

**UNIVERSITA' DEGLI STUDI DI NAPOLI
"FEDERICO II"**

SCUOLA DI MEDICINA E CHIRURGIA



Dipartimento di Medicina Clinica e Chirurgia

**DOTTORATO DI RICERCA IN TERAPIE AVANZATE
BIOMEDICHE E CHIRURGICHE – 31° Ciclo**

Direttore: Prof. Giovanni Di Minno

TESI DI DOTTORATO

**"EFFECTS OF DIFFERENT DIETARY INTERVENTIONS ON
NON-ALCOHOLIC FATTY LIVER DISEASE."**

RELATORE

Ch. mo Prof.
Gabriele RICCARDI

CANDIDATO

Dott.
Giuseppe DELLA PEPA

ANNO ACCADEMICO 2017-2018

SUMMARY

1. GENERAL INTRODUCTION AND OUTLINE OF THE THESIS	1
2. OVERVIEW ON NON-ALCOHOLIC FATTY LIVER DISEASE	4
2.1. NAFLD definition	4
2.2. NAFLD and cardiometabolic risk	4
2.3. Pathogenesis of NAFLD	5
2.4. Diagnosis of NAFLD	9
2.5. Management of NAFLD: hypocaloric diet and physical activity	10
3. DIETARY INTERVENTIONS AND NON-ALCOHOLIC FATTY LIVER DISEASE IN ISOCALORIC CONDITIONS	12
3.1. Dietary fatty acids	12
3.1.1. Saturated fatty acids	12
3.1.2. Monounsaturated and n-6 polyunsaturated fatty acids	13
3.1.3. n-3 polyunsaturated fatty acids	14
3.2. Carbohydrates	15
3.2.1. Low glycemic-index carbohydrate and fibre rich diets	15
3.2.2. Fructose/other simple sugars	23
3.3. Proteins	24
3.4. Other dietary components	25
3.4.1. Polyphenols	25
3.4.2. Vitamin E	28
3.4.3. Vitamin C	30
3.4.4. Vitamin D	32
4. AIM AND PERSONAL RESEARCH AREAS	35
5. CLINICAL TRIALS	36
5.1. ETHERPATH COHORT	36
5.2. FRUCTOSE TRIAL	50
5.3. MEDEA TRIAL	74
6. CONCLUSIONS	88
7. REFERENCES	90

1. GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

For its critical position between the systemic circulation and the blood flow of the gastrointestinal tract – mediated by the portal vein – the liver plays an essential role in the intermediary metabolism, transforming dietary nutrients into the major chemical elements crucial for life and human health. Conversely, many nutrients and the overall dietary composition can influence liver function. In fact, an excessive intake of refined carbohydrate and saturated fats, an increased consumption of fructose and other simple sugars, and the spread of high-calorie Western diets have been associated with a dramatic increase in overweight/obesity and insulin resistance and, more recently, also with non-alcoholic fatty liver disease (NAFLD) [1]. Noteworthy, the excess of adiposity, in particular abdominal adiposity, and insulin resistance are the major contributors to the development of several cardiometabolic abnormalities strictly related to the increased risk of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). Interestingly, it is important to underline that the most recent evidence from longitudinal studies suggests that NAFLD itself can be considered as an independent cardiometabolic risk factor beyond the classical cardiometabolic risk factors [2]. Currently, lifestyle interventions – including strategies to reduce body weight and to increase regular physical activity – represent the mainstay of NAFLD management. Total caloric intake plays a very important role in the development of NAFLD; however, apart from the caloric restriction alone, modifying the quality of the diet – modulating either the macro or micronutrient composition – can also markedly affect the clinical evolution of NAFLD, offering a more realistic and feasible treatment alternative.

Recently, considerable interest has been placed on the possible beneficial effect of some dietary components on NAFLD. Generally, hypercaloric diets, especially rich in trans/saturated fat and cholesterol, high consumption of red and processed meat, and fructose-sweetened beverages seem to increase the risk of NAFLD development, whereas a high consumption of foods or beverages rich in bioactive compounds of plant origin, such as whole grains, legumes, fruits, vegetables, nuts, coffee and tea, have shown preventive and therapeutic effects on NAFLD. The relationship between the intake of some dietary components and the improvement in NAFLD could be related, at least in part, to some bioactive compounds able to improve glucose and lipid metabolism, insulin resistance, hepatic fat content, sub-clinical inflammatory status, and oxidative stress.

Consequently, it is conceivable that the combination of these foods in a dietary model such as a "Portfolio diet", inspired to a Mediterranean Diet model in which more beneficial dietary component are included – i.e. low glycemic index (GI) carbohydrates, vegetable fiber, monounsaturated fatty acids (MUFA), n-6 and n-3 polyunsaturated fatty acids (PUFA), polyphenols, and vitamins – could maximize the impact on prevention and treatment of NAFLD.

In this scenario, it is important to define which dietary components are able to prevent and treat NAFLD to design dietary interventions to test. Therefore, the present project was designed to assess: (1) the relationships between liver fat content and metabolic, inflammatory and nutritional factors in a homogeneous cohort of individuals at high cardio-metabolic risk; (2) the effects of fructose intake on liver fat content and other cardiometabolic risk factors in a large cohort

of abdominally obese men; (3) the effects of a Portfolio diet versus an isoenergetic diet rich in MUFA on liver fat content in patients with T2DM.

2. OVERVIEW ON NON-ALCOHOLIC FATTY LIVER DISEASE

2.1. NAFLD definition

NAFLD is characterized by an excessive accumulation of lipids in >5% of hepatocytes, primarily in the form of triglycerides, in the absence of a considerable alcohol ingestion (ethanol intake ≤ 30 g/day for men and ≤ 20 g/day for women), and ruling out other causes of liver injury, i.e. hepatitis B/C virus infection, drugs assumption or environmental toxins, genetic or metabolic diseases, extrahepatic or nutritional conditions [3]. The term NAFLD incorporates an extensive spectrum of histologic liver abnormalities, varying from simple triglyceride accumulation in hepatocytes – non-alcoholic fatty liver (NAFL) or steatosis – to non-alcoholic steatohepatitis (NASH), characterized by the additional presence of inflammation and tissue injury. NASH can evolve to fibrosis, which can lead to cirrhosis and hepatocellular carcinoma [4].

2.2. NAFLD and cardiometabolic risk

NAFLD is the most common chronic liver disease in the industrialized world with a 15–30% prevalence reported in the general population [5]. Interestingly, based on the diagnostic criteria utilized and the clinical characteristics of the different populations, its prevalence varies widely. In particular, the prevalence of NAFLD is very high in individuals at high cardiometabolic risk. Cardiometabolic risk refers to a condition associated with an increased risk of developing CVD and T2DM as a consequence of the presence of interrelated alterations in metabolic and vascular functions, as well as dyslipidemia, hypertension, abdominal obesity, insulin resistance and hyperglycemia. All these abnormalities identify the

metabolic syndrome; consequently, the close association between NAFLD and the metabolic syndrome is not surprising [6,7]. In line with these observations, the prevalence of NAFLD is approximately 50% in hypertensive subjects, 70% in people with T2DM, and up to 90% in severely obese patients [8-10]. Dramatically, NAFLD is also the most prevalent form of chronic liver disease in childhood and very recent data indicate that nearly 70-80% of obese children may have NAFLD [11]. Given the increasing prevalence of obesity and metabolic syndrome, NAFLD will become one of the most important public health challenges in the next decades for its related complications. In particular, it should be considered that simple NAFL can progress to NASH in about 20-25% of cases, and nearly 20% of patients with NASH can develop fibrosis and cirrhosis [12]; in patients with cirrhosis, the cumulative incidence of hepatocellular carcinoma ranges from 2.4% to 12.8% over 3-7 years [13]. Beyond the liver-related complications, it is important to underline that NAFLD is also an emerging risk factor for T2DM and CVD [14,15], and that it has recently been associated with an increased risk of chronic kidney disease [16].

2.3. Pathogenesis of NAFLD

The mechanisms involved in NAFLD development and progression are not completely clear. The hypothesis of the “two-hit” model – for the first time proposed by Day *et al.* in 1998 – in the pathogenesis of NAFLD has been accepted for about one decade [17]. According to this model, the “first hit” is characterized by the accumulation of lipids – primarily in the form of triglycerides derived from esterification of free fatty acids and glycerol – in the hepatocytes.

The increased triglyceride liver content can be considered as a form of ectopic lipid accumulation, a condition strongly related to the imbalance between influx, synthesis, β -oxidation and export of free fatty acids in the liver [18]. In particular, Donnelly *et al.* clearly observed that 59% of the triglycerides present in the liver of patients with NAFLD derived from free fatty acids released from adipose tissue, 26% from *de novo lipogenesis*, and 15% from dietary lipids [19]. Also important is the low rate of β -oxidation of free fatty acids and the reduction in triglyceride export by very low density lipoprotein particles in a liver with increased fat content [20]. Insulin resistance plays a pivotal role in the “first-hit” and in liver triglyceride accumulation increasing the free fatty acids release from adipose tissue, reducing the glucose uptake from the skeletal muscle and favoring the hepatic influx of these metabolites; furthermore, insulin resistance increases *de novo lipogenesis* and reduces the synthesis and secretion of very low density lipoprotein [21].

The increase in liver triglyceride content is strongly associated with hepatocyte susceptibility to the damage promoted by the “second hit”. The “second-hit” can be promoted by lipid peroxidation, oxidative stress, inflammatory cytokines, and mitochondrial dysfunction. All together, these factors induce steatohepatitis and can lead to fibrosis, which can evolve to cirrhosis [22].

In the last few years, based on a large body of knowledge, the hypothesis of the “two-hit” model has been translated into the “multiple-hit” model. In fact, it appears reasonable that the simple “two hit” mechanism is too reductive and inadequate to explain the complex mechanisms involved in NAFLD development and progression; furthermore, only a minority of patients with NAFLD progress to

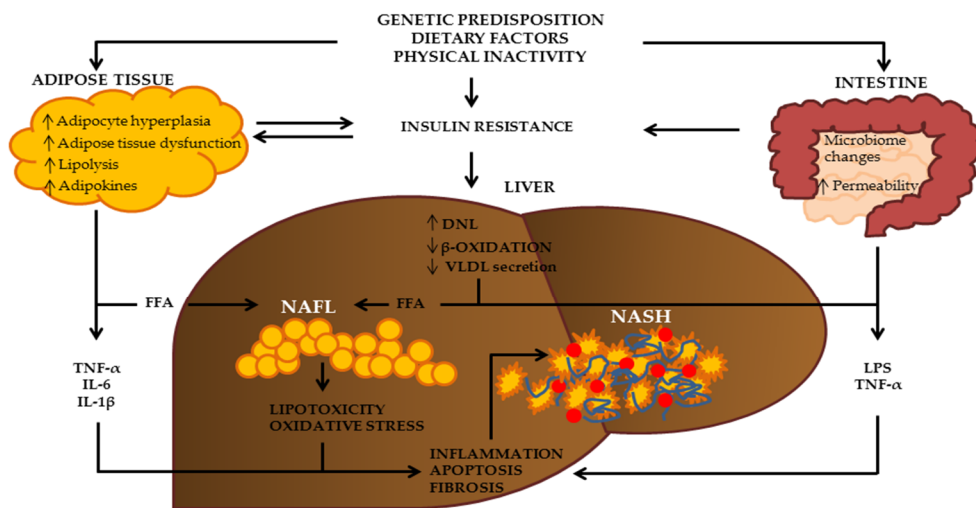
NASH or cirrhosis [1], and, on the other hand, steatohepatitis can be the initial alteration observed in the liver [23]. In fact, it has been observed that some patients with NASH have only a modest liver triglyceride content [23], suggesting that inflammation – in some conditions – can be the *primum movens* of the process.

The “multiple-hit” model provides a comprehensive model that takes into account the multiple factors and interactions involved in NAFLD [24]. Based on this model, dietary habits, insulin resistance, visceral adiposity, inflammatory state, oxidative stress, alteration in gut microbiome, and genetic predisposition, are all recognized risk factors for NAFLD development and progression.

In particular, the type of diet, other environmental factors and genetic predisposition play an important role in the development of insulin resistance, visceral obesity, and gut microbiome changes. Insulin resistance promotes steatosis with the mechanisms above discussed; adipose tissue is involved – beyond the free fatty acids efflux – in the production and secretion of the inflammatory cytokines and adipokines involved in NAFLD progression [25]. Changes in the gut microbiome related to dietary habits can influence energy homeostasis and systemic inflammation [24]; all these factors can aggravate oxidative stress and endoplasmic reticulum stress in hepatocytes, leading to hepatic inflammation [26]. Furthermore, genetic predisposition of single nucleotide polymorphisms in genes like Patatin-Like Phospholipase 3 (PNPLA3) or in Transmembrane 6 Superfamily Member 2 (TM6SF2) can aggravate liver injury [27], (**Figure 1**).

With respect to the strong relation between genetic predisposition and dietary habits, NAFLD represents an optimal example of disease by which nutrigenomics has allowed us to understand how nutrients can influence its development and progression by altering the expression of genes involved in inflammation, glucose and lipid metabolism [28].

Figure 1. Pathogenesis of NAFLD.



Based on the “multiple hit” model, dietary habits, insulin resistance, visceral adiposity, inflammatory state, oxidative stress, alteration in microbiome, and genetic predisposition, are all recognized risk factors for NAFLD. DNL: de novo lipogenesis; FFA: free fatty acids; IL-6: interleukin-6; IL-1 β : interleukin-1 β ; LPS: lipopolysaccharide; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; TNF- α : tumor necrosis factor- α .

Nutrigenomics focuses on identifying and understanding molecular interactions between nutrients/dietary bioactive compounds with the genome [29]. With regard to NAFLD, the PNPLA3 I148M polymorphism is a clear example of these possible interactions: individuals with the PNPLA3 I148M polymorphism are

more prone to develop steatosis when the intake of carbohydrates, in particular simple sugars, is elevated [30]. Briefly, PNPLA3 exerts a lipolytic activity on triglycerides and its up-regulation is mediated by carbohydrates [31]; in individuals with the PNPLA3 I148M polymorphism, high intake of carbohydrates induces the accumulation of the pathological protein – less able to hydrolyze the triglycerides – on the surface of lipid droplets and a consequent decreased secretion of triglyceride-rich lipoproteins from the liver [32]. Based on these observations individualized nutritional strategy takes into account also the genetic features of individuals may be more effective in the clinical management of NAFLD.

2.4. Diagnosis of NAFLD

Liver biopsy is still the *gold standard* for the diagnosis of NAFLD, and this invasive procedure – despite some limitation related to sampling variability and procedural potential risk – discerns simple NAFL from NASH [33]. However, in large population assessment or for disease monitoring, some non-invasive markers have been proposed. In terms of biochemical markers, it should be considered that serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are inaccurate markers of NAFLD and only moderately correlate with liver fat content [33].

For the identification of NAFL the best validated scores are represented by: the fatty liver index [34], the NAFLD liver fat score [35] and the Steato test [36], based on some biochemical markers and clinical parameters as liver enzymes, body mass index (BMI), waist circumference, presence of T2DM or metabolic

syndrome. In terms of instrumental evaluation, the first-step is represented by ultrasonography (US) [37,38], although it is limited by the possible interference of liver fibrosis on bright liver echo pattern and the very low sensitivity and specificity in individuals with BMI >40 kg/m². CT presents similar accuracy for NAFLD as US; however, it is limited by radiation exposure [33]. The proton magnetic resonance spectroscopy (¹H-MRS) can reveal a liver fat content as low as 1%, but it is limited by its high cost [33]. In terms of biochemical markers, cytokeratin-18 (CK-18) fragments, derived from hepatocytes apoptosis or death, are only modestly accurate; therefore, for the diagnosis of NASH, liver biopsy is still the only diagnostic procedure [39]. Several scores based on biochemical parameters have been proposed to evaluate liver fibrosis as. NAFLD Fibrosis Score, Fibrosis 4 Calculator, Enhanced Liver Fibrosis, and the Fibro Test are the most utilized [40]. Transient elastography is the instrumental imaging performed for the evaluation of liver fibrosis, but it has a high rate of false positive results [41].

2.5. Management of NAFLD: hypocaloric diet and physical activity

At present, lifestyle intervention – including strategies to reduce body weight and increase regular physical activity – represents the mainstay of NAFLD management. Recently, the Clinical Practice Guidelines for the management of NAFLD – proposed by a joint effort of the European Association for the Study of the Liver, the European Association for the Study of Obesity, and the European Association for the Study of Diabetes – recommended a 7–10% body weight loss in overweight/obese patients with NAFLD as a target to achieve [33,42]. A

similar target is proposed by the American Association for the Study of Liver Diseases [2].

Body weight loss in NAFLD can be achieved by hypocaloric diet alone or in combination with increased physical activity.

Although total calorie intake plays a very important role in both the development and the treatment of NAFLD, only modulating the quality of the diet, i.e. changing both the macro and the micronutrient composition, can also markedly affect the clinical evolution of NAFLD offering a more realistic and feasible treatment alternative.

To this regard, the Mediterranean diet – characterized by high consumption of olive oil as source of added fat, legumes, whole grains, fruits, vegetables, and fish; a low consumption of dairy products and meat; and a moderate alcohol assumption [43] – could represent an adequate therapeutic approach in NAFLD prevention and treatment and this dietary pattern has been recently recommended as appropriate for the management of NAFLD [33].

The beneficial effect of Mediterranean diet on many metabolic chronic diseases is largely supported by several epidemiological studies [44]. Recently, Zelber-Sagi *et al.* have comprehensively reviewed the evidence on this aspect [45] concluding that the adherence to the Mediterranean diet was significantly related to an improvement of NAFLD.

3. DIET COMPOSITION AND NON-ALCOHOLIC FATTY LIVER DISEASE IN ISOCALORIC CONDITIONS

3.1. Dietary fatty acids

3.1.1. Saturated fatty acids

Saturated fatty acids (SFA) contain no double bonds in the straight-chain hydrocarbon with varying length ranging from short chain length (volatile liquids) to chain lengths of 30 or more carbon atoms (waxy solids). The main food sources are animal fat products such as cream, cheese, butter, other whole milk dairy products and fatty meats and eggs, but also some vegetable fat, i.e. coconut and palm kernel oils.

Observational studies focusing on dietary habits of patients with NASH have suggested the possible negative influence of SFA, since their diets were richer in SFA than in other fatty acids compared to subjects with simple liver steatosis [46] or to the general population [47].

Along this line, controlled intervention trials demonstrated that increasing dietary SFA in isocaloric substitution of carbohydrates [48] or PUFA [49] increased hepatic and visceral fat accumulation in healthy subjects.

The detrimental effect of SFA on liver fat may be mediated by the increase in insulin resistance and oxidative stress, both associated with NAFLD. To date, studies *in vitro* and in animal models have shown that SFA could induce lipogenesis by promoting the transcription of peroxisome proliferator-activated receptor-gamma (PPAR- γ) coactivator-1 β and the sterol regulatory element-binding transcription factor 1c (SREBP-1c). In addition, they promote oxidative

stress, and apoptosis of hepatocytes [50,51], possibly leading to the progression of NAFLD to NASH [52].

3.1.2. Monounsaturated fatty acids and n-6 polyunsaturated fatty acids

MUFA contain one double bond in their aliphatic hydrocarbon chain. MUFA are mainly found in plant-based foods such as olive oil, canola oil, nuts, soy and avocado, and to a lesser extent in red meat and whole milk products.

PUFA contain more than one double bond in their chemical structure. There are two main PUFA groups with relevant biological functions and they are classified by the position of their first double bond counting from the methyl carbon: n-6 PUFA with their first unsaturated bond at carbon6 and n-3 PUFA at carbon3; their main dietary sources are flaxseed and some nuts.

Albeit scanty, the evidence available to date shows quite clearly the impact of MUFA on liver fat (**Table 1**). After an 8-week intervention with a high-MUFA diet (28% of total energy) *vs.* a high-carbohydrate/high-fiber/low-glycemic index diet (MUFA 16% of total energy), a 29% reduction of liver fat content, measured by ¹H-MRS, was observed in a group of T2DM subjects in comparison to a baseline diet moderately rich in SFA (13% of total energy) [53].

An even greater reduction (-39%) was observed in only 6 weeks by Ryan and colleagues [54] in a group of T2DM subjects with NAFLD assigned to an isocaloric Mediterranean diet (MUFA intake 23% of total energy) *vs.* a low-fat/high-carbohydrate diet (MUFA intake 8% of total fat). In a long-term intervention trial (24 weeks) [55], olive oil (MUFA 70%) and canola oil (MUFA 61%) consumption was compared to control oil (soybean or safflower oil such as

the most common oil used in the habitual diet, MUFA 15–24%) showing a remarkable reduction of fatty liver grading evaluated by US, with 66.7% and 76.7% of the participants in the olive and canola oil groups, respectively, reverting to normal liver grading after the intervention.

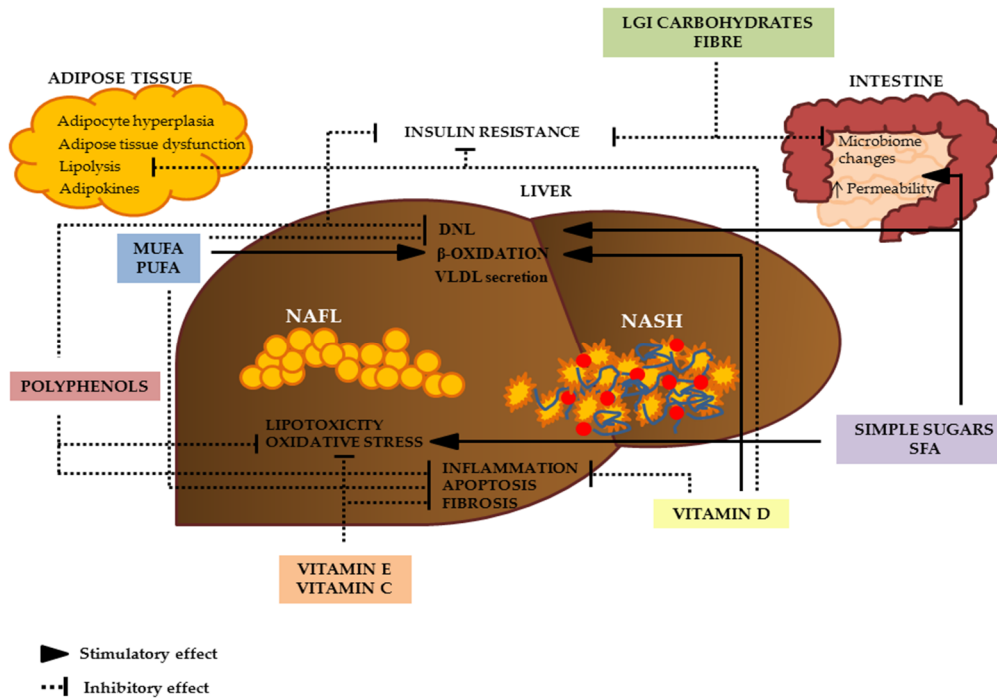
Although the evidence is rather convincing, the exact mechanism through which MUFA could affect hepatic triglycerides content is not completely clear. In *in vitro* and *in vivo* studies, MUFA have been shown to activate peroxisome proliferator-activated receptor- α (PPAR α) and PPAR γ [48], increasing lipid oxidation [57-59] and inhibiting lipogenesis [58,60], thus leading to a reduction in hepatic steatosis (**Figure 2**). On the other hand, MUFA can promote fatty acid deposition in adipose tissue rather than in the liver, enhancing the clearance of circulating triglyceride rich lipoproteins by lipoprotein lipase [61] with an improvement in blood lipid profile, insulin resistance and obesity-related inflammation [62,63]. As for the effect of n-6 PUFA on NAFLD, only one study has been conducted (**Table 1**). In a 10-week isocaloric randomized and controlled trial [64], participants were assigned either to a PUFA diet (sunflower oil and seeds) or to a saturated fat diet. After the intervention, liver steatosis assessed by ^1H -MRS was significantly reduced with the PUFA diet compared to the SFA diet (-26% vs. +8%, respectively).

Therefore, we can conclude that MUFA and n-6 PUFA seem to have beneficial effects on liver fat content in individuals at high cardiometabolic risk.

As for the possible mechanisms, PUFA are key regulators of the transcription of genes associated with lipid metabolism and mitochondrial β -oxidation (i.e. PPAR-

α and SREBP-1). Thus, increasing PUFA intake may lead to a reduction of lipogenesis in favor of an increased hepatic fatty oxidation [65] (**Figure 2**).

Figure 2. Possible sites of action of dietary nutrients in the nutritional treatment and prevention of NAFLD.



Nutrients and dietary composition can modulate many key aspects in the pathophysiology of NAFLD: simple sugars promote DNL, produce inflammation and activate cellular stress pathways. Contrarily, LGI meals can improve insulin resistance and can positively modulate the microbiome. SFA could induce lipogenesis, oxidative stress, and apoptosis of hepatocytes; conversely, MUFA and PUFA can improve FFA β -oxidation and can reduce DNL, improve insulin sensitivity and reduce inflammation. Polyphenols could inhibit DNL and increase FFA β -oxidation. Furthermore, polyphenols can improve insulin sensitivity, reduce the transcription of inflammatory cytokines, and can mitigate the oxidative stress involved in NAFLD progression. Vitamin C and vitamin E could avoid the progression of NAFLD and improve NASH acting as powerful antioxidants; furthermore, vitamin E could reduce plasma levels of cytokines involved in inflammation and liver fibrosis. Vitamin D can reduce the transcription of inflammatory cytokines and improve FFA β -oxidation. Furthermore, it has been observed that vitamin D increases adiponectin secretion, decreases lipolysis in adipose tissue, and improves insulin resistance. DNL: de novo lipogenesis; LGI: low glycemic index; MUFA: monounsaturated fatty acids; NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids

Table 1. Clinical trials on the effects of MUFA and n-6 PUFA on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with MUFA or n-6 PUFA			
					Liver imaging	Liver biomarkers	Liver scores	Liver Biopsy
MUFA								
Bozzetto et al. 2012 [53]	Randomized, controlled, parallel group	36 M/F, T2DM 58.7 years 29.7 kg/m ²	MUFA diet (MUFA 28% TE) vs high-CHO/fiber/low-GI diet (MUFA 16% TE)	8	↓ LIVER FAT (¹ H-MRS)	AST = ALT =	n.a.	n.a.
Ryan et al. 2013 [54]	Randomized, controlled, crossover	12 M/F, T2DM 55.0 years 32.0 kg/m ²	Mediterranean diet (MUFA 23% TE) vs low fat-high CHO (MUFA 8% TE)	6	↓ LIVER FAT (US)	AST = ALT =	n.a.	n.a.
Nigam et al. 2014 [55]	Randomized, controlled, parallel group	93 M 37.0 years 27.4 kg/m ²	olive oil (MUFA 70%) vs canola oil (MUFA 61%) vs soybean or safflower oil (MUFA 15–24%TE)	24	↓ LIVER FAT (US)	AST = ALT =	n.a.	n.a.
n-6 PUFA								
Bjeremo et al. 2012 [64]	Randomized, controlled, parallel group	61 M/F 56.5 years 30.2 kg/m ²	PUFA diet (linoleic acid 15% TE) vs SFA diet (butter 15% TE)	10	↓ LIVER FAT (¹ H-MRS)	AST n.a. ALT =	n.a.	n.a.

TE: total energy; = no changes; ↓ significant decrease.

BMI: body mass index; MUFA: monounsaturated fatty acids; n-6 PUFA: n-6 polyunsaturated fatty acids; vs: versus; T2DM: type 2 diabetes mellitus; M: male; F: female; CHO: carbohydrates; GI: glycemic index; SFA: saturated fatty acids; ¹H-MRS: proton magnetic resonance spectroscopy; ALT: alanine aminotransferase; AST: aspartate aminotransferase; n.a.: not assessed; US: ultrasonography.

3.1.3. n-3 polyunsaturated fatty acids

As reported above, n-3 PUFA is one of the two main PUFA groups with relevant biological functions. The most biologically relevant n-3 PUFA are α -linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The main dietary sources of n-3 PUFA are fish oil, flaxseed and some nuts. Several

studies on n-3 PUFA supplementation and NAFLD are available in individuals at high cardiometabolic risk. Overall, the available evidence still produces conflicting results (**Table 2**).

In a 24-week intervention trial, a complete fatty liver regression was observed after a 2 g/day of n-3 PUFA supplementation in the context of an American Heart Association (AHA) diet in 33.4% of the patients [66]. These findings were further confirmed by the results of the WELCOME study showing that the supplementation with 4 g/day of PUFA over 18 months significantly affected liver fat content in a dose-dependent manner as compared vs. placebo [67].

Table 2. Clinical trials on the effects of n-3 PUFA on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with n-3 PUFA			
					Liver Imaging	Liver biomarkers	Liver scores	Liver biopsy
Spadaro et al. 2008 [66]	Parallel group randomized, controlled	36 M/F 50.1 years 30.5 kg/m ²	2g/day vs. placebo	24	↓ LIVER FAT (US)	AST = ALT ↓	n.a.	n.a.
Scorletti et al. 2014 [67]	Double-blind, placebo-controlled	103 M/F 51.5 years 33.0 kg/m ²	4g/day (EPA 1.8 g, DHA 1.5 g) vs. placebo	72	↓ LIVER FAT (MRI)	AST = ALT =	n.a.	n.a.
Zhu et al. 2008 [68]	Double-blind, placebo-controlled	134 M/F 44.5 years 26.2 kg/m ²	6g/day vs. placebo	24	↓ LIVER FAT (US)	AST = ALT ↓	n.a.	n.a.
Vega et al. 2008 [69]	Crossover placebo-controlled	16 M/F 50.0 years 36.2 kg/m ²	9g/day (EPA 51.4%, DHA 23.9%) vs. placebo	8	= LIVER FAT (1H-MRS)	AST n.a. ALT n.a.	n.a.	n.a.
Argo et al. 2015 [70]	Double-blind, placebo-controlled	34 M/F 46.8 years 32.5 kg/m ²	3 g/day (EPA 35%, DHA 25%) vs. placebo	48	= LIVER FAT (MRI)	AST = ALT =	n.a.	= NASH score
Sanyal et al. 2014 [71]	Double-blind, placebo-controlled	243 M/F 48.7 years 34.8 kg/m ²	EPA 1.8 g/day vs. EPA 2.7 g/day vs. placebo	48	n.a.	AST = ALT =	n.a.	= NASH score

			placebo					
Cussons et al. 2009 [72]	Crossover placebo- controlled	25F 54.5 years 34.8 kg/m ²	4 g/day (EPA 27%, DHA 56%) vs. placebo (oleic acid 67%)	8	= LIVER FAT (¹ H-MRS)	AST n.a. ALT =	n.a.	n.a.

= no changes; ↓ significant decrease.

BMI: body mass index; M: male; F: female; n-3 PUFA: n-3 polyunsaturated fatty acids; US: ultrasonography; n.a.: not assessed; MRI: magnetic resonance imaging; ALT: alanine aminotransferase; AST: aspartate aminotransferase; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; ¹H-MRS: proton magnetic resonance spectroscopy; NASH: non-alcoholic steatohepatitis.

Similarly, increasing the amount of n-3 PUFA to 6 g/day vs placebo, in the context of an AHA diet, induced a full reversion of liver steatosis in 19.7% of participants, and an overall improvement of fatty liver grading in 53% of the study population after a 24-week intervention [68].

In contrast with the above studies, an 8-week supplementation with 9 g/day of fish oil vs. placebo did not affect hepatic triglyceride content measured by ¹H-MRS [69]; similarly, Argo *et al.* [70] did not detect any improvement of fatty liver in a group of subjects receiving 3g/day of fish oil for 12 months as compared with the placebo group. On the same line, a 12-months supplementation with EPA had no significant effects on the key features of NASH [71].

Cussons and colleagues [72] compared the effects of the daily consumption of n-3 PUFA or MUFA in a group of women with polycystic ovary syndrome, a condition associated with NAFLD. According to an 8-week crossover randomized and controlled trial, they consumed 4 g/day of n-3 PUFA and a placebo. Both arms reduced liver fat measured by hepatic ¹H-MRS.

As reported above, the efficacy of n-3 PUFA supplementation on liver fat content is still controversial. This lack of concordance may be due, at least in part, to the

largely different doses used in the trials (ranging from 0.25 to 6 g/day), the length of the exposure (from 2 to 18 months), and last but not least the imaging methods (US vs. $^1\text{H-MRS}$). Nevertheless, the only two studies looking at NASH features on liver biopsies showed no effect of n-3 PUFA.

To date, evidence of the mechanisms linking n-3 PUFA supplementation and NAFLD derives mainly from *in vitro* and animal studies. First of all, as reported for n-6 PUFA, increasing PUFA intake may increase fatty oxidation in the liver through the modulation of PPAR- α and SREBP-1 [65]. On the other hand, EPA and DHA are important modulators of the inflammatory pathway and, consequently, may inhibit pro-inflammatory eicosanoid production by inflammatory cells related to hepatic injury in NAFLD (**Figure 2**).

3.2. Carbohydrates

3.2.1. Low glycemic-index carbohydrate and fibre rich diets

The association between high carbohydrate intake, high GI carbohydrate consumption, insulin resistance and liver fat accumulation has been found in animal models and observational studies [73,74]. In particular, in a cross-sectional study, the prevalence of high-grade liver steatosis increased significantly across quartiles of high GI versus low GI diets [75]. In fact, available carbohydrates produce an increase in serum levels of glucose in the postprandial state that can be used for the synthesis of new triglycerides through *de novo lipogenesis* in the liver [76]. The consumption of foods with high GI promotes insulin resistance, a condition strongly related to NAFLD [77], and the negative effect of a high GI diet on liver fat content can be observed in few days [78]. Conversely, low GI

meals could have beneficial effect on NAFLD. In fact, low GI foods, especially foods rich in fibre, can decrease glucose absorption, reducing hepatic influx of glucose and *de novo lipogenesis* [79]; in addition, the fibre content of low GI foods can positively act on the gut microbiome, a possible mediator by which nutrients may influence liver fat content [80] (**Figure 2**).

Although GI seems to be an important factor in NAFLD prevention and treatment, few clinical trials have investigated the effect of low GI or low glycemic load (GL) at isocaloric conditions on NAFLD in patients at high cardiometabolic risk (**Table 3**).

Table 3. Clinical trials on the effects of different types of carbohydrates (low glycemic index diet, oligofructose, simple sugars) on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study Design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with carbohydrates, oligofructose and simple sugars			
					Liver Imaging	Liver biomarkers	Liver scores	Liver biopsy
Low glycemic index diet								
Fraser et al. 2008 [84]	Open label, quasi- randomized, controlled	259 M/F T2DM 56 years 31.5 kg/m ²	ADA diet (CHO: 50-55%, fat: 30%, protein: 20%) <i>or</i> LGI diet (CHO: 50-55%, fat: 30%, protein: 15-20%) <i>or</i> MM diet (CHO: 35%, fat: 45%, protein: 15- 20%)	52	n.a.	ALT ↓	n.a.	n.a.
Utzschnei der et al. 2012 [81]	Randomized parallel, double- bind,	35 M/F 68.9 years 27.5 kg/m ²	LSAT diet (23% fat, 7% saturated fat, GI<55) vs HSAT diet (43% fat, 24% saturated fat, GI>70)	4	↓ LIVER FAT (¹ H-MRS)	AST = ALT =	n.a.	n.a.

Ramon-Krauel et al. 2013 [83]	Randomized parallel	16 M/F 12.8 years 32.6 kg/m ²	LGI diet (CHO: 40%, fat: 35-40%, protein: 15-20%) vs LF diet (CHO: 55-60%, fat: 30%, protein: 15-20%)	24	= LIVER FAT (¹ H-MRS)	AST = ALT =	n.a.	n.a.	
Misciagna et al. 2016 [82]	Randomized parallel-group, controlled	98 M/F 47.5 years 31.5 kg/m ²	LGI diet (CHO: 50%, fat: 30%, protein: 15-20%) vs control (diet based on INRAN guidelines)	24	↓ LIVER FAT (US)	AST = ALT =	n.a.	n.a.	
Oligofructose									
Daubiol et al. 2005 [89]	Randomized double-blind, crossover controlled	7 M NASH 54.5 years 29.1 kg/m ²	Oligofructose (16 g/day) vs maltodextrine	8	= LIVER FAT (US)	AST ↓ ALT =	n.a.	n.a.	
Fructose/Simple sugars									
Johnston et al. 2011 [110]	Randomized double-blind	32 M 33.9 years 29.4 kg/m ²	Fructose (25% TE) vs Glucose (25% TE)	2	= LIVER FAT (¹ H-MRS)	AST = ALT =	n.a.	n.a.	
Bravo et al. 2013 [103]	Randomized parallel-group	64 M/F 42.1 years 27.2 kg/m ²	HFCS (8%, 18% or 30% of the calories required for weight maintenance) vs Sucrose (8%, 18% or 30% of the calories required for weight maintenance)	10	= LIVER FAT (CT)	n.a.	n.a.	n.a.	
Maersk et al. 2012 [102]	Randomized parallel-group	47 M/F 38.7 years 32.0 kg/m ²	Regular cola (1 L/day) or Milk (1 L/day) or Diet cola (1 L/day) or Water (1 L/day)	24	↑ LIVER FAT (¹ H-MRS)	n.a.	n.a.	n.a.	

TE: total energy; = no changes; ↓ significant decrease; ↑ significant increase. BMI: body mass index; M: male; F: female; T2DM: type 2 diabetes mellitus; ALT: alanine aminotransferase; AST: aspartate aminotransferase; n.a.: not assessed; ADA: American Diabetes Association; CHO: carbohydrates; LGI: low glycemic index; MM: Mediterranean modified; US: ultrasonography; ¹H-MRS: proton magnetic resonance spectroscopy; CT: computed tomography; LGI: low glycemic index; INRAN: Italian National Research Institute for Foods and Nutrition; LSAT: low-fat/low-saturated fat/low-glycemic index diet; HSAT: high-fat/high-saturated fat/high-glycemic index diet; GI: glycemic index; LF: low fat; HFCS: high-fructose corn syrup.

Three studies evaluated the effects of low GI diets on liver fat compared to diets with higher GI, and two of them found a significant reduction in liver fat – evaluated by ¹H-MRS in one and by US in the other [81,82]; no change was observed in the third study performed in obese children [83]. In none of these three studies was there any change with respect to liver enzymes; on the other hand, a reduction in ALT was reported after a low GI diet and a Mediterranean diet compared to a control diet in one intervention trial performed in patients with T2DM in which only liver enzymes were analyzed [84].

The fibre content of foods is one of the most important factors related to GI. Dietary fibre is defined as a non-digestible food carbohydrates; based on water solubility it can be classified into soluble – pectins, fructans, oligosaccharides and gums – and insoluble – hemicellulose, cellulose and lignin – and it is widely found in fruits, vegetable, whole grains and legumes [85]. Some epidemiological studies have shown that fibre intake in NAFLD patients is lower than in healthy individuals [86-88].

However, if we exclude the trials in which fibre was part of multifactorial dietary changes, only limited research regarding the effects of fibre alone on NAFLD has been done. We have found only one study evaluating the effects of a non-digestible carbohydrate, oligofructose (**Table 3**). In this trial, a decrease in ALT and AST was found after 16 g of oligofructose compared to maltodextrine in patients with NASH although no change in liver fat was detected at US [89].

Trying to draw some conclusions from the trials evaluating the effects of low GI diets on NAFLD, the few data available indicate that the low GI may have some

role within the context of a diet characterized by other favorable changes such as, *in primis*, the reduction of saturated fatty acids.

3.2.2. Fructose/other simple sugars

The intake of simple sugars increases liver fat content in animal models [90,91] and epidemiological studies suggest an association between consumption of soft drinks and NAFLD development in humans [92-94].

Simple sugars, in particular fructose, has been shown to promote hepatic lipogenesis by stimulating SREBP-1c and carbohydrate response element-binding protein (ChREBP), the major transcription factors of many enzymes involved in *de novo lipogenesis* [95-97]. Furthermore, it has been observed that fructose and glucose consumption – in addition to stimulating SREBP-1c and ChREBP – may produce inflammation and activate cellular stress pathways [98,99] (**Figure 2**).

Many clinic trials have investigated the effect of simple sugars – mainly fructose, glucose and sucrose – on NAFLD in healthy individuals and in those at high cardiometabolic risk [100-107], and two meta-analyses on this issue were carried out [108,109]. Briefly, the first meta-analysis reported that in healthy subjects using high doses of fructose – in terms of 104-220 g/day – in a hypercaloric diet increased both liver fat content and serum ALT levels while did not produce any effect in isocaloric conditions [108]. Similar findings were reported by the second meta-analysis where it was observed that the excess of added sugar intake in a hypercaloric diet compared with a eucaloric control diet increased liver fat content [109]. Only three trials have looked specifically at the effect of simple sugars intake as part of an isocaloric diet in overweight/obese subjects (**Table 3**).

Johnston *et al.* investigated the effects of glucose- or fructose-sweetened beverages providing 25% of energy requirements during an isocaloric period of 2 weeks. At the end of treatment, in overweight patients with NAFLD, serum ALT and AST levels, and liver fat content evaluated by ¹H-MRS were unchanged [110]. Similar findings were reported by Bravo *et al.* who investigated the effects of three different levels of sucrose or high-fructose corn syrup (55% fructose) at 8%, 18%, or 30% of the calories required for weight maintenance in overweight patients with NAFLD. At the end of 10-week intervention, liver fat content evaluated by CT was unchanged [103]. On the other hand, Maersk *et al.* compared the effects of four different drinks – 1 L/day of regular cola, or isocaloric semi-skim milk, or aspartame-sweetened diet cola or water – in obese subjects with NAFLD. After 24 weeks of treatment, drinking regular cola resulted in a higher amount of liver fat content, evaluated by ¹H-MRS [102].

In conclusion, even if data are still limited, it seems that simple sugars, at least within the context of an isocaloric diet, do not have a marked deleterious influence on liver fat in overweight individuals, while frankly obese subjects may be more sensitive to the exposure to simple sugars even within an isocaloric diet.

3.3. Proteins

Limited evidence on the effect of proteins on NAFLD is available. In animal models, a reduction in liver fat content was observed when protein intake was increased [111].

A very recent analysis of The Rotterdam Study, a large epidemiological study, showed that total protein intake, in particular proteins of animal origin, was

associated with higher odds of NAFLD in overweight subjects (OR= 1.50; 95% CI 1.17-1.92) [112]; similarly, a cross-sectional evaluation of the Israeli National Health and Nutrition Survey showed that the intake of meat was significantly associated with an increased risk for NAFLD (OR= 1.37, 95% CI 1.04–1.83) [113]. The effect of protein intake on NAFLD has been evaluated only in few controlled clinical trials, generally adopting hypocaloric diets [114-116]. Therefore, it is not possible to draw any conclusion about the possible effect of proteins *per se* on NAFLD.

3.4. Other dietary components

3.4.1. Polyphenols

Polyphenols represent a great variety of secondary plant metabolites and, based on their chemical structure, they can be divided into two major categories: flavonoid and non-flavonoids. About 8.000 phenolic compounds in the plant kingdom have been discovered. Vegetables, cereal grain, fruits, and some beverages – tea, coffee, red wine, beer – are good sources of polyphenols [117]. Mean total dietary intake of polyphenols was 1193 ± 510 mg/day [118]. These natural compounds are powerful antioxidants, in addition to having many other properties such as anti-inflammatory, anti-mutagenic, and immunomodulatory activities [117]. Phenolic compounds have received growing interest over the last few years and epidemiological studies have shown an inverse correlation between high polyphenol consumption and incidence of many chronic metabolic diseases, including obesity, insulin resistance, and CVD [119]. A randomized controlled trial in individuals at high cardiometabolic risk showed that diets naturally rich in

polyphenols improved fasting and postprandial dyslipidemia and reduced oxidative stress [120]. Recently, beneficial effects of polyphenols on NAFLD have been reported in animal models [121].

Polyphenols could prevent liver fat accumulation and NAFLD progression through several mechanisms. In *in vitro* and animal models it has been observed that polyphenols may reduce hepatic lipogenesis and increase free fatty acid oxidation. In particular, polyphenols can decrease the transcription of SREBP-1c [122] and increase transcription of PPAR- α . Moreover, polyphenols can improve insulin sensitivity and reduce the transcription of inflammatory cytokines [123-125]. All these molecular pathways can be indirectly modulated by the effect of polyphenols on the activation of AMP-activated protein kinase [126]. Finally, the antioxidant properties of phenolic compounds in reducing oxidative stress involved in NAFLD progression should be also considered [121] (**Figure 2**). Whereas from these animal and *in vitro* studies it can be argued that polyphenols may have positive influence on different aspects of NAFLD, the controlled intervention trials in humans have produced discordant results (**Table 4**).

The effects of mixed phenolic compounds have been investigated by two trials [127,128]. Chang *et al.* [127] evaluated in overweight NAFLD patients the effects of 150 mg/day of polyphenols compared to placebo. After 12 weeks of treatment, in the polyphenol group a significant 15% reduction in fatty liver score was observed, with no changes in AST or ALT levels. In a trial conducted by Guo *et al.* [128], young NAFLD patients were given 250 mL of bayberry juice or placebo twice daily for 4 weeks. No significant differences in the serum levels of AST and ALT between the groups were observed.

Table 4. Clinical trials on the effects of polyphenols supplementation on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with polyphenols			
					Liver imaging	Liver biomarkers	Liver scores	Liver biopsy
Suda et al. 2008 [129]	Double-blind, randomized, placebo-controlled	38 M 43.0 years 25.4 kg/m ²	Anthocianins (400 mg/day) vs placebo	8	n.a.	AST n.a. ALT ↓	n.a.	n.a.
Sakata et al. 2013 [130]	Double-blind, randomized, placebo-controlled	17 M/F 50.6 years 29.0 kg/m ²	Cathechin (1,080 mg/day) vs placebo	12	↓ LIVER FAT (CT)	AST n.a. ALT ↓	n.a.	n.a.
Chang et al. 2013 [127]	Double-blind, randomized, placebo-controlled	36 M/F 37.9 years 31.2 kg/m ²	Flavonoids, anthocyanins, phenolic acid (150 mg/day) vs placebo	12	↓ LIVER FAT (US)	AST = ALT =	↓ FS	n.a.
Guo et al. 2014 [128]	Double-blind, randomized, crossover, placebo-controlled	44 M/F 21.2 years 25.4 kg/m ²	Phenolic acids, anthocyanins (1,350 mg/day) vs placebo	4	n.a.	AST = ALT = TPS ↓ CK-18 ↓	n.a.	n.a.
Poulsen et al. 2013 [131]	Double-blind, randomized, placebo-controlled	24 M 38.3 years 34.2 kg/m ²	Resveratrol (500 mg/day) vs placebo	4	= LIVER FAT (¹ H-MRS)	AST n.a. ALT =	n.a.	n.a.
Faghihzadeh et al. 2014 [134]	Double-blind, randomized, placebo-controlled	50 M/F 45.1 years 28.5 kg/m ²	Resveratrol (500 mg/day) vs placebo	12	↓ LIVER FAT (US) = LIVER FIBROSIS (TEL)	AST = ALT ↓ CK-18 ↓	n.a.	n.a.
Chychay et al. 2014 [133]	Double-blind, randomized, placebo-controlled	20 M 48.1 years 31.5 kg/m ²	Resveratrol (3,000 mg/day) vs placebo	8	= LIVER FAT (¹ H-MRS)	AST* ↑ ALT* ↑ CK-18 =	n.a.	n.a.
Chen et al. 2014 [123]	Double-blind, randomized, placebo-controlled	60 M/F 44.3 years 25.7 kg/m ²	Resveratrol (600 mg/day) vs placebo	12	= LIVER FAT (US)	AST ↓ ALT ↓ CK-18 ↓ FGF-21 ↓	n.a.	n.a.
Heebøll et al. 2016 [132]	Double-blind, randomized, placebo-controlled	28 M (46% NASH) 43.3 years 31.9 kg/m ²	Resveratrol (1,500 mg/day) vs placebo	24	= LIVER FAT (¹ H-MRS)	AST = ALT =	n.a.	= NASH

* at week 6; = no changes; ↓ significant decrease; ↑ significant increase. BMI: body mass index; M: male; F: female; ALT: alanine aminotransferase; AST: aspartate aminotransferase; n. a.: not assessed; US: ultrasonography; CT: computed tomography; ¹H-MRS: proton magnetic resonance spectroscopy; TEL: transient elastography; FS: fatty liver score; CK-18: Cytokeratin-18; FGF-21: fibroblast growth factor 21; NASH: non-alcoholic steatohepatitis.

Two more trials evaluated the effects of specific polyphenols, such as anthocyanins and catechins. Authors observed a significant reduction in the serum levels of ALT versus placebo [129]. In the second study, the consumption of the highest dose of catechins significantly decreased serum ALT level by 42.1% and improved liver fat content with a liver-to-spleen CT attenuation ratio that increased from 92% to 102% [130].

Resveratrol is currently one of the more extensively studied polyphenols, and five trials have been conducted on NAFLD. In two of these studies resveratrol at the dose of 500 mg/day and 1,500 mg/day, respectively, did not induce any change in the different liver outcomes evaluated [131,132]. In another intervention trial, where a higher dose of resveratrol was used – 3,000 mg/day – a transient increase in ALT and AST was observed at week 6, with no change at the end of the intervention in liver enzymes or in liver fat [133]; conversely, two trials showed some beneficial effects [123,134].

Therefore, based on these data, catechins and anthocyanins seem to have some beneficial influence on liver fat, but this needs to be confirmed by additional evidence. As for resveratrol, the results are so discordant that no definite conclusion can be drawn.

3.4.2. Vitamin E

The term vitamin E refers to eight lipid-soluble compounds – four tocotrienols and four tocopherols – with powerful antioxidant properties. These essential vitamins are synthesized in vegetables and are largely present in seeds, nuts, vegetable oils, green leafy vegetables and fortified cereals [135, 136]. Vitamin E

plays a key role in many physiological functions: it is one of the most powerful antioxidant and acts as free radical scavenger; it is also involved in the regulation of platelet aggregation, protein kinase C activation, immune function, gene expression, and other metabolic processes [137].

Vitamin E could avoid the progression of NAFLD and improve NASH by virtue of its antioxidant capacity and as free radical scavenger. It has been observed that vitamin E reduces the inflammatory pathway in NASH by several mechanisms, beyond its “simple” antioxidant activity; in particular, vitamin E could improve superoxide dismutase activity and could decrease the transcription of many genes related to inflammation and liver fibrosis [138-141]; it has been also reported that vitamin E could improve insulin sensitivity [142] (**Figure 2**).

The possible effects of vitamin E supplementation on NAFLD have been assessed in different intervention trials and the results of these trials have been examined in two meta-analyses [143,144]. Briefly, vitamin E supplementation in patients with NAFLD reduces significantly liver enzymes, liver steatosis, inflammation and hepatocellular ballooning compared to control treatments. Moreover, in patients with NASH, vitamin E supplementation seems to reduce fibrosis as well. Despite the positive results of these meta-analyses, it is important to underline some limitations such as the variability in daily dosage of vitamin E, the length of treatment, and the small sample size of the studies, except for the PIVENS and the TONIC studies [145,146]. Furthermore, some concerns must be underlined about the possible negative effects of high doses of vitamin E (>400 IU/day) on all-cause mortality [147].

3.4.3. Vitamin C

Vitamin C is a soluble vitamin and its major dietary forms – L-ascorbic and dehydroascorbic acids – are largely found in vegetables and fresh fruits [148]. Vitamin C plays a key role in many physiological functions for human health – it is essential for the activity of the enzymes implicated in the synthesis of catecholamines, carnitine and collagen – and, in addition, it is a powerful antioxidant and acts as a free radical scavenger [149].

In the last few years, a noteworthy epidemiological literature has shown an inverse correlation between vitamin C deficiency and some chronic diseases, as obesity, hypertension, and CVD [150]. The results of epidemiological studies are conflicting about a possible relation between vitamin C and NAFLD. In fact, Ferolla *et al.* [151] reported that patients with NAFLD were unable to achieve the optimal intake of vitamin C, and similar findings were reported by Musso *et al.* [47] and Canbakan *et al.* [152], who analyzed the intake of vitamin C in patients with NASH. Conversely, in other cross-sectional studies no relation between dietary vitamin C intake and presence of NAFLD or NASH was observed [153-155]. These conflicting results may be related to ethnicity and differences in disease grade (NAFL or NASH); furthermore, it should be considered that in many studies the dietary intake of vitamin C was considered with no evaluation of plasma vitamin C levels.

Theoretically, vitamin C could play a beneficial role in NAFLD by acting as powerful antioxidant and as free radical scavenger. In *in vitro* models, vitamin C can reduce reactive oxygen species formation and improve the activity of glutathione peroxidase and superoxide dismutase [156]. Furthermore, vitamin C

can promote the production of adiponectin – an adipose tissue protein apparently able to decrease insulin resistance and inflammation in humans [157] (**Figure 2**).

To the best of our knowledge, no clinical trial has investigated the effect of vitamin C supplementation alone on NAFLD, while some clinical trials have evaluated the effects of the combination of vitamins C and E (**Table 5**).

Table 5. Clinical trials on the supplementation of vitamins C + E on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with vitamin C plus vitamin E			
					Liver Imaging	Liver biomarkers	Liver scores	Liver biopsy
Harrison et al. 2003 [164]	Double- blind, randomized, placebo- controlled	45 M/F, NASH 51.3 years 32.7 kg/m ²	Vitamin C (1,000 mg/day) + Vitamin E (1,000 IU/day) vs placebo	24	n.a.	AST = ALT =	n.a.	= NASH ↓ FIBROSIS
Ersöz et al. 2005 [166]	Open-label, randomized	57 M/F (15% NASH) 47.1 years 28.4 kg/m ²	Vitamin C (500 mg/day) + Vitamin E (600 IU/day) vs UDCA (10 mg/kg/day)	24	= LIVER FAT (US)	AST = ALT =	n.a.	n.a.
Nobili et al. 2006 [167]	Double- blind, randomized, placebo- controlled	90 M/F (26% NASH) 12.1 years 25.0 kg/m ²	Vitamin C (500 mg/day) + Vitamin E (600 IU/day) vs placebo	52	= LIVER FAT (US)	AST = ALT =	n.a.	n.a.
Nobili et al. 2008 [165]	Open-label, randomized, placebo- controlled	53 M/F 11.9 years 25.8 kg/m ²	Vitamin C (500 mg/day) + Vitamin E (600 IU/day) vs placebo	104	n.a.	AST = ALT =	n.a.	= LIVER FAT = NASH = FIBROSIS ↓ NAFLD activity score

= no changes; ↓ significant decrease. BMI: body mass index; NASH: non-alcoholic steatohepatitis; M: male; F: female; ALT: alanine aminotransferase; AST: aspartate aminotransferase; n.a. : not assessed; UDAC: ursodeoxycholic acid; IU: international unit; US: ultrasonography; NAFLD non-alcoholic fatty liver disease.

The results of these trials were not concordant, two studies showing a reduction in fibrosis and NAFLD activity score evaluated on liver biopsy [158,159], two studies showing no effects on liver fat [160,161].

3.4.4. Vitamin D

Vitamin D is a lipid-soluble compound found in few foods such as fatty fish, fish liver oils, and dairy products; it is also produced in the skin after ultraviolet irradiation. Vitamin D₂ and vitamin D₃ – also defined ergocalciferol and cholecalciferol – are the two main forms of vitamin D. Vitamin D plays a prominent role in calcium and phosphorus metabolism and is essential for bone health, promoting bone growth and remodeling. In the last decade, it has become evident that vitamin D also presents extra-skeletal effects, including metabolic effects, neuromuscular and immune functions [162].

A growing body of literature has shown that serum levels of vitamin D are inversely associated with insulin resistance, metabolic syndrome, CVD, T2DM and NAFLD [163-165]. A meta-analysis of observational studies showed that subjects with NAFLD were 26% more likely to present vitamin D deficit than controls [166]. Vitamin D receptors are widely expressed in the liver and can explain the possible effect of vitamin D on NAFLD. Vitamin D may down-regulate the expression of the NF- κ B – involved in the transcription of inflammatory cytokines – and improve the expression of PPAR- α in the liver [167]. Furthermore, it has been observed that vitamin D increases adiponectin secretion and decreases lipolysis in adipose tissue [168], improves the expression of GLUT-4 receptor in skeletal muscle [169] and promotes insulin secretion

[170]. All these effects – mediated by the specific vitamin D receptor – could reduce liver fat content (**Figure 2**).

To date, very few clinical trials have investigated the effects of vitamin D supplementation on NAFLD (**Table 6**).

Table 6. Clinical trials on the effects of vitamin D supplementation on NAFLD in individuals at high cardiometabolic risk.

Author [reference]	Study design	Study population Participants Age BMI	Intervention and doses	Duration weeks	Observed effects with vitamin D			
					Liver Imaging	Liver biomarkers	Liver scores	Liver Biopsy
Sharifi et al. 2014 [177]	Double-blind, randomized, placebo-controlled, parallel	53 M/F 42.1 years 30.3 kg/m ²	Cholecalciferol (3,570 IU/day) vs placebo	16	= LIVER FAT (US)	AST = ALT =	n.a.	n.a.
Barchetta et al. 2016 [178]	Double-blind, randomized, placebo-controlled	65 M/F T2DM 58.6 years 30.0 kg/m ²	Cholecalciferol (2,000 IU/day) vs placebo	24	= LIVER FAT (¹ H-MRS)	AST = ALT = CK-18 = P3NP =	= FLI	n.a.
Lorvand Amiri et al. 2016 [179]	Double-blind, randomized, placebo-controlled	73 M/F 41.9 years 30.3 kg/m ²	Cholecalciferol (1,000 IU/day) + hypocaloric diet vs placebo + hypocaloric diet	12	↓ LIVER FAT (US)	AST ↓ ALT ↓	n.a.	n.a.

= no changes; ↓ significant decrease. BMI: body mass index; M: male; F: female; T2DM: type 2 diabetes mellitus; ALT: alanine aminotransferase; AST: aspartate aminotransferase; n.a.: not assessed; US: ultrasonography; ¹H-MRS: proton magnetic resonance spectroscopy; IU: international unit; CK-18: Cytokeratin-18; P3NP: N-terminal Procollagen III Propeptide; FLI: fatty liver index.

Two of them [171,172] showed no effect of vitamin D supplementation on liver enzymes, liver fat content or hepatic biomarkers of injury and fibrogenesis. In the third one [173], the effect of vitamin D supplemented to a hypocaloric diet was evaluated in comparison to a hypocaloric diet. Liver enzymes and liver fat content

– evaluated by US – were significantly reduced by vitamin D independently of weight loss, which was similar in the two groups.

In conclusion, based on the available evidence in humans, we can conclude that data are reasonably convincing as for the possible effects of dietary macronutrients on liver fat content. In fact, SFA increase liver fat content and replacing SFA with MUFA or n-6 PUFA reduces liver fat, while the effectiveness of n-3 PUFA supplementation is still controversial. In terms of other dietary components (polyphenols) and micronutrients, data are not yet convincing, and any effect would refer especially to liver inflammation and fibrosis more than to fat content. As for the role of dietary vitamins on NAFLD prevention, only for vitamin E supplementation, data are rather convincing even if there might be concerns on high vitamin E supplementation – considering its possible negative effects on all cause-mortality.

Therefore, precise recommendations on the composition of the diet to be used for the prevention and treatment of NAFLD have not been proposed. More carefully conducted intervention studies are needed: it is very likely that the “optimal diet” for NAFLD should be based on different dietary modifications, i.e. a multifactorial diet or “Portfolio diet”, able to act both on the deposition of excess fat in the liver and the other pathways leading from liver fat deposition to NASH and fibrosis. However, this hypothesis needs to be substantiated by appropriate intervention studies in humans.

4. AIM AND PERSONAL RESEARCH AREAS

NAFLD is the most prevalent chronic liver disease. Evidence supports that dietary pattern may play a pivotal role in the development of NAFLD. However, there is no consensus regarding the best dietary intervention for its prevention or treatment. In this scenario, it is important to define which dietary components are useful to prevent and treat NAFLD in order to design dietary interventions with higher adherence and efficacy in the management of the disease. Consequently, my personal research area has covered three major experimental lines:

1. To evaluate in a more comprehensive and multifactorial manner the relationship of liver fat with metabolic, dietary and inflammatory factors in a cohort of individuals characterized by high cardiovascular risk with no evidence of hepatic disease and with homogenous anthropometric characteristics and nutritional habits (*HETHERPATHS cohort*).
2. To assess the effects of fructose (75 g day/ for 12 weeks in the form of drink together with the habitual *ad libitum* diet) on liver fat content in a large cohort of abdominally obese men with other cardiometabolic risk factors (*FRUCTOSE trial*).
3. To evaluate the effects of 8-weeks intervention with an isoenergetic Portfolio diet rich in MUFA, n-3 and n-6 PUFA, prebiotic fibre, polyphenols, vitamins, and low GI carbohydrates versus an isoenergetic diet rich in MUFA (control diet) on liver fat content in patients with T2DM and NAFLD (*MEDEA trial*).

5. CLINICAL TRIALS

5.1. HETHERPATS cohort

Background

NAFLD is an independent risk factor for CVD [14,15]. However, the cause of the relationships between NAFLD, other metabolic diseases and cardiovascular risk are not clear. Many nutritional, metabolic and inflammatory factors have been advocated as putative mechanisms for these associations and, therefore, for NAFLD pathogenesis. High caloric intake predisposes to liver steatosis and is also the main cause of other cardiovascular risk factors independently associated with fatty liver disease such as obesity and T2DM [4]. Beside over-nutrition, qualitative nutritional factors can play an important role in modulating liver fat content [3]. Among the metabolic factors, insulin resistance showed the strongest association with liver fat in many studies [21]. Moreover, since the prevalence of NAFLD is much higher in T2DM [10], also β -cell dysfunction may play a role in the natural history of fatty liver [174,175]. Recently, attention has been paid also to a possible role of GLP-1 [176]. Treatment with GLP-1 analogs ameliorated liver steatosis, suggesting that also gastrointestinal hormones may play a role in its pathogenesis [177]. Alterations of fasting and postprandial lipoprotein metabolism may also contribute to hepatic fat accumulation [21]. The role of inflammation in the natural history of NAFLD has been extensively discussed in the last few years [24,17]. The association between NAFLD and most of the above mentioned factors has been investigated in previous studies generally focusing on different, selected aspects of this complex issue.

Aim

In the present study, we aimed to evaluate with a more comprehensive, multifactorial manner the relationship of liver fat with metabolic, dietary and inflammatory factors in a cohort of individuals characterized by high cardiovascular risk with no evidence of hepatic disease and with homogenous anthropometric characteristics and nutritional habits.

Materials and Methods

Subjects. Eighty-six individuals of both sexes, aged 35-70 y, with overweight or obesity (BMI 27-35 m/kg²), high waist circumference (men > 102 cm, and women >88 cm) and meeting at least one criterion for metabolic syndrome diagnosis according to the NCEP/ATP III [178], were recruited at the obesity outpatient clinic of the Federico II University Hospital in order to participate in a nutritional intervention study [120]. Data presented in this paper refer to the subjects who underwent liver fat evaluation by ultrasound at baseline (n=70). Exclusion criteria were: fasting plasma triglycerides \geq 400 mg/dl, fasting cholesterol >270 mg/dl, cardiovascular events (myocardial attack or stroke) during the 6 months prior to the study, T2DM, and regular intensive exercise. The participants had no evidence of A, B, or C virus or autoimmune hepatitis; clinical signs or symptoms of inborn errors of metabolism; history of toxins or drugs known to induce hepatitis or any other chronic disease; or use of drugs able to influence inflammation and lipid and glucose metabolism (including statins). Inclusion and exclusion criteria were assessed by interviews, clinical examination and routine laboratory tests. Before inclusion in the study, participants were screened for diabetes status by an OGTT

performed by capillary blood glucose testing. Then they underwent baseline OGTT performed by venous blood glucose testing. Only if participants had no diabetes at screening and baseline tests were included in the study.

Study design, experimental procedures. The participants were randomly assigned to one of four nutritional interventions as previously described in details [120]. The design of the trial was approved by the Federico II University Ethics Committee, complied with the Helsinki Declaration guidelines, and was registered at Clinical-Trials.gov, trial registry #NCT01154478. All participants provided their written informed consent. Before the intervention, body weight, height, and waist circumference were measured according to standardized procedures. Dietary habits were evaluated from a 7-day food record filled in by participants before the start of the intervention and collected by an expert dietitian in occasion of the run-in visit. After a 12-h overnight fast, the participants underwent a 75 g OGTT, with blood sampling at 0, 15, 30, 60, 90, 120, 150 and 180 min. Two days later, after a 12-h overnight fast, they underwent a 1000 kcal test meal with blood sampling at 0, 60, 120, 240 and 360 min. Test-meal composition was similar to the diet assigned to the subjects for the trial: (a) control, low in long chain n-3PUFA (LCn3, 0.94 g) and polyphenols (50 mg), (b) rich in LCn3 (2.31 g) and low in polyphenols (50 mg), (c) rich in polyphenols (770 mg) and low in LCn3 (0.92 g), or (d) rich in LCn3 (2.31 g) and polyphenols (770 mg). All other components of the test meals were similar. Meals were composed of rice, butter, parmesan cheese, bresaola, and white bread, with intakes of olive oil, salmon and decaffeinated green tea differing in order to obtain a similar

composition as the diet in the trial [120]. Blood drawn in EDTA- or EDTA and aprotinin (for GLP-1 assay) tubes was centrifuged and plasma stored at -80°C until measurement. For assessment of hepatic/renal echo intensity ratio (H/R), ultrasound images of both right liver lobe and right kidney were obtained in sagittal view with the patient in lateral position. A region of interest (ROI) of 2x2 cm in the liver parenchyma was selected so that no blood vessels or other focal hypo/hyperechogenicity was crossed to obtain a sample of liver parenchyma alone, avoiding liver lesions. Another ROI of 0.5x0.5 cm was identified in the right renal cortex with no vessels, renal sinus or medulla. The mean echo intensity within the two ROIs was measured. Then, the average intensity of hepatic ROI was divided by the average intensity of renal cortex ROI to calculate the ultrasound hepatic/renal ratio. The use of hepatic/renal ratio for the determination of liver fat content was previously validated against H^1 -MRS [179].

Laboratory methods. Chylomicrons (Svedberg flotation unit >400) and large VLDLs (Svedberg flotation unit 60e400) were isolated from plasma by discontinuous density-gradient ultracentrifugation, as previously described [180]. HDLs were isolated from plasma by the phosphotungstic acid/magnesium chloride precipitation method. LDL cholesterol was calculated by the Friedewald formula. Plasma glucose, cholesterol and triglyceride concentrations were assayed by enzymatic colorimetric methods (ABX Diagnostics, Montpellier, France; Roche Diagnostics, Milan, Italy) on a Cobas Mira Autoanalyzer (ABX Diagnostics, Montpellier, France). Plasma insulin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA; DIAsource Immuno Assays

S.A., Nivelles, Belgium) on Triturus Analyzer (Diagnostics Grifols, S.A., Barcelona, Spain). Plasma active GLP-1 was assayed by a nonradioactive, highly specific sandwich enzyme-linked immunosorbent assay method (Merck-Millipore, Darmstadt, Germany) [181]. Hs-CRP plasma concentrations were determined by a high sensitivity immunoturbidimetric method (Roche Molecular Biochemicals, Mannheim, Germany) with a functional sensitivity of 0.11 mg/L. Intra- and inter-assay variability were, respectively, 0.3% and 1.9%. Plasma interleukins and growth factors were measured using a specific kit for IL-1b, IL-4, IL-6, IL-10, IL-12, IL-17, TNF- α , IFN- γ , granulocyte colony-stimulating factor (G-CSF), basic fibroblast growth factor (FGF) and vascular endothelial growth (VEGF) factor (Bio-Rad Laboratories SRL, Segrate e Italy).

Calculations. Fasting insulin resistance was evaluated by the homeostasis assessment method of the insulin resistance [HOMA-IR = (fasting glucose, mg/dl) x (fasting insulin, mU/l)/405] [182]. After glucose load, insulin action was evaluated by the 180-minutes oral glucose insulin sensitivity method (OGIS) [182]. Post-glucose insulin secretion capacity was calculated as β -cell function [insulin AUC/glucose AUC ratio] [182]. Total (AUC) and incremental (iAUC) areas under the curve after the glucose load and the test-meal were calculated by the trapezoidal method.

Statistical analysis. Data are expressed as mean \pm SD or median and IQR. Variables not normally distributed were analyzed after logarithmic transformation. Bivariate Pearson's correlations were calculated to examine the

relation between liver fat content and metabolic, dietary and anthropometric variables. All variables found to correlate significantly with liver fat content, in addition to age, BMI, and sex were entered into a multiple regression analysis in a stepwise fashion having liver fat as the dependent variable. The possible influence of the different test-meals and the glucose tolerance status was accounted for by including in the model as dummy variables three indicator variables for test-meals (rich in LCn3, rich in polyphenols, rich in LCn3 and polyphenols) and three indicator variables for glucose tolerance status (IFG, IGT, IFG+IGT). The reference groups, that we compared the other groups against, were the control test-meal group and the normal glucose tolerant group, respectively. A $P < 0.05$ was considered statistically significant. Statistical analysis was performed according to standard methods using the Statistical Package for Social Sciences software 20.0 (SPSS/PC; SPSS, Chicago, IL, USA).

Results

The main characteristics of our cohort are shown in **Table 7**. Participants were equally distributed between sex, and were overweight/obese and moderately insulin resistant as shown by HOMA-IR mean values. Twenty participants had impaired fasting glucose (IFG), 7 impaired glucose tolerance (IGT), and 18 IFG+IGT. The average hepatic/renal ratio in the cohort was 1.43 (0.76) (median (IQR)) with an overall prevalence of liver steatosis of 17% considering an H/R cut-off point of 2.2 for steatosis diagnosis [179]. On the average, the participants had healthy dietary habits as shown by the composition of their habitual diet (**Table 8**).

Table 7. Main characteristics of the participants.

Sex (M/F)	29/41
Age (y)	54 ± 9 (39-70)
Body weight (kg)	86 ± 12 (60-115)
BMI (kg/m ²)	31 ± 3 (25-38)
Waist circumference (cm)	103 ± 8 (89-127)
Fasting plasma cholesterol (mg/dl)	195 ± 30 (124-255)
Fasting HDL cholesterol (mg/dl)	43 ± 11 (24-72)
Fasting LDL cholesterol (mg/dl)	118 ± 26 (71-198)
Fasting plasma triglyceride (mg/dl)	103 (67)
Fasting plasma glucose (mg/dl)	103 ± 11 (75-129)
Fasting plasma insulin (mU/l)	19 ± 6 (5-32)
HOMA-IR	4.8 ± 1.8 (1.0-9.5)
IFG (n)	20
IGT (n)	7
IFG + IGT (n)	18
SBP (mm Hg)	120 (14)
DBP (mm Hg)	73 (10)
Hepatic/renal echo intensity ratio	1.43 (0.76)
Participants with liver steatosis (n)	12

Data are M±SD (range) or median (IQR).

HOMA-IR: homeostasis assessment method of the insulin resistance; IFG: Impaired Fasting Glucose; IGT: Impaired Glucose Tolerance; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Table 8. Composition of the habitual diet of the study participants (n = 70) as evaluated by 7-day food record.

Energy (kcal/day)	1706 (593)
Protein (% DEI)	17 ± 2 (12-27)
Total fat (% DEI)	33 ± 5 (20-43)
SFA (% DEI)	10 ± 2 (6-15)
MUFA (% DEI)	15 ± 3 (7-21)
PUFA (% DEI)	3.8 (0.93)
Carbohydrate (% DEI)	50 ± 5 (39-62)
Sugars (% DEI)	17 ± 4 (38-155)
Fiber (g/day)	20 ± 6 (6-17)
Vitamin C (mg/day)	103 (105)
Vitamin E (mg/day)	8.9 (4.1)
Polyphenols (mg/day)	552 (417)
b-carotene (mg/day)	2192 (2239)
FRAP (mmol eF2+/kg FW3)	187 (58)
Alcohol (g/day)	3.3 ± 6.7

Data are M ± SD (range) or median (IQR).

DEI: daily energy intake; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids. FRAP: ferric reducing antioxidant potential.

Bivariate correlations. Many anthropometric, metabolic, inflammatory and dietary variables were related to liver fat content (**Table 9**). Liver fat content was positively correlated with waist circumference, insulin resistance (HOMA-IR), fasting levels of plasma glucose and insulin, post-challenge insulin and glucose iAUCs and a dynamic index of β -cell function, and negatively correlated with the OGTT-derived index of insulin sensitivity (OGIS). Postprandial GLP-1 levels, either as iAUC or GLP-1 concentration 120 min after meal, were inversely related to hepatic/renal ratio. The pro-inflammatory cytokines and growth factors, IL-17, IFN- γ , IL-4, TNF- α , FGF and GCSF, but not Hs-CRP, were positively and significantly correlated with liver fat. Fasting HDL cholesterol was inversely related with liver fat. Postprandial incremental areas of cholesterol and triglyceride concentrations in large VLDL and total cholesterol in plasma were

directly associated with hepatic fat content. Among dietary factors, polyphenol, fiber intake, and Ferric Reducing Antioxidant Potential (FRAP) were significantly and negatively related with liver fat content.

Multiple regression analysis. All variables that correlated significantly with liver fat content (**Table 9**) plus age, sex, BMI, test-meal type and glucose tolerance status were included in multiple regression analysis.

Table 9. Bivariate Spearman correlations between hepatic/renal ratio and metabolic, inflammatory and dietary variables.

Variable	r coefficient	p value	Variable	r coefficient	p value
Anthropometric parameters			Dietary variables		
Body mass index	0.196	0.104	Daily energy intake	0.156	0.205
Waist circumference	0.338	0.004	Carbohydrates (% DEI)	0.115	0.352
Fasting glucose metabolism			Sugars (% DEI)	-0.120	0.328
Plasma glucose	0.243	0.042	Protein (% DEI)	-0.107	0.387
Plasma insulin	0.410	0.000	Total fat (% DEI)	-0.052	0.675
HOMA-IR	0.433	0.000	SAFA (% DEI)	0.112	0.362
OGTT indices			MUFA (% DEI)	-0.131	0.287
Plasma glucose 3h-iAUC	0.350	0.004	PUFA (% DEI)	-0.158	0.198
Plasma insulin 3h-iAUC	0.413	0.001	n-6/n-3	-0.229	0.060
OGIS	-0.489	0.000	Polyphenols	-0.258	0.034
β -Cell Function	0.270	0.025	FRAP	-0.257	0.035
Post-prandial GLP-1			Fiber	-0.284	0.019
GLP-1 3h-iAUC	-0.339	0.004	Inflammatory variables		
GLP-1 t120	-0.334	0.005	Hs-CRP	0.064	0.599
Lipid metabolism			IFN- γ	0.273	0.023
Fasting plasma chol	-0.029	0.812	IL-17	0.319	0.008
Fasting plasma triglyceride	0.214	0.076	IL-4	0.322	0.007
Fasting HDL chol	-0.411	0.000	TNF- α	0.263	0.035
PP plasma chol 6h-iAUC	0.270	0.024	FGF	0.299	0.017
PP IVLDL1 chol 6h-iAUC	0.303	0.011	GCSF	0.252	0.041
PP plasma tg 6h-iAUC	0.201	0.096			
PP VLDL1 tg 6h-iAUC	0.292	0.015			

DEI: daily energy intake; OGIS: oral glucose insulin sensitivity; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids; FRAP: ferric reducing antioxidant potential; Hs-CRP: high sensitivity C-reactive protein; TNF- α : tumor necrosis factor- α ; IFN- γ : interferon- γ ; IL: interleukin; FGF: fibroblast growth factor; GCSF: granulocyte colony-stimulating factor, PP: postprandial, chol: cholesterol, tg: triglycerides.

As shown in **Table 10**, OGIS was the best predictor of liver fat content predicting about 30% of liver fat variability. By stepwise analysis, also postprandial GLP-1, HDL cholesterol levels and IFN- γ were independent predictors of liver fat content.

Table 10. Stepwise regression analysis of independent contribution of metabolic, dietary and inflammatory variables to liver fat content.

	β	R ²	p value
Step 1		0.341	
OGIS	-0.595		0.000
Step 2		0.450	
OGIS	-0.499		0.000
GLP-1 t120	-0.355		0.002
Step 3		0.526	
OGIS	-0.390		0.000
GLP-1 t120	-0.381		0.000
HDL Cholesterol	-0.305		0.004
Step 4		0.558	
OGIS	-0.352		0.001
GLP-1 t120	-0.344		0.001
HDL Cholesterol	-0.323		0.002
IFN- γ	0.205		0.036

OGIS: oral glucose insulin sensitivity; IFN- γ : interferon- γ . Variables included in the model were: age, BMI, sex, test-meal type, glucose tolerance status, and those founded significantly correlated with liver fat at bivariate analysis as listed in Table 3.

Discussion

This study shows that in a population at high cardiometabolic risk homogeneous for metabolic characteristics and dietary habits, and with a relatively low prevalence of liver steatosis, there was a significant variability in liver fat content related to dietary intakes, indices of fasting and post-load glucose metabolism, postprandial lipid concentrations and inflammatory markers. In particular, the best

independent predictors of liver fat content in our cohort were OGIS, postprandial GLP-1, HDL cholesterol concentrations and fasting IFN- γ . The relationship between liver steatosis and insulin resistance/sensitivity has been clearly demonstrated by several studies in various populations with NAFLD: T2DM patients [10], individuals with metabolic syndrome [6], and otherwise healthy people [21]. Kahl *et al.* [183] evaluated this relationship for a range of hepatic fat content non diagnostic for liver steatosis showing, in line with our results, that both fasting and dynamic indices of glucose metabolism were strongly related with liver fat. Therefore, insulin-resistance may be not only a consequence of hepatic lipid accumulation but also one of its pathophysiological determinants. Interestingly, in our cohort, postchallenge insulin sensitivity (OGIS) was a better predictor of liver fat content than the fasting insulin resistance index (HOMA-IR). This may suggest that hepatic fat accumulation is mainly driven by iterative overflow conditions for hepatic pathways of energy clearance, such as postchallenge/postprandial states.

Also fasting HDL cholesterol levels were independently associated with liver fat content in our cohort. Since HDL cholesterol level is a very good marker of insulin resistance, this could explain its strong relationship with liver fat that has been already reported [184]. However, in our study the relationship between liver fat and HDL cholesterol was independent of other factors including insulin resistance; therefore, other mechanisms should be considered including the higher catabolic rate of ApoA1 that, as recently shown, is independently associated with liver fat content [185].

In our study, postprandial levels of GLP-1 were significantly, independently related to hepatic fat content. This finding – lower postprandial GLP-1 concentrations in people with higher liver fat – is in line with previous observations that individuals with NAFLD had significantly lower GLP-1 levels after glucose load than healthy subjects [186] and treatment with GLP-1 analogs decreased liver fat content [177]. Moreover, in vitro and animal studies showed that GLP-1 has direct effects on hepatocytes through the activation of a GLP-1 receptor regulating glucose metabolism in the liver and protecting against hepatocellular injuries [187]. Moreover, in human studies, the GLP-1 agonist exenatide inhibits postprandial absorption of chylomicrons that are a main source of hepatic triglyceride accumulation [188]. This would further confirm the “postprandial genesis” of liver steatosis. Inflammatory markers as well significantly predicted liver fat content in the participants in our study. Several previous studies reported a positive relationship between non-specific markers of systemic inflammation such as TNF- α , IL-6 and IL-1 and liver steatosis [24]. However, scanty data are available on the relationship between metabolic liver disease and markers of adaptive immunity. In particular, we observed that liver fat content correlated with circulating levels of IFN- γ independently of other factors, including other inflammatory markers. This observation is of particular relevance if we consider the recent evidence associating obesity with markers of adaptive immunity, particularly the activation of T-helper lymphocytes by endogenous stimulus, such as lipopolysaccharide deriving from gut microbiota [25,189]. This suggests, in line with the parallel hits hypothesis [24], that inflammation related with adaptive immunity activity may act as a *primum movens* in the natural

history of NAFLD, playing a relevant role also in accumulating fat in the liver. In our study, liver fat was associated also with dietary factors (dietary fiber, polyphenols, FRAP) and metabolic abnormalities, *in primis* the concentrations of cholesterol and triglycerides in plasma and large VLDL. These associations were not present at multiple regression analysis suggesting that their relationship with liver fat may be mediated by their strict relation with other mechanisms, such as insulin resistance, inflammation and GLP-1.

This study has some strengths and limitations. A strength is that we evaluated the relationships between liver fat and different putative pathogenic pathways in a very homogeneous population of individuals at high cardiometabolic risk with a relatively low level of liver steatosis. The very similar characteristics of our participants allowed us to recognize coherent interrelationships among different pathophysiologic aspects, suggesting that in addition to insulin resistance, innate immunity and postprandial metabolism alterations also play a relevant role in the determinism of NAFLD. Although the cross-sectional design did not allow us to approach cause-effect relationship issues, the results certainly underline that NAFLD pathophysiology includes the vast majority of pathogenic factors involved in the determinism of CVD. Therefore, the clinical relevance of this condition is not limited to its direct health impact, but also represents the macroscopic synthesis of the molecular mechanisms that trigger the atherosclerotic process. Moreover, the higher predictive value of postprandial compared to fasting variables (e.g. OGIS vs. HOMA; postprandial vs. fasting GLP-1) suggests that the main pathophysiological pathways of liver steatosis act in the postprandial phase. Possible limitations of our study are the small sample

size and the fact that we did not use the gold standard for noninvasive measurement of liver fat, i.e. H^1 -MRS. However, the ultrasound hepatic/renal ratio was validated versus H^1 -MRS [179]. Moreover, all the strong and consistent relationships between hepatic/renal ratio and metabolic measurements observed in this study further confirm the method reliability.

In conclusion, insulin resistance, systemic inflammation and postprandial GLP-1 were the main determinants of liver fat in a selected cohort of people at high cardio-metabolic risk with a rather low level of liver steatosis, explaining altogether about 30% of liver fat variability. The different factors implicated in the pathogenesis of NAFLD are also involved in the pathogenesis of atherosclerosis, suggesting that NAFLD may represent the tip of the iceberg of the complex metabolic derangements leading to CVD.

5.2. FRUCTOSE trial

Background

Among nutritional factors, epidemiologic studies suggesting a possible link between sugar sweetened beverages and CVD risk factors such as T2DM, obesity, hypertension and dyslipidemia [190–197]. In addition, overconsumption of dietary sugars as fructose has been linked to NAFLD. Fructose has been shown to promote hepatic lipogenesis by stimulating SREBP-1c and ChREBP, two master transcriptional regulators of DNL [19, 192, 1198-200]. Fructose also leads to ATP depletion and suppression of hepatic mitochondrial fatty acid oxidation, thus favoring liver fat storage [13,95-96]. Excess consumption of fructose may also operate indirectly by delivering extra energy leading to weight gain and ectopic fat depots including liver fat content [97,190,195,197,97,201]. Recent meta-analyses have identified several limitations in clinical studies investigating the adverse metabolic effects of fructose (such as small sample sizes, short intervention periods, variable doses of fructose intake and studies in healthy lean subjects with low baseline liver fat content) [202-204]. These limitations may explain the discrepant conclusions of the trials studying the effect of fructose consumption on liver fat content [201]. It appears that a hypercaloric fructose diet increases liver fat content in obese subjects [90]. However, it still remains unclear whether this is due to direct metabolic effects of fructose or merely a result of increased energy intake. Support for direct metabolic effects of fructose comes from studies showing that dietary sugars, in particular fructose, increase DNL and liver fat in humans [94,96,108, 205-208].

Aim

The aim of this study was to assess the effects of fructose (75 g day for 12 weeks served as a lemonade together with habitual ad libitum diet) on liver fat content measured by magnetic resonance examinations in a large cohort of abdominally obese men with and without other cardiometabolic risk factors. We also analyzed changes in body composition, dietary intake, an extensive panel of cardiometabolic risk markers, hepatic DNL as well as responses of postprandial lipids to a standardized oral fat tolerance test (OFTT).

Materials and Methods

Subjects. A total of 82 obese healthy men were recruited to the study (Clinical Trials NCT01445730) at four centers: in Helsinki, Finland; Naples, Italy; Quebec, Canada; and Gothenburg, Sweden. Subjects were recruited via newspaper advertisements. Inclusion criteria were as follows: men with large waist circumference (>96 cm), BMI between 27 and 40 kg/m², stable weight (\pm 3 kg) over the preceding 3 months, low-density lipoprotein (LDL) cholesterol <4.5 mmol/L and serum triglycerides (TG) <5.5 mmol/L. Exclusion criteria were as follows: age <20 years or >65 years, BMI or lipid levels outside the inclusion ranges, smoking, alcohol consumption over 2 doses day (i.e. 20 g pure alcohol), T2DM, CVD, hormonal therapy, hepatic and renal diseases, gastroenterological, thyroid or haematological abnormalities, and any chronic disease requiring medication except for controlled hypertension. None of the subjects used any medication or hormones known to influence lipoprotein metabolism. The study design was approved by the local ethics committees, and each subject gave

written informed consent before participation in the study. All studies were performed in accordance with the Declaration of Helsinki for clinical trials.

Fructose intervention. Subjects underwent a 12-week fructose intervention period, during which they consumed 75 g of fructose daily (303 kcal), administered as three 330-mL bottles. The carbonated beverages were prepared as 7.6% (w/w) solutions and flavoured with lemon aroma (produced for this study by Nokian Panimo Oy, Finland). Subjects were instructed to consume the beverages together with the three main daily meals whilst continuing their habitual ad libitum diet during the intervention. The fructose-sweetened beverage was well tolerated. Of the 82 subjects, two subjects discontinued the intervention study: one developed a skin rash which was considered as possible allergic reaction to flavouring. The rash disappeared after the discontinuation. The other subject discontinued the study in response to his dentist's advice. In addition, the data from magnetic resonance examinations at baseline or after the fructose intervention were not sufficient from nine subjects for technical reasons to allow the analyses of different fat depots. Thus, 71 subjects completed the full study protocol. The subjects' weight and height were measured in the study center after an overnight fast and barefoot with underwear. The waist circumference was recorded at the midpoint between the lower rib margin and the iliac crest. Three consecutive readings were taken, and the mean was recorded. A qualified nutritionist gave detailed verbal and written instructions for filling in the food records. The compliance was assessed based on weekly reporting of adherence to fructose beverages on a compliance sheet where the subjects indicated the number of

beverages missed during the week. The dietician contacted (i.e. face to-face visits, phone calls or email messages) the subjects once per week to monitor weight and compliance. Each subject kept a 3-day food record (2 work days and 1 day off) before the fructose intervention period and again within 2 weeks before completing the intervention period. Participants were not required to weigh foods but were asked to measure the volume of foods consumed with household measurements or to indicate the weight of the products. After completing the food records, participants met with the local dietitians to review the food records for completeness. Energy and nutrient intake were calculated by linking the food intake information with local food composition databases. The 3-day energy and nutrient values were averaged to obtain mean intakes for each subject.

Study design. The protocol included two separate study visits before the fructose intervention period: OFTT and magnetic resonance examinations. These visits were repeated within 2 weeks before completing the 12-week fructose intervention period. A standardized OFTT was performed in the morning after an overnight fast. The subjects received a fat-rich meal (927 kcal) consisting of bread, butter, cheese, ham, boiled eggs, fresh red pepper, low-fat (1%) milk, orange juice and tea or coffee (63 g carbohydrate, 56 g fat and 40 g protein). Blood samples were drawn before and at 2, 3, 4, 6 and 8 h after the meal. During the test, only water was allowed ad libitum and the subjects remained physically inactive. The participants abstained from alcohol and physical exercise for 2 days before each examination. In a subgroup of 56 subjects, DNL was analyzed before and at 4 and 8 h after the meal during the OFTT. A blood sample was drawn as a

background sample in the week before the OFTT. Subjects received 2 g/kg body weight deuterated water $^2\text{H}_2\text{O}$ (Larodan Fine Chemicals, Sweden) which was consumed in two servings together with evening meal on the day before the OFTT [209]. ^1H -MRS was performed using a 1.5-T whole-body device to determine liver fat content [210,211]. Magnetic resonance imaging was used to determine subcutaneous abdominal and intra-abdominal fat expressed as volumes [212]. A standardized protocol was used at all centers, and all analyses of the imaging results were performed by one person. Subjects were advised to fast for 4 h before imaging.

Laboratory methods. Lipoprotein fractions [chylomicrons (Sf > 400), large very low-density lipoprotein (VLDL1) particles (Sf 60–400) and smaller VLDL2 particles (Sf 20–60)] from blood samples drawn before and during the OFTT were separated by density gradient ultracentrifugation. As all fractions contain both apolipoprotein (apo) B48 and apoB100 particles, they were further analyzed using SDS-PAGE [213]. TG and cholesterol concentrations in total plasma and in lipoprotein fractions were analyzed by automated enzymatic methods using the Konelab 60i analyzer (Thermo Fisher Scientific, Finland). Fasting and postprandial apoB48 levels in total plasma were measured by ELISA (Shibayagi Co., Shibukawa, Japan). Fasting and postprandial concentrations of glucose (hexokinase method, Roche Diagnostic Gluco-quant, Germany) and insulin (electrochemiluminescence with Roche sandwich immunoassay on a Cobas autoanalyser) were measured after the fat-rich meal. Plasma levels of apoC-III, fibroblast growth factor 21 (FGF-21) and adiponectin were measured by ELISA

(R&D Systems, Minneapolis, USA), and 3-hydroxybutyrate concentrations were measured by an enzymatic method with β -hydroxybutyrate FS kit (DiaSys Diagnostic Systems, Holzheim, Germany) on a Konelab 60i analyser (Thermo Fisher Scientific, Finland).

Statistical analysis. Statistical analyses were performed using R version 3.1.1 for Windows, Stata (version 13.0, Stata Corporation, TX, USA) and GraphPad Prism version 7. For all variables, P values were calculated using the Wilcoxon signed-rank test or the Mann–Whitney U-test. As many clinical variables could not be assumed to be normally distributed, these nonparametric tests were used. Correlation coefficients and their corresponding P values were calculated using Spearman’s rank test. A P value <0.05 was considered statistically significant. Stepwise regression analysis was performed using the ‘step’ function in R. Bidirectional elimination was used in the selection of variables. The underreporting of energy intake was evaluated by determining the ratio of reported energy intake to estimated basal metabolic rate (BMR). The BMR was estimated from the age- and gender-specific equations proposed by Schofield [214]. A cut-off value of 0.9 was used to identify extreme underreports of energy intake [215]. All analysis involving energy intake was subjected to a sensitivity analysis in which we excluded under reporters, but because results were virtually unchanged, only the results including all subjects are presented. To elucidate the mechanisms for the responses of liver fat to the diet intervention, we divided the subjects into three groups according to their change in liver fat after fructose. Group 1 (n = 22) had reduced liver fat content (from $7.2 \pm 1.4\%$ at baseline to 5.5

$\pm 1.3\%$ after fructose feeding), Group 2 ($n = 20$) had no or minimal liver fat change, and Group 3 ($n = 29$) gained liver fat (from $8.5 \pm 1.2\%$ to $11.2 \pm 1.2\%$).

Results

Study cohort characteristics at baseline. Baseline data of the 71 men who completed the 12-week fructose intervention are shown in **Table 11**. The subjects showed a wide range of BMI (25.6 – 38.3 kg/m²), liver fat (0.3 – 24.8%) and other adiposity indices. BMI correlated modestly with visceral fat ($r = 0.28$, $P = 0.02$) but not with liver fat content. Visceral fat ($r = 0.35$, $P = 0.004$) but not waist circumference or subcutaneous fat correlated significantly with liver fat. The HOMA index averaged $2.8 \pm 1.8\%$ with a large range (0 – 8.5%) and correlated modestly with liver fat ($r = 0.31$, $P = 0.008$), baseline DNL ($r = 0.36$, $P = 0.008$) and FGF-21 ($r = 0.33$, $P = 0.004$) but not with weight or visceral fat. Baseline DNL also correlated with fasting insulin ($r = 0.40$, $P = 0.003$) but not with fasting glucose nor with liver fat content. Of the 71 participants, 27 had elevated fasting TG levels (>1.7 mmol/L). These men had an overall worse cardiometabolic profile with higher HOMA index, blood glucose, apoC-III, FGF-21, and uric acid and lower adiponectin than subjects with normal fasting TG levels. However, the two groups had comparable weight and waist circumference and did not significantly differ in either liver fat content ($6.5 \pm 1.0\%$ vs. $6.9 \pm 0.9\%$) or visceral and subcutaneous fat depots. There was a trend for a higher VAT/SAT ratio in the subjects with elevated TG levels ($P = 0.058$).

Table 11. Characteristics of the study subjects (n = 71), before and after fructose feeding. The data are mean ± SD. Ranges are indicated as minimum and maximum values. Changes of the means after versus before are shown with ± SD. P-values have been calculated using the Wilcoxon signed-rank test

	Before		After		Change Mean ± SD	P-value
	Mean ± SD	Range	Mean ± SD	Range		
Age (years)	49.1 ± 10	21–65				
Weight (kg)	99.4 ± 12	65.2–135	100.5 ± 12	66.8–137	1.1 ± 1.7	<0.0001
BMI (kg m ⁻²)	30.6 ± 2.9	25.6–38.3	30.9 ± 3	26.2–38.6	0.3 ± 0.53	<0.0001
Waist (cm)	109.3 ± 8.2	94–133	109.8 ± 8.1	96–131	0.5 ± 2.8	0.009
Liver fat (%)	6.66 ± 6.1	0.3–24.8	7.33 ± 6.6	0.6–27.7	0.67 ± 2.2	0.008
Systolic BP (mmHg)	132 ± 13	108–170	135 ± 14	113–180	3 ± 10.7	0.02
Diastolic BP (mmHg)	86 ± 8.8	68–113	87.3 ± 9.6	69–111	1.3 ± 6.9	0.18
Heart rate (bpm)	64.2 ± 10	47–105	65.5 ± 11	45–90	1.3 ± 8.7	0.25
Glucose (mmol L ⁻¹)	5.41 ± 0.46	4.3–6.5	5.46 ± 0.43	4.4–6.9	0.05 ± 0.42	0.46
Insulin (pmol L ⁻¹)	83.7 ± 51	21–261	97.2 ± 71	6–387	13.5 ± 44.6	0.004
HOMA-IR	2.78 ± 1.8	0.75–8.5	3.44 ± 2.6	0.2–14.1	0.66 ± 1.9	0.002
DNL (μmol L ⁻¹)	12.3 ± 10	0.06–47.4	16.5 ± 11	0.1–51.7	4.2 ± 11.7	0.005
FFA (μmol L ⁻¹)	428 ± 130	127–688	427 ± 130	173–838	-1 ± 143	0.79
Triglycerides (mmol L ⁻¹)	1.56 ± 0.9	0.6–4.7	1.67 ± 0.88	0.5–4.3	0.11 ± 0.57	0.02
Tot-chol (mmol L ⁻¹)	4.79 ± 0.8	3.0–6.8	4.86 ± 0.84	3.1–7.3	0.07 ± 0.38	0.12
LDL-chol (mmol L ⁻¹)	3.27 ± 0.79	1.4–5	3.34 ± 0.86	1.2–5.9	0.07 ± 0.35	0.21
HDL-chol (mmol L ⁻¹)	1.12 ± 0.32	0.6–2.1	1.12 ± 0.32	0.5–2	0 ± 0.11	0.85
ApoC-III (mg dL ⁻¹)	10.4 ± 5.1	1.8–28.5	11.2 ± 5	1.1–26	0.8 ± 3.1	0.006
FGF-21 (pg mL ⁻¹)	180 ± 140	20–632	226 ± 310	42.1–728	46 ± 224	0.07
β-OH butyrate (mg dL ⁻¹)	0.699 ± 0.55	0.1–2.6	0.542 ± 0.48	0.1–3.7	-0.16 ± 0.66	0.01
Adiponectin (ng mL ⁻¹)	4.48 ± 2.7	1.3–12.3	4.25 ± 2.3	1.4–12.1	-0.23 ± 0.85	0.09
Uric acid (μmol L ⁻¹)	380 ± 66	244–510	385 ± 64	228–556	5 ± 48.3	0.23
Liver fat (%)	6.66 ± 6.1	0.3–24.8	7.33 ± 6.6	0.6–27.7	0.67 ± 2.2	0.008
Visceral fat (cm ³)	2540 ± 999	538–5434	2597 ± 1020	612–5397	57 ± 404	0.30
Subcutaneous fat (cm ³)	4136 ± 1370	1820–8491	4201 ± 1420	1832–8389	65 ± 324	0.10
VAT/SAT ratio	0.68 ± 0.32	0.089–1.78	0.69 ± 0.32	0.1–1.7	0.01 ± 0.11	0.47

ALT, Alaninaminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. Significant of p-values <0.05 are bold.

Effects of fructose feeding on diet composition. Dietary intake calculated from 3-day food records at baseline and during the fructose intervention period are shown in **Table 12**. As expected, the consumption of 75 g of fructose per day caused significant changes in the macronutrient composition. However, because the study subjects adjusted their diets, the increased energy intake during the fructose intervention was small (only 54 kcal) and did not reach statistical significance. The proportion of energy from total carbohydrates was higher, but that of sucrose, protein, total fat and saturated and unsaturated fatty acids was lower during the fructose intervention period. Although the average intake of total fat reduced significantly by 6.6 ± 3.3 g day, there were large variations in the individual changes of saturated fat intake during fructose intervention (from -33.5 to 28.6 g/day). The energy provided by fructose was $2.5 \pm 0.2\%$ of the total energy intake at baseline and increased to $14.7 \pm 0.3\%$ during the intervention. During the fructose diet, $12.9 \pm 0.3\%$ of energy was from added fructose and $1.8 \pm 0.1\%$ of energy was from fructose present in the habitual diet. Intake of cholesterol, total fibre and alcohol was not significantly different between baseline and the fructose intervention period (**Table 12**).

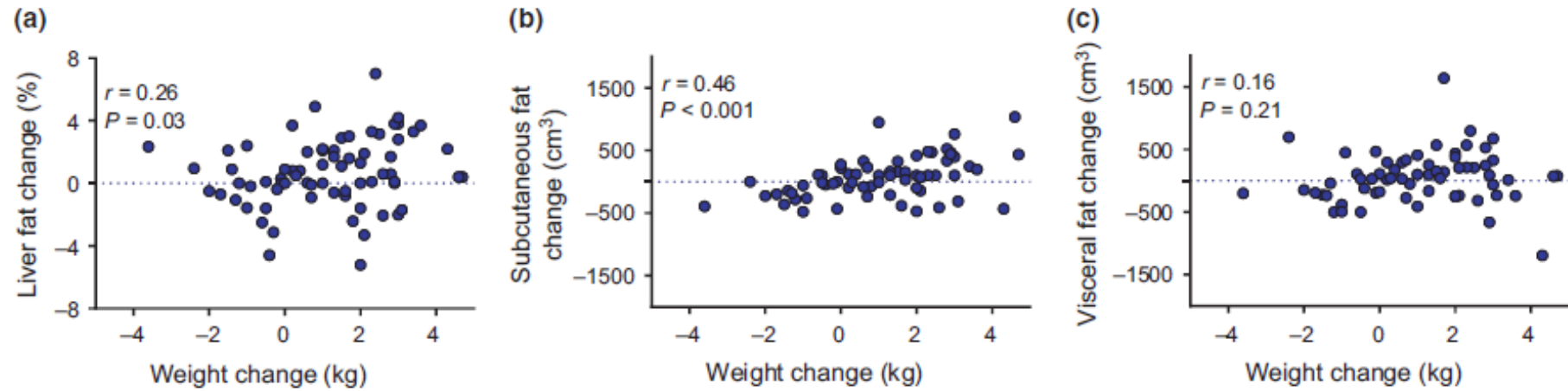
Effects of fructose feeding on body composition. Fructose intervention resulted in minor but significant increases in weight ($1.1 \pm 1.7\%$) and waist circumference ($0.67 \pm 2.5\%$) (**Table 11**). Changes in waist circumference correlated positively with changes in weight ($r = 0.53$, $P < 0.001$) and subcutaneous fat area ($r = 0.40$, $P = 0.002$) but only modestly with changes in visceral fat ($r = 0.29$, $P = 0.02$) and liver fat ($r = 0.30$, $P = 0.01$). There was a large variation in individual weight

Table 12. Reported dietary intake before and after fructose intervention. Values are shown as mean \pm SD. P-values have been calculated using the Wilcoxon signed-rank test and refer to differences between baseline and after fructose intervention.

	Baseline	After	Change	P-value
Energy (kcal day ⁻¹)	2367 \pm 560	2421 \pm 493	54 \pm 453	0.14
Carbohydrates (% of EI)	43.6 \pm 8.2	48.7 \pm 7.7	5.2 \pm 6.9	<0.0001
Protein (% of EI)	17.5 \pm 3.3	15.7 \pm 2.9	-1.8 \pm 3.4	<0.0001
Fat (% of EI)	34.8 \pm 6.5	31.6 \pm 6.2	-3.2 \pm 6.2	<0.0001
Fat (g day ⁻¹)	92 \pm 29	85.4 \pm 26	-6.6 \pm 27	0.046
Saturated fatty acids (% of EI)	12.2 \pm 3.7	10.6 \pm 3.2	-1.6 \pm 3.1	<0.0001
Saturated fatty acids (g day ⁻¹)	32.7 \pm 14	28.8 \pm 12	-3.9 \pm 12	0.017
Monounsaturated fatty acids (% of EI)	12.5 \pm 2.9	11.7 \pm 2.5	-0.8 \pm 3	0.09
Monounsaturated fatty acids (g day ⁻¹)	32.9 \pm 11	31.9 \pm 10	-1 \pm 11	0.60
Polyunsaturated fatty acids (% of EI)	5.3 \pm 1.7	4.9 \pm 1.5	-0.4 \pm 1.7	0.08
Polyunsaturated fatty acids (g day ⁻¹)	13.9 \pm 5.4	13.3 \pm 5.1	-0.6 \pm 5.9	0.49
Fructose (g day ⁻¹)	14.3 \pm 7.9	85.9 \pm 6.9	71.5 \pm 8.3	<0.0001
Sucrose (g day ⁻¹)	42.2 \pm 28	34.1 \pm 21	-8.0 \pm 22	0.005
Cholesterol (mg day ⁻¹)	321 \pm 138	320 \pm 147	-2 \pm 133	0.97
Fibre (g day ⁻¹)	23.1 \pm 8.9	21.2 \pm 8.1	-2 \pm 8.5	0.11
Alcohol (g day ⁻¹)	9.5 \pm 14	9.3 \pm 15	-0.1 \pm 9	0.53

EI, Energy intake. Significant of p-values <0.05 are bold.

Figure 3. Correlation between weight change and change of liver fat content (a), weight change and subcutaneous fat area change (b), and weight change and visceral fat area change (c) in response to fructose feeding. Change in subcutaneous fat area shows the closest correlation to weight change.



response to the fructose intervention: the majority ($n = 37$) gained weight (>1 kg), 26 subjects remained weight stable and eight subjects lost weight (>1 kg). There were no significant differences in total energy intake between these three groups during the diet intervention and no correlation between changes of energy intake and body weight (data not shown). Liver fat content was increased by about 10% after the 12-week fructose intervention (6.7 ± 0.7 vs. $7.3 \pm 0.8\%$, $P < 0.01$), but no significant changes were seen in visceral or subcutaneous fat depots (**Table 11**). There was a positive correlation between changes in liver fat and weight ($r = 0.26$, $P = 0.03$) after fructose feeding (**Figure 3a**). Notably, changes of subcutaneous fat correlated strongly with changes of weight ($r = 0.46$, $P < 0.001$) (**Figure 3b**), but no correlation was observed between changes of visceral fat and weight ($r = 0.16$, NS). Changes of liver fat content correlated also with respective changes of waist circumference ($r = 0.30$, $P = 0.01$), subcutaneous fat ($r = 0.37$, $P = 0.002$), insulin ($r = 0.25$, $P = 0.04$) and HOMA ($r = 0.31$, $P = 0.01$). We observed no correlation between the changes of saturated fat intake and liver fat ($r = 0.18$, $P = 0.14$). Next, we performed a multivariate regression analysis of seven selected parameters (changes in subcutaneous fat, FGF-21, apoC-III, saturated fat intake, fructose intake, DNL and total fat intake) to test their explanatory power for changes of liver fat in response to the diet intervention. The whole model explains 27% of the variance (adjusted R-squared), but no individual variable explained more than 5% of the variance alone. To further elucidate the mechanisms for the responses of liver fat to the diet intervention, we divided the subjects into three groups according to their change in liver fat after fructose. Group 1 ($n = 22$) had reduced liver fat content (from $7.2 \pm 1.4\%$ at baseline to $5.5 \pm 1.3\%$ after fructose feeding),

Group 2 (n = 20) had no or minimal liver fat change, and Group 3 (n = 29) gained liver fat (from $8.5 \pm 1.2\%$ to $11.2 \pm 1.2\%$). Adverse changes of cardiometabolic risk factors were most common in Group 3 (**Table 13**). Next, we analyzed differences in diet that could explain the different responses between Group 1 and Group 3. We showed that subjects who gained most liver fat had slightly lower fructose intake at baseline than subjects who lost liver fat (11.0 ± 1.3 g/L vs. 16.4 ± 2.0 g/L). In addition, these subjects increased significantly calorie intake ($P = 0.03$) probably due to less clear reduction in saturated fat intake. However, the actual difference of changes between the two groups showed only a nonsignificant trend towards higher intake of energy and saturated fat after fructose feeding. To clarify the impact of genetic polymorphisms, we analyzed three polymorphisms that are known to modify the liver fat metabolism: PNPLA3, TM6SF2 and MBOAT7. We showed that increased numbers of risk alleles correlated with increased liver fat content before diet intervention (**Figure 4a**). However, there was no difference in the number of risk alleles between Group 1 and Group 3 (**Fig. 4b**). Furthermore, there were no differences in liver fat change in response to the diet intervention between individuals with and without risk alleles (**Fig. 4c**). Thus, these three polymorphisms did not explain the different responses between Group 1 and Group 3.

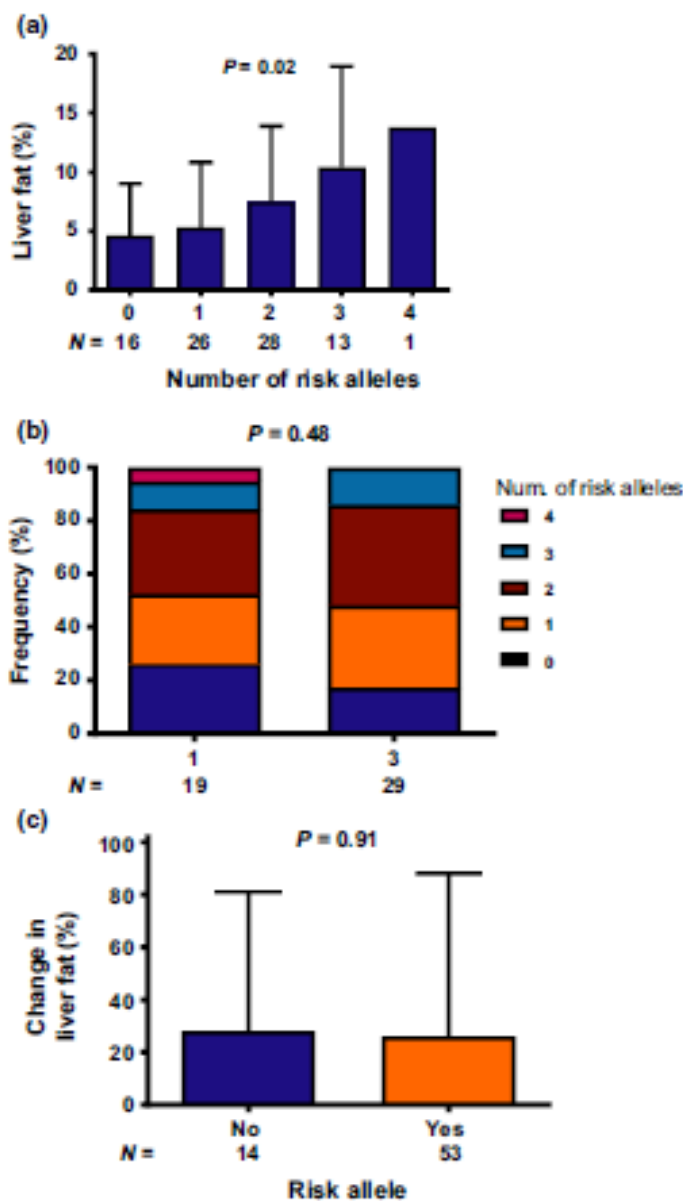
Effect of fructose feeding on cardiometabolic risk markers and hepatic lipid metabolism. We next analyzed the impact of the diet intervention on postprandial lipid responses. At baseline, plasma TG and apoB48 levels increased at early time points after the fat-rich meal (**Figure 5**). Importantly, baseline apoC-III showed

Table 13. Cardiometabolic risk factors in subjects with reduced or increased liver fat. Group 1 (n = 22) had reduced liver fat content (from 7.2 ± 1.4% at baseline to 5.5 ± 1.3% after fructose feeding) and Group 3 (n = 29) gained liver fat (from 8.5 ± 1.2% to 11.2 ± 1.2%). Data are shown as mean ± SD. P-values have been calculated using the Wilcoxon signed-rank test and refer to differences between baseline and after fructose intervention

Parameters	Group 1				Group 3			
	Before	After	Change	P-value	Before	After	Change	P-value
Weight (kg)	97.2 ± 12	97.8 ± 12	0.62 ± 1.6	0.09	101.9 ± 14	103.3 ± 14	1.46 ± 1.8	0.0004
Liver fat (%)	7.2 ± 6.4	5.5 ± 6	-1.7 ± 1.4	<0.0001	8.5 ± 6.6	11.2 ± 6.7	2.7 ± 1.3	<0.0001
BMI (kg m ⁻²)	29.8 ± 2.8	30 ± 2.7	0.20 ± 0.49	0.08	31.6 ± 3.2	32.1 ± 3.3	0.46 ± 0.56	0.0005
Waist (cm)	107.4 ± 7.6	107.6 ± 7.1	0.27 ± 1.4	0.47	112.7 ± 9	113.8 ± 8.9	1.15 ± 2.5	0.006
Systolic BP (mmHg)	136 ± 13	139 ± 18	3.6 ± 10	0.45	129 ± 13	135 ± 11	6.0 ± 11	0.01
Heart rate (bpm)	66.3 ± 12	65.9 ± 11	-0.33 ± 28	0.82	66 ± 8.6	68.5 ± 11	2.5 ± 7.7	0.10
Glucose (mmol L ⁻¹)	5.36 ± 0.48	5.36 ± 0.37	0.004 ± 0.46	0.99	5.4 ± 0.39	5.57 ± 0.45	0.16 ± 0.42	0.10
Insulin (pmol L ⁻¹)	74.6 ± 35	82.3 ± 63	7.7 ± 47	0.49	101 ± 60	125 ± 84	23.8 ± 48	0.007
HOMA-IR	2.51 ± 1.5	2.83 ± 2.2	0.32 ± 1.6	0.68	3.24 ± 2.1	4.53 ± 3.2	1.3 ± 2.3	0.001
DNL (μmol L ⁻¹)	14.3 ± 9.8	15.8 ± 13	1.5 ± 15	0.68	12.6 ± 11	17.9 ± 9.8	5.4 ± 11	0.03
Triglycerides (mmol L ⁻¹)	1.8 ± 1.1	1.8 ± 0.96	-0.002 ± 0.73	0.63	1.59 ± 0.85	1.77 ± 0.89	0.18 ± 0.59	0.049
ApoC-III (mg dL ⁻¹)	11.6 ± 6.5	12.2 ± 5.4	0.54 ± 4.1	0.21	10.2 ± 4.7	11.5 ± 5.4	1.3 ± 2.7	0.02
FGF-21 (pg mL ⁻¹)	210 ± 210	311 ± 520	101 ± 380	0.12	194 ± 96	225 ± 130	31.7 ± 120	0.28
FFA (μmol L ⁻¹)	423 ± 120	408 ± 99	-14.8 ± 120	0.53	419 ± 140	466 ± 150	47.1 ± 160	0.16
β-OH butyrate (mg dL ⁻¹)	0.65 ± 0.36	0.51 ± 0.28	-0.14 ± 0.4	0.21	0.64 ± 0.5	0.62 ± 0.69	-0.018 ± 0.77	0.27
Adiponectin (ng mL ⁻¹)	4.54 ± 2.8	4.25 ± 2.6	-0.29 ± 0.5	0.02	4.27 ± 2.6	4.05 ± 2	-0.22 ± 1	0.49
Uric acid (μmol L ⁻¹)	387 ± 70	373 ± 70	-14.3 ± 48	0.26	390 ± 62	399 ± 58	9.7 ± 53	0.22

Significant of p-values <0.05 are bold.

Figure 4. The different response to the diet intervention is independent from PNPLA3, TM6SF2 and MBOAT7. a) Increased number of risk alleles associates with increased liver fat content before diet intervention. b) No differences in the number of risk alleles between Group 1 and Group 3. Group 1 (n = 22) had reduced liver fat content (from $7.2 \pm 1.4\%$ at baseline to $5.5 \pm 1.3\%$ after fructose feeding) and Group 3 (n = 29) gained liver fat (from $8.5 \pm 1.2\%$ to $11.2 \pm 1.2\%$). c) Individuals without or with risk allele do not have differences in liver fat change in response to the diet intervention. Data are shown as mean value and standard deviation (continuous traits) or as percentage. P value was calculated by linear regression analysis (a), ordinal regression (b) or Mann–Whitney nonparametric test for independent samples (c). Risk alleles: TM6SF2 T; PNPLA3 G; MBOAT7 T.



strong positive correlations with both baseline TG (**Figure 6a**) and TG AUC (**Figure 6b**). At baseline, both fasting TG and TG AUC (after a fat-rich meal) correlated negatively with plasma adiponectin ($r = -0.37$ and -0.42 , respectively, $P < 0.001$), but the correlation between TG AUC and liver fat content was only modest ($r = 0.30$, $P = 0.01$). The responses of both plasma total TG and apoB48 levels to the fat-rich meal were higher after fructose feeding (**Figure 5**). Both plasma TG and apoB48 were augmented by fructose feeding for up to 120 min after the high-fat meal. The overall responses of plasma total TG, measured as AUC as well as iAUC were increased after fructose feeding. We did not observe any significant changes in AUCs of TG and apoB48 in chylomicrons, VLDL1 or VLDL2 fractions after fructose feeding. Fructose feeding induced significantly higher systolic blood pressure values (but not heart rate), fasting insulin and HOMA index (**Table 11**). Both fasting serum TG levels and apoC-III increased significantly after fructose intervention (**Table 11**). Importantly, apo CIII showed strong positive correlations with TG and postprandial TG AUCs after fructose feeding (**Figure 6c,d**). In addition, the apo CIII change after fructose feeding correlated significantly with changes of TG and TG AUCs (**Figure 6e,f**). Fructose feeding was not associated with significant changes in fasting FFA, FGF-21 or uric acid concentrations (**Table 11**), or in glucose or insulin AUCs during an OGTT (data not shown). Notably, we observed a strong correlation between liver fat and FGF-21 values before (**Figure 7a**) and after (**Figure 7b**) fructose feeding. Fructose feeding resulted in significant increases in DNL in the fasting state (12.3 vs. 16.5% de novo palmitic acid in VLDL1, $P < 0.01$) and also at 4 and 8 h postprandially. In contrast, fructose feeding resulted in a significant decrease in

fasting levels of β -hydroxybutyrate ($P = 0.005$), a surrogate marker of hepatic lipid β -oxidation. We further observed an inverse relationship between the changes in DNL and β -hydroxybutyrate in response to fructose intervention ($r = -0.42$, $P = 0.002$). Importantly, the increase in DNL correlated positively with the respective change of postprandial TG AUC ($r = 0.43$, $P = 0.001$). Thus, the fructose-stimulated DNL may contribute to the increased postprandial TG responses. We next selected seven variables (changes of apo CIII, DNL, insulin, HOMA, weight, saturated fat intake and total fat intake) and tested their explanatory power for TG AUC change after fructose intervention in a multivariate regression analysis. The whole model explains 73.7% of the variance (adjusted R-squared). Multiple regression analysis identified the apo C-III change as the strongest predictor for the change of TG AUC followed by those of DNL, insulin and HOMA change. Importantly, apo C-III alone explained 59% of the variance, whereas DNL alone explained 16% of the variance in changes in TG AUC.

Figure 5. Responses of plasma TG and apoB48 after a fat-rich mixed meal before and after fructose feeding. The P-values have been calculated using the Wilcoxon signed-rank test. * $P < 0.05$. The AUC before versus after for TG (a) and (b) apoB48 are shown.

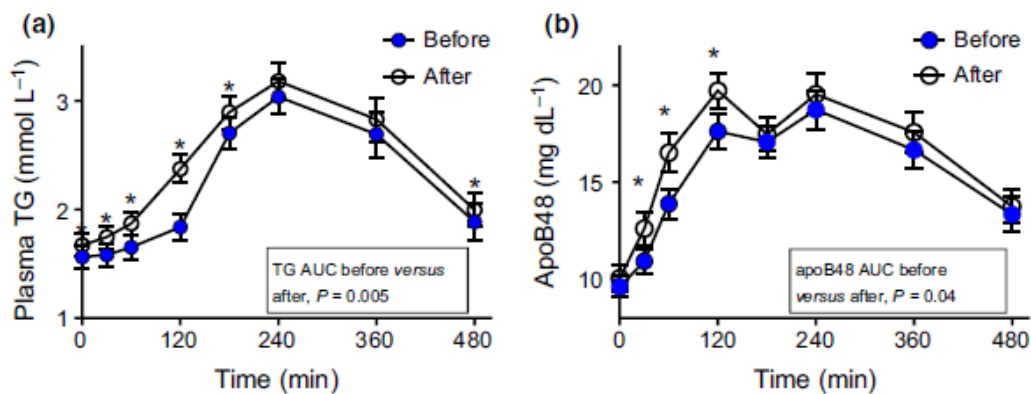


Figure 6. Correlations of plasma apoC-III and TG and TG AUCs after a fat-rich meal, before (a + b) and after fructose feeding (c + d). ApoC-III changes versus TG and TG AUCs changes after fructose feeding (e + f). Correlation coefficients and their corresponding P-values were calculated using Spearman's rank test

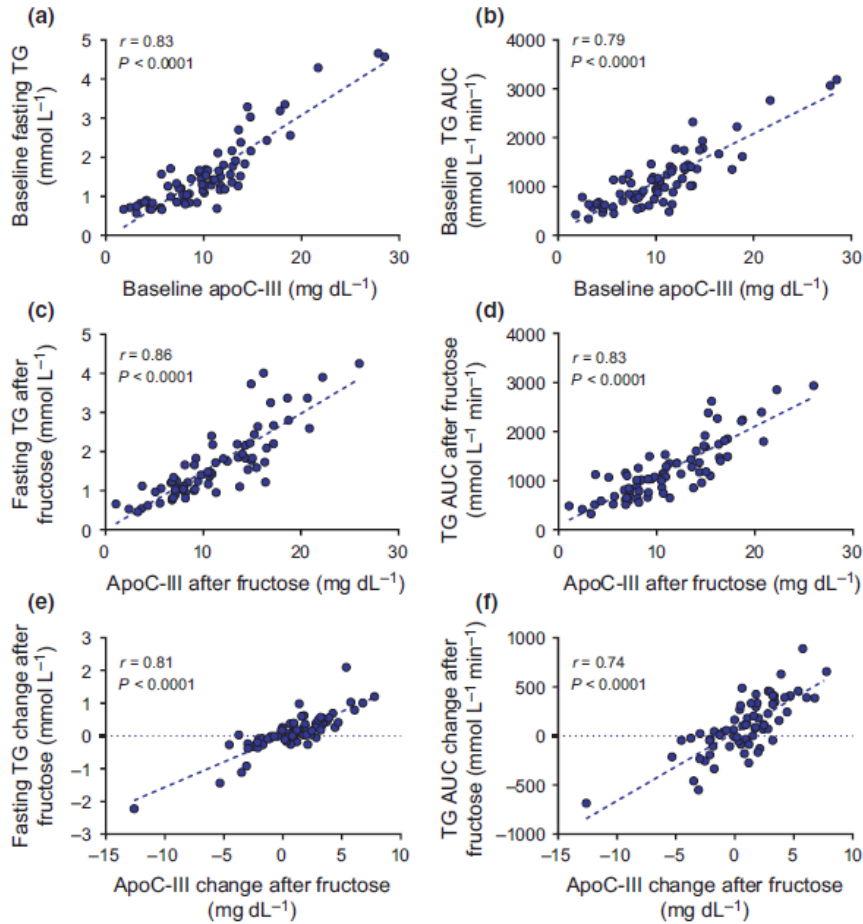
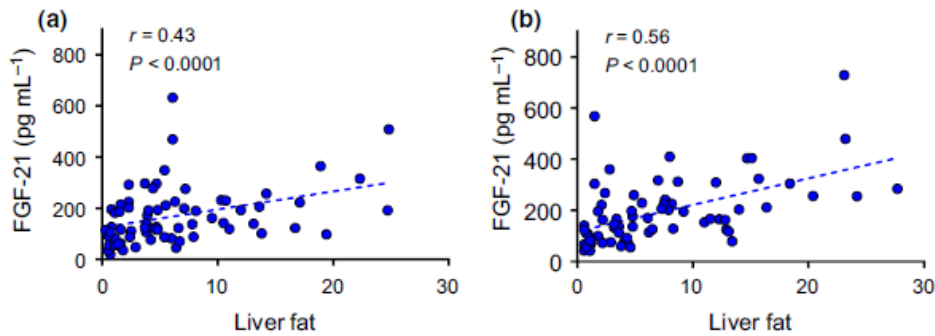


Figure 7. Correlations of fasting plasma FGF-21 and liver fat content before (a) and after fructose feeding (b). Correlation coefficients and their corresponding P-values were calculated using Spearman's rank test.



Discussion

A central finding in this study is that a real-world daily consumption of fructose-sweetened beverages for 12 weeks had significant but modest adverse effects on multiple cardiometabolic risk factors. We also report that the fructose consumption significantly increased liver fat content and hepatic DNL and decreased levels of β -hydroxybutyrate (indicating decreased hepatic β -oxidation). Interestingly, the individual changes of liver fat were highly variable in subjects with the same weight change (**Figure 3**). However, the average relative increase in liver fat (10%) was more pronounced than the significant but low relative increases in weight (1.1%) and waist circumference (0.5%). Importantly, the adverse changes of cardiometabolic risk factors seemed to cluster more with the increase in liver fat than with the weight gain. Our intervention was intended to be hypercaloric, in which the fructose-sweetened beverages were consumed in addition to the habitual diet. The fructose dose (75 g/day, corresponding to 13% of the energy intake) was slightly higher than the mean consumption of fructose in the United States (55 g/day) [215, 216], but comparable to the dose that is habitually consumed by some high risk groups, including adolescents in Western societies [217]. Despite our intention to undertake a hypercaloric intervention study, the reported energy intake was not significantly higher after fructose intervention despite the small but significant increase in weight and waist circumference. The fact that fructose sweetened beverages provided an excess of 300 calories but the daily energy intake increased only by an average of 54 calories indicates that the study subjects reduced their energy intake from other food and beverages. Indeed, the subjects reported significantly decreased intake of

saturated fat and sucrose during fructose feeding. Recently, it was reported that 10-week consumption of fructose-sweetened beverages was linked to reduced resting energy expenditure [218]. This would further increase weight gain if total energy intake is not reduced accordingly. Earlier studies have shown that the size and distribution of fat depots varied significantly also according to the saturation of the fat that was consumed [219]. Rosqvist *et al.* recently tested whether overeating a diet rich in additional PUFA would reduce formation of ectopic fat compared with overeating a diet high in saturated fatty acids (SFA) [220]. The results show that the SFA diet induced a significant increase in liver fat relative to the PUFA diet [220]. In our study, the subjects reduced their intake of dietary saturated fat, which may have counteracted the stimulatory effect of fructose intake on the liver fat accumulation. The reduction in saturated fat was more prominent in subject who lost liver fat than in those who gained liver fat. A critical question is whether the fructose consumption directly increased liver fat content? Enhanced DNL is reported to contribute significantly to the hepatic triacylglycerols in NAFLD [221]. Fructose (and sucrose in sugar sweetened beverages) acutely and chronically promotes hepatic lipogenesis by stimulating SREBP-1c and ChREBP [19,192,198,199,205] and suppresses mitochondrial fatty acid oxidation [95,96,197]. These processes synergistically promote hepatic storage of lipids and secretion of triglyceride rich VLDL particles [200, 206, 208, 222-224]. Our observation that DNL was increased during fructose feeding for 12 weeks is consistent with earlier shorter studies [225-228] and compliments the study by Stanhope *et al.* [223] showing that increased DNL is maintained during chronic intake of high-fructose liquids. In our study, fructose beverages reduced

β -hydroxybutyrate (a surrogate marker of hepatic lipid oxidation). Cox *et al.* recently reported decreased postprandial fat oxidation in overweight/obese subjects who consumed fructose beverages at 25% of energy requirements for 10 weeks as part of energy balanced *ad libitum* diet [218]. These changes of energy fluxes were not seen in subjects consuming glucose-sweetened beverages at 25% energy requirements [218]. Notably, DNL also increased significantly, but liver fat changes were not quantitated in these studies [218,223]. Fructose is absorbed via the portal vein and delivered at much higher concentrations to the liver compared to other tissues [207]. Thus, high fructose consumption forces the liver to adapt its metabolism against liver toxicity. Key pathways that could be altered include the storage of excess lipids in hepatocytes resulting in steatosis, and the packaging of triacylglycerols in VLDL to remove extra lipids from the liver. The fact that increases in DNL correlated with increases in postprandial TG AUC after fructose intervention suggests that there is a direct link between increased hepatic triacylglycerol synthesis and assembly and secretion of VLDL. Increased fat oxidation is another adaptive mechanism that prevents liver fat accumulation [196]. Our observation of a reduction in β -hydroxybutyrate during fructose feeding indicates reduced ability of the liver to shuttle fatty acids to oxidation and ketone body formation. FGF21 is considered to be a major regulator of body energy metabolism promoting fatty acid oxidation but suppressing DNL [229,230]. We observed strong correlations between fasting FGF-21 levels and the liver fat content in line with earlier studies [231,232] and a negative correlation with b-OH butyrate ($r = -0.28$, $P < 0.017$). We observed a nonsignificant trend for increase in fasting FGF-21 levels after fructose feeding in

these abdominally obese subjects. Fructose has earlier been reported to increase acutely the response of FGF-21 [233]. In this study, FGF-21 increased about 3.4-fold after acute intake of 75 g of fructose, but so far no data exist on the effects of more chronic intake of fructose. The lack of effect on fasting uric acid could be due to the fact that the greatest effects of fructose-containing sugars on uric acid are observed in the postprandial and not the fasting state [234]. Postprandial dyslipidemia is a key feature of the atherogenic lipid profile in abdominally obese subjects and is exaggerated in those with hypertriglyceridemia. Acute and short term as well as more chronic intake of high fructose promotes elevation of both fasting and postprandial triglyceride levels in healthy subjects as well as in those with the metabolic syndrome [100, 105, 235-241]. The data from meta-analyses suggest that these effects on postprandial lipids are induced by both hypercaloric and isocaloric diets [242,243]. We performed here thorough analyses of triglycerides, apoB48 and apoB100 in serum and different triglyceride-rich lipoprotein fractions to clarify the responses to fructose feeding. We showed that fructose feeding aggravated the increases in both total triglycerides and apoB48 at early time points after a fat-rich meal. However, the differences in total responses of plasma triglycerides and apoB48 AUC after the fructose feeding remained marginal. Interestingly, we observed a significant rise of apo C-III during fructose feeding in line with recent results by Stanhope *et al.* [244]. These data are consistent with the possibility that fructose, like glucose, stimulates the expression of apoC-III via ChREBP. We also showed that apoC-III was a strong predictor for postprandial serum TG AUC, both before and after fructose feeding, supporting a role for apo C-III in the clearance of triglyceride-rich lipoproteins [245]. Our

study has several strengths. First, the study group was larger than in any previous mechanistic study using magnetic resonance examinations and stable isotopes to elucidate the adverse effects of fructose on cardiometabolic risk factors. Secondly, the study subjects were genotyped for three key risk alleles for fatty liver development to determine whether they played a role in liver fat responses to fructose. Thirdly, the duration of the study was longer than in earlier acute or short-term mechanistic studies. Fourthly, the amount of fructose was similar to the habitual consumption in the USA and Middle East. An important limitation is that we do not have a control group to specifically disentangle the metabolic effect(s) of weight gain versus fructose. Additional potential weaknesses are that fructose served in beverages may not induce the same metabolic responses as when fructose is ingested as a part of sucrose or in natural compounds; pure fructose may be absorbed less efficiently than sucrose [217]. We cannot confirm that the study subjects really consumed the daily recommended dose of fructose as our compliance assessment was indirect and did not include a measure of blood fructose concentration. Overall the reported compliance was good when recognizing all caveats. Both poor adherence to fructose intake and less absorption of fructose would result in less robust metabolic effects of fructose consumption than actually were seen in our study.

In conclusion, our data demonstrate that the adverse cardiometabolic effects of fructose consumption over a 12-week period were significant but modest. However, these detrimental cardiometabolic effects may be exacerbated over a longer period time as occurs in the real life. Thus, our results should be interpreted in the context of chronic overconsumption of sugar-sweetened beverages

containing fructose amongst heavy consumers who are common across the globe. Our study also indicates that there are remarkable individual differences in susceptibility to visceral adiposity/liver fat deposition and that such differences play a role in modulating the health hazard associated with chronic consumption of fructose-containing beverages.

5.3. MEDEA trial

Background

NAFLD is the most common chronic liver disease in the industrialized world and its prevalence has been reported to be in the 15–30% range in the general population in various countries. The prevalence approaches 70–90% in people with obesity and T2DM and it can be considered the hepatic component of the metabolic syndrome [246]. Patients with T2DM are particularly susceptible to more severe forms of NAFLD [246,247], by mechanisms that are still incompletely understood, and have a higher progression to hepatocellular carcinoma [248,249]. Furthermore, the presence of NAFLD in T2DM aggravates the metabolic profile, insulin sensitivity, and dyslipidemia [250]. It is important to underline that NAFLD in T2DM is also related to an increased prevalence of cardiovascular events and microvascular complications including chronic kidney disease and retinopathy [251,252]. Lifestyle modifications remain the therapy of choice for NAFLD also in individuals with T2DM. In recent years, there has been a growing interest in studies concerning the beneficial effects of dietary nutrients on NAFLD since these components have several advantages such as being widely available, while having low or minimal side effects. However, few studies examining optimal dietary strategies for NAFLD in T2DM are available.

As discussed above, data are reasonably convincing as for the possible effects of dietary macronutrients on liver fat content. In fact, SFA increase liver fat content and replacing SFA with MUFA or n-6 PUFA reduces liver fat, while the effectiveness of n-3 PUFA supplementation is still controversial. In terms of other

dietary components as polyphenols and micronutrients, beneficial effects would refer especially to liver inflammation and fibrosis more than to fat content.

Consequently, it is conceivable that a diet rich in all micro- and macronutrients (a Portfolio diet) with beneficial effects on NAFLD is more effective than a monofactorial dietary intervention, such as the only increase of MUFA. So far, no studies have evaluated the effect of this kind of diet naturally rich in low GI carbohydrates, vegetable fiber, MUFA, n-3 and n-6 PUFA and polyphenols on liver fat in patients with T2DM.

Aim

To evaluate the effects of a 8-weeks intervention with an isoenergetic Portfolio diet rich in MUFA, n-3 and n-6 PUFA, prebiotic fibre and polyphenols, and with low GI carbohydrates versus an isoenergetic diet rich in MUFA on NAFLD in patients with T2DM.

Materials and Methods

Subjects. Thirty-one individuals of both sexes, aged 35–75 y, with a large BMI (in kg/m²; 25–35) and waist circumference (men \geq 102 cm; women \geq 88 cm) were recruited from patients referred to the Diabetes outpatient clinic of the Federico II University Hospital. Health status and medical history were assessed by interviews, clinical examinations, and routine laboratory tests. Inclusion criteria were as follows: T2DM in satisfactory blood glucose control with diet or drug not affecting liver fat content (metformin, DDP4-i, sulfonylurea, repaglinide), stable weight (\pm 3 kg) over the preceding 3 months, glycated hemoglobin $<$ 7,5%,

evidence of steatosis both on ultrasonography at ultrasonography, stable therapy with lipid-lowering drugs, LDL cholesterol < 130 mg/dL and serum triglycerides <350 mg/dL. Exclusion criteria were as follows: treatment with insulin or other glucose-lowering drugs affecting liver fat content (as GLP-1 analogues, SGLT2 inhibitors, insulin). Any acute or chronic liver disease of genetic or infectious origin, any acute or chronic disease seriously affecting the health status, use of alcoholic beverages (more than 1 serving/day), hormonal therapy. The study design was approved by the local ethics committees, and each subject gave written informed consent before participation in the study. All procedures were performed in accordance with the Declaration of Helsinki for clinical trials.

Study design. The study was based on a randomized, controlled, parallel group design and consisted of a 2–4 week run-in period, during which the participants were stabilized on their own diet, and a 8-wk test period. At the end of the run-in period, the participants were randomly assigned to one of two groups: one group consumed a Portfolio diet (naturally rich in MUFA, n-3 and n-6 PUFA, prebiotic fibre, polyphenols, vitamins, and low GI carbohydrates) and the other group consumed a MUFA diet (naturally rich in MUFA). The randomization was performed with stratification for sex, age and BMI (25–30, 30–35 kg/m²), and T2DM therapy by use of random allocation software. Allocation was carried out by personnel not involved in the study; therefore the investigators and the dieticians were aware of the group allocation of the participant only after the randomization process had been performed. During the study, participants were advised not to change their body weight and lifestyle habits such as exercise and

alcohol consumption and not to change their medications unless necessary. As shown in **Table 14**, the assigned diets differed only in n-3 and n-6 PUFA, vitamin D, vitamin C, glycemic index, glycemic load, fibre, and polyphenol contents and were similar in all other characteristics as macronutrient composition, MUFA and other micronutrients. Dietary composition was derived from the tables of the Italian National Research Institute for Food and Nutrition [253], whereas polyphenol contents and the Oxygen Radical Absorbance Capacity index were calculated according to USDA tables [254]. The main dietary sources of carbohydrates were represented by based on wholegrain products including whole wheat bread, whole wheat pasta, barley kernels, legumes in the Portfolio diet, while MUFA diet contained commercial products based on refined cereals such as wheat bread, rice, pasta, and breakfast cereals. The main dietary source of MUFA was extra-virgin olive oil in both diet, while the main sources of n-3 and n-6 PUFA were represented by were salmon, dentex, and anchovies and nuts in the Portfolio diet. Main source of polyphenols were represented by decaffeinated green tea, decaffeinated coffee, artichokes, onions, spinach, rocket. The initial assigned energy intake of the diet was determined based on the individual's habitual energy intake evaluated by a 7-d food record, adjusted for body weight and clinical judgment of the dietitians, in order to take care of a possible underreporting, common in overweight/obese individuals. To improve dietary adherence, meals and beverages were provided to the participants for the whole study period in amounts sufficient to cover their household consumption. Meals were prepared in a qualified catering service under the surveillance of the dietitians. Adherence to diets was evaluated by a 7-d dietary record at baseline, 4

wk, and 8 wk and was reinforced by the dietitians through counseling every week and phone calls every 2–3 d. Participants allocated to the two diet were considered compliant with the treatment if intakes were > 80% of those assigned.

Table 14. Nutrient composition of the experimental diets.

		MUFA	Portfolio
Energy	Kcal/day	1972	1977
Proteins	% TE	18	18
Lipids	% TE	41	41
SAFA	% TE	7	6
MUFA	% TE	28	26
PUFA	% TE	4	5,8
n-3	g/day	1,1(0,5%)	2,6 (1,2%)
n-6	g/day	7,4 (3,4%)	9,6 (4,4%)
Cholesterol	mg	184	121
Carbohydrates	% TE	41	41
Sugars	% TE	11	9,8
Fiber	(g/1000 Kcal)	10	24
Glycemic Index	(%)	58,5	47
Glycemic Load	-	125	94
Vitamin E	mg	22	23,6
Vitamin D	mcg	1	5,3
Vitamin C	mg	88	254
Total ORAC	μmolT	7501	14187
Polyphenols	mg	376	2715,5

ORAC= Oxygen Radical Absorbance Capacity; TE= Trolox Equivalent

Experimental procedures. At baseline and after the 8-wk intervention, body weight, height, waist circumference, and blood pressure were measured according to standardized procedures. After a 12-h overnight fast, blood samples were

collected for the measurement of plasma concentrations of glycated hemoglobin, ALT, AST, and GGT.

Laboratory methods. Glycated hemoglobin was determined by high-performance liquid chromatography (HPLC). ALT, AST, and GGT activities were determined by colorimetric methods according to the International Federation of Clinical Chemistry recommendations.

Liver fat evaluation. Liver fat content was measured by ¹H-MRS performed on a 3T MR scanner (Intera; Philips Medical Systems, Best, the Netherlands) equipped for proton spectroscopy acquisitions in the morning at fasting, at baseline and after the 8-wk intervention. Sagittal, coronal, and axial slices covering the whole liver were preliminarily taken to position the spectroscopy acquisition voxel. A single voxel of 8 cc ($2 \times 2 \times 2 \text{mm}^2$) was placed within the right lobe avoiding major blood vessels, intrahepatic bile ducts, and subcutaneous fat tissue. The proton spectrum was acquired using an eight-channel phased array body coil after shimming over the volume of interest using an unsuppressed water PRESS sequence with the following parameters: TE = 35 ms, TR = 3000 ms, NSA=1, spectral width 2000 Hz. Total spectra acquisition time was 16 s and thus acquired in breath hold to avoid movement artifacts and spectra broadening. Spectra were analyzed using LCModel software (version 6.2-1; <http://s-provencher.com>) that fits in vivo metabolite spectra using model resonances acquired under comparable scanning conditions from multiple compounds in standard phantom solutions. Concentration values in arbitrary units of water peak (signal of water) and the sum

of lipid peak at 1.3, 0.9, and 1.6 ppm (signal of fat) were considered for fat liver quantification, including both methyl and methylene groups of triglyceride molecule. Signal decay was corrected for the different T2 decay of water and fat using mean T2 relaxation times of 50 and 60 ms for water and fat, respectively. Hepatic fat percentage was calculated using the following formula: $100 \cdot S_f / (S_f + S_w)$ [255], where S_f is signal of fat and S_w is signal of water. These values represent a relative quantity of water and fat in the volume of interest. To convert these values into absolute concentrations (weight per volume) expressed as percent fat, equations validated by Longo et al. [256] were applied.

Sample size calculation and statistical analysis. In order to detect a 30% difference in liver fat content between treatments with a 80% power at 5% significance level, 46 patients (23 for each group) had to be studied. The expected changes in liver fat content after treatment correspond to the differences observed between obese patients with or without T2DM in a previous study [53].

Energy intake and nutrient composition at the end of the run-in period and during the intervention were calculated from the food records; the intakes during the intervention were expressed as mean of three food records completed at 4, 8 and 12 weeks. The results for continuous variables were presented as mean \pm standard error (mean \pm SEM), unless otherwise stated. Variables with skewed distributions by Shapiro-Wilks test were normalized with a logarithmic or square root transformation.

A paired-samples t test was used to examine the changes within each group. Repeated measures ANOVA was used to evaluate differences between Portfolio

diet and Mufa diet calculated as absolute changes (8 wk value - baseline value). A t-test for unpaired data was used for the differences in liver fat content measured as percent variation (end values – baseline values × 100/baseline values).

For all analyses, the level of statistical significance was set at $p = 0.05$ (two tails).

Data were analyzed using SPSS for Windows 20.0 (SPSS Inc., Chicago IL).

Preliminary results

The preliminary results on the first 31 individuals are reported.

Characteristics of participants at baseline. As shown in **Table 15**, the participants allocated to the two intervention groups were comparable for age, BMI, waist circumference, blood pressure, diabetes drug treatment, plasma values of HbA1c, AST, ALT, GGT, as well as liver fat content.

Table 15. Baseline characteristics of the 2 groups of participants in the dietary intervention study.

	MUFA (n=15)	Portfolio (n=16)	P
Sex (M/F)	11/4	11/5	
Age (y)	61.7±5.2	64.4±56.5	0.213
Body weight (kg)	87.0±14.6	84.2±9.7	0.528
BMI (kg/m ²)	30.7±3.4	31.0±3.3	0.793
Waist circumference (cm)	107.2±8.9	105.5±9.2	0.608
Systolic blood pressure (mm Hg)	129.1±11.7	135.1±33.6	0.518
Dyastolic blood pressure (mm Hg)	79.8±7.4	80.5±7.5	0.797
HbA1c (%)	6.6±0.5	6.4±0.5	0.306
Liver fat (%)	11.5±9.6	8.6±8.0	0.371
AST (U/l)	21.0±10.2	18.7±6.7	0.461
ALT (U/l)	28.9±12.6	22.3±10.9	0.132
GGT (U/l)	27.8±13.8	29.2±15.0	0.792

Mean ± SD (all such values).

Compliance with dietary intervention. The composition of the diets strictly reflected the dietary composition assigned per protocol in the two groups (data not show). As expected, the diets were significantly different in n-3 and n-6 PUFA, vitamin D, vitamin C, GI carbohydrates, glycemic load, fibre, and polyphenol content. No differences in macronutrients content were observed between the 2 dietary groups. All subjects were within the ranges of intakes defined for good compliance for each dietary component.

Effects of dietary intervention on anthropometric parameters, blood pressure, HbA1c, and liver enzymes. At the end of the intervention, there was a small significant weight loss with both diets (Mufa Diet: $-0,6 \pm 0,3$ kg vs. Port Diet: -1.4 ± 0.5 kg), but the difference in weight change between the groups was not significant ($p = 0.232$). Similarly, at end of the intervention, there was a small significant reduction in BMI with both diets (Mufa Diet: $-0,2 \pm 0,1$ m/kg² vs. Port Diet: -0.5 ± 0.1 m/kg²), but the difference between the groups was not significant ($p = 0.202$).

No differences in waist circumference, blood pressure, HbA1c, AST, ALT, GGT at end of intervention in the two groups and between groups were observed (**Table 16**).

Effects of dietary intervention liver fat. In the absence of baseline differences in hepatic fat content between the two groups, liver fat did not change significantly after Mufa diet (11.5 ± 9.6 vs 10.0 ± 9.3 %; $P=0.071$), while it significantly

decreased after the Portfolio Diet (8.6 ± 8.0 vs. $4.8 \pm 4.7\%$; $P = 0.006$), with a significant difference between groups ($P= 0.022$) (**Table 16**).

Table 16. Anthropometrics, metabolic characteristics, and liver fat before and after the 8-week interventions.

	MUFA diet (n=15)			PORT diet (n=16)			p for § Δ
	baseline	8-week	Δ	baseline	8-week	Δ	
Body weight (kg)	87.0±14.6	86.3±14.3	-0.6*	84.2±9.7	82.7±9.4	-1.4*	0.720
BMI (kg/m ²)	30.9±3.4	30.4±3.3	-0.2*	31.0±3.2	30.4±3.2	-0.5*	0.850
Waist circumference (cm)	107.2±8.9	106.9±9.0	-0.3	105.5±9.2	104.7±9.0	-0.7	0.400
Systolic blood pressure (mmHg)	129.1±11.7	127.4±14.4	-1.6	135.1±33.5	127.6±11.8	-7.4	0.760
Diastolic blood pressure (mmHg)	79.8±7.3	75.3±9.7	-4.5	80.5±7.4	75.9±8.9	-4.6	0.840
HbA1c (%)	6.6±0.5	6.5±0.5	-0.1	6.4±0.5	6.2±0.6	-0.2	0.300
Liver fat (%)	11.5±9.6	10.0±9.3	-1.5	8.6±8.0	4.8±4.7	-3.8*	0.002
AST (U/l)	21.0±10.2	21.0±8.5	0.0	18.7±6.7	16.1±4.9	-2.6	0.059
ALT (U/l)	28.9±12.6	25.3±8.6	-3.6	22.3±10.9	21.8±9.2	-0.5	0.810
GGT (U/l)	27.8±13.8	25.6±12.0	-2.2	29.2±15.0	26.3±16.3	-2.9	0.830

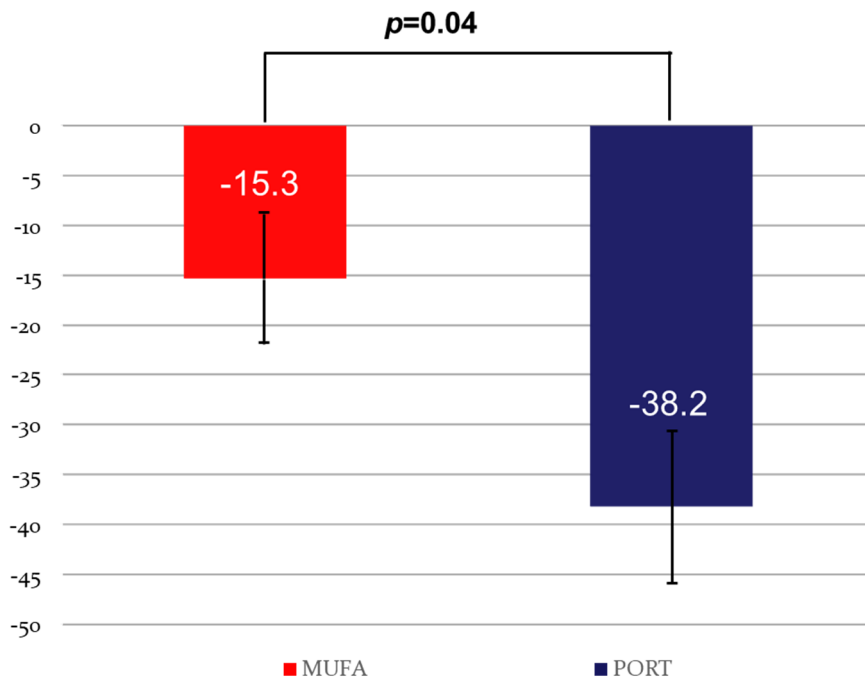
Mean ± SD (all such values), Δ: change of the parameters calculated as 8 week-baseline values.

* $p < 0.05$ paired sample t-test (8-week vs. baseline), corrected for body weight loss.

§Differences between the two groups (MUFA vs. PORT) were evaluated by one way ANOVA.

Measured as percent variations (end values – baseline values × 100/baseline values), hepatic fat was significantly reduced by the Portfolio diet (–38.2%) more than by the Mufa Diet (–15.3%) ($P=0.04$ by ANOVA) (**Figure 8**).

Figure 8. Percent changes (end values – baseline values × 100/baseline values) in liver fat content after the 8-week intervention in the two groups.



Data are expressed as means ± SE; *P < 0.05 vs. Portfolio diet.

Discussion

Preliminary results our trial have demonstrated that in just 8 weeks, a Portfolio diet naturally rich in n-3 and n-6 PUFA, prebiotic fibre, polyphenols, and with low GI carbohydrates, can induce a relative reduction in liver fat, compared with a diet rich in MUFA.

To our knowledge, this the first trial to demonstrate in a randomized, controlled study, a reduction in liver fat with a Portfolio diet in T2DM. We have compared the Portfolio diet with a naturally rich MUFA diet since our previous data showed that an isocaloric diet enriched in MUFA in replacement of saturated fat and fiber was associated with a clinically relevant reduction of hepatic fat content in T2DM [53].

As discussed above, data are reasonably convincing as for the possible effects of dietary macronutrients and micronutrients on liver fat content. In fact, SFA increase liver fat content and replacing SFA with MUFA or n-6 PUFA reduces liver fat, while the effectiveness of n-3 PUFA supplementation is still controversial. In terms of other dietary components as polyphenols and micronutrients, beneficial effects would refer especially to liver inflammation and fibrosis more than to fat content. Consequently, we have demonstrated that the combination of these foods in a dietary model such as a "Portfolio diet", inspired to a Mediterranean Diet model in which more beneficial dietary component are included could be more effective in the prevention and treatment of NAFLD, compared to a single dietary component.

In spite of isoenergetic study design, a small significant weight loss with both diets has been observed, however, this did not influence the results because analyses were corrected for body weight loss.

Based on our preliminary results, it is conceivable to infer that a diet rich in all micro- and macronutrients with beneficial effects on NAFLD is more effective than a monofactorial dietary intervention, such as the only increase of MUFA.

The lack of changes seen in the liver function tests may have been a result of the small sample size or the short duration of the study; however, it does demonstrate a lack of sensitivity of these tools as markers of liver inflammation, as it has been previously demonstrated [33].

Taking into account the different components of our Portfolio diet, different mechanism could explain the liver fat reduction observed. From a mechanistic point of view and as evidenced both in animal and human studies, the hepatic fat-

lowering effects of n-3 and n-6 PUFA could be mainly due to their anti-inflammatory and anti-fibrotic properties, to the up-regulation of hepatic lipolysis and fat oxidation and to the down-regulation of hepatic lipogenesis [65]. MUFAs may exert their beneficial effects on liver steatosis through the improvement of postprandial hepatic fatty acid oxidation [48] as well as through the stimulation of lipoprotein lipase activity in adipose tissue and the resulting enhancement of triglyceride (TAG) clearance [57-60]. In contrast, the effects of fibre intake on NAFLD could be associated with their ability to modulate the gut microbiota, leading to the subsequent attenuation of de novo fatty acid synthesis in the liver [80]. On the other hand, low GI carbohydrates have been shown to induce a lower rise in postprandial glucose and insulin levels, leading to decreased activation of hepatic lipogenesis [79]. Regarding vitamins, those that have been demonstrated to be more effective for NAFLD patients are vitamin E and vitamin D. Specifically, different studies have reported that the mechanisms of action of these vitamins include decreased oxidative stress, lipid peroxidation, inflammation, fibrosis, lipid uptake and lipogenesis [138-141,157]. Likewise, the metabolic processes involved in the anti-steatotic effects of polyphenols could be the inhibition of hepatic lipogenesis, oxidative stress and inflammation and the improvement of fatty acid oxidation [122-125]. Finally, by shaping gut microbiota composition and decreasing endotoxaemia, probiotics have been shown to decrease liver oxidative stress, inflammation and lipogenesis [80].

This study has some strengths and limitations. This was a randomized controlled trial with a rigorous follow-up of dietary adherence. Hepatic fat content was measured by the gold standard ¹H-MRS. A limitation is that only Caucasian

patients in good metabolic control were studied, and therefore our results may not be extended to T2DM patients of other ethnic groups or with poorer control. Nevertheless, there are no indications that worse metabolic control could negatively influence the effects of a Portfolio diet on hepatic fat content.

Therefore, in light of our new findings, we suggest that an increase in the intake of MUFAs and also n-3 PUFAs, particularly as a replacement for saturated fat and as a higher proportion of low GI carbohydrates in the diet, is beneficial to NAFLD patients.

These preliminary results are clinically relevant and have implications for the nutritional management of fatty liver, suggesting that a Portfolio diet might be the preferential approach. In fact it is very likely that the “optimal diet” for NAFLD should be based on the synergic and/or complementary action of different food compounds able to act both on the deposition of excess fat in the liver and the other pathways leading from liver fat deposition to NASH and fibrosis.

6. CONCLUSIONS

The aim of my research project, developed in these three years, has been to evaluate firstly the relationships between liver fat content and metabolic, inflammatory and nutritional factors in individuals at high cardio-metabolic risk; furthermore, the adverse effects of fructose intake on liver fat content and other cardiometabolic risk factors in obese men has been evaluated; finally the effects of a Portfolio diet on liver fat content in patients with T2DM has been performed.

In the ETHERPATS cohort we have observed that insulin resistance, systemic inflammation and postprandial GLP-1 were the main determinants of liver fat in people at high cardio-metabolic risk, explaining altogether about 30% of liver fat variability. The different factors implicated in the pathogenesis of NAFLD are also involved in the pathogenesis of atherosclerosis, suggesting that NAFLD may represent the tip of the iceberg of the complex metabolic derangements leading to CVD.

The data of the FRUCTOSE trial demonstrate that the adverse cardiometabolic effects of fructose consumption over a 12-week period are significant but modest. However, these detrimental cardiometabolic effects may be exacerbated over a longer period of exposition as occurs in the real life. Thus, our results should be interpreted in the context of chronic overconsumption of sugar-sweetened beverages containing fructose amongst heavy consumers who are frequent in human populations across the globe. Our study also indicates that there are remarkable individual differences in the susceptibility to visceral adiposity/liver fat deposition and that such differences play a role in modulating the health hazard associated with chronic consumption of fructose-containing beverages.

Finally, the MEDEA trial demonstrates that an isocaloric Portfolio diet – naturally rich in MUFA, n-3 and n-6 PUFA, prebiotic fibre and polyphenols, and with low GI carbohydrate foods – compared with a diet enriched only in MUFA is able to induce a more relevant reduction of hepatic fat content in T2DM patients.

These results are clinically relevant and have implications for the nutritional management of fatty liver, suggesting that a Portfolio diet might be the preferential choice. In fact, it is very likely that the “optimal diet” for NAFLD should be based on the synergic and/or complementary action of different food components able to act both on the deposition of excess fat in the liver and on the other pathways leading from liver fat deposition to NASH and fibrosis.

7. REFERENCES

1. Haas, J.T.; Francque, S.; Stael; B. Pathophysiology and mechanisms of nonalcoholic fatty liver disease. *Annu. Rev. Physiol.* **2016**, *78*, 181–205.
2. Francque, S.M.; van der Graaff, D.; Kwanten, W.J. Non-alcoholic fatty liver disease and cardiovascular risk: Pathophysiological mechanisms and implications. *J. Hepatol.* **2016**, *65*, 425–443.
3. Chalasani, N.; Younossi, Z.; Lavine, J.E.; Diehl, A.M.; Brunt, E.M.; Cusi, K.; Charlton, M.; Sanyal, A.J. The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* **2012**, *55*, 2005-2023.
4. Neuschwander-Tetri, B.A.; Caldwell, S.H. Nonalcoholic Steatohepatitis: Summary of an AASLD Single Topic Conference. *Hepatology* **2003**, *37*, 1202–1219.
5. Younossi, Z.M.; Koenig, A.B.; Abdelatif, D.; Fazel, Y.; Henry, L.; Wymer, M. Global epidemiology of nonalcoholic fatty liver disease-meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **2016**, *64*, 73–84.
6. Yang, K.C.; Hung, H.F.; Lu, C.W.; Chang, H.H.; Lee, L.T.; Huang, K.C. Association of non-alcoholic fatty liver disease with metabolic syndrome independently of central obesity and insulin resistance. *Sci. Rep.* **2016**, *6*, 27034.

7. Bozzetto, L.; Annuzzi, G.; Ragucci, M.; Di Donato, O.; Della Pepa, G.; Della Corte, G.; Griffo, E.; Anniballi, G.; Giacco, A.; Mancini, M.; Rivellese, AA. Insulin resistance, postprandial GLP-1 and adaptive immunity are the main predictors of NAFLD in a homogeneous population at high cardiovascular risk. *Nutr. Metab. Cardiovasc. Dis.* **2016**, *26*, 623-629.
8. López-Suárez, A.; Guerrero, J.M.; Elvira-González, J.; Beltrán-Robles, M.; Cañas-Hormigo, F.; Bascuñana-Quirell, A. Nonalcoholic fatty liver disease is associated with blood pressure in hypertensive and nonhypertensive individuals from the general population with normal levels of alanine aminotransferase. *Eur. J. Gastroenterol. Hepatol.* **2011**, *23*, 1011–7.
9. Blachier, M.; Leleu, H.; Peck-Radosavljevic, M.; Valla, D.C.; Roudot-Thoraval, F. The burden of liver disease in Europe: a review of available epidemiological data. *J. Hepatol.* **2013**, *58*, 593–608.
10. Yamazaki, H.; Tsuboya, T.; Tsuji, K.; Dohke, M.; Maguchi, H. Independent association between improvement of nonalcoholic fatty liver disease and reduced incidence of type 2 diabetes mellitus. *Diabetes Care* **2015**, *38*, 1673–1679.
11. Bush, H.; Golabi, P.; Younossi, Z.M. Pediatric Non-Alcoholic Fatty Liver Disease. *Children* **2017**, *4*, 48.
12. Angulo, P. Long-term mortality in nonalcoholic fatty liver disease: is liver histology of any prognostic significance? *Hepatology* **2010**, *51*, 373–375.
13. Bellentani, S. The epidemiology of non-alcoholic fatty liver disease. *Liver International* **2017**, *37*, S81–84

14. Byrne, C.D.; Targher, G. NAFLD: a multisystem disease. *J. Hepatol.* **2015**, *62*, S47–64.
15. Bozzetto, L.; Prinster, A.; Mancini, M.; Giacco, R.; De Natale, C.; Salvatore, M.; Riccardi, G.; Rivellesse, AA.; Annuzzi, G. Liver fat in obesity: role of type 2 diabetes mellitus and adipose tissue distribution. *Eur. J. Clin. Invest.* **2011**, *41*, 39–44.
16. Marcuccilli, M.; Chonchol, M. NAFLD and Chronic Kidney Disease. *Int. J. Mol. Sci.* **2016**, *17*, 562.
17. Day, C.P.; James, O.F. Steatohepatitis: a tale of two “hits”? *Gastroenterol.* **1998**, *114*, 842–845.
18. Dowman, J.K.; Tomlinson, J.W.; Newsome, P.N. Pathogenesis of non-alcoholic fatty liver disease. *Q. J. Med.* **2010**, *103*, 71–83.
19. Donnelly, K.L.; Smith, C.I.; Schwarzenberg, S.J.; Jessurun, J.; Boldt, M.D.; Parks E.J. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* **2005**, *115*, 1343–51.
20. Nguyen, P.; Leray, V.; Diez, M.; Serisier, S.; Le Bloc’h, J.; Siliart, B.; Dumon, H. Liver lipid metabolism. *J. Anim. Physiol. Anim. Nutr.* **2008**, *92*, 272–83.
21. Alam, S.; Mustafa, G.; Alam, M.; Ahmad, N. Insulin resistance in development and progression of nonalcoholic fatty liver disease. *World J. Gastrointest. Pathophysiol.* **2016**, *7*, 211–217.
22. Basaranoglu, M. From fatty liver to fibrosis: A tale of “second hit”. *World J. Gastroenterol.* **2013**, *19*, 1158–1165.

23. Tiniakos, D.G.; Vos, M.B.; Brunt, E.M. Nonalcoholic fatty liver disease: Pathology and pathogenesis. *Annu. Rev. Pathol.* **2010**, *5*, 145–171.
24. Buzzetti, E.; Pinzani, M.; Tsochatzis, E.A. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Metab.* **2016**, *65*, 1038–48.
25. Kitade, H.; Chen, G.; Ni, Y.; Ota, T. Nonalcoholic Fatty Liver Disease and Insulin Resistance: New Insights and Potential New Treatments. *Nutrients* **2017**, *9*, 387.
26. Mokhtari, Z.; Gibson, D.L.; Hekmatdoost, A. Nonalcoholic Fatty Liver Disease, the Gut Microbiome, and Diet. *Adv. Nutr.* **2017**, *8*, 240–252.
27. Sookoian, S.; Pirola, C.J. Genetic predisposition in nonalcoholic fatty liver disease. *Clin. Mol. Hepatol.* **2017**, *23*, 1–12.
28. Dongiovanni, P.; Valenti, L. A Nutrigenomic Approach to Non-Alcoholic Fatty Liver Disease. *Int. J. Mol. Sci.* **2017**, *18*, 1534.
29. Naska, A.; Trichopoulou, A. Nutrigenomics: The Genome–Food Interface. *Environ. Health. Perspec.* **2007**, *115*,: A582–A589.
30. Davis, J.N.; Le, K.A.; Walker, R.W.; Vikman, S.; Spruijt-Metz, D.; Weigensberg, M.J.; Allayee, H.; Goran, M.I. Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in PNPLA3 and high dietary carbohydrate and sugar consumption. *Am. J. Clin. Nutr.* **2010**, *92*, 1522–1527.
31. Anstee, Q.M.; Day, C.P. The genetics of NAFLD. *Nat. Rev. Gastroenterol. Hepatol.* **2013**, *10*, 645–55.
32. Pirazzi, C.; Adiels, M.; Burza, M.A.; Mancina, R.M.; Levin, M.; Ståhlman, M.; Taskinen, M.R.; Orho-Melander, M.; Perman, J.; Pujia, A.; Andersson,

- L.; Maglio, C.; Montalcini, T.; Wiklund, O.; Borén, J.; Romeo, S. Patatin-like phospholipase domain-containing 3 (PNPLA3) I148M (rs738409) affects hepatic VLDL secretion in humans and in vitro. *J. Hepatol.* **2012**, *57*, 1276–82.
33. European Association for the Study of the Liver (EASL). Electronic address: easloffice@easloffice.eu; European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO). EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J. Hepatol.* **2016**, *64*, 1388–1402.
34. Bedogni, G.; Bellentani, S.; Miglioli, L.; Masutti, F.; Passalacqua, M.; Castiglione, A.; Tiribelli, C. The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol.* **2006**, *6*, 33.
35. Kotronen, A.; Peltonen, M.; Hakkarainen, A.; Sevastianova, K.; Bergholm, R.; Johansson, L.M.; Lundbom, N.; Rissanen, A.; Ridderstråle, M.; Groop, L.; Orho-Melander, M.; Yki-Järvinen, H. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. *Gastroenterology* **2009**, *137*, 865–72.
36. Poynard, T.; Lassailly, G.; Diaz, E.; Clement, K.; Caiazzo, R.; Tordjman, J.; Munteanu, M.; Perazzo, H.; Demol, B.; Callafe, R.; Pattou, F.; Charlotte, F.; Bedossa, P.; Mathurin, P.; Ratziu, V. Performance of biomarkers FibroTest, ActiTest, SteatoTest, and NashTest in patients with severe obesity: meta-analysis of individual patient data. *PLoS One* **2012**; *7*, e30325.

37. Saadeh, S.; Younossi, Z.M.; Remer, E.M.; Gramlich, T.; Ong, J.P.; Hurley, M.; Mullen, K.D.; Cooper, J.N.; Sheridan, M.J. The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology* **2002**, *123*, 745–750.
38. Mancini, M.; Prinster, A.; Annuzzi, G.; Liuzzi, R.; Giacco, R.; Medagli, C.; Cremone, M.; Clemente, G.; Maurea, S.; Riccardi, G.; Rivellese, AA.; Salvatore, M. Sonographic hepatic-renal ratio as indicator of hepatic steatosis: comparison with (1)H magnetic resonance spectroscopy. *Metabolism*. **2009**, *58*, 1724-30.
39. Guo, J.; Friedman, S.L. Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. *Fibrogenesis Tissue Repair* **2010**, *3*, 21.
40. European Association for the Study of the Liver, Asociacion Latinoamericana para el Estudio del Hgado. EASL-ALEH Clinical Practice Guidelines: non-invasive tests for evaluation of liver disease severity and prognosis. *J. Hepatol.* **2015**, *63*,237–264.
41. Wong, V.W.; Vergniol, J.; Wong, G.L.; Foucher, J.; Chan, H.L.; Le Bail, B.; Choi, P.C.L.; Kowo, M.; Hung Chan. A.; Merrouche, W.; Sung, J.J.; de Lédinghen, V. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. *Hepatology* **2010**, *51*, 454–462.
42. Toshimitsu, K.; Matsuura, B.; Ohkubo, I.; Niiya, T.; Furukawa, S.; Hiasa, Y.; Kawamura, M.; Ebihara, K.; Onji, M. Dietary habits and nutrient intake in non-alcoholic steatohepatitis. *Nutrition* **2007**; *23*, 46-52.

43. Naska, A.; Trichopoulou, A. Back to the future: the Mediterranean diet paradigm. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 216–219.
44. Romagnolo, D.F.; Selmin, O.I. Mediterranean Diet and Prevention of Chronic Diseases. *Nutr. Today* **2017**, doi: 10.1097/NT.0000000000000228.
45. Zelber-Sagi, S.; Salomone, F.; Mlynarsky, L. The Mediterranean dietary pattern as the diet of choice for non-alcoholic fatty liver disease: Evidence and plausible mechanisms. *Liver Int.* **2017**, *37*, 936–949.
46. Allard, J.P.; Aghdassi, E.; Mohammed, S.; Raman, M.; Avand, G.; Arendt, B.M.; Jalali, P.; Kandasamy, T.; Prayitno, N.; Sherman, M.; Guindi, M.; Ma, D.W.; Heathcote, J.E. Nutritional assessment and hepatic fatty acid composition in non-alcoholic fatty liver disease (NAFLD): a cross-sectional study. *J. Hepatol* **2008**; *48*, 300-307.
47. Musso, G.; Gambino, R.; De Michieli, F.; Cassader, M.; Rizzetto, M.; Durazzo, M.; Fagà, E.; Silli, B.; Pagano, G. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* **2003**, *37*, 909-916.
48. Westerbacka, J.; Lammi, K.; Häkkinen, A.M.; Rissanen, A.; Salminen, I.; Aro, A.; Yki-Järvinen, H. Dietary fat content modifies liver fat in overweight nondiabetic subjects. *J Clin Endocrinol Metab* **2005**; *90*, 2804-2809.
49. Rosqvist, F.; Iggman, D.; Kullberg, J.; Cedernaes, J.; Johansson, H.E.; Larsson, A.; Johansson, L.; Ahlström, H.; Arner, P.; Dahlman, I.; Risérus, U. Overfeeding polyunsaturated and saturated fat causes distinct effects on liver and visceral fat accumulation in humans. *Diabetes* **2014**; *63*, 2356-2368.

50. Cao, J.; Dai, D.L.; Yao, L.; Yu, H.H.; Ning, B.; Zhang, Q.; Cheng, W.H.; Shen, W.; Yang, Z.X. Saturated fatty acid induction of endoplasmic reticulum stress and apoptosis in human liver cells via the PERK/ATF4/CHOP signaling pathway. *Mol. Cell. Biochem.* **2012**, *364*, 115–129.
51. Lin, J.; Yang, R.; Tarr, P.T.; Wu, P.H.; Handschin, C.; Li, S.; Yang, W.; Pei, L.; Uldry, M.; Tontonoz, P.; Newgard, C.B.; Spiegelman, B.M. Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. *Cell* **2005**, *120*, 261–273.
52. Leamy, A.K.; Egnatchik, R.A.; Young, J.D. Molecular Mechanisms and the Role of Saturated Fatty Acids in the Progression of Non-Alcoholic Fatty Liver Disease. *Prog. Lipid. Res.* **2013**, *52*, 165–174.
53. Bozzetto, L.; Prinster, A.; Annuzzi, G.; Costagliola, L.; Mangione, A.; Vitelli, A.; Mazzeola, R.; Longobardo, M.; Mancini, M.; Vigorito, C.; Riccardi, G.; Rivellese, A.A. Liver fat is reduced by an isoenergetic MUFA diet in a controlled randomized study in type 2 diabetic patients. *Diabetes Care* **2012**, *35*, 1429-1435.
54. Ryan, M.C.; Itsiopoulos, C.; Thodis, T.; Ward, G.; Trost, N.; Hofferberth, S.; O'Dea, K.; Desmond, P.V.; Johnson, N.A.; Wilson, A.M. The Mediterranean diet improves hepatic steatosis and insulin sensitivity in individuals with non-alcoholic fatty liver disease. *J. Hepatol.* **2013**, *59*, 138-143.
55. Nigam, P.; Bhatt, S.; Misra, A.; Chadha, D.S.; Vaidya, M.; Dasgupta, J.; Pasha, Q.M. Effect of a 6-month intervention with cooking oils containing a high concentration of monounsaturated fatty acids (olive and canola oils)

- compared with control oil in male Asian Indians with nonalcoholic fatty liver disease. *Diabetes Technol. Ther.* **2014**, *16*, 255-261.
56. Varela, L.M.; Ortega-Gomez, A.; Lopez, S.; Abia, R.; Muriana, F.J.; Bermudez, B. The effects of dietary fatty acids on the postprandial triglyceride-rich lipoprotein/apoB48 receptor axis in human monocyte/macrophage cells. *J. Nutr. Biochem.* **2013**, *24*, 2031-2039.
57. DeLany, J.P.; Windhauser, M.M.; Champagne, C.M.; Bray, G.A. Differential oxidation of individual dietary fatty acids in humans. *Am. J. Clin. Nutr.* **2000**, *72*, 905-911.
58. Ferramosca, A.; Savy, V.; Zara, V. Olive oil increases the hepatic triacylglycerol content in mice by a distinct influence on the synthesis and oxidation of fatty acids. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 62-69.
59. Bozzetto, L.; Costabile, G.; Luongo, D.; Naviglio, D.; Cicala, V.; Piantadosi, C.; Patti, L.; Cipriano, P.; Annuzzi, G.; Rivellese, A.A. Reduction in liver fat by dietary MUFA in type 2 diabetes is helped by enhanced hepatic fat oxidation. *Diabetologia* **2016**, *59*, 2697-2701.
60. Hussein, O.; Grosovski, M.; Lasri, E.; Svalb, S.; Ravid, U.; Assy, N. Monounsaturated fat decreases hepatic lipid content in non-alcoholic fatty liver disease in rats. *World. J. Gastroenterol.* **2007**, *13*, 361-368.
61. Rivellese, A.A.; Giacco, R.; Annuzzi, G.; De Natale, C.; Patti, L.; Di Marino, L.; Minerva, V.; Costabile, G.; Santangelo, C.; Masella, R. Effects of monounsaturated vs. saturated fat on postprandial lipemia and adipose tissue lipases in type 2 diabetes. *Clin. Nutr.* **2008**, *27*, 133-141.

62. Zivkovic, A.M.; German, J.B.; Sanyal, A.J. Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. *Am. J. Clin. Nutr.* **2007**, *86*, 285-300.
63. Juárez-Hernández, E.; Chávez-Tapia, N.C.; Uribe, M.; Barbero-Becerra, V.J. Role of bioactive fatty acids in nonalcoholic fatty liver disease. *Nutr. J.* **2016**, *15*, 72.
64. Bjermo, H.; Iggman, D.; Kullberg, J.; Dahlman, I.; Johansson, L.; Persson, L.; Berglund, J.; Pulkki, K.; Basu, S.; Uusitupa, M.; Rudling, M.; Arner, P.; Cederholm, T.; Ahlström, H.; Risérus U. Effects of n-6 PUFAs compared with SFAs on liver fat, lipoproteins, and inflammation in abdominal obesity: a randomized controlled trial. *Am. J. Clin. Nutr.* **2012**, *95*, 1003-1012.
65. Nobili, V.; Alisi, A.; Musso, G.; Scorelitti, E.; Calder, P.C.; Byrne, C.D. Omega-3 fatty acids: Mechanisms of benefit and therapeutic effects in pediatric and adult NAFLD. *Crit. Rev. Clin. Lab. Sci.* **2016**, *53*, 106–120.
66. Spadaro, L.; Magliocco, O.; Spampinato, D.; Piro, S.; Oliveri, C.; Alagona, C.; Papa, G.; Rabuazzo, A.M.; Purrello, F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Digest. Liver. Dis.* **2008**, *40*, 194–199.
67. Scorletti, E.; Bhatia, L.; McCormick, K.G, Clough, G.F.; Nash, K.; Hodson, L.; Moyses, H.E.; Calder, P.C.; Byrne, C.D.; WELCOME Study. Effects of purified eicosapentaenoic and docosahexaenoic acids in non-alcoholic fatty liver disease: results from the WELCOME study. *Hepatology* **2014**, *60*, 1211–1221.

68. Zhu, F.S.; Liu, S.; Chen, X.M.; Huang, Z.G.; Zhang, D.W. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. *World. J. Gastroenterol.* **2008**, *14*, 6395–6400.
69. Vega, G.L.; Chandalia, M.; Szczepaniak, L.S.; Grundy, S.M. Effects of N-3 Fatty Acids on Hepatic Triglyceride Content in Humans. *J. Invest. Med.* **2008**, *56*, 780–785.
70. Argo, C.K.; Patrie, J.T.; Lackner, C.; Henry, T.D.; de Lange, E.E.; Weltman, A.L.; Shah, N.L.; Al-Osaimi, A.M.; Pramoonjago, P.; Jayakumar, S.; Binder, L.P.; Simmons-Egolf, W.D.; Burks, S.G.; Bao, Y.; Taylor, A.G.; Rodriguez, J.; Caldwell, S.H. Effects of n-3 fish oil on metabolic and histological parameters in NASH: a double-blind, randomized, placebo-controlled trial. *J. Hepatol.* **2015**, *62*, 190–197.
71. Sanyal, A.J.; Abdelmalek, M.; Suzuki, A.; Cummings, W.; Chojkier, M. No Significant Effects of Ethyl-eicosapentanoic Acid on Histologic Features of Nonalcoholic Steatohepatitis in a Phase 2 Trial. *Gastroenterology* **2014**, *147*, 377–384.
72. Cussons, J.A.; Watts, G.F.; Mori, T.A.; Stuckey, B.G. Omega-3 Fatty Acid Supplementation Decreases Liver Fat Content in Polycystic Ovary Syndrome: A Randomized Controlled Trial Employing Proton Magnetic Resonance Spectroscopy. *J. Clin. Endocrinol. Metab.* **2009**, *94*, 3842–3848.
73. Schugar, R.C.; Crawford, P.A. Low-carbohydrate ketogenic diets, glucose homeostasis, and nonalcoholic fatty liver disease. *Curr. Opin. Clin. Nutr. Metab. Care.* **2012**, *15*, 374–80.

74. Da Silva-Santi, L.G.; Masetto Antunes, M.; Martins Caparroz-Assef, S.; Carbonera, F.; Nunes Masi, L.; Curi, R.; Visentainer, J.V.; Barbosa Bazotte, R. Liver Fatty Acid Composition and Inflammation in Mice Fed with High-Carbohydrate Diet or High-Fat Diet. *Nutrients* **2016**, *8*, 682.
75. Valtuena, S.; Pellegrini, N.; Ardigo, D.; Del Rio, D.; Numeroso, F.; Scazzina, F.; Monti, L.; Zavaroni, I.; Brighenti, F. Dietary glyceimic index and liver steatosis. *Am. J. Clin. Nutr.* **2006**, *84*,136–142.
76. Moon, Y.A. The SCAP/SREBP Pathway: A Mediator of Hepatic Steatosis. *Endocrinol. Metab.* **2017**, *32*, 6-10.
77. Shirani, F; Zaribaf, F; Esmailzadeh, A. Dietary glyceimic index and glyceimic load in relation to cardiovascular disease risk factors: A review of current evidence. *J. Health Syst. Res.* **2015**, *10*, 641-654.
78. Bawden, S.; Stephenson, M.; Falcone1, Y.; Lingaya1, M.; Ciampi, E.; Hunter, K.; Bligh, F.; Schirra, J.; Taylor, M.; Morris, P.; Macdonald, I.; Gowland, P.; Marciani, L.; Aithal, G.P. Increased liver fat and glycogen stores after consumptionof high versus low glycaemic index food: A randomized crossover study. *Diabetes. Obes. Metab.* **2017**, *19*, 70–77.
79. Rusul, E.; Dragomir, A., Posea, M. Metabolic effects of low glycaemic index diets. *Nutr. J.* **2009**, *8*, 5.
80. Parnell, J.A.; Reimer, A.R. Prebiotic fiber modulation of the gut microbiota improves risk factors for obesity and the metabolic syndrome. *Gut microbes.* **2012**, *3*, 29–34.
81. Utzschneider, K.M.; Bayer-Carter, J.L.; Arbuckle, M.D.; Tidwell, J.M.; Richards, T.L.; Craft, S. Beneficial effect of a weight-stable, low-fat/low-

- saturated fat/low-glycaemic index diet to reduce liver fat in older subjects. *Br. J. Nutr.* **2013**, *109*, 1096–104.
82. Misciagna, G.; Del Pilar Díaz, M.; Caramia, D.V.; Bonfiglio, C.; Franco, I.; Noviello, M.R.; Chiloiro, M.; Abbrescia, D.I.; Mirizzi, A.; Tanzi, M.; Caruso, M.G.; Correale, M.; Reddavid, R.; Inguaggiato, R.; Cisternino, A.M.; Osella, A.R. Effect of a Low Glycemic Index Mediterranean Diet on Non-Alcoholic Fatty Liver Disease. A Randomized Controlled Clinical Trial. *J. Nutr. Health Aging.* **2016**, *21*, 404–412.
83. Ramon-Krauel, M.; Salsberg, S.L.; Ebbeling, C.B.; Voss, S.; Mulkern M.R.; Cooke, E.A.; Sarao, C.; Jonas, M.M.; Ludwig, D.S. Low-Glycemic-Load versus Low-Fat Diet in the Treatment of Fatty Liver in Obese Children. *Child. Obes.* **2013**, *9*, 252–260.
84. Fraser, A.; Abel, R.; Lawlor, D.A.; Fraser, D.; Elhayany, A. A modified Mediterranean diet is associated with the greatest reduction in alanine aminotransferase levels in obese type 2 diabetes patients: results of a quasi-randomised controlled trial. *Diabetologia* **2008**, *51*, 1616–1622.
85. Dhingra, D.; Michael, M.; Rajput, H.; Patil, R.T. Dietary fibre in food: a review. *J. food sci. technol.* **2012**, *49*, 255–266.
86. Cortez-Pinto, H.; Jesus, L.; Barros, H.; Lopes, C.; Moura, M.C.; Camilo, M.E. How different is the dietary pattern in non-alcoholic steatohepatitis patients? *Clin. Nutr.* **2006**, *25*, 816–23.
87. Kim, C.H.; Kallman, J.B.; Bai, C.; Pawloski, L.; Gewa, C.; Arsalla, A.; Sabatella, M.E.; Younossi, Z.M. Nutritional assessments of patients with non-alcoholic fatty liver disease. *Obes. Surg.* **2010**, *20*, 154–60.

88. Zolfaghari, H.; Askari, G.; Siassi, F.; Feizi, A.; Sotoudeh, G. Intake of Nutrients, Fiber, and Sugar in Patients with Nonalcoholic Fatty Liver Disease in Comparison to Healthy Individuals. *Int. J. Prev. Med.* **2016**, *7*, 98.
89. Daubioul, C.A.; Horsmans, Y.; Lambert, P.; Danse, E.; Delzenne, N.M. Effects of oligofructose on glucose and lipid metabolism in patients with nonalcoholic steatohepatitis: results of a pilot study. *Eur. J. Clin. Nutr.* **2005**, *59*, 723–726.
90. Rippe, J.M.; Angelopoulos, T.J. Fructose-containing sugars and cardiovascular disease. *Adv. Nutr.* **2015**, *6*, 430–9.
91. Nunes, P.M.; Wright, A.J.; Veltien, A.; van Asten, J.J.; Tack, C.J.; Jones, J.G.; Heerschap, A. Dietary lipids do not contribute to the higher hepatic triglyceride levels of fructose-compared to glucose-fed mice. *FASEB J.* **2014**, *28*, 1988–1997.
92. Nseir, W.; Nassar, F.; Assy, N. Soft drinks consumption and nonalcoholic fatty liver disease. *World J. Gastroenterol.* **2010**, *16*, 2579–88.
93. Abdelmalek, M.F.; Suzuki, A.; Guy, C.; Unalp-Arida, A.; Colvin, R.; Johnson, R.J.; Diehl, A.M. Increased fructose consumption is associated with fibrosis severity in patients with nonalcoholic fatty liver disease. *Hepatology* **2010**, *5*, 1961–1971.
94. Ma, J.; Fox, C.S.; Jacques, P.F.; Speliotes, E.K.; Hoffmann, U.; Smith, C.E.; Saltzman, E.; McKeown, N.M. Sugar-sweetened beverage, diet soda, and fatty liver disease in the Framingham Heart Study cohorts. *J. Hepatol.* **2015**, *63*, 462–469.

95. Dekker, M.J.; Su, Q.; Baker, C.; Rutledge, A.C.; Adeli, K. Fructose: a highly lipogenic nutrient implicated in insulin resistance, hepatic steatosis, and the metabolic syndrome. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *299*, E685–94.
96. Rebollo, A.; Roglans, N.; Alegret, M.; Laguna, J.C. Way back for fructose and liver metabolism: bench side to molecular insights. *World J. Gastroenterol.* **2012**, *18*, 6552–9.
97. Basaranoglu, M.; Basaranoglu, G.; Bugianesi, E. Carbohydrate intake and nonalcoholic fatty liver disease: fructose as a weapon of mass destruction. *Hepatobiliary Surg. Nutr.* **2015**, *4*, 109–16.
98. Bernardes, N.; Ayyappan, P.; De Angelis, K.; Bagchi, A.; Akolkar, G.; da Silva Dias, D.; Belló-Klein, A. Excessive consumption of fructose causes cardiometabolic dysfunctions through oxidative stress and inflammation. *Can. J. Physiol. Pharmacol.* **2017**, *10*, 1–13.
99. Mohamed, J.; Nazratun Nafizah, A. H.; Zariyantey, A.H. Budin, S.B. Mechanisms of Diabetes-Induced Liver Damage The role of oxidative stress and inflammation. *Sultan Qaboos Univ. Med. J.* **2016**, *16*, e132–e141.
100. Ngo Sock, E.T.; Le, K.A.; Ith, M.; Kreis, R.; Boesch, C.; Tappy, L. Effects of a short-term overfeeding with fructose or glucose in healthy young males. *Br. J. Nutr.* **2010**, *103*, 939–943.
101. Silbernagel, G.; Machann, J.; Unmuth, S.; Schick, F.; Stefan, N.; Haring, H.U.; Fritsche, A. Effects of 4-week very-high-fructose/glucose diets on insulin sensitivity, visceral fat and intrahepatic lipids: An exploratory trial. *Br. J. Nutr.* **2011**, *106*, 79–86. 39.

102. Maersk, M.; Belza, A.; Stodkilde-Jorgensen, H.; Ringgaard, S.; Chabanova, E.; Thomsen, H.; Pedersen, S.B.; Astrup, A.; Richelsen, B. Sucrose-sweetened beverages increase fat storage in the liver, muscle, and visceral fat depot: A 6-month randomized intervention study. *Am. J. Clin. Nutr.* **2012**, *95*, 283–289.
103. Bravo, S.; Lowndes, J.; Sinnott, S.; Yu, Z.; Rippe, J. Consumption of sucrose and high-fructose corn syrup does not increase liver fat or ectopic fat deposition in muscles. *Appl. Physiol. Nutr. Metab.* **2013**, *38*, 681–688.
104. Lecoultré, V.; Egli, L.; Carrel, G.; Theytaz, F.; Kreis, R.; Schneiter, P.; Boss, A.; Zwyzgart, K.; Le, K.-A.; Bortolotti, M. Effects of fructose and glucose overfeeding on hepatic insulin sensitivity and intrahepatic lipids in healthy humans. *Obesity* **2013**, *21*, 782–785.
105. Aeberli, I.; Gerber, P.A.; Hochuli, M.; Kohler, S.; Haile, S.R.; Gouni-Berthold, I.; Spinass, G.A.; Berneis, K. Low to moderate sugar-sweetened beverage consumption impairs glucose and lipid metabolism and promotes inflammation in healthy young men: a randomized controlled trial. *Am. J. Clin. Nutr.* **2011**, *94*, 479–485.
106. Lê, K.A.; Faeh, D.; Stettler, R.; Ith, M.; Kreis, R.; Vermathen, P.; Boesch, C.; Ravussin, E.; Tappy, L. A 4-wk high-fructose diet alters lipid metabolism without affecting insulin sensitivity or ectopic lipids in healthy humans. *Am. J. Clin. Nutr.* **2006**, *84*, 1374–1379.
107. Taskinen, M.R.; Söderlund, S.; Bogl, L.H.; Hakkarainen, A.; Matikainen, N.; Pietiläinen, K.H.; Räsänen, S.; Lundbom, N.; Björnson, E.; Eliasson, B.; Mancina, R.M.; Romeo, S.; Alméras, N.; Della Pepa, G.; Vetrani, C.; Prinster, A.; Annuzzi, G.; Rivellesse, A.; Després, J.P.; Borén, J. Adverse effects of

- fructose on cardiometabolic risk factors and hepatic lipid metabolism in subjects with abdominal obesity. *J. Intern. Med.* **2017**
DOI: 10.1111/joim.12632.
108. Chiu, S.; Sievenpiper, J.L.; de Souza, R.J.; Cozma, A.I.; Mirrahimi, A.; Carleton, A.J.; Ha, V.; Di Buono, M.; Jenkins, A.L.; Leiter, L.A.; Wolever, T.M.S.; Don-Wauchope, A.C.; Beyene, J.; Kendall, C.W.C.; Jenkins, D.J.A. Effect of fructose on markers of non-alcoholic fatty liver disease (NAFLD): a systematic review and meta-analysis of controlled feeding trials. *Eur. J. Clin. Nutr.* **2014**, *68*, 416–423.
109. Ma, J.; Karlsen, M.C.; Chung, M.; Jacques, P.F.; Saltzman, E.; Smith, C.E.; Fox, C.S.; McKeown, N.M. Potential link between excess added sugar intake and ectopic fat: a systematic review of randomized controlled trials. *Nutr. Rev.* **2015**, *74*, 18–32.
110. Johnston, R.D.; Stephenson, M. C.; Crossland, H.; Cordon, S.M.; Palcidi, E.; Cox, E.F.; Taylor, M.A.; Aithal, G.P.; Macdonald, I.A. No difference between high-fructose and high-glucose diets on liver triacylglycerol or biochemistry in healthy overweight men. *Gastroenterology* **2013**, *145*, 1016–1025.
111. Pichon, L.; Huneau, J.F.; Fromentin, G.; Tome, D. A high-protein, high-fat, carbohydrate-free diet reduces energy intake, hepatic lipogenesis, and adiposity in rats. *J. Nutr.* **2006**, *136*, 1256–60.
112. Alferink, L.; Kieft-deJong, J.C.; B.; Schoufour, J.D.; Taimr, P.; Ikram, A.M.; Janssen, H.L.A.; Metselaar, H.J.; Franco, O.H.; Murad, S.D. Animal protein is the most important macronutrient associated with non-alcoholic

- fatty liver disease in overweight participants: The Rotterdam Study. *J. Hepatol.* **2017**, Volume 66, S50.
113. Zelber-Sagi, S.; Nitzan-Kaluski, D.; Halpern, Z.; Oren, R. Long term nutritional intake and the risk for non-alcoholic fatty liver disease (NAFLD): A population based study. *J. Hepatol.* **2007**, 47, 711–7.
114. Bortolotti, M.; Maiolo, E.; Corazza, M.; Van Dijke, E.; Schneider, P.; Boss, A.; Carrel, G.; Giusti, V.; Lê, K.A.; Chong, D.G.Q.; Buehler, T.; Kreis, R.; Boesch, C.; Tappy, L. Effects of a whey protein supplementation on intrahepatocellular lipids in obese female patients. *Clin. Nutr.* **2011**, 30, 494–498.
115. Duarte, S.M.B.; Faintuch, J.; Stefano, J.T.; Oliveira, M. B. S. D.; Mazo, D. F. D. C.; Rabelo, F.; Vanni, D.; Nogueira, M.A.; Carhillo, F.J.; Oliveira, C. P. M. S. D. Hypocaloric high-protein diet improves clinical and biochemical markers in patients with nonalcoholic fatty liver disease (NAFLD). *Nutr. Hosp.* **2014**, 29, 94–101.
116. Kani, A.H.; Alavian, S.M.; Esmailzadeh, A.; Adibi, P.; Azadbakht, L. Effects of a novel therapeutic diet on liver enzymes and coagulating factors in patients with non-alcoholic fatty liver disease: A parallel randomized trial. *Nutrition* **2014**, 30, 814–821.
117. Lima, G.P.P.; Vianello, F.; Corrêa, C.R.; da Silva Campos, R.A.; Borguini, M.G. Polyphenols in Fruits and Vegetables and Its Effect on Human Health. *Food Nutr. Sci.* **2014**, 5, 1065–1082.

118. Pérez-Jiménez, J.; Fezeu, L.; Touvier, M.; Arnault, N.; Manach, C.; Hercberg, S.; Galan, P.; Scalbert, A. Dietary intake of 337 polyphenols in French adults. *Am. J. Clin. Nutr.* **2011**, *93*, 1220–1228.
119. Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid. Redox. Signal* **2013**, *18*, 1818–1892.
120. Annuzzi, G.; Bozzetto, L.; Costabile, G.; Giacco, R.; Mangione, A.; Anniballi, G.; Vitale, M.; Vetrani, C.; Cipriano, P.; Della Corte, G.; Pasanisi, F.; Riccardi, G.; Rivellese, AA. Diets naturally rich in polyphenols improve fasting and postprandial dyslipidemia and reduce oxidative stress: a randomized controlled trial. *Am. J. Clin. Nutr.* **2014**, *99*, 463-471.
121. Salomone, F.; Godos, J.; Zelber-Sagi, S. Natural anti-oxidants for nonalcoholic fatty liver disease: molecular targets and clinical perspectives. *Liver Int.* **2016**, *36*, 5–20.
122. Liu, J.F.; Ma, Y.; Wang, Y.; Du, Z.; Shen, J.K., Peng, H.L. Reduction of lipid accumulation in HepG2 cells by luteolin is associated with activation of AMPK and mitigation of oxidative stress. *Phytother. Re.* **2011**, *25*, 588–596.
123. Chen, S.; Zhao, X.; Ran, L.; Wan, J.; Wang, X.; Qin, J.; Shu, F.; Gao, Y.; Yuan, L.; Zhang, Q.; Mi, M. Resveratrol improves insulin resistance, glucose and lipid metabolism in patients with non-alcoholic fatty liver disease: a randomized controlled trial. *Dig. Liver Dis.* **2015**, *47*, 226–232.

124. Rodriguez-Ramiro, I.; Vauzour, D.; Minihane, A.M. Polyphenols and non-alcoholic fatty liver disease: impact and mechanisms. *Proc. Nutr. Soc.* **2016**, *75*, 47–60.
125. Bozzetto, L.; Annuzzi, G.; Pacini, G.; Costabile, G.; Vetrani, C.; Vitale, M.; Griffo, E.; Giacco, A.; De Natale, C.; Cocozza, S.; Della Pepa, G.; Tura, A.; Riccardi, G.; Rivellese, A.A. Polyphenol-rich diets improve glucose metabolism in people at high cardiometabolic risk: a controlled randomised intervention trial. *Diabetologia* **2015**, *58*, 1551–60.
126. Shang, J.; Chen, L.L.; Xiao, F.X.; Sun, H.; Ding, H.C.; Xiao, H. Resveratrol improves non-alcoholic fatty liver disease by activating AMP-activated protein kinase. *Acta Pharmacol. Sin.* **2008**, *29*, 698–706.
127. Chang, H.C.; Peng, C.H.; Yeh, D.M.; Kao, E.S.; Wang, C.J. Hibiscus sabdariffa extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. *Food Funct.* **2014**, *5*, 734–739.
128. Guo, H.; Zhong, R.; Liu, Y.; Jiang, X.; Tang, X.; Li Z.; Xia, M.; Ling, W. Effects of bayberry juice on inflammatory and apoptotic markers in young adults with features of non-alcoholic fatty liver disease. *Nutr.* **2014**, *30*, 198–203.
129. Suda, I.; Ishikawa, F.; Hatakeyama, M.; Miyawaki M.; Kudo T.; Hirano, K.; Ito, A.; Yamakawa, O.; Horiuchi, S. Intake of purple sweet potato beverage affects on serum hepatic biomarker levels of healthy adult men with borderline hepatitis *Eur. J. Clin. Nutr.* **2008**, *62*, 60–67.
130. Sakata, R.; Nakamura, T.; Torimura, T.; Ueno, T.; Sata, S. Green tea with high-density catechins improves liver function and fat infiltration in non

- alcoholic fatty liver disease (NAFLD) patients: A double-blind placebo-controlled study *Int. J. Mol. Med.* **2013**, *32*, 989–994.
131. Poulsen, M.M.; Vestergaard, P.F.; Clasen, B.F.; Radko, Y.; Christensen, L.P.; Stødkilde-Jørgensen, H.; Møller, N.; Jessen, N.; Pedersen, S.B.; Jørgensen J.O.L. High-Dose Resveratrol Supplementation in Obese Men: An Investigator-Initiated, Randomized, Placebo-Controlled Clinical Trial of Substrate Metabolism, Insulin Sensitivity, and Body Composition. *Diab.* **2013**, *62*, 1186–1195.
132. Heebøll, S.; Kreuzfeldt, M.; Hamilton-Dutoit, S.; Poulsen, M.P.; Stødkilde-Jørgensen, H.; Møller, H.J.; Jessen, N.; Thorsen, K.; Hellberg, Y.K.; Pedersen, S.B.; Grønbæk, H. Placebo-controlled, randomised clinical trial: high dose resveratrol treatment for non-alcoholic fatty liver disease. *Scand. J. Gastroenterol.* **2016**, *51*, 456–463.
133. Chachay, V.S.; Macdonald, G.A.; Martin, J.H.; Whitehead, J.P.; O’Moore–Sullivan, T.M.; Lee, P.; Franklin, M.; Klein, K.; Taylor, P.J.; Ferguson, M.; Coombes, J.S.; Thomas, J.P.; Cowin, J.C.; Kirkpatrick, C.M.J.; Prins, J.B.; Hickman I.J. Resveratrol Does Not Benefit Patients With Nonalcoholic Fatty Liver Disease. *Clin. Gastroenterol. Hepatol.* **2014**, *12*, 2092–2103.
134. Faghihzadeh, F.; Adibi, P.; Rafiei, R.; Hekmatdoost, A. Resveratrol supplementation improves inflammatory biomarkers in patients with nonalcoholic fatty liver disease. *Nutr. Res.* **2014**, *34*, 837–843.
135. Niki, E.; Traber, M.G. A history of vitamin E. *Ann. Nutr. Metab.* **2012**, *61*, 207–12.

136. Traber, M.G. Vitamin E regulatory mechanisms. *Annu. Rev. Nutr.* **2007**, *27*, 347–62.
137. Rizvi, S.; Raza, S.T.; Ahmed, F.; Ahmad, A.; Abbas, S.; Mahdi, F. The Role of Vitamin E in Human Health and Some Diseases. *SQU Med. J.* **2014**, *14*, e158–e165.
138. Nan, Y.M.; Wu, W.J.; Fu, N.; Liang, B.L.; Wang, R.Q.; Li, L.X.; Zhao, S.X.; Zhao, J.M.; Yu, J. Antioxidants vitamin E and 1-aminobenzotriazole prevent experimental non-alcoholic steatohepatitis in mice. *Scand. J. Gastroenterol.* **2009**, *44*, 1121–31.
139. Phung, N.; Pera, N.; Farrell, G.; Leclercq, I.; Hou, J.Y.; George, J. Pro-oxidant-mediated hepatic fibrosis and effects of antioxidant intervention in murine dietary steatohepatitis. *Int. J. Mol. Med.* **2009**, *24*, 171–80.
140. Soden, J.S.; Devereaux, M.W.; Haas, J.E.; Gumprich, E. Dahl, R.; Gralla, J.; Traber, M.G.; Sokol, R.J. Subcutaneous vitamin E ameliorates liver injury in an in vivo model of steatocholestasis. *Hepatology* **2007**; *46*, 485–95.
141. Hasegawa, T.; Yoneda, M.; Nakamura, K.; Makino, I.; Terano, A. Plasma transforming growth factor-beta1 level and efficacy of alpha-tocopherol in patients with non-alcoholic steatohepatitis: a pilot study. *Aliment. Pharmacol. Ther.* **2001**, *15*, 1667–72.
142. Williams, D.B.; Wan, Z.; Frier, B.C.; Bell, R.C.; Field, C.J.; Wright, D.C. Dietary supplementation with vitamin E and C attenuates dexamethasone-induced glucose intolerance in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2012**, *302*, 49–58.

- 143.Sato, K.; Goshō, M.; Yamamoto, T.; Kobayashi, Y.; Ishii, N.; Ohashi, T.; Nakade, Y.; Ito, K.; Fukuzawa, Y.; Yoneda, M. Vitamin E has a beneficial efficacy on nonalcoholic fatty liver disease: a meta-analysis of randomized controlled trials. *Nutr.* **2015**, *31*, 923–30.
- 144.Ji, H.F.; Sun, Y.; Shen, L. Effect of vitamin E supplementation on aminotransferase levels in patients with NAFLD, NASH and CHC: Results from a meta-analysis. *Nutr.* **2014**, *30*, 986–991.
- 145.Sanyal, A.J.; Chalasani, N.; Kowdley, K.V.; McCullough, A.; Diehl, A.M.; Bass, N.M.; Neuschwander-Tetri, B.A.; Lavine, J.E.; Tonascia, J.; Unalp, A.; Van Natta, M.; Clark, J.; Brunt, E.M.; Kleiner, D.E.; Hoofnagle, J.H.; Robuck, P.R. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N. Engl. J. Med.* **2010**, *362*, 1675–85.
- 146.Lavine, J.E.; Schwimmer, J.B.; Van Natta, M.L.; Molleston, J.P.; Murray, K.F.; Rosenthal, P.; Abrams, S.H.; Scheimann, A.O.; Sanyal, A.J.; Chalasani, N.; Tonascia, J.; Unalp, A.; Clark, J.M.; Brunt, E.M.; Kleiner, D.E.; Hoofnagle, J.H.; Robuck, P.R. Nonalcoholic Steatohepatitis Clinical Research Network. Effect of vitamin E or metformin for treatment of nonalcoholic fatty liver disease in children and adolescents: the TONIC randomized controlled trial. *JAMA* **2011**, *305*, 1659–68.
- 147.Miller, E. R.; Pastor-Barriuso, R.; Dalal, D.; Riemersma, R.A.; Appel, L.J.; Guallar, E. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann. Intern. Med.* **2005**, *42*, 37–46.

148. Duarte, T.L.; Lunec, J. Review: when is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radic. Res.* **2005**, *39*, 671–686.
149. Naidu, K.A. Vitamin C in human health and disease is still a mystery? *Nutr. J* **2003**, *2*:7.
150. Lykkesfeldt, J.; Poulsen, H.E. Is vitamin C supplementation beneficial? Lessons learned from randomized controlled trials. *Brit. J. Nutr.* **2010**, *103*, 1251–1259.
151. Ferolla, S.M.; Ferrari, T.C.; Lima, M.L.; Reis, T.O.; Tavares, W.C., Jr.; Couto, O.F.; Vidigal, P.V.; Fausto, M.A.; Couto, C.A. Dietary patterns in brazilian patients with nonalcoholic fatty liver disease: A cross-sectional study. *Clinics* **2013**, *68*, 11–17.
152. Canbakan, B.; Senturk, H.; Tahan, V.; Hatemi, I.; Balci, H.; Toptas, T.; Sonsuz, A.; Velet, M.; Aydin, S.; Dirican, A.; *et al.* Clinical, biochemical and histological correlations in a group of non-drinker subjects with non-alcoholic fatty liver disease. *Acta Gastroenterol. Belg.* **2007**, *70*, 277–284.
153. Da Silva, H.E.; Arendt, B.M.; Noureldin, S.A.; Therapondos, G.; Guindi, M.; Allard, J.P. A cross-sectional study assessing dietary intake and physical activity in canadian patients with nonalcoholic fatty liver disease vs. healthy controls. *J. Acad. Nutr. Diet.* **2014**, *114*, 1181–1194.
154. Madan, K.; Bhardwaj, P.; Thareja, S.; Gupta, S.D.; Saraya, A. Oxidant stress and antioxidant status among patients with nonalcoholic fatty liver disease (NAFLD). *J. Clin. Gastroenterol.* **2006**, *40*, 930–935.

155. Mager, D.R.; Patterson, C.; So, S.; Rogenstein, C.D.; Wykes, L.J.; Roberts, E.A. Dietary and physical activity patterns in children with fatty liver. *Eur. J. Clin. Nutr.* **2010**, *64*, 628–635.
156. Valdecantos, M.P.; Perez-Matute, P.; Quintero, P.; Martinez, J.A. Vitamin C, resveratrol and lipoic acid actions on isolated rat liver mitochondria: all antioxidants but different. *Redox Rep.* **2010**, *15*, 207–216.
157. Rose, F.J.; Webster, J.; Barry, J.B.; Phillips, L.K.; Richards, A.A.; Whitehead, J.P. Synergistic effects of ascorbic acid and thiazolidinedione on secretion of high molecular weight adiponectin from human adipocytes. *Diabetes Obes. Metab.* **2010**, *12*, 1084–1089
158. Harrison, S.A.; Torgerson, S.; Hayashi, P.; Ward, J.; Schenker, S. Vitamin E and vitamin C treatment improves fibrosis in patients with nonalcoholic steatohepatitis. *Am. J. Gastroenterol.* **2003**, *98*, 2485–2490.
159. Nobili, V.; Manco, M.; Devito, R.; di Ciommo, V.; Comparcola, D.; Sartorelli, M.R.; Piemonte, F.; Marcellini, M.; Angulo, P. Lifestyle intervention and antioxidant therapy in children with nonalcoholic fatty liver disease: A randomized, controlled trial. *Hepatology* **2008**, *48*, 119–128.
160. Ersöz, G.; Gunsar, F.; Karasu, Z.; Akay, S.; Batur, Y.; Akarca, U.S. Management of fatty liver disease with vitamin E and C compared to ursodeoxycholic acid treatment. *Turkish J. Gastroenterol.* **2005**, *16*, 124–128.
161. Nobili, V.; Manco, M.; Devito, R.; Ciampalini, P.; Piemonte, F.; Marcellini, M. Effect of vitamin E on aminotransferase levels and insulin resistance in children with non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **2006**, *24*, 1553–1561.

162. Cianferotti, L.; Bertoldo, F.; Bischoff-Ferrari, H.A.; Bruyere, O.; Cooper, C.; Cutolo, M.; Kanis J.A.; Kaufman, J.M.; Reginster, J.Y.; Rizzoli, R.; Brandi, M.L. Vitamin D supplementation in the prevention and management of major chronic diseases not related to mineral homeostasis in adults: research for evidence and a scientific statement from the European society for clinical and economic aspects of osteoporosis and osteoarthritis (ESCEO). *Endocr.* **2017**, *56*, 245–261.
163. Pittas, A.G.; Chung, M.; Trikalinos, T.; Mitri, J.; Brendel, M.; Patel, K.; Lichtenstein, A.H.; Lau, J.; Balk, E.M. Systematic review: Vitamin D and cardiometabolic outcomes. *Ann. Intern. Med.* **2010**, *152*, 307–314.
164. Barchetta, I.; Angelico, F.; Ben, M.D.; Baroni, M.G.; Pozzilli, P.; Morini, S.; Cavallo M.G. Strong association between non-alcoholic fatty liver disease (NAFLD) and low 25 (OH) vitamin D levels in an adult population with normal serum liver enzymes. *BMC Med.* **2011**, *9*, 85.
165. Annuzzi, G.; Della Pepa, G.; Vetrani, C. Vitamin D and cardiovascular disease: is there evidence to support the bandwagon? *Curr. Atheroscler. Rep.* **2012**, *14*, 525-34.
166. Eliades, M.; Spyrou, E.; Agrawal, N.; Lazo, M.; Brancati, F.L.; Potter, J.J.; Koteish, A.A.; Clark, J.M.; Guallar, E.; Hernaez, R. Meta-analysis: vitamin D and non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* **2013**, *38*, 246–254.
167. Ning, C.; Liu, L.; Lv, G.; Yang, Y.; Zhang, Y.; Yu, R.; Wang, Y.; Zhu, J. Lipid metabolism and inflammation modulated by Vitamin D in liver of diabetic rats. *Lipids Health Dis.* **2015**, *14*, 31.

168. Shi, H.; Norman, A.W.; Okamura, W.H.; Sen, A.; Zemel, M.B. 1 α ,25-Dihydroxyvitamin D₃ modulates human adipocyte metabolism via nongenomic action. *Faseb J.* **2011**, *15*, 2751–2753.
169. Zhou, Q.G.; Hou, F.F.; Guo, Z.J.; Liang, M.; Wang, G.B.; Zhang, X. 1,25-Dihydroxyvitamin D improved the free fatty-acid-induced insulin resistance in cultured C2C12 cells. *Diab. Metab. Res. Rev.* **2004**, *24*, 459–64.
170. Nagpal, J.; Pande, J.N.; Bhartia, A. A double-blind randomized placebo controlled trial of the short-term effect of vitamin D₃ supplementation on insulin sensitivity in apparently healthy, middle-aged, centrally obese men. *Diabet. Med.* **2009**, *26*, 19–27.
171. Sharifi, N.; Amani, R.; Hajjani, E.; Cheraghian, B. Does vitamin D improve liver enzymes, oxidative stress, and inflammatory biomarkers in adults with non-alcoholic fatty liver disease? A randomized clinical trial. *Endocr.* **2014**, *47*, 70–80.
172. Barchetta, I.; Del Ben, M.; Angelico, F.; Di Martino, M.; Fraioli, A.; La Torre, G.; Saule, R.; Perri, L.; Morini, S.; Tiberti, C.; Bertocchini, L.; Cimini, F.A.; Panimolle, F.; Catalano, C.; Baroni, M.G.; Cavallo, M.G. No effects of oral vitamin D supplementation on non-alcoholic fatty liver disease in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *BMC Med.* **2016**, *14*, 92.
173. Lorvand Amiri, H.; Agah, S.; Mousavi, S.N.; Hosseini, A.F.; Shidfar, F. Regression of Non-Alcoholic Fatty Liver by Vitamin D Supplement: A Double-Blind Randomized Controlled Clinical Trial. *Arch. Iran. Med.* **2016**, *19*, 631–8.

174. Rijkeljkhuizen, J.M.; Doesburg, T.; Girman, C.J.; Mari, A.; Rhodes, T.; Gastaldelli, A. et al. Hepatic fat is not associated with beta-cell function or postprandial free fatty acid response. *Metabolism* **2009**, *58*:196-203.
175. Finucane, F.M.; Sharp, S.J.; Hatunic, M.; Sleight, A.; De Lucia Rolfe, E.; Aihie Sayer, A. et al. Liver fat accumulation is associated with reduced hepatic insulin extraction and beta cell dysfunction in healthy older individuals. *Diabetol. Metab. Syndr.* **2014**, *6*: 43.
176. Alssema, M.; Rijkeljkhuizen, J.M.; Holst, J.J.; Teerlink, T.; Scheffer, P.G.; Eekhoff, E.M. et al. Preserved GLP-1 and exaggerated GIP secretion in type 2 diabetes and relationships with triglycerides and ALT. *Eur. J. Endocrinol.* **2013**, *169*: 421-430.
177. Cuthbertson, D.J.; Irwin, A.; Gardner, C.J.; Daousi, C.; Purewal, T.; Furlong, N. et al. Improved glycaemia correlates with liver fat reduction in obese, type 2 diabetes, patients given glucagon-like peptide-1 (GLP-1) receptor agonists. *PLoS One* **2012**, *7*: e50117.
178. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation* **2002**;106: 3143421.
179. Mancini, M.; Prinster, A.; Annuzzi, G.; Liuzzi, R.; Giacco, R.; Medagli, C. et al. Sonographic hepatic-renal ratio as indicator of hepatic steatosis:

- comparison with (1)H magnetic resonance spectroscopy. *Metabolism* **2009**,58:1724-1730.
- 180.Rivellese, A.A.; De Natale, C.; Di Marino, L.; Patti, L.; Iovine, C.; Coppola, S. et al. Exogenous and endogenous postprandial lipid abnormalities in type 2 diabetic patients with optimal blood glucose control and optimal fasting triglyceride levels. *J. Clin. Endocrinol. Metab.* **2004**, 89: 2153-2159.
- 181.Di Marino, L.; Griffo, E.; Maione, S.; Mirabella, M. Active glucagon-like peptide-1 (GLP-1): storage of human plasma and stability over time. *Clin. Chim. Acta.* **2011**, 412:1693-1694.
- 182.Pacini, G.; Mari, A. Methods for clinical assessment of insulin sensitivity and beta-cell function. *Best. Pract. Res. Clin. Endocrinol. Metab.* **2003**, 17: 305-322.
- 183.Kahl, S.; Straßburger, K.; Nowotny, B.; Livingstone, R.; Klüppelholz, B.; Keßel, K.; Hwang, J.H. et al. Comparison of liver fat indices for the diagnosis of hepatic steatosis and insulin resistance. *PLoS One* **2014**; 9: e94059.
- 184.Chatrath, H.; Vuppalanchi, R.; Chalasani, N. Dyslipidemia in patients with nonalcoholic fatty liver disease. *Semin. Liver. Dis.* **2012**, 32: 22-29.
- 185.Vergès B, Adiels M, Boren J, Barrett PH, Watts GF, Chan D, et al. Interrelationships between the kinetics of VLDL subspecies and HDL catabolism in abdominal obesity: a multicenter tracer kinetic study. *J. Clin. Endocrinol. Metab.* **2014**; 99:4281-4290.
- 186.Bernsmeier, C.; Meyer-Gerspach, A.C.; Blaser, L.S.; Jeker, L.; Steinert, R.E.; Heim, M.H. et al. Glucose-induced glucagon-like peptide 1 secretion is

- deficient in patients with non- alcoholic fatty liver disease. *PLoS One* **2014**; 9: 87488.
- 187.Seino, Y.; Yabe, D. Glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1: incretin actions beyond the pancreas. *J. Diabetes Invest.* **2013**; 4: 108-130.
- 188.Xiao, C.; Bandsma, R.H.; Dash, S.; Szeto, L.; Lewis, G.F. Exenatide, a glucagon-like peptide-1 receptor agonist, acutely inhibits intestinal lipoprotein production in healthy humans. *Arterioscler Thromb. Vasc. Biol.* **2012**; 32:1513-1519.
- 189.Kisseleva, T. Does interleukin-17 play the villain in non-alcoholic steatohepatitis? *Hepatology* **2014**; 59:1671-1672.
- 190.Tappy, L.; Le, K.A. Does fructose consumption contribute to non-alcoholic fatty liver disease? *Clin. Res. Hepatol. Gastroenterol.* **2012**, 36: 554–60.
- 191.Vos, M.B.; Lavine, J.E. Dietary fructose in nonalcoholic fatty liver disease. *Hepatology* **2013**, 57: 2525-2531.
- 192.Moore, J.B.; Gunn, P.J.; Fielding, B.A. The role of dietary sugars and de novo lipogenesis in non-alcoholic fatty liver disease. *Nutrients* **2014**, 6: 5679-5703.
- 193.Griffin, B.A. Relevance of liver fat to the impact of dietary extrinsic sugars on lipid metabolism. *Proc. Nutr. Soc.* **2015**, 74: 208-214.
- 194.Jin, R.; Vos, M.B. Fructose and liver function—is this behind nonalcoholic liver disease? *Curr. Opin. Clin. Nutr. Metab. Care* **2015**, 18: 490-495.

195. Sievenpiper, J.L.; de Souza, R.J.; Cozma, A.I.; Chiavaroli, L.; Ha, V.; Mirrahimi, A. Fructose vs. glucose and metabolism: do the metabolic differences matter? *Curr. Opin. Lipidol.* **2014**, *25*: 8-19.
196. Hodson, L.; McQuaid, S.E.; Humphreys, S.M. et al. Greater dietary fat oxidation in obese compared with lean men: an adaptive mechanism to prevent liver fat accumulation? *Am. J. Physiol. Endocrinol. Metab.* **2010**, *299*: E584-592.
197. Malik, V.S.; Hu, F.B. Fructose and Cardiometabolic Health: what the Evidence From Sugar-Sweetened Beverages Tells Us. *J. Am. Coll. Cardiol.* **2015**, *66*: 1615-1624.
198. Flannery, C.; Dufour, S.; Rabol, R.; Shulman, G.I.; Petersen, K.F. Skeletal muscle insulin resistance promotes increased hepatic de novo lipogenesis, hyperlipidemia, and hepatic steatosis in the elderly. *Diabetes* **2012**, *61*: 2711-2717.
199. Diraison, F.; Moulin, P.; Beylot, M. Contribution of hepatic de novo lipogenesis and re-esterification of plasma non esterified fatty acids to plasma triglyceride synthesis during non-alcoholic fatty liver disease. *Diabetes. Metab.* **2003**, *29*: 478-85
200. Bantle, J.P.; Raatz, S.K.; Thomas, W.; Georgopoulos, A. Effects of dietary fructose on plasma lipids in healthy subjects. *Am. J. Clin. Nutr.* **2000**, *72*: 1128-1134.
201. Sanders, F.W.; Griffin, J.L. De novo lipogenesis in the liver in health and disease: more than just a shunting yard for glucose. *Biol. Rev. Camb. Philos. Soc.* **2016**, *91*: 452-468.

202. Perry, R.J.; Samuel, V.T.; Petersen, K.F.; Shulman, G.I. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature* **2014**, *510*: 84-91.
203. Jensen-Urstad, A.P.; Semenkovich, C.F. Fatty acid synthase and liver triglyceride metabolism: housekeeper or messenger? *Biochim. Biophys. Acta* **2012**, *1821*: 747-753.
204. Stanhope, K.L. Sugar consumption, metabolic disease and obesity: the state of the controversy. *Crit. Rev. Clin. Lab. Sci.* **2016**, *53*: 52-67.
205. Ma, J.; Karlsen, M.C.; Chung, M. et al. Potential link between excess added sugar intake and ectopic fat: a systematic review of randomized controlled trials. *Nutr. Rev.* **2016**; *74*: 18-32
206. Chung, M.; Ma, J.; Patel, K.; Berger, S.; Lau, J.; Lichtenstein, A.H. Fructose, high-fructose corn syrup, sucrose, and nonalcoholic fatty liver disease or indexes of liver health: a systematic review and meta-analysis. *Am. J. Clin. Nutr.* **2014**; *100*: 833-849.
207. Chong, M.F.; Fielding, B.A.; Frayn, K.N. Metabolic interaction of dietary sugars and plasma lipids with a focus on mechanisms and de novo lipogenesis. *Proc. Nutr. Soc.* **2007**, *66*: 52-59.
208. Hochuli, M.; Aeberli, I.; Weiss, A. et al. Sugar-sweetened beverages with moderate amounts of fructose, but not sucrose, induce Fatty Acid synthesis in healthy young men: a randomized crossover study. *J. Clin. Endocrinol. Metab.* **2014**, *99*: 2164-2172.
209. Faeh, D.; Minehira, K.; Schwarz, J.M.; Periasamy, R.; Park, S.; Tappy, L. Effect of fructose overfeeding and fish oil administration on hepatic de novo

- lipogenesis and insulin sensitivity in healthy men. *Diabetes* **2005**; *54*: 1907-1913.
- 210.Aarsland, A.; Chinkes, D.; Wolfe, R.R. Contributions of de novo synthesis of fatty acids to total VLDL-triglyceride secretion during prolonged hyperglycemia/hyperinsulinemia in normal man. *J. Clin. Invest.* **1996**, *98*: 2008-2017.
- 211.Softic, S.; Cohen, D.E.; Kahn, C.R. Role of Dietary Fructose and Hepatic De Novo Lipogenesis in Fatty Liver Disease. *Dig. Dis. Sci.* **2016**, *61*: 1282-1293.
- 212.Schwarz, J.M.; Noworolski, S.M.; Wen, M.J. et al. Effect of a High Fructose Weight-Maintaining Diet on Lipogenesis and Liver Fat. *J. Clin. Endocrinol. Metab.* **2015**, *100*: 2434-2442.
- 213.Diraision, F.; Pachiardi, C.; Beylot, M. In vivo measurement of plasma cholesterol and fatty acid synthesis with deuterated water: determination of the average number of deuterium atoms incorporated. *Metabolism* **1996**, *45*: 817-821.
- 214.Lundbom, J.; Hakkarainen, A.; Soderlund, S.; Westerbacka, J.; Lundbom, N.; Taskinen, M.R. Long-TE ¹H MRS suggests that liver fat is more saturated than subcutaneous and visceral fat. *NMR Biomed.* **2011**; *24*: 238-245.
- 215.Taskinen, M.R.; Adiels, M.; Westerbacka, J. et al. Dual metabolic defects are required to produce hypertriglyceridemia in obese subjects. *Arterioscler. Thromb. Vasc. Biol.* **2011**, *31*: 2144-2150.
- 216.Ryysy, L.; Hakkinen, A.M.; Goto, T. et al. Hepatic fat content and insulin action on free fatty acids and glucose metabolism rather than insulin

- absorption are associated with insulin requirements during insulin therapy in type 2 diabetic patients. *Diabetes* **2000**, *49*: 749-758.
217. Matikainen, N.; Manttari, S.; Westerbacka, J. et al. Postprandial lipemia associates with liver fat content. *J. Clin. Endocrinol. Metab.* **2007**, *92*: 3052-3059.
218. Schofield, W.N. Predicting basal metabolic rate, new standards and review of previous work. *Hum. Nutr. Clin. Nutr.* **1985**, *39*: 5-41.
219. Goldberg, G.R.; Black, A.E.; Jebb, S.A. et al. Critical evaluation of energy intake data using fundamental principles of energy physiology: 1. Derivation of cut-off limits to identify under recording. *Eur. J. Clin. Nutr.* **1991**, *45*: 569-581.
220. Vos, M.B.; Kimmons, J.E.; Gillespie, C.; Welsh, J.; Blanck, H.M. Dietary fructose consumption among US children and adults: the Third National Health and Nutrition Examination Survey. *Medscape J. Med.* **2008**, *10*: 160.
221. Laughlin, M.R.; Bantle, J.P.; Havel, P.J. et al. Clinical research strategies for fructose metabolism. *Adv. Nutr.* **2014**, *5*: 248-259.
222. Cox, C.L.; Stanhope, K.L.; Schwarz, J.M. et al. Consumption of fructose-sweetened beverages for 10 weeks reduces net fat oxidation and energy expenditure in overweight/obese men and women. *Eur. J. Clin. Nutr.* **2012**, *66*: 201-208.
223. Yki-Jarvinen, H. Nutritional Modulation of Non-Alcoholic Fatty Liver Disease and Insulin Resistance. *Nutrients* **2015**, *7*: 9127-9138.

- 224.Lambert, J.E.; Ramos-Roman, M.A.; Browning, J.D.; Parks, E.J. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology* **2014**, *146*: 726-735.
- 225.Ameer, F.; Scandiuzzi, L.; Hasnain, S.; Kalbacher, H.; Zaidi, N. De novo lipogenesis in health and disease. *Metabolism* **2014**, *63*: 895-902.
- 226.Stanhope, K.L.; Schwarz, J.M.; Keim, N.L. et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J. Clin. Invest.* **2009**, *119*: 1322-1334.
- 227.Parks, E.J.; Skokan, L.E.; Timlin, M.T.; Dingfelder, C.S. Dietary sugars stimulate fatty acid synthesis in adults. *J. Nutr.* **2008**, *138*: 1039-1046.
- 228.Chong, M.F.; Fielding, B.A.; Frayn, K.N. Mechanisms for the acute effect of fructose on postprandial lipemia. *Am. J. Clin. Nutr.* **2007**; *85*, 1511-1520.
- 229.Chong, M.F.; Hodson, L.; Bickerton, A.S. et al. Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am. J. Clin. Nutr.* **2008**, *87*, 817-823.
- 230.Hudgins, L.C.; Parker, T.S.; Levine, D.M.; Hellerstein, M.K. A dual sugar challenge test for lipogenic sensitivity to dietary fructose. *J. Clin. Endocrinol. Metab.* **2011**; *96*: 861-868.
- 231.Theytaz, F.; de Giorgi, S.; Hodson, L. et al. Metabolic fate of fructose ingested with and without glucose in a mixed meal. *Nutrients* **2014**, *6*: 2632-2649.

232. Sevastianova, K.; Santos, A.; Kotronen, A. et al. Effect of short term carbohydrate overfeeding and long-term weight loss on liver fat in overweight humans. *Am. J. Clin. Nutr.* **2012**, *96*: 727-734.
233. Ni, B.; Farrar, J.S.; Vaitkus, J.A.; Celi, F.S. Metabolic Effects of FGF21: thermoregulation and Beyond. *Front. Endocrinol.* **2015**, *6*: 148.
234. Kharitonkov, A.; DiMarchi, R. FGF21 Revolutions: recent Advances Illuminating FGF21 Biology and Medicinal Properties. *Trends Endocrinol. Metab.* **2015**, *26*: 608-617.
235. Inagaki, T. Research Perspectives on the Regulation and Physiological Functions of FGF21 and its Association with NAFLD. *Front Endocrinol (Lausanne)* **2015**, *6*: 147.
236. Liu, J.; Xu, Y.; Hu, Y.; Wang, G. The role of fibroblast growth factor 21 in the pathogenesis of non-alcoholic fatty liver disease and implications for therapy. *Metabolism* **2015**, *64*: 380-390.
237. Dushay, J.R.; Toschi, E.; Mitten, E.K.; Fisher, F.M.; Herman, M.A.; Maratos-Flier, E. Fructose ingestion acutely stimulates circulating FGF21 levels in humans. *Mol. Metab.* **2015**, *4*: 51-57.
238. Johnson, R.J.; Nakagawa, T.; Sanchez-Lozada, L.G. et al. Sugar, uric acid, and the etiology of diabetes and obesity. *Diabetes* **2013**, *62*: 3307-3315.
239. Stanhope, K.L.; Bremer, A.A.; Medici, V. et al. Consumption of fructose and high fructose corn syrup increase postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young men and women. *J. Clin. Endocrinol. Metab.* **2011**, *96*: E1596-1605.

240. Jackson, K.G.; Walden, C.M.; Murray, P. et al. A sequential two meal challenge reveals abnormalities in postprandial TAG but not glucose in men with increasing numbers of metabolic syndrome components. *Atherosclerosis* **2012**, *220*: 237-243.
241. Lowndes, J.; Sinnett, S.; Yu, Z.; Rippe, J. The effects of fructose containing sugars on weight, body composition and cardiometabolic risk factors when consumed at up to the 90th percentile population consumption level for fructose. *Nutrients* **2014**, *6*: 3153-3168.
242. David Wang, D.; Sievenpiper, J.L.; de Souza, R.J. et al. Effect of fructose on postprandial triglycerides: a systematic review and meta-analysis of controlled feeding trials. *Atherosclerosis* **2014**; *232*: 125-133.
243. Chiavaroli, L.; de Souza, R.J.; Ha, V. et al. Effect of Fructose on Established Lipid Targets: a Systematic Review and Meta Analysis of Controlled Feeding Trials. *J. Am. Heart. Assoc.* **2015**, *4*: e001700.
244. Stanhope, K.L.; Medici, V.; Bremer, A.A. et al. A dose-response study of consuming high-fructose corn syrup-sweetened beverages on lipid/lipoprotein risk factors for cardiovascular disease in young adults. *Am. J. Clin. Nutr.* **2015**, *101*: 1144-1154.
245. Boren, J.; Watts, G.F.; Adiels, M. et al. Kinetic and Related Determinants of Plasma Triglyceride Concentration in Abdominal Obesity. Multicenter Tracer Kinetic Study. *Arterioscler. Thromb. Vasc. Biol.* **2015**, *35*: 2218-2224.
246. Hazlehurst JM, Woods C, Marjot T, Cobbold JF, Tomlinson JW. Non-alcoholic fatty liver disease and diabetes. *Metabolism* **2016**; *65*:1096–1108.

247. Koliak C, Roden M. Hepatic energy metabolism in human diabetes mellitus, obesity and non-alcoholic fatty liver disease. *Mol Cell Endocrinol* **2013**; 379: 35–42
248. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* **2004**; 126:460–468
249. Wang C, Wang X, Gong G, et al. Increased risk of hepatocellular carcinoma in patients with diabetes mellitus: a systematic review and metaanalysis of cohort studies. *Int J Cancer* **2012**; 130:1639–1648
250. Lomonaco R, Bril F, Portillo-Sanchez P, et al. Metabolic impact of nonalcoholic steatohepatitis in obese patients with type 2 diabetes. *Diabetes Care* **2016**; 39: 632–638
251. Williams KH, Shackel NA, Gorrell MD, McLennan SV, Twigg SM. Diabetes and nonalcoholic Fatty liver disease: a pathogenic duo. *Endocr Rev.* **2013**; 34:84-129.
252. Targher G., Bertolini L., Rodella S., Zoppini G., Lippi G., Day C. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and proliferative/laser-treated retinopathy in type 2 diabetic patients. *Diabetologia.* 2008;51:444–450.
253. Carnovale E, Marletta L. Tabelle di Composizione degli Alimenti Aggiornamento **2000** Inran. [Food Composition Database – Update by the National Research Institute for Food and Nutrition in 2000.] Milan, Italy: EDRA, 2000 (in Italian).
254. USDA database for the flavonoid content of selected foods. Release 2.1. Washington, DC: USDA, **2007**.

255. Longo R, Pollesello P, Ricci C, et al. Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* **1995**; 5: 281–285.
256. Longo R, Ricci C, Masutti F, et al. Fatty infiltration of the liver. Quantification by ¹H localized magnetic resonance spectroscopy and comparison with computed tomography. *Invest Radiol* **1993**; 28: 297–302.