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Facultad de Farmacia y Nutrición

Follow-up of two personalized energy-restricted dietary strategies in subjects with nonalcoholic fatty liver disease: underlying nutritional and lifestyle factors

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"La ciencia, a pesar de sus progresos increíbles, no puede ni podrá nunca explicarlo todo. Cada vez ganará nuevas zonas a lo que hoy parece inexplicable. Pero las rayas fronterizas del saber, por muy lejos que se eleven, tendrán siempre delante un infinito mundo de misterio"

Gregorio Marañón

A mis padres

A mi familia

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List of abbreviations

2D-SWE	2-dimensional shear wave elastography
AASLD	American Association for the Study of Liver Diseases
ALT	Alanine aminotransferase
ANCOVA	Analysis of covariance
AST	Aspartate aminotransferase
AHA	American Heart Association
AIP	Atherogenic index of plasma
ALA	α-linolenic acid
APRI	Aspartate aminotransferase-to-Platelet Ratio Index
ARFI	Acoustic radiation force impulse
BARD	BMI, aspartate aminotransferase/alanine aminotransferase ratio, diabetes score
BDI-II	Beck's Depression Inventory-II
BMI	Body mass index
DASH	Dietary approaches to stop hypertension
DHA	Docosahexaenoic acid
DXA	Dual-energy X-ray absorptiometry
EPA	Eicosapentaenoic acid
EASD	European Association for the Study of Diabetes
EASL	European Association for the Study of the Liver
EASO	European Association for the Study of Obesity
FAME	Fatty acid methyl esters
FIB-4	Fibrosis-4
FLI	Fatty Liver Index
FLiO	Fatty Liver in Obesity study
FFA	Free fatty acids
FFQ	Food frequency questionnaire
FGF-21	Fibroblast growth factor 21
GEE	Generalized estimating equations
GGT	Gamma-glutamyltransferase

HCC	Hepatocellular carcinoma
HDL-c	High-density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HSI	Hepatic Steatosis Index
IL-6	Interleukin 6
LAP	Lipid Accumulation Product
LDL-c	Low-density lipoprotein cholesterol
LECT2	Leukocyte cell-derived chemotaxin 2
MedDiet	Mediterranean diet
MetS	Metabolic syndrome
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MUFA	Monounsaturated fatty acids
NAFL	Nonalcoholic fatty liver
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NFS	NAFLD-Fibrosis Score
OSAS	Obstructive sleep apnea syndrome
OWLiver	One Way Liver S. L.
PNPLA3	Patatin-like phospholipase domain-containing 3
PREDIMED	Prevention with Mediterranean diet
PSQI	Pittsburgh Sleep Quality Index
PUFA	Polyunsaturated fatty acids
RBP4	Retinol-binding protein 4
RESMENA	Reduction of metabolic syndrome in Navarra
SFA	Saturated fatty acids
STAI	State Trait Anxiety Inventory
TAC	Total antioxidant capacity
TEV	Total energy value
TG	Triglycerides

TM6SF2	Transmembrane 6 Superfamily Member 2
TNF- α	Tumor necrosis factor alpha
TyG	Triglycerides/Glucose index
WC	Waist circumference

Abstract

Nonalcoholic fatty liver disease (NAFLD) prevalence is estimated around 25% worldwide being related to the growing rates of obesity, type 2 diabetes mellitus, and cardiovascular disease. NAFLD onset and progression are complex and have shown relationships with multiple environmental contributors, including unbalanced diets. A link between NAFLD and some psychological traits and inadequate sleep has been suggested, but the nature of these relationships is poorly understood. The current management of NAFLD relies on lifestyle modifications that promote weight loss by means of changes in the diet and/or physical activity. In this context, this research had the following objectives: 1) To evaluate the long-term effects of two personalized energy-restricted dietary strategies on weight loss, anthropometric measurements, biochemical determinations, and hepatic status in subjects with overweight or obesity and NAFLD within a follow-up of 24 months (Chapter 1); 2) To assess the potential relationships of different nutritional factors with hepatic outcomes of individuals with overweight or obesity and NAFLD following personalized energy-restricted dietary strategies (Chapter 2 and 3); 3)To analyze the potential relationships between well-being factors including psychological traits and sleep features with hepatic health markers of individuals with overweight or obesity and NAFLD (Chapter 4 and 5). Regarding the first objective, the results suggested that both dietary strategies may be suitable alternatives for NAFLD management. However, the Fatty Liver in Obesity strategy seemed to provide greater and more persistent metabolic and hepatic benefits. In relation to the second objective, the decrease in liver fat content was associated with a greater weight loss and a higher adherence to the Mediterranean Diet (MedDiet) and dietary Total antioxidant capacity (TAC) after 6 months of intervention. Moreover, the adherence to dietary strategies for 6 months led to changes in erythrocyte membrane omega-3 fatty acid composition, which in turn were associated with changes in hepatic markers (liver stiffness and liver iron content). Concerning the third objective, dietary strategies produced benefits not only in hepatic status but also in depressive and anxiety symptoms, which were positively associated with anthropometric and hepatic determinations. In addition, NAFLD subjects showed poorer sleep features compared to non-NAFLD controls. Likewise, higher sleep disturbances were associated with more probability of having NAFLD, while more sleep disturbances and worse sleep quality were associated with higher values of liver stiffness in NAFLD subjects. In conclusion, healthy energy-restricted dietary strategies with high adherence to MedDiet, as well as TAC and omega-3 fatty acids seem to be effective to improve different metabolic and hepatic markers in subjects with NAFLD. Moreover, beneficial effects on depressive and anxiety symptoms are promoted with these approaches fostering improvements in overall health. In addition to dietary factors, psychological traits and characteristics of sleep pattern should receive attention in the design and implementation of strategies for the prevention and management of NAFLD.

Resumen

La prevalencia de la enfermedad del hígado graso no alcohólico (EHGNA) se estima en alrededor del 25% en el mundo y está relacionada con las tasas crecientes de obesidad, diabetes mellitus tipo 2 y enfermedad cardiovascular. El inicio y la progresión de la EHGNA son complejos y han mostrado relaciones con múltiples factores ambientales, incluidas las dietas desequilibradas. Se ha sugerido un vínculo entre la EHGNA y algunos rasgos psicológicos y el sueño inadecuado, sin embargo, la naturaleza de estas relaciones es poco comprendida. El manejo actual de la EHGNA se basa en modificaciones del estilo de vida que promueven la pérdida de peso mediante cambios en la dieta y / o actividad física. En este contexto, esta investigación tuvo los siguientes objetivos: 1) Evaluar los efectos a largo plazo de dos estrategias dietéticas personalizadas con restricción energética sobre la pérdida de peso, las mediciones antropométricas, las determinaciones bioquímicas y el estado hepático de sujetos con sobrepeso u obesidad y EHGNA dentro de un seguimiento de 24 meses (Capítulo 1). 2) Evaluar las posibles relaciones entre diferentes factores nutricionales y los resultados hepáticos de personas con sobrepeso u obesidad y EHGNA siguiendo estrategias dietéticas personalizadas de restricción energética (capítulos 2 y 3); 3) Analizar las posibles relaciones entre factores de bienestar, incluidos los rasgos psicológicos y las características del sueño, con los marcadores de salud hepática de personas con sobrepeso u obesidad y EHGNA (Capítulos 4 y 5). En cuanto al primer objetivo, los resultados sugirieron que ambas estrategias dietéticas pueden ser alternativas adecuadas para el manejo de la EHGNA. Sin embargo, la estrategia Fatty Liver in Obesity pareció proporcionar beneficios metabólicos y hepáticos mayores y más persistentes. Con relación al segundo objetivo, la disminución del contenido de grasa hepática se asoció con una mayor pérdida de peso y una mayor adherencia a la Dieta Mediterránea (MedDiet) y la Capacidad Antioxidante Total (TAC) dietética a los 6 meses de intervención. Además, la adherencia a las estrategias dietéticas durante 6 meses provocó cambios en la composición de ácidos grasos omega-3 de la membrana eritrocitaria, que a su vez se asociaron con cambios en los marcadores hepáticos (rigidez hepática y contenido de hierro en el hígado). En cuanto al tercer objetivo, las estrategias dietéticas produjeron beneficios no solo en el estado hepático, sino también en los síntomas depresivos y de ansiedad, los cuales se asociaron positivamente con determinaciones antropométricas y hepáticas. Además, los sujetos con EHGNA mostraron peores características de sueño en comparación con los controles sin EHGNA. Asimismo, mayores alteraciones del sueño se asociaron con una mayor probabilidad de tener EHGNA, mientras que más alteraciones del sueño y una peor calidad de este se asociaron con valores más altos de rigidez hepática en sujetos con EHGNA. En conclusión, las estrategias dietéticas saludables restringidas en energía con alta adherencia a MedDiet y TAC, así como ácidos grasos omega-3 parecen ser efectivas para mejorar diferentes marcadores metabólicos y hepáticos en sujetos con EHGNA. Además, los efectos beneficiosos sobre los síntomas depresivos y de ansiedad se promueven con estos enfoques que promoviendo mejoras en la salud general. Adicionalmente a los factores dietéticos, los rasgos psicológicos y las características del patrón de sueño deben recibir atención en el diseño e implementación de estrategias para la prevención y el manejo de la EHGNA.

Table of content

INTRODUCTION ***1***

1. Nonalcoholic fatty liver disease **2**

1.1 Definition 2

1.2 Prevalence and natural history 2

1.3 Etiology and Physiopathology 4

1.4 Diagnosis 8

1.5 Metabolic comorbidities associated with NAFLD 13

2. Lifestyle factors associated with nonalcoholic fatty liver disease **17**

2.1 Dietary features 17

2.2 Physical inactivity 21

2.3 Sleep features 22

2.4 Psychological factors 23

3. Management of nonalcoholic fatty liver disease **23**

3.1 Weight loss 24

3.2 Dietary approaches 24

3.3 Fatty acids 25

3.4 Other dietary and lifestyle factors 26

3.5 Pharmacotherapy 27

HYPOTHESIS AND OBJECTIVES ***29***

1. Hypothesis **30**

1. General objective **30**

2. Specific objectives 31

SUBJECTS AND METHODS 32

1. Study design 34

2. Study participants 35

3. Dietary interventions 36

4. Anthropometric, body composition and biochemical determinations 37

5. Composition of erythrocyte membranes 38

6. Assessment of liver status 39

7. Lifestyle assessment: Diet and physical activity 40

8. Lifestyle assessment: Sleep quality, depressive symptoms and anxiety 40

9. Statistical analyses 42

RESULTS 45

- CHAPTER 1: Effects of two personalized dietary strategies during a 2-year intervention in subjects with nonalcoholic fatty liver disease: a randomized trial 46

- CHAPTER 2: The Metabolic and Hepatic Impact of Two Personalized Dietary Strategies in Subjects with Obesity and Nonalcoholic Fatty Liver Disease: The Fatty Liver in Obesity (FLiO) Randomized Controlled Trial 72

- CHAPTER 3: Effects of a 6-month dietary-induced weight loss on erythrocyte membrane omega-3 fatty acids and hepatic status of subjects with nonalcoholic fatty liver disease: The Fatty Liver in Obesity study 89

- **CHAPTER 4: Preserved improvements on depressive and anxiety symptoms in subjects with nonalcoholic fatty liver disease during a 24-month energy-restricted dietary intervention.....105**

- **CHAPTER 5: Association between sleep disturbances and liver status in obese subjects with nonalcoholic fatty liver disease: A comparison with healthy controls.....132**

DISCUSSION*.....*148

1. **Rationale of the study.....149**
2. **Longtime effects of dietary interventions on metabolic and hepatic status 151**
3. **Effects of weight loss, MedDiet adherence and dietary TAC on NAFLD 154**
4. **Omega-3 PUFA and hepatic status after 6 months of dietary intervention 157**
5. **Depressive and anxiety symptoms during a 24-month follow-up of energy-restricted dietary strategies for NAFLD treatment 161**
6. **Sleep disturbances and NAFLD.....164**
7. **Strengths and limitations.....167**
8. **General conclusion.....169**

CONCLUSIONS*.....*171

REFERENCES*.....*174

APPENDICES 199

INTRODUCTION

1. Nonalcoholic fatty liver disease

1.1 Definition

Nonalcoholic fatty liver disease (NAFLD) is a term that encompasses a continuum of liver abnormalities (Figure 1) characterized by an excessive hepatic fat accumulation (Marchesini, Day, et al., 2016). In addition, the definition of NAFLD requires the absence of secondary causes of fat accumulation in the liver, such as significant consumption of alcohol (>21 standard drinks per week in men and >14 standard drinks per week in women), long-term use of steatogenic medication, or monogenic hereditary disorders (Chalasani et al., 2018).

Histologically, NAFLD is typically classified into two categories: nonalcoholic fatty liver (NAFL), also referred as simple steatosis, and nonalcoholic steatohepatitis (NASH) (Brown & Kleiner, 2016). NAFL is defined as the presence of steatosis in ≥5% of hepatocytes without the evidence of hepatocellular injury and can progress to NASH (Stefan et al., 2019), while in NASH hepatic steatosis is accompanied by inflammation with hepatocyte injury and with or without variable degrees of fibrosis (Anstee et al., 2019; Miller, 2020). NASH covers a wide spectrum of disease severity including cirrhosis and hepatocellular carcinoma (HCC), which may be developed in a small proportion of individuals (Marchesini, Day, et al., 2016).

1.2 Prevalence and natural history

The prevalence of NAFLD is heterogeneous in different studies, mainly due to the use different diagnostic methods and the heterogeneity of the evaluated populations (Bullón-Vela et al., 2018). The majority of the available epidemiological data on NAFLD are based on ultrasonography, which detect liver fat in a semi quantitative manner (Mitra et al., 2020). The estimated global prevalence of NAFLD is about 24%-25% of the general adult population with the highest rates in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States of America (24%) and Europe (23%), whereas Africa (14%) shows lower rates (Younossi, Anstee, et al., 2018). The prevalence of NASH in the general population is unknown, but it is estimated to be about 3-5% in the adult

population of the United States, while there are no precise data regarding the incidence rates of NAFLD, although it has almost certainly increased given the rise in the prevalence of obesity since the early 1960s (Fazel et al., 2016). Concerning the available data, the estimated incidence of NAFLD is reported to be between 28 and 52 per 1000 person-years (Younossi et al., 2016).

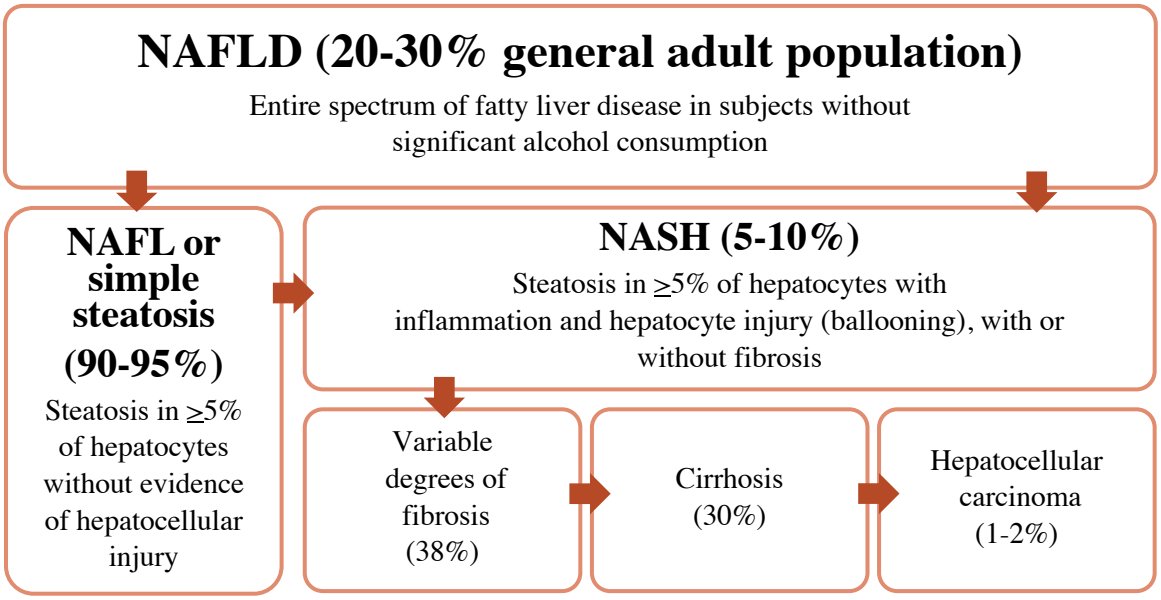


Figure 1. Spectrum and natural history of nonalcoholic fatty liver disease. Adapted from Buzzetti et al., 2016; Chalasani et al., 2018; Marchesini, Day, et al., 2016. Abbreviations: nonalcoholic fatty liver disease (NAFLD), nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH).

The components of metabolic syndrome, especially visceral obesity, are important risk factors for the development of NAFLD, while the risk factors for developing NASH include the presence of diabetes, hypertension, dyslipidemia and obesity (Fazel et al., 2016; Younossi, Anstee, et al., 2018). The natural history of NAFLD shows that even when the prevalence in the general population is high, the majority of individuals have NAFL or simple steatosis (Figure 1). However, 5-10% of the subjects with NAFLD diagnosis will develop NASH and of these cases, 30% will progress to cirrhosis, while 1-2% will develop HCC (Buzzetti et al., 2016). The disease becomes especially relevant since the prevalence of NAFLD usually increases with the prevalence of obesity and it is currently one of the most important causes of liver disease in the world, being expected to be the main cause of end-stage liver disease in the next decades (Younossi, Anstee, et al., 2018). Additionally,

NAFLD is considered serious and costly, because individuals with this condition have a high risk for liver-related and cardiovascular morbimortality (Koutoukidis et al., 2019).

1.3 Etiology and Physiopathology

The underlying mechanisms for the development of NAFLD are complex and involve multiple factors (Bullón-Vela et al., 2018). NAFLD has shown a strong association with insulin resistance and metabolic syndrome features, including obesity, type 2 diabetes mellitus, dyslipidemia and hypertension (Stefan et al., 2019). Notably, the pathogenic drivers are not likely to be identical in all the individuals with NAFLD and a high heterogeneity has been found in the mechanisms that lead to the disease and accompanying clinical manifestations (Friedman et al., 2018). Accordingly, different theories have been formulated, but the disease still not fully understood (Arab et al., 2018).

The "two-hit hypothesis" proposed by Day and James in 1998, states that the accumulation of lipids in the liver (steatosis) provides a "first hit" that sensitize the liver to oxidative stress, considered the "second hit". The oxidative stress or "second hit" is derived from the elevated lipotoxicity in the liver, which leads to necroinflammation and fibrogenesis, causing NASH (Day & James, 1998). However, this theory is nowadays considered an over-simplification of the complex development and progression of NAFLD (Anstee et al., 2019).

The "multiple parallel hits hypothesis" is currently the most accepted approach to explain the pathogenesis of NAFLD (Arab et al., 2018). This hypothesis considers multiple parallel factors, which synergistically act in subjects with a susceptible genetic or epigenetic background to induce NAFLD (Figure 2), including insulin resistance, adipose tissue dysfunction, dietary factors and gut microbiota, among others (Tilg & Moschen, 2010; Buzzetti et al., 2016). In subjects with genetic predisposition or epigenetic modifications, these factors may have influence on the fat content of hepatocytes and on the promotion of a liver inflammatory environment, leading to a chronic inflammation state of the liver through heterogenous pathways, which may contribute to the progression of hepatocellular death, activation of hepatic stellate cells and fibrous matrix deposition (Buzzetti et al., 2016).

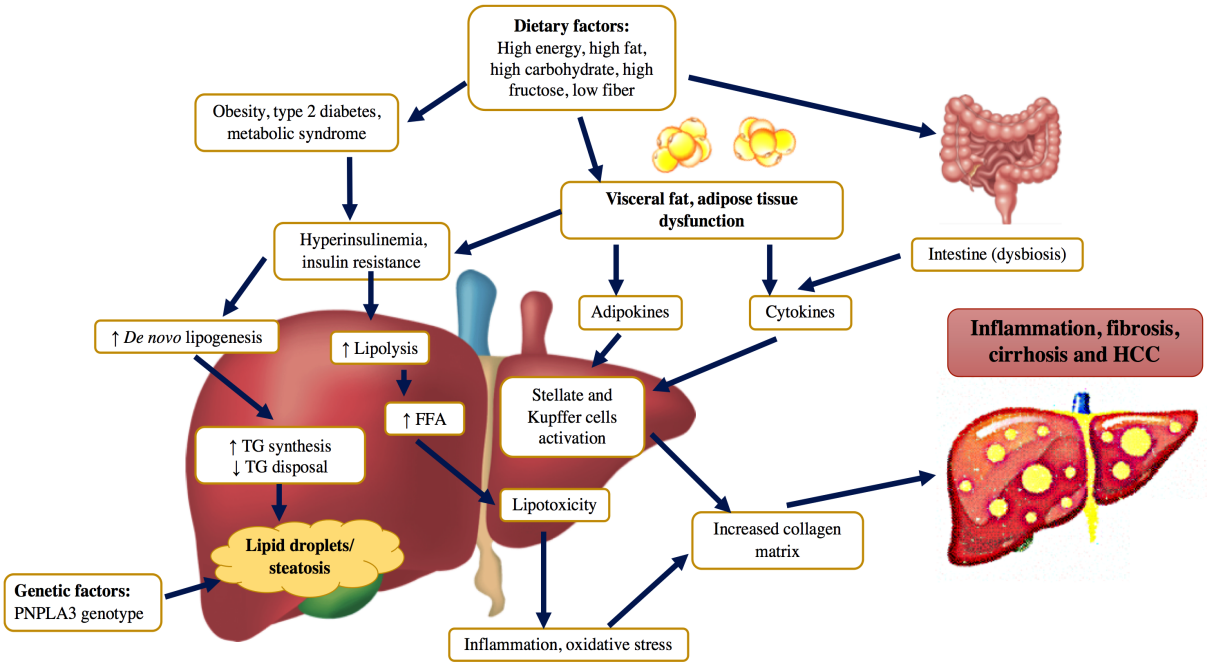


Figure 2. Multiple factors contributing the development and progression of NAFLD. Adapted from Buzzetti et al., 2016; Byrne & Targher, 2015). Abbreviations: free fatty acids (FFA), hepatocellular carcinoma (HCC), patatin-like phospholipase domain-containing 3 (*PNPLA3*), triglycerides (TG).

The role of insulin resistance is considered central to the pathophysiological processes of NAFLD, given that it results in an increased hepatic *de novo* lipogenesis and impaired inhibition of the lipolysis of adipose tissue, resulting in an increase in hepatic fat accumulation through an elevated delivery of free fatty acids to the liver (Musso et al., 2018; Bessone et al., 2019). Additionally, insulin resistance promotes adipose tissue disfunction, which leads to an altered production and secretion of adipokines and inflammatory cytokines (Bullón-Vela et al., 2018).

When the hepatic capacity to handle carbohydrates and fatty acids is overwhelmed, triglycerides and a variety of lipid metabolites are accumulated in the liver inducing mitochondrial dysfunction with oxidative stress, production of reactive oxygen species and stress of the endoplasmic reticulum, which produces injury and death of the hepatocytes, leading to fibrogenesis and genomic instability that prompts cirrhosis and HCC (Buzzetti et al., 2016; Friedman et al., 2018). Notably, different lipid species have shown to mediate hepatic lipotoxicity, including free fatty acids, triglycerides, free cholesterol, lysophosphatidyl cholines, ceramides and bile acids (Mendez-Sanchez et al., 2018).

Several fatty acids may have differential roles in the pathogenesis and progression of NAFLD (Musso et al., 2018). For instance, palmitic acid has shown to be lipotoxic, induce fat accumulation and promote metabolic stress and apoptosis of hepatocytes, while oleic acid may reduce some of these effects (Bruschi et al., 2020; Zeng et al., 2020). In addition, omega-3 polyunsaturated fatty acids (PUFA) are precursors of anti-inflammatory eicosanoids and seems to participate in the elimination of fat from hepatocytes (Mendez-Sanchez et al., 2018). Interestingly, other less studied compounds, such as the pentadecanoic acid (odd-chain fatty acid) have been suggested to regulate liver injury (Yoo et al., 2017; Venn-Watson et al., 2020).

Increasing researches suggest that the gut microbiota through the gut-liver axis might contribute to the development and differential progression of NAFLD (Adams et al., 2017; Hu et al., 2020). Several mechanisms have been proposed to explain these potential relationships. For instance, the dysbiosis induced by the dysregulation of gut endothelial barrier function that allows the translocation of bacterial components may contribute to the initiation of hepatic inflammation and promote lipogenesis and fibrosis (Safari & Gérard, 2019; Hu et al., 2020). Furthermore, an altered gut microbiota profile has been associated with systemic inflammation in patients with NAFLD-related cirrhosis with HCC, suggesting a role of microbiota in the process of hepatocarcinogenesis derived from NASH (Ponziani et al., 2019; Takakura et al., 2019).

Current knowledge shows that genetic polymorphisms can influence NAFLD development and progression (Table 1), mainly by alterations in hepatic free fatty acids, oxidative stress and inflammation (Arab et al., 2018). The available findings suggest that the genetic predisposition to NAFLD is the result of a combination of several variants, which might influence different steps of the metabolism of lipids and carbohydrates in the liver (Di Costanzo et al., 2018).

Table 1. Genetic variants associated with the development and progression of NAFLD.

Gene	Variant	Function	Phenotype
PNPLA3	rs738409 C>G	Lipid droplets remodeling	↑ NAFLD, NASH, fibrosis, HCC
TM6SF2	rs58542926 C>T	VLDL secretion	↑ NAFLD, NASH, fibrosis
LYPLAL1	rs12137855 C>T	Triglycerides catabolism	↑ NAFLD
GCKR	rs780094 A>G rs1260326 C>T	Regulation of de novo lipogenesis	↑ NAFLD, NASH, fibrosis
APOB	Several	VLDL secretion	↑ NAFLD, NASH, fibrosis, HCC
MTTP	Several	VLDL secretion	↑ NAFLD
LPIN1	rs13412852 C>T	Regulation of lipid metabolism	↓ NASH, fibrosis
SOD2	rs4880 C>T	Mitochondrial antioxidant	↑ fibrosis
UCP2	rs695366 G>A	Mitochondrial lipid metabolism Oxidative phosphorylation	↓ NASH
ENPP1	rs1044498 A>C	Insulin signaling inhibitor	↑ fibrosis
IRS1	rs1801278 A>C	Insulin signaling	↑ fibrosis
IL28B	rs12979860 C>T	Innate immunity	↓ fibrosis
KLF6	rs3750861 G>A	Regulation of de novo lipogenesis; fibrogenesis	↓ fibrosis
MERTK	rs4374383 G>A	Innate immunity, hepatic stellate cells activation	↓ fibrosis
Irisin	rs3480 A>G	Hepatic stellate cells activation	↓ fibrosis
SH2B1	rs7359397 C>T	VLDL secretion, insulin sensitivity	↑ NASH

Adapted from Eslam, Valenti and Romeo, 2018. Abbreviations: nonalcoholic fatty liver disease (NAFLD), nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC),very low-density lipoproteins (VLDL).

The Patatin-like phospholipase domain containing 3 (*PNPLA3*) *I148M* genotype has been identified as the major common determinant of NAFLD, being considered as a risk factor for both NASH presence and fibrosis severity and has shown a strong association with an increase in hepatic fat content and with elevated serum levels of hepatic transaminases (Fazel et al., 2016; Arab et al., 2018; Eslam et al., 2018). Moreover, epidemiological studies have shown large variability in the prevalence of NAFLD and NASH among different ethnicities,

which is in part attributed to the variation in the prevalence of *PNPLA3* genotypes (Eslam et al., 2018; Younossi, Anstee, et al., 2018). The Transmembrane 6 Superfamily Member 2 (*TM6SF2*) *E167K* variant has also shown to increase the susceptibility to NAFLD (Di Costanzo et al., 2018). Recently, a higher risk of developing a severe stage of NAFLD was found in carriers of the *SH2B1 rs7359397* genetic variant (Perez-Diaz-Del-Campo et al., 2020).

Finally, hepatic iron overload is observed in approximately one third of patients with NAFLD and has been suggested as a factor that potentially induces inflammation and oxidative stress contributing to the development of NASH (Aigner et al., 2015; Marmur et al., 2018). However, other researchers have suggested that iron is not as hepatotoxic as is commonly assumed and that the claims that assign a causal role to iron overload in hepatic damage should be carefully considered (Bloomer & Brown, 2019).

1.4 Diagnosis

Before the initiation of exhaustive diagnostic test for NAFLD, it is important to exclude other etiologies for steatosis and coexisting common causes of chronic liver disease (Younossi, Loomba, et al., 2018). According to the American Association for the Study of Liver Diseases (AASLD), the diagnosis of NAFLD requires the following conditions (Chalasani et al., 2018):

1) Presence of hepatic steatosis determined by histology or by imaging.

2) Absence of significant alcohol consumption (>21 standard drinks per week in men and >14 standard drinks per week in women).

3) Absence of competing etiologies for hepatic steatosis (excessive alcohol consumption, hepatitis C, parenteral nutrition, etc.).

4) Absence of coexisting causes of chronic liver disease (Hemochromatosis, autoimmune liver disease, viral hepatitis, Wilson's disease, drug-induced liver injury, among others).

1.4.1 Invasive methods

To the date, liver biopsy is a widely recognized procedure for the diagnosis and classification of NAFLD that allows to differentiate NAFL (simple steatosis) from NASH (Marchesini, Day, et al., 2016; Younossi, Loomba, et al., 2018). Thus, it is considered the reference for identifying these end points and for assessing liver fibrosis (Perakakis et al., 2019).

The distinctive histopathological features that are investigated when diagnosing NAFLD by liver biopsy include the hepatocellular accumulation of triglycerides, hepatocellular injury in the centrilobular location, cytoskeletal damage presented as hepatocellular ballooning, parenchymal inflammation with predominance of macrophages and lymphocytes (although neutrophils may be present in severe cases), and perisinusoidal fibrosis found as deposition of collagen in the space of Disse (Younossi, Loomba, et al., 2018).

The drawbacks of liver biopsy limit its use in all patients with suspected NAFLD (Sumida et al., 2014). The sample harvested by liver biopsy may be a cause of interpretation errors, since the obtained liver fraction represents the $50{,}000^{th}$ part of hepatic volume and the liver is not uniformly affected in NAFLD; the technique has a poor acceptability by the patients given its invasiveness with potential risk of complications, and the cost of its performance is elevated, among other limitations (Andronescu et al., 2018).

1.4.2 Non- or minimally invasive methods

The limitations in the use of liver biopsy has fostered the development of alternative non-invasive strategies, which have been an area of intensive investigation over the past years (Castera et al., 2019).

Non-invasive markers of NAFLD should have the following objectives in order to reduce the need for liver biopsy (Marchesini, Day, et al., 2016):

1) To identify the risk of NAFLD among individuals with increased metabolic risk in primary care settings.

2) To identify those individuals with worse prognosis in secondary and tertiary care settings

3) To monitor the progression of the disease.

4) To predict the response to therapeutic interventions.

In clinical practice, non-invasive methods are widely applied instead of biopsy for the diagnosis of NAFLD and have importance for the tracking of the disease process and the monitoring of treatment effects (Zhou et al., 2019).

Non-invasive methods rely on two different approaches that are complementary: a "physical" approach based on the determination of liver fat, liver stiffness and other hepatic parameters or a "biological" approach based on the quantification of biomarkers in serum samples (Castera et al., 2019). A variety of serum markers, radiographical modalities and non-invasive predictive algorithms have been reported or are undergoing research, but to the date, most of these approaches have suboptimal performance (Younossi, Loomba, et al., 2018).

Imaging techniques

Different imaging techniques have been used to determine liver steatosis (Altamirano et al., 2020). One of the most widely used is ultrasonography, considered the first-line imaging test and diagnostic method in clinical practice for individuals with suspected NAFLD (Zhou et al., 2019). Magnetic resonance (MR) techniques are considered well-developed alternatives to liver biopsy for the measurement of the liver lipid fraction. Magnetic resonance imaging (MRI) is sensitive to lipid and iron deposition in the liver, being able to detect and quantify the hepatic and iron fat content (Sharma et al., 2014). Studies have suggested that MRI proton density fat fraction is more sensitive than liver biopsy in evaluating changes in hepatic fat and may be utilized in the setting of clinical trials (Castera et al., 2019). Magnetic resonance spectroscopy (MRS) is another MR-based technique that indirectly measures the chemical composition of the liver with a high sensitivity and specificity for the quantification

of hepatic triglyceride content (Lee, 2020). However, the use of MRS is limited by its availability and the need for expertise in protocol prescription, data collection and spectral analysis (Younossi, Loomba, et al., 2018). Other techniques for the evaluation of liver steatosis are the Controlled attenuation parameter, which is based on ultrasonic signals measured by the FibroScan®, and the computed tomography which has been used in clinics since 1970 to evaluate fatty liver (Zhou et al., 2019).

Liver stiffness is an intrinsic physical property of liver parenchyma that is used as a surrogate marker of hepatic fibrosis (Younossi, Loomba, et al., 2018; Castera et al., 2019). Ultrasound-based techniques (sonoelastography) are non-invasive, easily accessible and available at point-of care alternative tools for assessing liver fibrosis (Lee et al., 2017). The pioneer and most widely used technique for the evaluation of chronic liver disease and associated fibrosis is transient elastography, but newer elastography modalities integrated in conventional ultrasound systems, such as acoustic radiation force impulse (ARFI) or 2-dimensional shear wave elastography (2D-SWE) are emerging (Castera et al., 2019).

Serum biomarkers and scores

Numerous investigations have tried to identify non-invasive biomarkers or to develop diagnostic scores to differentiate between NAFL and NASH and to stage hepatic fibrosis (Perakakis et al., 2019). Liver enzymes *per se* are not considered reliable and accurate predictors of NAFLD (Piazzolla & Mangia, 2020). A recent systematic review and meta-analysis found that 25% of NAFLD patients and 19% of NASH individuals had normal alanine aminotransferase (ALT) values (Ma et al., 2020). Instead of liver enzymes alone, they are used as items of predictive indexes or scores (Table 2), along with other factors such as age, sex, Body mass index (BMI), triglyceride levels and the presence of diabetes (Castera et al., 2019; Younossi, Loomba, et al., 2018).

Several scores have been proposed for the detection of steatosis and fibrosis (Table 2). The most widely used scores for steatosis include the Fatty Liver Index (FLI), Hepatic Steatosis Index (HSI), Lipid Accumulation Product (LAP), and the NAFLD Liver Fat Score, while

the most common scores for liver fibrosis, include the Fibrosis-4 (FIB-4), Aspartate aminotransferase-to-Platelet Ratio Index (APRI), which have been originally designed for hepatitis C evaluation, and others specific for NAFLD, such as the BARD (BMI, aspartate aminotransferase/alanine aminotransferase ratio, diabetes score), the BAAT (BMI, Age, ALT and triglycerides) or the NAFLD fibrosis score (Castera et al., 2019; Cheah et al., 2017).

Table 2. Components of commonly used indexes and scores for the diagnosis of liver steatosis or fibrosis in subjects with nonalcoholic fatty liver disease.

	Hepatic steatosis				**Hepatic fibrosis**				
Index	**FLI**	**HSI**	**LAP**	**NALFD-LFS**	**APRI**	**FIB-4**	**Fibrometer NAFLD**	**BARD score**	**NFS**
Items (n)	4	3	3	4	2	4	7	3	6
Age						x	x		x
Sex			x						x
BMI	x	x						x	
Diabetes		x		x				x	x
Platelet count					x	x	x		x
AST					x	x	x		
ALT						x	x		
AST/ALT ratio		x		x				x	x
GGT	x								
Triglycerides	x		x						
Other components	WC		WC	MetS and insulin			Glucose, ferritin and ALT		Albumin

Adapted from Castera et al, 2019. Abbreviations: alanine aminotransferase (ALT), Aspartate aminotransferase-to-Platelet Ratio Index (APRI), aspartate aminotransferase (AST), Body mass index, aspartate aminotransferase/alanine aminotransferase ratio, diabetes score (BARD), Body mass index (BMI), Fibrosis-4 (FIB-4), Fatty Liver Index (FLI), gamma-glutamyl transferase (GGT), Hepatic Steatosis Index (HSI), Lipid Accumulation Product (LAP), metabolic syndrome (MetS), Nonalcoholic fatty liver disease (NAFLD), NAFLD-Liver Fat Score (NAFLD-LFS), NAFLD-Fibrosis Score (NFS), waist circumference (WC).

Other biomarkers and diagnostic approaches

Various hepatokines are being studied as potential biomarkers for NAFLD, such as fibroblast growth factor 21 (FGF-21), retinol-binding protein 4 (RBP4), fetuin A, fetuin B, leukocyte cell-derived chemotaxin 2 (LECT2) and selenoprotein P, among others (Meex & Watt, 2017).

Some predictive models for diagnosing NAFLD or grading steatosis or fibrosis are propriety formulas, including FibroTest, Fibrometer, Hepascore (Castera et al., 2019), and OWLiver test, among others (Alonso et al., 2017).

Increasing evidence suggest that epigenetic factors, such as differential DNA methylation and circulating cell-free DNA methylation signatures in plasma may potentially stratify subjects with NAFLD into mild versus severe fibrosis (Younossi, Loomba, et al., 2018).

Other approaches under research include the use of lipidomic, proteomic, metabolomic and microbiome markers, as well as circulating microRNAs, considered potential biomarkers for NAFLD severity due to their stability (Zhou et al., 2019). For instance, the OWLiver test is noninvasive validated metabolomic method, which is able to differentiate between a normal liver, simple steatosis or NASH with high accuracy (Alonso et al., 2017).

1.5 Metabolic comorbidities associated with NAFLD

The pathophysiology and progression of NAFLD is influenced by multiple biological and environmental factors, being the origin of the "multiple parallel-hit" model of NAFLD (Buzzetti et al., 2016). Each individual with this condition has been dynamically affected by a different pathogenic combination during the life, which includes specific genetic polymorphisms and epigenetic modifications, dietary factors, lack of physical activity, obesity, insulin resistance, dysregulation of adipokines, lipotoxicity, dysbiosis of the gut microbiota, endocrine disruptions, among others (Polyzos et al., 2019).

The comorbid manifestations of NAFLD extend beyond the liver, with a wide range of extrahepatic manifestations (Velarde-Ruiz Velasco et al., 2019). A high prevalence of

metabolic comorbidities in subjects with NAFLD/NASH (Table 3) has been reported (Marchisello et al., 2019). There is evidence of a strong link of this condition with type 2 diabetes mellitus, chronic kidney disease, atherosclerosis, hypertension and cardiovascular disease (Rosato et al., 2019), while more recent research has shown pathological correlations between NAFLD and conditions such as hypothyroidism, obstructive sleep apnea and polycystic ovarian syndrome, among others (Mantovani et al., 2020).

Table 3. Prevalence of metabolic comorbidities in subjects with NAFLD and NASH.

	NAFLD	NASH
Obesity	51%	82%
Type 2 diabetes mellitus	23%	47%
Metabolic syndrome	41%	71%
Hyperlipidemia/dyslipidemia	69%	72%
Hypertension	39%	68%

Adapted from Marchisello et al., 2019. Abbreviations: nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH).

1.5.1 Obesity

The obesity epidemic is closely related with the growing prevalence and severity of NAFLD, given that has been associated not only with simple steatosis, but also with advanced disease including NASH, cirrhosis and hepatocellular carcinoma (Polyzos et al., 2019). However, this association is not fully understood, and several reviews and meta-analysis have contributed to the elucidation of the impact of the interplay between obesity and NAFLD and the potential differences between the progression of obese and non-obese NAFLD in the recent years. For instance, a meta-analysis that included 21 cohort studies (13 prospective and 8 retrospective studies) with more than 381,000 participants found that obesity independently led to a 3.5-fold increase in the risk of NAFLD development compared with normal weight subjects. In addition, a degree-dependent relationship between BMI and risk of NAFLD was suggested (Li et al., 2016). Other meta-analysis compared clinical characteristics between obese and non-obese individuals with NAFLD according to specific BMI cut-off points for different ethnicities to define obesity and found that obese-NAFLD

may have higher transaminase levels, higher steatosis degree and an increased risk of liver fibrosis and metabolic complications than those subjects with NAFLD but without obesity (Lu et al., 2018). Nonetheless, the consequences of lean or non-obese NAFLD should not be underestimated because those cases may also present advance liver disease (Sookoian & Pirola, 2018).

1.5.2 Insulin resistance and diabetes mellitus

Insulin resistance is defined as the failure of insulin to stimulate the transport of glucose into target cells (Asrih & Jornayvaz, 2015). NAFLD and insulin resistance have shown a close relationship, with NAFLD present in up to two thirds of the patients with type 2 diabetes mellitus (Khan et al., 2019), while the prevalence of NASH is estimated in 37.3% (Younossi, Golabi, et al., 2019). This association has functional relevance, since the presence of both NAFLD and type 2 diabetes mellitus increases the likelihood of developing diabetes complications as well as the risk of more severe progression of NAFLD, including cirrhosis, hepatocellular carcinoma and death (Hazlehurst et al., 2016). The key hypothetical mechanisms that link insulin resistance with NAFLD involve different adipokines such as leptin and adiponectin, the adipose tissue inflammation promoted by cytokines (interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- α), etc.), the rise in lipogenesis, the augmented levels of serum free fatty acids and the increased mitochondrial fatty acid oxidation, which may promote the generation of toxic lipid intermediates leading to impaired insulin signaling (Khan et al., 2019).

1.5.3 Metabolic syndrome

Metabolic syndrome (MetS) is a cluster of cardio-metabolic abnormalities generally triggered by the expansion of the visceral adipose tissue, which includes insulin resistance (with or without impaired glucose metabolism and type 2 diabetes mellitus), atherogenic dyslipidemia and high blood pressure (Lonardo et al., 2015). Importantly, MetS is a precursor of cardiovascular disease and shares pathogenic mechanism with NAFLD, involving the aforementioned interaction of adipokines, cytokines, inflammatory factors and

insulin resistance (Yang et al., 2016). Accordingly, NAFLD is often referred to as the "hepatic manifestation" of MetS (Rosato et al., 2019). Indeed, a growing body of evidence supports the idea of a bidirectional relationship between NAFLD and the features of the MetS with insulin resistance as the central pathophysiological process common to both conditions (Wainwright & Byrne, 2016). However, NAFLD is not currently recognized as a component of the MetS but has been recommended as an additional criterion (Yang et al., 2016).

1.5.4 Cardiovascular disease

The leading cause of death among individuals with NAFLD is cardiovascular disease, with men being at higher risk than women (Mullish et al., 2018). Besides the classical cardiovascular risk factors associated with metabolic syndrome (insulin resistance, hypertension, atherogenic dyslipidemia, and obesity), many non-traditional and emerging risk factors have been identified in subjects with NAFLD (Figure 3), including proinflammatory cytokines (C-reactive protein, IL-6, TNF-α), procoagulant factors (fibrinogen, plasminogen and vascular adhesion molecules) and hyperuricemia (Rosato et al., 2019). Moreover, genetic studies evaluating the association between NAFLD susceptibility genes and cardiovascular disease suggest that plasma lipids are relevant mediators between both entities, which may have important therapeutic consequences (Brouwers et al., 2019). Notably, NAFLD seems to affect anatomical structures of the heart, conferring and increased risk of cardiomyopathy, cardiac valve calcification and arrhythmia. Thus, based on the evidence, subjects with NAFLD should be carefully monitored for cardiovascular events (Rosato et al., 2019).

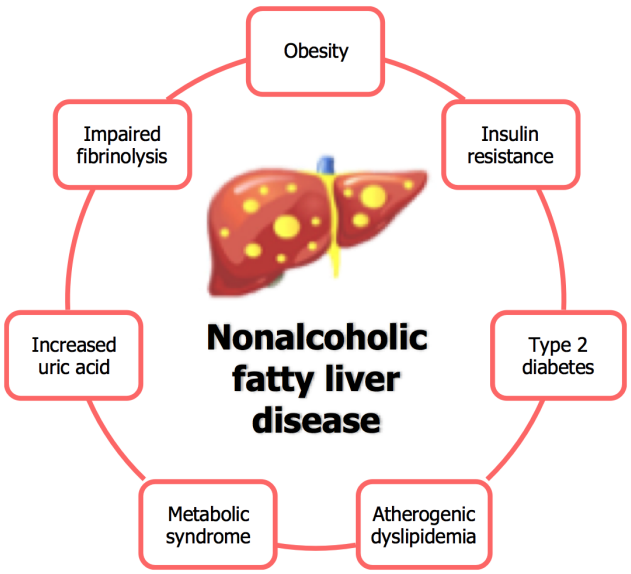


Figure 3. Cardiovascular risk factors in subjects with NAFLD. Adapted from Lonardo et al., 2016.

2. *Lifestyle factors associated with nonalcoholic fatty liver disease*

2.1 Dietary features

The habitual diet plays an important role in the development and progression of NAFLD, with the potential to contribute as a risk or as a protective factor (Marchesini, Petta, et al., 2016). The diet may be responsible for about 15% of the liver triglyceride content in NAFLD subjects (Marchisello et al., 2019). Factors such as a high calorie intake and the excessive consumption of saturated fats, refined carbohydrates (mainly fructose) have been associated with NAFLD development (Cantoral et al., 2019).

2.1.1 Dietary components

The macronutrient composition of the diet is associated with NAFLD and NASH, independently of energy intake (Perdomo et al., 2019). Saturated fatty acids (SFA), trans fats, simple sugars (sucrose and fructose) and animal protein may have damaging hepatic effects, while monounsaturated fatty acids (MUFA), omega-3 PUFA, plant-based proteins and dietary fiber seems to be beneficial for the liver (Berná & Romero-Gomez, 2020).

Dietary fatty acids participate in hepatic lipogenesis and may have a dual role in the pathogenesis of hepatic steatosis, given that are involved in its development and in preventing or reversing the accumulation of fat in the liver (Juárez-Hernández et al., 2016). The role of PUFA in the progression of NAFLD has been studied, mainly focused on omega-3 and omega-6. Linoleic acid (18:2n-6) is metabolized to arachidonic acid (20:4n-6) and their metabolic products are considered proinflammatory and prothrombotic, while α-linolenic acid (ALA; 18:3n-3) is metabolized to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which are thought to modulate the lipid composition of the liver, increasing anti-inflammatory mediators and decreasing insulin resistance (Berná & Romero-Gomez, 2020). Elevated dietary intake of lipids with high consumption of SFA has also been associated with NAFLD and hepatic inflammation (Kechagias et al., 2020).

In addition, the development and progression of NAFLD is affected by certain dietary sugars, particularly fructose (Vos & Lavine, 2013). Fructose acts as a substrate and as an inducer of hepatic *de novo* lipogenesis, and moreover, its excessive intake is associated with the release of several key mediators by the liver, leading to alterations in the communication between this organ, the gut, muscles and adipose tissue (Jegatheesan & De Bandt, 2017). The current evidence suggests that the predisposition to NAFLD is related with the metabolism of fructose by fructokinase C, resulting in ATP consumption, nucleotide turnover and uric acid production that mediate the accumulation of fat in the liver (Jensen et al., 2018). The intake of fructose seems to stimulate appetite by elevating the levels of ghrelin and by acting in the endocannabinoid signaling pathway of the central nervous system (Marchisello et al., 2019). Importantly, the effects of fructose are more severe when it is consumed as a disaccharide, such as sucrose which is composed by equal parts of fructose and glucose, or when it is associated with lipids (Jegatheesan & De Bandt, 2017). Fructose is present in honey and fruits, but its major source are sucrose and high fructose corn syrup, especially from sugary sweetened beverages (Jensen et al., 2018).

The role of protein and amino acids intake in the development and progression of NAFLD is insufficiently understood, possibly due to the variability of the composition and origin of food sources of this nutrients and to the different methodologies used in research (Berná & Romero-Gomez, 2020), among other factors. Moreover, both insufficient (Ampong et al.,

2020) and excessive protein intake (Alferink et al., 2019) may have deleterious effects on liver health, and it has been suggested that proteins and amino acids may have differential impact on hepatic status, depending on the stage of NAFLD (Galarregui et al., 2020). Specifically, an elevated consumption of red/and or processed meat has been associated with higher probability of having NAFLD and insulin resistance (Zelber-Sagi et al., 2018), and with more liver iron content, which may have a role in the progression of the disease by increasing oxidative stress (Recaredo et al., 2019). Notably, a recent study showed that a high protein intake was independently associated with more active and severe histological disease activity in subjects with NAFLD, but the effect of animal or vegetable protein was not differentiated (Lang et al., 2020). In addition, a study showed that the dietary intake of aromatic amino acids, branched-chain amino acids and sulfur amino acids was positively associated with liver fat content and other NAFLD-related features (Galarregui et al., 2020).

Little is known regarding the relationship between different forms of dietary iron in the development of NAFLD, but a recent case-control study in Chinese subjects found that a high consumption of animal-derived iron was associated with an elevated risk of NAFLD, while a high intake of iron from plant-based foods was associated with a decreased NAFLD risk (Peng et al., 2019).

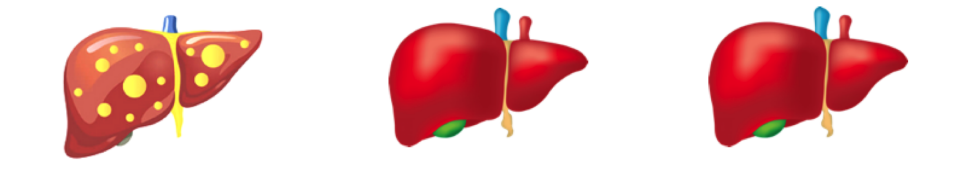
On the other hand, dietary total antioxidant capacity (TAC) is considered a useful indicator of diet quality and has been proposed as a tool to evaluate the potentially beneficial effects of antioxidants in mixed diets (Galarregui et al., 2018). In this sense, some investigations have reported a relationship between a high dietary TAC and a reduced risk of NAFLD (Salehi-Sahlabadi et al., 2020). Interestingly, a research found that subjects with NASH evaluated by liver biopsy who had a higher dietary TAC presented less ballooned hepatocytes compared to individuals with lower dietary TAC (de Oliveira et al., 2019).

Finally, decreased levels of serum zinc, copper, vitamin A, C, D, E and carotenoids have been found in subjects with NAFLD (Berná & Romero-Gomez, 2020).

2.1.2 Dietary patterns

Previous research has shown that the Western dietary pattern (Figure 4), characterized by high fat and high carbohydrate consumption, and by insufficient intake of vitamins may promote insulin resistance and its associated hepatotoxicity (Nivukoski et al., 2020).

The Mediterranean diet (MedDiet) has shown several effects on health, the majority of them related to the prevention of cardiovascular disease (Ros et al., 2014). Accordingly, the MedDiet is one of the most studied traditional dietary patterns with potential beneficial effects for the prevention of NAFLD (Martínez-González & Bastarrika, 2020). The traditional MedDiet (Figure 4) is an alimentary model characterized by high intake of vegetables, fruits, legumes, whole cereals and nuts, a moderate ingestion of fish and dairy products; moreover, the MedDiet promotes a moderate consumption of alcohol (mainly red wine during the meals) and a high intake of olive oil, in contrast with a low saturated fatty acid intake (Khalatbari-Soltani et al., 2019). Specific compounds present in the MedDiet, such as polyphenols, fiber, carotenoids, and omega-3 PUFA has been proposed as the responsible for the putative beneficial effects of this dietary pattern on liver status (Bullón-Vela et al. 2020). A cross-sectional study which included more than 13,000 adults evaluated two population-based cohorts from Switzerland and England, finding that a higher adherence to MedDiet was associated with a lower prevalence of liver steatosis (Khalatbari-Soltani et al., 2019).



	NAFLD	Healthy liver	Healthy liver
Dietary pattern	Western diet	Mediterranean diet	DASH diet
Representative foods	- Processed foods - Red and processed meat - Sugary beverages - Snacks - Cakes and biscuits - Eggs - Butter	- Extra virgin olive oil - Vegetables and fruits - Cereals, legumes, nuts - Moderate intake of fish and other meat, dairy products and red wine - Low intake of eggs and sweets	- Fruits and vegetables - Whole grains - Legumes, Fish, poultry, nuts - Low-fat dairy products - Reduced sodium - Fresh and minimally processed food
Nutrients	**High:** - Energy intake - SFA - Animal protein - Sugar and fructose - Cholesterol **Low:** - PUFA - Fiber	**High:** - MUFA - PUFA - Vegetable protein - Fiber - Polyphenols and carotenoids **Low:** - SFA and cholesterol - Sugar and fructose	**High:** - Vegetable protein - Fiber - Polyphenols and carotenoids **Low:** - Total fat and cholesterol - Salt (Sodium) - Sugar and fructose

Figure 4. Main dietary patterns associated with NAFLD. Adapted from Berná & Romero-Gomez, 2020. Abbreviations: monounsaturated fatty acids (MUFA), nonalcoholic fatty liver disease (NAFLD), polyunsaturated fatty acids (PUFA), saturated fatty acids (SFA).

The Dietary approaches to stop hypertension (DASH) diet was designed in the 1990s with the objective to regulate blood pressure (Vogt et al., 1999). DASH diet (Figure 4) is rich in fruits, vegetables, whole grains, fish, poultry, nuts, legumes and low-fat dairy products, while a reduced intake of sodium, added sugars, saturated and total fats, and red and processed meat is recommended (Riazi et al., 2019). This approach has shown beneficial effects on other metabolic disorders, such as cardiovascular disease, type 2 diabetes mellitus, and more recently an inverse association between a DASH-style diet and the risk of NAFLD has been reported (Hekmatdoost et al., 2016).

2.2 Physical inactivity

Current epidemiological and clinical evidence shows that the level of physical activity, exercise behavior and aerobic capacity can independently influence the risk for hepatic steatosis (Stevanović et al., 2020). Observational studies have identified lower levels of

physical activity as a risk factor for developing NAFLD, including a recent cross-sectional study using data of more than 42,000 individuals from the Lifelines cohort, which showed that higher levels of habitual moderate-to-vigorous physical activity were dose dependently associated with a lower risk of NAFLD and that the association was stronger in older adults and in subjects with diabetes (Byambasukh et al., 2019). Most of the current research in this topic suggest that the crosstalk between skeletal muscle, adipose tissue and the liver regulates intrahepatic fat storage by diverse mechanisms involving myokines, which are cytokines and other peptides with autocrine, paracrine and endocrine actions (Berzigotti et al., 2016).

2.3 Sleep features

Insufficient sleep has been associated with poor health outcomes, including diabetes mellitus, hypertension, cardiovascular disease, coronary heart disease, obesity and all-cause mortality (Itani et al., 2017), while a long-sleep duration has shown an association with mortality, incident diabetes mellitus, cardiovascular disease, coronary heart disease and obesity (Jike et al., 2018). Similarly, a U-shaped distribution curve has been suggested for the relationship between sleep duration and NAFLD (Imaizumi et al., 2015). Nevertheless, the studies regarding the association between sleep characteristics and NAFLD, have shown inconsistent and often contradictory outcomes. For instance, two systematic reviews and meta-analysis drew conflicting results. The first one found a small significant increase in the risk of NAFLD among subjects with short sleep duration (Wijarnpreecha et al., 2016), while the other study did not show an association between either short or long sleep duration and fatty liver disease risk (Shen et al., 2016).

In addition, other specific features of sleep pattern analysis, including the time required to fall asleep or the daytime sleepiness may have a relevant role in the development and progression of NAFLD (Bernsmeier et al., 2015). However, if sleep alterations are a cause or a consequence of the dysfunction of the liver in subjects with NAFLD, or if the relationship is bidirectional, remains unclear.

2.4 Psychological factors

Chronic liver diseases have been long associated with depression, but the underlying mechanisms of this association remain largely unknown. In addition, the nature of this relationship may vary depending on the specific pathology and the severity of the liver disease (Huang et al., 2017). Two recent studies evaluated the association between NAFLD and depression in different populations. The first one was a cross-sectional study which used the 2007-2016 National Health and Nutrition Examination Survey database among adults in the United States; the analysis of more than 10,400 participants showed that the prevalence of depression was higher in subjects with NAFLD than in those without the liver disease (Kim et al., 2019). The second study was carried out with a sample of more than 120,000 participants from Korea and found that the presence and severity of NAFLD was significantly associated with depressive symptoms (Jung et al., 2019). The production of pro-inflammatory cytokines, and the increase in cortisol and epinephrine levels have been proposed as a possible mechanism that link NAFLD with depression, as well as the close correlation of NAFLD with obesity and diabetes mellitus, both of which have been strongly associated with depressive symptoms (Huang et al., 2017).

Interestingly, other symptoms and psychological traits which potentially affect quality of life, including anxiety, cognitive impairment and loss of self-esteem have as well been reported in subjects with NAFLD (Golabi et al., 2016). Nevertheless, few studies regarding psychological factors in individuals with NAFLD have been published.

3. Management of nonalcoholic fatty liver disease

Currently, no drug therapy or medical procedure have been approved for the treatment of NAFLD (Mahjoubin-Tehran et al., 2020). Therefore, the management is focused on lifestyle modifications aiming at controlling body weight and cardiometabolic risk factors related to the metabolic syndrome (Katsagoni et al., 2017).

3.1 Weight loss

Lifestyle modification that leads to weight loss (hypocaloric diets and/or increased physical activity) is the cornerstone approach for NAFLD treatment (Miller, 2020). Caloric restriction to induce a negative energy balance has been associated with the improvement of NAFLD and the resolution of hepatic steatosis, regardless of the macronutrient composition of the diet (Worm, 2020). On the other hand, behavioral weight loss programs, weight loss pharmacotherapy, and bariatric surgery lead to weight reduction and have favorable cardiometabolic effects, but their association with improvements in NAFLD remains unclear (Koutoukidis et al., 2019).

The current guidance statements of the AASLD indicate that a weight loss of at least 3%-5% of body weight seems necessary to improve hepatic steatosis, but a greater weight loss in the range of 7%-10% is deeded to improve the majority of the histopathological characteristics of NASH, including fibrosis (Chalasani et al., 2018), while the clinical practice guidelines for the management of NAFLD of the European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) recommends a weight loss target of 7%-10% for lifestyle interventions in overweight or obese subjects with NAFLD (Marchesini, Day, et al., 2016). On the other hand, a systematic review and meta-analysis of 22 randomized clinical trials with 2588 participants evaluated the effect of interventions aiming at weight loss in subjects with NAFLD, including behavioral programs with diet alone or accompanied by exercise, pharmacotherapy and bariatric surgery. The researchers found that weight loss interventions were associated with improvement in liver steatosis, but no statistically significant change was observed in histologic liver fibrosis or NAFLD fibrosis score (Koutoukidis et al., 2019).

3.2 Dietary approaches

Several therapeutic diets, such as the low carbohydrate diets, the DASH diet and the MedDiet have been matter of investigation for the treatment of NAFLD (Moosavian et al., 2019; Perdomo et al., 2019).

A sub-study of the CENTRAL 18-month trial showed that a Mediterranean/low-carbohydrate diet induced a greater reduction in hepatic fat content determined by MRI compared to a low-fat diet (Gepner et al., 2019). In addition, a 12-week duration study showed that hepatic steatosis significantly decreased to a similar degree by both *ad-libitum* low-fat diet and MedDiet, even with only a 2% of weight loss (Properzi et al., 2018). Notably, a recent systematic review of 10 randomized controlled trials, which involved more than 850 adults concluded that the MedDiet could improve NAFLD severity parameters, although inconsistencies among the included studies were found (Moosavian et al., 2019).

The DASH diet has some similarities with the MedDiet and has shown beneficial effects on NAFLD. For instance, a randomized controlled trial with duration of 8 weeks which included 60 overweight participants with NAFLD, found a significant decrease in body weight, triglycerides level, hepatic enzymes, insulin sensitivity, oxidative stress and inflammation biomarkers in the DASH diet group, compared to the controls who received a conventional calorie-restricted diet (Razavi Zade et al., 2016).

Finally, it has been suggested that the Paleolithic diet (based on a high intake of fruits, nuts, vegetables, fish, eggs and meat, and a minimum consumption of dairy products, grains, legumes, and processed foods) may have beneficial effects in the prevention of cardiometabolic diseases (de la O et al., 2020). A research in postmenopausal women with obesity compared the effects of an *ad libitum* Paleolithic diet with a conventional low-fat diet on liver fat determined by proton resonance spectroscopy within a period of two years and found that body weight and hepatic fat content were significantly reduced in both groups at 6 and at 24 months, with a more pronounced effect of Paleolithic diet at 6 months (Otten et al., 2016). However, insufficient studies regarding the effects of this dietary pattern have been carried out in subjects with NAFLD.

3.3 Fatty acids

A reduction in the consumption of saturated fatty acids, total fats and trans fatty acids is strongly is recommended for the management of NAFLD (Ahmed et al., 2019). In the contrary, studies in which extra virgin olive oil was a major source of MUFA suggest that

its intake may improve fatty liver disease, although it is difficult to isolate the effects of MUFA from other components present in extra virgin olive oil, such as polyphenols (Berná & Romero-Gomez, 2020)

In relation to omega-3 PUFA, a meta-analysis which included 11 randomized controlled trials concluded that the supplementation with this family of fatty acids, especially with DHA had a favorable effect in the treatment of NAFLD, by reducing triglycerides, ALT and aspartate aminotransferase (AST) concentrations, while a marginal reduction in liver fat content was observed (Guo et al., 2018). However, the effects of supplementation with omega-3 PUFA still producing inconclusive results, especially regarding liver fibrosis (He et al., 2016; Yan et al., 2018). Likewise, current evidence suggests that omega-3 PUFA supplementation may be effective in the early stages of NAFLD, but not in individuals with more severe NAFLD (Yang et al., 2019). The guidance statements of the AASLD point out that omega-3 PUFA should not be used as a specific treatment of NAFLD or NASH, but they may be used to treat hypertriglyceridemia in NAFLD subjects (Chalasani et al., 2018). The inconclusive results on omega-3 could be due to differences in methodology, the duration of the trials, the level of intake of PUFA, their sources, the EPA/DHA relationships, the chemical composition of omega-3 and the genetic background of the participants, among other possible causes (Berná & Romero-Gomez, 2020). In general, the consumption of omega-3 is preferable than other forms of PUFA, but well-designed studies with a large sample-size are needed to obtain the optimal recommendations of these group of fatty acids, either as a supplement or as a part of the dietary treatment of NAFLD (Guo et al., 2018; Perdomo et al., 2019).

3.4 Other dietary and lifestyle factors

Fiber contains components that are not considered as essential nutrients but could be important mediators in human health (Cantero et al., 2017). Dietary fiber may provide a benefit to NAFLD individuals through the modulation of gut microbiota, since it has shown to promote a reduction in body weight, serum aminotransferases and to improve glycolipid metabolism (Perdomo et al., 2019). Moreover, the type of dietary fiber may differentially

impact the hepatic status. For instance, a higher intake of fiber derived from fruit has been associated with lower levels of transaminases and FLI (Cantero et al., 2017).

The impact of different foods, such as dairy products or coffee remains unclear (Berná & Romero-Gomez, 2020). Regarding coffee, a meta-analysis of epidemiological studies found that regular coffee intake was significantly associated with a reduced risk of NAFLD and a decreased risk of developing hepatic fibrosis in subjects with previously diagnosed NAFLD (Hayat et al., 2020). On the contrary, other recent research with large sample size did not supported a causal relationship between coffee consumption and the risk of NAFLD (Zhang et al., 2020). Concerning dairy products, a randomized controlled trial in women with obesity, metabolic syndrome and NAFLD found that the intake of yogurt was better than milk for the decrease of insulin resistance and liver fat, possibly by decreasing serum lipids, inflammation and oxidative stress (Chen et al., 2019). Nevertheless, people usually consume different quantities of a variety of food groups and the number of large clinical trials is limited (Berná & Romero-Gomez, 2020). Therefore, more studies are needed to determine if these specific foods could provide additional benefits in the treatment of NAFLD.

Similarly, limited data support the benefit of some probiotics which may be related to the modulation of liver function and the reduction of dysbiosis and hepatic fat (Eslamparast et al., 2017). Thus, more studies are needed to translate these findings to clinical practice.

Finally, the additive impact of exercise with nutritional interventions on hepatic steatosis is not well stablished. Exercise may only have a marginal additional effect on liver steatosis in the context of a significant dietary-induced weight loss. However, exercise is likely to promote cardiovascular improvements, which is an important goal in the treatment of NAFLD patients (Hallsworth & Adams, 2019).

3.5 Pharmacotherapy

Therapeutic approaches aiming at the reduction of liver steatosis, blockade of hepatic cell death, suppression of hepatic immune cells and inhibition of the fibrogenic activity of

hepatic stellate cells are considered promising strategies for the management of NAFLD based on the "multiple parallel hits hypothesis" (Kim & Lee, 2018).

To the date, there are no approved pharmacotherapies for NASH by the Federal Drug Administration or the European Medicines Agency and the necessity for effective anti-fibrotic treatment is still unmet (Sumida et al., 2020). Notably, a previous review concluded that there is very low-quality evidence regarding the effectiveness of pharmacological treatments for individuals with NAFLD including those with NASH and further well designed randomized clinical trials are needed (Lombardi et al., 2017). However, the guidelines from the United States, Europe and Japan recommend the use of pioglitazone in conjunction with vitamin E for biopsy-proven NASH with or without type 2 diabetes mellitus (Sumida et al., 2020).

In this context, the current pharmacological approach of NAFLD is often focused on the comorbidities associated with the disease, such as diabetes mellitus, obesity and dyslipidemia, to regulate cardiometabolic risk, glycaemia and liver function (Jeznach-Steinhagen et al., 2019; Stefan et al., 2019). Meanwhile, several ongoing phase 2 and 3 major clinical trials are investigating promising agents such as obeticholic acid (Farnesoid X receptor agonist), Selonsertib (Apoptosis-signal regulating kinase 1 inhibitor), Emricasan (Caspase inhibitor) and Semaglutide (Glucagon like peptide 1 agonist), among others (Friedman et al., 2018; Yoo et al., 2019).

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

The pathogenesis of NAFLD is influenced by multiple risk factors including obesity, insulin resistance, unbalanced dietary intake, and low physical activity, among others. Moreover, NAFLD is considered a multisystem disease with a broad spectrum of extra-hepatic manifestations. In addition, the understanding of the link between NAFLD and other aspects such as psychological traits and the characteristics of sleep pattern is still limited. The current treatment of NAFLD relies on lifestyle modifications mainly focused on the achievement of a negative energy balance by means of dietary and or/physical activity interventions. Therefore, we proposed the following hypotheses: First, well-designed healthy personalized dietary strategies aiming at weight loss according to the AASLD recommendations provide beneficial effects on general health and hepatic status of subjects with overweight or obesity and NAFLD, being alternatives for the management of this condition. Second, specific nutritional factors such as the adherence to MedDiet, dietary TAC, and omega-3 PUFA contribute to explain the effects of energy-restricted dietary interventions in subjects with overweight or obesity and NAFLD. Third, psychological traits and sleep features are associated with liver-related outcomes from individuals with overweight or obesity and NAFLD.

1. General objective

The main purpose of this research was to study the effects of two personalized energy-restricted dietary strategies on anthropometry, body composition, general metabolic markers, hepatic status, and psychological traits of individuals with overweight or obesity and NAFLD during a 24-month follow-up, as well as to understand the potential relationships of dietary and well-being factors with NAFLD features.

2. Specific objectives

1) To evaluate the long-term effects of two personalized energy-restricted dietary strategies on weight loss, anthropometric measurements, body composition, biochemical determinations, and hepatic status within a follow-up of 24 months in subjects with overweight or obesity and NAFLD (Chapter 1).

2) To assess the potential relationships of different nutritional factors with hepatic outcomes of individuals with overweight or obesity and NAFLD following personalized energy-restricted dietary strategies (Chapter 2 and 3).

3) To analyze the potential relationships between well-being factors including psychological traits and sleep features with hepatic health markers of individuals with overweight or obesity and NAFLD (Chapter 4 and 5).

SUBJECTS AND METHODS

The present work has been carried out within the Fatty Liver in Obesity (FLiO) project. The proposed objectives have been addressed in different chapters (Figure 5), where each chapter corresponds to an article. Chapter 1 to 4 included longitudinal data from different follow-up evaluations, while Chapter 5 analyzed baseline data from the FLiO project. A brief explanation of the design used in each chapter will be presented.

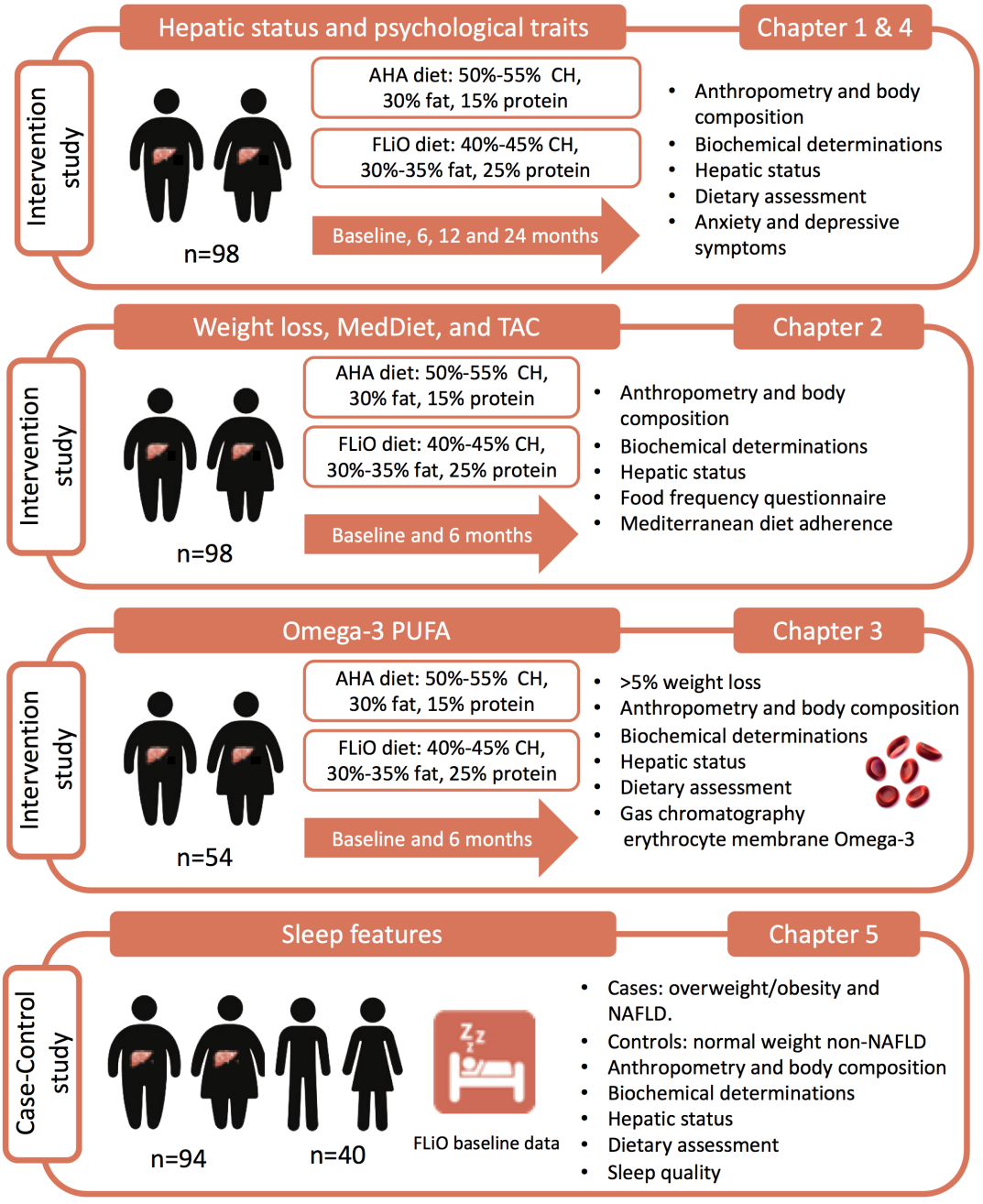


Figure 5. Overview of the experimental design conducted within each chapter. Abbreviations: American Heart Association (AHA), carbohydrates (CH), Fatty Liver in Obesity (FLiO), nonalcoholic fatty liver disease (NAFLD).

1. Study design

The FLiO randomized controlled trial was designed to compare the effects of two weight loss dietary strategies with different nutritional features on hepatic status, anthropometric measurements, body composition and biochemical markers in overweight or obese subjects with ultrasonography-proven liver steatosis.

The participants were randomly assigned to the American Heart Association (AHA) group or the FLiO group (Marin-Alejandre et al., 2019). The intervention had a total duration of two years with a complete evaluation of the participants at baseline and after 6, 12 and 24 months (Figure 6). The assessment of the participants at each point of the study included anthropometric measurements, body composition by dual energy X-Ray absorptiometry (DXA), biochemical determinations, and evaluation of the liver by ultrasonography, ARFI elastography, transient elastography, and MRI. Qualified radiologists and hepatologists performed the assessment of liver status at the University of Navarra Clinic. In addition, fasting blood samples were properly collected, processed, and stored at -80 ºC for further analyses.

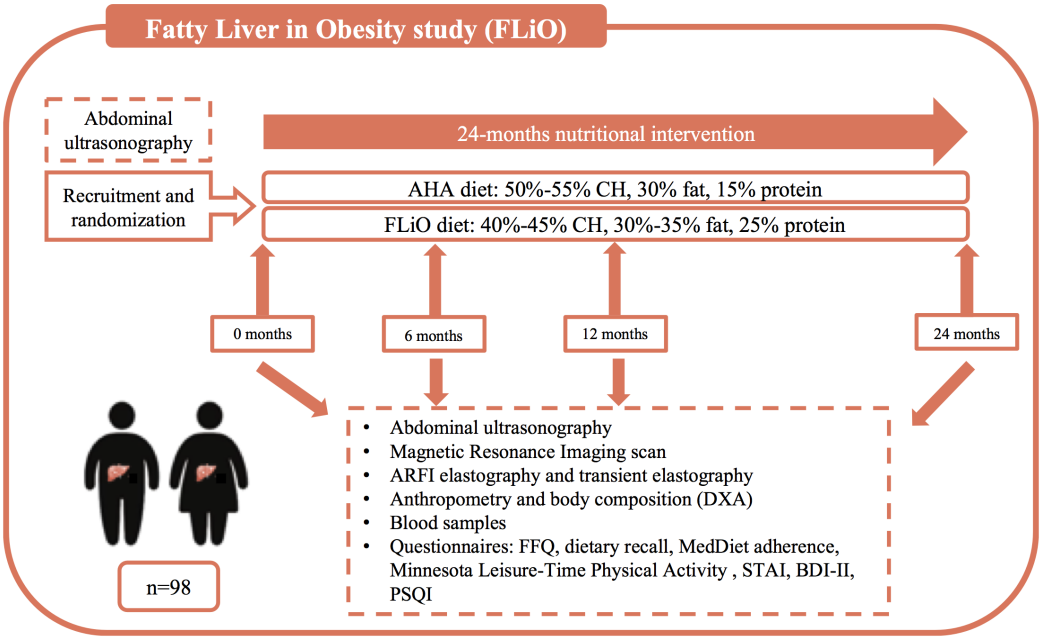


Figure 6. Overview of the FLiO study. Abbreviations: American Heart Association (AHA), Acoustic radiation force impulse (ARFI), Beck's Depression Inventory-II (BDI-II), carbohydrates (CH), food frequency questionnaire (FFQ), Pittsburgh Sleep Quality Index (PSQI) and State Trait Anxiety Inventory (STAI).

The trial was approved by the Research Ethics Committee of the University of Navarra (ref. 54/2015) and was registered in www.clinicaltrials.gov (FLiO: Fatty Liver in Obesity study; NCT03183193). Each subject gave a written informed consent prior to the enrollment in the study. All the procedures were performed in accordance with the Declaration of Helsinki and the study was conducted following the CONSORT 2010 guidelines (Moher et al., 2010).

2. Study participants

The participants of the study were recruited between June 2016 and June 2017 in Navarra, Spain. A total of 228 subjects were evaluated to determine the presence of liver steatosis by abdominal ultrasonography. A total of 98 men and women with overweight or obesity (BMI $\geq$ 27.5 kg/m^2 to <40 kg/m^2) between 40-80 years old fulfilled the selection criteria and started the trial (Marin-Alejandre et al., 2019). Two individuals from the AHA group were excluded due to important alterations in the initial assessment of biochemical parameters, which required medical management. Therefore, the study started with 48 participants in AHA group and 50 participants in FLiO group. The flowchart of the participants along the study is shown (Figure 7). Exclusion criteria included the presence of known hepatic disease other than NAFLD, excessive alcohol consumption (>21 units of alcohol per week for men and >14 per women (Sanyal et al., 2011), weight loss $\geq$ 3kg in the last 3 months, endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), pharmacological treatment with immunosuppressants, cytotoxic agents, corticosteroids (or other drugs that could potentially cause liver steatosis or alteration in hepatic tests) according to accepted criteria (Chalasani et al., 2018), active autoimmune disease or requiring pharmacological treatment, the use of weight modifiers and severe psychiatric disorders, and finally, the lack of autonomy or inability to follow the diet, as well as the difficulties in following the scheduled visits.

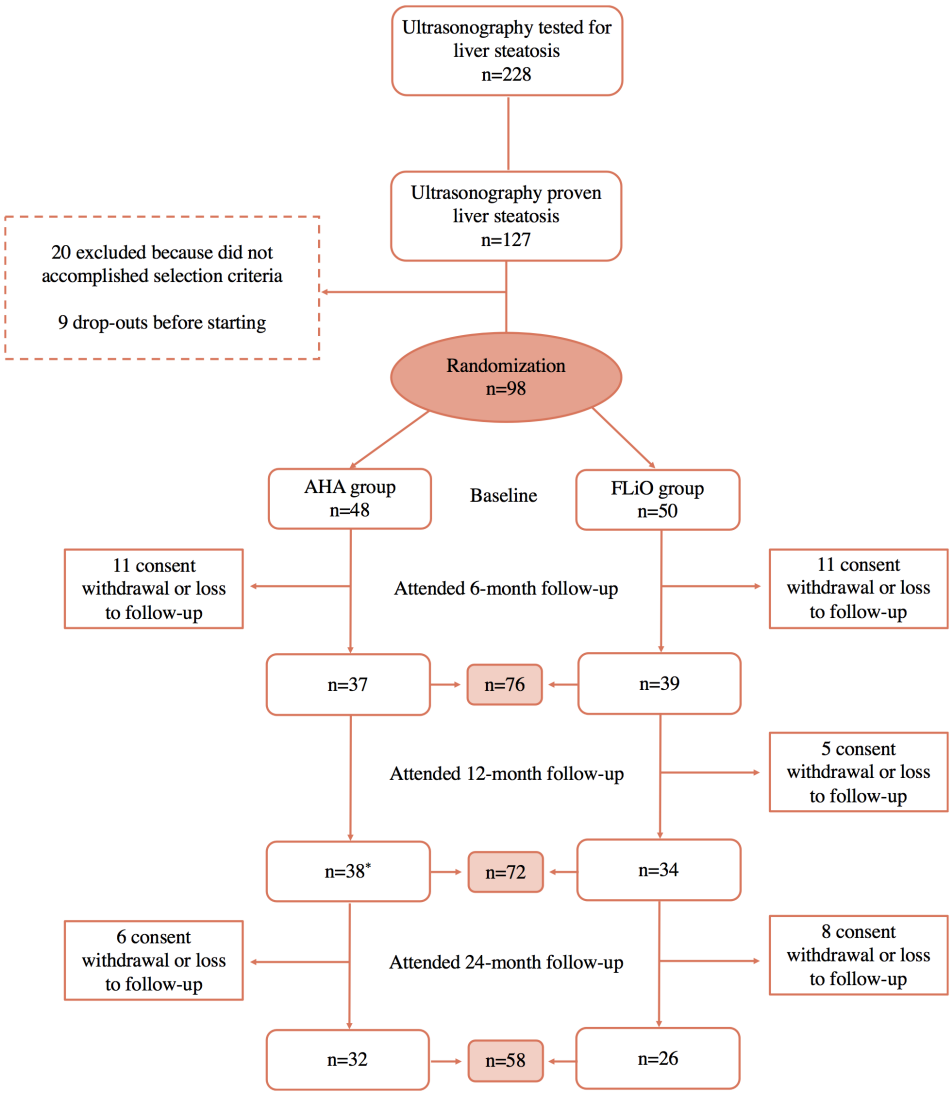


Figure 7. Flowchart of the participants in the FLiO study American Heart Association (AHA). Fatty Liver in Obesity (FLiO). * One participant did not attend to 6-month follow-up visit but did attend to 12-month follow-up visit.

3. Dietary interventions

Two different diets were prescribed and compared according to the allocation group. Both diets applied an energy restriction of 30% of the total energy requirements of each participant with the objective to achieve a loss of at least 3%-5% of the initial body weight, in accordance with the recommendations of the AASLD (Chalasani et al., 2018). The energy prescription for each participant was estimated using the equation of the Institute of

Medicine to calculate the resting metabolic rate, as elsewhere described (Trumbo et al., 2002). One diet was based on the guidelines of the AHA (De La Iglesia et al., 2014) which propose 3-5 meals/day with a conventionally balanced distribution of macronutrients in relation of the total caloric value: 50%-55% from carbohydrates, 15% from proteins and 30% from lipids with a healthy fatty acid profile. On the other hand, the FLiO diet was designed with a higher meal frequency of 7 meals/day. The macronutrient distribution according to the total energy value was: 40%-45% carbohydrates (preferring those with low glycemic index), 25% proteins (predominantly from vegetable sources), and 30%-35% from lipids favoring extra virgin olive oil and omega-3 polyunsaturated fatty acids to the detriment of saturated and trans fats (Marin-Alejandre et al., 2019). The FLiO diet proposed a high adherence to the MedDiet, involving an increased quantity of natural antioxidants based on previous studies of this research group (Lopez-Legarrea et al., 2013). Moreover, a 7 days menu plan was provided to the participants in both groups.

4. Anthropometric, body composition and biochemical determinations

Anthropometric and body composition evaluation was carried out after overnight fasting at the Metabolic Unit of the University of Navarra. Body weight, height and waist circumference were determined as elsewhere described (De La Iglesia et al., 2014). Blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, the Netherlands) and DXA body composition (Lunar iDXA, General Electric Healthcare, encore 14.5, Madison, WI, USA) were assessed following the instructions of the manufacturers.

Blood samples for biochemical determinations were properly collected after an overnight fasting of 8-10 hours. The samples were processed at the Laboratory of Biochemistry of the University of Navarra Clinic (Pamplona, Spain). Blood glucose, insulin, total cholesterol, high-density lipoprotein cholesterol (HDL-c), triglycerides, AST, ALT and gamma-glutamyltransferase (GGT) concentrations were determined on an autoanalyzer with specific commercial kits following the instructions of the company (Cobas 8000, Roche Diagnostics, Basel, Switzerland).

BMI was calculated as the body weight in kilograms divided by the squared height in meters (kg/m^2) as reported by the World Health Organization and Spanish Society for the Study of Obesity (Pérez-Rodrigo et al., 2006).

The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was calculated as HOMA-IR = (insulin (μU/mL) x glucose (mmol/L))/22.5. The low-density lipoprotein cholesterol (LDL-c) levels were calculated using the Friedewald formula (Friedewald et al., 1972). The Triglycerides/Glucose index (TyG) was calculated as ln[triglycerides (mg/dL) × glucose (mg/dL)/2] (Navarro-González et al., 2016) and the Atherogenic index of plasma (AIP) as log [triglycerides/HDL-c)] (Wang et al., 2018).

5. Composition of erythrocyte membranes

Isolation of erythrocyte membranes from peripheral blood

Erythrocyte membranes were isolated following previously described methodology (Harris et al., 2012). The fatty acid composition was evaluated in unwashed packed erythrocytes isolated from blood samples and drawn into 10ml plasma-EDTA tubes after overnight fasting of 8-10 hours. The samples were obtained and centrifugated, and the plasma and buffy coat were separated from erythrocytes. After the collection, erythrocytes were immediately frozen at -80 ºC. Samples were freeze-dried and transported to the Portuguese Institute for the Sea and Atmosphere (IPMA, IP).

Analysis of fatty acid profile in erythrocyte membranes

Fatty acid methyl esters (FAME) were prepared from the freeze-dried erythrocytes by acid-catalysis transesterification using the methodology described by Bandarra *et al* (Bandarra et al., 2002). Samples were injected into a Scion 456-GC gas chromatograph (West Lothian, UK), equipped with an auto sampler with a flame ionization detector at 250 °C. FAME were identified by comparing their retention time with those of Sigma–Aldrich standard (PUFA-

3, Menhaden oil). The limit of detection was 1 mg/100g. Results were calculated in percentage of total fatty acids on the basis of peak areas.

6. Assessment of liver status

The presence of hepatic steatosis was determined by means of ultrasonography (Siemens ACUSON S2000 and S3000, Erlangen, Germany) in accordance with previously described methodologies (Lee & Park, 2014; Cantero et al., 2019).

ARFI elastography and transient elastography were performed to assess liver stiffness as a surrogate marker of hepatic fibrosis (Liu et al., 2015). ARFI elastography was carried out along with the ultrasonography. Briefly, ten valid determinations were performed on each participant and the median value of liver stiffness was computed (Cassinotto et al., 2016). Transient elastography was carried out by means of FibroScan® (Echosens, Paris, France) as previously described (Cantero et al., 2019).

MRI (Siemens Aera 1.5 T) was used to determine the fat and iron content of the liver by Dixon technique as described elsewhere (Cantero et al., 2019). The whole evaluation was carried out under fasting conditions at the of the University of Navarra Clinic. The radiologist was blinded to treatment allocation, clinical information and laboratory data.

The Hepatic Steatosis Index or HSI was calculated using the following formula (Lee et al., 2010):

$$HSI = 8 \times (ALT/AST\ ratio) \pm BMI\ (\pm 2, \text{if female}; \pm 2, \text{if diabetes mellitus}).$$

HSI values < 30 rule out NAFLD with a sensitivity of 93.1%, while values > 36 detect NAFLD with a specificity of 92.4% (Lee et al., 2010).

The Fatty Liver Index or FLI was computed using serum triglycerides, BMI, waist circumference, and GGT concentrations using the following formula (Bedogni et al., 2006).

$$FLI = (e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745}) * 100$$

FLI values ranges from o to 100. A FLI <30 rule out liver steatosis and values >60 indicate liver steatosis with a good accuracy (Bedogni et al., 2006).

7. Lifestyle assessment: Diet and physical activity

Information regarding the diet and physical activity of the participants was collected at each timepoint of the study (baseline, 6, 12 and 24 months). The dietary intake was registered with a semi-quantitative food frequency questionnaire (FFQ). The FFQ consisted of 137-item and was previously validated in Spain for energy and nutrient intake (Fernández-Ballart et al., 2010). Each item in the FFQ included a typical portion size and the frequencies of consumption were registered in nine categories that ranged from "Never or almost never" to "$\geq$ 6 times/day". The composition of the food items was derived from accepted Spanish food composition tables (Moreiras et al., 2009) as previously described (Galarregui et al., 2018; Recaredo et al., 2019). The adherence to the MedDiet was evaluated using a 17-point screening questionnaire (Martínez-González et al., 2019). The final score ranged from 0 to 17 and a higher punctuation indicated a better adherence to the MedDiet (Galmes-Panades et al., 2019). Physical activity was estimated with the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire (Elosua et al., 1994, 2000).

8. Lifestyle assessment: Sleep quality, depressive symptoms and anxiety

Sleep features, including sleep duration, sleep efficiency, total time in bed, sleep disturbances and sleep quality, were assessed using the validated Spanish version of the Pittsburgh Sleep Quality Index (PSQI) (Royuela et al., 1997). This tool has shown a strong reliability and validity and has been used in a wide variety of samples (Doi et al., 2000;

Zhong et al., 2015; Mollayeva et al., 2016). The PSQI consists of 19 self-administered questions that generate 7 component scores with subscales ranging from 0 to 3. The sum of these components scores leads to a global score ranging from 0 to 21, for the assessment of sleep quality. A punctuation of more than 5 in the global score identifies "poor sleepers", while a punctuation of 5 or lower identifies "good sleepers" (Buysse et al., 1989). Thus, poorer sleep quality is characterized by higher scores (Royuela et al., 1997; Hita-Contreras et al., 2014). Short sleep duration was defined as a self-reported sleep time ≤ 6 h per night, according to previous studies (Kant & Graubard, 2014; Imaizumi et al., 2015; Miyake et al., 2015).

Depressive symptoms were evaluated using the Spanish adaptation of the Beck's Depression Inventory-II (BDI-II) (Sanz et al., 2003). This self-reporting questionnaire has the purpose to evaluate the severity of depressive symptoms in adults and adolescents. The instrument contains 21 items, each of them with four possible responses ranging from 0 to 3 according to the severity of the symptom. The sum of all items leads to a total punctuation ranging from 0 to 63 with higher values indicating more severe depressive symptomatology (Sanz et al., 2003).

The validated Spanish adaptation of the State Trait Anxiety Inventory (STAI) was used to evaluate anxiety symptoms (Spielberger, 1971; Guillén-Riquelme & Buela-Casal, 2011). This instrument consists of two independent subscales with 20 self-administered items in each of them with answer options on Likert-type scale ranging from 0 to 3. One subscale corresponds to State Anxiety, which evaluates the current level of anxiety responding to how the participant feels "right now". The second subscale evaluates Trait Anxiety, which is understood as a personality factor that predisposes an individual to suffer from anxiety. The sum of all items within each subscale leads to the total subscale score ranging from 0 to 60, with higher values indicating greater state or trait anxiety according to the subscale evaluated (Guillén-Riquelme & Buela-Casal, 2011).

9. Statistical analyses

The primary outcome of the study was the change in body weight, given that the current recommendations of the AASLD to ameliorate NAFLD features are focused on weight loss (Chalasani et al., 2018). The sample size was calculated to achieve a significant outcome with a 95% confidence interval ($\alpha = 0.05$) and a statistical power of 80% ($\beta = 0.8$). A total of 36 participants per study group was estimated with this approach, but 50 subjects were included in each arm of the study considering an estimated drop-out rate of 35%-40% according to the experience of the research group. The group assignment of the participants was performed using random numbers generated by computer.

The differences between the effects of the two dietary interventions during the entire trial (baseline, 6, 12 and 24 months), as well as the repeated measures overtime within each dietary group were analyzed using linear mixed model approach (Chapter 1 and 4). Linear regression analyses were performed to evaluate the potential relationship between the psychological features and anthropometric and hepatic parameters at each follow-up visit of the study (Chapter 4). The regression analyses were carried out in the pooled sample, as well as separated by sex. The final models were adjusted for age, sex (in the pooled sample), dietary group, energy intake and physical activity.

Concerning the analyses of the effects of the intervention strategies on metabolic and hepatic status after 6 months of follow up (Chapter 2) the statistical analysis was carried out as follows: The mean value (standard deviation) was reported for the studied variables. The normality of the distribution of the evaluated variables was assessed by the Shapiro–Wilk test. The differences between the groups were compared by means of Student's *t*-test or the Mann–Whitney *U* test when appropriate. The differences between the beginning and the end of the intervention period within each group were analyzed by a paired Student's *t*-test or Wilcoxon signed-rank test as appropriate. Categorical variables were compared using a Chi-squared test. Linear regression analyses were used to evaluate the potential association between the anthropometry, body composition, and components of the diet with hepatic status variables. Multilinear regression models were adjusted for potential confounders considering the group of intervention, age, sex, physical activity, and energy intake. The median value of the MedDiet Adherence questionnaire was used to classify the participants

into low adherence (<50th percentile) or high adherence (≥50th percentile). The differences between the two groups were assessed by Student's *t*-test or a Mann–Whitney *U* test.

The analysis of the effects of the intervention strategies on omega-3 fatty acids and hepatic status after 6 months of follow up (Chapter 3) was performed as follows: The differences between baseline and 6-month data in the pooled simple of subjects with a weight loss >5% were analyzed by the paired Student's *t*-test, assuming the normality of the distribution of the variables. The comparison between the changes in the 50th percentile (P50) groups (<P50 vs. ≥P50) was carried out by means of the Student's *t*-test or the Mann–Whitney *U* test when appropriate. The distribution of the variables was evaluated with the Shapiro-Wilk test. Pearson's or Spearman´s correlation analyses were performed according to the distribution of the data in order to evaluate the relationships between the change in erythrocyte membrane fatty acids and the change in hepatic parameters. Multivariate linear regression models were adjusted for potential confounders (age, sex, dietary group and, change in body weight) as appropriate.

For the comparison between NAFLD participants and non-NAFLD controls (Chapter 5) the statistical analysis was performed as follows: variable distribution was assessed using the Shapiro-Wilk test. Data were presented as mean ± standard deviation or mean ± standard error. Groups were compared by the Student's *t*-test for unpaired samples when data followed a normal distribution and the Mann-Whitney U test when data did not show a normal distribution. Comparisons between groups were adjusted by BMI using Analysis of covariance (ANCOVA). Categorical variables were compared using the Chi-squared test. To evaluate the potential association between sleep features and hepatic status, the Pearson's correlation coefficient or the Spearman's rho were performed for parametric and non-parametric variables, respectively. Logistic regression models were set up to evaluate the risk of NAFLD (dependent variable) associated with sleep quality variables (independent variables). Linear regression analyses were performed to assess the influence of sleep characteristics in the variability of liver stiffness as determined by ARFI. Multilinear regression models were adjusted for potential confounding factors (age, sex, physical activity, smoking status, and others when indicated).

All *p*-values presented are two-tailed and a $p<0.05$ was considered statistically significant. Statistical analyses were carried out using the software Stata version 12.0 (StataCorp, College Station, TX, USA). Graphs were generated using GraphPad Prism 6 (Graph-Pad Software, San Diego, CA, USA).

RESULTS

CHAPTER 1

Effects of two personalized dietary strategies during a 2-year intervention in subjects with nonalcoholic fatty liver disease: a randomized trial

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Abstract

Background and objectives: Nonalcoholic fatty liver disease (NALFD) management is focused on lifestyle modifications, but long-term maintenance is a considerable challenge for many individuals. This study aimed to evaluate the long-term effects of two personalized energy-restricted dietary strategies on weight loss, metabolic and hepatic outcomes in overweight/obese subjects with NAFLD.

Methods: Ninety-eight subjects from the Fatty Liver in Obesity (FLiO) study (NCT03183193) were randomly assigned to the American Heart Association (AHA) or the FLiO dietary group in a 2-year controlled trial. Anthropometry, body composition (DXA), biochemical parameters, and hepatic status (ultrasonography, Magnetic Resonance Imaging, and elastography) were assessed at baseline, 6, 12, and 24 months.

Results: Both the AHA and FLiO diets significantly reduced body weight at 6 (-9.2% vs. -9.8%), 12 (-6.4% vs. -9.3%) and 24 months (-4.7% vs. -7.3%), respectively, with significant improvements in body composition, biochemical and liver determinations throughout the intervention. At the end of the follow-up, the FLiO group showed a greater decrease in waist circumference (p=0.028), Triglycerides/Glucose index (p=0.021), Atherogenic index of plasma (p=0.019), ALT (p=0.038), liver stiffness (p=0.016), and Fatty Liver Index (p=0.021), and a greater increase in adiponectin (p=0.008) compared to the AHA group.

Conclusions: The AHA and FLiO diets were able to improve body weight and body composition, as well as metabolic and hepatic status of participants with overweight/obesity and NAFLD within a 2-year follow-up. These findings show that both strategies are suitable alternatives for NAFLD management. However, the FLiO strategy may provide greater and more persistent benefits in metabolic and hepatic parameters.

Keywords: NAFLD, obesity, diet, AHA, FLiO, fatty liver.

Abbreviations: NALFD, nonalcoholic fatty liver disease; FLiO, Fatty Liver in Obesity; AHA, American Heart Association; NASH, nonalcoholic steatohepatitis; MedDiet, Mediterranean Diet; BMI, Body Mass Index; DXA, dual energy X-Ray absorptiometry; ARFI, acoustic radiation force impulse; TE; transient elastography; MRI, magnetic resonance imaging; AASLD, American Association for the Study of Liver Diseases; HDL-c, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; LDL-c, low-density lipoprotein cholesterol; TyG, Triglycerides/Glucose index; AIP, Atherogenic index of plasma; HIS, Hepatic Steatosis Index; FLI, Fatty Liver Index; FFQ, food frequency questionnaire; GEE, generalized estimating equations; PUFA, Polyunsaturated fatty acids; SFA, saturated fatty acids;

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently considered as the most common form of chronic liver disease in the majority of the world regions with a prevalence that parallels the worldwide increase of obesity [1–3]. NAFLD encompasses a spectrum of chronic liver diseases characterized by the excessive accumulation of intrahepatic triglycerides that occurs in the absence of significant consumption of alcohol [4]. This condition ranges from simple steatosis to nonalcoholic steatohepatitis (NASH) when inflammation and hepatocyte injury accompanies triglyceride retention, to a variable degree of hepatic fibrosis, to cirrhosis and/or to hepatocellular carcinoma in latter stages of the disease [5]. NAFLD is strongly associated with obesity, insulin resistance, type 2 diabetes mellitus, and cardiovascular disease [4]. The natural history of NAFLD is highly variable and reflects diverse mechanisms that converge in the onset and development of this disease [6], including environmental factors (diet and exercise), microbiome, and genetic risk factors [3].

Lifestyle modifications aiming at weight loss through dietary and physical activity interventions remain the cornerstone therapy for the treatment of subjects with NAFLD [7]. Caloric restriction plays a fundamental role in the reduction of body weight, visceral, subcutaneous, and hepatic fat, being the most important element in the dietary treatment of

NAFLD [8]. Additionally, the interventions based on the Mediterranean Diet (MedDiet) seems to be the most supported nutritional approach for NAFLD management [7,9]. However, the maintenance of weight loss is a considerable challenge for many individuals [8], and there is a lack of studies evaluating the long-term effects of dietary interventions on liver status features of subjects with NAFLD [10].

In this context, the aim of this study was to evaluate the long-term effects of two personalized energy-restricted dietary strategies on weight loss, anthropometric measurements, biochemical determinations, and hepatic status within a 2-year follow-up in overweight or obese subjects with NAFLD.

2. Materials and methods

Study participants

The participants of the study were recruited between June 2016 and June 2017 in Navarra, Spain. In total, 228 subjects were evaluated to determine the presence of liver steatosis by abdominal ultrasonography. A total of 98 men and women with overweight or obesity (Body Mass Index (BMI) > 27.5 kg/m2 to <40 kg/m2) between 40-80 years old fulfilled the selection criteria and started the trial (Figure 1). Exclusion criteria included the presence of known hepatic disease other than NAFLD, excessive alcohol consumption (>21 units of alcohol per week for men and >14 per women [11]), weight loss > 3kg in the last 3 months, endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), pharmacological treatment with immunosuppressants, cytotoxic agents, corticosteroids (or other drugs that could potentially cause liver steatosis or alteration in hepatic tests) [12], severe psychiatric disorders, active autoimmune disease or requiring pharmacological treatment, the use of weight modifiers, and the lack of autonomy or inability to follow the diet, as well as the difficulties in following the scheduled visits. The trial was approved by the Research Ethics Committee of the University of Navarra (ref. 54/2015) and registered at www.clinicaltrials.gov (FLiO: Fatty Liver in Obesity study; NCT03183193). Each subject gave written informed consent prior to enrollment in the study. All the participants were

blinded to the group of allocation and the professionals in charge of hepatic assessment and other determinations were blinded to the dietary treatment, clinical information and laboratory data. All the procedures were performed in accordance with the Declaration of Helsinki and the study was conducted following the CONSORT 2010 guidelines.

Overview of the study protocol

This randomized controlled trial was designed to compare the effects of two weight loss dietary strategies with different nutritional features on liver status, anthropometric measurements, body composition and biochemical markers in overweight or obese subjects with ultrasonography proven liver steatosis. The participants were randomly assigned to the American Heart Association (AHA) group or the Fatty Liver in Obesity (FLiO) group (Figure 1). The intervention had a total duration of two years with a complete evaluation of the participants at baseline and after 6, 12, and 24 months. The assessment of the participants at each point of the study included anthropometric measurements, body composition by dual energy X-Ray absorptiometry (DXA), biochemical determinations, and evaluation of the liver by ultrasonography, acoustic radiation force impulse (ARFI) elastography, transient elastography (TE), and magnetic resonance imaging (MRI). Qualified radiologist and hepatologist performed the assessment of the liver status at the University of Navarra Clinic (CUN, Pamplona, Spain). In addition, fasting blood samples were properly collected, processed, and stored at -80 ºC for further analyses.

Dietary interventions

Two different diets were prescribed and compared according to the group of allocation. Both diets applied an energy restriction of 30% of the total energy requirements of each participant with the objective to achieve a loss of at least 3%-5% of the initial body weight, in accordance with the recommendations of the American Association for the Study of Liver Diseases (AASLD) [12]. The energy prescription for each participant was estimated using the equation of the Institute of Medicine to calculate the resting metabolic rate, as elsewhere described [13]. One diet was based on the guidelines of the AHA [14] which propose 3-5 meals/day with a conventionally balanced distribution of macronutrients in relation to the total caloric value: 50%-55% from carbohydrates, 15% from proteins and 30% from lipids

with a healthy fatty acid profile. On the other hand, the FLiO diet was designed with a higher meal frequency (7 meals/day). The macronutrient distribution according to the total energy value was: 40%-45% carbohydrates (preferring those with low glycemic index), 25% proteins (predominantly from vegetable sources), and 30%-35% from lipids (favoring extra virgin olive oil and Omega-3 polyunsaturated fatty acids to the detriment of saturated and trans fats). The FLiO diet proposed a high adherence to the MedDiet, involving an increased quantity of natural antioxidants based on previous studies of this research group [15]. Moreover, a 7 days menu plan was provided to the participants in both groups.

Anthropometry, body composition and biochemical evaluation

Anthropometric and body composition evaluation was carried out at the Metabolic Unit of the University of Navarra. Body weight, height, and waist circumference were determined as elsewhere described [14]. Blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, the Netherlands) and DXA body composition (Lunar iDXA, encore 14.5, Madison, WI, USA) were assessed following the instructions of the manufacturers. Blood samples for biochemical determinations were properly collected after overnight fasting of 8-10 hours. The samples were processed at the Laboratory of Biochemistry of the University of Navarra Clinic (CUN, Pamplona, Spain). Blood glucose, insulin, total cholesterol, high-density lipoprotein cholesterol (HDL-c), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) concentrations were determined on an autoanalyzer with specific commercial kits and following the instructions of the company (Cobas 8000, Roche Diagnostics, Basel, Switzerland). Leptin and adiponectin concentrations were measured using in a Triturus autoanalyzer (Grifols, Barcelona, Spain) using specific ELISA kits (Demeditec; Kiel-Wellsee, Germany). BMI was calculated as the body weight divided by the squared height (kg/m2). The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) was computed as HOMA-IR = (insulin (μU/mL) x glucose (mmol/L))/22.5 [16]. The low-density lipoprotein cholesterol (LDL-c) levels were estimated using the Friedewald formula [17]. The Triglycerides/Glucose index (TyG) was calculated as ln[triglycerides (mg/dL) × glucose (mg/dL)/2] [18] and the Atherogenic index of plasma (AIP) as log [triglycerides/HDL-c)] [19].

Assessment of liver status

The presence of hepatic steatosis was determined by means of ultrasonography (Siemens ACUSON S2000 and S3000, Erlangen, Germany) in accordance with previously described methodology [20,21]. ARFI elastography and TE were performed to assess liver stiffness as a surrogate marker of hepatic fibrosis [22]. ARFI was carried out along with the ultrasonography. Briefly, ten valid determinations were performed on each participant and the median value of liver stiffness was computed [23]. TE was carried out through FibroScan® (Echosens, Paris, France) as previously described [21]. Finally, MRI (Siemens Aera 1.5 T) was used to determine the fat content of the liver by Dixon technique as elsewhere described [21]. The whole liver evaluation was carried out under fasting conditions at the of the University of Navarra Clinic.

Hepatic Steatosis Index (HSI) was calculated using the following formula [24]: HSI = 8 x (ALT/AST ratio) ± BMI (± 2, if female; ± 2, if diabetes mellitus). The Fatty Liver Index (FLI) was computed using serum triglycerides, BMI, waist circumference, and GGT concentrations using the formula elsewhere described [25].

Lifestyle assessment: Diet and physical activity

Information regarding the diet and physical activity of the participants was collected at each timepoint of the study (baseline, 6, 12, and 24 months). The dietary intake was registered with a semi-quantitative food frequency questionnaire (FFQ), which consisted of 137-item and was previously validated in Spain for energy and nutrient intake [26]. Each item in the FFQ included a typical portion size and the frequencies of consumption were registered in nine categories that ranged from “Never or almost never” to “ > 6 times/day”. The composition of the food items was derived from accepted Spanish food composition tables as previously described [16,27]. The adherence to the MedDiet was evaluated using a 17-point screening questionnaire [28]. The final score ranged from 0 to 17 and a higher punctuation indicated a better adherence to the MedDiet [29]. Physical activity was estimated using the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [30,31].

Randomization and statistical analyses

The primary outcome of the study was the change in body weight, given that the current recommendations of the AASLD to ameliorate NAFLD features are focused on weight loss [12]. The sample size was calculated to achieve a significant outcome with a 95% confidence interval ($\alpha = 0.05$) and a statistical power of 80% ($\beta = 0.8$). A total of 36 participants per study group was estimated with this approach, but 50 subjects were included in each arm of the study considering the estimated dropout rate of 35%-40% according to the experience of the research group. The group assignment of the participants was performed using random numbers generated by computer. The randomization and allocation were done by a physician not involved in the intervention, adherence, and follow-up of the subjects throughout the study and were concealed from the investigators and the participants. Two individuals from the AHA group were excluded due to important alterations in the initial assessment of biochemical parameters, which required medical management. Therefore, the study started with 48 participants in the AHA group and 50 participants in the FLiO group. The differences in primary and secondary outcomes between the effects of the two dietary interventions during the entire trial (baseline, 6, 12 and 24 months), as well as the repeated measures overtime within each dietary group were analyzed using linear mixed models. All p-values presented are two-tailed and a $p<0.05$ was considered statistically significant. Statistical analyses were carried out using the software Stata version 12.0 (StataCorp, College Station, TX, USA). Graphs were generated using GraphPad Prism 6 (Graph-Pad Software, San Diego, CA, USA).

3. Results

The age of the participants at the beginning of the study was 51.1 (9.8) years in the AHA group and 49.2 (8.9) years in the FLiO group (p=0.326), while sex distribution was 20 women/28 men in AHA group and 27 women/23 men in FLiO group (p=0.666). A total of 40 participants were lost after 2 years of follow-up (Figure 1). No statistical differences were found at baseline evaluation in anthropometry, body composition, general biochemical

determinations (Table 1), hepatic parameters (Table 2), or macronutrient intake (Table 3) between the two groups of intervention.

Both the AHA and FLiO groups achieved significant weight reductions at 6 (-9.2% vs. -9.8%), 12 (-6.4% vs. -9.3%) and 24 months (-4.7% vs. -7.3%) without significant differences between them (Figure 2). Significant improvements in anthropometry, body composition, and biochemical parameters were observed along the study in both dietary groups (Table 1). Notably, both groups maintained the reductions in glucose, insulin, HOMA-IR, and leptin during the follow-up of the study, without significant differences between the changes in AHA and FLiO diets. Meanwhile, AHA but not FLiO group maintained a reduction in systolic blood pressure at 24 months compared to baseline values. However, the FLiO group exhibited significantly greater reductions in triglycerides (p=0.045), TyG (p=0.008) and AIP (p=0.009) at 12 months, and in waist circumference (p=0.028), TyG (p=0.021) and AIP (p=0.019) at 24 months, compared to AHA group. Regarding adiponectin, significant increases within dietary groups along the study were observed, but the change was significantly greater in the FLiO group than in the AHA group at 6 (p=0.034) and 24 months (p=0.008).

Regarding hepatic evaluation, both groups showed significant improvements in the follow-up evaluations of the study (Table 2). Notably, both groups maintained the reductions in steatosis degree, FLI, and HSI along the study. However, the FLiO group maintained a significant decrease in ALT (p<0.001), liver fat (p<0.01), and TE liver stiffness (p<0.05) at 24 months compared to baseline values, while no significant changes were observed within the AHA group in the same parameters after the 24-month follow-up. Moreover, the FLiO group showed a significantly greater decrease in ARFI liver stiffness at 6 months (p=0.039), AST (p=0.034) and FLI (p=0.008) at 12 months, and ALT (p=0.038), TE liver stiffness (p=0.016) and FLI (p=0.021) at 24 months compared to the AHA group. In addition, significantly lower values of AST and FLI at 12 months, and liver fat and liver stiffness at 24 months were observed in the FLiO group compared to the AHA group (Figure 3).

Dietary evaluation (Table 3) showed expected differences between the AHA and FLiO groups regarding the changes in carbohydrate and protein intake at 6 and 12 months of the study. Although these differences were attenuated at 24 months. The reported increase in the

intake of polyunsaturated fatty acids (PUFA) was significantly greater in FLiO group compared to AHA group at all the follow-up visits of the study (6 months p=<0.001; 12 months p<0.001; 24 months p=0.002), while the reduction in the consumption of saturated fatty acids (SFA) was significantly greater in FLiO group at 24 months (p=0.019). Notably, the MedDiet adherence significantly increased in both groups at the different timepoints of the study and this change was significantly greater in FLiO group at 6 months (p=0.003) and marginally greater at 12 months (p=0.052) compared to AHA group. On the other hand, the AHA group showed significant increases in physical activity at all the follow-up visits of the study compared to baseline values, while the FLiO group only increased physical activity at 6 months. However, the changes in physical activity did not reveal statistical differences between the groups.

4. Discussion

After two years of follow-up in this randomized controlled trial that aimed to compare the effect of two personalized energy-restricted dietary strategies in overweight or obese participants with NAFLD, significant improvements were observed in both intervention groups. Body weight, body composition, glycemic profile, leptin, adiponectin, and hepatic status parameters showed significant improvements at all the follow-up visits of the study, compared to baseline values within both intervention groups. However, the FLiO group evidenced a greater and more persistent effect in the reduction of metabolic and hepatic parameters including ALT, liver stiffness and FLI, and a greater increase in adiponectin at 24 months compared to the AHA group. Notably, the differences between both treatments became more evident with the longer follow-up.

The current treatment of NAFLD relies on lifestyle modifications, mainly focused on diet and physical activity [33]. Moreover, the achievement of weight loss is considered essential in NAFLD management, especially for subjects with overweight or obesity [34,35]. For instance, the AASLD recommends a weight reduction of at least 3%-5% of body weight to reduce liver steatosis and propose a weight loss of 7%-10% to improve fibrosis and other histological features of NASH [12]. A recent systematic review and meta-analysis evaluated

the effect of interventions aiming at weight loss in subjects with NAFLD, including behavioral programs with diet alone or accompanied by exercise, pharmacotherapy, and bariatric surgery [35]. The studies had a median duration of 6 months and researchers found a consistent association between weight loss interventions and the improvement in a variety of hepatic biomarkers. However, although clear evidence showed that weight loss interventions were associated with improvement in liver steatosis, no statistically significant change was observed in histologic liver fibrosis or NAFLD fibrosis score [35]. This aspect becomes relevant since the degree of fibrosis is considered the strongest predictor correlating NAFLD progression with life-threatening complications [36]. In the current study, both groups achieved the weight loss recommendations of the AASLD for the reduction of liver steatosis at all the timepoints of the study without significant differences between them, but only the FLiO group maintained the weight loss percentage recommended for the improvement of fibrosis [12] at 12 and 24 months follow-up. Accordingly, the FLiO group showed a persistent decrease in TE liver stiffness (a surrogate marker of liver fibrosis) along the study with a more pronounced decreased at 24 months compared to the AHA group. In addition, dietary factors beyond caloric restriction and weight loss may have influence in the amelioration of liver fibrosis and longer periods of treatment and follow-up may be needed to observe consistent changes induced by the diet in this outcome.

The long-term adherence to dietary treatments, and the maintenance of body weight and lifestyle modifications is a considerable challenge for many individuals [37]. Previous studies have found that weight loss is largely unsuccessful in NAFLD patients in ambulatory care settings and that frequent clinical encounters are associated with a successful weight reduction [38]. In the present research, although both groups significantly reduced body weight at all the time points of the study compared to baseline values, a tendency to regain weight was observed. Thus, periodical reinforcement of behavioral changes in lifestyle interventions may be needed to maintain the beneficial metabolic and hepatic effects evidenced in short and medium-term trials [35]. Moreover, the optimal duration of follow-up in patients with NAFLD is still undetermined [5].

In this context, few studies about the effect of long-term interventions on hepatic outcomes have been published. For instance, a research in postmenopausal women with obesity

compared the effects of an ad libitum Paleolithic diet with a conventional low-fat diet on liver fat determined by proton resonance spectroscopy within a period of 2 years and found that body weight and liver fat content were significantly reduced in both groups at 6 and 24 months, with a more pronounced effect of Paleolithic diet at 6 months [10]. Another study evaluated the 2-year effects of a lifestyle intervention with diet and exercise counseling on liver status of middle age and elderly men with NAFLD, compared with usual care. The results showed a greater improvement in steatosis degree evaluated by ultrasonography, ALT, and FLI in the intervention group [39]. Our results contribute to this growing body of evidence, showing a variety of beneficial metabolic and hepatic long-term effects in individuals following the AHA or the FLiO diet (both energy-restricted strategies) and evaluated by different noninvasive techniques, including ultrasonography, MRI, elastography and fatty liver indexes. However, the FLiO strategy may provide greater and more persistent benefits in the evaluated outcomes.

Dietary patterns such as the MedDiet, the Dietary Approaches to Stop Hypertension (DASH) diet, and the Paleolithic diet have shown favorable effects for the management of some features of NAFLD, with a larger body of scientific evidence for the first pattern [10,40,41]. A sub-study of the CENTRAL 18-month trial showed that a Mediterranean/low-carbohydrate diet induced a greater reduction in hepatic fat content determined by MRI compared to a low-fat diet [42], while a recent systematic review of randomized controlled trials concluded that the MedDiet could improve NAFLD severity parameters, although inconsistencies among the included studies were found [40]. According to our findings, both groups significantly improved their adherence to MedDiet, which may have contributed to the beneficial effects observed in the two arms of the study. However, the FLiO diet was originally designed with a higher adherence to MedDiet and a significant difference in the changes in the MedDiet score between the two groups was observed at 6 months. In addition, the increase in the intake of PUFA was significantly greater in FLiO group at all the timepoints of the study compared to AHA group. These factors, along with weight loss, may have contributed to the greater improvements in the FLiO group evidenced in the longer follow-up (12 and 24 months).

On the other hand, insulin resistance is implicated in both the pathogenesis of NAFLD and the progression from simple steatosis to NASH [43]. Accordingly, the modulation of insulin resistance by means of dietary changes and/or use of medication has been proposed as a potential strategy for the treatment of NAFLD [43,44]. In addition, leptin and adiponectin are two adipokines with opposite effects in the pathogenesis of NAFLD that are associated with insulin resistance [45]. Leptin seems to promote insulin resistance and hepatic fibrogenesis, while adiponectin has shown to reduce insulin resistance and to have anti-steatoic and anti-inflammatory effects by increasing free fatty acid oxidation and decreasing gluconeogenesis, and de novo lipogenesis, among other mechanisms [46]. Notably, both groups in this research maintained the beneficial significant effects on glycemic profile and insulin resistance evaluated by HOMA-IR, at all the follow-up visits. Moreover, leptin evidenced a decrease within both dietary groups along the study when compared to baseline values, while adiponectin significantly increased in the two groups with a significantly greater improvement in FLiO at 6 and 24 months. Hence, both strategies may represent feasible options to improve glucose metabolism, insulin resistance, and related adipokines in individuals with NAFLD, although the FLiO diet seems to promote a higher increase in adiponectin, which may entail greater benefits for metabolic and hepatic status.

Interestingly, a bidirectional association between NAFLD and hypertension has been observed and it has been suggested that the change in fatty liver status overtime might influence the incidence of hypertension [47]. In this regard, the AHA group showed a more pronounced and persistent decrease in systolic blood pressure, although significant difference was not reached when the changes in the two groups were compared. This finding might be expected since the AHA recommendations on diet and lifestyle are intended to reduce cardiovascular risk and aim for normal blood pressure [48]. Thus, the AHA diet might be a suitable option for individuals with coexisting NAFLD and hypertension.

Some limitations of the present study should be acknowledged. First, the dietary data was obtained using self-reported information of the participants by means of an FFQ, which may produce some bias in the assessment of the diets. Second, liver status was evaluated using only non-invasive techniques instead of histological analyses, which is considered the most reliable approach for detecting NASH and fibrosis [49]. However, we carried out a

compressive evaluation of the liver by means of validated and widely used techniques for the assessment of liver steatosis (ultrasonography and MRI[20]) and liver stiffness (ARFI elastography and TE [50]), in addition to blood biomarkers and hepatic indexes, which facilitates the translation of these findings to clinical and public health settings. The major strengths of the study include the design as a randomized trial, the follow-up for 24 months and that both strategies included personalized diets and promoted the adoption of behavioral changes and a healthy lifestyle with individual follow-up of the participants.

Conclusion

This 2-year follow-up trial in overweight/obese participants with NAFLD showed that both personalized energy-restricted dietary strategies, the AHA and the FLiO diets, were able to produce a weight loss within the range recommended by the AASLD for the treatment of liver steatosis. Moreover, both diets promoted the improvement in anthropometry, body composition, glycemic profile, leptin, adiponectin and hepatic status parameters. Thus, these approaches may be suitable alternatives for personalized dietary treatment of NAFLD. However, the FLiO strategy might provide greater and more persistent improvements in metabolic and hepatic status.

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Table 1. Characteristics at baseline and after 6, 12, and 24 months of dietary intervention in subjects with NAFLD according to the group of intervention.

	AHA diet				FliO diet						
	Baseline† (n=48)	6 months (n=37)	12 months (n=38)	24 months (n=32)	Baseline† (n=50)	6 months (n=39)	12 months (n=34)	24 months (n=26)	Δ p-Value[a]	Δ p-Value[b]	Δ p-Value[c]
Anthropometry and body composition											
Weight (kg)	94.4 (14)	84.2 (13)***	87.3 (15)***	89.8 (16)***	95.1 (14)	86.6 (13)***	86.3 (13)***	89.1 (13)***	0.985	0.537	0.684
BMI (kg/m^2)	33.7 (4)	30.2 (4)***	31.2 (5)***	32.1 (5)***	33.3 (4)	30.1 (4)***	30.1 (4)***	30.8 (4)***	0.479	0.131	0.200
Waist circumference (cm)	110 (10)	99 (15)***	102 (11)***	108 (13)	108 (9)	99 (10)***	98 (10)***	102 (11)***	0.798	0.061	0.028
Total fat mass (%)	42.7 (6)	37.0 (7)***	38.6 (8)***	40.1 (7)*	42.3 (6)	37.8 (8)***	37.9 (8)***	39.2 (8)*	0.968	0.456	0.803
VAT (kg)	2.4 (1)	1.6 (1)***	1.9 (1)***	2.2 (1)***	2.2 (1)	1.5 (1)***	1.6 (1)***	1.7 (1)**	0.755	0.237	0.313
Lean mass (kg)	52.5 (10)	51.3 (9)***	51.6 (10)***	51.9 (10)***	53.1 (9)	51.9 (8)***	51.6 (9)***	52.4 (8)***	0.947	0.982	0.984
SBP (mmHg)	133 (14)	119 (23)***	127 (16)*	126 (16)*	128 (15)	123 (15)	124 (18)	126 (15)	0.271	0.518	0.705
DBP (mmHg)	87 (8)	79 (8)***	80 (9)***	79 (9)***	86 (9)	82 (8)***	82 (11)**	81 (9)***	0.567	0.330	0.556
Biochemical determinations											
Total cholesterol (mg/dL)	192 (40)	177 (43)*	180 (35)	185 (45)	197 (35)	185 (41)*	180 (34)**	193 (38)	0.727	0.850	0.852
HDL cholesterol (mg/dL)	51.9 (14)	53.0 (14)	52.8 (14)	50.2 (13)	53.8 (12)	54.7 (12)	57.3 (13)*	57.6 (14.1)	0.577	0.114	0.050
LDL cholesterol (mg/dL)	114 (37)	103 (37)	104 (30)	108 (34)	118 (30)	112 (35)	104 (29)**	112 (31)	0.523	0.918	0.859
Triglycerides (mg/dL)	129 (66)	99 (41)**	117 (49)	135 (76)	129 (62)	91 (59)***	93 (41)***	115 (82)	0.346	0.045	0.153
Glucose (mg/dL)	104 (18)	95 (14)***	98 (23)***	101 (21)**	101 (14)	93 (11)***	94 (19)***	94 (22)**	0.797	0.309	0.416
Insulin (mU/L)	17.5 (9)	11.2 (7)***	12.8 (7)***	13.5 (7)***	16.6 (7)	11.2 (7)***	12.3 (8)**	10.3 (6)***	0.931	0.984	0.227
HbA1c (%)	5.8 (0.6)	5.6 (0.6)**	5.7 (0.8)	5.8 (0.8)	5.7 (0.8)	5.4 (0.3)**	5.6 (0.6)**	5.6 (0.5)	0.538	0.324	0.546
HOMA-IR	4.6 (3)	2.7 (2)***	3.3 (2)***	3.4 (2)***	4.2 (2)	2.6 (2)***	3.0 (3)**	2.6 (2)***	0.785	0.818	0.315
TyG	8.7 (0.5)	8.4 (0.4)***	8.6 (0.5)**	8.7 (0.6)	8.7 (0.5)	8.2 (0.5)***	8.3 (0.5)***	8.4 (0.7)***	0.063	0.008	0.021
AIP	0.8 (0.6)	0.6 (0.6)**	0.8 (0.6)	0.9 (0.7)	0.8 (0.6)	0.4 (0.6)***	0.4 (0.6)***	0.5 (0.8)**	0.082	0.009	0.019
Leptin (ng/mL)	37.1 (27)	22.3 (19)***	23.2 (17)***	27.1 (18)*	38.8 (31)	23.3 (20)***	20.3 (15)***	29.0 (17)**	0.750	0.267	0.966
Adiponectin (µg/mL)	6.7 (2)	8.0 (3)*	8.3 (4)**	8.4 (3)**	6.6 (2)	9.5 (4)***	8.8 (3)***	10.6 (3)***	0.034	0.520	0.008

Data presented as mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Heart Association (AHA). Fatty Liver in Obesity (FLiO). Body Mass Index (BMI). Visceral adipose tissue (VAT). Systolic blood pressure (SBP). Diastolic blood pressure (DBP). High density lipoprotein (HDL). Low density lipoprotein (LDL). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR). Triglyceride-glucose index (TyG). Atherogenic index of plasma (AIP). †No significant differences were found at baseline between AHA and FLiO groups. Comparison of the changes between AHA and FLiO groups: [a] Baseline vs. 6 months. [b] Baseline vs. 12 months. [c] Baseline vs. 24 months. Comparison within dietary groups (baseline vs. 6, 12 and 24 months): *p<0.05, **p<0.01, ***p<0.001.

Table 2. Hepatic parameters at baseline and after 6, 12, and 24 months of dietary intervention in subjects with NAFLD according to the group of intervention.

	AHA diet				FLiO diet						
	Baseline† (n=48)	6 months (n=37)	12 months (n=38)	24 months (n=32)	Baseline† (n=50)	6 months (n=39)	12 months (n=34)	24 months (n=26)	Δ p-Value[a]	Δ p-Value[b]	Δ p-Value[c]
AST (IU/L)	25.5 (11)	21.6 (6)**	25.1 (9)	25.7 (8)	23.9 (8)	21.9 (9)	20.6 (8)*	22.8 (7)	0.568	0.034	0.248
ALT (IU/L)	33.1 (17)	22.9 (9)***	25.7 (11)***	31.1 (18)	33.3 (18)	21.7 (9) ***	24.4 (13)***	21.8 (7) ***	0.839	0.919	0.038
GGT (IU/L)	40.9 (29)	28.3 (23)**	31.4 (20)**	36.5 (40)	33.6 (24)	26.4 (43)	25.1 (19)	20.8 (12)	0.903	0.709	0.110
Steatosis degree	1.5 (0.7)	0.8 (0.7)***	1.0 (0.9)***	0.9 (0.9)***	1.5 (0.6)	0.7 (0.6)***	0.6 (0.7)***	0.5 (0.6)***	0.379	0.081	0.156
Liver fat (%)	7.4 (5)	3.8 (3)**	5.1 (4) *	6.8 (5)	7.0 (5)	2.8 (3)***	5.6 (6)*	4.5 (3)**	0.756	0.940	0.284
ARFI Liver stiffness (m/s)	1.9 (1)	2.0 (1)	2.0 (1)	1.8 (1)	1.8 (1)	1.7 (1)	1.9 (1)	1.9 (1)	0.039	0.816	0.619
TE Liver stiffness (kPa)	5.2 (2)	5.0 (2)	4.4 (1)*	4.8 (2)	4.7 (2)	4.6 (1)	4.4 (2)	3.7 (1)*	0.253	0.931	0.016
FLI	80.4 (16)	54.4 (24)***	62.7 (24)***	69.2 (26)**	76.9 (21)	47.9 (24)***	51.0 (26)***	56.8 (27)***	0.066	0.008	0.021
HSI	45.1 (5)	39.9 (5)***	40.7 (7)***	42.4 (7)***	45.2 (5)	39.1 (4)***	40.5 (5)***	39.7 (5)***	0.456	0.946	0.077

Data presented as mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Heart Association (AHA). Fatty Liver in Obesity (FLiO). Aspartate aminotransferase (AST). Alanine aminotransferase (ALT). Gamma-glutamyl transferase (GGT). Acoustic Radiation Force elastography (ARFI). Transient elastography (TE). Fatty Liver Index (FLI). Hepatic Steatosis Index (HSI). †No significant differences were found at baseline between AHA and FLiO groups. Comparison of the changes between AHA and FLiO groups: [a] Baseline vs. 6 months. [b] Baseline vs. 12 months. [c] Baseline vs. 24 months. Comparison within dietary groups (baseline vs. 6, 12 and 24 months): *p<0.05, **p<0.01, ***p<0.001.

Table 3. Dietary intake and physical activity at baseline and after 6, 12, and 24 months of dietary intervention in subjects with NAFLD according to the group of intervention.

	AHA diet				FLiO diet						
	Baseline† (n=48)	6 months (n=37)	12 months (n=38)	24 months (n=32)	Baseline† (n=50)	6 months (n=39)	12 months (n=34)	24 months (n=26)	Δ p-Value[a]	Δ p-Value[b]	Δ p-Value[c]
Total energy (kcal/day)	2730 (867)	2170 (474)**	2163 (727)**	2235 (760)**	2521 (1000)	1816 (569)***	2042 (998)**	2380 (907)	0.079	0.294	0.611
Carbohydrates (% TEV)	43.4 (7)	45.0 (7)	44.4 (7)	41.7 (8)	42.8 (7)	39.0 (7)**	40.9 (8)	40.8 (9)	0.001	0.024	0.582
Proteins (% TEV)	16.8 (3)	18.5 (3)	18.9 (5)*	18.5 (4)*	17.6 (4)	22.1 (4)***	21.4 (5)***	19.7 (4)**	<0.001	0.004	0.237
Lipids (% TEV)	37.0 (7)	34.5 (6)	34.0 (6)*	37.0 (7)	36.9 (8)	36.5 (9)	35.5 (7)	37.3 (8)	0.305	0.402	0.925
MUFA (% TEV)	17.8 (5)	17.5 (4)	16.8 (5)	18.8 (5)	17.4 (4)	16.1 (6)	14.9 (4)**	17.6 (5)	0.170	0.063	0.312
PUFA (% TEV)	5.5 (2)	5.2 (2)	5.5 (2)	6.1 (2)	5.4 (2)	9.0 (5)***	8.5 (4)***	8.8 (4)***	<0.001	<0.001	0.002
SFA (% TEV)	10.6 (2)	9.4 (2)*	9.2 (2)**	10.3 (2)	10.6 (3)	8.7 (3)***	8.7 (2)***	8.6 (2)**	0.293	0.530	0.019
Fiber (g)	24.8 (9)	30.4 (10)*	31.6 (12)***	27.9 (12)	23.0 (8)	25.9 (8)	28.8 (14)*	33.6 (12)***	0.114	0.171	0.104
Fiber (g/1000 kcal)	9.8 (3)	14.4 (4)***	14.8 (5)***	12.8 (4)***	9.7 (4)	14.9 (4)***	14.1 (5)***	14.6 (4)***	0.353	0.668	0.174
Glycemic load	165 (78)	117 (41)***	123 (48)**	117 (54)***	147 (78)	84 (34)***	99 (41)***	125 (74)*	0.022	0.037	0.745
Meal frequency	4.5 (0.9)	4.8 (0.8)	4.6 (1.0)	4.6 (1.2)	4.8 (0.9)	5.8 (1.0)***	5.5 (1.2)***	5.6 (1.1)***	<0.001	0.001	<0.001
MedDiet adherence score	6.2 (2)	10.8 (3)***	10.4 (3)***	9.8 (2)***	5.8 (2)	12.6 (3)***	11.8 (3)***	10.9 (3)***	0.003	0.052	0.226
PA (METs-min/week)	2685 (2112)	4463 (3031)***	4578 (3988)***	4308 (3677)***	3120 (2136)	4158 (2532)*	3434 (2242)	3868 (3603)	0.691	0.208	0.395

Data presented as mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Heart Association (AHA). Fatty Liver in Obesity (FLiO). Total energy value (TEV). Monounsaturated fatty acids (MUFA). Polyunsaturated fatty acids (PUFA). Saturated fatty acids (SFA). Mediterranean diet (MedDiet). Physical Activity (PA). Metabolic equivalent of the task (METs). †No significant differences were found at baseline between AHA and FLiO groups. Comparison of the changes between AHA and FLiO groups: [a] Baseline vs. 6 months. [b] Baseline vs. 12 months. [c] Baseline vs. 24 months. Comparison within dietary groups (baseline vs. 6, 12 and 24 months): *p<0.05, **p<0.01, ***p<0.001.

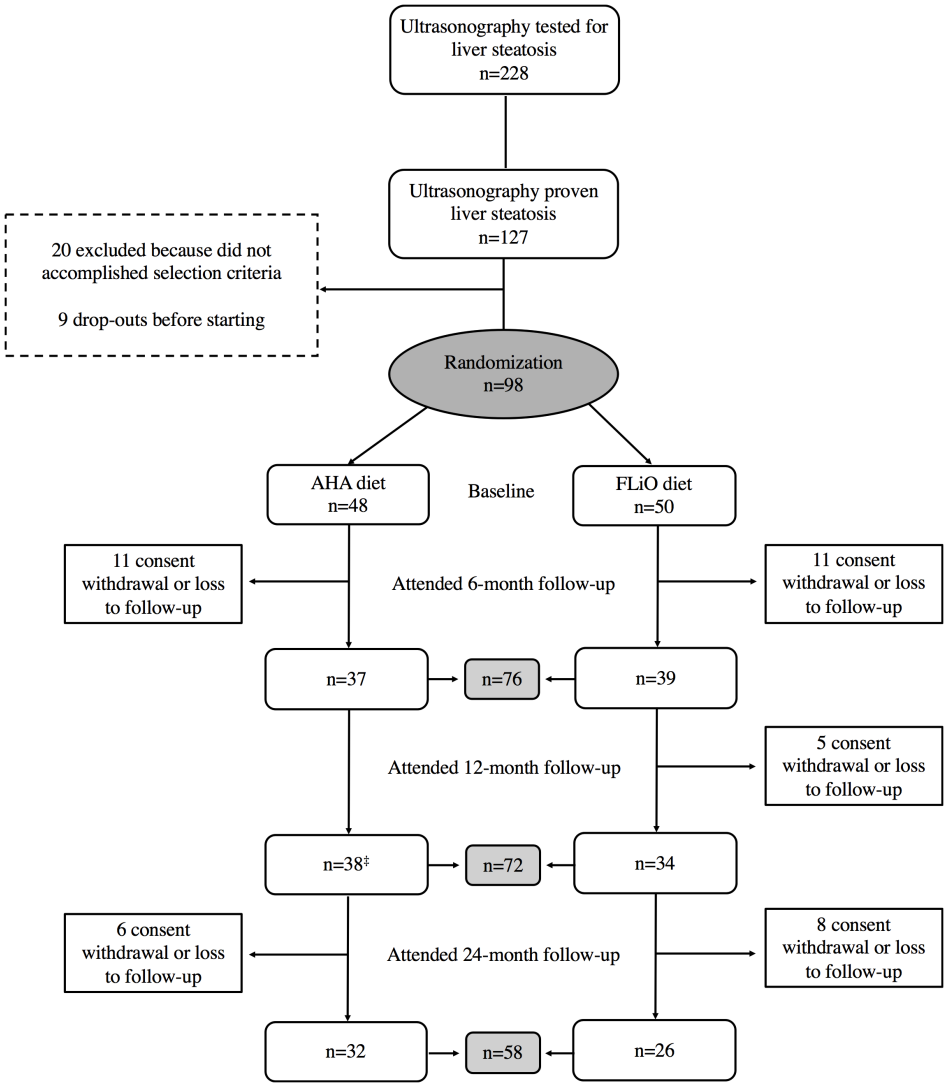

Figure 1. Flowchart of the participants in the FLiO study. American Heart Association (AHA). Fatty Liver in Obesity (FLiO). ‡ One participant did not attend to 6-month follow-up visit but did attend to 12-month follow-up visit.

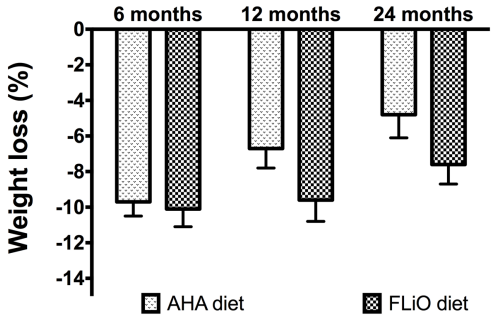

Figure 2. Comparison of weight loss (%) between AHA and FLiO groups at different timepoints of the study. Data presented as mean (SEM). American Heart Association (AHA). Fatty Liver in Obesity (FLiO).

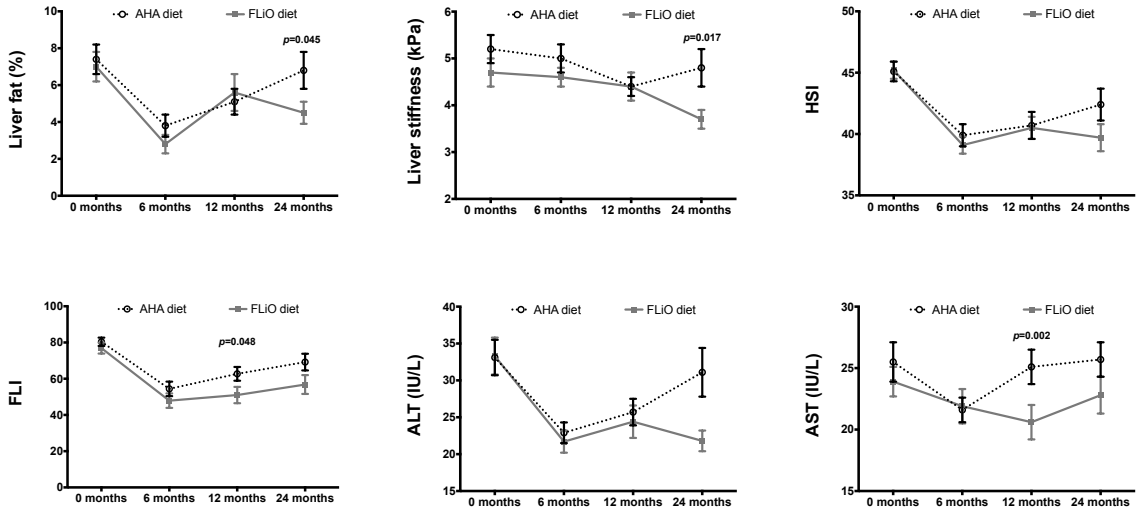

Figure 3. Comparison of liver status parameters between AHA and FLiO group at each timepoint of the study. Data presented as mean (SEM). American Heart Association (AHA). Fatty Liver in Obesity (FLiO). Fatty Liver Index (FLI). Hepatic Steatosis Index (HSI). Aspartate aminotransferase (AST). Alanine aminotransferase (ALT).

CHAPTER 2

The Metabolic and Hepatic Impact of Two Personalized Dietary Strategies in Subjects with Obesity and Nonalcoholic Fatty Liver Disease: The Fatty Liver in Obesity (FLiO) Randomized Controlled Trial

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The Metabolic and Hepatic Impact of Two Personalized Dietary Strategies in Subjects with Obesity and Nonalcoholic Fatty Liver Disease: The Fatty Liver in Obesity (FLiO) Randomized Controlled Trial

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Abstract: The prevalence of nonalcoholic fatty liver disease (NAFLD) is increasing worldwide. NAFLD management is mainly focused on weight loss, but the optimal characteristics of the diet demand further investigation. This study aims to evaluate the effects of two personalized energy-restricted diets on the liver status in overweight or obese subjects with NAFLD after a 6 months follow-up. Ninety-eight individuals from the Fatty Liver in Obesity (FLiO) study were randomized into two groups and followed different energy-restricted diets. Subjects were evaluated at baseline and after 6 months. Diet, anthropometry, body composition, and biochemical parameters were evaluated. Liver assessment included ultrasonography, Magnetic Resonance Imaging, elastography, and determination of transaminases. Both dietary groups significantly improved their metabolic and hepatic markers after the intervention, with no significant differences between them. Multivariate regression models evidenced a relationship between weight loss, adherence to the Mediterranean Diet (MedDiet), and a decrease in liver fat content, predicting up to 40.9% of its variability after 6 months. Moreover, the antioxidant capacity of the diet was inversely associated with liver fat content. Participants in the

group with a higher adherence to the MedDiet showed a greater reduction in body weight, total fat mass, and hepatic fat. These results support the benefit of energy-restricted diets, high adherence to the MedDiet, and high antioxidant capacity of the diet for the management of NAFLD in individuals with overweight or obesity.

Keywords: Obesity; NAFLD; dietary intervention; AHA; FLiO; fatty liver; Mediterranean Diet

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as an excessive accumulation of hepatic fat in the absence of consumption of significant amounts of alcohol or other related causes of secondary hepatic steatosis [1,2]. The worldwide prevalence of NAFLD is rising parallelly to the obesity burden and type 2 diabetes mellitus epidemics [3,4]. The pathogenesis of NAFLD is complex, and multiple environmental and genetic factors are involved in its development and progression [5]. The background of genetic predisposition may include variants in genes, such as the Patatin-like phospholipase domain-containing 3 (PNPLA3) or the Transmembrane 6 superfamily member 2 (TM6SF2) [5,6]. Other important factors involved are obesity, dietary intake, physical inactivity, insulin resistance, gut microbiota dysbiosis, epigenetic impairments, among others [4,7,8]. Indeed, NAFLD is considered not only a liver disturbance but also a multisystem disease that is related to type 2 diabetes mellitus, cardiovascular disease, and chronic kidney disease [9]. This condition can lead to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and, finally, hepatocellular carcinoma [10], which will be the leading cause for liver transplantation in the next few years [11].

Regarding the management of NAFLD, lifestyle modifications focused on weight loss remain the cornerstone of therapy [12]. Strategies like changes in dietary patterns and exercise have demonstrated benefits for the prevention of the onset and progression of this morbid condition [13,14]. Caloric restriction apparently plays a major role in weight loss and in hepatic fat reduction, so it is considered a central element in nutritional interventions for subjects with NAFLD [15]. Currently, 3%–5% loss in body weight is often recommended to improve liver steatosis, with greater medical improvements of liver status when the weight loss is higher [2,16]. However, the evidence for nutritional factors, characteristics of the diet, and dietary strategies for NAFLD treatment remains inconclusive and demands further investigation [14,15,17].

In this context, the objective of this study was to evaluate the effects of two personalized energy-restricted dietary strategies differing in several dietary factors (macronutrients, fiber, meal frequency, total antioxidant capacity, and Mediterranean diet adherence) on liver status, as assessed by imaging techniques and biochemical markers in overweight and obese subjects with NAFLD after a 6 months follow-up.

2. Materials and Methods

2.1. Study Participants

Ninety-eight (55 Male and 43 Female) overweight or obese (BMI $\geq$ 27.5 kg/m^2 to $<$ 40 kg/m^2) adults between 40–80 years old and with hepatic steatosis confirmed by abdominal ultrasonography were enrolled in the study. A total of 76 of the participants completed the evaluation after 6 months (Figure 1). Exclusion criteria included the presence of known liver disease other than NAFLD, $\geq$3 kg of body weight loss in the last 3 months, excessive alcohol consumption (>21 and >14 units of alcohol per week for men and women, respectively [18], endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), pharmacological treatment with immunosuppressants, cytotoxic agents, systemic corticosteroids (or other drugs that could potentially cause hepatic steatosis or altering liver tests) [2], active autoimmune diseases or requiring pharmacological treatment, the use of weight modifiers and

severe psychiatric disorders, and the lack of autonomy or an inability to follow the diet, as well as difficulties in following the scheduled visits. This information was obtained in a clinical interview of the subjects before their participation in the study. Each individual gave written informed consent prior to their enrollment. All the procedures performed were in accordance with the Declaration of Helsinki. This trial was approved by the Research Ethics Committee of the University of Navarra (ref. 54/2015) and appropriately registered in www.clinicaltrails.gov (FLiO: Fatty Liver in Obesity study; NCT03183193).

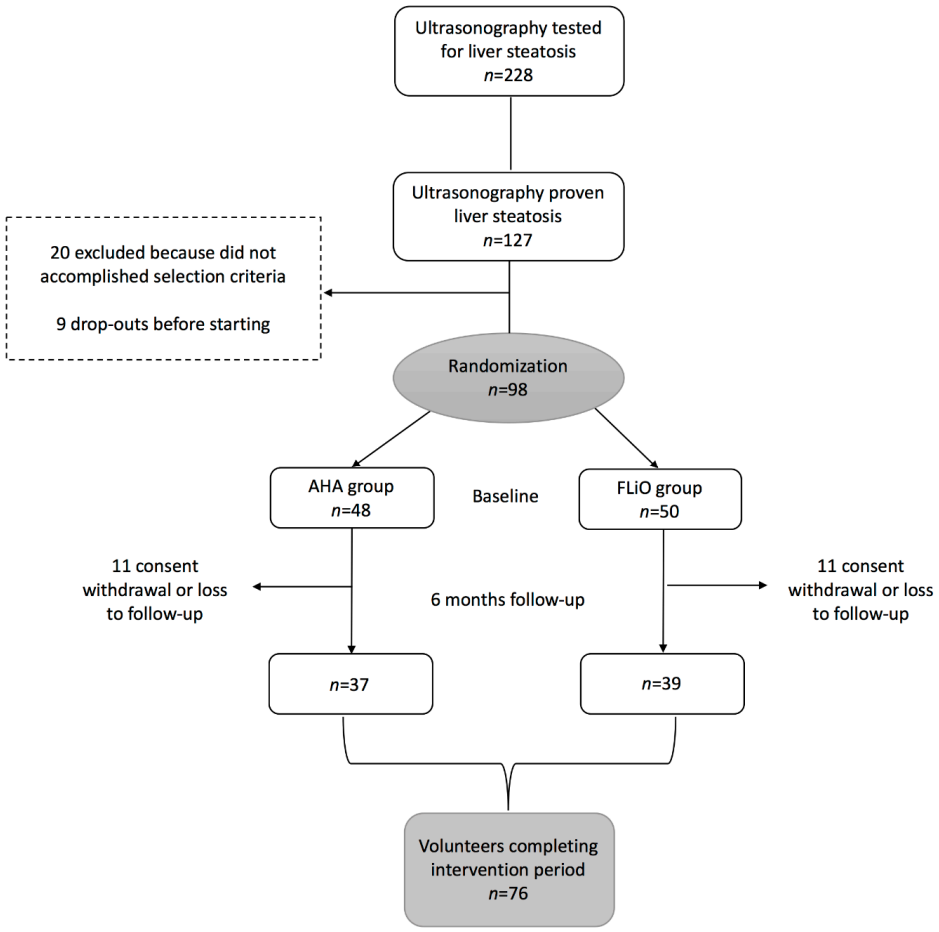

Figure 1. Flowchart of participants in the Fatty Liver in Obesity (FLiO) study.

2.2. Study Protocol

This randomized controlled trial was designed to compare the effects of two dietary strategies for weight loss with different nutritional characteristics for hepatic status, as well as for anthropometric measurements, body composition, and biochemical markers, in overweight or obese subjects with ultrasonography proven liver steatosis. The intervention had a duration of 6 months, and the participants were randomly assigned to the American Heart Association (AHA) or the Fatty Liver in Obesity (FLiO) group (Figure 1). A comprehensive assessment was carried out at the baseline and

at the endpoint of the study, including anthropometric measurements, body composition by dual energy X-Ray absorptiometry (DXA), biochemical determinations, and evaluation of the liver using ultrasonography, Acoustic Radiation Force Impulse (ARFI) elastography, and Magnetic Resonance Imaging (MRI). The imaging evaluations of the liver were performed by a qualified radiologist and hepatologist at the University of Navarra Clinic. Additionally, fasting blood samples were properly collected, processed, and stored at −80 °C for further analyses. A step-based physical activity recommendation of 10,000 steps/day was given to the participants [19]. Physical activity was estimated using the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [20]. The energy expenditure in physical activity was estimated assuming the value of 1 MET = 3.5 mL/kg/min, as previously described [21], and the ratio between energy intake and energy expenditure was computed.

2.3. Dietary Intervention

Two energy-restricted diets were prescribed and compared. Both diets applied a 30% energy restriction of the total energy requirements of each participant in order to achieve a loss of at least 3%–5% of the initial body weight, according to the objective of the American Association for the Study of Liver Diseases (AASLD) [2]. Resting metabolic rate was calculated using the equation of the Institute of Medicine, as elsewhere described [22], in order to estimate the energy prescription for each subject. The control diet was based on the guidelines of the American Heart Association (AHA) [23], which suggest 3–5 meals/day, with a conventionally balanced distribution of macronutrients (50%–55% of total caloric value from carbohydrates, 15% from proteins, and 30% from lipids) and a healthy fatty acid profile. On the other hand, the FLiO diet was designed with a higher meal frequency (7 meals/day), including breakfast, lunch, dinner, two snacks in the morning, and two snacks in the afternoon. The established macronutrient distribution was 40%–45% of the total caloric value from carbohydrates (low glycemic index), 25% from proteins (mainly from vegetable sources), and 30%–35% from lipids (extra virgin olive oil and Omega-3 fatty acids to the detriment of saturated and trans fats). Moreover, the dietary pattern included a high adherence to the Mediterranean Diet (MedDiet), subsequently involving an increased quantity of natural antioxidants [24]. Participants were provided with a 7 days menu plan in both groups. A semiquantitative food frequency questionnaire (FFQ) of 137 items previously validated in Spain for energy and nutrient intake [25] was used to assess the diets of the participants at baseline and after 6 months of the study. The mean antioxidant capacity values of the foods contained in each item of the FFQ were used to estimate the total antioxidant capacity (TAC) of the diet, as previously described [26]. The adherence to the MedDiet was assessed with a 17-point screening questionnaire, with a final score ranging from 0 to 17 and a higher score indicating a better adherence to the MedDiet [27].

2.4. Anthropometric, Body Composition and Biochemical Assessment

The determination of anthropometric measurements (body weight, height, and waist circumference), body composition by DXA (Lunar iDXA, encore 14.5, Madison, WI, USA), and blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, the Netherlands) was carried out under fasting conditions at the Metabolic Unit of the University of Navarra following standardized procedures, as previously described [28]. Body Mass Index (BMI) was calculated as the body weight divided by the squared height (kg/m^2). Biochemical determinations, including blood glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), and triglyceride (TG) concentrations were measured on an autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific commercial kits. Insulin, C-reactive protein (CRP), leptin, and adiponectin concentrations were measured using specific ELISA kits (Demeditec; Kiel-Wellsee, Germany) in a Triturus autoanalyzer (Grifols, Barcelona, Spain). Insulin resistance was estimated using the Homeostasis Model Assessment Index (HOMA-IR), which was calculated using the formula

elsewhere described [28]. Finally, the Fatty Liver Index (FLI) was computed using serum TG, BMI, waist circumference, and GGT concentrations, as previously published [29]. FLI values <30 rule out liver steatosis and values ≥60 indicate liver steatosis [30].

2.5. Imaging Techniques for the Assessment of Liver Status

The entire hepatic assessment was performed under fasting conditions by qualified staff at the University of Navarra Clinic. Ultrasonography (Siemens ACUSON S2000 and S3000) was carried out to determine the presence of hepatic steatosis in accordance with the previously described methodology [31–33]. In addition, ARFI elastography was performed along with the ultrasonography in order to assess liver stiffness. The median value of liver stiffness was obtained from the ten valid ARFI measurements that were performed on each participant [34]. The same experienced radiologist executed all the ultrasonographic evaluations at the department of Ultrasonography and Radiology. Finally, an MRI (Siemens Aera 1.5 T) was used to determine the hepatic volume and the fat content of the liver (Dixon technique), as described elsewhere [31].

2.6. Statistical Analyses

The sample size was calculated with weight loss as a primary outcome, given that the current recommendations of the AASLD are focused on weight loss to ameliorate NAFLD features [2]. In this sense, and according to previous studies [28], the sample size was calculated to detect a difference of 1.0 (1.5 kg) between the AHA and FLiO groups in their reduction of weight, with a 95% confidence interval (α = 0.05) and a statistical power of 80% (β = 0.8). This approach estimated a total of 36 participants per study group but considering the estimated dropout rate of 20%–30% (according to the experience of the research group), 50 subjects were included in each group. However, two subjects were excluded from the AHA group due to important alterations in the initial evaluation of biochemical parameters, which indicated that they required medical treatment. Consequently, this trail started with 98 subjects (n = 48 in AHA group and n = 50 in FLiO group). The mean value (standard deviation) is reported for the studied variables. The normality of the distribution of the evaluated variables was assessed by the Shapiro–Wilk test. The differences between the groups were compared by means of Student's t-test or the Mann–Whitney U test when appropriate. The differences between the beginning and the end of the intervention period within each group were analyzed by a paired Student's t-test or Wilcoxon signed-rank test when appropriate. Categorical variables were compared using a Chi-squared test. Linear regression analyses were used to evaluate the potential association between the anthropometry, body composition, and components of the diet with the hepatic status variables. Multivariate linear regression models were adjusted for potential confounders considering the group of intervention, age, sex, physical activity, and energy intake. The median value of the MedDiet Adherence questionnaire was used to classify the participants into low adherence (<50th percentile) or high adherence (≥50th percentile). The differences between the two groups were assessed by Student's t-test or a Mann–Whitney U test. Analyses were carried out using Stata version 12.0 software (StataCorp, College Station, TX, USA). All p-values presented are two-tailed and were considered statistically significant at $p < 0.05$.

3. Results

After 6 months of nutritional intervention, a total of 76 participants completed the evaluation. Both the AHA and FLiO groups exhibited a significant body weight loss (−9.7% (5.0) vs. −10.1% (6.5); p = 0.757). Furthermore, BMI and the rest of the anthropometrical measurements were significantly lowered in both dietary groups. No statistically significant differences were found between the intervention groups for these variables (Table 1). Regarding the biochemical parameters, both dietary groups showed improvements in total cholesterol, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and triglycerides concentrations. Fasting glucose, insulin, HOMA-IR, and leptin had a significant reduction in both groups, while adiponectin and C-reactive

protein only showed significant improvements in the FLiO group (Table 1). However, the changes from baseline to 6 months of intervention for all these variables did not differ between both dietary strategies.

Table 1. Characteristics at baseline and after 6 months of dietary intervention in subjects with NAFLD according to the group of intervention.

	AHA			FLiO				
	Baseline (n = 48)	**6 months (n = 37)**	***p*-Value [a]**	**Baseline (n = 50)**	**6 months (n = 39)**	***p*-Value [a]**	**Baseline *p*-Value [b]**	**Δ *p*-Value [c]**
Age (years)	51.1 (9.8)		-	49.2 (8.9)		-	0.326	-
Sex (Women/Men)	20/28		-	27/23		-	0.666 [e]	-
Anthropometry and body composition								
Weight (kg)	94.4 (14.4)	84.2 (13.1)	<0.001	95.1 (14.0)	86.6 (13.2)	<0.001	0.791	0.621
BMI (kg/m^2)	33.7 (4.0)	30.2 (4.1)	<0.001	33.3 (3.5)	30.1 (3.6)	<0.001	0.645	0.929
Waist circumference (cm)	109.7 (9.6)	98.7 (14.8)	<0.001	108.3 (9.2)	99.4 (9.5)	<0.001	0.462	0.614
Total fat mass (%)	42.7 (6.0)	37.0 (7.4)	<0.001	42.3 (6.2)	37.8 (7.8)	<0.001	0.759	0.857
Visceral adipose tissue (kg)	2.5 (1.1)	1.6 (0.8)	<0.001	2.3 (1.1)	1.5 (0.8)	<0.001	0.235	0.330
SBP (mmHg)	133 (14.3)	119 (23.2)	0.003	128 (15.2)	123 (15.3)	0.009	0.089	0.111
DBP (mmHg)	87 (8.2)	79 (7.9)	<0.001	86 (9.3)	82 (8.3)	<0.001	0.797	0.661
Biochemical parameters								
Total cholesterol (mg/dL)	192 (40.4)	177 (42.9)	0.089	197 (34.7)	185 (41.3)	0.051	0.514	0.362
HDL cholesterol (mg/dL)	51.9 (14.3)	53.0 (13.8)	0.073	53.8 (12.1)	54.7 (12.0)	0.251	0.436	0.858
LDL cholesterol (mg/dL)	114 (37.3)	103 (36.7)	0.264	118 (30.4)	112 (35.4)	0.265	0.576	0.934
Triglycerides (mg/dL)	128.6 (65.7)	98.6 (41.4)	0.003	129.1 (61.9)	90.6 (58.5)	<0.001	0.960	0.103
Fasting glucose (mg/dL)	103.8 (17.9)	94.7 (14.4)	<0.001	101.4 (13.5)	93.0 (10.8)	<0.001	0.912	0.204
Insulin (mU/L)	17.5 (9.4)	11.2 (7.2)	<0.001	16.6 (7.4)	11.2 (7.3)	<0.001	0.615	0.788
HOMA-IR	4.6 (2.8)	2.7 (2.2)	<0.001	4.2 (2.1)	2.6 (1.9)	<0.001	0.623	0.581
Leptin (ng/mL)	37.1 (27.0)	20.8 (15.7)	<0.001	38.8 (31.1)	22.3 (17.1)	<0.001	0.934	0.770
Adiponectin (µg/mL)	6.7 (2.2)	8.0 (3.0)	0.118	6.6 (2.2)	9.5 (3.7)	<0.001	0.887	0.100
C-reactive protein (mg/dL)	0.65 (1.9)	0.32 (0.4)	0.250	0.40 (0.6)	0.18 (0.2)	<0.001	0.710	0.559

Data are presented as the mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Health Association (AHA). Fatty Liver in Obesity (FLiO). Body Mass Index (BMI). Systolic blood pressure (SBP). Diastolic blood pressure (DBP). High density lipoprotein (HDL). Low density lipoprotein (LDL). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR). [a] Comparison within dietary groups (baseline and after 6 months). [b] Baseline differences between the AHA and FLiO groups. [c] Comparison of changes (baseline and 6 months) between the AHA and FLiO groups. [e] Chi-squared test for baseline differences between the AHA and FLiO groups.

MRI evidenced a significant reduction in hepatic volume and hepatic fat content after 6 months of follow-up in both groups (Table 2). A significant reduction in liver enzymes (AST, ALT, and GGT) was observed in both groups, with the exception of AST, which significantly decreased only in the AHA group (Table 2). On the other hand, liver stiffness did not show a significant change after this period in any of the groups (Table 2). A significant reduction was found in the Fatty Liver Index with both dietary interventions. Notably, there were no mentionable statistical differences in the changes of liver markers between the groups (Table 2).

Table 2. Liver parameters at baseline and after 6 months of dietary intervention in subjects with NAFLD, according to the group of intervention.

	AHA			FLiO				
	Baseline (n = 48)	**6 months (n = 37)**	***p*-Value [a]**	**Baseline (n = 50)**	**6 months (n = 39)**	***p*-Value [a]**	***Baseline* *p*-Value [b]**	**Δ *p*-Value [c]**
AST (IU/L)	25.5 (11.0)	21.6 (6.1)	<0.001	23.9 (8.3)	21.9 (8.5)	0.302	0.487	0.116
ALT (IU/L)	33.1 (16.8)	22.9 (8.5)	<0.001	33.3 (17.9)	21.7 (9.2)	<0.001	0.759	0.474
GGT (IU/L)	40.9 (29.0)	28.3 (23.0)	<0.001	33.6 (23.9)	26.4 (42.6)	<0.001	0.174	0.692
Hepatic volume (mL)	1797 (433)	1633 (316)	<0.001	1758 (406)	1563 (330)	<0.001	0.721	0.636
Liver fat (%)	7.4 (5.3)	3.8 (3.3)	<0.001	7.0 (5.4)	2.8 (3.1)	<0.001	0.468	0.706
Liver stiffness (m/s)	1.9 (0.8)	2.0 (0.7)	0.177	1.8 (0.8)	1.7 (0.6)	0.203	0.588	0.062
FLI	80.4 (15.6)	54.4 (23.7)	<0.001	76.9 (21.2)	47.9 (24.1)	<0.001	0.654	0.123

Data are presented as the mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Health Association (AHA). Fatty Liver in Obesity (FLiO). Asparate aminotransferase (AST). Alanine aminotransferase (ALT). Gamma-glutamyl transferase (GGT). Fatty Liver Index (FLI). [a] Comparison within dietary groups (baseline and after 6 months). [b] Baseline differences between the AHA and FLiO groups. [c] Comparison of the changes (baseline and 6 months) between the AHA and FLiO groups.

Furthermore, there were no significant differences at baseline in relation to dietary intake for both dietary groups (Table 3). As expected, the changes in meal frequency, percentage of protein, PUFA, and TAC were significantly higher in the FLiO group, while the percentage of carbohydrates significantly decreased. Notably, the adherence to the MedDiet significantly increased in both groups, but this change was significantly higher ($p = 0.002$) in the FLiO group. Similar results were found when the intake at 6 months was compared. Regarding energy intake, there was a significant difference between both groups at 6 months. However, the change from baseline to the endpoint was not statistically significant between them. On the other hand, physical activity significantly increased in both intervention groups, but the difference between them ($p = 0.326$) was not statistically significant (Table 3). When comparing the ratio of energy intake to the energy expenditure in physical activity, there was no significant difference between the groups (Table 3).

Table 3. Dietary intake and physical activity at baseline and after 6 months of dietary intervention in subjects with NAFLD according to the group of intervention.

	AHA			FLiO					
	Baseline (*n* = 48)	**6 months (*n* = 37)**	***p*-Value [a]**	**Baseline (*n* = 50)**	**6 months (*n* = 39)**	***p*-Value [a]**	**Baseline *p*-Value [b]**	**Δ *p*-Value [c]**	**6 months *p*-Value [d]**
Total energy (kcal/day)	2730 (867)	2170 (474)	0.002	2521 (1000)	1816 (569)	<0.001	0.148	0.536	0.012
Meal frequency	4.5 (0.9)	4.8 (0.8)	0.045	4.8 (0.9)	5.8 (1.0)	<0.001	0.199	0.022	0.001
Carbohydrates (% TEV)	43.4 (7)	45.0 (7)	0.336	42.8 (7)	39.0 (7)	0.015	0.685	0.048	<0.001
Proteins (% TEV)	16.8 (3)	18.5 (3)	0.047	17.6 (4)	22.1 (4)	<0.001	0.285	0.018	<0.001
Lipids (% TEV)	37.0 (7)	34.5 (6)	0.111	36.9 (8)	36.5 (9)	0.973	0.927	0.444	0.292
MUFA (% TEV)	17.8 (5)	17.5 (4)	0.682	17.4 (4)	16.1 (6)	0.353	0.785	0.623	0.088
PUFA (% TEV)	5.5 (2)	5.2 (2)	0.704	5.4 (2)	9.0 (5)	0.001	0.727	0.001	<0.001
SFA (% TEV)	10.6 (2)	9.4 (2)	0.032	10.6 (3)	8.8 (3)	0.024	0.838	0.572	0.102
Fiber (g/1000 kcal)	9.8 (3)	14.4 (4)	<0.001	9.7 (4)	14.9 (4)	<0.001	0.586	0.380	0.577
Glycemic load	165 (78)	117 (41)	0.003	147 (78)	83.8 (34)	<0.001	0.181	0.667	<0.001
TAC (mmol/1000 kcal)	4.9 (2)	4.6 (2)	0.426	4.2 (2)	5.7 (3)	0.022	0.332	0.044	0.264
MedDiet adherence score	6.2 (2)	10.8 (3)	<0.001	5.8 (2)	12.6 (3)	<0.001	0.370	0.002	0.002
PA (METs—min/week)	2685 (2112)	4463 (3031)	<0.001	3120 (2136)	4158 (2532)	0.039	0.258	0.326	0.809
Ratio Energy intake/Energy expenditure in PA	7.1 (6)	3.0 (2)	0.001	5.8 (7)	2.7 (2)	0.033	0.088	0.298	0.502

Data are presented as the mean (SD). Nonalcoholic fatty liver disease (NAFLD). American Health Association (AHA). Fatty Liver in Obesity (FLiO). Total energy value (TEV). Monounsaturated fatty acid (MUFA). Polyunsaturated fatty acid (PUFA). Saturated (SFA). Total antioxidant capacity (TAC). Mediterranean diet (MedDiet). Physical Activity (PA). Metabolic equivalent of the task (METs). [a] Comparison within dietary groups (baseline and after 6 months). [b] Differences between the AHA and FLiO groups at baseline. [c] Comparison of the changes (baseline and 6 months) between the AHA and FLiO groups. [d] Differences between the AHA and FLiO groups at 6 months of intervention.

Given that the changes in anthropometric clinical and biochemical parameters did not statistically differ between AHA and FLiO groups and that both approaches displayed similar outcomes for relevant variables of liver status, we considered both dietary strategies as equally nutritionally effective (Tables 2 and 3), and, consequently, the two groups were merged and evaluated as one sample for the subsequent analyses.

Linear regression analyses (adjusted by group of intervention, age, sex, 6 months physical activity, and 6 months energy intake) were performed in order to evaluate the factors potentially involved with hepatic status parameters after the 6 months of the dietary intervention (Table 4). Notably, weight loss percentage was significantly associated with improvements in ALT, a reduction of liver fat percentage, FLI, and liver stiffness. Parallelly, the decrease in total fat mass was significantly associated with a reduction in liver fat content, FLI, and liver stiffness, while the decrease in visceral adipose tissue seemed to influence liver stiffness. Regarding dietary factors, the increase in the adherence to MedDiet seemed to play a role in the decrease of liver fat percentage, while its association with lower values of ALT and liver stiffness was only marginally significant. Additionally, the decrease in the lipid percentage of the diet was associated with a reduction in hepatic fat content. Finally, the change in dietary fiber consumption was inversely associated with FLI.

Table 4. Regression analyses of the hepatic status parameters after 6 months of dietary intervention as dependent variables and changes in anthropometric, biochemical, and dietary factors as independent variables.

	Weight Loss (%)		Δ Visceral Adipose Tissue (kg)		Δ Total Fat Mass (%)		Δ Adiponectin (µg/mL)		Δ C-Reactive Protein (mg/dL)		Δ MedDiet Adherence		Δ Proteins (%)		Δ Lipids (%)		Δ Meal Frequency		Δ TAC (mmol/1000 kcal)		Δ Fiber (g/1000 kcal)		Δ Glycemic Load	
	β	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*B*	*p*
AST (IU/L)	−0.191	0.263	1.672	0.059	0.086	0.742	0.151	0.565	3.389	0.224	−0.307	0.320	−0.096	0.682	0.124	0.272	−0.325	0.806	−0.642	0.194	0.001	0.995	0.008	0.531
ALT (IU/L)	−0.369	0.046	1.580	0.089	0.311	0.260	−0.010	0.972	6.991	0.021	−0.626	0.062	−0.093	0.719	0.191	0.123	−0.062	0.964	−0.287	0.602	−0.246	0.372	−0.001	0.906
GGT (IU/L)	−0.691	0.113	0.785	0.745	1.129	0.095	0.117	0.862	13.146	0.089	−0.799	0.312	−0.242	0.699	−0.314	0.292	0.085	0.981	−0.749	0.572	−1.112	0.103	0.029	0.391
Hepatic Volume (mL)	−5.489	0.450	15.68	0.664	1.503	0.899	−3.840	0.731	162.95	0.096	−3.444	0.770	2.780	0.761	3.543	0.437	−41.099	0.320	0.388	0.984	0.888	0.931	−0.597	0.274
Liver fat (%)	−0.252	<0.001	0.123	0.753	0.338	0.004	−0.166	0.080	1.377	0.174	−0.396	0.001	−0.111	0.268	0.098	0.046	−0.227	0.549	−0.238	0.282	−0.155	0.167	0.005	0.369
FLI	−2.340	<0.001	3.402	0.078	3.590	<0.001	−0.1.45	0.069	11.966	0.199	−0.962	0.313	−0.308	0.668	0.319	0.353	−5.156	0.172	−0.391	0.795	−1.872	0.015	0.018	0.645
Liver Stiffness (m/s)	−0.043	0.004	0.168	0.044	0.070	0.003	−0.010	0.679	0.140	0.589	−0.053	0.061	0.016	0.451	−0.009	0.375	−0.147	0.214	−0.006	0.887	−0.034	0.143	−0.0002	0.980

Models were adjusted by diet (group of intervention), age, sex, 6 months physical activity, and 6 months energy intake. Mediterranean diet (MedDiet). Total antioxidant capacity (TAC). Asparate aminotransferase (AST). Alanine aminotransferase (ALT). Gamma-glutamyl transferase (GGT). Fatty Liver Index (FLI).

To explore this issue in a more comprehensive manner, a linear regression analysis was used. The effect of nutritional factors on liver fat content after 6 months of dietary treatment was analyzed by linear univariate regression and subsequently a multivariate analysis (Table S1). In addition to the global MedDiet adherence, the TAC of the diet was the only nutritional factor that showed a consistent, significant, and inverse association with liver fat content.

According to the relationships observed in the previous analyses, the influence of weight loss, adherence to MedDiet, and TAC on liver fat content is summarized in Table 5. Models of these main factors that are unadjusted and adjusted by potential confounders are presented. In model 5 and model 6, both weight loss and adherence to the MedDiet were included along with the rest of the adjusting variables. In model 5, MedDiet adherence remained statistically significant, while in model 6, both variables showed statistical significance and explained up to 40.9% of the variation in liver fat content (Adjusted R^2 = 0.409; p-model < 0.001) after the 6 months intervention. In model 7 and model 8, the TAC of the diet was included instead of its MedDiet adherence. Model 7 illustrates the inverse association between the TAC and liver fat content, even after an adjustment for weight loss, and explains up to 30.5% of the variation in liver fat (Adjusted R^2 = 0.305; p-model < 0.001).

Table 5. Regression analyses of liver fat percentage at 6 months as the dependent variable and MedDiet adherence, total antioxidant capacity, and weight loss percentages after the dietary intervention as independent variables.

Liver Fat (%) after 6 Months of Treatment		β	p	Adjusted R^2	p-Model
Unadjusted model	6 months MedDiet adherence	−0.465	<0.001	0.186	<0.001
Model 1	6 months MedDiet adherence	−0.585	<0.001	0.319	<0.001
Unadjusted model	6 months TAC (mmol/1000 kcal)	−0.351	0.028	0.061	0.027
Model 2	6 months TAC (mmol/1000 kcal)	−0.306	0.102	0.114	0.047
Unadjusted model	Weight loss (%)	−0.257	<0.001	0.188	<0.001
Model 3	Weight loss (%)	−0.252	<0.001	0.259	<0.001
Model 4	Weight loss (%)			0.366	<0.001
	5%–10%	−3.410	0.001		
	>10%	−5.033	<0.001		
Model 5	6 months MedDiet adherence	−0.437	0.007	0.344	<0.001
	Weight loss (%)	−0.133	0.087		
Model 6	6 months MedDiet adherence	−0.325	0.031	0.409	<0.001
	Weight loss (%)				
	5%–10%	−2.490	0.021		
	>10%	−3.638	0.002		
Model 7	6 months TAC (mmol/1000 kcal)	−0.351	0.037	0.305	<0.001
	Weight loss (%)	−0.262	<0.001		
Model 8	6 months TAC (mmol/1000 kcal)	−0.297	0.056	0.398	<0.001
	Weight loss (%)				
	5%–10%	−3.410	0.001		
	>10%	−5.009	<0.001		

All models were adjusted by dietary group (group of intervention), age, sex, 6 months physical activity and 6 months energy intake. Mediterranean diet (MedDiet). Total antioxidant capacity (TAC).

Because the adherence to the MedDiet was an important dietary factor significantly associated with an improvement in hepatic status variables even after multiple adjustments in the regression analyses, the whole sample was categorized into two groups according to the median value of the

adherence to the MedDiet questionnaire (low adherence <12 points, n = 30, and high adherence ≥12 points, n = 46). The percentage of changes in anthropometric and hepatic status variables were compared between these two groups (Figure 2). The group with a higher adherence to the MedDiet showed significantly greater reductions in body weight, BMI, total fat mass, liver fat, and FLI.

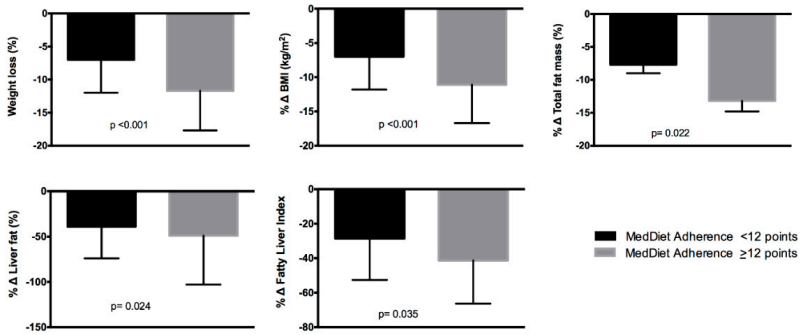

Figure 2. Weight loss percentage and percentage of changes in selected variables according to the median score of the Mediterranean Diet (MedDiet) adherence questionnaire (<12 points n = 30 and > 12 points n = 46) after 6 months of dietary intervention.

4. Discussion

This randomized controlled trial compared the effects of two personalized energy-restricted dietary strategies on anthropometry, body composition, biochemical determinations, and the non-invasive parameters of liver status in overweight or obese subjects with ultrasonography proven liver steatosis after 6 months of intervention. The main results evidenced the important association between weight loss, TAC, and MedDiet adherence, with an improvement in hepatic status variables such as liver fat content, FLI, and liver stiffness, regardless of the group of intervention and under the prescription of an energy-restricted balanced diet.

The pathogenesis of NAFLD involves a variety of components where dietary factors seem to be of key importance and have been associated with weight gain, obesity, and NAFLD development. Such factors include a high intake of calories and an excessive consumption of saturated fats, refined carbohydrates, and fructose, all distinctive features of the Western diet [35,36]. The important association between NAFLD and obesity emphasizes the role of dietary intake in the development and progression of NAFLD, since its prevalence rises with an increase in BMI [4]; in general, weight gain leads to the accumulation of fat in the liver [37]. Accordingly, lifestyle interventions focusing on weight loss by means of a reduction in caloric intake and/or an increase in energy expenditure are currently the first line treatment for subjects with NAFLD [2,38,39].

In the present study, both groups achieved comparable results in the evaluated variables, including weight loss, and the reduction in liver fat content and hepatic volume was evaluated by MRI, transaminases, and FLI. The findings with no significant differences in the changes between the intervention groups (even when dietary intake presented the expected statistical differences) may be explained by the energy restriction of 30% applied to both diets and the consequently similar weight loss of more than 9% in both groups, which agrees with the objectives proposed by the AASLD for the treatment of subjects with NAFLD. The AASLD recommends a weight loss of at least 3%–5% of body weight to reduce liver steatosis, while a loss of 7%–10% may be needed to improve fibrosis and the other histological characteristics of NASH [2,40]. Moreover, both dietary strategies in this trail were carefully designed and based on previously studied healthy dietary approaches, which have shown beneficial effects in the management of cardiovascular risk factors. The AHA diet was based on the

AHA guidelines, and the FLiO diet was based on the RESMENA diet [28,41], instead of a comparison with a control group without active intervention or receiving only general recommendations.

On the other hand, several authors have proposed that dietary pattern analysis is the most realistic approach to evaluate the associations between diet and disease [42,43]. Accordingly, we evaluated the adherence to the MedDiet, as well as individual dietary features.

The MedDiet has shown a variety of health effects mainly related to the prevention of cardiovascular disease [44]. Thus, its potential repercussions in NAFLD development and treatment continue to be studied. A recent cross-sectional study with more than 13,000 individuals evaluated two population-based cohorts of adults from Switzerland and England and found that a greater adherence to the MedDiet was associated with a lower prevalence of hepatic steatosis [45]. Meanwhile, a study that compared subjects with NAFLD to an age, sex, and BMI matched control group found that a higher adherence to the MedDiet was not associated with a lower likelihood of having NAFLD but was associated with lower insulin resistance and less severe liver disease [46]. Recently, a randomized controlled trail compared a group that only followed a MedDiet, a group that followed a "Mediterranean lifestyle" (diet, exercise, and sleep recommendations), and a group that only received general dietary guidelines. The MedDiet group had significantly lowered liver stiffness values, while the subjects with the "Mediterranean lifestyle" prescription had significantly reduced ALT levels and liver stiffness in comparison with the third group [17]. In our study, most of the individual characteristics of the diet showed a small association with liver status after the dietary intervention, while a greater increase in the TAC and a higher adherence to the MedDiet (along with weight loss) seemed to be more decisive factors for additional benefits in the treatment of subjects with NAFLD. In the regression analyses, the TAC of the diet and the adherence to the MedDiet were significant and inversely associated with less hepatic fat content, even when the models included the percentage of weight loss.

Given that weight reduction through lifestyle changes is not a feasible alternative for all individuals with NAFLD, an increasing number of studies have been carried out to determine if certain dietary or lifestyle modifications might have beneficial effects on liver status even in the absence of weight loss [39,47]. These dietary modifications are focused on following a "high quality" and a "healthy" diet and aim to avoid foods typically present in the Western dietary pattern [15]. In a recent study with a duration of 12 weeks, hepatic steatosis was significantly diminished to a similar degree by both *ad libitum* low-fat and Mediterranean diets, even with only a 2% body weight loss [48].

Regarding liver stiffness, the results of the ARFI elastography did not significantly change in the whole sample after the intervention period and the differences between the groups did not reach statistical significance. Interestingly, liver stiffness decreased only in the FLiO group, although the difference in the changes was only marginally significant ($p = 0.062$). The remission or the stabilization of fibrosis is considered to be a positive aspect in clinical trials with NAFLD subjects [34]. Even when our results were derived from a medium-term intervention (6 months), the period of follow-up may have not been long enough to evidence an improvement in liver stiffness assessed by ARFI elastography. Previous studies have found an improvement in liver stiffness [49] while others have found no changes after dietary treatment [48]. Notably, in the regression analyses, a lower liver stiffness at the endpoint of the intervention was associated with a loss of body weight, visceral adipose tissue, and total fat mass and was marginally associated with a higher adherence to the MedDiet.

Inflammation plays a major role in the evolution of NAFLD; liver steatosis accompanied by hepatic inflammation (NASH) increases the risk of progression to fibrosis and cirrhosis [11]. Moreover, an association between adiponectin levels and liver fibrosis in subjects with chronic liver disease, including those with NAFLD, has been suggested. However, this relationship varies widely among the different liver diseases, and conflicting results are reported in the literature, so the nature of these associations remains poorly understood [50]. On the other hand, there is evidence that weight loss is a central factor in reducing the pro-inflammatory markers in obese or overweight individuals and that a hypocaloric diet has anti-inflammatory effects independent of the diet's composition [51]. Interestingly, even when the weight loss percentage and the changes in the rest of the variables evaluated in our

study were not significantly different between the groups, inflammatory markers (such as adiponectin and C-reactive protein) significantly improved in the FLiO group but not in the AHA group. These results might be related to the higher adherence to the MedDiet and the significant increase of the TAC in subjects following the FLiO diet, which entails a higher intake of fruits and vegetables and a "healthier profile" of fatty acids. A recent review and meta-analysis concluded that a higher intake of fruits and vegetables leads to a reduction in proinflammatory mediators [52]. Thus, the effect of weight loss in inflammatory markers might be greater when accompanied by a higher intake of fruits and vegetables, and we hypothesize that in a longer follow-up, the FLiO diet (with a higher adherence to de MedDiet and a higher TAC) may provide greater improvements. Due to the limited evidence on this issue, more well-designed trails specifically aiming to evaluate the interplay between inflammatory markers and dietary features in the treatment of NAFLD are needed, especially regarding NASH and liver fibrosis.

There are some limitations in this study that should be acknowledged. First, NAFLD was evaluated using non-invasive techniques instead of a liver biopsy, which is currently the most reliable approach for detecting steatohepatitis and fibrosis in NAFLD subjects. Nevertheless, liver biopsy is limited by cost, sampling errors, and procedure-related complications [2]. However, we carried out a compressive evaluation of liver status, and the design of this study was based on validated and widely used imaging techniques for the assessment of liver steatosis (MRI and ultrasonography [33]) and liver stiffness (ARFI elastography [53]). Second, the evaluation of the diet was carried out using self-reported information of the participants by means of an FFQ, which may produce a bias in dietary evaluation. On the other hand, the major strengths of this study include that it is a randomized controlled trial (considered to be the gold standard for evaluating the efficacy and safety of dietary interventions). Additionally, the implemented strategies consisted of personalized diets with individual follow-ups and tended to promote long term behavioral changes and a healthy lifestyle.

5. Conclusions

The data in this study suggest that, in the context of energy restriction, both the AHA and the FLiO diets may be valid options for the dietary treatment of NAFLD in overweight or obese subjects. Moreover, a higher TAC and adherence to the MedDiet might provide additional benefits to weight loss in the treatment of obesity and associated comorbidities, such as NAFLD.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/10/2543/s1, Table S1: Regression analyses of liver fat percentage at 6 months as dependent variable and selected dietary components after the dietary intervention as independent variables.

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CHAPTER 3

Effects of a 6-month dietary-induced weight loss on erythrocyte membrane omega-3 fatty acids and hepatic status of subjects with nonalcoholic fatty liver disease: The Fatty Liver in Obesity study

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Original Research

Effects of a 6-month dietary-induced weight loss on erythrocyte membrane omega-3 fatty acids and hepatic status of subjects with nonalcoholic fatty liver disease: The Fatty Liver in Obesity study

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KEYWORDS:
NAFLD;
Omega-3;
ALA;
EPA;
Liver stiffness;
Liver iron;

BACKGROUND: Omega-3 polyunsaturated fatty acids (PUFAs) have been suggested as important biomolecules in the management of nonalcoholic fatty liver disease (NAFLD).

OBJECTIVE: This study aimed to evaluate the effect of 6-month weight loss diets on erythrocyte membrane omega-3 PUFA composition of NAFLD adults, and to evaluate the potential relationship between erythrocyte membrane omega-3 PUFAs and hepatic health markers.

METHODS: In this secondary analysis of the Fatty Liver in Obesity study, erythrocyte membranes were analyzed by gas chromatography in 54 subjects with liver steatosis detected by ultrasonography

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Weight loss;
FLiO

who achieved a weight loss >5% after the follow-up. Baseline and 6-month evaluation included hepatic acoustic radiation force impulse elastography and magnetic resonance imaging, anthropometry, body composition, and biochemical determinations.

RESULTS: After the follow-up, α-linolenic acid (ALA) proportion significantly increased in erythrocyte membranes, whereas eicosapentaenoic acid (EPA) showed no statistical difference and docosapentaenoic acid decreased. Both the changes in erythrocyte membrane ALA and EPA were positively associated with dietary ALA. Regression analyses evidenced that the changes in erythrocyte membrane ALA and EPA were inversely associated with the changes in liver stiffness and liver iron content, respectively.

CONCLUSION: The adherence to weight loss strategies for 6 months led to changes in erythrocyte membrane omega-3 PUFA composition, which in turn were associated with changes in hepatic markers, suggesting that these fatty acids accompany the improvements in the liver during a dietary treatment. These findings show that beyond weight loss, the composition of the diet has an important role in the management of NAFLD.

Introduction

The spectrum of nonalcoholic fatty liver disease (NAFLD) encompasses adverse lipid-associated liver conditions that begin as simple steatosis and can progress to nonalcoholic steatohepatitis (NASH) with variable degrees of liver fibrosis, which can potentially lead to cirrhosis or hepatocellular carcinoma.[1] According to growing evidence, NAFLD is the result of a multifactorial process, which includes lifestyle, dietary, and genetic factors, among others,[2] and involves a variety of metabolic pathways that can operate at different stages of the disease.[3] When the liver capacity to metabolize, store, and export free fatty acids as triglycerides is surpassed by free fatty acids flux from the periphery and/or by hepatic de novo lipogenesis, hepatic lipotoxicity can occur.[4] Furthermore, lipotoxicity effects in the liver are characterized by an abnormal cellular lipid composition, which promotes lipid accumulation, dysfunction of organelles, cell injury, and hepatocyte dysfunction or hepatocyte cell death.[3,4]

In contrast, the synthesis of polyunsaturated fatty acids (PUFAs), including omega-3 PUFAs, cannot occur endogenously; therefore, those fatty acids must be supplied from dietary sources.[5] Omega-3 PUFAs participate in diverse body processes, such as the modulation of the fluidity and permeability of cell membranes[6] and the synthesis of precursors of anti-inflammatory molecules, among others.[5] Moreover, omega-3 PUFAs have been associated with the prevention of metabolic alterations or chronic diseases, including obesity and type 2 diabetes mellitus,[7] as well as with a reduced risk of cardiovascular disease.[8]

In NAFLD, PUFA appears to modulate the activity of genes involved in lipid metabolism, redox balance, and fibrogenesis through the interaction with nuclear receptors and transcription factors.[3,9] In addition, differential mechanisms of omega-3 PUFA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), to modulate hepatic steatosis have been suggested,[10] whereas α-linolenic acid (ALA) has shown some beneficial hepatic effects in murine models.[11] However, the effects of supplementation with omega-3 PUFA still producing inconclusive evidence, especially regarding liver fibrosis.[12,13] Likewise, current evidence suggests that omega-3 PUFA supplementation may be effective in the early stages of NAFLD, but not in individuals with more severe stages of the disease.[14] Moreover, the hepatic effects of dietary omega-3 PUFA have been less studied than supplementation and the potential mechanisms induced by dietary changes that promote the improvement of a variety of hepatic outcomes are still not fully understood.[15]

As there is no approved pharmacological treatment for NAFLD and lifestyle modifications, including diet and physical exercise pointing at weight loss remain the cornerstone therapy for this condition,[16,17] omega-3 PUFAs may play a relevant role in the dietary management of NAFLD.[18] Thus, the aim of this study was to evaluate the effect of 6-month weight loss diets on erythrocyte membrane omega-3 PUFA composition of subjects with NAFLD, and to evaluate the potential relationship between erythrocyte membrane omega-3 PUFA and hepatic health markers.

Materials and methods

Subjects and study protocol

The present research is a secondary analysis of the Fatty Liver in Obesity (FLiO) study (Fig. 1), which was a randomized parallel trial designed to compare the effect of 2 dietary strategies for weight loss on liver status, anthropometric measurements, body composition, and biochemical markers of subjects with overweight or obesity and ultrasonography-proven liver steatosis (all the selection criteria are elsewhere described).[19] The current analysis was carried out in a subsample of 54 participants who achieved a weight loss of more than 5% of the initial body weight after 6 months of follow-up. The cut-off point was set up considering this value as an indicator of good adherence to the treatment, given that both diets applied

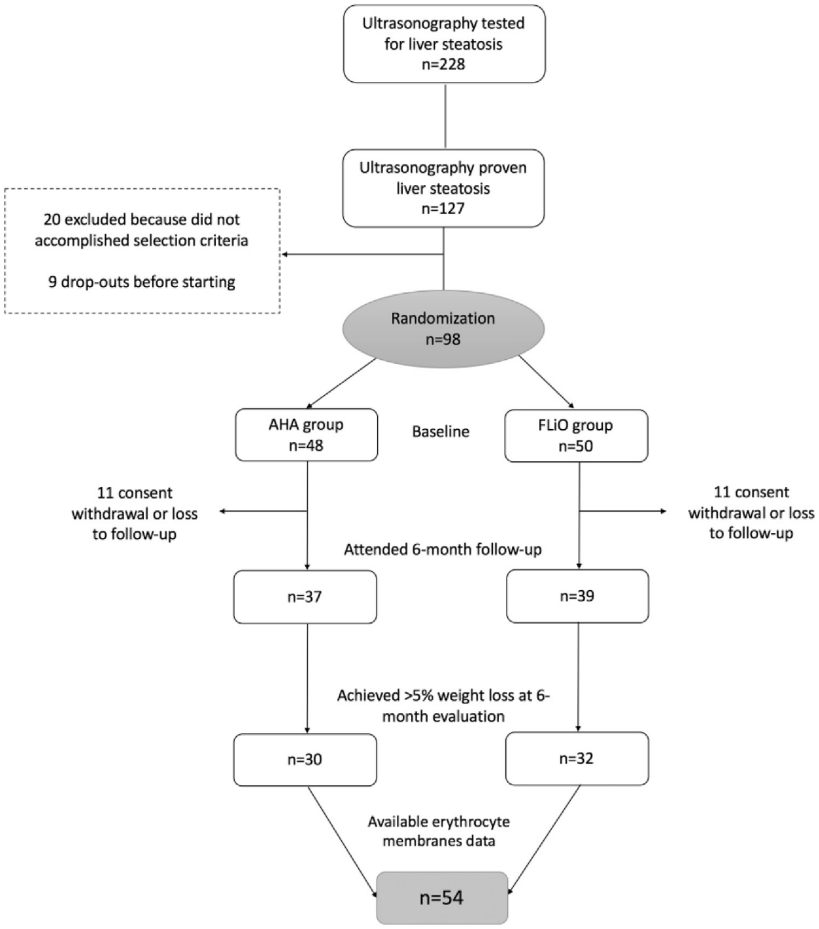

Figure 1 Flowchart of the study participants. AHA, American Heart Association; FLiO, Fatty Liver in Obesity.

an energy restriction of 30% of the total requirements of each participant to achieve the recommendations of the American Association for the Study of Liver Diseases, which propose a weight loss of at least 3% to 5% to improve hepatic steatosis.[20] Thus, in accordance with previous research, subjects with nonadherence were removed.[21] At the beginning of the study, the participants were randomly assigned to the American Heart Association (AHA) or the FLiO group. All the subjects were blinded to the group of allocation and the professionals in charge of liver assessment and other determinations were blinded to the dietary treatment of each participant, clinical information, and laboratory data. Briefly, one diet was based on the AHA guidelines[22] with a conventionally balanced macronutrient distribution of 50% to 55% from carbohydrates, 15% from proteins, and 30% from lipids (with a healthy fatty acid profile) of the total caloric value. The FLiO diet proposed a high adherence to the Mediterranean Diet and was designed with a macronutrient distribution of 40% to 45% from carbohydrates, 25% from proteins (predominantly from vegetable sources and seafood), and 30% to 35% from lipids (favoring extra virgin olive oil and omega-3 PUFA consumption).[19]

The trial was approved by the Research Ethics Committee of the University of Navarra (ref. 54/2015) and was appropriately registered in www.clinicaltrials.gov (FLiO; NCT03183193). Each participant gave written informed consent before the enrollment in the study. All the procedures performed were in accordance with the Declaration of Helsinki and the study was conducted following the CONSORT 2010 guidelines.

Anthropometric, body composition, biochemical, and dietary intake assessment

The assessment of anthropometrical measurements and body composition was carried out after overnight fasting at

the Metabolic Unit of the University of Navarra. Body weight, height, and waist circumference were determined as previously described.[22] Body composition was determined by dual-energy X-ray absorptiometry following the instructions of the manufacturer (Lunar iDXA, enCORE 14.5, Madison, WI). Body mass index was calculated as the body weight divided by the squared height (kg/m^2). Blood samples for biochemical assessment were properly collected after overnight fasting of 8 to 10 hours and processed at the Laboratory of Biochemistry of the University of Navarra Clinic (CUN, Pamplona, Spain). Blood glucose, insulin, total cholesterol, high-density lipoprotein cholesterol, triglycerides, aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyltransferase concentrations were determined on an autoanalyzer with specific commercial kits and following the instructions of the company (cobas 8000, Roche Diagnostics, Basel, Switzerland). The Homeostatic Model Assessment of Insulin Resistance was calculated as previously described,[23] and the low-density lipoprotein cholesterol levels were calculated using the Friedewald formula.[24] The dietary intake of the participants was estimated using a semiquantitative food frequency questionnaire of 137 items previously validated in Spain for energy and nutrient intake[25] and the consumption of omega-3 PUFA was derived as previously reported.[26]

Evaluation of liver status

Liver steatosis was determined by Ultrasonography (Siemens ACUSON S2000 and S3000) in accordance with previously described procedures.[27] Liver stiffness was assessed using acoustic radiation force impulse (ARFI) elastography (Siemens ACUSON S2000 and S3000) following the guidelines of the manufacturer. The median value of liver stiffness in each patient was obtained from 10 valid ARFI elastography measurements.[28] Magnetic resonance imaging (Siemens Aera 1.5 T) was used to determine hepatic volume, liver fat, and liver iron content (Dixon method) following standardized procedures.[27] All the evaluation of the liver was carried out under fasting conditions by qualified staff at the University of Navarra Clinic.

Isolation of erythrocyte membranes from peripheral blood

Erythrocyte membranes were isolated following previously described methodology.[29] The fatty acid composition was evaluated in unwashed packed erythrocytes isolated from blood samples and drawn into 10-mL plasma-EDTA tubes after overnight fasting of 8 to 10 hours. The samples were obtained and centrifugated, and the plasma and buffy coat were separated from erythrocytes. After the collection, erythrocytes were immediately frozen at −80°C. Samples were freeze-dried and transported to the Portuguese Institute for the Sea and Atmosphere (IPMA, IP).

Analysis of fatty acid profile in erythrocyte membranes

Fatty acid methyl esters were prepared from the freeze-dried erythrocytes by acid-catalysis transesterification using the methodology described by Bandarra et al.[30] Samples were injected into a Scion 456-GC gas chromatograph (West Lothian, UK), equipped with an autosampler with a flame ionization detector at 250°C. Fatty acid methyl esters were identified by comparing their retention time with those of Sigma–Aldrich standard (PUFA-3, Menhaden oil). The limit of detection was 1 mg/100 g. Results were calculated in percentage of total fatty acids on the basis of peak areas.

Statistical analyses

The mean value and standard deviations were reported for the studied variables. The sample size of the FLiO study was calculated as previously described.[19] The differences between baseline and 6-month data were analyzed by the paired Student t test, assuming the normality of the distribution of the variables. The comparison between the changes in the 50th percentile (P50) groups ($<$P50 vs $\geq$P50) was carried out by means of Student t test or the Mann–Whitney U test when appropriate. The distribution of the variables was evaluated with the Shapiro–Wilk test. Pearson or Spearman correlation analyses were performed according to the distribution of the data to evaluate the relationships between the change in erythrocyte membrane fatty acids and the change in hepatic parameters. Multivariate linear regression models were adjusted for potential confounders (age, sex, dietary group, and change in body weight) as appropriate.

All P values presented are 2-tailed and a $P < .05$ was considered to be statistically significant. Statistical analyses were carried out using the software Stata version 12.0 (StataCorp, College Station, TX).

Results

The average age of the 54 participants was 51.3 (10) years and the sample consisted of 63% men and 37% women. After 6 months, dietary treatments improved most of the variables related to anthropometry, body composition, general biochemistry, and hepatic status (Table 1). When comparing the subjects according to the median of weight loss (9.35 kg), those participants with greater weight loss achieved significantly greater improvements in anthropometric and body composition parameters, insulin, triglycerides, and liver stiffness. In contrast, participants on or above the median of erythrocyte membrane ALA increase showed a significantly greater decrease in waist circumference, total fat mass, liver iron content, and liver stiffness.

The analysis of the food frequency questionnaire showed a significant reduction in total energy, carbohydrates (g/d),

Table 1 Characteristics of the participants at baseline and after 6 months and categorized by the 50th percentile (P50) of the changes in body weight and erythrocyte membrane ALA

					Δ Body weight		Δ Erythrocyte membrane ALA	
n = 54	Baseline	6 mo	Difference	*P* value	<*P50* (9.35 kg)	≥*P50* (9.35 kg)	<*P50* (0.007%)	≥*P50* (0.007%)
Anthropometry and body composition								
Weight (kg)	96.1 (13)	84.7 (12)	−11.4 (5)	<.001	−7.4 (2)	−15.3 (5)***	−9.5 (4)	−13.0 (6)
BMI (kg/m^2)	33.6 (4)	29.7 (4)	−3.9 (2)	<.001	−2.7 (1)	−5.2 (2)***	−3.4 (1)	−4.5 (2)
WC (cm)	110 (9)	99 (9)	−11 (6)	<.001	−8.3 (4)	−14.5 (6)***	−9.1 (6)	−13.8 (5)**
Total fat mass (%)	41.7 (6)	36.4 (8)	−5.3 (4)	<.001	−3.0 (2)	−7.7 (3)***	−4.1 (3)	−6.3 (4)*
General biochemistry								
Glucose (mg/dL)	105 (19)	94 (14)	−11 (12)	<.001	−10.9 (12)	−11.4 (11)	−10.4 (10)	−10.7 (12)
Insulin (mU/L)	17.8 (8)	10.7 (7)	−7.1 (8)	<.001	−4.8 (9)	−9.6 (6)*	−5.3 (8)	−8.1 (8)
HOMA-IR	4.7 (2)	2.6 (2)	−2.1 (2)	<.001	−1.6 (3)	−2.6 (2)	−1.6 (2)	−2.4 (2)
TC (mg/dL)	193 (40)	174 (37)	−19 (22)	.001	−17.2 (18)	−21.1 (25)	−17.2 (18)	−22.1 (26)
HDL-c (mg/dL)	52 (14)	54 (13)	2.0 (9)	.146	−0.44 (8)	4.0 (9)	2.8 (7)	0.36 (10)
LDL-c (mg/dL)	113 (35)	101 (32)	−12.0 (23)	<.001	−11.1 (17)	−13.0 (28)	−12.0 (21)	−12.2 (27)
Triglycerides (mg/dL)	139 (64)	90 (40)	−49.0 (54)	<.001	−33.6 (42)	−64.3 (61)*	−46.4 (49)	−55.2 (62)
Hepatic status								
AST (IU/L)	25.6 (10)	21.4 (8)	−4.2 (11)	.005	−5.9 (9)	−0.9 (8)	−3.6 (8)	−3.3 (10)
ALT (IU/L)	33.7 (16)	21.3 (8)	−12.4 (13)	<.001	−15.8 (17)	−9.0 (7)	−11.0 (10)	−15.2 (16)
GGT (IU/L)	40.8 (31)	23.6 (19)	−17.2 (25)	<.001	−17.0 (28)	−17.4 (22)	−12.7 (26)	−22.7 (26)
Liver fat (%)	7.2 (5)	2.6 (2)	−4.6 (4)	<.001	−5.1 (5)	−4.2 (3)	−4.2 (4)	−5.2 (5)
Liver iron (%)	36.2 (11)	30.2 (10)	−6.0 (8)	<.001	−6.1 (5)	−4.7 (6)	−3.0 (5)	−8.9 (9)**
Liver stiffness (m/s)	1.8 (0.7)	1.8 (0.6)	0.02 (0.9)	.865	0.4 (0.7)	−0.3 (0.9)**	0.4 (0.7)	−0.4 (1.0)*

ALA, α-linolenic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-c, low-density lipoprotein cholesterol; P50, 50th percentile; SD, standard deviation; TC, total cholesterol; WC, waist circumference.

Change between baseline and 6 mo (Δ).

Data presented as mean (SD).

*$P < .05$, **$P < .01$, and ***$P < .001$ for the comparison between each group (<P50 vs ≥P50).

Table 2 Dietary intake of the participants at baseline and after 6 mo

n = 54	Baseline	6 mo	*P* value
Total energy (kcal/d)	2806 (1041)	1941 (607)	<.001
Carbohydrates (% TEV)	42.8 (7.6)	43.0 (7.6)	.922
Proteins (% TEV)	16.7 (3.1)	20.3 (4.1)	<.001
Lipids (% TEV)	37.6 (7.5)	34.3 (7.1)	.011
MUFAs (% TEV)	18.2 (4.9)	16.2 (4.9)	.014
PUFAs (% TEV)	5.3 (1.7)	6.6 (3.7)	.016
SFAs (% TEV)	10.7 (2.5)	9.0 (3.3)	.006
ALA (g/1000 kcal/d)	0.53 (0.2)	0.82 (0.6)	.012
Marine omega-3 (g/1000 kcal/d)	0.22 (0.1)	0.36 (0.2)	.002
Omega-6 (g/d)	13.3 (7.0)	10.9 (9.3)	.106
Iron intake (mg/d)	19.0 (7.5)	16.0 (4.3)	.005

ALA, α-linolenic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SD, standard deviation; SFAs, saturated fatty acids; TEV, total energy value.

Data presented as mean (SD).

total lipids, monounsaturated fatty acids, and saturated fatty acids (SFAs) intake after 6 months. As expected, the reported ingestion of proteins had a significant increase in the percentage of total energy value, although the ingestion decreased in grams per day. The proportion of PUFA, and the proportion of ALA and marine omega-3 PUFA per 1000 kcal significantly increased after the 6-month period. Regarding iron intake, a significant decrease was observed (Table 2).

Regarding the fatty acid composition of erythrocyte membranes, significant changes were found (Table 3). ALA significantly increased, whereas EPA showed no significant differences and DHA significantly decreased. In addition, the total of SFA significantly increased, whereas the total of PUFA, omega-6, and the Omega-3 Index significantly decreased. Results analyzed by dietary group in anthropometry, body composition, biochemical parameters, hepatic status (Supplementary table 1), dietary intake (Supplementary table 2), and erythrocyte membrane fatty acid composition are presented (Supplementary table 3).

Table 3 Fatty acid composition (%) of erythrocyte membranes of the participants at baseline and after 6 mo

n = 54	Baseline	6 mo	*P* value
Omega-3 PUFA			
18:3n3 (ALA)	0.059 (0.01)	0.072 (0.02)	.002
20:5n3 (EPA)	0.49 (0.2)	0.53 (0.2)	.194
22:6n3 (DHA)	4.9 (1.3)	4.5 (1.0)	.009
Omega-3 Index (EPA + DHA)	5.4 (1.4)	5.0 (1.2)	.027
Total MUFA	18.0 (1.7)	18.6 (1.8)	.050
Total SFA	39.0 (2.4)	41.2 (2.4)	<.001
Total PUFA	41.5 (3.4)	38.8 (3.5)	<.001
Total omega-3	11.5 (2.0)	10.9 (1.8)	.052
Total omega-6	29.8 (2.5)	27.7 (2.8)	<.001

ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SD, standard deviation; SFAs, saturated fatty acids.

Data presented as mean (SD).

Notably, the changes observed in specific erythrocyte membrane omega-3 PUFA were associated with dietary fatty acid consumption (Supplementary table 4). The change in ALA per 1000 kcal was strongly associated with the change in erythrocyte membrane ALA and moderately associated with the change in erythrocyte membrane EPA, whereas no statistical association with DHA was observed. Furthermore, the analysis of the potential relationship between the changes in hepatic iron and iron intake did not show a statistical association.

Correlation analysis showed a negative and significant association between the change in liver iron content (Fig. 2) and the change in ALA ($r = -0.422$, $P = .003$), EPA ($r = -0.436$, $P = .001$), DHA ($r = -0.352$, $P = .012$), Omega-3 Index ($r = -0.378$, $P = .006$), and total omega-3 PUFA ($r = -0.372$, $P = .007$) in erythrocyte membranes. Moreover, the change observed in liver stiffness showed an inverse association with the change in ALA ($r = -0.414$, $P = .005$). In contrast, the decrease in liver fat content showed a marginal relationship with the change in DHA ($r = -0.265$, $P = .062$). No significant associations were found between the change in erythrocyte membrane specific or total omega-3 PUFA and other hepatic status variables (data not shown).

Interestingly, the change in liver stiffness was negative and significantly associated with the increase in the proportion of dietary ALA per 1000 kcal ($r = -0.393$, $P = .013$), whereas no other association between the reported dietary intake of omega-3 PUFAs and liver status markers was observed.

As liver iron content and liver stiffness showed significant associations with erythrocyte membrane fatty acid composition, the relationship between these liver parameters and body weight loss was as well evaluated (Fig. 3). No statistical association was observed between the decrease in body weight and the reduction in liver iron content

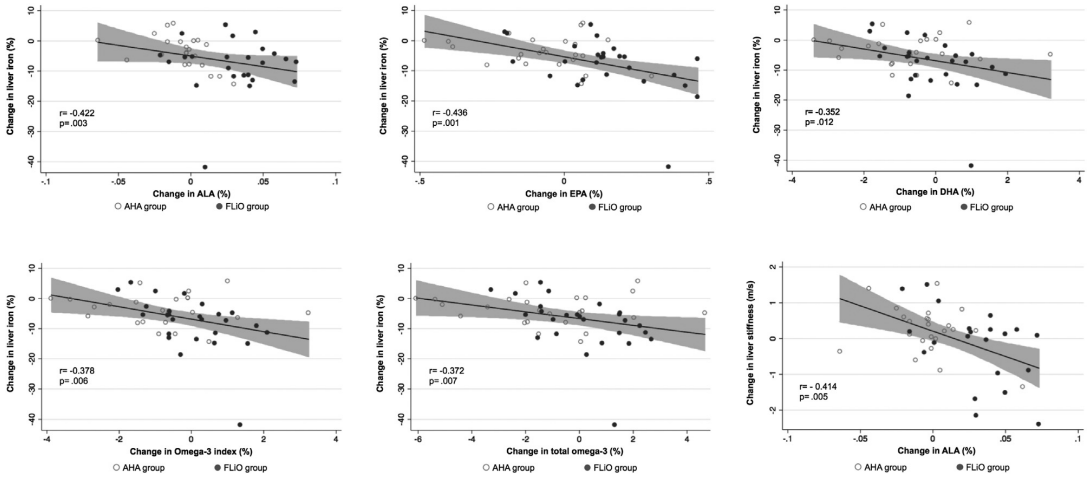

Figure 2 Correlation plots between the change (Δ) in liver parameters and the change (Δ) in the proportion of fatty acids from erythrocyte membranes. AHA, American Heart Association; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FLiO, Fatty Liver in Obesity.

($r = -0.037$, $P = .795$), whereas the change in liver stiffness was positive and significantly associated with the decrease in body weight ($r = 0.400$, $P = .004$).

Multilinear regression analyses (Table 4) showed the negative and significant association between the change in liver stiffness and the change in erythrocyte membrane ALA ($P = .032$), where the Model 2 predicted up to 24.8% of the variation in liver stiffness change (adjusted $R^2 = 0.248$; p-Model = 0.006). The analyses also corroborated the negative and significant association between the change in liver iron content and the change in erythrocyte membrane EPA ($P = .010$; adjusted $R^2 = 0.177$; p-Model = 0.017) after adjustment for body weight change and other confounding variables (Table 5). The presence of type II diabetes mellitus and tobacco use were also considered as two different cofactors in the preliminary data analysis. However, no statistically significant differences were observed when considering these variables. In contrast, the associations between the change in liver iron content and the change in erythrocyte membrane ALA, DHA, and total omega-3 were no longer significant after the adjustments.

In addition, correlation analyses revealed that the change in liver stiffness was positive and significantly associated with erythrocyte membrane total omega-6 PUFA ($r = 0.285$, $P = .049$). However, there were no significant associations of omega-6 with other liver status variables and the previous relationship was attenuated after multiple adjustments in regression models (data not shown).

Discussion

After a 6-month follow-up of participants with overweight/obesity and NAFLD following weight loss diets, improvements in most of the evaluated variables, including hepatic status parameters were observed. Those subjects who achieved a greater weight loss showed a greater decrease in adiposity markers, triglycerides, insulin, and liver stiffness. In addition, significant changes in the proportion of specific omega-3 PUFAs (ALA and DHA) in erythrocyte membranes were observed after the 6 months. The increase in erythrocyte membrane ALA was negatively associated with the change in liver stiffness, whereas the change in EPA was associated with the reduction of liver iron content. However, the changes in liver stiffness and erythrocyte membrane EPA did not reach statistical significance after the 6-month follow-up.

The fatty acid composition of blood, diverse tissues, and cell types (including erythrocytes) can be objectively measured and reflects the interplay between dietary intake of fatty acids and endogenous lipid metabolism, which is strongly genetically regulated.[31–33] Regarding subjects with NAFLD, a modified lipidomic profile has been observed, suggesting a relationship between the change in the concentration and quality of lipids in blood, liver tissue, and cell membranes, and the pathogenesis and progression of steatosis or steatohepatitis.[34,35] A recent meta-analysis reported that the pooled estimate of blood and/or liver DHA content in 10 case–control studies was significantly lower in NAFLD subjects compared with controls, whereas monounsaturated fatty acids and SFAs were significantly inferior in the controls.[34] The existing literature regarding erythrocyte membrane composition in subjects with NAFLD is very limited and reports a wide range of fatty acid proportions, which may depend on genetic and environmental factors, the dietary background of the participants,[6] and/or the stage of the liver disease.[36] It is important to note that most of the trials evaluating the fatty acid composition of

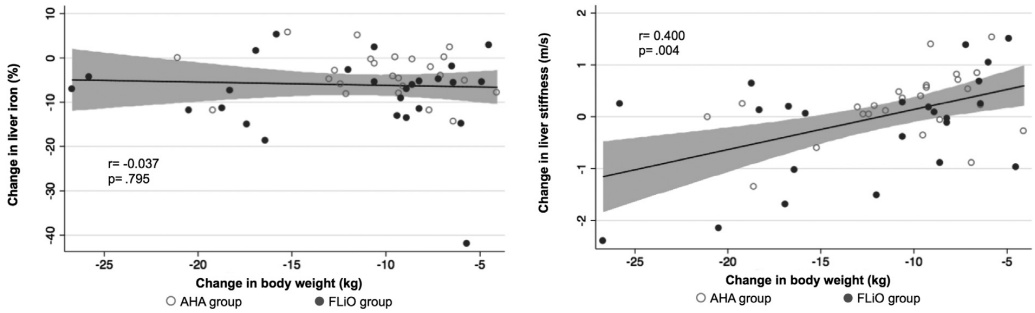

Figure 3 Correlation plots between the change (Δ) in liver parameters and the change (Δ) in body weight. AHA, American Heart Association; FLiO, Fatty Liver in Obesity.

erythrocyte membranes have been carried out in subjects with stable body weight and that the effect of pathological conditions, which may have an impact on fatty acid metabolism, such as diabetes mellitus or hepatic disease,[37] as well as the impact of weight loss dietary treatment on different lipid pools has been insufficiently studied. In this context, the fatty acid composition of erythrocyte membranes of the participants in the present research is in agreement with the findings in older Spanish adults at high cardiovascular risk from the PREDIMED study.[38]

In the present research, a significant increase in ALA and a significant decrease in omega-6 PUFAs, DHA, and Omega-3 Index in erythrocyte membranes were found after the follow-up. The erythrocyte membrane DHA content in healthy persons who do not regularly take DHA supplements has been reported within a range of 1.9% to 7.9%,[39] which is comparable with the findings of the present study both at baseline and after 6 months. Regarding the Omega-3 Index, a cut-off point of ≤4% has been associated with the highest risk of death from coronary heart disease.[40] Nevertheless, the utility of this Index in the prediction of NAFLD remains unclear.[41] Conversely, a previous study in a sample of Polish individuals with NAFLD found that a 6-month dietary treatment increased the proportion of EPA ($P < .055$), DHA ($P < .05$), and Omega-3 Index ($P < .05$) in erythrocyte membranes, whereas no significant change was observed in ALA. The diet was prescribed with 55% to 65% of the total energy requirements from carbohydrates, 20% to 30% from fat mainly of vegetable origin with olive and rapeseed oil as the main sources of fat and without omega-3 PUFA supplementation, and 1.0 g/kg of body mass/24 h of protein.[42] However, the baseline values of DHA and Omega-3 Index in the subjects of such study were under the lower cut-off points reported in the literature.[39,41] Thus, an increase in these biomarkers after following the dietary recommendations could have been expected.

ALA is essential in the human diet and is the precursor for the synthesis of EPA and DHA.[43] The main dietary sources of ALA include chia seeds, flaxseeds, walnuts, rapeseed (canola) oil, soybeans, and its respective oils, whereas the main dietary source of EPA and DHA is fatty fish.[11,44,45] The oxidation rate of dietary ALA ranges from 15% to 35%, whereas only a proportion of <1% is converted to DHA, primarily in the liver.[46] Accordingly, we performed correlation analyses between the change in specific fatty acids of erythrocyte membranes and the change in the dietary intake of fatty acids (Supplementary table 4). The analyses revealed a positive significant association between the change in the consumption of ALA and the change in the proportion of erythrocyte membrane ALA and EPA, whereas the change in the intake of marine omega-3 (EPA + DHA) showed a positive marginal association with the change in erythrocyte membrane DHA.

Table 4 Regression analysis with the change in liver stiffness as the dependent variable and the change in erythrocyte membrane ALA as independent variable

Δ Liver stiffness	Model 1				Δ Liver stiffness	Model 2			
	B	95% CI	*P* value			*B*	95% CI	*P* value	
Δ ALA	−14.3	−22.4; −6.3	.001	*p*-Model	Δ ALA	−10.7	−20.6; −1.0	.032	*p*-Model
Age	0.006	−0.02; 0.03	.617	0.007	Age	0.001	−0.02; 0.03	.926	0.006
Sex	0.243	−1.5; 1.5	.334		Sex	0.127	−0.4; 0.7	.627	
				Adjusted R^2	Dietary group	−0.011	−0.6; 0.6	.969	Adjusted R^2
				0.201	Δ Body weight	0.050	0.002; 0.1	.040	0.248

ALA, α-linolenic acid; CI, confidence interval.
Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, dietary group, and change in body weight. Change between baseline and 6 mo (Δ).

Table 5 Regression analysis with the change in liver iron as the dependent variable and the change in erythrocyte membrane EPA and Omega-3 Index as independent variables

	Model 1					Model 2			
Δ Liver iron	*B*	95% CI	*P* value		Δ Liver iron	*B*	95% CI	*P* value	
Δ EPA	−16.1	−25.9; −6.4	.002	*p*-Model	Δ EPA	−15.0	−26.4; −3.7	.010	*p*-Model
Age	0.112	−0.1; 0.3	.291	0.003	Age	0.123	−0.1; 0.3	.262	0.017
Sex	−0.697	−4.8; 3.4	.736		Sex	−0.313	−4.6; 4.0	.884	
				Adjusted R^2	Dietary group	−0.967	−5.7; 3.8	.683	Adjusted R^2
				0.201	Δ Body weight	−0.148	−0.5; 0.2	.451	0.177
Δ Omega-3 Index	−1.9	−3.4; −0.4	.013	*p*-Model	Δ Omega-3 Index	−1.6	−3.2; −0.2	.047	*p*-Model
Age	0.164	−0.1; 0.4	.130	0.021	Age	0.158	−0.1; 0.4	.159	0.051
Sex	−0.928	−5.2; 3.4	.666		Sex	−0.338	−4.8; 4.1	.879	
				Adjusted R^2	Dietary group	−2.5	−7.1; 1.9	.256	Adjusted R^2
				0.134	Δ Body weight	−0.132	−0.5; 0.3	.515	0.125

EPA, eicosapentaenoic acid; CI, confidence interval.
Model 1: adjusted for age and sex. Model 2: adjusted for age, sex, dietary group, and change in body weight. Change between baseline and 6 months (Δ).

These findings, along with the unexpected decrease of DHA in erythrocyte membranes, suggest that other processes might influence erythrocyte membrane fatty acid composition during weight loss treatment of subjects with NAFLD. Such processes may include the retroconversion of DHA to EPA,[47] the regulation of the transference of fatty acids to the liver,[48] and/or the lipid remodeling of cell membranes[6] and lipid droplets of hepatocytes,[49] which among other biological processes warrant further research. For instance, a lipidomic analysis of murine hepatocytes revealed that lipid droplets from fasted murine phenotype presented an enriched profile in triglyceride molecular species with very long-chain PUFA residues while high-fat diet phenotype presented less unsaturated triglycerides.[50]

Studies in murine models have shown beneficial effects of ALA on NAFLD features.[11,51,52] A research found that the substitution of linoleic acid with ALA or omega-3 long-chain PUFAs prevented steatosis and dyslipidemia by inhibiting lipogenesis and increasing insulin sensitivity in male rats with high-fat high-fructose induced NASH,[51] whereas other assays reported that the supplementation with ALA reduced hepatic steatosis and insulin resistance, which was associated with the modulation of inflammatory and endoplasmic reticulum stress responses.[52] Moreover, a study evaluated the effect of chia seed consumption (known for its high content in ALA) in rats with acute dyslipidemia and steatohepatitis, reporting improvements in biochemical determinations and histological characteristics of NASH.[11] However, in other studies, supplementation with ALA has not shown beneficial effects on the liver.[10]

In the present study, a strong association between the increase in dietary and erythrocyte membrane ALA was found, and the increase in both variables was inversely associated with liver stiffness. Nevertheless, the values of this last variable did not change significantly in the pooled sample or in each dietary group after the 6-month follow-up (although a tendency to a decrease in liver stiffness was observed in the FLiO group, but not in AHA group). Several factors may contribute to these results; notably, the beneficial effects of lifestyle interventions (diet alone or diet with exercise) and weight loss on liver steatosis and transaminases seem consistent in the short-to-medium term.[53,54] However, the effect of these approaches on hepatic fibrosis and surrogate markers such as liver stiffness is less clear. The lack of consistent findings may be explained in part by the reduced duration of the interventions,[55] the different composition of dietary prescriptions,[56] the NAFLD stage of the study participants,[14] and/or the different methods used to determine hepatic fibrosis or liver stiffness. Thus, the nature and possible implications of the association between the changes in ALA and liver stiffness deserves further investigation.

In contrast, excessive iron accumulation has been found in various hepatic diseases and could accelerate the progression of liver fibrosis to cirrhosis and hepatocellular carcinoma by different mechanism regardless of the

etiology.[57] Hepatic iron overload is observed in approximately one-third of patients with NAFLD and has been suggested as a factor that potentially induces inflammation and oxidative stress contributing to the development of NASH, along with the decrease in copper levels.[58,59]

In the present study, liver iron content significantly decreased after the 6-month follow-up and this shift was inversely associated with the change in the proportion of ALA, EPA, DHA, Omega-3 Index, and total omega-3 PUFA in erythrocyte membranes. The association remained significant for EPA after multiple adjustments, and this effect seemed to be independent of weight loss. EPA and DHA in different lipid pools are well-validated markers of habitual oily fish consumption.[60] Interestingly, an association between higher fish consumption and lower ferritin concentrations, a valid indicator of iron status in humans,[61] and a positive association between liver iron content and red and processed meat intake has been reported in individuals with NAFLD.[23] Thus, the inverse association between the increase in EPA and other omega-3 PUFA, and the decrease in liver iron may be related with a higher consumption of fish and lower intake of red meat during the follow-up as suggested by the dietary intake. In addition, there is evidence suggesting that weight loss may help to reestablish iron homeostasis in subjects with overweight or obesity and that the effects are positively associated with the intervention duration.[62] However, the research has been focused on blood biomarkers rather than the hepatic content of iron, thus, the possible association between weight loss and hepatic iron may require a longer follow-up to be manifested. Moreover, weight loss may have differential effects on liver iron content depending on the characteristics of the strategy used to achieve weight reduction.

We also evaluated the potential relationship between the change in total dietary intake of iron and the change in liver iron content, but no statistically significant association was found. The joint effect of several factors participating in iron homeostasis may explain this lack of association. Importantly, the homeostasis of iron is influenced by diet composition, genetics, physiological, and pathophysiological aspects.[63] A variety of dietary factors such as the type of dietary iron (heme and nonheme iron), the presence of enhancers (eg, ascorbic acid), and/or inhibitors of iron absorption (eg, phytates, tannins, and polyphenols) affect the bioavailability of this element.[64] Moreover, NAFLD itself, inflammation processes, and obesity may have an impact on iron metabolism and storage,[65] and a cross-talk between iron and lipid metabolism has been described in different diseases.[66] For instance, a study in male Wistar rats showed that an iron-rich diet-induced oxidative stress and a reduction in the hepatic desaturation capacity of fatty acids, with depletion of long-chain PUFA in different tissues.[67] However, other researchers have suggested that iron is less hepatotoxic than is generally assumed and that the claims that assign a causal role to iron overload in liver injury should be carefully considered.[68] Accordingly, we did not find a significant association between liver iron and liver stiffness.

Overall, the characteristics of the weight loss diets beyond the total iron intake, and the pathophysiological condition of the study participants may have conjointly influenced the change observed in liver iron content and the association with other outcomes. Therefore, the dietary and pathophysiological mechanisms that may be involved in the potential relationship between omega-3 PUFAs, weight loss, iron accumulation, and liver stiffness in subjects with NAFLD deserve other specifically designed investigation, but this research opens the way to understand the beneficial role of fatty acids in recovery from liver damage.

A loss of at least 3% to 5% of body weight is recommended to improve hepatic steatosis, whereas a greater weight loss (7%–10%) may be needed to improve fibrosis and most histopathological features of NASH.[20] The results of this study support the evidence regarding the importance of weight loss in the improvement of hepatic status variables, such as liver stiffness, and other clinical and biochemical determinations in subjects with overweight or obesity and NAFLD. Moreover, the importance of weight loss achievement by means of a well-designed dietary strategy was manifested in this trial. Notably, improvements in the quality of diet could also affect the clinical evolution of NAFLD, even without caloric restriction.[69] Thus, omega-3 PUFA intake from food sources appears as a central element to carefully consider in the design of dietary strategies for NAFLD management, which may contribute to the regression or attenuation of the progression of liver fibrosis.

To the best of our knowledge, this is the first study with a dietary approach in NAFLD participants that reports a consistent association between the change in specific PUFA of omega-3 series in erythrocyte membranes and the change in liver stiffness and hepatic iron content without fatty acid supplementation, under an energy-restricted dietary prescription with a healthy fatty acid profile. However, some limitations should be mentioned. First, liver status was evaluated by noninvasive techniques instead of liver biopsy and histopathologic assessment. Nevertheless, magnetic resonance imaging has been found to be highly accurate and sensitive to changes in liver fat content.[27] Similarly, liver stiffness is a surrogate marker that has shown a good correlation with liver fibrosis[70] and can be measured using different noninvasive techniques with a comparable diagnostic performance for staging fibrosis in patients with NAFLD, including ARFI, transient, and supersonic shear imaging elastography.[71] Second, the fatty acid composition of erythrocyte membranes does not necessarily indicate the concentration of fatty acids in the hepatic structure as well as in adipose tissue or other lipid pools. Nonetheless, the fatty acid composition of erythrocyte membranes seems to be a sensitive proxy of dietary intake and appears to be associated with the liver response to dietary treatment. Third, the sample size was relatively small given that the participants of this research were a

subsample from the FLiO study. However, important statistical associations were found. Also, despite that type I and II errors cannot be discarded, the outcomes are plausible and in concordance with comparable scientific data. Finally, as there were no significant effects on the pooled sample of the 2 weight loss diets on liver stiffness after the 6-month follow-up, the current findings would warrant further well-designed clinical studies to specifically evaluate the effects of ALA on liver health markers in subjects with NAFLD.

Conclusions

The adherence to personalized weight loss strategies for 6 months led to changes in erythrocyte membrane omega-3 PUFA composition, which in turn were associated with changes in hepatic markers, suggesting that these fatty acids accompany the improvements in the liver during a dietary treatment. These findings show that beyond weight loss, the composition of the diet has an important role in the management of NAFLD.

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Appendix

Supplementary Table 1 Anthropometrical, biochemical and hepatic features at baseline and after 6 mo of follow-up in subjects with NAFLD according to the group of intervention

	AHA (n = 25)		FLiO (n = 29)		
n = 54	Baseline	6 mo	Baseline	6 mo	Δ *P* value†
Anthropometry and body composition					
Weight (kg)	95.8 (14.8)	85.3 (14.0)***	95.9 (12.0)	84.0 (10.6)***	.850
BMI (kg/m^2)	33.8 (4.3)	30.1 (4.3)***	33.5 (3.6)	29.4 (3.3)***	.792
Waist circumference (cm)	111.3 (10.0)	100.1 (11.1)***	108.6 (7.4)	97.3 (7.4)***	.938
Total fat mass (%)	42.2 (4.9)	36.1 (6.3)***	42.4 (6.9)	37.0 (8.3)***	.979
Biochemical parameters					
Glucose (mg/dL)	110 (21)	96 (16)***	100 (16)	92 (11)***	.149‡
Insulin (mU/L)	18.5 (8.1)	11.3 (6.8)***	16.5 (7.6)	10.0 (6.8)***	.699
HOMA-IR	5.1 (2.5)	2.8 (2.2)***	4.2 (2.2)	2.4 (1.8)***	.499
Total cholesterol (mg/dL)	190 (47)	177 (43)**	197 (33)	176 (34)***	.246
HDL cholesterol (mg/dL)	50.0 (15.8)	52.0 (13.6)	53.5 (11.6)	55.7 (13.1)	.945
LDL cholesterol (mg/dL)	111 (43)	102 (39)	118 (26)	104 (28)**	.416
Triglycerides (mg/dL)	141 (69)	101 (36)***	133 (62)	79 (39)***	.336
Hepatic status					
AST (IU/L)	27.6 (11.4)	22.0 (6.7)**	23.9 (9.3)	21.0 (9.2)	.989‡
ALT (IU/L)	35.2 (11.6)	22.9 (8.5)***	32.2 (18.9)	19.7 (7.4)***	.114
GGT (IU/L)	50.5 (34.4)	30.3 (24.7)**	32.7 (24.8)	18.0 (9.8)***	.158‡
Liver fat (%)	8.3 (6.1)	3.4 (2.7)***	5.6 (3.6)	1.8 (1.3)***	.741
Liver iron (%)	37.5 (10.2)	33.9 (10.9)**	34.6 (11.3)	27.2 (7.4)***	.011‡
Liver stiffness (m/s)	1.9 (0.7)	2.0 (0.6)	1.8 (0.8)	1.6 (0.5)	.092

AHA, American Heart Association; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FLiO, Fatty Liver in Obesity; GGT, gamma-glutamyltransferase; HDL, high-density lipoprotein; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; LDL, low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; SD, standard deviation.

Data are presented as the mean (SD).

*$P < .05$, **$P < .01$ and ***$P < .001$ for the comparison between baseline and 6-month values within each group.

†Comparison of changes (baseline and 6 mo) between the AHA and FLiO groups.

‡Adjusted for baseline value.

Supplementary Table 2 Dietary intake at baseline and after 6 mo of follow-up in subjects with NAFLD according to the group of intervention

	AHA (n = 25)		FLiO (n = 29)		
n = 54	Baseline	6 mo	Baseline	6 mo	Δ *P* value†
Total energy (kcal/d)	2832 (904)	2084 (539)**	2782 (1173)	1810 (646)***	.649
Carbohydrates (% TEV)	43.0 (8.2)	46.1 (6.9)	42.6 (7.2)	40.0 (7.1)	.038
Proteins (% TEV)	16.9 (3.3)	18.3 (3.6)	16.5 (2.9)	22.2 (3.8)***	.002
Lipids (% TEV)	37.5 (7.7)	34.0 (6.1)	37.7 (7.4)	34.6 (8.1)	.850
MUFA (% TEV)	18.6 (5.6)	17.5 (4.2)	17.9 (4.3)	14.9 (5.4)*	.263
PUFA (% TEV)	4.9 (1.2)	4.6 (1.4)	5.6 (2.1)	8.5 (4.1)**	.002
SFA (% TEV)	10.9 (2.6)	9.7 (2.3)	10.4 (2.4)	8.4 (3.9)**	.152
ALA (g/1000 kcal/d)	0.50 (0.2)	0.44 (0.2)	0.55 (0.3)	1.18 (0.8)***	<.001
Marine omega-3 (g/1000 kcal/d)	0.23 (0.1)	0.32 (0.2)	0.23 (0.2)	0.40 (0.3)*	.404
Omega-6 (g/d)	12.6 (6.1)	7.8 (3.3)***	14.2 (7.9)	13.9 (12.0)	.140
Iron intake (mg/d)	19.6 (2.0)	16.8 (1.0)	18.5 (1.1)	15.2 (0.8)**	.256

AHA, American Heart Association; ALA, α-linolenic acid; FLiO, Fatty Liver in Obesity; MUFA, monounsaturated fatty acids; NAFLD, nonalcoholic fatty liver disease; PUFA, polyunsaturated fatty acids; SD, standard deviation; SFA, saturated fatty acids; TEV, total energy value.

Data are presented as the mean (SD).

*$P < .05$, **$P < .01$ and ***$P < .001$ for the comparison between baseline and 6-mo values within each group.

†Comparison of the changes (baseline and 6 mo) between the AHA and FLiO groups.

Supplementary Table 3 Fatty acid composition of erythrocyte membranes (%) at baseline and after 6 mo of follow-up in subjects with NAFLD according to the group of intervention

	AHA group (n = 25)		FLiO group (n = 29)		
n = 54	Baseline	6 mo	Baseline	6 mo	Δ *P* value†
Omega-3 PUFA					
18:3n3 (ALA)	0.06 (0.02)	0.05 (0.01)	0.05 (0.01)	0.08 (0.03)***	<.001
20:5n3 (EPA)	0.5 (0.2)	0.4 (0.2)	0.5 (0.2)	0.6 (0.2)**	<.001
22:6n3 (DHA)	4.8 (1.4)	4.1 (1.3)*	5.0 (1.2)	4.8 (0.7)	.121
Omega-3 Index (EPA + DHA)	5.3 (1.5)	4.5 (1.4)*	5.5 (1.4)	5.4 (0.8)	.052
Total MUFA	18.1 (2.1)	18.8 (2.3)	18.0 (1.4)	18.4 (1.2)	.637
Total SFA	38.9 (2.5)	41.7 (3.3)**	39.0 (2.4)	40.8 (1.2)**	.471
Total PUFA	41.4 (3.6)	38.0 (4.7)**	41.6 (3.3)	39.5 (1.8)**	.367
Total omega-3	11.5 (2.0)	10.3 (2.1)*	11.5 (2.1)	11.4 (1.2)	.066
Total omega-6	29.7 (2.6)	27.4 (3.5)***	29.8 (2.4)	27.9 (2.0)***	.869

AHA, American Heart Association; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FLiO, Fatty Liver in Obesity; MUFA, monounsaturated fatty acids; NAFLD, nonalcoholic fatty liver disease; PUFA, polyunsaturated fatty acids; SD, standard deviation; SFA, saturated fatty acids.

Data presented as mean (SD).

*$P < .05$, **$P < .01$ and ***$P < .001$ for the comparison between baseline and 6-mo values within each group.

†Comparison of the changes (baseline and 6 mo) between the AHA and FLiO groups.

Table S4 Correlation analyses between the change in specific fatty acids in erythrocyte membranes and the change in the consumption of fatty acids as evaluated with the FFQ

Δ Erythrocyte membrane fatty acids (%)	Δ FFQ	*rho*	*P* value
ALA	ALA (g/1000 kcal/d)	0.723	<.001
EPA	ALA (g/1000 kcal/d)	0.333	.026
	Marin Omega-3 (g/1000 kcal/d)	0.138	.370
DHA	ALA (g/1000 kcal/d)	0.127	.409
	Marin Omega-3 (g/1000 kcal/d)	0.280	.065
Total Omega-3	Σ ALA + Marine Omega-3 (g/1000 kcal/d)	0.195	.203

ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire.

Change between baseline and 6 months (Δ).

CHAPTER 4

Preserved improvements on depressive and anxiety symptoms in subjects with nonalcoholic fatty liver disease during a 24-month energy-restricted dietary intervention

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CHAPTER 5

Association between sleep disturbances and liver status in obese subjects with nonalcoholic fatty liver disease: A comparison with healthy controls

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Association between Sleep Disturbances and Liver Status in Obese Subjects with Nonalcoholic Fatty Liver Disease: A Comparison with Healthy Controls

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Abstract: The relevance of sleep patterns in the onset or evolution of nonalcoholic fatty liver disease (NAFLD) is still poorly understood. Our aim was to investigate the association between sleep characteristics and hepatic status indicators in obese people with NAFLD compared to normal weight non-NAFLD controls. Ninety-four overweight or obese patients with NAFLD and 40 non-NAFLD normal weight controls assessed by abdominal ultrasonography were enrolled. Hepatic status evaluation considered liver stiffness determined by Acoustic Radiation Force Impulse elastography (ARFI) and transaminases. Additionally, anthropometric measurements, clinical characteristics, and biochemical profiles were determined. Sleep features were evaluated using the Pittsburgh Sleep Quality Index (PSQI). Hepatic status parameters, anthropometric measurements, and clinical and biochemical markers differed significantly in NAFLD subjects compared to controls, as well as sleep efficiency, sleep disturbance score, and sleep quality score. In the NAFLD group, a higher prevalence of short sleep duration ($p = 0.005$) and poor sleep quality ($p = 0.041$) were found. Multivariate-adjusted odds ratio (95% confidence interval) for NAFLD considering sleep disturbance was 1.59 (1.11–2.28). Regression models that included either sleep disturbance or sleep quality predicted up to 20.3% and 20.4% of the variability of liver stiffness, respectively, and after adjusting for potential confounders.

Current findings suggest that sleep disruption may be contributing to the pathogenesis of NAFLD as well as the alteration of the liver may be affecting sleep patterns. Consequently, sleep characteristics may be added to the list of modifiable behaviors to consider in health promotion strategies and in the prevention and management of NAFLD.

Keywords: Obesity; NAFLD; sleep; sleep duration; sleep disruption; Pittsburgh Sleep Quality Index

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent cause of hepatic disease around the world, which will putatively emerge as the most important cause of end-stage liver disease in the next decades [1]. NAFLD is described as the excessive accumulation of hepatic fat in the absence of history of alcohol abuse or other causes of secondary hepatic steatosis [2,3]. Multiple factors are involved in the complex pathogenesis of NAFLD, such as obesity, lipotoxicity, inflammation, unbalanced dietary intake, low physical activity, gut microbiota, and socioeconomic aspects, which might contribute to the development of this burden, along with genetic predisposition [1,4–7]. Currently, it is suggested that the link between NAFLD, metabolic syndrome and its individual components, including hypertension, type 2 diabetes mellitus, and cardiovascular disease, is more complex than previously believed and that NAFLD may be both a cause and a consequence of the metabolic syndrome [8]. Additionally, NAFLD can potentially lead from simple steatosis to the development of nonalcoholic steatohepatitis (NASH), a condition that often progresses to fibrosis, cirrhosis, and hepatocellular carcinoma, although these entities may often follow an asymptomatic course [1,9,10].

On the other hand, inadequate sleep has been associated with poor health outcomes [11], such as obesity [12], type 2 diabetes mellitus [12,13], cardiometabolic diseases [14], and all-cause mortality [11], as well as to the risk [15] and progression [16] of NAFLD. Sleep disruption has been reported to modify feeding behaviors and timing of food intake, to promote obesity, and to alter insulin sensitivity in adipose tissues in both humans and murine models [16–18]. Moreover, it is proposed that these metabolic alterations related to disrupted sleep patterns may be partially mediated by changes in gut microbiota [17,19]. Regarding the association of sleep duration and NAFLD, conflicting and often inconsistent results have been reported [15,20–22]. Thus, further investigation is needed to clarify this relationship.

Furthermore, specific characteristics concerning the sleep pattern analysis, such as time to fall asleep or daytime sleepiness, might have a relevant role in the onset and evolution of NAFLD [16]. However, it is unclear if sleep disruption is a cause or a consequence of the liver dysfunction in NAFLD patients.

Currently, the association of the characteristics of sleep patterns with the onset and progression of liver steatosis are still inconclusive and poorly understood. Moreover, the number of studies which investigate sleep features other than sleep duration remain scarce. Therefore, the aim of this study was to evaluate the relationships between sleep quality and hepatic status in obese patients with NAFLD compared to normal weight non-NAFLD controls considering sleep duration, sleep efficiency, sleep disturbance, and overall sleep quality scores.

2. Materials and Methods

2.1. Subjects

Overweight or obese (Body Mass Index (BMI) $\geq$ 25 kg/m^2) participants with NAFLD were included in the study. A total of 40 healthy normal weight (BMI < 25.0 kg/m^2) participants were also recruited as a control group. Patients with NAFLD were participants of the Fatty Liver in Obesity (FLiO) study (evaluated at baseline) who accurately completed the sleep questionnaires. The presence

of hepatic steatosis was determined by abdominal ultrasonography (Siemens ACUSON S2000 and S3000) in the absence of other causes of liver disease reported by the subjects in a face to face clinical interview. These causes include excessive alcohol consumption, hepatitis C, parenteral nutrition, medications, and Wilson's disease, among others [3]. The ultrasonography assessment consisted of a qualitative visual evaluation of liver echogenicity, measurements of the difference between the kidneys and the liver in the echo amplitude, and determination of the clarity of the structures of the intrahepatic vessels [23,24]. All the ultrasound examinations were performed and evaluated by the same qualified radiologist at the department of Ultrasonography and Radiology of the University of Navarra. Exclusion criteria included the following conditions: Known liver disease other than NAFLD, weight loss $\geq$ 3kg in the last 3 months, alcohol consumption > 21 and > 14 units of alcohol a week for men and women, respectively [25], endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), drug treatment with immunosuppressants, cytotoxic agents, systemic corticosteroids, or other drugs that could potentially cause hepatic steatosis or alteration of liver tests [3], presence of active autoimmune diseases or requiring pharmacological treatment, use of weight modifiers, and presence of severe psychiatric disorders. This information was declared by the subjects in the clinical interview before their enrollment in the study. All the procedures performed in the study were in accordance with the Declaration of Helsinki. Written informed consent was obtained from each individual prior to participation in the study, which was approved by the Research Ethics Committee of the University of Navarra (ref. 54/2015). The study protocol was properly registered in www.clinicaltrails.gov (FLiO: Fatty Liver in Obesity study; NCT03183193).

2.2. Anthropometric and Biochemical Measurements

Anthropometric measurements (body weight, height and waist circumference), body composition by dual-energy x-ray absorptiometry (DXA, Lunar iDXA, encore 14.5, Madison, WI, USA), and blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, The Netherlands) were determined in fasting conditions under previously described procedures [26] at the Metabolic Unit of the University of Navarra. Fasting blood samples were collected, processed (15 min; 3500 rpm; 5 °C), and stored at −80 °C until the analyses were performed. Blood glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), and triglycerides (TG) concentrations were determined by means of an autoanalyzer (Pentra C-200; HORIBA ABX, Madrid, Spain) following standardized procedures. Insulin, C-reactive protein (CRP), leptin, and adiponectin concentrations were determined using specific Enzyme-Linked ImmunoSorbent Assay (ELISA) kits (Demeditec; Kiel-Wellsee, Germany) in another autoanalyzer (Triturus; Grifols, Barcelona, Spain). Hepatic Steatosis Index (HSI) was calculated using the following formula [27]: (HSI) = 8 × (ALT/AST ratio) ± BMI (± 2, if female; ±2, if diabetes mellitus). HSI values < 30 rule out NAFLD with a sensitivity of 93.1%, while values > 36 detect NAFLD with a specificity of 92.4% [27]. Smoking status categorized participants as smokers or non-smokers. Those who reported as smoking at least sporadically during the last year were considered as smokers while those who reported as having stopped smoking at least one year before their enrolment in the study and those who had not smoked during their life were defined as non-smokers. The validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire was used to assess physical activity [28].

2.3. Liver Stiffness Assessment

Acoustic Radiation Force Impulse (ARFI) elastography (Siemens ACUSON S2000 and S3000) was the method used to assess liver stiffness [29]. The measurements were taken under fasting conditions and ARFI was carried out along with the ultrasonography when assessing liver steatosis. The median value of the 10 ARFI valid measurements that were performed in each patient was registered and used for further analyses [9].

2.4. Sleep Quality Assessment

Sleep characteristics (sleep duration, sleep efficiency, total time in bed, sleep disturbances and sleep quality) were assessed in both groups using the validated Spanish version of the Pittsburgh Sleep Quality Index (PSQI) as reported elsewhere [30]. This tool has shown a strong reliability and validity and has been used in a wide variety of samples [31–33]. The PSQI consists of 19 self-administered questions that generate 7 component scores with subscales ranging from 0 to 3. The sum of these components scores leads to a global score ranging from 0 to 21, for the assessment of sleep quality. A punctuation of more than 5 in the global score identifies "poor sleepers", while a punctuation of 5 or lower identifies "good sleepers" [34]. Thus, poorer sleep quality is characterized by higher scores [30,35]. Short sleep duration was defined as a self-reported sleep time $\leq$ 6 h per night [15,22,36].

2.5. Dietary Assessment

The diet of the participants was assessed with a semiquantitative food frequency questionnaire (FFQ) of 137 items, previously validated in Spain for energy and nutrient intake. The nutrient composition of the food items was derived from accepted Spanish food composition tables [37,38].

2.6. Statistical Analyses

Variable distribution was assessed by means of the Shapiro-Wilk test. Data were presented as mean $\pm$ standard deviation or mean $\pm$ standard error. Groups were compared by the Student's *t*-test for unpaired samples when data followed a normal distribution and the Mann-Whitney U test when data did not show a normal distribution. Comparison between groups was adjusted by BMI using Analysis of covariance (ANCOVA). Categorical variables were compared using the Chi-squared test. To evaluate the relationship between variables, the Pearson's correlation coefficient or the Spearman's rho were performed for parametric and non-parametric variables, respectively. Logistic regression models were set up to evaluate the risk of NAFLD (dependent variable) associated with sleep quality variables (independent variables). A linear regression analysis was performed to assess the influence of sleep characteristics in the variability of liver stiffness measured by ARFI. Multiple variable linear regression models were adjusted for potential confounders considering age, sex, physical activity, smoking status, and others when indicated. Software Stata version 12.0 (StataCorp, College Station, TX, USA) was used for the analyses. A p-value < 0.05 was considered statistically significant and all p-values presented were two-tailed.

3. Results

3.1. Characteristics of the Participants

A total of 94 patients with NAFLD and 40 normal weight controls were included in the study, whose main anthropometrical, clinical, and biochemical features are reported (Table 1). NAFLD patients were older than controls and the sex distribution was different between groups with more women in the control group than in the NAFLD group. Therefore, further analyses were adjusted by age and sex when required. Anthropometric and body composition measurements were significantly higher in NAFLD participants than in controls. Regarding lipid profile, HDL cholesterol was significantly lower, and triglycerides were significantly higher in NAFLD patients as compared to the control group. There were significant differences in glycemic profile (glucose, insulin, and HOMA-IR) and other metabolic parameters, such as leptin, ALT, AST, and ARFI values, in the NAFLD group compared to the control group. Adiponectin concentrations were significantly higher in the control group as compared to the NAFLD group.

Table 1. Anthropometry, clinical features, and biochemical parameters of participants with ultrasound diagnosed NAFLD (n = 94) and controls (n = 40).

	Controls Mean ± SD	NAFLD Mean ± SD	p	Controls # Mean ± SE	NAFLD # Mean ± SE	p #
Age (years)	41.5 ± 9.8	51.4 ± 8.9	<0.001	39.4 ± 9.8	51.4 ± 8.9	<0.001
Weight (kg)	62.1 ± 10.0	94.6 ± 13.6	<0.001	-	-	-
BMI (kg/m^2)	22.1 ± 1.8	33.7 ± 3.9	<0.001	-	-	-
Sex (men/women)	14/26	53/41	0.023	-	-	-
SBP (mmHg)	108 ± 17	132 ± 15	<0.001	114 ± 4	129 ± 2	0.005
DBP (mmHg)	69 ± 10	87 ± 9	<0.001	72.8 ± 2.3	85.3 ± 8	<0.001
Waist circumference (cm)	75.6 ± 7.3	109.0 ± 8.8	<0.001	90.7 ± 1.5	102.7 ± 0.8	<0.001
DXA Total adipose tissue (%)	26.8 ± 7.6	42.3 ± 6.5	<0.001	33.4 ± 1.7	39.6 ± 0.9	0.006
DXA Visceral fat mass (kg)	0.2 ± 0.21	2.3 ± 1.08	<0.001	0.3 ± 0.25	2.3 ± 0.13	<0.001
Total cholesterol (mg/dL)	191.5 ± 30.6	192.4 ± 39.5	0.906	183.7 ± 9.7	196.2 ± 5.0	0.344
HDL cholesterol (mg/dL)	63.1 ± 11.7	51.8 ± 14.3	<0.001	56.5 ± 3.5	54.5 ± 1.8	0.682
LDL cholesterol (mg/dL)	114.7 ± 26.9	113.8 ± 35.7	0.896	114.7 ± 26.9	113.8 ± 35.7	0.607
Triglycerides (mg/dL)	68.8 ± 40.4	135.4 ± 77.9	<0.001	86.0 ± 18.1	128.1 ± 9.4	0.086
Fasting glucose (mg/dL)	85.4 ± 6.6	106.4 ± 31.1	<0.001	104.3 ± 6.6	98.5 ± 3.5	0.511
Insulin (mU/L)	4.3 ± 2.0	18.6 ± 10.7	<0.001	12.2 ± 2.2	15.3 ± 1.1	0.312
HOMA-IR	0.9 ± 0.5	5.1 ± 4.8	<0.001	4.7 ± 1.5	3.6 ± 0.5	0.416
Leptin (ng/mL)	10.0 ± 8.0	40.1 ± 33.5	<0.001	43.7 ± 6.5	25.7 ± 3.4	0.043
Adiponectin (µg/mL)	13.5 ± 4.7	6.8 ± 2.3	<0.001	11.9 ± 0.8	7.5 ± 4.2	<0.001
C-reactive protein (mg/dL)	0.47 ± 0.6	0.45 ± 0.6	0.853	0.72 ± 0.2	0.34 ± 0.1	0.061
AST (IU/L)	21.4 ± 6.4	24.5 ± 9.9	0.035	19.9 ± 2.4	25.3 ± 1.2	0.094
ALT (IU/L)	17.2 ± 13.5	33.7 ± 18.2	<0.001	17.9 ± 4.5	33.5 ± 2.3	0.010
Hepatic Steatosis Index (HSI)	29.6 ± 3.1	45.4 ± 45.4	<0.001	-	-	-
ARFI liver stiffness (m/s)	1.34 ± 0.2	1.86 ± 0.7	<0.001	1.82 ± 0.1	1.65 ± 0.8	0.396

Nonalcoholic fatty liver disease (NAFLD); Body Mass Index (BMI); Systolic blood pressure (SBP); Diastolic blood pressure (DBP); Homeostasis model assessment of insulin resistance (HOMA-IR); Asparate aminotransferase (AST); Alanine aminotransferase (ALT); Acoustic radiation force impulse (ARFI); # Adjusted by BMI.

Due to the differences in BMI between the two groups, an analysis adjusted by BMI was carried out to corroborate that the discrepancies observed in the parameters could be attributed to the presence or absence of hepatic steatosis (Table 1). After this analysis, only the differences in blood pressure, anthropometric measurements, leptin, adiponectin, and ALT were significant.

Regarding dietary intake and lifestyle factors (Table 2), there were no significant differences between groups in total energy consumption (p = 0.906) and macronutrient distribution (carbohydrates, p = 0.922; proteins, p = 0.212; lipids, p = 0.159). Nevertheless, the total ingestion of dietary fiber (p = 0.001) and vegetables (p < 0.001) were higher among the controls, while the intake of meat products (p = 0.019) was higher in the NAFLD group. Notably, physical activity (p < 0.001), was significantly lower in the NAFLD subjects, compared to the controls. Finally, there was no significant difference in smoking status (p = 0.080) between groups.

Sleep characteristics of patients with NAFLD compared to normal weight controls are shown (Figure 1a). When classifying sleep duration as ≤6 h or >6 h, short sleep duration was more prevalent in the NAFLD group than in controls (51% vs. 25%; p = 0.005). Moreover, sleep disturbance scores and sleep quality scores (total PSQI score) were significantly higher in patients with NAFLD, and when differentiating those with total PSQI scores of 5 or less ("good sleepers") from those with scores > 5 ("poor sleepers"), poor sleep was more frequent in NAFLD patients than in controls (54% vs. 35%; p = 0.041). Sleep efficiency was significantly lower in the NAFLD group compared to the control group (p = 0.028). After the analysis was adjusted by BMI when the data were considered as continuous variables, there were no differences between groups.

Nutrients **2019**, *11*, 322

Table 2. Daily nutrient intake and lifestyle factors of participants with ultrasound diagnosed NAFLD (n = 94) and controls (n = 40).

	Controls Mean ± SD	NAFLD Mean ± SD	p
Energy and macronutrients			
Total energy (kcal)	2677 ± 749	2697 ± 1089	0.906
Carbohydrates (%)	43 ± 6.6	43 ± 7.0	0.922
Proteins (%)	16 ± 3.2	17 ± 3.8	0.212
Lipids (%)	39 ± 5.5	37 ± 7.0	0.159
Dietary fiber (g)	33 ± 16	25 ± 9	0.001
Food groups			
Fruit (g)	345 ± 179	290 ± 197	0.141
Vegetables (g)	431 ± 236	285 ± 120	<0.001
Legumes (g)	21 ± 14	21 ± 10	0.738
Fish (g)	99 ± 46	88 ± 45	0.232
Meat products (g)	154 ± 80	190 ± 78	0.019
Micronutrients			
Vitamin A (µg)	1526 ± 650	1119 ± 893	0.014
Vitamin C (mg)	250 ± 76	192 ± 98	0.001
Vitamin D (µg)	8 ± 3.8	6 ± 4.0	0.041
Vitamin E (mg)	12 ± 4.1	10 ± 4.3	0.017
Vitamin B9 (µg)	445 ± 132	360 ± 151	0.003
Marine Omega-3 (g)	0.90 ± 0.5	0.62 ± 0.5	0.003
Lifestyle factors			
Physical Activity (METs-min/week)	5801 ± 4225	3049 ± 2440	<0.001
Smokers (%)	35.0	20.7	0.080

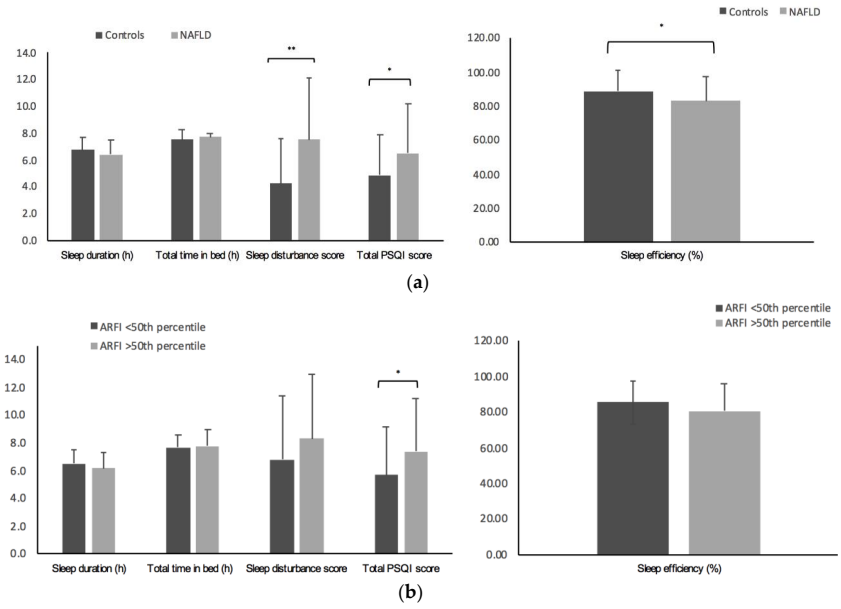

Figure 1. (**a**) Sleep characteristics of patients with NAFLD (n = 94) and normal weight controls (n = 40). (**b**) Sleep characteristics of patients with NAFLD and liver stiffness < 50th percentile (n = 46) vs. liver stiffness > 50th percentile (n = 46). Liver stiffness assessed by Acoustic Radiation Force Impulse (ARFI) elastography. Data expressed as mean ± SD CI95%, * $p < 0.05$, ** $p < 0.01$.

Table 4. Regression analysis of sleep characteristics and liver stiffness assessed by ARFI in patients with NAFLD.

		β (95% IC)	*p*	Adjusted R^2	*p* Model
Sleep duration ≤6 h or >6 h	Model 1	−0.30 (−0.57; −0.02)	0.034		
	Model 2	−0.28 (−0.56; −0.002)	0.048	0.028	0.135
	Model 3	−0.25 (−0.54; 0.02)	0.078	0.099	0.022
	Model 4	−0.22 (−0.53; 0.08)	0.154	0.058	0.123
	Model 5	−0.27 (−0.55; 0.01)	0.059	0.188	0.002
	Model 6	−0.22 (−0.50; 0.06)	0.114	0.205	<0.001
Sleep Efficiency	Model 1	−0.01 (−0.02; −0.0002)	0.045		
	Model 2	−0.009 (−0.01; 0.0005)	0.063	0.023	0.165
	Model 3	−0.008 (−0.01; 0.001)	0.087	0.097	0.023
	Model 4	−0.008 (−0.02; 0.002)	0.131	0.061	0.114
	Model 5	0.008 (−0.01; 0.0008)	0.075	0.184	0.002
	Model 6	−0.005 (−0.01; 0.004)	0.248	0.195	0.001
Total time in bed	Model 1	0.02 (−0.11; 0.15)	0.750		
	Model 2	0.01 (−0.12; 0.15)	0.805	−0.014	0.646
	Model 3	0.002 (−0.13; 0.13)	0.974	0.064	0.072
	Model 4	−0.01 (−0.16; 0.13)	0.863	0.031	0.233
	Model 5	−0.01 (−0.14; 0.11)	0.868	0.150	0.007
	Model 6	−0.02 (−0.14; 0.10)	0.781	0.182	0.002
Sleep disturbance score	Model 1	0.03 (0.001; 0.06)	0.037		
	Model 2	0.02 (−0.001; 0.06)	0.064	0.023	0.166
	Model 3	0.02 (−0.002; 0.05)	0.069	0.102	0.020
	Model 4	0.04 (0.005; 0.07)	0.024	0.097	0.042
	Model 5	0.04 (0.005; 0.07)	0.024	0.203	0.001
	Model 6	0.03 (0.004; 0.07)	0.081	0.212	<0.001
Sleep quality (Total PSQI score)	Model 1	0.04 (0.004; 0.07)	0.029		
	Model 2	0.03 (−0.0002; 0.07)	0.051	0.027	0.142
	Model 3	0.04 (0.006; 0.08)	0.022	0.123	0.009
	Model 4	0.04 (0.002; 0.09)	0.039	0.086	0.057
	Model 5	0.04 (0.006; 0.08)	0.023	0.204	0.001
	Model 6	0.03 (−0.005; 0.07)	0.085	0.211	<0.001

Model 1: unadjusted variable. Model 2: adjusted for age and sex. Model 3: adjusted for age, sex, smoking, fat mass (%), physical activity (METs). Model 4: adjusted for age, sex, smoking, fat mass (%), physical activity (METs) and total energy intake (kcal/day). Model 5: adjusted for age, sex, smoking, fat mass (%), physical activity (METs) and coughing or snoring. Model 6: adjusted for age, sex, smoking, BMI, physical activity (METs) and coughing or snoring.

3.4. Correlation of Sleep Characteristics and Variables Related to Hepatic Status (S1)

The relationship between sleep characteristics and the variables related to hepatic status in NAFLD patients and lean controls was separately analyzed in both groups. ARFI values were inversely correlated with sleep duration and sleep efficiency and positively associated to sleep disturbance and sleep quality (total PSQI score). These associations were only observed in the NAFLD group. When analyzing transaminases, no significant associations were found in the NAFLD group, while in the control group, ALT levels were inversely associated to sleep duration and AST concentration was positively associated to total PSQI score. The BMI was inversely associated to sleep duration and sleep efficiency in the NAFLD group. Android fat mass was also inversely associated with sleep duration in NAFLD subjects while no significant association was observed in the control group. Adiponectin concentration was positively associated with sleep duration in the NAFLD group and with the sleep disturbance score in the control group.

4. Discussion

There are well known risk factors for NAFLD, including obesity, type 2 diabetes mellitus, and metabolic syndrome [3]. However, this condition is influenced by multiple aspects, including genetic, demographic, clinical, and environmental determinants [39]. In this context, the evaluation of the possible influence of other putative factors is gaining relevance. Thus, in the present study, we analyzed the association between sleep disturbances and liver status in obese subjects with NAFLD compared with lean controls. We found that hepatic status, anthropometric measurements, and clinical and biochemical markers as well as some dietary components significantly differed in NAFLD subjects compared to the control group. Furthermore, sleep efficiency, sleep disturbance, and sleep quality differed between groups too. Moreover, a higher prevalence of short sleep duration and a poorer sleep quality were found among subjects with NAFLD. This significance was more robust in relation to short sleep duration than to sleep quality. Additionally, sleep disturbance was associated with a higher risk of NAFLD. Interestingly, sleep quality and sleep disturbance predicted the variability of liver stiffness after adjustment by potential confounders in NAFLD subjects.

Our results corroborate that blood pressure, anthropometric measurements, leptin, adiponectin, and ALT differ in NAFLD individuals compared to subjects without NAFLD, as found in previous studies [16,40,41], even when adjusting by BMI. Anyway, some of the differences between obese individuals with NAFLD and lean control subjects may be attributed to differences in BMI and not to their hepatic status. This study did not contemplate a group of obese subjects without NAFLD. However, obesity is closely related to NAFLD [42]. In an Italian population, an ultrasonographical NAFLD prevalence of 91% was reported in subjects with obesity (BMI $\geq$ 30 kg/m^2), 67% in overweight individuals (BMI 25–30 kg/m^2), and 25% in normal weight persons (BMI 18–25 kg/m^2). Hence, prevalence of NAFLD rises with BMI [1]. Consequently, it is a more common scenario to find subjects with obesity and NAFLD than obese subjects without NAFLD.

To evaluate the differences in diet between both groups, we used a semiquantitative FFQ. Interestingly, there were no significant differences between groups in total energy and macronutrient consumption, but NAFLD subjects reported a lower intake of fiber and vegetables and a higher ingestion of meat products, while physical activity was higher in the control group. There are some factors that may explain the similar energy intake reported by both groups. First, the NAFLD patients were older than the control group, thus a decrease in energy requirements might be expected; second, the control group reported a significantly higher physical activity, therefore, this factor could contribute to higher energy requirements. Third, the underreporting of food intake may be more frequent in individuals with excessive body weight than in lean persons [43]. On the other hand, previous studies support that fiber may play an important role in liver diseases and that diverse liver damage markers are lower in subjects with higher fiber consumption [4,44]. Regarding meat ingestion, it has been described that patients with high Fatty Liver Index have a higher red meat intake [45] and that red meat may be associated with an increased risk of chronic liver disease [46]. Those findings suggest that not only energy intake, but also other dietary components and lifestyle factors have an influence on hepatic health.

Additionally, dietary intake has been proposed as a mediator of the association between short sleep duration, obesity and related comorbidities [18]. Thus, a relationship of short sleep duration with a higher total energy intake has been found [47,48] and a trend towards higher fat intake is suggested [47,49]. Therefore, this may contribute to an increased risk of obesity related chronic diseases [18]. However, we did not find significant correlations between sleep characteristics and dietary factors.

The PSQI is a widely used self-report questionnaire that evaluates subjective sleep quality, which has shown good reliability and validity for both healthy and clinical groups with diverse conditions, including mental and health problems, in different age groups and in a variety of cultural contexts [50]. In this study, sleep indicators, such as sleep duration, sleep efficiency, sleep disturbance, and sleep quality, were poorer in patients with NAFLD, but these differences could not be ascribed to NAFLD

itself, thus BMI may play an important role in the interplay between sleep features and hepatic steatosis. In this context, previous research found that sleep duration was shortened and sleep quality was poor in patients with NAFLD [16]. However, other studies did not report significant differences in sleep duration between NAFLD subjects and healthy controls [51]. In addition, two recent systematic reviews and meta-analysis drew conflicting conclusions based on their evaluation. One of them found a small, but significant increase in the risk of NAFLD with short sleep duration [52], while the other found that short or long sleep duration was not significantly associated with the risk of fatty liver disease [53]. The lack of consensus in the definition of short sleep duration [18] and the different methods used to register sleep patterns that include subjective and objective measurements [54,55] may be in part responsible for inconsistencies in the results. Moreover, self-perceived sleep quality represents a challenge to define, to measure, and to classify because there is no generally accepted reference or gold standard for this construct [31]. In our sample, the analysis showed an increased risk of NAFLD in relation to sleep indicators, however, only the sleep disturbance score remained significant after multiple adjustment. It is important to mention that when adjusting sleep features for multiple variables in the logistic regression models, age, physical activity, and insulin were also significant. These results may indicate that sleep duration as well as other characteristics of sleep patterns should be specifically considered when evaluating NAFLD and when developing strategies for the treatment of this condition, along with the traditionally contemplated risk factors.

The biophysiological mechanisms underlying the association between short sleep duration and NAFLD are not completely understood, but there is compelling evidence that insufficient sleep promotes weight gain, obesity, insulin resistance, metabolic syndrome, and diabetes mellitus [56]. In addition, the reduction of leptin [57,58] and elevation of ghrelin [58] have been suggested as effects of sleep insufficiency. Thus, short sleep duration may be related to the distribution, timing, and behavior of the intake [48,58]. Furthermore, it has been suggested that insufficient sleep may predispose to NAFLD by means of proinflammatory markers and stress response [56].

Regarding liver stiffness, in patients with NAFLD, an association was found with short sleep duration, sleep efficiency, sleep disturbance, and overall sleep quality. In the multivariable analysis, the inclusion of coughing or snoring was considered as a confounding variable because it is associated with the risk of obstructive sleep apnea syndrome (OSAS) [59–61], which is the most studied sleep disorder and has been related to liver fibrosis [62–64]. Several studies have associated OSAS with hepatic inflammation and it is suggested that the chronic intermittent hypoxia in these individuals may play a role in the pathogenesis of NAFLD and in the progression from steatosis to steatohepatitis, cirrhosis, and hepatocellular carcinoma [42]. In our NAFLD sample, only three patients reported a previous diagnosis of OSAS, but it should be considered that OSAS affects 35–45% of individuals with obesity and that these two conditions often coexist and share common molecular mechanisms that lead to metabolic alterations [42]. Consequently, OSAS may be undiagnosed in a percentage of the individuals in our sample and sleep characteristics in NAFLD subjects are described in this study independent of the etiology. In addition, when the regression model considered BMI instead of the percentage of fat, the sleep variables were not significant. Nevertheless, fat percentage reflects body composition more accurately than BMI. However, in our analysis, the association of sleep disturbance and sleep quality was consistent and independent of "coughing or snoring", but it is important to note that the prediction of the model was considerably raised when including this last variable. Therefore, these findings suggest that liver stiffness may be associated with different characteristics of sleep patterns besides only those derived from the risk of OSAS.

On the other hand, a previous study found an association between daytime sleepiness and the degree of liver fibrosis assessed by biopsies in NAFLD patients [16], being recognized that the risk of excessive daytime sleepiness is high among subjects with established liver cirrhosis [65,66]. In this case, the alteration of sleep may not be a cause, but a consequence of the hepatic disfunction. Likewise, daytime sleepiness is considered a symptom of OSAS [59]. However, in our study, daytime sleepiness was not significantly associated with liver stiffness (Table S1). These results may be explained by the

diversity of methods used to assess liver fibrosis, which includes liver biopsies, ARFI elastography, transient elastography, among others. Furthermore, daytime sleepiness was registered using PSQI, which only includes one question regarding feelings of sleepiness in contrast to other instruments, such as the Epworth Sleepiness Scale, a frequently used instrument to auto-report the habitual chances of falling asleep in a variety of common situations [67].

There are some limitations in the present study that should be mentioned: Firstly, due to the cross-sectional design of the study, causal inferences cannot be made. Secondly, since matching of the age and sex of the control group with NAFLD subjects was a methodological difficulty, multivariable analysis, including age and sex as covariables, was performed to overcome this situation. Thirdly, sleep and dietary evaluations were carried out using self-reported information of the participants. Thus, subjective measures may produce some biases. Fourthly, the screening of participants, including information about competing causes of liver disease (endocrine disorders, infection with hepatitis virus, among others), was based on a clinical interview rather than a specific laboratory assessment and the diagnosis and evaluation of NAFLD was based on imaging techniques rather than histological methods. Fifthly, we could not perform a diagnostic evaluation of OSAS in our patients. Sixthly, this study included a lean control group without NAFLD and an obese group with NAFLD. A methodological drawback should be noted, since the control group is non-obese. Additionally, this study did not include a group matched for BMI without NAFLD. Therefore, it could not be confirmed that sleep disturbances were due exclusively to NAFLD and not to the excessive body weight.

To our knowledge, few studies have detailed this variety of sleep features in subjects with NAFLD and analyzed the relationship of these variables with ARFI assessed liver stiffness. Based on the findings reported here, further studies should analyze more comprehensively the association between sleep characteristics and NAFLD, not only taking into consideration sleep duration, but other sleep features, such as sleep disturbance, sleep efficiency and overall sleep quality, among others, which may have impact on the development and progression of NAFLD. Moreover, interventional strategies focused on sleep behavior changes may be contemplated in subjects with NAFLD. Additionally, to examine the consistency of the results, further studies should compare data measured by objective (actigraphy, polysomnography, etc.) and subjective sleep parameters in patients with NAFLD and differentiate those subjects with OSAS from those without OSAS because general disagreements in the associations may depend on the method used for the sleep evaluation [68,69] and situation, which may contribute to explain the discrepancies reported in the literature. Furthermore, subjects should be better characterized, taking into consideration other emerging conditions that are also associated with NAFLD, such as hypothyroidism [70] and other endocrinopathies, osteoporosis, colorectal cancer, and psoriasis [3]. Finally, since the liver plays an important role in the regulation of hormones, such as melatonin, sleep disturbances could be evaluated from the perspective of the consequences of NAFLD.

5. Conclusions

This data supports the association of sleep characteristics with the development and progression of NAFLD. Sleep disruption may be contributing to the pathogenesis of NAFLD as well as the alteration of the liver may be affecting sleep parameters. These findings suggest that sleep quality and related factors may be added to the list of modifiable behaviors to consider in health promotion strategies and in the prevention and management of NAFLD in diverse clinical settings.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/2/322/s1, Table S1: Correlations of sleep characteristics and hepatic status related variables of patients with nonalcoholic fatty liver disease and controls.

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DISCUSSION

1. Rationale of the study

NAFLD affects around 25% of the worldwide general adult population (Younossi, Marchesini, et al., 2019), with a prevalence ranging from 20% to 40% in high-income countries (Atabaki-Pasdar et al., 2020), which is usually parallel to the prevalence of obesity (Younossi, Anstee, et al., 2018). From a public health point of view, NAFLD is globally an important burden on morbidity, mortality, and health care utilization (Mitra et al., 2020). Nevertheless, a lack of national policies, awareness campaigns and epidemiological registries has been recently documented in European countries (Lazarus et al., 2019). The development of NAFLD has shown a close relationship with lifestyle determinants such as excessive caloric intake and low physical activity (Hallsworth & Adams, 2019). Furthermore, the pathophysiology of NAFLD is complex and seems to involve multiple factors including insulin resistance, inflammation, lipotoxicity, and gut microbiota dysbiosis, in addition to obesity and genetic contributors (Buzzetti et al., 2016). Likewise, this condition has a variety of manifestations described in all ethnicities and occurring in both sexes (Perumpail et al., 2017).

Regarding the management of NAFLD, several drugs have been or are being investigated with some promising results, but currently no pharmacological treatments have been approved for this disease and the optimal approach remains uncertain (Lombardi et al., 2017; Mahjoubin-Tehran et al., 2020). Therefore, lifestyle modifications aiming at weight reduction through dietary and physical activity interventions remain the cornerstone therapy for the treatment of NAFLD in subjects with overweight or obesity (Saeed et al., 2019). In this sense, randomized controlled trials are the gold standard for the assessment of the efficacy and safety of dietary interventions (Moher et al., 2010). However, there is a lack of randomized trials evaluating the effects of dietary interventions on liver status and other biological and psychological features of subjects with NAFLD in the long-term (Otten et al., 2016). Thus, the research and the development of strategies for the personalized dietary management of NAFLD is necessary to provide significant and persistent beneficial effects in the individuals suffering from this condition and in the burden that this disease represents for healthcare systems (Allen et al., 2018; Ratziu et al., 2019).

In addition, the knowledge about the different factors that potential participate in the development, progression and/or response to the personalized management of NAFLD is still insufficient and deserves further investigation. Such potential contributors may include specific characteristics of the diet, sleep features and psychological components, among others (Berná & Romero-Gomez, 2020). In this regard, case-control studies have advantages such as speed and efficiency compared to other observational designs and are often used to investigate factors contributing to the etiology of diseases (Knol et al., 2008).

Regarding hepatic evaluation, liver biopsy is often difficult to perform due to various limitations of the procedure (Tapper & Loomba, 2018). Therefore, other accurate methods which are non- or minimally invasive can be used, including MRI, abdominal ultrasonography, different elastography techniques, as well as hepatic indexes, among others (Younossi, Loomba, et al., 2018). In relation to nutritional evaluation, dietary intake is often evaluated by previously validated food frequency or diet quality questionnaires (Fernández-Ballart et al., 2010; Martínez-González et al., 2019). In addition, analytic methods are used to determine different nutritional compounds in biological samples (Ferreri et al., 2016). On the other hand, psychological traits and sleep characteristics are commonly assessed by using self-reporting validated questionnaires such as the BDI-II for depressive symptoms, STAI for anxiety and PSQI for sleep features (Royuela, Angel & Macías Fernández, 1997; Sanz et al., 2003; Guillén-Riquelme & Buela-Casal, 2011).

In this context, the present research was focused on the assessment of the long-term effects of two personalized energy-restricted dietary strategies on weight loss, anthropometric measurements, body composition, general metabolic markers, hepatic status evaluated by non- or minimally invasive methods, and psychological features of subjects with overweight or obesity and NAFLD within a 24-month follow-up. Moreover, this work aimed to expand the understanding of different potentially underlying factors in the course of NAFLD, including the adherence to the MedDiet, dietary TAC, omega-3 PUFA, depressive and anxiety symptoms, and sleep features.

2. *Longtime effects of dietary interventions on metabolic and hepatic status*

The cornerstone therapy for the treatment of individuals with NAFLD is currently based on lifestyle modifications focused on weight loss through dietary and physical activity (Saeed et al., 2019). The achievement of weight loss in NAFLD management is especially important for subjects with overweight or obesity (Rinella & Sanyal, 2016; Koutoukidis et al., 2019), but the adherence to dietary treatments for long periods and the maintenance of weight loss and lifestyle modifications is a considerable challenge for many individuals (Properzi et al., 2018). A negative energy balance is considered the most important factor in the dietary management of this condition, given that plays a fundamental role in the decrease of body weight and body fat, including visceral adipose tissue and liver fat (Ross et al., 2020). However, few trials have assessed the results of dietary interventions on hepatic features of NAFLD during long periods (Otten et al., 2016). In this context, the present study aimed to evaluate the long-term effects of two personalized energy-restricted dietary strategies on weight loss, anthropometric measurements, body composition, general biochemical determinations, and hepatic status within a 2-year follow-up in overweight or obese subjects with NAFLD.

A statistical mixed model approach was used to assess the effects of the dietary interventions when analyzing all the follow-up evaluations of the study, given that is a powerful method that takes all available data into account and that is recommended for longitudinal clinical trials with missing values (Ma et al., 2012). After the follow-up significant improvements were observed within both intervention groups in body weight, anthropometric measurements, body composition, and metabolic and hepatic status along the study. Notably, the differences between both dietary treatments became more evident with the longer follow-up, especially in hepatic markers where the FLiO group showed greater and more persistent benefits.

A systematic review and meta-analysis of Koutoukidis et al., 2019 assessed the effect of weight loss interventions in subjects with NAFLD (behavioral programs with diet alone or accompanied by exercise, pharmacotherapy, and bariatric surgery). Although the review evidenced that weight loss interventions were associated with the reduction of liver fat, no

statistically significant change was observed in histologic liver fibrosis or NAFLD fibrosis score (Koutoukidis et al., 2019). Interestingly, most of the clinical trials evaluating the effects of lifestyle management of NAFLD have a duration between 3 and 12 months, which is considered relatively short (Hallsworth & Adams, 2019) and the duration may not be enough to observe the amelioration of hepatic fibrosis as suggested by other researchers (Properzi et al., 2018). These backgrounds have relevance since the degree of fibrosis is considered the strongest predictor correlating NAFLD progression with life-threatening complications (Anstee et al., 2019). In this sense, both intervention groups in the current trial achieved the weight loss recommendations of the AASLD for the reduction of liver steatosis (3%-5%) at all the timepoints of the study, but only the FLiO group maintained the weight loss percentage recommended for the improvement of fibrosis (7%-10%) at the end of the follow-up. Similarly, only the FLiO group showed a continuous decrease in TE liver stiffness which was maintained at the end of the intervention. All these findings suggest that longer periods of treatment and follow-up may be needed to observe consistent changes induced by the diet in hepatic fibrosis. Moreover, dietary factors in addition to weight loss might have influence on the amelioration of liver fibrosis deserving further investigation.

On the other hand, leptin and adiponectin are adipokines with opposite effects in the pathogenesis of NAFLD and are associated with insulin resistance (Mikami et al., 2020). Leptin seems to promote insulin resistance and hepatic fibrogenesis, while adiponectin has shown to reduce insulin resistance and to have anti-steatoic and anti-inflammatory effects by increasing free fatty acid oxidation and decreasing gluconeogenesis, and *de novo* lipogenesis, among other mechanisms (Polyzos et al., 2016). Notably, both the AHA and FLiO groups maintained the beneficial significant effects on glycemic profile and insulin resistance evaluated by HOMA-IR, at all the follow-up visits of the study. Moreover, leptin decreased within both dietary groups along the study when compared to baseline values, while adiponectin increased in the two groups, but this improvement was significantly greater in the FLiO group at the end of the intervention. Therefore, both the AHA and the FLiO strategies may represent feasible options to improve glucose metabolism, insulin resistance, and related adipokines in individuals with NAFLD. However, the FLiO diet seems to promote a higher increase in adiponectin, which may entail greater benefits for metabolic and hepatic status overtime.

Interestingly, the AHA group showed a more pronounced and persistent decrease in systolic blood pressure, although no statistically significant difference was reached when comparing the change in the two groups. This finding might be expected given that the AHA recommendations on diet and lifestyle are intended to reduce cardiovascular risk and aim for normal blood pressure (Lichtenstein et al., 2006). Those results may be of importance, since a bidirectional association between NAFLD and hypertension has been observed and it has been suggested that the change in fatty liver status overtime might influence the incidence of hypertension (Hadi et al., 2019). Hence, the AHA diet may be a suitable option for individuals with coexisting NAFLD and hypertension.

Importantly, previous research has found that weight loss is largely unsuccessful in patients with NAFLD in ambulatory care settings and that frequent clinical encounters are associated with a successful weight reduction (Dudekula et al., 2014). In this sense, although both groups in the current study significantly reduced body weight at all the time points of the trial compared to baseline values, a tendency to regain weight was observed. However, some hepatic benefits and insulin resistance improvements were preserved. These results are in accordance with a previous research that suggested that despite regaining weight, the improvements in hepatic features and insulin resistance can be maintained (Haufe et al., 2013). However, periodical reinforcement of behavioral changes in lifestyle interventions may be needed to preserve the beneficial effects evidenced in short and medium-term trials (Koutoukidis et al., 2019).

Finally, since few studies evaluating the effect of long-term interventions on hepatic outcomes have been published, the current results contribute to this body of evidence showing a variety of beneficial long-term effects in subjects following the AHA or the FLiO diets, with the use of different noninvasive techniques for hepatic assessment.

3. *Effects of weight loss, MedDiet adherence and dietary TAC on NAFLD*

Dietary factors associated with weight gain and obesity (high calorie intake and excessive consumption of saturated fats, refined carbohydrates, and fructose, among others) seems to be of key importance in the pathogenesis of NAFLD (Cantoral et al., 2019; Mazidi et al., 2020). Accordingly, strategies such as the changes in dietary patterns and exercise have been proposed for the prevention of the onset and progression of this disease (Cantero et al., 2017; Oh et al., 2017). However, current knowledge suggests that there is no single optimal dietary strategy for NAFLD treatment, thus more investigation is needed to improve the recommendations for the lifestyle management of NAFLD (Katsagoni et al., 2018; Berná & Romero-Gomez, 2020). In this context, the aim of this study was to assess the effects of two personalized energy-restricted dietary strategies differing in several dietary factors (macronutrients, fiber, meal frequency, TAC, and MedDiet adherence) on liver status evaluated by non-invasive techniques and biochemical markers in overweight/obese subjects with NAFLD after a 6-month follow-up and to analyze the potential relationships of liver fat content with weight loss and dietary factors.

After the 6-month follow-up period, both the AHA and the FLiO group achieved comparable results in weight loss and other metabolic and hepatic parameters, including liver fat content and hepatic volume, even when the dietary intake presented the expected differences according to the dietary prescription. The energy restriction of 30% applied in both diets and the similar weight loss of more than 9% in both groups, may explain the comparable findings with both strategies during this period. Moreover, the AHA diet was based on the AHA guidelines (Van Horn et al., 2016), while the FLiO diet was based on the diet of the Reduction of metabolic syndrome in Navarra (RESMENA) project (De La Iglesia et al., 2014). Thus, both dietary strategies in this study were carefully designed and based on previously investigated healthy dietary approaches instead of a comparison with a control group with no active intervention or receiving only general recommendations (Marin-Alejandre et al., 2019).

A recent systematic review of randomized controlled trials showed that the MedDiet could improve parameters of NAFLD severity, but inconsistencies among the included studies

were found (Moosavian et al., 2019). Accordingly, the current research evaluated the adherence to the MedDiet in addition to the other dietary characteristics considering both intervention groups as one sample. Most of the individual dietary features displayed a small association with hepatic status after the dietary intervention, while a greater increase in the TAC of the diet and a higher adherence to the MedDiet, along with weight loss, appeared to be more decisive factors for the achievement of beneficial outcomes in the treatment of NAFLD (Marin-Alejandre et al., 2019). In this sense, dietary pattern analysis has been proposed as the most realistic approach for the assessment of the relationships between diet and disease (Hu, 2002; Varraso et al., 2012). Notably, the dietary TAC and the adherence to the MedDiet showed an inverse significant association with hepatic fat content, in the regression analyses, even when the models included the percentage of weight loss as adjustment variable (Marin-Alejandre et al., 2019). These findings suggest that the whole quality of the diet may have beneficial effects on the liver of subjects with overweight or obesity and NAFLD in addition to caloric restriction.

Inflammation has an important role in the pathophysiology of NAFLD (Kupčová et al., 2019). For instance, liver fat accumulation accompanied by hepatic inflammation (NASH) rises the risk of progression to fibrosis and cirrhosis (McGettigan et al., 2019). Additionally, there is evidence that weight reduction is a major factor in decreasing pro-inflammatory markers in obese or overweight subjects and that a hypocaloric diet has anti-inflammatory effects independent of the dietary composition (Bianchi, 2018). Interestingly, markers of inflammation (such as adiponectin and C-reactive protein) showed significant improvements in the FLiO group but not in the AHA group, even when the percentage of weight reduction and the changes in other variables assessed in this study were not significantly different between the two groups (Marin-Alejandre et al., 2019). These outcomes may have a relation with the higher adherence to the MedDiet and the significant elevation of the TAC of the diet of the subjects following the FLiO diet, which entails a higher consumption of fruits and vegetables and a healthier profile of fatty acids. A review and meta-analysis found that a higher intake of fruits and vegetables led to a decrease in proinflammatory mediators (Hosseini et al., 2018). Therefore, the effect of weight loss on inflammatory markers might be more pronounced when accompanied by a higher intake of fruits and vegetables. Accordingly, it was hypothesized that the FLiO diet might provide greater improvements in

metabolic and hepatic outcomes in a longer follow-up (Marin-Alejandre et al., 2019), which was confirmed when the data of the 24 months was analyzed.

On the other hand, the remission or the stabilization of fibrosis is considered a positive feature in clinical trials with NAFLD subjects (Cassinotto et al., 2016). Previous studies have found an improvement in liver stiffness as a surrogate marker of liver fibrosis (Abenavoli et al., 2017), while other investigations have found no changes after following a dietary intervention (Properzi et al., 2018). In the current study liver stiffness assessed by ARFI elastography only showed a statistical tendency to improvement in FLiO group compared to AHA group (later mixed linear model approach displayed a statistically significant difference). Notably, the regression analyses showed that a lower liver stiffness at the 6 months of the trial was associated with a higher loss of body weight accompanied by lower visceral adipose tissue, and lower total fat mass. Those findings were in accordance with the 7-10% weight loss suggested by the AASLD to improve fibrosis compared with the weight loss of 3%-5% recommended to reduce liver fat (Chalasani et al., 2018). Interestingly, a marginal association with a higher adherence to the MedDiet was as well observed (Marin-Alejandre et al., 2019). Thus, the additive benefits of weight loss and an improvement in diet quality on liver stiffness might be speculated.

Overall, these results support the benefit of energy-restricted diets, high adherence to the MedDiet, and high dietary TAC for the management of NAFLD in individuals with overweight or obesity. However, given that the decrease of body weight through lifestyle modifications is not a feasible option for all the subjects with NAFLD, and that a growing number of researches have been carried out to determine if certain dietary or lifestyle changes might have benefits on hepatic status even without caloric restriction (Della Pepa et al., 2017; Katsagoni et al., 2017), the dietary strategies proposed in the current trial could be tested to evaluate if they provide hepatic benefits in the absence of weight loss.

4. *Omega-3 PUFA and hepatic status after 6 months of dietary intervention*

The synthesis of PUFA, including omega 3, cannot occur endogenously; thus, those fatty acids must be obtained from the diet (Das, 2008). Omega-3 PUFA participate in diverse processes of the body, such as the modulation of the fluidity and permeability of cell membranes (Ferreri et al., 2016) and the synthesis of precursors of anti-inflammatory molecules, among others (Das, 2008). Moreover, these biomolecules have been associated with the prevention obesity, type 2 diabetes mellitus, cardiovascular disease and other metabolic alterations or chronic diseases (Harris et al., 2018; González-Becerra et al., 2019). Specifically, in subjects with NAFLD a modified lipidomic profile involving omega-3 PUFA has been reported, suggesting a relationship between the change in the concentration and quality of circulating blood lipids, liver tissue and cell membranes, and the pathogenesis and progression of this disease (Notarnicola et al., 2017; Guo et al., 2018). Since lifestyle modifications pointing at weight loss remain the cornerstone therapy for NAFLD (Plaz Torres et al., 2019; Yoo et al., 2019), omega-3 PUFA may play a relevant role the dietary management of this condition (Perdomo et al., 2019). On the other hand, the fatty acid content of blood, diverse tissues and cell types, such as erythrocytes, can be objectively measured and reflects the interplay between dietary intake of fatty acids and endogenous lipid metabolism, which is strongly genetically regulated (Patel et al., 2010; Kröger et al., 2015; Lankinen et al., 2018).

In this context, the current analysis was carried out in a subsample of 54 participants of the FLiO study who achieved a weight loss of more than 5% of the initial body weight after 6 months of follow-up, with the aim to evaluate the effect of the weight loss diets (AHA and FLiO) after this period on erythrocyte membrane omega-3 PUFA composition of subjects with NAFLD, and to evaluate the potential relationship between erythrocyte membrane omega-3 PUFA and hepatic markers. The cut-off point of more than 5% of weight loss was set up considering this value as an indicator of good adherence to the treatment, given that both diets applied an energy restriction of 30% of the total requirements of each participant to achieve the recommendations of the AASLD (Marin-Alejandre et al., 2020). Thus, in

accordance with previous research, subjects with nonadherence were removed (Shiovitz et al., 2016).

After 6 months of follow-up, significant changes in erythrocyte membrane omega-3 proportions were found. A significant increase in ALA was observed, while the increase in EPA did not reach statistical significance, and DHA significantly decreased. Few studies have reported the effects of dietary interventions in omega-3 PUFA composition of erythrocyte membranes of subjects with NAFLD. In contrast with the findings of the current research, a previous trial in Polish individuals with NAFLD found that a 6-month dietary treatment with 20-30% of the total energy requirements from fat (mainly of vegetable origin with olive and rapeseed oil) and without omega-3 PUFA supplementation significantly increased the proportion of DHA, while a marginal significant increase in EPA was found and ALA did not change significantly (MacIejewska et al., 2018). However, an increase in DHA could have been expected in such study, since the baseline values of the participants were under the lower cut-off points reported in the literature (Parker et al., 2015; de Castro & Calder, 2018). These outcomes differ from the values observed in the current investigation, where the DHA content of erythrocyte membranes both at baseline and after 6 months was within the range of 1.9% to 7.9%, which has been previously reported in healthy persons who do not regularly take DHA supplements (de Castro & Calder, 2018). Notably, the existing literature regarding the composition of erythrocyte membranes in individuals with NAFLD is limited and reports a wide range of fatty acid proportions, which may depend on genetic and environmental factors, the dietary background of the participants (Ferreri et al., 2016), and/or the stage of the liver disease (Cansanção et al., 2018). In this context, the fatty acid composition of erythrocyte membranes of the participants of the present work was in agreement with the findings in older Spanish adults at high cardiovascular risk from the PREDIMED study (Muralidharan et al., 2019).

ALA is essential in the human diet and is the precursor for the synthesis of EPA and DHA (Burdge, 2006). Chia seeds, flaxseeds, walnuts, rapeseed (canola) oil, soybeans and its respective oils are the main sources of ALA, while the main dietary source of EPA and DHA is fatty fish (Gebauer et al., 2006; Ríos-Hoyo et al., 2017; Fernández-Martínez et al., 2019). In this sense, positive significant associations between the change in ALA consumption and

the changes in erythrocyte membrane ALA and EPA were found, while the change in the intake of marine omega-3 (EPA+DHA) showed a positive marginal association with the change in erythrocyte membrane DHA. These findings, along with the unexpected decrease of DHA in erythrocyte membranes, suggest that other processes such as the retroconversion of DHA to EPA (Jump et al., 2018), the regulation of the transference of fatty acids to the liver (Mitsche et al., 2018), and/or the lipid remodeling of cell membranes (Ferreri et al., 2016) and lipid droplets of hepatocytes (Ruhanen et al., 2014), may influence erythrocyte membrane fatty acid composition during weight loss treatment of individuals with NAFLD. Interestingly, a lipidomic analysis of murine hepatocytes revealed that lipid droplets from fasted murine presented an enriched profile in triglyceride molecular species with very long-chain PUFA residues, while hepatocytes from high-fat diet murine presented less unsaturated triglycerides (Chitraju et al., 2012).

An important finding of the current investigation was the inverse association between the increase in dietary and erythrocyte membrane ALA and liver stiffness. Nevertheless, the values of this last variable did not change significantly in the pooled sample or in each dietary group after the 6-month follow-up (although a tendency to a decrease in liver stiffness was observed in the FLiO group, but not in AHA group). Studies in murine models have displayed beneficial effects of ALA on different characteristics of NAFLD (Gonçalves et al., 2018; Jeyapal et al., 2018; Fernández-Martínez et al., 2019). A research found that the supplementation with ALA reduced hepatic steatosis and insulin resistance, which was associated with the modulation of inflammatory and endoplasmic reticulum stress responses (Gonçalves et al., 2018), while other study evaluated the effect of chia seed consumption (known for its high content in ALA) in rats with acute dyslipidemia and steatohepatitis, reporting improvements in biochemical determinations and histological characteristics of NASH (Fernández-Martínez et al., 2019). Nevertheless, other researches have failed to demonstrate beneficial effects of ALA supplementation on hepatic features (Hong et al., 2019). PUFA appears to modulate the activity of genes involved in lipid metabolism, redox balance, and fibrogenesis through the interaction with nuclear receptors and transcription factors (Juárez-Hernández et al., 2016; Musso et al., 2018).

Notably, the beneficial effects of lifestyle interventions and weight loss on liver steatosis and transaminases seem consistent in the short to medium term (Katsagoni et al., 2017; Koutoukidis et al., 2019), but the effect of these approaches on hepatic fibrosis and surrogate markers (e.g., liver stiffness) is less clear, maybe due to the reduced duration of the interventions (Properzi et al., 2018), the different composition of diets (Eslamparast et al., 2017), the NAFLD stage of the study participants (Yang et al., 2019), among other factors. Thus, the nature and possible implications of the association between the changes in ALA and liver stiffness deserves further investigation.

Excess iron accumulation has been found in various hepatic diseases and it is suggested that could accelerate the progression of liver fibrosis to cirrhosis and hepatocellular carcinoma by different mechanism regardless of the etiology (Mehta et al., 2019). In the current study, liver iron significantly decreased after 6 months of follow-up and this shift was inversely associated with the change in the proportion of EPA, but not with the total dietary intake of iron. The joint effect of several factors participating in iron homeostasis may explain these findings. Importantly, the homeostasis of iron is influenced by diet composition, genetics, physiological and pathophysiological aspects (Anderson & Frazer, 2017). A variety of dietary factors such as the type of iron (heme and non-heme), the presence of enhancers (e.g., ascorbic acid), and/or inhibitors of iron absorption (e.g., phytates, tannins, and polyphenols) affect the bioavailability of this element (Ems et al., 2020). On the other hand, EPA and DHA in different lipid pools are well-validated markers of habitual oily fish consumption (Cuparencu et al., 2019). Interestingly, an association between higher fish intake and lower ferritin concentrations, an accurate indicator of iron status in humans (Ju & Ha, 2016), and a positive association between liver iron content and red and processed meat ingestion has been reported in individuals with NAFLD (Recaredo et al., 2019). In this sense, the inverse association between the increase in EPA and the decrease in liver iron observed in this study may be related with a higher consumption of fish and lower intake of red meat during the follow-up. In addition, NAFLD itself, inflammation processes and obesity may have an impact on iron metabolism and storage (Britton et al., 2016), and there is evidence suggesting that weight loss may help to reestablish iron homeostasis in subjects with overweight or obesity (Teng et al., 2020). Furthermore, a crosstalk between iron and lipid metabolism has been reported in different diseases (Rockfield et al., 2018). Interestingly, a research in male

Wistar rats exhibited that an iron-rich diet induced oxidative stress and a reduction in the hepatic desaturation capacity of fatty acids, with a decrease of long-chain PUFA in different tissues (Valenzuela et al., 2018).

Overall, the composition of the weight loss diets (omega-3 PUFA, type of iron, enhancers and inhibitors of absorption, etc.) along with the pathophysiological condition of the study participants may have influenced the change observed in liver iron and its association with other outcomes. Therefore, the dietary and pathophysiological mechanisms that may be involved in the potential relationship between omega-3 PUFA and iron accumulation in subjects with NAFLD deserve further investigation.

This trial manifested the importance of weight loss achievement by means of a well-designed dietary strategy and that the improvements in the quality of diet related to omega-3 PUFA status could also affect the clinical evolution of NAFLD. Thus, omega-3 PUFA intake from food sources appears as a central element to carefully consider in the design of dietary strategies for NAFLD management, which may contribute to the regression or attenuation of the progression of liver fibrosis with energy-restricted diets.

5. Depressive and anxiety symptoms during a 24-month follow-up of energy-restricted dietary strategies for NAFLD treatment

NAFLD has a close relationship with obesity and diabetes mellitus, while these both conditions have been strongly associated with depressive symptoms (Huang et al., 2017). Interestingly, recent epidemiological evidence has shown significant associations between NAFLD and depression (Jung et al., 2019; Kim et al., 2019). Moreover, other symptoms and psychological traits which potentially affect quality of life, including anxiety, cognitive impairment and loss of self-esteem have as well been reported in subjects with NAFLD (Golabi et al., 2016). However, few studies regarding psychological factors and the possible effect of lifestyle interventions on psychological traits in individuals with NAFLD have been reported. In this context, the aim of this substudy was to assess the long-term effects of two personalized energy-restricted dietary strategies on depressive and anxiety symptoms, and

to analyze the potential relationship of these symptoms with anthropometric and hepatic determinations in overweight or obese subjects with NAFLD within a 24-month follow-up.

After 24 months of following personalized energy-restricted dietary strategies, participants showed significant reductions in depressive and Trait anxiety symptoms without statistical differences between the changes in the AHA and the FLiO group for these psychological traits. Regarding State anxiety, no significant changes were found along the intervention, in agreement with previous studies (Rodriguez-Lozada et al., 2019). Although the research in this topic is still limited, two recent epidemiological studies with large sample sizes reported significant associations between NAFLD and depressive symptoms (Jung et al., 2019; Kim et al., 2019). Other study evaluated data across 5 European countries and found that subjects with NASH presented significantly greater proportions of anxiety, depression, and sleep difficulties, compared to matched general population, while the proportions were similar to those of individuals with type 2 diabetes mellitus (Balp et al., 2019). In agreement with the findings of the current study, a meta-analysis reported reductions in depressive symptoms of subjects who participated in dietary randomized controlled trials with different objectives such as weight loss or the improvement of nutrient intake (Firth et al., 2019). Interestingly, significant reductions in the symptoms of depression were observed in the trials that primarily aimed to reduce body weight or fat intake, and in those that specified the involvement of a nutrition professional in the delivery of dietary interventions (Firth et al., 2019). Regarding anxiety symptoms, some studies have reported significant decreases after weight loss treatment in subjects with obesity and/or metabolic syndrome (Perez-Cornago, Ramírez, et al., 2014; Rodriguez-Lozada et al., 2019), while other studies have not shown effects on anxiety (Ibero-Baraibar et al., 2015; Firth et al., 2019). Additionally, changes in body weight, BMI, and body composition have shown associations with the decrease in depressive (Simon et al., 2010; Perez-Cornago, de la Iglesia, et al., 2014; Cameron et al., 2019;) and/or anxiety symptoms (Perez-Cornago, Mansego, et al., 2014).

Interestingly, both depressive and anxiety symptoms showed consistent positive associations with body weight, BMI and fatty liver indexes at different timepoints of the study. Moreover, some less consistent relationships with liver fat content were observed. To our knowledge, no studies have reported an association between non-invasive fatty liver markers and

depressive and anxiety symptoms in participants with NAFLD under weight loss treatments. However, both indexes include BMI in the respective algorithms (Bedogni et al., 2006; Lee et al., 2010), which might contribute to explain the similar persistent association with depressive and anxiety symptoms, while MRI evaluation only disclosed a weak relationship between liver fat content and psychological traits. These findings suggest that BMI and not the accumulation of fat in the liver *per se* contribute to the relationship between NAFLD, depressive and anxiety symptoms.

The findings of subgroup analyses by sex displaying a stronger relationship between anthropometric and hepatic determinations with depressive and anxiety symptoms in women than in men showed consistency with the scientific literature. A prospective cohort study in women with a follow-up of 7 years found that being underweight or obese at baseline was associated with future onset of depression (Martin-Rodriguez et al., 2016). In addition, obesity-related inflammation has been shown to exert sex-specific effects on somatic depressive symptoms, suggesting sex as a key biological factor to consider in the relationship between obesity and depression (Kohn et al., 2019). Interestingly, previous research has reported significantly greater improvements in symptoms of depression and anxiety from studies of dietary interventions with female samples (Firth et al., 2019). However, no significant differences in the changes in psychological traits between sex subgroups were observed during the current study.

Chronic liver diseases have been long associated with depression, but the underlying mechanisms remain largely unknown and the nature of this relationship may vary depending on the specific pathology and the severity of the liver disease (Huang et al., 2017). Moreover, NAFLD is closely associated with obesity and the prevalence of both conditions seems to increase parallelly (Younossi, Anstee, et al., 2018), while several studies have evidenced a bidirectional association between obesity and depression showing that the presence of one increases the risk of developing the other (Milano et al., 2020). Thus, the possible mechanisms associating obesity and depression involve the hypothalamic-pituitary-adrenal axis dysregulation, inflammatory processes, oxidative stress, insulin resistance, and endocrine dysfunction (Jantaratnotai et al., 2017). Some of these mechanisms are as well observed in the pathophysiology of NAFLD (Buzzetti et al., 2016). Thus, it has been

suggested that the association between depression and NAFLD may be mediated by the metabolic condition of individuals, with a particular interest on insulin resistance (Jung et al., 2019). Moreover, growing evidence suggests that NAFLD and NASH may manifest in the central nervous system as cerebrovascular alteration, brain insulin resistance, and neuroinflammation (Colognesi et al., 2020). In the current study, associations between depressive symptoms and HOMA-IR in the pooled sample and in the women subgroup were observed at baseline, while no significant association was found between anxiety and HOMA-IR.

Future investigations should evaluate potential differences in depressive and anxiety symptoms between simple steatosis and more advanced stages of NAFLD, given that the alterations of liver function may have an effect on psychological traits as suggested by research in other liver diseases (Lee et al., 2013).

6. Sleep disturbances and NAFLD

The studies regarding the association between sleep duration and NAFLD, have shown conflicting and frequently inconsistent results (Kim et al., 2013; Imaizumi et al., 2015; Miyake et al., 2015; Liu et al., 2016). In addition, there have been published few investigations analyzing the possible link between NAFLD and sleep characteristics different from sleep duration. Thus, the understanding of the relationship between sleep features and the development and progression of NAFLD is still insufficient and inconclusive. Therefore, one of the objectives of this dissertation was to evaluate the potential relationships between sleep quality and hepatic status in obese participants with NAFLD compared to normal weight non-NAFLD controls considering a variety of sleep features along with overall sleep quality scores obtained from the PSQI.

The inconsistencies in scientific literature regarding the link between NAFLD and sleep features might be explained by the absence of consensus in the definition of short sleep duration (Dashti et al., 2015) and by the variety of approaches used to register sleep patterns, being available subjective and objective methods (Marino et al., 2013; Cespedes et al., 2016). Importantly, self-perceived sleep quality is difficult to measure, to categorize and

even to define, given that this construct lacks an accepted general reference or gold standard (Mollayeva et al., 2016). However, the PSQI has been extensively used for the self-reporting of subjective sleep quality and has displayed good reliability and validity for both healthy and clinical samples with a variety of disorders in diverse frameworks (Guo et al., 2016).

In the present research, some of the dietary components evaluated differed significantly between the two groups, with lower consumption of fiber and vegetables and higher intake of meat products in the NAFLD group, while there were no significant differences in total energy and macronutrient consumption. Previous research supports that fiber may be relevant in hepatic diseases and that a variety of markers of liver damage are lower in those subjects with more intake of fiber (Cantero et al., 2017; Eslamparast et al., 2017). On the other hand, sleep duration, sleep efficiency, sleep disturbance, and sleep quality, were poorer in NAFLD participants than in control group. Nevertheless, some of the differences between obese individuals with NAFLD and lean controls might be explained by the dissimilar BMI and not by the liver condition. Thus, BMI may play a role in the interplay between sleep features and NAFLD. Additionally, sleep disturbances were associated with a higher risk of NAFLD. When adjusting logistic regression models for multiple variables, age, physical activity, and insulin were also statistically significant. These results suggest that sleep duration as well as other sleep features should be specifically considered when evaluating subjects with NAFLD and when developing strategies for the prevention or treatment of this condition, along with the traditionally contemplated risk factors (Trovato et al., 2016).

Dietary intake has been proposed as a mediator of the relationship between the reduced duration of sleep, obesity and associated diseases (Dashti et al., 2015). Interestingly, a link between short sleep duration and an elevated total energy consumption has been previously reported (Grandner et al., 2010; Stern et al., 2014). Similarly, a recent research found that sleep quality was associated with specific characteristics of the diet, such as a high intake of sugar-sweetened beverages, even after adjusting by psychological status in young males (Matsunaga et al., 2020). Consequently, these links may contribute to an increased risk of chronic diseases related to obesity (Dashti et al., 2015). Nevertheless, in the current study no significant associations between sleep features and the evaluated dietary factors were found. Additionally, compelling evidence has shown that inadequate sleep fosters an increase in

body weight, obesity, insulin resistance, metabolic syndrome, and diabetes mellitus, possibly involving proinflammatory and stress mediators (Peng et al., 2017). Nonetheless, more research is needed to clarify the biophysiological mechanisms underlying the relationship between inadequate sleep and NAFLD.

Regarding the associations between liver stiffness and sleep features in the group with NAFLD, the inclusion of the "coughing or snoring" item in the multivariable analysis was considered as a confounding variable given that this symptom is associated with the risk of obstructive sleep apnea syndrome (OSAS) (Khassawneh et al., 2009; Faria et al., 2015; Chiu et al., 2017). Several investigations have found a relationship between OSAS and liver inflammation and it has been suggested that the chronic intermittent hypoxia in subjects with this condition may have importance in the development of NAFLD and in the progression from simple steatosis to NASH, cirrhosis, and hepatocellular carcinoma (Paschetta et al., 2015). Importantly, 35–45% of subjects with obesity are affected by OSAS and these two conditions often coexist and have common molecular mechanisms that may lead to metabolic alterations (Paschetta et al., 2015). However, only three participants of the current study reported a previous diagnosis of OSAS and the association between liver stiffness and sleep features (sleep disturbance and sleep quality) was consistent and independent of the "coughing or snoring" item, but it should be considered that the prediction of the model was considerably increased when this last variable was included. Thus, the current findings suggest that liver stiffness might have an association with different features of sleep pattern beyond OSAS. Interestingly, a recent retrospective population-based cohort study found a relationship between sleep disorders and NAFLD both in subjects with and without OSAS (Wei et al., 2020).

In summary, these findings support the existence of a relationship between sleep characteristics and hepatic status, suggesting that sleep quality and related features may be considered as potentially modifiable factors in the prevention and management of NAFLD. Based on these findings, further studies should analyze more comprehensively the possible association between sleep and NAFLD taking into account several sleep characteristics and disorders beyond sleep duration, such as sleep disturbance, sleep efficiency, overall sleep quality, among others. Moreover, further research in subjects with NAFLD should compare

objective (actigraphy, polysomnography, etc.) and subjective (questionnaires) sleep parameters, and differentiate those participants with OSAS from those without OSAS given that general disagreements in the outcomes may be found depending on the approaches used to assess sleep features (Okifuji & Hare, 2011; Herbert et al., 2017).

7. Strengths and limitations

There are various considerations of the present research that should be mentioned as strengths or limitations. First, the liver status of the participants was evaluated by means of non- or minimally invasive techniques instead of histological analyses of liver biopsy, which is considered the most reliable approach for differentiating NAFL or simple steatosis from NASH and fibrosis (Younossi, Loomba, et al., 2018). The use of liver biopsy has some important limitations in research and clinical practice, including the elevated cost and the low acceptability by the patients due to the invasiveness of the procedure and the potential risk of complications (Long et al., 2020). Thus, in this study was carried out a comprehensive hepatic evaluation by means of validated and widely used non- or minimally invasive methods for the assessment of liver steatosis and liver stiffness, including ultrasonography, MRI (Lee & Park, 2014), ARFI elastography, transient elastography (Bota et al., 2013), blood biomarkers and hepatic indexes, which facilitates the translation of these findings to clinical and public health settings. MRI has shown to be accurate and sensitive in the detection of changes in liver fat content and treatment response in clinical trials (Piazzolla & Mangia, 2020). Similarly, liver stiffness is a surrogate marker that has shown a good correlation with liver fibrosis (Liu et al., 2015) and can be measured using different non-invasive techniques with a comparable diagnostic performance for staging fibrosis in patients with NAFLD, including ARFI and transient elastography (Lee et al., 2017).

Second, the assessment of dietary intake, sleep features, depressive symptoms and anxiety was carried out only with self-reported information of the participants, which may produce some bias in the data obtained. However, all the instruments used in this study have been previously validated in Spanish population and are commonly used in clinical and research evaluations.

Third, the sample size was relatively small (specially concerning the analyses of erythrocyte membrane composition and the 24-month evaluation). However, the likelihood of type-II errors is reduced given that important statistical differences and associations were found despite this issue. In addition, the fatty acid composition of erythrocyte membranes does not necessarily indicate the concentration of fatty acids in the hepatic structure as well as in adipose tissue or other lipid pools.

Fourth, regarding the analysis of sleep characteristics and the accompanying relationships with liver status, causal inferences cannot be made due to the cross-sectional design of the study. Moreover, matching the age and sex of the control group with NAFLD subjects was a methodological difficulty. Nevertheless, multivariate analysis with age and sex as covariables was performed to overcome this situation. In addition, this research did not include a group without NAFLD matched for BMI. Therefore, it could not be confirmed that sleep disturbances were explained exclusively by NAFLD and not by the excessive body weight.

Finally, it should be considered that the participants of this investigation lived in a Mediterranean country. Thus, the generalizability and applicability of the current findings may be limited by biological, cultural and/or economic characteristics of different populations, among other factors. Moreover, individuals with severe psychological or psychiatric disorders were not included in the trial. Thus, the findings of this research may not be generalizable to subjects with severe depression or anxiety, which require clinical assessment and management.

In addition, an important strength of this research is the analyses of both cross-sectional data comparing subjects with NAFLD with non-NAFLD controls and longitudinal data from an intervention study designed as a randomized controlled trial, which is considered the gold standard for evaluating the efficacy and safety of dietary interventions (Moher et al., 2010). Other relevant strength is that both the AHA and the FLiO strategies included personalized diets and promoted the adoption of behavioral changes and a healthy lifestyle with individual follow-up of the participants for 24 months. The last important strength is that the AHA diet, a well-recognized healthy dietary approach, was used as a reference reinforcing the evidence

that the positive results obtained with the FLiO diet are of great significance for the lifestyle management of NAFLD.

8. General conclusion

This investigation successfully displayed the beneficial metabolic, hepatic, and psychological effects of two personalized energy-restricted dietary strategies in participants with NAFLD and excessive body weight. Moreover, the research provides evidence for the study of the potential relevance of dietary, lifestyle, and psychological factors in the development, progression, and/or management of NAFLD, including the adherence to MedDiet, dietary TAC, the omega-3 PUFA intake, the characteristics of sleep pattern, and the symptoms of depression and anxiety.

The importance of weight loss by means of energy restriction in the context of two different healthy dietary patterns for the treatment of NAFLD was shown in this study. Therefore, these strategies represent alternatives with concrete recommendations for the personalized dietary management of NAFLD. Both the AHA and the FLiO strategies produced similar and significant improvements within a 6-month follow-up. However, some data suggested a greater improvement in hepatic and metabolic status in the FLiO group in the longer follow-up. Weight loss, adherence to MedDiet, and dietary TAC were apparently the most relevant factors in the reduction of liver fat content during the dietary interventions. Additionally, the intake and incorporation to erythrocyte membranes of specific omega-3 PUFA showed an inverse relationship with liver stiffness and liver iron content. These factors, along with energy restriction, may be of importance not only for the reduction of hepatic fat content but for the potential influence on the amelioration of hepatic inflammation and hepatic fibrosis, which represent more advanced stages of the disease and can lead to worse health outcomes.

Moreover, depressive and anxiety symptoms decreased after following the dietary interventions showing that well-designed dietary interventions could exert beneficial effects on the overall health of the participants. However, it should be noted that an important consistent association between these psychological features and body weight, BMI, and fatty

liver indexes was observed especially in women during all the duration of the trial. These findings suggest that psychological features should be periodically monitored and taken into account in the design and implementation of dietary interventions in order to promote not only improvements in liver status but also in the mental health of individuals with NAFLD. Finally, sleep features evidenced a relationship with hepatic status, suggesting that the characteristics of sleep pattern and the promotion of sleep hygiene should be taken into account in the interventions targeted to prevent or ameliorate NAFLD.

CONCLUSIONS

Conclusions

1) Both the AHA and the FLiO strategies produced significant improvements in body weight and body composition, as well as in biochemical and liver determinations during the 24-month follow-up. These findings suggest that both strategies are suitable alternatives for NAFLD management. However, the FLiO strategy seemed to provide greater and more persistent metabolic and hepatic benefits compared to the AHA strategy at the end of the study.

2) The decrease in liver fat content after 6 months of intervention was associated not only with a greater weight loss percentage, but also with a higher adherence to the MedDiet and dietary TAC. These results support the benefit of energy-restricted diets with high adherence to the MedDiet, and high TAC for the management of NAFLD in individuals with overweight or obesity.

3) The adherence to dietary strategies for 6 months led to changes in erythrocyte membrane omega-3 PUFA composition, which in turn were associated with changes in hepatic markers (liver stiffness and liver iron content), suggesting that these fatty acids accompany the hepatic improvements during the dietary treatment of NAFLD. These findings show that beyond weight loss, the fatty acid composition of the diet has a putative role in NAFLD management.

4) Both the AHA and the FLiO dietary strategies significantly decreased depressive and anxiety symptoms during the study without significant differences between groups. Consistent associations between psychological scores, BMI and hepatic indexes were found along the trial, mainly in women. These findings suggest that well-designed dietary strategies may produce benefits both in hepatic and psychological status and that psychological traits should be periodically monitored in subjects with NAFLD and overweight or obesity, especially in women.

5) Participants with NAFLD showed impaired sleep features compared to normal weight non-NAFLD controls. Moreover, higher sleep disturbances were associated with higher probability of having NAFLD, while more sleep disturbances and worse sleep quality

were associated with higher values of liver stiffness in NAFLD subjects. These findings support the existence of a relationship between sleep characteristics and hepatic status, suggesting that sleep quality and related features may be considered as potentially modifiable factors in the prevention and management of NAFLD.

6) Strategies including healthy energy-restricted dietary patterns with high adherence to MedDiet, TAC and omega-3 fatty acids seem to be effective to improve different markers of metabolic and liver status in subjects with NAFLD. Moreover, beneficial effects on depressive and anxiety symptoms are promoted with these approaches fostering improvements in overall health. In addition to dietary factors, psychological traits and characteristics of sleep pattern should receive specific attention in the design and implementation of strategies for the prevention and management of NAFLD.

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APPENDICES

Other publications

1. **Title:** Interplay of Glycemic Index, Glycemic Load, and Dietary Antioxidant Capacity with Insulin Resistance in Subjects with a Cardiometabolic Risk Profile

   Authors: Cristina Galarregui, María Ángeles Zulet, Irene Cantero, Bertha Araceli Marín-Alejandre, José Ignacio Monreal, Mariana Elorz, Alberto Benito-Boillos, José Ignacio Herrero, Josep Antoni Tur, Itziar Abete and José Alfredo Martínez

   Journal: International Journal of Molecular Sciences

   ISSN: 1422-0067

   Year: 2018

2. **Title:** Ultrasound/elastography techniques, lipidomic and blood markers compared to magnetic resonance imaging in non-alcoholic fatty liver disease adults

   Authors: Irene Cantero, Mariana Elorz, Itziar Abete, Bertha Araceli Marin, Jose Ignacio Herrero, Jose Ignacio Monreal, Alberto Benito, Jorge Quiroga, Ana Martínez, Mª Pilar Huarte, Juan Isidro Uriz-Otano, Josep Antoni Tur, John Kearney, J. Alfredo Martinez, M. Angeles Zulet

   Journal: International Journal of Medical Sciences

   ISSN: 1449-1907

   Year: 2019

3. **Title:** Association between Different Animal Protein Sources and Liver Status in Obese Subjects with Non-Alcoholic Fatty Liver Disease: Fatty Liver in Obesity (FLiO) Study

   Authors: Gregorio Recaredo, Bertha Araceli Marin-Alejandre, Irene Cantero, J. Ignacio Monreal, José Ignacio Herrero, Alberto Benito-Boillos, Mariana Elorz, Josep A. Tur, J. Alfredo Martínez, M. Angeles Zulet and Itziar Abete

   Journal: Nutrients

   ISSN: 2072-6643

   Year: 2019

4. **Title:** Dietary intake of specific amino acids and liver status in subjects with nonalcoholic fatty liver disease: fatty liver in obesity (FLiO) study

Authors: Cristina Galarregui, Irene Cantero, Bertha Araceli Marin-Alejandre, J. Ignacio Monreal, Mariana Elorz, Alberto Benito-Boillos, José Ignacio Herrero, Víctor de la O, Miguel Ruiz-Canela, Helen Hermana M. Hermsdorff, Josefina Bressan, Josep A. Tur, J. Alfredo Martínez, M. Angeles Zulet and Itziar Abete

Journal: European Journal of Nutrition

ISSN: 1436-6215

Year: 2020

5. **Title:** Association of the *SH2B1* RS7359397 gene polymorphism with steatosis severity in subjects with obesity and non-alcoholic fatty liver disease

   Authors: Nuria Perez-Diaz-del-Campo, Itziar Abete, Irene Cantero, Bertha Araceli Marin-Alejandre, J. Ignacio Monreal, Mariana Elorz, José Ignacio Herrero, Alberto Benito-Boillos, Jose I. Riezu-Boj, Fermín I. Milagro, Josep A. Tur, J. Alfredo Martinez and M. Angeles Zulet

   Journal: Nutrients

   ISSN: 2072-6643

   Year: 2020

6. **Title:** Predictive Value of Serum Ferritin in Combination with Alanine Aminotransferase and Glucose Levels for Noninvasive Assessment of NAFLD: Fatty Liver in Obesity (FLiO) Study

   Authors: Cristina Galarregui, Bertha Araceli Marin-Alejandre, Nuria Perez-Diaz-Del-Campo, Irene Cantero, J. Ignacio Monreal, Mariana Elorz, Alberto Benito-Boillos, José Ignacio Herrero, Josep A. Tur, J. Alfredo Martínez, M. Angeles Zulet, Itziar Abete

   Journal: Diagnostics

   ISSN: 2075-4418

   Year: 2020