeated measurements in two young lean and two middle-aged overweight individuals. **B** Relative change in hepatic glycogen in the morning after an overnight fast of 12h compared to postprandial in the evening in young lean volunteers (n=3).

Prolonging overnight fast does not modulate hepatic glycogen in NAFL

Interestingly, the overnight change in hepatic glycogen upon a 9.5h (+4.1 ± 3.3%) and 16h (-2.4 ± 4.7%) overnight fast was not significantly different (figure 3A) in volunteers with NAFL. Consistently, the overnight change in liver volume did not significantly differ upon 9.5h (-1.6 ± 2.5%) compared to 16h fasting (-4.4 ± 4.0%) (figure 3B).

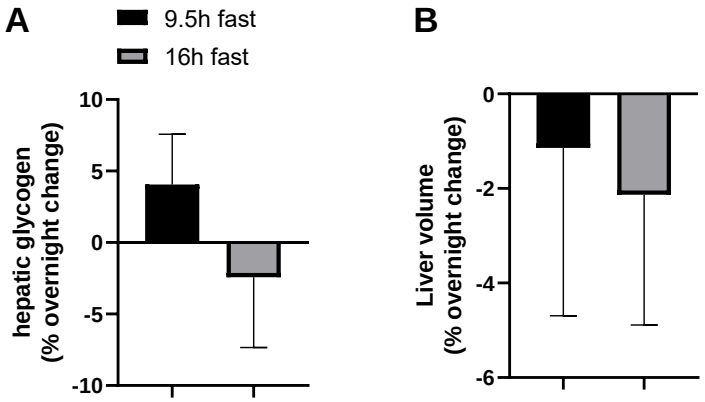

Figure 3: Relative change in A) hepatic glycogen (n=10) and B) liver volume (n=6) from 2 pm in the afternoon to 6.30 am in the morning upon 9.5h and 16h overnight fasting.

Fat oxidation is not increased upon prolonged overnight fasting in NAFL

We next determined whether prolonged overnight fasting changes substrate oxidation during the night. Sleeping metabolic rate (SMR) was lower in the 16h overnight fast (4.9 ± 0.2 kJ/min) compared to the 9.5h overnight fast (5.3 ± 0.2 kJ/min, p<0.001; figure 4A), probably indicating diet-induced thermogenesis from the late evening meal. This higher overnight SMR upon 9.5 hours of fasting was accompanied by an increase in carbohydrate oxidation, which was higher in the 9.5h overnight fast (3.4 ± 0.3 kJ/min,) compared to the 16h overnight fast (2.7 ± 0.2 kJ/min, p=0.024; figure 4B), whereas fat oxidation was not significantly affected by prolonged overnight fasting compared to 9.5 overnight fasting (2.2 ± 0.2 kJ/min vs. 2.1 ± 0.1 kJ/min; figure 4C). As a consequence, respiratory exchange ratio (RER) tended to be higher during the 9.5h overnight fast (0.88 ± 0.01 vs 0.85 ± 0.01; p=0.053; figure 4D). In order to limit the direct effect of the meal and to test the hypothesis that prolonged fasting would lead to a favourable switch towards fat oxidation during the night, we divided the overnight fast in two parts; 12-3 am and 3-5.30 am. However, in neither of the two fasting conditions a decrease in RER was found, suggesting these participants did not completely enter the fasted state, even not after prolonged, 16h of fasting. On the contrary, during the 9.5h overnight fast RER actually significantly increased during the night (12-3 am: 0.87 ± 0.01, 3-5.30 am: 0.89 ± 0.01, p=0.004; figure 4E), probably reflecting postprandial effects. These results indicate that prolonging an overnight fast by 6.5 hours in people with NAFL fails to increase fat oxidation overnight.

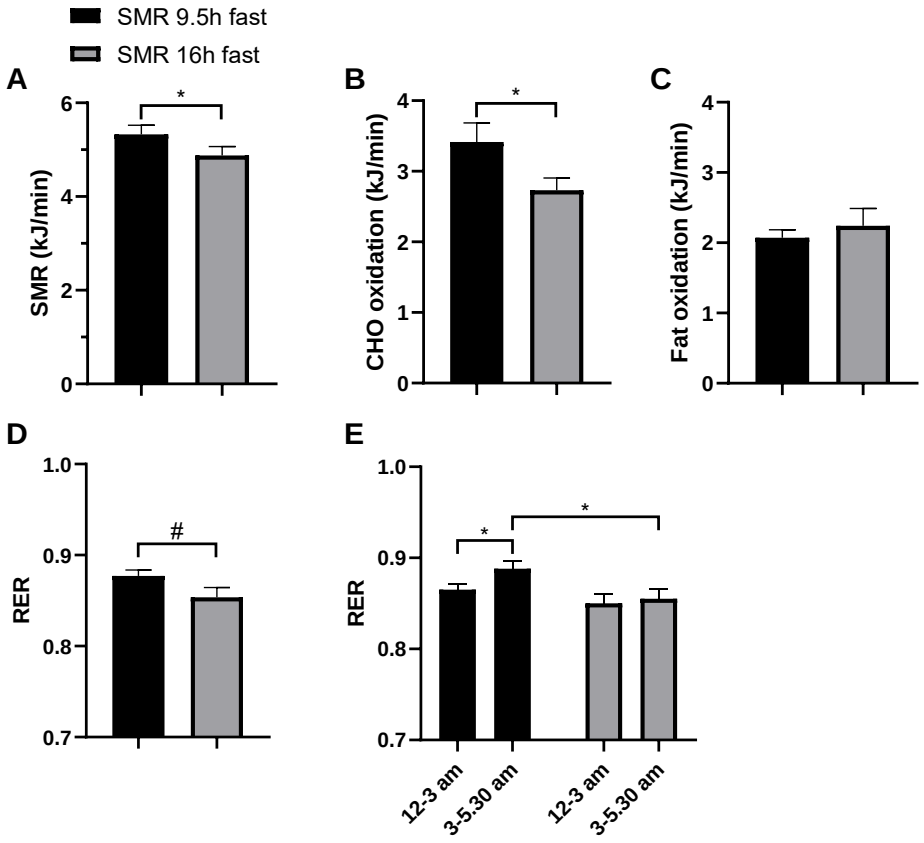

Figure 4: Sleeping metabolic rate (SMR) and substrate oxidation during 9.5h and 16h overnight fasting. A SMR, **B** Carbohydrate (CHO) oxidation, **C** Fat oxidation, **D** Respiratory exchange ratio(RER) during a 9.5h and 16h overnight fast (n=10). **E** RER during the first (12-3 am) and second half (3-5.30 am) of the night in the 9.5h and 16h fasting condition (n=10). * p<0.05, # p<0.10.

After the overnight fast in the morning at 8:30 AM, energy expenditure and substrate use were also measured in the fasted state and upon a meal. Energy expenditure, RER, carbohydrate and fat oxidation in the fasted state (t=-15) were all comparable between the 9.5h and 16h fasting condition (figure 5A, B, C, D). No significant main effect of fasting condition was found on the meal responses in EE, RER, carbohydrate and fat oxidation (figure 5A, B, C, D). Thus, postprandially substrate oxidation is also not influenced by a prolonged overnight fast.

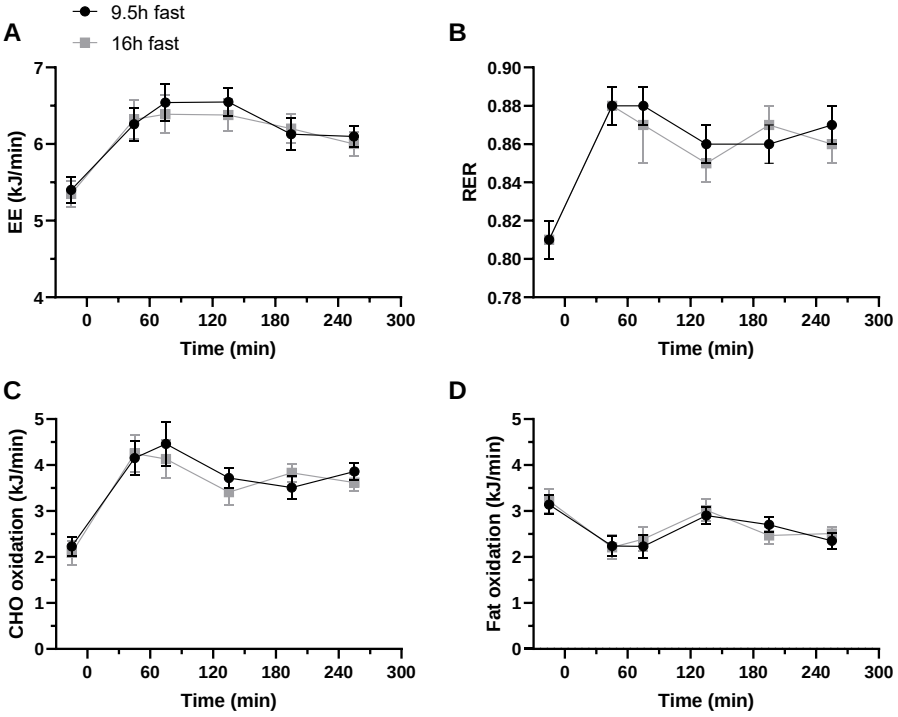

Figure 5: Energy expenditure and substrate oxidation during the meal test upon a 9.5h and 16h overnight fast. **A** Energy expenditure (EE), **B** Respiratory exchange ratio, **C** Carbohydrate oxidation, **D** Fat oxidation during the meal test after a 9.5h and 16h overnight fast (n=10).

Prolonging overnight fasting time does not improve hepatic lipid stores in NAFL

We next investigated if prolonged overnight fasting for 5 days would influence liver fat content and composition. Therefore, participants were asked to adhere to the same timing of eating/fasting at home for 5 days (day 2 until day 7). After this period, we quantified hepatic lipid composition and content. Participants complied to both fasting protocols, as can be seen from the glucose monitoring results during the last evening and night (figure 6). In the long overnight fast condition (early dinner) glucose peaked around 6 pm, whereas during the short overnight fast condition (late dinner) glucose peaked around 11 pm. Prolonging overnight fasting time, while keeping daily calorie intake similar, did not result in significant changes in liver fat content (figure 7A) nor composition (figure 7B, C, D) after 5 days at home on day 7 of the protocol.

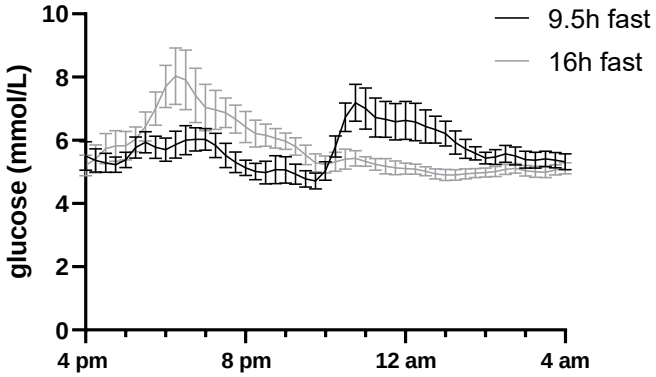

Figure 6: Glucose levels during the last overnight fast at home. Glucose in interstitial fluid during the last overnight fast at home for the 9.5h and 16h fasting condition.

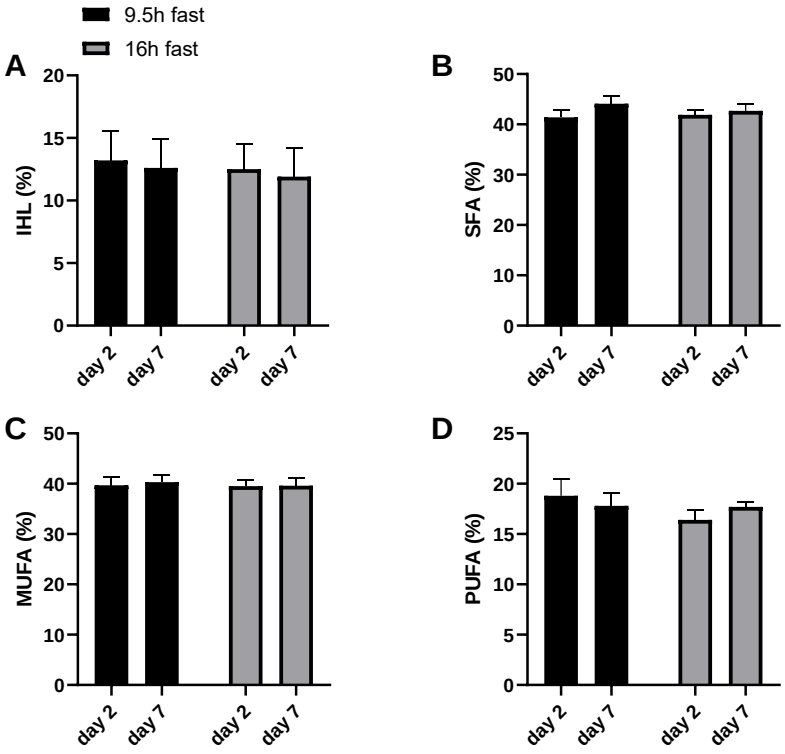

Figure 7: Liver fat content and composition after 9.5h and 16h overnight fasting. A Liver fat content, **B** hepatic SFA fraction, **D** hepatic MUFA fraction, and **D** hepatic PUFA fraction after one night 9.5h and 16h overnight fasting (day 2) and after six nights following the 9.5h and 16h fasting protocol at home (day 7) (n=10).

DISCUSSION

Time-restricted eating has previously been shown to improve metabolic health in volunteers with pre-diabetes, even in energy balance (11). The beneficial effects may at least partially be due to stronger fluctuations in hepatic glycogen. Whether prolonging overnight fasting decreases hepatic glycogen levels in individuals with NAFL and whether this has an acute effect on metabolic parameters related to lipid metabolism in these individuals has not been investigated so far. Here we showed that one night of prolonged overnight fasting by 6.5 hours did not affect hepatic glycogen. In addition, prolonging overnight fasting did not have acute effects on whole-body fat oxidation during the night and upon a breakfast meal. Similarly, continuing the prolonged overnight fasts for 5 days did not influence hepatic lipid saturation nor hepatic lipid content.

We hypothesized that hepatic glycogen would decrease more strongly by prolonging overnight fasting time, so that the change in glycogen would be more negative in the condition with prolonged fasting. However, upon one night of prolonged overnight fasting, glycogen did not change. We should note that unlike earlier studies, where the effect of fasting time on glycogen depletion was investigated (6, 7, 20), in the current set-up, total carbohydrate and energy intake between the afternoon and the morning glycogen scans were identical in both conditions, and solely the timing of the meals changed. Therefore, It is likely that in the 9.5h fast condition, glycogen stores decrease in the afternoon and evening until the late dinner because of limited energy intake. Between lunch and (late) evening meal only a small snack is consumed. In such a situation, glycogen levels right before late dinner at 11 pm are lower compared to the glycogen levels right before dinner in the 16h fasting condition at 4.30 pm. Therefore, it could be that at the end of the night hepatic glycogen stores were filled to a similar extent and thus, hepatic glycogen reductions in the morning were not different compared to baseline in the afternoon. This would mean that if TRE and control conditions are compared while being truly isoenergetic, more pronounced glycogen depletion cannot explain the beneficial metabolic effects.

Previously, it has been shown that overnight fasting leads to reduced hepatic glycogen content in healthy overweight individuals and T2D patients (6-8). Reductions ranging from -19% to -55% in controls and -18% to -40% in T2D patients have been reported compared to postprandial levels, depending on the time of fasting (10 hours to 17 hours). In contrast to these findings, we did not find reduced hepatic glycogen levels in the morning, in individuals with NAFL. This may suggest that hepatic glycogen stores might be more inert in NAFL. Moreover, glycogen levels even slightly increased in the morning in some individuals, especially upon the 9.5h fast. While a low glycogen depletion may be characteristic for

NAFL, the timing of the hepatic glycogen measurements may also have contributed to the findings and may explain that in some cases the glycogen level was even increased in the morning. Timing of the glycogen scans were chosen to be at fixed timepoints in both study arms to avoid circadian influences on glycogen and after the same caloric intake during the day. To this end, hepatic glycogen levels were not determined at peak levels (4-5 hours after the evening meal high in calories and carbohydrates), but before, at 2 pm in both arms. Therefore, the size of the evening meal (50% of daily energy intake, carbohydrate-rich) may have contributed to the fact that glycogen in the morning was slightly higher than at 2 pm, after a light lunch. Indeed, it has been reported before that 10h after a carbohydrate-rich meal, hepatic glycogen levels can still be higher compared to fasting values (20).

The regular depletion of hepatic glycogen may improve metabolic health by increasing metabolic flexibility. In healthy people, substrate use switches to fat oxidation upon fasting. However, in the current study, during the night, no significant increase in fat oxidation was observed upon prolonging fasting time in volunteers with NAFL. Furthermore, even in the long fasting period of 16h, fat oxidation was not stimulated (as we found no increase in fat oxidation in the course of the night), which we would have expected based on studies in healthy lean people (21). Such metabolic inflexibility has been reported before in obese (22), pre-diabetic (23) and diabetic (24) people under fasting conditions in the morning and postprandially. Furthermore, it has recently been shown that fat oxidation does not increase during the night in overweight pre-diabetic volunteers (25), suggesting that fluctuations in fat oxidation over 24h are diminished in people with disturbed metabolic health. This ‘inertia’ in whole-body substrate oxidation is in line with the lack in glycogen fluctuations over time and no difference in glycogen between periods. The observed increase in energy expenditure in the form of carbohydrate oxidation during the night likely reflects postprandial effects of the late dinner in the 9.5h fasting condition, as these measurements were started 1 hour after late dinner.

As glucose oxidation rates remain high throughout the night, and glycogen stores are not depleted, the question arises, what the origin is of the glucose that is oxidized. Increased hepatic glucose output mediated by gluconeogenesis might be used as a possible source for carbohydrate oxidation in individuals with NAFL. Studies reporting an increased gluconeogenesis, but similar glycogenolysis, in NAFL compared to individuals without NAFL support this hypothesis (26, 27). This increase in gluconeogenesis could be driven by increased substrate availability (e.g. glycerol, lactate and pyruvate) and upregulation of gluconeogenic enzymes. Increased availability of pyruvate has been shown in NAFL, together with increased rates of pyruvate carboxylase flux, driving substrate to the gluconeogenic pathway (27). Furthermore, NAFL is associated with increased adipose tissue

insulin resistance (28), which can contribute to increased glycerol flux and thereby provide gluconeogenic substrate. Also in patients with Type 2 diabetes, who are often characterised by increased liver fat storage, gluconeogenic substrate availability is increased (29, 30). Therefore, the fact that glycogen was not decreased in the current study may be characteristic for the study population having NAFL, however, this requires further study.

We hypothesized that prolonging overnight fasting time for 5 days would reduce hepatic fat content and saturation, as a result of an expected increase in fat oxidation and reduction in DNL. However, consistent with our finding that one night of prolonged overnight fasting did not change glycogen depletion, we did not find changes in liver fat content and no indications of changes in DNL after five days of prolonging overnight fasting time. Still, we should be aware that especially in people with NAFL it is difficult to pick up small differences, as a small relative change in hepatic fat content or fat composition may represent a big change in absolute terms. Changes in substrate oxidation have been reported after prolonging fasting by 2.5 days in healthy lean and obese volunteers (31, 32). In rodents, intermittent fasting and TRE interventions, that were more extreme and/or of longer duration (8-12 weeks), have shown to reduce hepatic steatosis (33, 34). Similarly, TRE and alternate day fasting interventions of longer duration result in beneficial effects on metabolic health in obese (35) and in people with NAFL (36). However, such interventions usually also lead to weight loss, as a result of reduced calorie intake. As such, it is unknown if the reported effects are due to metabolic changes, mediated by fasting per se or by weight reductions. Here, we used isocaloric conditions and thus the effect of prolonging fasting time during 5 days was studied independent of weight loss. To date, only one study has shown that increasing fasting time results in beneficial metabolic effects independent of weight loss, showing improvements in insulin sensitivity, β cell responsiveness and blood pressure (11). In that trial it was not investigated whether increasing fasting time improved parameters related to liver fat content and metabolism.

In conclusion, we found no effect of acutely prolonging the overnight fast on hepatic glycogen content in individuals with NAFL. Furthermore, fat oxidation during the night was not increased upon this prolonged fast. Interestingly, a fast of 16h did not result in higher fat oxidation rates compared to a short, 9.5h fast, indicating that these individuals did not fully reach the fasting state. Similar, postprandial substrate oxidation after one prolonged overnight fast was unchanged. Upon 5 days of prolonged overnight fasting, hepatic lipid content and composition did not improve. These results suggest that hepatic substrate metabolism is disturbed in individuals with NAFL and it would therefore be of great interest to further investigate the effect of this fasting intervention on hepatic glycogen and substrate metabolism in healthy volunteers without NAFL.

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CHAPTER 5

The effect of a low versus high glycemic index and saturated fat diet on liver fat content and substrate metabolism

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In preparation

ABSTRACT

The type of dietary fat and carbohydrate, specifically the degree of fat saturation and the glycemic index (GI), have been suggested to modulate the intrahepatic lipid content (IHL). However, up to now, human studies are limited to proof of principle studies with exaggerated differences in diet composition, which are not comparable to dietary patterns of the general population. Here, we investigated whether a two-week low vs. high GI/saturated fat (SFA) diet, with similar macronutrient composition, reduces liver fat content and whether this is paralleled by increased whole-body fat oxidation, decreased glycemic response and reduced hepatic glycogen levels (ClinicalTrials.gov ID: NCT04054297). To this end, 10 overweight/obese volunteers participated in a randomized cross-over trial, following a two-week high GI/SFA diet and a two-week low GI/SFA diet with a wash-out period of 4 weeks. At the end of each 2-week dietary intervention period, we measured IHL by proton magnetic resonance spectroscopy, fasted hepatic glycogen content by carbon MRS, glycemic response by using continuous glucose measurements, and substrate oxidation overnight as well as in the morning upon a meal using whole-body respirometry. We show that IHL was lower after a two-week low vs. high GI/SFA diet. Furthermore, the glycemic response tended to be reduced after the low vs. high GI/SFA diet and there was a diet x time effect for postprandial fat oxidation, indicating increased fat oxidation early after the meal in the low vs. high GI/SA diet. Hepatic glycogen content in the morning and whole-body substrate oxidation during the night were similar between the two interventions. These results indicate that realistic reductions in both GI and SFA content beneficially affects IHL in overweight/obese subjects.

INTRODUCTION

Obesity is a major health problem worldwide and its prevalence still continues to rise (1). In fact, in the last 45 years, obesity rates nearly tripled (1). Obesity has been associated with excessive fat storage in the liver, referred to as Non-Alcoholic Fatty Liver (NAFL). In obese people NAFL prevalence rates as high as 50-70% have been reported (2). A healthy diet is a cornerstone in the prevention and treatment of obesity and related metabolic conditions such as NAFL. Low calorie diets, resulting in weight loss have consistently shown to decrease intrahepatic liver fat (IHL) content (3-5). But even without an energy deficit, changes in macronutrient content (for example replacing dietary fat by carbohydrate) have been shown to be effective in reducing IHL (6, 7).

Importantly, not only the amount of dietary fat and carbohydrate, but also their quality seems to be of significance. Specifically, high amounts of saturated fat (SFA) and carbohydrate-rich foods with a high glycemic index (GI), are thought to increase IHL, but human data is yet scarce. When a diet high in SFA was compared to an isocaloric diet high in poly-unsaturated fat (PUFA) (8-11), IHL was higher after the high SFA diet. However, these diets were studied under overfeeding conditions (8, 10, 11) and/or were using enriched food items (8, 9, 11) and thus not reflecting dietary patterns of the general population. One study compared a high GI diet with a low GI diet and reported higher liver fat content after high GI diet (12). The diets compared in this study were however extreme in terms of GI and therefore, also not comparable to dietary patterns of the general population. Thus, it would be of interest to study whether a diet low in GI and low in SFA compared to a high GI/SFA diet is beneficial for IHL when dietary composition is reflecting typical dietary patterns and food choices of the general population, while energy and macronutrient content are similar.

In addition, the underlying mechanisms involved in modulation of IHL by such diets are of interest. Unraveling these mechanisms will give insight in the targets that can be used to treat and prevent NAFL. It is suggested that the degree of fat saturation may affect fat oxidation, as stable isotope studies have shown that exogenous SFA are oxidized to a lesser extent than unsaturated fatty acids (13-15) and similarly, acute meal challenge studies have reported lower whole-body fat oxidation when consuming SFA compared to mono-unsaturated fatty acids (MUFA) (16, 17). High GI diets, on the other hand, could induce liver fat accumulation through increased glycemic and insulinemic responses (12), and increased hepatic glycogen stores (12), conditions that promote *de novo* lipogenesis and reduce fat oxidation.

Here, we investigated whether a two-week low vs. high GI/SFA diet, with similar macronutrient content, reduces IHL and whether this is paralleled by an increase in whole-

body fat oxidation, decreased glycemic response and reduced hepatic glycogen levels. Using proton magnetic resonance spectroscopy (^{1}H-MRS), we determined liver fat content at the end of a two-week low GI/SFA and two-week high GI/SFA diet. In addition, at the end of both interventions hepatic glycogen content was measured by carbon MRS (^{13}C-MRS), glycemic response by using continuous glucose measurements, and substrate oxidation overnight as well as in the morning upon a meal using whole-body respirometry. We show that a two-week low vs. high GI/SFA diet reduces IHL. Also, glycemic response was lower and there was a diet x time effect for postprandial fat oxidation, indicating an increased fat oxidation early after the meal in the low vs. high GI/SA diet. We did not observe differences between diets in hepatic glycogen or nocturnal whole-body substrate oxidation.

METHODS

This study was conducted at the Maastricht University Medical Center, the Netherlands, between August 2019 and December 2020, and was approved by the institutional Medical Ethical Committee. The study was performed in accordance with all ethical regulations regarding research with human participants and all participants provided written informed consent.

Participants

Ten overweight/obese (BMI 27-38 kg/m^2) volunteers, aged 45-75 years were recruited for this study. This study population was chosen because of its increased risk for the development of NAFL. Participant characteristics are shown in table 1. Female study participants were postmenopausal. Exclusion criteria were engagement in more than 2 hours of structured exercise per week, unstable body weight (weight loss or gain more than 3 kg in 3 months preceding enrollment), alcohol consumption of more than 2 units per day, smoking, contra-indication for MRI, being vegetarian, vegan or food intolerant to common foods, use of medication known to interfere with the outcome parameters, diabetes or other active disease.

Table 1: Participant characteristics.

	Participants (n=10)
Age (years)	66 ± 7
BMI (kg/m²)	29.6 ± 2.1
Sex (f/m)	3 / 7
Body fat (%)	36.8 ± 7.1
Plasma glucose (mmol/L)	5.4 ± 0.3
Plasma TG (mmol/L)	1.25 ± 0.41
ALT (U/L)	28 ± 12
AST (U/L)	26 ± 8
Gamma-GT (U/L)	27 ± 15

Data are presented as mean ± SD, n=10.

Clinical Study Design

Volunteers participated in a randomized cross-over trial, following a two-week high GI/SFA diet and a two-week low GI/SFA diet (figure 1) with a wash-out period of 4 weeks. Each dietary intervention period started with a short visit to the research facilities for body weight measurement, instructions on the assigned diet and to receive food products relevant to the assigned diet. Volunteers followed the diet for one week and visited the research facilities again to monitor their body weight and adherence to the diet. At the end of each dietary intervention period, at day 14, volunteers visited the research facilities for 1.5 day for study measurements. During this visit IHL, hepatic glycogen content and substrate oxidation (overnight and post-meal) were determined. Two days before the study measurements, participants were instructed to refrain from physical exercise. During the entire study period, volunteers were encouraged to adhere to their normal lifestyle, except for the change in dietary composition as part of the study design.

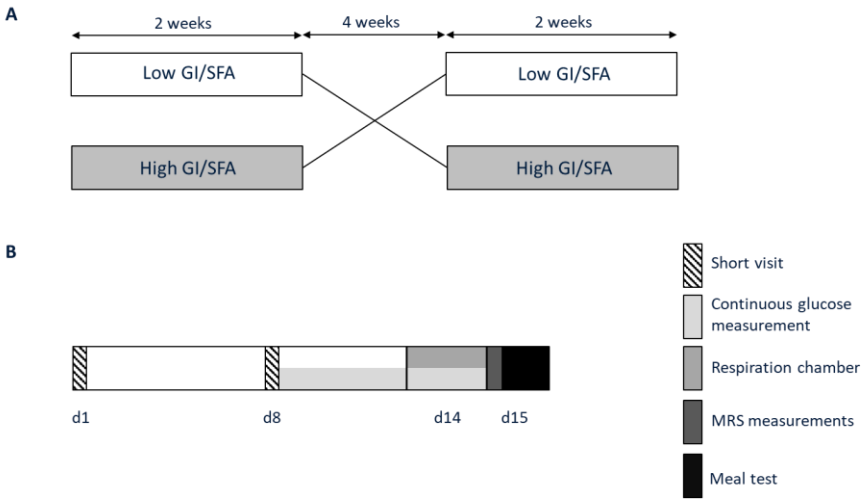

Figure 1: Study protocol. A Participants followed two dietary interventions in a randomized order; a two-week high GI/SFA diet and a two-week low GI/SFA, interspersed by a four-week washout. **B** At the end of each dietary intervention, volunteers visited the university for study measurements (day 14-15). At the start and after one week of each of the intervention periods participants visited the research facilities for instructions, body weight and food intake evaluation (short visit d1 and d8).

Diets

At the start of the dietary periods, energy needs were estimated using Harris-Benedict equations adjusted for age and physical activity level (PAL). For <50y, 50-70y and >70y a PAL-value of 1.5, 1.4, 1.3 was used respectively (18). The outcome was translated into dietary guidelines for each participant in order to maintain body weight throughout the study. These guidelines consisted of daily menus that participants were asked to consume. Non-perishable food items were provided for the whole two-week period. Participants were instructed to buy the rest of the food products as indicated on a food-product shopping list. Checklists were divided into breakfast, lunch and evening meal and snacks. Participants were asked to adhere to the dietary menus and kept track of their food intake by using these dietary checklists. Adherence was monitored by evaluation of the dietary checklist and body weight after one week and at the end of the dietary interventions. In case of weight loss or gain after one week, the researcher provided additional counselling and if necessary, the number of calories provided was adapted to ensure participants stayed in energy balance. Diets were comparable in macronutrient content, however, differed in SFA content and GI. GI tables of Foster-Powell et al. (19) were used to estimate GI and design the diets. The resulting nutrient composition of the two diets is shown in table 2. The distribution of energy intake throughout the day was as follows: breakfast 25 En%, lunch 25

En%, dinner 40 En% and snack 10 En%. Breakfast and lunch were light meals and dinner was a hot meal, which reflects the typical meal pattern in The Netherlands.

Table 2: Mean daily nutrient compositions of the low GI/SFA and high GI/SFA diet.

Daily nutrients	Low GI/SFA	High GI/SFA
Total carbohydrates (E%)	57.5	56.1
GI	36	60
Total fat (E%)	27.4	29.5
Saturated fatty acids (E%)	5.4	14.7
Total protein (E%)	15.7	14.5

Measurement of hepatic fat content

At day 15, at 7.15 am after an overnight fast, hepatic lipid content was determined by ^{1}H-MRS. All ^{1}H-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 32-channel sense cardiac/torso coil (Philips Healthcare, Best, Netherlands). All spectra were obtained by using a STEAM sequence (20) with the following parameters; repetition time (TR) 4500 ms/echo time (TE) 20 ms/ mixing time (TM) 16 ms, spectral bandwidth 2000 Hz and data points 2048. For the hepatic lipid spectra, VAPOR water suppression (21) was applied and an additional water reference scan was obtained. The number of averages was 128 for the hepatic lipid spectra. We used a voxel size of 30 x 30 x 30 mm. All spectra were post-processed with a custom-written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States.) script as previously described (22). Lipid content was calculated after T_2 correction as ratio of the CH_2 peak relative to the sum of the unsuppressed water resonance and CH_2 peak, and converted to weight/weight percentage.

Measurement of hepatic glycogen

At day 15 at 06.30 am after an overnight fast, hepatic glycogen was determined by ^{13}C-MRS. All ^{13}C-MRS experiments were performed on a 3T MR system (Achieva 3T-X Philips Healthcare, Best, Netherlands) by using a 21x24cm ^{13}C quadrature detection surface coil (RAPID Biomedical GmbH, Germany). Hepatic glycogen levels were measured by acquiring non-localized ^{13}C MR spectra (FID; TR: 280ms; NSA: 4096). Data analysis was performed with an in-house developed Matlab script. Phase-correction and baseline correction was applied in the high GI/SFA and low GI/SFA spectra simultaneously with the aim to minimize differences between spectra in regions without signal, and therefore ensure identical pre-

processing of both spectra. The AUC of glycogen signal was determined by integration of +-5 PPM around the C1 of glycogen at 100.5 PPM.

Respiration chamber measurement

Participants stayed in a respiration chamber during the last day and night of each dietary intervention period (from 07.30 am onwards on day 14 until the morning of day 15). The metabolic chamber is a small room with a bed, toilet, television, computer, and access to water in which oxygen consumption and carbon dioxide production were measured continuously in sampled room air by whole-room indirect calorimetry (Omnical, Maastricht Instruments, Maastricht, The Netherlands) (23). Volunteers received all meals matching their assigned diet (breakfast at 8.00 am, lunch at 12.00 pm, dinner at 4.30 pm and snack at 6.30 pm). Calculated GI was 45 vs. 52 for breakfast, 33 vs 57 for lunch, 43 vs. 63 for dinner and 33 vs. 64 for snack, in the low and high GI/SFA diets respectively. During the overnight stay in the respiration chamber, sleeping metabolic rate (SMR), respiratory exchange ratio (RER), fat oxidation and carbohydrate oxidation were assessed. Energy expenditure was calculated based on the measured averaged oxygen and carbon dioxide concentrations in the inspired and expired gases with the assumption that protein oxidation was negligible, using the Weir equation (24). SMR was calculated as the lowest average 3-h energy expenditure during the sleeping period. During this 3h period also RER, carbohydrate and fat oxidation were determined. RER was also determined for the early (12.00 am - 3.00 am) and late (3.00 am - 5.30 am) phase of the night. Carbohydrate oxidation and fat oxidation rates were calculated according to Péronnet and Massicotte (25). Participants were instructed to go to sleep at 11.00 pm. At 5.45 AM the next morning participants were woken up and left the chamber in an overnight fasted state. In two occasions technical problems occurred during the measurements, data from these volunteers were unreliable and excluded from analyses.

Meal test

After the MRS measurements in the morning of day 15, a meal test was performed. A breakfast (25% of daily energy need) matching the assigned diet with respect to GI and SFA content was provided to the participants at 8:30 AM (t=0). We chose to use a meal matching the diet, and therefore different meals for the two periods, because we were mainly interested in the postprandial effects of the specific diets. Before the meal (t=-15), and at t=45, t=75, t=135, t=195, t=255 minutes, indirect calorimetry measurements using a ventilated hood system (Omnical, Maastricht Instruments, Maastricht University) were

performed. VO_2 and VCO_2 were used at these timepoints to assess energy expenditure (24), respiratory exchange ratio (RER), fat and carbohydrate oxidation (25).

Continuous glucose monitoring

During the short visit after 1 week of the diet, a continuous glucose monitoring sensor (FreeStyle Libre, Abbott) was placed at the back of the upper arm. Interstitial fluid glucose was measured every 15 minutes by the sensor. Measurements took place up to day 15, when the sensor was removed in the morning before the MRS measurements. In three volunteers the sensor fell off and therefore, the measurement was incomplete, these were excluded from analyses.

Body composition

Body mass and body volume were assessed during the first visit (d1) of the first dietary intervention using air-displacement plethysmography (ADP) using the Bod Pod device (Cosmed, Italy, Rome) according to the manufacturer's instructions (26). Thoracic gas volume was predicted based on equations included in the Bod Pod software (version 4.2.0). From these data, body fat percentage was calculated as described by Siri (27).

Statistical analyses

Results were expressed as mean ± SEM. Participant characteristics were expressed as mean ± SD. Continuous variables were tested for normality. Nocturnal RER was not normally distributed and for this variable Wilcoxon signed-rank test was used. All other variables were normally distributed, for these variables changes between diets were assessed by paired-sample t-test. Changes across the time course during the meal tests and changes across the time course during the continuous glucose measurements on day 14 were assessed using two-way repeated-measures ANOVA to evaluate any significant main effect of diet and timepoint, and any interaction between diet and timepoint. Pearson correlation was performed to identify correlations. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 27.0 for Windows.

RESULTS

IHL was lower after a two-week low- compared to a high GI/SFA diet

Body weight significantly decreased in both diets (high GI/SFA: pre: 90.5 ± 4.8kg, post: 89.4 ± 4.7kg, p=0.02 and low GI/SFA diet: pre: 90.8 ± 4.7kg, post: 89.4 ± 4.6kg, p=0.01, figure 2A), while the decrease did not differ between diets (-1.2 ± 0.4 in High GI/SFA and -1.4 ± 0.3kg in Low GI/SFA; p=0.40, figure 2A). IHL was significantly lower (-27%) after the two-week low GI/SFA diet compared to the two-week high GI/SFA diet (p=0.04, figure 2B). After the high GI/SFA diet IHL ranged from 0.5-6.9% weight/weight and after the low GI/SFA diet IHL ranged from 0.7-5.1% weight/weight. The difference in IHL after the two-week low vs. high GI/SFA intervention did not correlate with differences in body weight change.

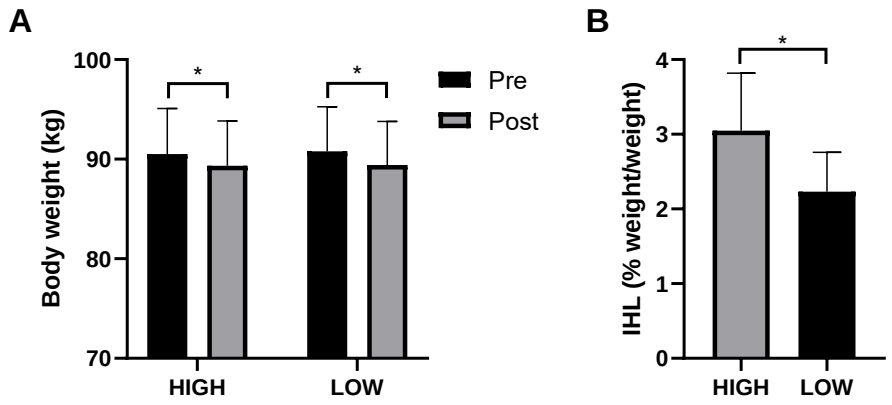

Figure 2: Body weight and intrahepatic lipid content upon a two-week high vs. low GI/SFA diet. A Body weight pre and post two-week high vs. low GI/SFA diet (n=10), **B** IHL after a two week high vs. low GI/SFA diet (n=10). * p<0.05.

Dietary low GI/SFA reduced the 24h pattern of glycemia without changes in glycogen

To investigate the mechanisms underlying how a lower GI may affect liver fat, we assessed glucose levels continuously in the second week of the intervention and determined whether hepatic glycogen was different between the dietary periods on day 15. We chose day 14 to compare the 24-hour interstitial glucose pattern, as on that day volunteers were in the respiration chamber and therefore meal timing was exactly aligned, omitting the noise that can be introduced by variations of meal timing. There was a tendency for a significant main effect of diet for interstitial glucose levels during day 14 (p=0.09, figure 3A). Differences in

glycemic response were especially apparent after lunch, with lower average glucose levels during 3h post-meal in the low GI/SFA diet (p=0.03, figure 3B). Hepatic glycogen content was similar after the low- compared to the high GI/SFA diet (figure 4).

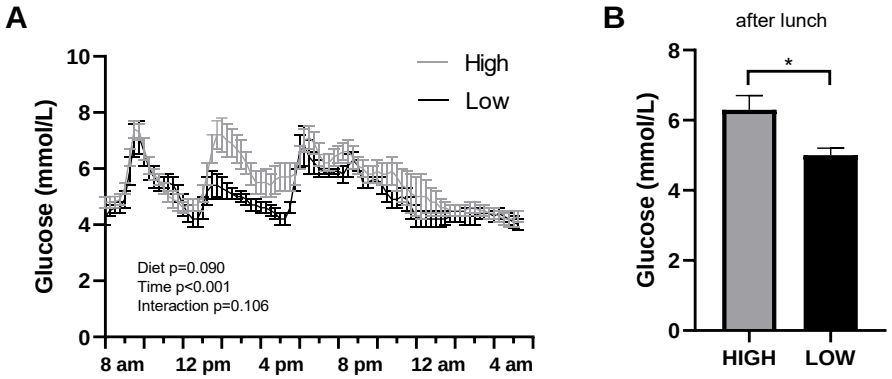

Figure 3: Continuous glucose values on day 14 after two weeks of a low GI/SFA vs. high GI/SFA diet. A 15 minute glucose measurements in interstitial fluid on day 14 of the high and low GI/SFA diets (n=7), **B** 3h-average glucose in interstitial fluid upon lunch on day 14 of the high- and low GI/SFA diets (n=7). * p<0.05.

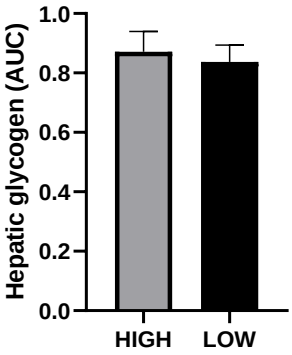

Figure 4: Fasting hepatic glycogen on day 15 after two weeks of a low GI/SFA vs. high GI/SFA diet (n=10).

Whole-body substrate oxidation was not changed by dietary GI/SFA

We next determined whether whole-body substrate oxidation, which was measured during the night and the following morning postprandially after breakfast, was influenced by dietary GI/SFA content. Whole-body substrate oxidation and SMR during the night were not different between the dietary interventions (figure 5). In the high GI/SFA diet, RER was not different between the early versus late part of the night, but RER tended to increase during the night in the low GI/SFA diet (3-5.30am vs 12-3am; p=0.08, figure 5C).

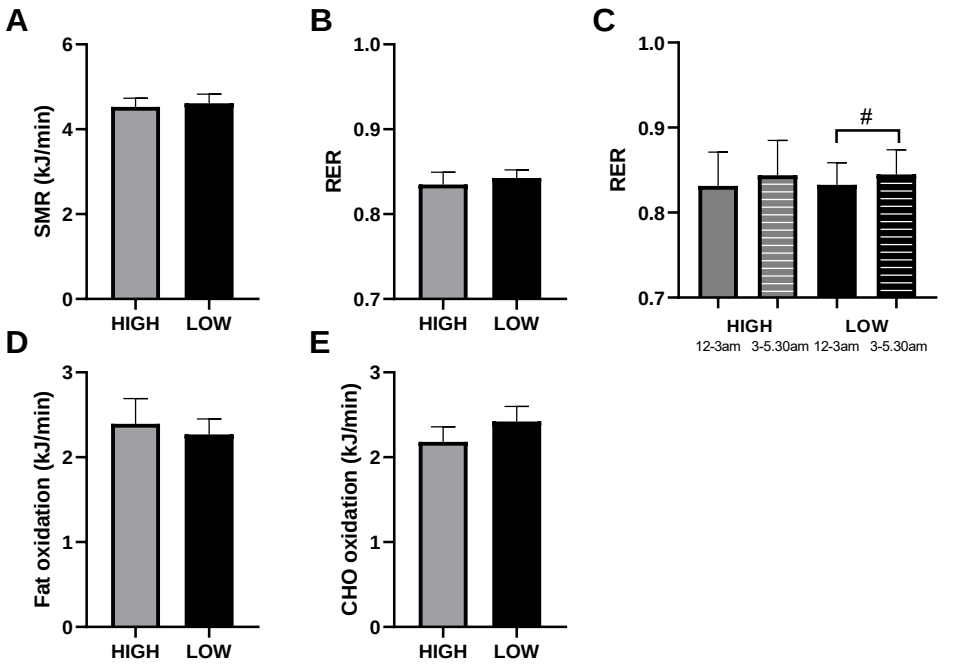

Figure 5: Sleeping metabolic rate (SMR) and overnight substrate oxidation on day 14 after two weeks of a high and low GI/SFA diet. A Sleeping metabolic rate (SMR), **B** Respiratory exchange ratio (RER) during 3h period with lowest energy expenditure, **C** RER between 12-3am and 3-5.30am, **D** Fat oxidation, and **E** Carbohydrate (CHO) oxidation during 3h period with lowest energy expenditure after a high and low GI/SFA diet (n=8).

Under fasting conditions in the morning (t=-15 before the meal), substrate oxidation was similar (figure 6) between interventions. Postprandially, after a breakfast matching the corresponding diet, there was a significant diet x time interaction effect for fat oxidation (p=0.04, figure 6D), showing higher fat oxidation in the early postprandial phase after the low vs. high GI/SFA diet.

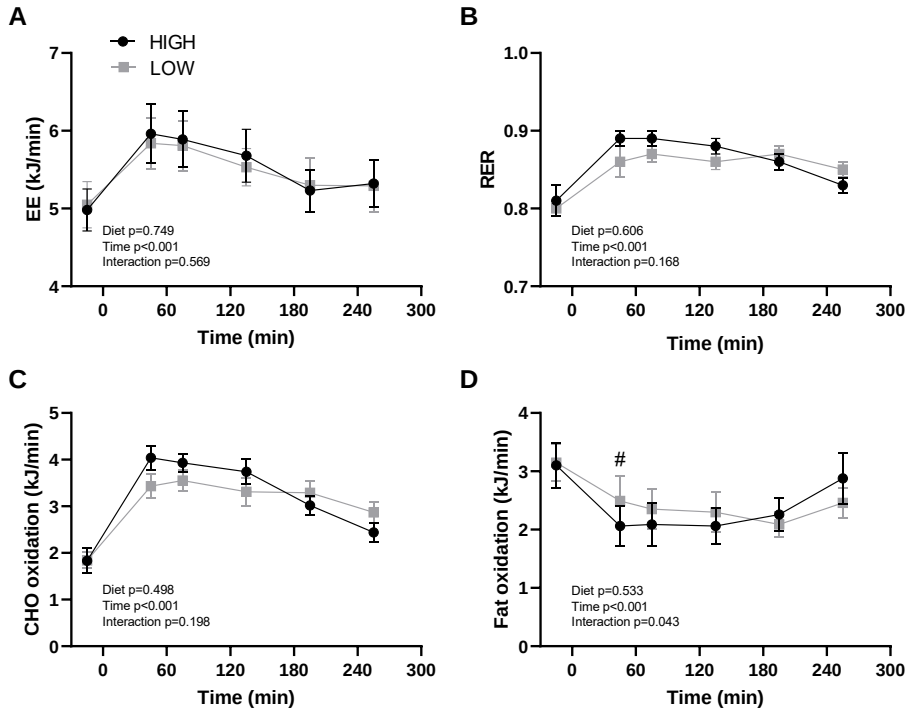

Figure 6: Energy expenditure and substrate oxidation upon a breakfast meal corresponding to the previous 14d high and low GI/SFA diet. A Energy expenditure (EE), **B** Respiratory exchange ratio (RER), **C** Carbohydrate (CHO) oxidation, **D** Fat oxidation during the meal test after two weeks of a high and low GI/SFA diet (n=10). # p<0.10 for low vs. high GI/SFA.

DISCUSSION

Low dietary SFA and low GI are two aspects of healthy nutrition as generally recommended by health authorities, and both have been shown to influence liver fat storage (8-12). These studies were however performed under overfeeding conditions, were using enriched food items or used extreme differences in GI. Here, we combined low GI and low SFA on the one hand and high GI/high SFA on the other hand, thus comparing a healthy to an unhealthy diet in terms of GI and SFA, while using realistic food choices. International guidelines regarding SFA are maximal 10% of daily energy intake; here we compared 5 vs 15 En% from SFA. GI used in our diets was 36 vs 60, corresponding to a low (<55) and medium GI (55-70). We showed that a two-week low compared to high GI/SFA diet, with similar macronutrient content, has beneficial effects on IHL and glycemic response, and leads to a higher fat oxidation early in the postprandial phase, while substrate oxidation overnight and fasting hepatic glycogen were similar after the two diets.

We here showed that with adaptations in SFA and GI within a typical Dutch diet, IHL can be reduced by 27% already after 2 weeks. As we chose to use diets that are similar to diets typically consumed by the general population, the results suggest that GI and fat saturation are important factors in determining IHL storage even when consumed within normal ranges of dietary intake. The 27% reduction in IHL as shown here is substantial, as such an effect is comparable to reductions found in a similar study population after 12 weeks of a supervised exercise program (28% decrease) (28) and after 7% weight loss following a 30% energy restricted diet (31% decrease upon carbohydrate restriction, 23% decrease upon fat restriction) (29). Despite the dietary counseling and adaptations made to dietary energy content in case volunteers lost weight in the first week, body weight was reduced by on average ~1.3kg on both diets. However, as body weight was reduced to a similar extent on both diets and the difference in IHL observed between diets did not correlate with differences in body weight change, the difference in IHL observed after the two diets is independent from differences in body weight.

We hypothesized that a lower glycemic response could underly a reduction in IHL. Previously, by definition, differences in glycemic response have been observed acutely in healthy volunteers after consuming low compared to high GI meals (12, 30). However, the GI of the high GI diets was more extreme in these studies (Bawden et al; high GI 84, low GI 32, and Morgan et al; high GI 84, low GI 34). In the current study, we investigated the glucose levels throughout the day at the end of each dietary period. A distinction in glucose pattern could especially be made upon lunch, with a lower glycemic response on the low compared to high GI/SFA diet. The fact that this was especially visible after lunch may be explained by differences in GI of the meals throughout the day, with a bigger difference in calculated GI for lunch compared to breakfast and dinner.

One of the possible mechanisms involved in a reduced IHL through lower glycemic response is a reduction in hepatic glycogen levels. By lowering hepatic glycogen levels, the capacity to store glucose as glycogen is increased, which prevents shuttling glucose to the DNL pathway. Therefore, regular depletion of hepatic glycogen is believed to be key for good metabolic health. Contrary to our expectations, we did not observe changes in liver glycogen content after an overnight fast between diets. Although not much human data is available, one earlier study investigated liver glycogen in response to high GI and showed that after 7 days liver glycogen levels in the fasted state were not significantly influenced by dietary GI (12). Postprandially, they did find increased hepatic glycogen levels with higher dietary GI. It is therefore possible that possible differences in glycogen levels over the day were masked by the predominating effect of the overnight fast on reducing glycogen levels, and future studies are needed that determine hepatic glycogen levels over the day.

No differences were found in whole-body substrate oxidation during the night after two weeks of low compared to high GI/SFA diet. Remarkably, we found that RER did not reduce during the night; this inflexibility in switch from carbohydrate to fat oxidation during the night, that is usually seen in healthy volunteers (31), matches observations in pre-diabetic individuals (32) and individuals with NAFL (unpublished data). Moreover, we found a tendency for RER to even increase during the night after the low GI/SFA diet. Postprandially, after breakfast, we found an interaction effect between time and diet for fat oxidation, indicating a different response in fat oxidation between diets over the course of the meal test. In line with this, meal challenge experiments have shown an increased whole-body fat oxidation with unsaturated fatty acids compared to saturated fatty acids when investigated acutely (16, 17) or after one week diet (33). It should also be noted that we here investigated fat oxidation on whole-body level. To investigate if an increased fat oxidation contributed to decreased IHL, it would be valuable to determine if liver specific fat oxidation is changed by dietary GI/SFA content.

Luukkonen et al. showed that overfeeding saturated fatty acids increased adipose tissue lipolysis and overfeeding unsaturated fatty acids decreased adipose tissue lipolysis (10). As adipose tissue lipolysis is the main determinant for hepatic NEFA uptake and around 60% of liver lipids are derived from NEFA uptake (34, 35), the effects on IHL found here may also partly be mediated by changes in NEFA uptake as a result of differences in the amount of dietary saturated fatty acids. Thus, next to the possible pathways that could underly the changes in IHL considered here (DNL and fat oxidation), also other pathways contributing to liver fat accumulation, (i.e. NEFA uptake, dietary fat storage and VLDL-TG secretion) could be influenced by dietary GI/SFA content and should be taken into account in future studies.

In conclusion, a two-week low compared to high GI/SFA diet resulted in lower IHL in overweight/obese volunteers. This was accompanied by a lower glycemic response and increased fat oxidation early in the postprandial phase, which might underly the change in liver fat content, while fasting hepatic glycogen and overnight whole-body substrate oxidation was similar between diets. These results indicate that reducing both GI and SFA content in a diet typically consumed by the general population can lower IHL already within two weeks. The exact mechanisms underlying these reductions in IHL remain to be elucidated.

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CHAPTER 6

Hepatic water and lipid T2 relaxation times are associated with level of steatosis - implications for absolute fat quantification

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Submitted

CHAPTER 7

Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans

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ABSTRACT

Background: Nicotinamide riboside (NR) is a NAD^+ precursor that boosts cellular NAD^+ concentrations. Preclinical studies have shown profound metabolic health effects after NR supplementation.

Objective: Investigate the effects of six weeks NR supplementation on insulin sensitivity, mitochondrial function and other metabolic health parameters in overweight and obese volunteers.

Design: A randomized, double-blinded, placebo-controlled, cross-over intervention study was conducted in thirteen healthy overweight and obese men and women. Participants received six weeks NR (1000 mg/day) and placebo supplementation, followed by broad metabolic phenotyping, including hyperinsulinemic-euglycemic clamps, magnetic resonance spectroscopy, muscle biopsies, and assessment of *ex vivo* mitochondrial function and *in vivo* energy metabolism.

Results: Markers of increased NAD^+ synthesis – NAAD and MeNAM – were elevated in skeletal muscle after NR compared to placebo. NR increased body fat free mass (62.65 ± 2.49 vs. 61.32 ± 2.58, 1.34 ± 0.50 % in NR, placebo, and the change respectively, p=0.02) and increased sleeping metabolic rate. Interestingly, acetylcarnitine concentrations in skeletal muscle were increased upon NR (4558 ± 749 vs. 3025 ± 316 and 1533 ± 683 pmol/mg dry weight in NR, placebo, and the change respectively, p=0.04) and the capacity to form acetylcarnitine upon exercise was higher in NR compared to placebo (2.99 ± 0.30 vs. 2.40 ± 0.33 and 0.53 ± 0.21 mmol/kg wet weight in NR, placebo, and the change respectively, p=0.01). However, no effects of NR were found on insulin sensitivity, mitochondrial function, hepatic and intramyocellular lipid accumulation, cardiac energy status, cardiac ejection fraction, ambulatory blood pressure, plasma markers of inflammation or energy metabolism.

Conclusion: NR supplementation of 1000 mg/day for six weeks in healthy overweight and obese men and women increased skeletal muscle NAD^+ metabolites, affected skeletal muscle acetylcarnitine metabolism, and induced minor changes in body composition and sleeping metabolic rate. However, no other metabolic health effects were observed.

INTRODUCTION

Nicotinamide riboside (NR) is a naturally occurring vitamin B3 present in the human diet, acts as a NAD^+ precursor (1), and is suggested to improve mitochondrial function and insulin sensitivity (2). NR acts via activation of the NAD^+-dependent sirtuin enzyme family, thereby regulating oxidative metabolism (3-7). *In vitro* experiments have shown the successful NAD^+ restoring capability of NR supplementation and the subsequent increase oxidative gene expression in skeletal muscle cells (8, 9). Results from *in vivo* mouse models have shown improvements in insulin sensitivity and oxidative energy metabolism, including enhanced metabolic flexibility, increased aerobic exercise capacity and indications of improved mitochondrial biogenesis (9-13). In these mouse models NR seems to specifically act on muscle, liver, heart and brown adipose tissue (9, 14). Reports indicate that NAD^+ metabolism including NAD^+ concentrations are decreased in the obese and older population (15-17). The number of human interventions with NR is small and the evidence that NR may have beneficial effects in humans is limited. Human pharmacokinetic studies showed increased circulatory NAD^+ metabolite concentrations in whole blood, PBMC and urine, after various dosages of NR supplementation (18-23), varying from 300 to 2000 mg/day, indicating that NR supplementation is able to increase the NAD^+ pool in humans. So far, three randomized placebo-controlled NR supplementation studies have been performed in humans, in which the effect of NR supplementation on human metabolic health was investigated. Dollerup et al. (21, 24), investigated the effect of 2000 mg/day NR for 12 weeks in insulin resistant middle-aged obese men. No effects of NR supplementation were found on insulin sensitivity, muscle mitochondrial function or metabolic flexibility (21, 24). In addition, Martens et al. (20), investigated the effect of 1000 mg/day NR for six weeks in healthy normal weight middle-aged and older men and women. Besides a trend in a reduction in a reduced arterial stiffness and lower blood pressure after NR supplementation, no effects were found on a wide variety of outcomes indicative of metabolic function, glucose metabolism, motor function and exercise capacity (20). Furthermore, Elhassan et al. (25) investigated the effects of 1000 mg/day NR for 3 weeks in older men and showed increased skeletal muscle NAD^+ metabolite concentrations, however again no effect on skeletal muscle mitochondrial function was observed. To date, the effect of NR supplementation on both insulin sensitivity and skeletal muscle mitochondrial function has only been recently published in one human clinial trial (21, 24). Based on the promising preclinical findings reported until 2017, we designed a double blinded randomized placebo controlled cross-over study in which we aimed to investigate the effect of 1000 mg/day NR supplementation on metabolic health in healthy obese and overweight men and women. The primary focus was on insulin sensitivity and muscle mitochondrial function, and secondary outcomes were related to energy metabolism and skeletal muscle

NAD^+ metabolites. Therefore, our study expands the limited knowledge of the effect of NR supplementation on human metabolic health and aims to investigate the translational value of previous promising preclinical findings.

METHODS

The study was conducted in accordance with the principles of the declaration of Helsinki and approved by the Ethics Committee of the Maastricht University Medical Center. The study was registered at https://clinicaltrials.gov (NCT02835664). All participants provided written informed consent before screening.

Participants

Recruitment and data collection took place between December 2016 and December 2018 in Maastricht, The Netherlands. Thirteen participants completed the study, two dropped out because of personal or medical reasons (see Supplementary figure 1). Men and postmenopausal women were included. Participants underwent a screening including assessment of blood biochemistry, electrocardiography, anthropometric measurements and a questionnaire (including Baecke physical activity questionnaire (26)) to evaluate eligibility. Inclusion criteria were 45-65 years of age, BMI 27-35 kg/m^2, sedentary lifestyle (<3 h exercise per week), non-smoking for at least 6 months, alcohol use of $\leq$ 2 servings per day, stable body weight for at least 6 months and having no active diseases.

Study design

The study had a randomized controlled, double blinded, placebo controlled, cross-over design. Simple randomization was applied with use of computer-generated random numbers. Each participant received two interventions each of which lasted six weeks. During the first six weeks the participant was randomized to receive either NR supplementation or placebo. This six-week period was followed by a washout period of four to seven weeks. At the end of the washout period the participant crossed over to the treatment they did not receive during the first six weeks. In both intervention arms participants underwent exactly the same tests to determine the effect of the intervention (figure 1). These tests were performed in the end of week five and during week six. During the entire study period no changes in lifestyle (no change in diet, physical activity level or medication or supplement use) were allowed. Compliance was checked on a weekly basis by pill count. Furthermore, a fasted blood sample was taken, and body weight was monitored. During supplementation periods, any adverse effects and side effects were noted.

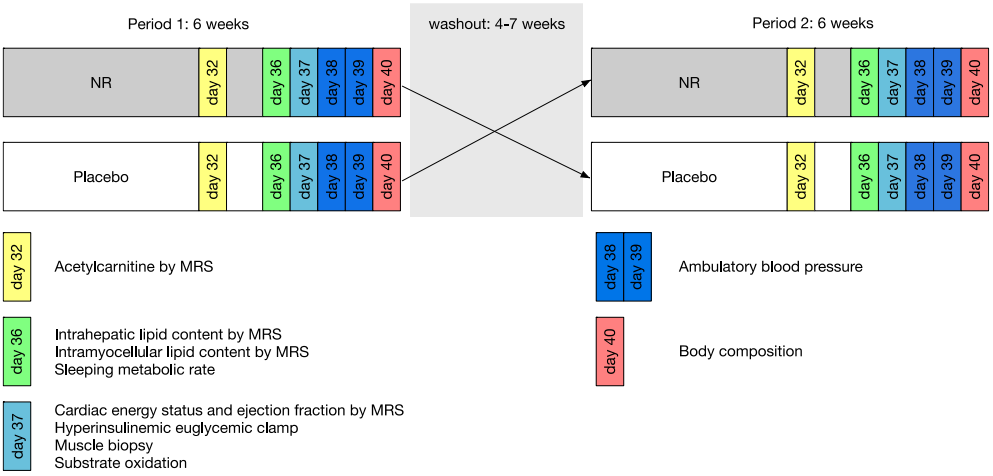

Figure 1: Study design. In this cross-over study, participants were randomly assigned to start with six weeks of NR supplementation or six weeks of placebo treatment. After a washout period of four-to-seven weeks, participants entered the other intervention arm such that all participants served as their own control. Participants were studied in week five (day 32) and in week six (day 36 – 40) of each of the two interventions.

Determination of sleeping metabolic rate

At day 36 of each intervention, participants received a standardized dinner at 18:30 h after which they remained fasted and entered a metabolic chamber at 19:30 h. The metabolic chamber is a small room with a bed, toilet, television, computer and access to water in which oxygen consumption and carbohydrate production were measured continuously in sampled room air. During the overnight stay in the respiration chamber sleeping metabolic rate was assessed. Participants decided themselves what time they went to sleep. At 06:00 h the next morning participants were woken up and left the chamber in an overnight fasted state.

Hyperinsulinemic euglycemic clamp

To determine insulin sensitivity, a two-step hyperinsulinemic euglycemic clamp with co-infusion of D-[6.6-$^{2}H_2$] glucose tracer (0.04 mg/kg/min) was performed on day 37 of both interventions (27). Insulin was infused, starting at 10:30 h, at 10 mU/m^2/min for 2.5 h to assess hepatic insulin sensitivity and subsequently increased to 40 mU/m^2/min for 2 h to measure whole body insulin sensitivity. Blood was frequently sampled to measure glucose concentrations directly from arterialized blood and tracer-enriched 20% glucose was co-infused at a variable rate to maintain euglycemia (~5.0 mmol/l) and reach steady state condition. During the steady state of 30 minutes, blood samples were collected, and

substrate utilization was measured using indirect calorimetry. Due to technical failures, one participant was excluded from the clamp analysis.

Skeletal muscle biopsies

On day 37 of each intervention, a muscle biopsy was taken at 8:30 h after an overnight stay in the respiration chamber from the m. vastus lateralis under local anesthesia (1% lidocaine, without epinephrine) using the Bergström technique (28). The muscle biopsy was divided in several parts. One part was immediately frozen in melting isopentane for biochemical analyses. The remaining part was used for mitochondrial respiration analysis.

Skeletal muscle NAD^+ metabolites

NAD^+ content was determined in muscle biopsies using an enzymatic spectrophotometric cycling assay based on the coupled reaction of malate and alcohol dehydrogenases, as previously described (29). NAD^+ metabolites were measured in muscle biopsies through metabolomics. Freeze-dried muscle tissue (2-4 mg) was transferred to 2 mL tubes, and then 425 µL water, 500 µL methanol and 75 µL of internal standards mixture (see Supplementary table 1) was added to each sample. Samples were homogenized using TissueLyser II (Qiagen; 5 min at 30/s), followed by addition of 1000 µL chloroform and thorough mixing. After centrifugation, the top layer containing the polar phase was transferred to 1.5 mL tubes and dried in a vacuum evaporator at 60 °C. Dried samples were reconstituted in 100 µL methanol/water (6/4, v/v) and analyzed in an Aquity UPLC system (Waters) coupled to an Impact IITM Ultra-High Resolution qTOF mass spectrometer (Bruker). Chromatographic separation of the compounds was achieved using a SeQuant ZIC-cHILIC column (PEEK 100 x 2.1 mm, 3 µm particle size, Merck, Darmstadt, Germany) at 30 °C. The LC method consisted in a gradient running at 0.25 mL/min from 100% mobile phase B (9:1 acetonitrile:water with 5 mM ammonium acetate pH 6.8) to 100% mobile phase A (1:9 acetonitrile:water with 5 mM ammonium acetate pH 6.8) in 28 minutes, followed by a re-equilibration step at 100% B of 5 minutes. MS data were acquired both in negative and positive ionization modes in full scan mode over the range of m/z 50-1200. Due to limited sample availability, NAD^+ content was measured in muscle biopsies of 8 participants and NAD^+ metabolomics was measured in muscle biopsies of 12 participants.

Skeletal muscle mitochondrial respiration and protein content

From the muscle biopsies, permeabilized muscle fibers were prepared as described elsewhere (30). Thereafter *ex vivo* mitochondrial respiration was determined by measuring oxygen consumption rate upon addition of several substrates using high-resolution respirometry (Oxygraph, OROBOROS Instruments, Innsbruck, Austria) as described previously (31). All measurements were performed in quadruplicate and the integrity of the outer mitochondrial membrane was assessed in every experiment by the addition of cytochrome C (10 µmol/L) upon maximal coupled respiration. The average cytochrome C response in the included traces was 1.7 ± 0.03 % and traces with a cytochrome C response above 15% were excluded from statistical analyses. Data is presented per mg wet weight. Oxphos complex protein content was measured in skeletal muscle biopsies of 12 participants as previously described (32).

Skeletal muscle acylcarnitine concentrations

2-4 mg of freeze-dried muscle tissue was homogenized 1 mL 80% acetonitrile containing 50 µL of the internal standards. After centrifugation at 16000g, the resulting supernatant was dried under a stream of nitrogen at 40 °C and derivatized by addition of 1-propanol/acetylchloride (4/1, v/v) during 15 minutes at 60 °C. After evaporation under nitrogen at 40 °C, samples were redissolved in pure acetonitrile. Determination of the propylated acylcarnitines in the medium was performed by mass spectrometry in an Acquity UPLC System (Waters) coupled to a Quattro Premier XE Tandem Quadrupole Mass Spectrometer (Waters).

VO_2peak

Maximal oxygen consumption (VO_2peak) was assessed during an incremental cycling test on an ergometer (Lode Excalibur Sport, Groningen, The Netherlands) (33), in the first week of the first supplementation period. After a warming-up of five minutes at 75 Watt, the power was increased every 2.5 minutes by 50 Watt until 80% of the maximally calculated heart rate was reached, then the power was increased every 2.5 minutes by 25 Watt until exhaustion. The highest average oxygen consumption over 25 seconds was used as the VO_2peak, reflecting physical fitness of the participant.

MR spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) was used to quantify intrahepatic lipid content (IHL), intramuscular lipid content (IMCL) and skeletal muscle acetylcarnitine concentrations. Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) was used for assessment of phosphocreatine (PCr) to adenosine triphosphate (ATP) ratio as a marker of the energy status and *in vivo* mitochondrial function of the heart muscle. Magnetic resonance imaging was used for determination of cardiac left ventricle ejection fraction (EF). All measurements were performed on a 3.0T whole body scanner (Achieva Tx, Philips Healthcare, Best, The Netherlands).

IHL & IMCL: IHL quantification took place on day 36 of each intervention at 17:00 h. Participants were fasted for at least 3 hours. Spectra were acquired as described before (34). Values are given as T2 corrected ratios of the CH_2 peak relative to the unsuppressed water peak, expressed as percentage. Consecutive, IMCL was measured in the m. tibialis anterior of the left leg, as reported earlier (34). Values are given as T1- and T2-corrected ratios of the CH_2 peak relative to the unsuppressed water peak, expressed as percentage. Due to analytical problems only 9 participants could be included in the analyses of IMCL.

Acetylcarnitine: Acetylcarnitine concentrations in skeletal muscle were acquired on day 32 of each intervention at 17:00 h in the evening. Participants were fasted for at least 3 hours and were asked to refrain from strenuous physical activity 48 hours prior to the measurement. Resting skeletal muscle Acetylcarnitine concentrations were measured using a T1-editing method, as described earlier (35). Additionally, Acetylcarnitine concentrations were measured after 30 minutes exercise (70% maximal output on an ergometer). Acetylcarnitine values were converted to absolute concentrations as described earlier (36). The creatine peak was used as a reference.

Cardiac PCr/ATP ratio & ejection fraction: PCr/ATP ratio was quantified by ^{31}P-MRS on day 37 of each intervention at 06:30 h, using an ISIS sequence. Participants were positioned prone and headfirst in the MRI. A $^{1}H^{31}P$ surface heart coil was placed beneath the participants chest. The voxel of interest was carefully placed around the left ventricle of the heart. Spectra were acquired during the end-systolic phase (NSA = 96, number of points = 2048, bandwidth = 3000 Hz) with a repetition time of 5-8 heartbeats, depending on heart rate. PCr and ATP resonances were quantified using a custom written MATLAB (MATLAB 2014b, The MathWorks, Inc., Natick, Massachusetts, United States) script and values were corrected for T1 saturation and expressed as ratio of PCr over gamma-ATP. Due to technical errors, only 11 participants are included in the analyses of PCr/ATP ratio. Left ventricular size was measured for 12 participants based of MRI images of the heart. Left ventricular EF

was calculated from the end diastiolic volume (EDV) and end systolic volume (ESV) according to the Biplane Ellipsoid Model, as described earlier (37).

Ambulatory blood pressure

Ambulatory blood pressure (BP) was measured (Mobil-O-Graph, I.E.M., Stolberg, Germany) on day 38 and 39 of each intervention for two days and one night (36-hours). Mean systolic (SBP) and diastolic (DBP) blood pressure during day time and night time, and night time dipping were calculated as previously described (38). Night was defined as the mean time going to bed of all participants (23:38 h) until the mean time waking up of all participants (8:05 h). Due to technical failures night time BP could only by obtained from 12 participants.

Body composition

Body composition was determined on day 40 of each intervention at 08:30 h after an overnight fast of at least 10 hours. Body mass and body volume were assessed using air-displacement plethysmography using the BodPod device (Cosmed, Italy, Rome) according to the manufacturer's instructions (39) and previously reported (40).

Blood sampling and analyses

Glucose (Hk-CP, Axonlab, Amsterdam, The Netherlands) and free fatty acids (FFA) (NEFA-HR, WAKO chemicals, Neuss, Germany) were analyzed enzymatically in EDTA plasma using a Pentra 400 (Horiba, Montpellier, France). Triglycerides (Sigma, Zwijndrecht, The Netherlands), cholesterol (CHOD-PAP, Roche Diagnostics, Mannheim, Germany) and HDL-C (CHOD-PAP, Roche Diagnostics, Mannheim,Germany) after precipitation of apoB-containing lipoproteins with phosphotungstic acid and magnesium ions, were analyzed in serum also using a Pentra 400. All samples from one participant were analyzed within one run. LDL-C was calculated for 12 participants according to the Friedewald equation (41). In a subset of 7 participants (age 60 ± 3 years; BMI 30.0 ± 1.7 kg/m^2; n=2 women) inflammatory cytokine concentrations were measured on a Luminex® 200TM system using an inflammation 20-plex human Procartaplex panel (eBioscience, EPX200-12185-901) containing markers for sE-Selectin; ICAM-1/CD54; IL-1α; IL-4; IL-12p70; IL-17A/CTLA-8; IP-10/CXCL10; MCP-1/CCL2; MIP-1α/CCL3; MIP-1β/CCL4; sP-Selectin; TNFα.

Calculations

Energy expenditure was calculated based on the measured averaged oxygen and carbon dioxide concentrations in the inspired and expired gasses with the assumption that protein oxidation was negligible, using the Weir equation (42, 43). Sleeping metabolic rate was calculated as the lowest average 3h energy expenditure during the sleeping period. Glucose oxidation and fat oxidation rates were calculated according to Perronet et al. (42, 43). Steele's single pool non-steady state equations were used to calculate the rate of glucose appearance (Ra) and the rate of glucose disappearance (Rd) during the clamp (44). Volume of distribution was assumed to be 0.160 l/kg for glucose. The change in insulin-stimulated glucose disposal (delta Rd) was calculated by the difference between Rd under insulin-stimulated conditions and Rd under basal non-insulin-stimulated conditions. Endogenous glucose production (EGP) was calculated as Ra minus exogenous glucose infusion rate. Non-oxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation.

Sample size

The sample size was determined based on demonstrating the statistical superiority of NR on insulin stimulated skeletal muscle glucose disposal compared with placebo. Twelve participants were required to achieve 80 % power with an alpha of 5 %, an assumed treatment difference of within person changes of 3.25 μmol/kg/min, and an assumed SD of within person changes of 3.60 μmol/kg/min for a one-group paired t-test for a hyperinsulinemic euglycemic clamp. A dropout of 20% was taken into account, so 15 participants were recruited. The expected effect size and SD was based on previous research within our research group (45).

Statistical analyses

Data are reported as mean ± SE, unless otherwise stated. Data are presented for n=13, unless otherwise indicated. Differences between interventions were analyzed with a two-tailed paired Students t-test for parametric data and with a Wilcoxon test for non-parametric data. A two-tailed $p<0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS version 23.0 for MacOS.

RESULTS

Participant population and study compliance

Thirteen healthy overweight and obese men and women (age 59 ± 5 years; BMI 30.2 ± 2.6 kg/m^2 [mean ± SD]; 7 women) participated in the study. Participants were non-smokers, had no active diseases, used no medication or supplements interfering with the study outcomes, had a sedentary lifestyle according to the Baecke questionnaire (7.51 ± 1.16 arbitrary units [mean ± SD]) and an average VO_2peak of 27.0 ± 5.7 ml/min/kg [mean ± SD] (see Supplementary table 2). NR at 1000 mg/day was well tolerated and no adverse events or side effects were reported. Surplus NR and placebo supplements were returned by the participants and compliance rate was calculated as the proportion of capsules ingested relative to the prescribed number. The mean compliance rate during the six-week NR period was 99.3 ± 1.8% [mean ± SD] and during the six-week placebo period was 99.1 ± 2.2% [mean ± SD]. Participants were instructed to maintain their habitual diet and physical activity pattern during the entire study, this was confirmed by a stable body weight of 85.2 ± 3.8 kg [mean ± SD] after six weeks of NR supplementation compared to 85.5 ± 3.8 kg [mean ± SD] upon six weeks placebo (p=0.55).

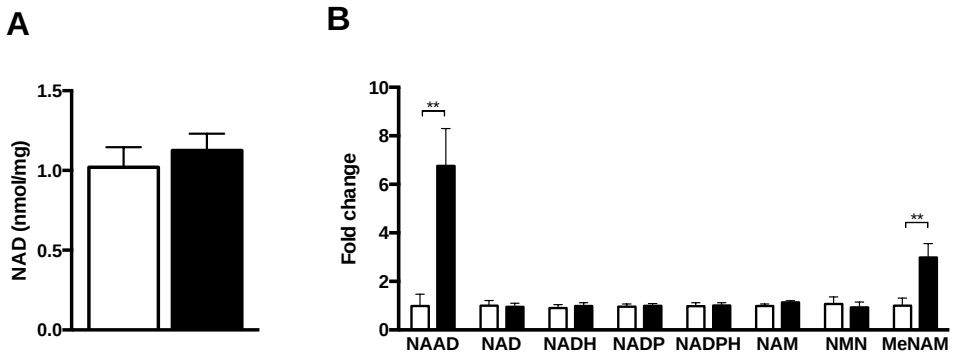

Figure 2: NAD$^+$ metabolites in skeletal muscle after NR and placebo supplementation. A NAD$^+$ concentrations measured in skeletal muscle biopsies by enzymatic assay, n=8. **B** NAD$^+$ metabolites measured in skeletal muscle biopsies by mass spectrometry, n=12. Black bars is NR, open bars is placebo. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. **p<0.01.

NAD^+ metabolites in skeletal muscle

Compliance was further checked by analysis of NAD^+-derived metabolites in muscle biopsy samples collected after six weeks of NR and placebo, ~14-16 hours after supplement intake. First skeletal muscle NAD^+ content was measured. Quantitative analyses of NAD^+ concentrations using enzymatic cycling assay in skeletal muscle showed that NAD^+ content was not different between NR and placebo (1.019 ± 0.126 vs. 1.125 ± 0.106, 0.106 ± 0.105 nmol/mg dry weight in NR, placebo, and the change respectively, p=0.34, n=8, figure 2A). The lack of increase in NAD^+ may indicate that NAD^+ flux is increased without elevated steady state NAD^+ concentrations. Therefore, we next performed metabolomics to check if NAD^+ and NAD^+ metabolites in skeletal muscle were affected by NR. We confirmed that NR supplementation had no effect on skeletal muscle NAD^+ content itself (0.96 ± 0.15 fold change in NR compared to placebo, p=0.91, n=12, figure 2B). However, oral NR supplementation significantly increased two main markers of enhanced NAD^+ metabolism – nicotinic acid adenine dinucleotide (NAAD, 6.8 ± 1.5 fold change p=<0.01, n=12, Figure 2B) and methylnicotinamide (MeNAM, 3.0 ± 0.6 fold change p=<0.01, n=12, figure 2B), confirming that indeed NR was amplifying skeletal muscle NAD^+ metabolism without affecting steady state. Moreover, NADH, NADP, NADPH, NAM and NMN concentrations remained unchanged (p=0.73, p=0.79, p=0.75, p=0.25 and p=0.97 respectively, n=12, see figure 2B).

Mitochondrial respiration in skeletal muscle

Supplementation with NR did not result in any change in mitochondrial respiration compared ot the placebo state. Respiration in the presence of substrate alone (state 2) (malate (M), malate + octanoyl carnitine (MO2) or malate + glutamate (MG2)) was not different between conditions (p=0.34, p=0.19, p=0.74 respectively, figure 3A). Furthermore, ADP stimulated (state 3) respiration on lipid-derived substrate (malate + octanoyl carnitine + ADP (MO3)) and upon complex I substrates (malate + glutamate + ADP (MG3)) was unchanged (p=0.67 and p=0.64 respectively, figure 3B and 3C). Respiration upon parallel electron input to both complex I and II (malate + octanoyl carnitine + glutamate (MOG3)) was not different between conditions (49.87 ± 2.80 vs. 50.92 ± 2.44 pmol/mg/s, -1.04 ± 2.58 in NR, placebo, and the change respectively, p=0.69, figure 3D). Similar results were observed when succinate was sequentially added in both experiments (MGS3: 74.27 ± 3.27 vs. 72.90 ± 4.64, 1.37 ± 5.01 pmol/mg/s in NR, placebo, and the change respectively, p=0.79; MOGS3: 75.30 ± 3.92 vs. 76.09 ± 3.31, -0.79 ± 4.36 pmol/mg/s in NR, placebo, and the change respectively, p=0.86, figure 3D). Maximal FCCP-induced uncoupled respiration (state u), reflecting the maximal capacity of the electron transport chain, was also

unchanged (p=0.81, figure 3E). Finally, state 4o respiration (reflecting proton leak) was similar after NR and placebo conditions (p=0.89, figure 3F). For a description of the different states, please see: https://www.bioblast.at/index.php/ MitoPedia: Respiratory states. Mitochondrial OXPHOS protein concentrations (Complex I 0.96 ± 0.21 vs. 0.91 ± 0.15, 0.05 ± 0.235 A.U. in NR, placebo, and the change respectively, p=0.84; Complex II 1.01 ± 0.17 vs. 0.78 ± 0.05, 0.23 ± 0.20 A.U. in NR, placebo, and the change respectively, p=0.64; Complex III 0.94 ± 0.19 vs. 0.92 ± 0.17, 0.02 ± 0.22 A.U. in NR, placebo, and the change respectively, p=0.93; Complex IV 1.02 ± 0.15 vs. 0.94 ± 0.10, 0.09 ± 0.14 A.U. in NR, placebo, and the change respectively p=0.55; Complex V 1.00 ± 0.17 vs. 0.79 ± 0.16, 0.21 ± 0.17 A.U. in NR, placebo, and the change respectively p=0.24) were similar after NR and placebo supplementation, indicating no effect of NR on mitochondrial content (figure 3G).

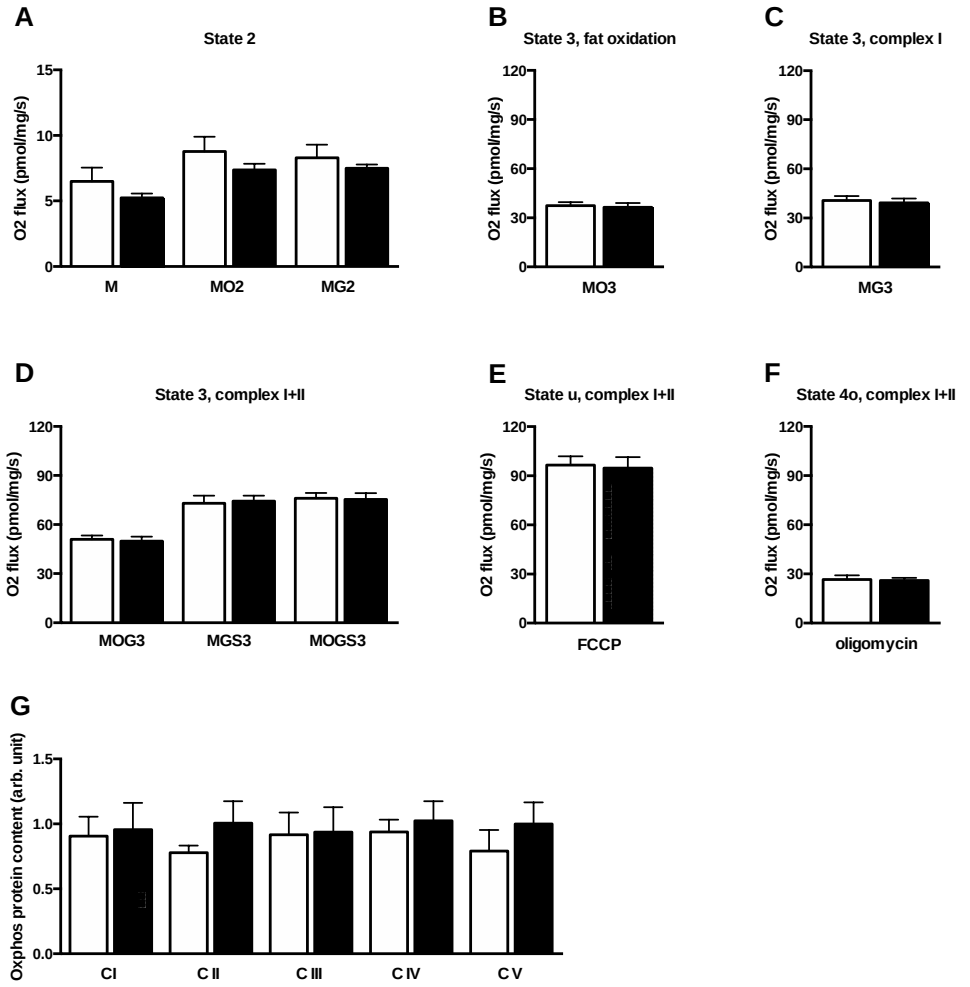

Figure 3: Skeletal muscle *ex vivo* mitochondrial respiratory capacity after NR and placebo supplementation. A State 2 respiration upon malate (M), malate + octanoyl carnitine (MO2) and malate + glutamate (MG2). **B** ADP stimulated state 3 respiration upon lipid derived substrate, malate + octanoyl carnitine + glutamate (MO3). **C** ADP stimulated state 3 respiration upon complex I substrates, malate + glutamate (MG3). **D** ADP stimulated state 3 respiration upon parallel electron input to both complex I and II, malate + octanoyl carnitine + glutamate (MOG3), malate + glutamate + succinate (MGS3), malate + octanoyl carnitine + glutamate + succinate (MOGS3). **E** State u: maximal FCCP-induced uncoupled respiration. **F** State 4o: Oligomycin induced respiration not coupled to ATP synthesis. **G** Protein content of individual complexes of the electron transport chain. Black bars is NR, open bars is placebo. N=12. Data are expressed as mean ± SE.

Acetylcarnitine concentrations in skeletal muscle

Quantification of acetylcarnitine concentration in skeletal muscle, measured by MRS technique in the early evening at 17:00 h after 3 hours fasting, revealed significantly lower baseline acetylcarnitine concentrations under NR supplementation compared to placebo (1.30 ± 0.16 vs. 1.80 ± 0.18, -0.53 ± 0.19 mmol/kg wet weight in NR, placebo, and the change respectively, p=0.02, figure 4A). Maximally stimulated acetylcarnitine concentrations, measured upon exercise, were not different between conditions (4.29 ± 0.29 vs. 4.20 ± 0.27, 0.00 ± 0.20 mmol/kg wet weight in NR, placebo, and the change respectively, p=0.67, figure 4A). Nonetheless, the capacity to increase acetylcarnitine formation, expressed as the change computed as the post exercise value minus the baseline value, was significantly higher in NR compared to placebo (2.99 ± 0.30 vs. 2.40 ± 0.33, 0.53 ± 0.21 mmol/kg wet weight in NR, placebo, and the change respectively, p=0.01, figure 4A). Based on these results, we decided to perform full acylcarnitine analysis in skeletal muscle biopsies taken at 8:30 h after an overnight fast. Remarkably, acetylcarnitine (C2) concentrations were significantly higher under NR supplementation compared to placebo (C2: 4558 ± 749 vs. 3025 ± 316, 1533 ± 683 pmol/mg dry weight in NR, placebo, and the change respectively, p=0.04; figure 4B). No differences were detected in free carnitine (C0), other short chain (C3 to C5), medium chain (C6 to C12) or long chain acylcarnitines (C13 to C20) between NR and placebo (p=0.25, p=0.27, p=0.99 and p=0.45 respectively; figure 4C).

Insulin sensitivity and substrate kinetics

Potential effects of NR on whole body and tissue-specific insulin sensitivity were assessed by a two-step hyperinsulinemic-euglycemic clamp. Whole body insulin stimulated glucose uptake, as expressed by the change in glucose disposal (delta Rd) from baseline to high insulin dose, was not different between NR and placebo (p=0.98, table 1). Hepatic insulin sensitivity, reflected by EGP suppression (EGP%) during the low insulin phase, was not affected by NR compared to placebo (p=0.30, table 1). NR had no effect on baseline substrate oxidation (carbohydrate oxidation p=0.84, and fat oxidation p=0.67). Furthermore, insulin stimulated carbohydrate oxidation or suppression of fatty acid oxidation, reflecting metabolic flexibility, was not different between NR and placebo (p=0.92 and p=0.70) (see table 1). In addition, non-oxidative glucose disposal (delta NOGD) during low and high insulin phase of the clamp remained unchanged between conditions (p=0.88 and p=0.99) (see table 1). Plasma FFA concentrations were suppressed by insulin to a similar extend between NR and placebo (p=0.97 and p=0.99 during low and high insulin phase respectively, table 1), indicating similar white adipose tissue insulin sensitivity.

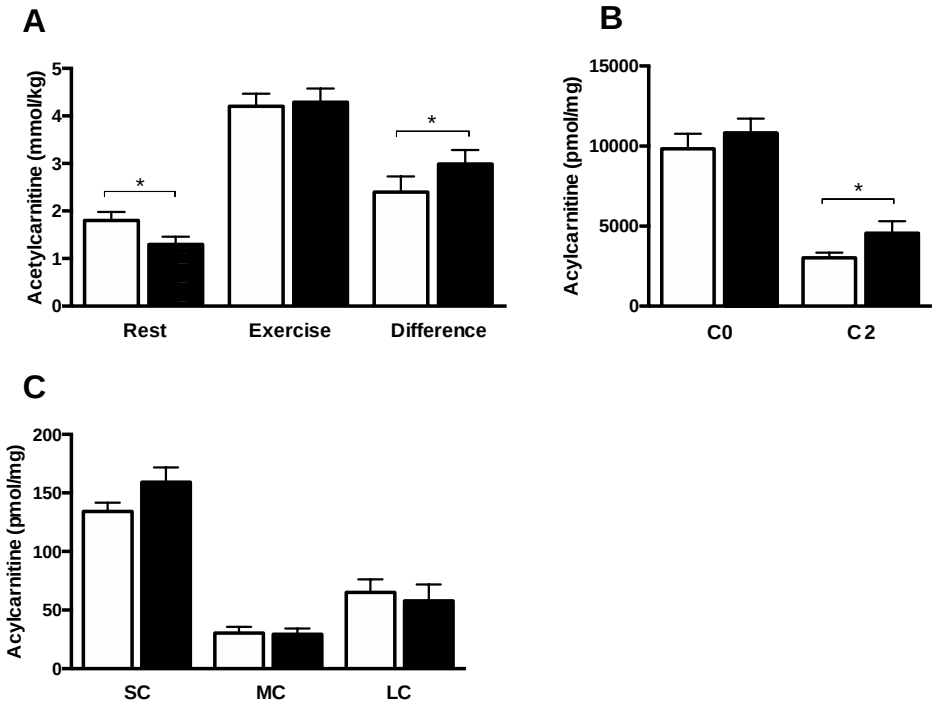

Figure 4: Skeletal muscle acylcarnitine concentrations measured in the morning and evening after NR and placebo supplementation. **A** Acetylcarnitine concentration measured by MRS in skeletal muscle in the evening during rest, after exercise and the capacity to form acetylcarnitine expressed as the difference between rest and exercise. **B** Free carnitine (C0) and acetylcarnitine (C2) concentrations measured in muscle biopsies in the morning during rest. **C** Sum of short chain acylcarnitines (SC), sum of medium chain acylcarnitines (MC) and sum of long chain acylcarnitines (LC) concentrations measured in biopsies during rest in the morning. Black bars is NR, open bars is placebo. N=13. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. *p<0.05.

Table 1: Insulin sensitivity and substrate kinetics

Parameter	Placebo	NR	Change	p-value
Ra (μmol/kg/min)[1]				
Baseline	9.32 ± 0.33	9.54 ± 0.78	0.22 ± 0.72	0.77
Low insulin	11.66 ± 0.93	11.74 ± 0.91	0.08 ± 0.42	0.85
High insulin	36.70 ± 3.40	37.60 ± 2.66	0.91 ± 2.24	0.69
Rd(μmol/kg/min)[1]				
Baseline	9.90 ± 0.53	9.52 ± 0.84	-0.38 ± 0.97	0.70
Low insulin	12.06 ± 1.00	12.36 ± 0.94	0.30 ± 0.45	0.51
High insulin	36.76 ± 3.36	36.32 ± 2.62	-0.45 ± 2.13	0.84
Delta baseline-low insulin	2.16 ± 1.07	2.84 ± 1.01	0.68 ± 1.03	0.47
Delta baseline-high insulin	26.86 ± 3.31	26.79 ± 2.86	-0.07 ± 2.17	0.98
EGP (μmol/kg/min)[1]				
Baseline	9.32 ± 0.33	9.54 ± 0.78	0.22 ± 0.72	0.77
Low insulin	2.70 ± 0.40	3.38 ± 0.56	0.68 ± 0.48	0.18
% suppression low insulin	70.44 ± 4.57	61.02 ± 7.05	-9.42 ± 7.13	0.30
High insulin	-0.04 ± 0.26	0.51 ± 0.56	0.55 ± 0.51	0.30
% suppression high insulin	99.78 ± 2.52	96.82 ± 4.65	-2.95 ± 4.28	0.50
NOGD (μmol/kg/min)[1]				
Baseline	5.27 ± 1.22	4.88 ± 1.06	-0.40 ± 1.18	0.74
Low insulin	3.77 ± 0.92	3.56 ± 0.75	-0.19 ± 0.63	0.76
High insulin	21.28 ± 2.84	20.85 ± 2.01	-0.43 ± 2.03	0.84
Delta baseline-low insulin	-1.51 ± 1.36	-1.30 ± 0.85	0.21 ± 1.34	0.88
Delta baseline-high insulin	16.01 ± 2.81	15.97 ± 2.04	-0.03 ± 1.83	0.99
Carbohydrate oxidation (μmol/kg/min)				
Baseline	4.42 ± 0.81	4.58 ± 0.55	0.16 ± 0.61	0.84
Low insulin	7.84 ± 0.69	8.79 ± 0.78	0.95 ± 0.76	0.23
High insulin	14.90 ± 0.96	14.99 ± 1.15	0.09 ± 0.89	0.92
Fat oxidation (μmol/kg/min)				
Baseline	3.78 ± 0.23	3.71 ± 0.20	-0.07 ± 0.17	0.67
Low insulin	2.73 ± 0.19	2.55 ± 0.17	-0.18 ± 0.21	0.41
High insulin	1.47 ± 0.23	1.38 ± 0.22	-0.09 ± 0.22	0.70
Plasma FFA (μmol/l)				
Baseline	555.34 ± 30.87	581.16 ± 32.08	25.83 ± 35.55	0.48
Low insulin	128.55 ± 21.74	128.03 ± 19.26	-0.52 ± 14.19	0.97
High insulin	49.84 ± 8.26	56.45 ± 14.80	6.61 ± 10.04	0.99
Respiratory exchange ratio				
Baseline	0.77 ± 0.01	0.77 ± 0.01	0.00 ± 0.01	0.83
Low insulin	0.82 ± 0.01	0.83 ± 0.01	0.01 ± 0.01	0.37
High insulin	0.91 ± 0.01	0.91 ± 0.01	0.00 ± 0.01	0.86

Ra, rate of appearance; Rd, rate of disappearance; EGP, endogenous glucose production; NOGD, non-oxidative glucose disposal; FFA, free fatty acids. [1]n=12. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

Plasma biochemistry and inflammatory markers

NR supplementation did not affect fasting plasma glucose, triglycerides, total cholesterol, HDL-C, LDL-C, or inflammatory markers, including chemokine, cytokine or cell-adhesion molecule concentrations (table 2). However, interleukin 1 alpha (IL-1α) tended to be lower after NR supplementation compared to placebo (1.61 ± 0.28 vs. 2.11 ± 0.35 respectively, p=0.06, table 2).

Table 2: Blood biochemistry

Parameter	Placebo	NR	Change	p-value
Glucose (mmol/l)	5.48 ± 0.14	5.44 ± 0.13	-0.04 ± 0.10	0.70
Triglycerides (mmol/l)	1.57 ± 0.35	1.63 ± 0.38	0.06 ± 0.08	0.24
Total cholesterol (mmol/l)	5.54 ± 0.30	5.55 ± 0.35	0.01 ± 0.11	0.99
HDL-C (mmol/l)	1.32 ± 0.12	1.32 ± 0.09	-0.00 ± 0.04	0.99
LDL-C (mmol/l)[1]	3.42 ± 0.18	3.37 ± 0.17	-0.05 ± 0.07	0.52
sE-selectin (pg/ml)[2]	32726 ± 5298	34765 ± 4354	686 ± 2068	0.99
sP-selectin (pg/ml)[2]	21428 ± 3662	26645 ± 4065	2624 ± 3739	0.58
ICAM-1 (pg/ml)[2]	92493 ± 19326	129236 ± 34547	35350 ± 27518	0.58
TNF-α (pg/ml)[2]	29.80 ± 6.38	31.85 ± 6.14	2.70 ± 1.97	0.22
IL-1α (pg/ml)[2]	2.11 ± 0.35	1.61 ± 0.28	-0.57 ± 0.24	0.06
IL-4 (pg/ml)[2]	8.04 ± 1.03	8.86 ± 1.41	0.71 ± 3.63	0.69
IL-12p70 (pg/ml)[2]	76.07 ± 2.73	78.89 ± 3.02	1.94 ± 2.82	0.81
IL-17α (pg/ml)[2]	6.36 ± 1.36	7.95 ± 2.09	1.76 ± 1.02	0.16
CXCL10 (pg/ml)[2]	3.71 ± 0.55	3.39 ± 0.50	-0.61 ± 0.40	0.22
CCL2 (pg/ml)[2]	127.39 ± 24.54	144.34 ± 47.86	21.42 ± 40.12	0.69
CCL3 (pg/ml)[2]	15.42 ± 8.59	17.04 ± 10.39	2.43 ± 1.92	0.30
CCL4 (pg/ml)[2]	23.89 ± 5.98	28.49 ± 7.51	4.52 ± 4.45	0.38

Blood samples were taken in week six of NR supplementation and placebo after an overnight fast. [1] n=12, [2] n=7. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

Body composition

After six weeks of NR and placebo supplementation, several changes in body composition were detected. Percentage fat free mass (FFM) was significantly higher after NR compared to placebo (62.65 ± 2.49 vs. 61.32 ± 2.58, 1.34 ± 0.50 % in NR, placebo, and the change respectively, p=0.02, figure 5B). In line with this, percentage fat mass (FM) was significantly lower after NR compared to placebo (37.35 ± 2.49 vs. 38.68 ± 2.58, -1.34 ± 0.50 % in NR, placebo, and the change respectively, p=0.02, figure 5B). However, total bodyweight remained unchanged (p=0.55, figure 5A).

Sleeping metabolic rate

Sleeping metabolic rate (SMR), measured during an overnight stay in a respiration chamber, was higher upon six weeks of NR compared to placebo (6.68 ± 0.30 vs. 6.49 ± 0.31, 0.19 ± 0.08 MJ/day in NR, placebo, and the change respectively, p=0.05, figure 5C). This could be explained by the increase in FFM and sleeping metabolic rate per FFM was not significantly different (0.127 ± 0.003 vs. 0.125 ± 0.003, 0.00 ± 0.00 MJ/FFM/day in NR, placebo, and the change respectively, p=0.48, figure 5E).

Ectopic lipid storage

Intrahepatic lipid content, measured by MRS, was not different between NR and placebo conditions (3.4 ± 1.2 vs. 3.4 ± 1.3, 0.0 ± 0.0 % in NR, placebo, and the change respectively, p=0.85). Furthermore, intramyocellular lipid content, measured by MRS technique in the m. tibialis anterior, was not affected by NR supplementation (0.5 ± 0.1 vs. 0.5 ± 0.1, 0.0 ± 0.1 % in NR, placebo, and the change respectively, p=0.50).

Cardiac function

To investigate if NR supplementation could affect cardiac energetics, we determined cardiac PCr/ATP ratios, which however were not affected by NR (p=0.90, table 3). No differences were observed in left ventricular end systolic volume (ESV), end diastolic volume (EDV), stroke volume (SV) and subsequently ejection fraction (EF) between NR and placebo (p=0.23, p=0.72, p=0.69 and p=0.24 respectively, table 3). NR supplementation had no effect on 24-hour systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP), and heart rate (HR) (p=0.56, p=0.39, p=0.40, p=0.60 and p=0.60 respectively, table 3). Separate analyses of day time and night time measurements did not reveal an effect of NR supplementation (see table 3). In addition, night time dipping of SBP and DBP was not affected by NR compared to placebo (p=0.53 and p=0.26 respectively, table 3).

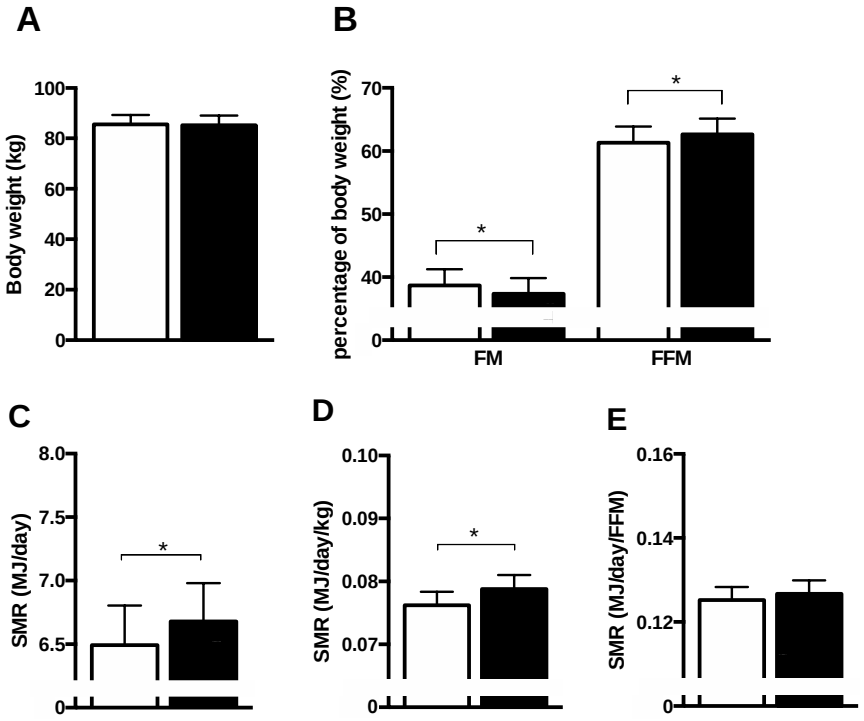

Figure 5: Body composition and sleeping metabolic rate after NR and placebo supplementation. **A** Bodyweight, fat mass (FM) and fat free mass (FFM) expressed in kilograms (kg). **B** FM and FFM expressed as percentage (%) of body weight. **C** Sleeping metabolic rate expressed (SMR) as MJ per day. **D** SMR corrected for body weight. **E** SMR corrected for FFM. Black bars is NR, open bars is placebo. Data are expressed as mean ± SE. N=13. P values are derived from the analysis of the mean within-person changes and the SEM of the within-group changes. *p<0.05.

Table 3: Cardiometabolic health parameters

Parameter	Placebo	NR	Change	p-value
MRS Cardiac left ventricle[2]				
Cardiac PCr/ATP ratio	1.29 ± 0.11	1.22 ± 0.09	-0.08 ± 0.14	0.90
MRI Cardiac left ventricle[1]				
EF (%)	71.2 ± 2.2	68.2 ± 2.1	-3.0 ± 2.4	0.24
ESV (ml)	35.0 ± 4.2	38.9 ± 3.9	4.0 ± 3.6	0.23
EDV (ml)	119.4 ± 8.2	121.2 ± 8.3	1.8 ± 5.1	0.72
SV (ml)	84.4 ± 5.6	82.3 ± 5.6	-2.1 ± 3.8	0.69
Ambulatory BP 36-hour[1]				
SBP (mmHg)	124.6 ± 2.4	126.6 ± 3.2	2.0 ± 2.3	0.56
DBP (mmHg)	77.1 ± 1.8	77.9 ± 2.0	0.8 ± 0.9	0.39
MAP (mmHg)	98.9 ± 1.7	100.2 ± 2.1	1.3 ± 1.5	0.40
PP (mmHg)	47.5 ± 2.6	48.6 ± 3.1	1.2 ± 2.2	0.60
HR (bpm)	76.4 ± 3.3	75.5 ± 3.6	-0.9 ± 1.7	0.60
Night time dipping SBP (%)	10.6 ± 2.4	12.4 ± 0.8	1.8 ± 2.8	0.53
Night time dipping DBP (%)	11.9 ± 2.4	14.8 ± 1.9	3.0 ± 2.5	0.26
Ambulatory BP daytime				
SBP (mmHg)	126.9 ± 2.3	129.0 ± 2.9	2.1 ± 2.3	0.54
DBP (mmHg)	79.5 ± 1.7	80.5 ± 1.9	1.0 ± 1.0	0.32
MAP (mmHg)	101.4 ± 1.5	102.7 ± 1.9	1.3 ± 1.4	0.39
PP (mmHg)	47.6 ± 2.7	48.5 ± 3.0	0.9 ± 2.1	0.68
HR (bpm)	78.3 ± 3.2	77.5 ± 3.5	-0.8 ± 1.7	0.67
Ambulatory BP night-time[1]				
SBP (mmHg)	113.5 ± 3.4	113.0 ± 3.1	-0.5 ± 3.7	0.90
DBP (mmHg)	69.4 ± 2.6	68.1 ± 2.3	-1.3 ± 1.5	0.41
MAP (mmHg)	89.6 ± 2.7	88.8 ± 2.4	-0.8 ± 2.5	0.75
PP (mmHg)	43.8 ± 2.5	45.2 ± 2.6	1.4 ± 2.3	0.57
HR (bpm)	65.6 ± 2.5	66.6 ± 2.9	1.0 ± 1.9	0.60

PCr, phosphocreatin; ATP, adenosine triphosphate; EF, ejection fraction; ESV, end systolic volume; EDV, end diastolic volume; SV, stroke volume; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; HR, heart rate. [1]n=12, [2]n=11. Data are expressed as mean ± SE. P values are derived from the analysis of the mean within-person changes and the SE of the within-group changes.

DISCUSSION

We hypothesized that NR supplementation in humans would increase NAD^+ availability and thereby would improve a broad range of metabolic health parameters, mainly via improving mitochondrial function. To investigate this hypothesis, we performed a randomized double-blinded placebo controlled cross-over study with detailed metabolic phenotyping in which we provided healthy overweight and obese men and women 1000 mg/day NR for six weeks. In line with our hypothesis, NR supplementation did significantly increase markers of NAD^+

metabolism – NAAD and MeNAM – in skeletal muscle. This effect was accompanied by small but statistically significant improvements in body composition, sleeping metabolic rate and skeletal muscle acetylcarnitine concentrations, and a trend towards increased circulatory IL-1α concentrations. No further effects on skeletal muscle mitochondrial function, hepatic and whole-body insulin sensitivity, substrate oxidation, cardiovascular health markers and ectopic lipid accumulation were observed. However, it should be noted that many outcomes have been tested in our study and no adjustments for multiple comparisons were performed, therefore the possibility of false positive findings cannot be excluded. These results suggest that NR, at the dose of 1000 mg/day for six weeks, did have, albeit relatively small, effects on metabolic parameters in humans, but was not effective in boosting muscle mitochondrial function or insulin sensitivity.

Animal studies showed that NR is able to increase plasma and tissue NAD^+ concentrations (9-14). Also in humans, NR is able to increase circulatory NAD^+ metabolites after several dosages ranging from 100 mg/day to 2000 mg/day (18-21). Here, we used a dose of 1000 mg/day for six weeks, and in line with other data presented we did not report side effects (18, 20). We investigated if NR supplementation was able to increase the NAD^+ metabolome in skeletal muscle tissue. In agreement with findings by Elhassan et al. (20) and Dollerup et al. (24), we show that NR supplementation increased NAD^+ metabolites NAAD and MeNAM in skeletal muscle, but without an increase in total NAD^+ content itself. NAAD is a highly sensitive biomarker of NR supplementation and increased NAD^+ synthesis rate in tissues (14). MeNAM is part of the NAD^+ degradation pathway and is a marker for increased NAD^+ flux. These results might suggest that NR increases NAD^+ turnover rate, without affecting steady state NAD^+ concentrations in skeletal muscle.

We hypothesized that a NR-stimulated increase in NAD^+ metabolism would lead to an increase in muscle mitochondrial function and a subsequent increase in human insulin sensitivity. However, in contrast to our hypothesis, skeletal muscle mitochondrial function was not elevated upon NR supplementation. This is in agreement with the findings of Elhassan et al. (25) and Dollerup et al. (24), who also reported no effect of NR on skeletal muscle mitochondrial function. Consistent with the lack of effect on mitochondrial function, we and others (21) did not observe improvements on insulin sensitivity upon NR supplementation. Although the limited duration of our and other studies may explain the lack of effect of NR on insulin sensitivity, we have previously shown that nutritional supplements like resveratrol can increase skeletal muscle mitochondrial function after four weeks of supplementation (45-47). Trammel et al. (48) and Dollerup et al. (21) suggested that the underlying pathway of metabolic improvements observed in obese mice was a decrease in hepatic lipid accumulation. In addition, Dollerup et al. (21) described a decrease

in hepatic lipid content in obese men with elevated baseline hepatic lipid content, although this was not significant. Here, we did not observe an effect of NR on hepatic lipid accumulation, which may be contributed to our study population which had in general a healthy hepatic lipid content (i.e. < 5% liver fat).

NR supplementation has also been suggested to improve cardiovascular health (20, 49). Therefore, we here examined the effect of NR on cardiovascular health via detailed cardiovascular phenotyping. In contrast to Martens et al. (20) but in accordance with Conze et al. (23), we did not observe an effect of NR on blood pressure values. Martens et al. observed a decrease in resting SBP and DBP after NR, whereas we measured 36-hour ambulatory blood pressure, which gives a better estimation of blood pressure values and gives a better indication of the risk of cardiovascular events (50, 51). Consistent with the lack of effect of NR on blood pressure we did not find effects of NR on cardiac energy status or cardiac ejection fraction. Of note, the cardiac status of our participants was considered 'healthy', and in rodents also no change in cardiac function after NR supplementation in control mice with a healthy cardiac function could be observed (49).

We reported an improvement in body composition by an increase in percentage fat free mass mirrored by a decrease in percentage fat mass, while body weight remained unchanged. An effect of NR on body composition in humans has not been reported before (20, 21). Interestingly, in six out of seven women NR did increase fat free mass and reduce fat mass whereas this was only the case in one out of six men, suggesting that there might be a gender difference in the effect of NR supplementation on body composition. Consistent with the effect of NR on body composition, we show that the sleeping metabolic rate was also affected by NR supplementation and a higher metabolic rate could potentially lead to a reduction in fat mass. The increase in sleeping metabolic rate was due to an increase in fat free mass, suggesting that the primary effect of these findings could be an effect of NR on fat free mass. Interestingly, it has previously been shown that NAD^+ metabolism and homeostasis is involved in maintaining muscle mass (13). Future studies should be designed to investigate if NR supplementation can indeed increase muscle mass in humans and investigate the underlying mechanisms.

Next to an effect of NR on muscle mass, NR supplementation enhanced the exercise-induced increase in acetylcarnitine. Moreover, acetylcarnitine concentrations measured in skeletal muscle biopsies obtained in the morning, showed significantly increased concentrations after NR supplementation. These data suggest that NR is able to increase skeletal muscle acetylcarnitine metabolism, which has been associated with metabolic flexibility and improved metabolic health (36). Remarkably, though, resting acetylcarnitine

concentrations measured using MRS three hours after lunch and before the exercise session were significantly lower after NR compared to placebo. It has previously been shown that meal consumption lowers acetylcarnitines in skeletal muscle (52, 53), but why NR would substantiate such meal-induced lowering in acetylcarnitine metabolism cannot be deducted from this study. Interestingly, a recently published study in obese mice showed an effect of combined supplementation of NR with L-carnitine and reported a reduction in fat mass percentage and hepatic steatosis (54), which matches with the positive outcome parameters of our and other studies (9, 21, 48). The exact link between NR metabolism and acetylcarnitine metabolism is however still unknown. Furthermore, the large gap between the clear metabolic improvements upon NR in mice and the lack of effects in humans might derive from the fact that mice studies applied a longer supplementation duration (8 to 15 weeks) (9, 10, 12, 48) compared to short-term supplementation in human trials (3 to 12 weeks) (21, 24, 25, 55).

In conclusion, we here show that NR supplementation of 1000 mg/day for six weeks in healthy overweight and obese men and women increased the NAD^+ metabolites NAAD and MeNAM in human skeletal muscle and increased skeletal muscle acetylcarnitine metabolism. In addition, NR induced improvements in body composition and increased sleeping metabolic rate. However, no other metabolic health effects were observed. We conclude that NR, at this dose and short duration, may not be beneficial in improving metabolic health in healthy overweight and obese men and women. However, further research is warranted into the effects of long-term NR supplementation on acetylcarnitine concentrations, sex specific improvements in body composition and metabolic health effects in humans.

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The authors' contributions were as follows: CR BH JA JH VSH EP PS designed the study. CR KR MM NC VdW conducted the experiments. BH provided medical responsibility. SA BS HE RZP performed analysis in plasma samples and muscle biopsies. CR JM VdW TvdW SA RZP VSH analyzed data. CR SA EL RZP RH JH LL VSH EP PS interpreted the data. CR EP PS wrote paper and all authors reviewed and accepted the last version of the manuscript. PS had primary responsibility for final content. The authors would like to thank the participants for their commitment to this study and ChromaDex Inc. for providing NIAGEN and placebo capsules for the study. PS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors declare no conflict of interest relevant to this article.

SUPPLEMENTARY MATERIAL FOR CHAPTER 7

Supplementary table 1: Internal standard mixture for NAD^+ targeted metabolomics in skeletal muscle biopsies

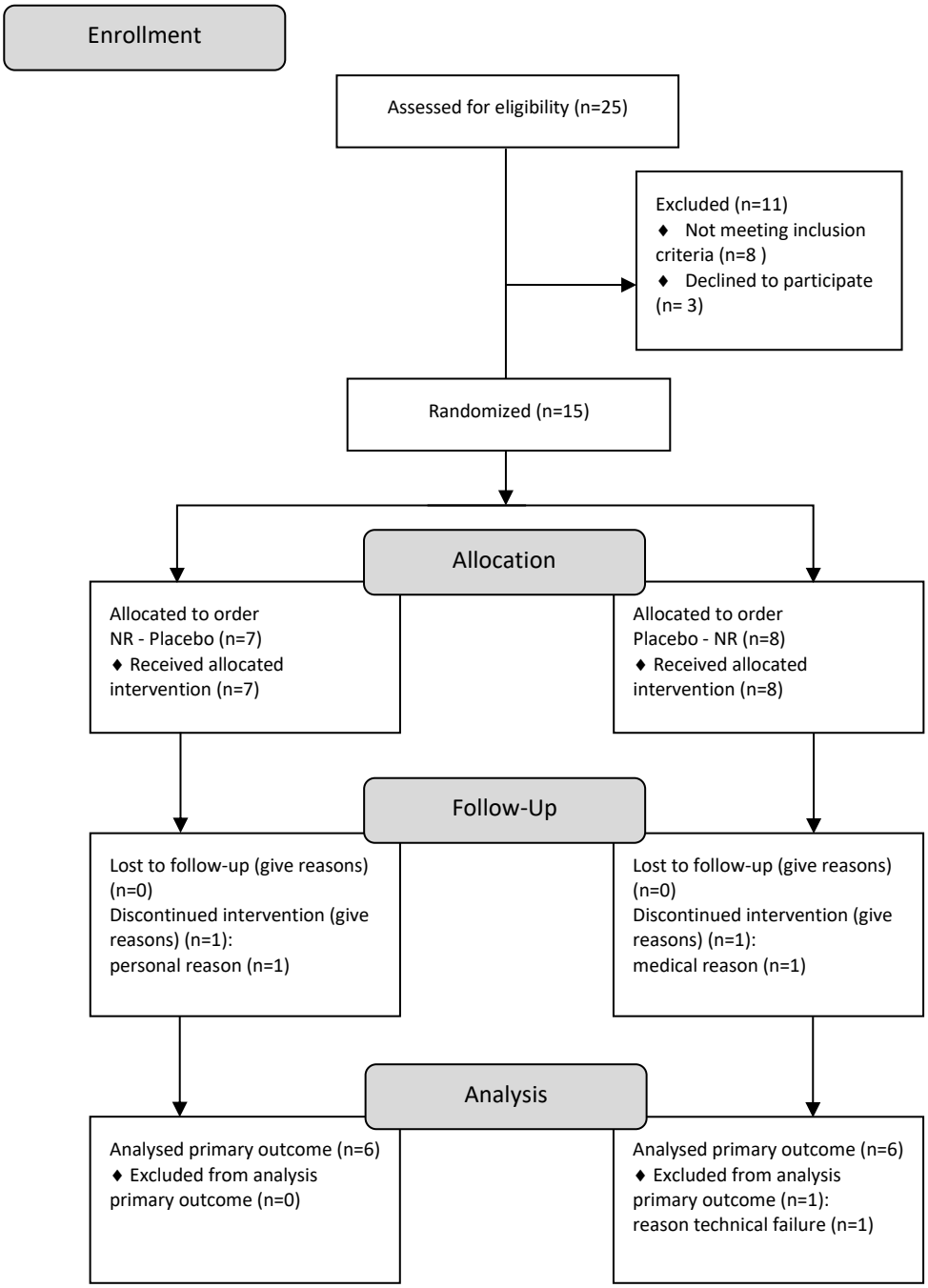
Internal standard	Concentration (µM)
Adenosine-$^{15}N_5$-monophosphate	100
Adenosine-$^{15}N_5$-triphosphate	1000
D_4-alanine	100
D_7-arginine	100
D_3-aspartic acid	100
D_4-citric acid	100
$^{13}C_1$-citrulline	100
$^{13}C_6$-fructose-1,6-diphosphate	100
Guanosine-$^{15}N_5$-monophosphate	100
Guanosine-$^{15}N_5$-triphosphate	1000
$^{13}C_6$-glucose	1000
$^{13}C_6$-glucose-6-phosphate	100
D_3-glutamic acid	100
D_5-glutamine	100
$^{13}C_6$-isoleucine	100
D_3-leucine	100
D_4-lysine	100
D_3-methionine	100
D_6-ornithine	100
D_5-phenylalanine	100
D_7-proline	100
$^{13}C_3$-pyruvate	100
D_3-serine	100
D_5-tryptophan	100
D_4-tyrosine	100
D_8-valine	100
$^{13}C_5$-nicotinamide adenine dinucleotide	100

Supplementary table 2: Participant characteristics

Parameter	Mean ± SD
Gender F/M	7/6
Age (years)	59 ± 5
Body weight (kg)	87.2 ± 13.6
Height (m)	1.70 ± 0.11
BMI (kg/m^2)	30.2 ± 2.6
VO_2peak (ml/min/kg)	27.0 ± 5.7
Physical activity level (Baecke score)	7.51 ± 1.16

BMI, body mass index; VO_2peak, peak oxygen consumption.

Supplementary figure 1: Flowchart participant inclusion



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CHAPTER 8

General discussion and conclusion

NAFL is the number one chronic liver condition worldwide (1). It is estimated that 25% of the adult population worldwide has NAFL (1). In obesity, NAFL rates are even higher, with reported prevalence ranging from 55% to 75% (2, 3). Fatty liver can progress into more severe conditions such as NASH and cirrhosis, which will ultimately increase the risk for hepatocellular carcinoma (4). The negative consequences of NAFL are however not only related to progressive liver disease, but also include cardiometabolic effects. In this respect, NAFL has been associated with cardiovascular disease and type 2 diabetes (5, 6). People with NAFL have been shown to have a reduced whole-body and hepatic insulin resistance, which is a major risk factor in the development of T2D (7-10). Little is known about the disturbances in substrate metabolism of the liver in NAFL even though such disturbances could play an important role in NAFL etiology and its contribution to cardiometabolic health. To study hepatic substrate metabolism and substrate storage, advanced (MR) methodologies can be instrumental. Therefore, the research in this PhD thesis focusses on the use of advanced magnetic resonance (MR) methodology to investigate hepatic substrate metabolism and to better understand the metabolic changes that take place during the development of NAFL. Specifically, the importance of hepatic lipid composition (i.e. lipid saturation) and hepatic glycogen are investigated.

How to measure different substrate stores in NAFL?

A technique that is of great value in determining hepatic substrate stores non-invasively is magnetic resonance spectroscopy (MRS). Using ^{1}H-MRS, information on intrahepatic lipid (IHL) storage can be acquired, whereas ^{13}C-MRS can be used to determine hepatic glycogen storage.

IHL *per se* has been negatively associated with metabolic health. However, there are also examples of dissociation between IHL and insulin sensitivity. For instance, increased ChREBP has been shown to promote IHL accumulation without worsening insulin sensitivity (11) and the presence of the rs738409 SNP in the PNPLA3 gene positively associates with hepatic fat storage, without being associated with insulin resistance (12). There is evidence that lipid composition may even be more important than content *per se*: the type of stored fat may also influence metabolic health. A high degree of palmitic (C16:0) acid in serum has been shown to be associated with impaired insulin sensitivity (13). In addition, differences in lipid composition have been shown in liver biopsies of people with and without NAFL (14, 15). Furthermore, high fractions of hepatic saturated fatty acids (SFA) might explain the associations seen between *de novo* lipogenesis (DNL) and poor metabolic health (16-18), as SFA is the main end product of DNL.

Therefore, determining liver fat composition (determination of SFA, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA)) in humans is of great interest. As taking biopsies to determine fat composition is not preferred because of its invasiveness, MRS could be applied as an excellent non-invasive alternative. Determining the hepatic fatty acid fractions is however challenging, because: 1) the resonances of unsaturated fatty acids are small, 2) resonances overlap and 3) imperfect suppression of the large water resonance leads to contamination of other resonances. In **chapter 3**, we set-up, validated and applied a novel MRS technique to non-invasively determine the fraction of hepatic SFA, MUFA and PUFA separately in humans, while addressing all the beforementioned difficulties. We validated our approach in oils with high resolution ^{13}C-NMR, in human subcutaneous adipose tissue by comparing MRS determined fat composition to MS-based values in biopsies, and by showing good reproducibility in liver in humans. This novel methodology enabled us to determine liver fat composition in healthy and metabolically compromised volunteers. Importantly, by using this new methodology, we could also determine the relation of the hepatic fatty acid composition to hepatic insulin sensitivity and *de novo* lipogenesis (DNL).

When quantifying IHL content *per se* one should keep in mind that different factors could influence the quantification. Determining IHL content requires accurate T_2 correction of MRS signals. Commonly, fixed water and lipid T_2 values are used (19-21). However, T_2 relaxation times might differ with increased levels of hepatic steatosis and therefore could influence IHL quantification. In **chapter 6** we therefore examined how variations in T_2 relaxation times affect the calculations of absolute IHL% (w/w). We showed that T_2 relaxation times are different with different levels of steatosis. Correction with a fixed average T_2 is introducing only very minor error as long as the investigated population is homogenous in terms of steatosis. However, in individuals with different levels of steatosis, using fixed average T_2 correction factors lead to an overestimation of liver fat content in people with high IHL content. In general, it would be the best approach to determine the T_2 relaxation times individually in each volunteer in order to prevent any bias in the calculations of IHL. This strategy however requires additional acquisitions of MR spectra at multiple echo times, which is time consuming. We showed that by using STEAM sequence with short echo time the bias in IHL content was minimized.

Combining ^{1}H-MRS with ^{13}C-MRS provides the opportunity to acquire additional information on liver substrate stores and metabolism, giving a broad picture of liver substrate metabolism, which can aid in elucidating NAFL etiology. ^{13}C-MRS can be used to determine hepatic glycogen content (22-26), another substrate that may be a key factor in determining substrate metabolism, specifically partitioning of fat towards oxidation or

storage. Hepatic glycogen changes rhythmically during the day, with increased levels postprandially and decreasing glycogen levels during fasting periods (22-24). Little is known about the dynamics of glycogen and its effects on fat and carbohydrate metabolism in NAFL. We used ^{13}C-MRS in **chapter 4** to determine the effect of one-night prolonged fasting on hepatic glycogen levels in individuals with NAFL.

How are liver substrate stores changed in NAFL?

IHL content is (by definition) increased in NAFL, however, next to this, also carbohydrate metabolism and glycogen storage may be affected and the application of both ^{1}H-MRS and ^{13}C-MRS can give a broad picture of hepatic metabolism. We applied our novel ^{1}H-MRS approach and ^{13}C-MRS to determine hepatic lipid composition and hepatic glycogen and elucidated their role in NAFL.

As expected, we found a strong positive correlation between DNL and hepatic SFA fraction (**chapter 3**). In contrast, MUFA fraction negatively correlated with DNL. These results suggest that the formation of SFA in the liver is not necessarily accompanied by subsequent desaturation to MUFA, as is generally believed. Indeed, animal studies do show upregulation of desaturation markers when lipogenic enzymes are upregulated (11); this may suggest that the formation of SFA from DNL may be handled differently in animals and humans. The positive association between DNL and hepatic SFA fraction was further confirmed by our finding that patients with glycogen storage disease type 1a (GSD1a), a genetic model of increased DNL (27), showed an increased hepatic SFA fraction compared to healthy overweight or obese volunteers. Moreover, the hepatic SFA fraction was also increased in people with NAFL and T2D patients, two populations that have been identified with high DNL rates before (16, 28, 29).

Next to changes in liver fat composition in NAFL, we also investigated hepatic glycogen in **chapter 4**, as another substrate that may serve as a key factor in the development of NAFL. We tested our hypothesis that an overnight reduction in hepatic glycogen would become more prominent when prolonging the overnight fast by 6.5 hours, in volunteers with NAFL. To this end, two protocols were used: an early dinner with a subsequent long fast and a late dinner with shorter overnight fast. Surprisingly, we found no significant reduction in hepatic glycogen either in the short or prolonged overnight fast. This indicates that the use of hepatic glycogen is reduced in people with NAFL. In **chapter 5** we modulated IHL content by decreasing dietary glycemic index (GI) and dietary SFA content, while macronutrient composition was kept similar, and investigated whether changes in hepatic glycogen were underlying these differences in IHL content. Decreasing dietary GI and SFA content did

however not affect hepatic glycogen levels in the morning after an overnight fast. Possibly, differences in glycogen levels that might have been present over the day were masked by the predominating effect of the overnight fast on reducing glycogen levels. From our studies it appears that in NAFL hepatic SFA fraction is increased and hepatic glycogen cycling is reduced. Reduced hepatic glycogen cycling could be a mechanism underlying the development of NAFL and increased saturation of hepatic fat, mediated through increased rates of DNL. A link between hepatic glycogen content and DNL has been suggested before. Overfeeding studies with carbohydrates show that carbohydrates are first used to replenish glycogen stores and when these glycogen stores have reached their maximal capacity DNL and carbohydrate oxidation are increased (30). Other indications can be found in GSD1a patients, which are characterised by increased rates of DNL as glycogen breakdown is hampered by a defect in glucose-6-phosphatase (27) and we show in **chapter 3** that these patients also have an increased hepatic SFA fraction. This connection between hepatic glycogen stores and DNL is also apparent in mice unable to synthesize glycogen, shuttling carbohydrates to the DNL pathway instead and end up with hepatic steatosis (31). Apart from a link to DNL, hepatic glycogen may also influence fat oxidation. Whole-body fat oxidation on a high-fat diet has been shown to be increased when glycogen levels are lowered by exhaustive exercise, as compared to a high-fat diet without preceding glycogen-lowering exercise (32). In **chapter 4** we show that during overnight fasting whole-body fat oxidation did not increase in individuals with NAFL, in line with the absence of fluctuations in hepatic glycogen. Such metabolic inflexibility has earlier also been shown in volunteers with pre-diabetes (33). Thus, a decreased cycling of hepatic glycogen as indicated by the results of **chapter 4** could promote DNL and reduce the switch to fat oxidation, both contributing to increased storage of hepatic fat, that will be relatively high in SFA. Furthermore, in **chapter 5** we showed that even in a healthy overweight population, not defined as prediabetic or having NAFL, the shift from carbohydrate to fat oxidation that is seen in healthy individuals does not take place during the night. This suggests that the inertia in metabolic flexibility takes place early in the development of metabolic disease, and therefore can be an essential target for interventions.

What are the metabolic consequences of altered substrate metabolism in NAFL?

As we have shown that hepatic SFA is increased in NAFL, possibly influenced by reduced fluctuations in hepatic glycogen, the question arises what the metabolic consequences of increased hepatic SFA are. Therefore, we investigated in **chapter 3** whether liver fat composition correlates to hepatic insulin sensitivity. We showed that the hepatic SFA fraction was negatively related to hepatic insulin sensitivity and that this correlation was

stronger than for total IHL. This suggests that it is the type of fat that is of great importance in determining hepatic insulin sensitivity, rather than the total IHL content. As we have also shown in **chapter 3** that the hepatic SFA fraction is a reflection of DNL and it has been shown before that also DNL is associated with hepatic insulin resistance (34), the question arises whether hepatic insulin resistance is due to the process of DNL or whether the accumulation of SFA *per se* causes hepatic insulin resistance. Diets high in SFA have been associated with decreased metabolic health before (35-37). Reducing the amount of dietary SFA may therefore have a therapeutic effect on metabolic health, as we showed in **chapter 5** by improvements in IHL content and glycemic response. The proposed question is of clinical relevance, as it will give novel insights towards the best strategy for prevention and treatment of hepatic insulin resistance. Next to interventions targeting DNL, such as low carbohydrate, low fructose and low GI diets or drugs such as ACC inhibitors, diets targeting liver fat saturation directly could be effective. Indeed, increased desaturation following lipogenesis is believed to be a rescue mechanism to reduce the negative effects exerted by SFA (11). Thus, use of drugs targeting liver fat saturation might be an effective strategy to improve metabolic health in people with NAFL and T2D. It is well-known that hepatic desaturases are under the control of PPAR and therefore, PPARs may be a potential drug target. PPAR α/δ stimulation has previously been shown to improve fasting plasma glucose, whole-body insulin sensitivity and especially hepatic insulin sensitivity (38, 39). Strikingly, these effects seem to be independent of reductions in liver fat *per se* and thus PPARs may improve metabolic health and specifically insulin resistance via decreased liver fat saturation.

Future perspectives

Using our novel MRS methodology, we showed that the hepatic SFA fraction is a reflection of DNL and that specifically this fraction may hamper hepatic insulin sensitivity (**chapter 3**). It is however important to keep in mind that these results are only associative and future research will have to show whether decreasing the amount of SFA in the liver has beneficial effects on metabolic health. Studies targeting hepatic fat saturation by diets or drugs could provide an answer to the question whether the relationship between hepatic SFA and hepatic insulin sensitivity is causal. If so, the use of PPAR agonists might prove to be an effective strategy to decrease hepatic fat saturation and thereby improve hepatic insulin sensitivity. These strategies, targeting hepatic SFA, will need to be investigated in the future. They are however only effective if the negative metabolic effects are mediated by hepatic SFA *per se*. In case increased hepatic SFA would merely be a marker of increased DNL and the process of DNL seems to be mediating the observed negative effects on hepatic insulin

sensitivity, interventions should specifically target DNL instead. To answer this pivotal question, the metabolic effects of interventions targeting DNL and hepatic SFA *per se* need to be compared. This can be done by dietary means, using a high fructose diet targeting DNL versus a high SFA diet increasing the hepatic SFA fraction *per se*.

Another interesting finding in this thesis that requires more investigation is the lack of change in hepatic glycogen upon prolonged overnight fasting in people with NAFL (**chapter 4**). This needs to be investigated in more detail to determine whether stimulating hepatic glycogen cycling is beneficial. It could be that specifically in NAFL, fluctuations in glycogen are reduced and that this is underlying the excessive storage of hepatic fat. It would therefore first of all be interesting to study how hepatic glycogen levels respond in healthy individuals without NAFL when following a similar prolonged fasting intervention as used in **chapter 4**. Furthermore, measuring gluconeogenesis and gluconeogenic substrate availability in healthy volunteers and volunteers with NAFL may help us to understand the complex metabolic interplay in NAFL. Increased hepatic gluconeogenesis, that could be driven by blunted postprandial inhibition of lipolysis, might be used as a source for hepatic glucose and thereby prevent the need for the use of hepatic glycogen overnight. To investigate whether hepatic gluconeogenesis is already elevated in the early fed-to-fasting transition period and whether this results in a blunted decline in hepatic glycogen overnight, hepatic gluconeogenesis and hepatic glycogen stores could be measured in healthy volunteers and volunteers with NAFL in the late evening and in the morning.

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Addendum

IMPACT

What is the main aim of the research described in this thesis and what are the most important results and conclusions?

The aim of this thesis is to investigate how fat and glucose metabolism are changed in non-alcoholic fatty liver (NAFL) and how such changes contribute to impaired health. Specifically, this thesis focusses on liver fat, with special attention for the composition of the fat, and hepatic glycogen. Fats in the liver are composed of glycerol and fatty acids and the fatty acids can be classified based on the number of double bonds in saturated, mono-unsaturated and poly-unsaturated fatty acids. In general, saturated fatty acids are believed to be harmful to your health. Glycogen is the stored form of glucose and the liver is an important organ for this storage. This stored form of glucose can be used as energy source by the body when needed, for example during fasting when the body is not getting glucose from food. Liver glycogen could play an important role in determining whether body fat will be used or stored. In this thesis, liver fat content and composition and liver glycogen are determined by magnetic resonance spectroscopy methodologies. Magnetic resonance spectroscopy is a non-invasive technique that can be used to study the metabolism of organs such as the liver, without the need for taking biopsies.

In **chapter 2** the available methods to study the different routes that contribute to storage and disposal of fat in the liver were reviewed. It appeared that most studies are using techniques that require specific expertise and are costly. Therefore, the number of studies investigating the importance of these storage and disposal routes in the development of NAFL are limited, and even less is known about how diet can influence these routes.

One of the routes that can contribute to NAFL is the formation of fat from carbohydrates, called *de novo* lipogenesis. This newly formed fat is mainly saturated fat. In **chapter 3,** a new magnetic resonance spectroscopy technique was set-up to measure the fraction of the saturated, mono-unsaturated and poly-unsaturated fat in the liver. Once it was possible to measure these fat fractions in the liver, the relationship with *de novo* lipogenesis and liver insulin sensitivity could be studied. Liver insulin sensitivity indicates how well the liver responds to the hormone insulin, which is essential in maintaining normal blood sugar levels. It was shown that the higher *de novo* lipogenesis, the larger the fraction of saturated fat in the liver. Also, it was shown that in people with a large fraction of saturated fat in the liver, the sensitivity for insulin was reduced. A next step will be to investigate whether these relationships are causal. For example, does insulin sensitivity improve when we lower the amount of saturated fat in the liver by medication or diet?

In **chapter 4**, it was investigated if extending the overnight fast by 6.5 hours has beneficial effects on the health of volunteers with NAFL. This was done by eating the last meal of the day at 4.30 pm and comparing this to eating the last meal of the day at 11pm. Extending fasting time for a few hours each day has been shown before to have beneficial health effects. This could be due to fluctuations in liver glycogen. From our results, it appeared that people with NAFL are not sensitive to an extended overnight fast as liver glycogen did not change. Normally, fat oxidation is increased with fasting, but also this we did not see in these volunteers with NAFL. Furthermore, when the volunteers continued to extend their overnight fast for 5 days, no effects were seen on the amount and composition of liver fat. But why is the effect of fasting disturbed in people with NAFL? New research has to be performed to answer this question, which can lead to novel insights to improve health.

Another possible way to improve health and liver fat storage is by diet. There are indications that the amount of saturated fat in the diet and the glycemic index of the diet can play an important role. The glycemic index (GI) indicates how quickly food can increase blood glucose levels; the higher the GI of food, the quicker the blood glucose level increases. In **chapter 5** it was tested in overweight and obese volunteers whether a two-week diet low in saturated fat and GI would reduce liver fat storage when compared to a two-week diet high in saturated fat and GI. Indeed, it appeared that by only reducing the saturated fat and GI content of the diet for 2 weeks, liver fat storage decreased.

In addition to these results, it was shown in **chapter 6** that factors related to the way of measuring liver fat content with magnetic resonance spectroscopy and the calculations used can have a big influence on the amount of liver fat content that is measured. In **chapter 7**, a supplement, nicotinamide riboside, was used to improve health of overweight and obese people using some of the techniques that were also applied in abovementioned studies. It appeared that some health aspects, such as body composition, were improved, but that liver fat content and insulin sensitivity were unchanged.

What is the contribution of the results to science and societal challenges?

Obesity rates are extremely high worldwide and NAFL is frequently present in people with obesity. NAFL is the most common cause of chronic liver disease and can progress to more severe liver disease, but is also often accompanied by type 2 diabetes and cardiovascular disease. At present, knowledge about liver metabolism in people with NAFL and the health consequences of disturbed liver metabolism are limited.

The results of the research described in this thesis contribute to our understanding of human liver metabolism and its importance for human health. Based on the results of this

research new targets can be found that are important for improving health. Future studies can extend the findings presented in this thesis by investigating if lowering saturated fat in the liver and promoting fluctuations in liver glycogen by diet, exercise or medication can improve health of people with NAFL and related diseases, such as type 2 diabetes. Also, lowering dietary saturated fat and GI could be implemented by food industry, health care professionals, and government agencies in order to prevent the development of NAFL or treat NAFL and related diseases. Ultimately, this will contribute to reducing health care costs and relieving the pressure on the health care system.

For whom are the results interesting and of relevance?

The results and conclusions presented in this thesis are interesting for other researchers, who can set-up new studies further investigating the exact role of liver glycogen, saturated fat and *de novo* lipogenesis in NAFL and how these factors contribute to the development of NAFL related diseases. These studies can make use of the techniques described in this thesis, specifically the technique to specifically determine saturated fatty acids in the liver is new and enables us and other researchers in the field to perform follow-up studies. Ultimately, this knowledge could help in the prevention and treatment of NAFL and related diseases. In terms of prevention, this knowledge would be of interest for people with overweight and obesity, as these are at increased risk for developing NAFL. Specific lifestyle advice, such as minimizing dietary saturated fat and GI intake, may help this group at risk to keep liver fat content low and composition beneficial. Improving the quality of food products by the food industry in terms of saturated fat and GI could also aid in the prevention of fatty liver development. Next to this, knowledge from future studies building on our results can lead to the development of new drugs for the treatment of NAFL, thereby reducing the risk for progressed liver disease and the development of related diseases such as type 2 diabetes in people with NAFL.

Other researchers will be informed about the results described in this thesis through publications in scientific journals and presentations at national and international conferences. Results will also be shared on websites, social media and participant information events, thereby informing the people at risk for the development of NAFL and the people with NAFL or related diseases.

SUMMARY

Non-alcoholic fatty liver (NAFL) is the leading cause of chronic liver disease and highly prevalent in obesity. NAFL is defined as intrahepatic lipids (IHL) exceeding 5% of liver weight, in absence of excessive alcohol consumption. NAFL can progress to more severe stages of liver disease, such as non-alcoholic steatohepatitis (NASH) and liver cirrhosis, conditions in which liver function is severely hampered and the risk for hepatocellular carcinoma is increased. Importantly, NAFL *per se* is already very strongly associated with metabolic diseases such as cardiovascular disease (CVD) and type II diabetes (T2D). Knowledge on the metabolic changes that take place during the development of NAFL and how NAFL contributes to cardiometabolic disease is limited, particularly because of the limitations in techniques available. Thus, advanced methodologies are needed to investigate metabolic processes in NAFL. Magnetic resonance spectroscopy (MRS) can be applied to acquire chemical information of tissues, thereby making it a useful technique to study hepatic substrate stores. The research presented in this thesis describes the investigation of liver substrate metabolism in NAFL using advanced MR methodology.

While the pathways determining IHL content have long been identified (direct fat storage from a meal, *de novo* lipogenesis (DNL), uptake of plasma non-esterified fatty acids (NEFA), mitochondrial fatty acid oxidation, and secretion into the circulations within VLDL-particles), knowledge on the contribution of each of these pathways to IHL content in humans is sparse. Such knowledge is however essential in order to develop strategies to prevent and treat NAFL. Therefore, in **chapter 2** we reviewed the techniques available to study the different pathways leading to IHL accumulation and the studies using these techniques to estimate the relative contribution of the different pathways or to study dietary modulation of these pathways.

Increased DNL may be one of the underlying pathways leading to NAFL. The end product of DNL are saturated fatty acids (SFA), and therefore high DNL rates might contribute to an increased hepatic SFA fraction. It is suggested that specifically these SFA, negatively influence metabolic health. Therefore, in **chapter 3** a novel MR technique was developed, validated and applied that enabled to non-invasively quantify the fractions of hepatic SFA, mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) separately, in healthy and metabolically compromised human volunteers. Using this methodology, we showed in **chapter 3** that DNL is positively associated with hepatic SFA fraction and is elevated in patients with NAFL and T2D. Furthermore, we showed that hepatic SFA fraction is strongly and negatively correlated with hepatic insulin sensitivity. These results suggest

that hepatic SFA fraction is reflecting DNL and suggest that specifically the hepatic SFA fraction may hamper hepatic insulin sensitivity.

Another key factor in hepatic substrate metabolism could be hepatic glycogen stores. Hepatic glycogen is thought to change dynamically, decreasing during fasting and being replenished upon feeding. It is suggested that the regular depletion of hepatic glycogen stores is underlying the beneficial effects seen with time restricting eating regimes. To study the importance of hepatic glycogen stores in NAFL, prolonged overnight fasting was used as a tool to modulate hepatic glycogen in volunteers with NAFL in **chapter 4**. We investigate if extending fasting time from 9.5h after the last meal to 16h after the last meal would acutely lower hepatic glycogen by using ^{13}C-MRS and thereby improve substrate oxidation in volunteers with NAFL. We showed that prolonging the overnight fast does not affect hepatic glycogen content. Interestingly, a prolonged fast of 16h did not result in higher fat oxidation rates during the night either, suggesting that these volunteers with NAFL did not fully reach a fasting state. Furthermore, prolonging the overnight fast for five days did not improve IHL content or composition. Altogether, these results suggest that hepatic substrate metabolism is not sensitive to extending acute fasting periods in individuals with NAFL.

A healthy diet is a cornerstone in the prevention and treatment of obesity and related metabolic conditions such as NAFL. Importantly, not only the amount of dietary fat and carbohydrate, but also their quality seems to be of significance. Specifically, high amounts of dietary SFA and carbohydrate-rich foods with a high glycemic index (GI), are thought to increase IHL, but human data is yet limited to proof of principle studies with exaggerated differences in diet composition. In **chapter 5,** it was investigated whether a two-week low vs. high GI/SFA diet, with similar macronutrient composition, reduces liver fat content and whether this is paralleled by increased whole-body fat oxidation, decreased glycemic response and reduced hepatic glycogen levels. To this end, overweight/obese volunteers participated in a randomized cross-over trial, following a two-week high GI/SFA diet and a two-week low GI/SFA diet with a wash-out period of 4 weeks. We showed that IHL was lower after a two-week low vs. high GI/SFA diet. Furthermore, the glycemic response tended to be reduced after the low vs. high GI/SFA diet and fat oxidation was increased early after the meal in the low vs. high GI/SFA diet. Hepatic glycogen content in the morning and whole-body substrate oxidation during the night were similar between the two interventions. These results indicate that realistic reductions in both GI and SFA content beneficially affect IHL in overweight/obese subjects.

Quantification of IHL could be influenced by different factors and it is important to understand how these can bias IHL quantification. In **chapter 6** it was evaluated how variations in T2 relaxation times affect the calculations of absolute IHL% (w/w) and to what extend the bias occurs while using different IHL quantification formulas. We showed that in individuals with different levels of steatosis, using fixed average T2 correction factors lead to an overestimation of IHL content in people with high IHL levels. However, by using STEAM sequence with short echo time the bias in IHL content can be minimized. Additionally, it was shown that the formula to express IHL is important, as it can introduce bias in individuals with high IHL levels.

High prevalence of overweight and obesity put large population groups at risk for NAFL and metabolic disease. Results from animal studies suggest that nicotamine riboside (NR), as an NAD+ precursor, can have metabolically beneficial effects, but human data is largely lacking. In **chapter 7,** the metabolic effect of six-week NR supplementation was assessed. We showed that NR supplementation increases skeletal muscle NAD+ metabolites, affects skeletal muscle acetylcarnitine metabolism, and induces minor changes in body composition and sleeping metabolic rate. No effects were observed in other measurements of metabolic health, including IHL stores and insulin sensitivity.

Overall, the studies described in this thesis investigated liver substrate metabolism in NAFL using advanced MR methodology, specifically focusing on the role of IHL composition and hepatic glycogen stores. From our studies it appears that in NAFL, hepatic SFA fraction is increased and hepatic glycogen cycling is reduced and furthermore, that high hepatic SFA fractions may hamper hepatic insulin sensitivity. Therefore, the hepatic SFA fraction and hepatic glycogen cycling could be interesting novel targets in the prevention and treatment of NAFL and other metabolic diseases.

SAMENVATTING

Niet-alcoholische vette lever (NAFL) is de belangrijkste oorzaak van chronische leverziekte en komt veel voor bij obesitas. NAFL wordt gedefinieerd als intrahepatische lipiden (IHL) van meer dan 5% van het levergewicht, zonder aanwezigheid van overmatig alcoholgebruik. NAFL kan ontwikkelen naar ernstigere stadia van leverziekte, zoals niet-alcoholische steatohepatitis (NASH) en levercirrose, aandoeningen waarbij de leverfunctie ernstig wordt belemmerd en het risico op hepatocellulair carcinoom toeneemt. Belangrijk is dat NAFL op zich al zeer sterk geassocieerd is met metabole ziekten zoals hart- en vaatziekten (CVD) en type II-diabetes (T2D). De kennis over de metabole veranderingen die plaatsvinden tijdens de ontwikkeling van NAFL en hoe NAFL bijdraagt tot cardiometabole ziekten is beperkt, vooral door beperkingen in de beschikbare technieken. Daarom zijn geavanceerde methodologieën nodig om metabole processen in NAFL te onderzoeken. Magnetische resonantie spectroscopie (MRS) kan worden toegepast om chemische informatie van weefsels te verkrijgen, waardoor het een bruikbare techniek is om substraatvoorraden in de lever te bestuderen. In dit proefschrift wordt het onderzoek naar het substraatmetabolisme in de lever bij NAFL met behulp van geavanceerde MR-methodologie beschreven.

Hoewel de routes die het IHL-gehalte bepalen al lang zijn geïdentificeerd (directe vetopslag na de maaltijd, *de novo* lipogenese (DNL), opname van vrije vetzuren (NEFA), mitochondriale vetoxidatie, en secretie in de circulatie in VLDL-deeltjes), is de kennis over de bijdrage van elk van deze routes aan het IHL-gehalte in de mens schaars. Dergelijke kennis is echter essentieel voor het ontwikkelen van strategieën om NAFL te voorkomen en te behandelen. Daarom hebben we in **hoofdstuk 2** een overzicht gegeven van de technieken die beschikbaar zijn om de verschillende routes die leiden tot IHL ophoping te bestuderen en van de studies die deze technieken gebruiken om de relatieve bijdrage van de verschillende routes te schatten of om de modulatie van deze routes door voeding te bestuderen.

Verhoogde DNL kan een van de onderliggende pathways zijn die tot NAFL leidt. Het eindproduct van DNL zijn verzadigde vetzuren (SFA), en daarom zouden hoge DNL-waarden kunnen bijdragen aan een verhoogde SFA fractie in de lever. Er wordt gesuggereerd dat juist deze SFA, een negatieve invloed hebben op de metabole gezondheid. Daarom werd in **hoofdstuk 3** een nieuwe MR techniek ontwikkeld, gevalideerd en toegepast die het mogelijk maakt om op een niet-invasieve manier de fracties van SFA, enkelvoudig onverzadigde vetzuren (MUFA) en meervoudig onverzadigde vetzuren (PUFA) afzonderlijk in de lever te kwantificeren, in gezonde en metabool gecompromitteerde vrijwilligers. Met behulp van

deze methodologie hebben wij in **hoofdstuk 3** aangetoond dat DNL positief geassocieerd is met de lever SFA fractie en verhoogd is bij patiënten met NAFL en T2D. Verder hebben we aangetoond dat de lever SFA fractie sterk en negatief gecorreleerd is met insulinegevoeligheid van de lever. Deze resultaten suggereren dat de lever SFA fractie een afspiegeling is van DNL en suggereren dat specifiek de lever SFA fractie de insulinegevoeligheid van de lever kan belemmeren.

Een andere belangrijke factor in het levermetabolisme zou de leverglycogeenvoorraad kunnen zijn. Aangenomen wordt dat leverglycogeen dynamisch verandert, afneemt tijdens het vasten en weer wordt aangevuld bij het eten. Er wordt gesuggereerd dat de regelmatige uitputting van de leverglycogeenvoorraden ten grondslag ligt aan de gunstige effecten die worden waargenomen bij 'time-restricted eating'. Om het belang van leverglycogeenvoorraden in NAFL te bestuderen, is in **hoofdstuk 4** langdurig vasten gedurende de nacht gebruikt als een middel om leverglycogeen in vrijwilligers met NAFL te moduleren. We hebben met behulp van ^{13}C-MRS onderzocht of het verlengen van de vastentijd van 9.5 uur na de laatste maaltijd tot 16 uur na de laatste maaltijd het leverglycogeen acuut zou verlagen en daarmee de substraatoxidatie zou verbeteren in vrijwilligers met NAFL. Wij toonden aan dat het verlengen van de gevaste tijd geen invloed heeft op het leverglycogeengehalte. Interessant is dat het verlengde vasten van 16 uur ook niet resulteerde in een hogere vetoxidatie gedurende de nacht, wat suggereert dat deze vrijwilligers met NAFL geen volledig gevaste toestand bereikten. Bovendien leidde het verlengd nachtelijke vasten gedurende vijf dagen niet tot een verbetering van het gehalte of de samenstelling van het IHL. Al met al suggereren deze resultaten dat het levermetabolisme niet gevoelig is voor het verlengen van de acute vastenperiode bij mensen met NAFL.

Een gezond dieet is een belangrijke pijler in de preventie en behandeling van obesitas en gerelateerde metabole aandoeningen zoals NAFL. Niet alleen de hoeveelheid vetten en koolhydraten in de voeding, maar ook de kwaliteit ervan lijkt belangrijk te zijn. Hoge hoeveelheden SFA in de voeding en koolhydraatrijke voedingsmiddelen met een hoge glycemische index (GI), zouden de IHL verhogen, maar humane data zijn nog beperkt tot 'proof of principle' studies met overdreven verschillen in voedingssamenstelling. In **hoofdstuk 5** werd onderzocht of een twee weken durend laag vs. hoog GI/SFA dieet, met vergelijkbare macronutriënt samenstelling, het vetgehalte in de lever verlaagt en of dit gepaard gaat met een verhoogde vetoxidatie, een verlaagde glycemische respons en verlaagde leverglycogeenniveaus. Hiertoe namen vrijwilligers met overgewicht of obesitas deel aan een gerandomiseerde cross-over studie, waarbij ze twee weken een hoog GI/SFA dieet volgden en twee weken een laag GI/SFA dieet met een wash-out periode van 4 weken.

Wij toonden aan dat het IHL lager was na een twee weken durend laag versus hoog GI/SFA dieet. Bovendien was de glycemische respons lager na het lage vs. hoge GI/SFA dieet en was de vetoxidatie kort na de maaltijd verhoogd bij het lage vs. hoge GI/SFA dieet. Het hepatische glycogeengehalte in de ochtend en de substraatoxidatie gedurende de nacht waren vergelijkbaar tussen de twee interventies. Deze resultaten wijzen erop dat realistische verlagingen van zowel de GI als de hoeveelheid SFA een gunstig effect hebben op het IHL bij mensen met overgewicht of obesitas.

Kwantificering van het IHL kan beïnvloed worden door verschillende factoren en het is belangrijk om te begrijpen hoe deze de IHL kwantificering kunnen vertekenen. In **hoofdstuk 6** is onderzocht hoe variaties in T2 relaxatietijden de berekeningen van het absolute IHL% (w/w) beïnvloeden en in hoeverre de bias optreedt bij gebruik van verschillende IHL kwantificeringsformules. We toonden aan dat bij personen met verschillende niveaus van steatose het gebruik van vaste gemiddelde T2-correctiefactoren leidt tot een overschatting van het IHL-gehalte bij personen met een hoog IHL-gehalte. Door gebruik te maken van een STEAM sequentie met korte echotijd kan de bias in het IHL gehalte echter geminimaliseerd worden. Bovendien werd aangetoond dat de formule om IHL uit te drukken belangrijk is, aangezien deze een bias kan introduceren bij personen met een hoog IHL gehalte.

Door de hoge prevalentie van overgewicht en obesitas loopt een groot deel van de bevolking een risico op NAFL en metabole ziekten. Resultaten van dierstudies suggereren dat nicotamine riboside (NR), als een NAD+ precursor, metabool gunstige effecten kan hebben, maar informatie over de effecten in de mens ontbreken grotendeels. In **hoofdstuk 7** werd het metabole effect van zes weken NR suppletie onderzocht. We toonden aan dat NR-suppletie de NAD+ metabolieten in de skeletspieren verhoogt, het acetylcarnitine metabolisme in de skeletspieren beïnvloedt, en kleine veranderingen in lichaamssamenstelling en slaapmetabolisme induceert. Er werden geen effecten waargenomen in andere metingen van metabole gezondheid, waaronder IHL-voorraden en insulinegevoeligheid.

In het algemeen hebben de in dit proefschrift beschreven studies het substraatmetabolisme van de lever bij NAFL onderzocht met behulp van geavanceerde MR-methodologie, waarbij de nadruk lag op de rol van de IHL-samenstelling en de leverglycogeenvoorraden. Uit onze studies blijkt dat bij NAFL de SFA fractie in de lever verhoogd en de leverglycogeencyclus verstoord is, en bovendien dat hoge SFA leverfracties de leverinsulinegevoeligheid kunnen belemmeren. Daarom zouden de lever SFA fractie en het lever glycogeen interessante nieuwe doelen kunnen zijn in de preventie en behandeling van NAFL en andere metabole ziekten.

ABOUT THE AUTHOR

Kay Roumans was born on January 14th 1992 in Meerssen, the Netherlands. After finishing secondary education at Stella Maris Meerssen in 2010, Kay started his study Biomedical sciences at Maastricht University. During his master he performed internships at the department of Human Biology, investigating the effect of bile acid supplementation on brown adipose tissue activity and energy expenditure, and studying the relation between skeletal muscle mitochondrial function and hepatic lipid accumulation in people with compromised metabolic health. In 2015, he completed his master's in biomedical sciences, with a specialization in Nutrition and Metabolism. In 2016, he started his PhD at Maastricht University at the department of Nutrition and Movement Sciences under supervision of prof. dr. Patrick Schrauwen, dr. Vera Schrauwen-Hinderling and dr. ir. Lucas Lindeboom. During his PhD he worked on the topic of substrate metabolism in people with NAFL within a TKI/Health Holland consortium with partners from Leiden University Medical Center and Unilever R&D. In this human research he applied both invasive techniques, such as hyperinsulinemic-euglycemic clamps, and non-invasive techniques, such as Magnetic Resonance Spectroscopy. Kay will continue his career as a postdoctoral researcher at the department of Nutrition and Movement Sciences and will perform a human intervention study to further unravel the role of *de novo* lipogenesis and hepatic saturated fatty acids in insulin resistance.

LIST OF PUBLICATIONS

1. **K.H.M. Roumans***, L. Lindeboom*, P. Veeraiah, C.M.E. Remie, E. Phielix, B. Havekes, Y.M.H. Bruls, M.C.G.J. Brouwers, M. Ståhlman, M. Alssema, H.P.F. Peters, R. de Mutsert, B. Staels, M-R. Taskinen, J. Borén, P. Schrauwen, V. B. Schrauwen-Hinderling. Hepatic saturated fatty acid fraction is associated with *de novo* lipogenesis and hepatic insulin resistance. Nat. Comm. 2020 Apr 20;11(1):1891. doi: 10.1038/s41467-020-15684-0.

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