Universidad de Navarra

Facultad de Farmacia y Nutrición

Nutrigenetic approaches for precision nutrition management of non-alcoholic fatty liver disease

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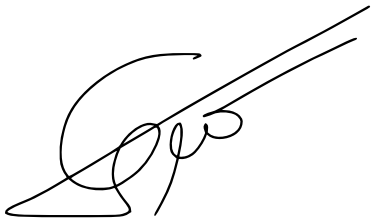
Memoria presentada por Dña. **Nuria Pérez Díaz del Campo** para aspirar al grado de Doctor por la Universidad de Navarra.



Nuria Pérez Díaz del Campo

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación y Fisiología de la Facultad de Farmacia y Nutrición de la Universidad de Navarra y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

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"Nada en la vida debe ser temido, solamente comprendido.
Ahora es el momento de comprender más, para temer menos"

Marie Curie

A *PDXM*: Papá, Mamá y Dani

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List of abbreviations

AAA: Aromatic Amino Acids

AASLD: American Association for the Study of Liver Diseases

AHA: American Heart Association

ALT: Alanine Aminotransferase

AST: Aspartate Aminotransferase

ATP: Adenosine Triphosphate

AUROC: Area Under the Receiver Operating Characteristics Curve

BARD: BMI, Aspartate Aminotransferase/Alanine Aminotransferase ratio, Diabetes Score

BCAA: Branched-Chain Amino Acids

BMI: Body Mass Index

CKD: Chronic Kidney Disease

CVD: Cardiovascular Disease

DASH: Dietary Approaches to Stop Hypertension

DHA: Docosahexaenoic Acid

DNA: Deoxyribonucleic Acid

DNL: *de novo* Lipogenesis

DXA: Dual-energy X-ray Absorptiometry

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

EPA: Eicosapentaenoic Acid

ER: Endoplasmic Reticulum

FFA: Free Fatty Acids

FGF-21: Fibroblast Growth Factor 21

FIB-4: Fibrosis-4

FLI: Fatty Liver Index

FLiO: Fatty Liver in Obesity

GGT: Gamma-Glutamyltransferase

GRS: Genetic Risk Score

GWAS: Genome Wide Association Study

HCC: Hepatocellular Carcinoma

HDL-c: High-Density Lipoprotein-cholesterol

HFC: Hepatic Fat Content

HOMA-IR: Homeostatic Model Assessment of Insulin Resistance

HSI: Hepatic Steatosis Index

IR: Insulin Resistance

LDL-c: Low-Density Lipoprotein-cholesterol

LSECs: Liver Sinusoidal Endothelial Cells

MAFLD: Metabolic (dysfunction) Associated Fatty Liver Disease

MDD: Major Depressive Disorder

MedDiet: Mediterranean Diet

MetS: Metabolic Syndrome

MRI: Magnetic Resonance Imaging

MUFA: Monounsaturated Fatty Acids

NAFL: Non-alcoholic Fatty Liver

NAFLD: Non-alcoholic Fatty Liver Disease

NAFLD-LFS: NAFLD-Liver Fat Score

NAS: Non-alcoholic Fatty Liver Disease Activity Score

NASH: Non-alcoholic Steatohepatitis

NFS: NAFLD-Fibrosis Score

OWLiver®-test: One Way Liver® S. L-test

POUNDS LOST trial: Preventing Overweight Using Novel Dietary Strategies trial

PUFA: Polyunsaturated Fatty Acids

RBP4: Retinol-Binding Protein 4

RNAs: Ribonucleic Acid

SAA: Sulfur Amino Acids

SFA: Saturated Fatty Acids

SNP: Single Nucleotide Polymorphism

TAG: Triacylglycerol

TE: Transient Elastography

TG: Triglycerides

TyG: Triglycerides/Glucose index

T2DM: Type 2 Diabetes Mellitus

UPR: Unfolded Protein Response

VLDL: Very Low-Density Lipoprotein.

WC: Waist Circumference

List of abbreviations of genes

***ADCY3*:** Adenylate cyclase 3

***ADIPOQ*:** Adiponectin, C1Q and collagen domain containing

***ADRB3*:** β-adrenergic receptor 3

APOB: Apolipoprotein B

***APOC3*:** Apolipoprotein C-III

***CD14*:** Cluster of differentiation 14

***ENPP1*:** Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1

***FADS1*:** Fatty acid desaturase 1

***FADS2*:** Fatty acid desaturase 2

***FGF21*:** Fibroblast growth factor 21

***FTO*:** Fat mass and obesity associated

***GCKR*:** Glucokinase regulatory protein

***GCLC*:** Glutamate-cysteine ligase catalytic subunit

***HSD17B13*:** 17-beta hydroxysteroid dehydrogenase 13

***IL-6*:** Interleukin 6

***IRS1*:** Insulin-receptor substrate 1

***IRS2*:** Insulin-receptor substrate 2

***KLF6*:** Kruppel-like factor

***LEPR*:** Leptin receptor

***LPIN1*:** Lipin 1

LYPLAL1: Lysophospholipase-like 1

***MBOTAT*:** Membrane-bound O-acyltransferase domain-containing 7

***NCAN*:** Neurocan

***NR1I2*:** Nuclear receptor subfamily 1 group Imember 2

***PCSK7*:** Proprotein convertase subtilisin/kexin Type 7

***PEMT*:** Phosphatidylethanolamine N-methyltransferase

***PNPLA3*:** Patatin-like phospholipase domain-containing protein 3

***PPAR*:** Peroxisome proliferator-activated receptor

***PPAR-α*:** Peroxisome proliferator-activated receptor alpha

PPARγ: Peroxisome proliferative activated receptor γ

***SH2B*:** Src-homology-2 B

***SH2B1*:** Src-homology-2 B adaptor protein 1

***SH2B2*:** Src-homology-2 B adaptor protein 2

***SH2B3*:** Src-homology-2 B adaptor protein 3

***SLC2A1*:** Solute carrier family 2 member 1

SOD2: Superoxide dismutase 2

SREBF2**:** Sterol regulatory element binding transcription factor 2

TCF7L2**:** Transcription factor 7-like 2

TM6SF2**:** Transmembrane 6 superfamily 2

TNF**:** Tumor necrosis factor

TNF-α: Tumor necrosis factor-α

UCP2**:** Uncoupling protein-2

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a rising epidemic affecting around 25% of the global population, in parallel with increasing worldwide rates of obesity and metabolic syndrome. NAFLD is a complex condition with a genetic component shared with other liver or related metabolic disorders. To date, healthy lifestyle modifications based on diet and physical activity are a cornerstone of the NAFLD therapy, where the genetic involvement appears to affect treatment outcomes by interacting with environmental factors. In this context, this research focused on the following objectives: 1) To analyze the association of the *SH2B1* rs7359397 gene polymorphism with steatosis severity in subjects with obesity and NAFLD (Chapter 1); 2) To evaluate the influence of the *SH2B1* rs7359397 genetic variant on changes in body composition, metabolic status and liver health after 6-month energy-restricted treatment in overweight/obese subjects with NAFLD (Chapter 2); 3) To assess three different genetic risk scores (GRSs) based on Fatty Liver Index (FLI), Magnetic Resonance Imaging (MRI) and lipidomic (OWLiver®-test) for a nutrigenetic personalized management of NAFLD after a 6-months weight-loss nutritional treatment (Chapter 3); and 4) To build a predictive model based on genetic and hepatic health information, deeming insulin resistance markers in order to personalize dietary treatment in overweight/obese subjects with NAFLD (Chapter 4). Regarding the first objective, the results suggested that the risk genotype concerning the *SH2B1* rs7359397 genetic variant was associated with higher homeostatic model assessment of insulin resistance, FLI and protein intake. while lower mono- unsaturated fatty acid and fiber intake was found. Moreover, individuals with the minor risk allele also showed a higher susceptibility of advanced stages of NAFLD. Considering the second objective, carriers of the minor allele of the *SH2B1* rs7359397 genetic variant showed a better response to a weight-loss dietary intervention in terms of hepatic health and liver status. Furthermore, adherence to Mediterranean dietary pattern rich in fiber and other components such as omega-3 fatty acids might boost these benefits. In relation to the third objective, three GRSs based on different diagnostic tools for detecting NAFLD were able to predict the improvement in liver health after a 6-month energy-restricted nutritional treatment. These associations were particularly influenced by factors such as insulin resistance, inflammatory biomarkers and specific nutrients. Concerning the fourth objective, the designed GRS was able to predict the change in FLI adjusted by diet, age and sex, allowing to personalize the most suitable diet for 72% of the volunteers. Similar models were also able to predict the changes on variables related to insulin resistance depending on diet. In conclusion, new diagnostics and personalized intervention approaches based on nutrigenetics instruments could help to improve precision nutrition management in subjects with NAFLD, reducing the severity, some associated comorbidities and impact on healthcare concerning this disease, as well as explaining the benefits of individualized prescribed dietary patterns.

Resumen

La enfermedad del hígado graso no alcohólico (EHGNA) es una epidemia creciente que afecta a alrededor del 25% de la población mundial, en paralelo con el aumento de las tasas globales de obesidad y síndrome metabólico. La EHGNA es una enfermedad compleja, que comparte un componente genético con trastornos hepáticos y metabólicos relacionados. En la actualidad, las modificaciones del estilo de vida saludable basadas en la dieta y la actividad física son el pilar de la terapia de esta enfermedad, donde la implicación de la genética parece afectar a los resultados del tratamiento al interactuar con los factores ambientales. En este contexto, esta investigación se centró en los siguientes objetivos: 1) Analizar la asociación del polimorfismo del gen *SH2B1* rs7359397 con la gravedad de la esteatosis en sujetos con obesidad y EHGNA (Capítulo 1); 2) Evaluar la influencia de la variante *SH2B1* rs7359397 en los cambios de la composición corporal, el estado metabólico y la salud hepática tras un tratamiento de restricción energética de 6 meses en sujetos con sobrepeso/obesidad y EHGNA (Capítulo 2); 3) Evaluar tres diferentes puntuaciones de riesgo genético (GRSs) basadas en el índice de hígado graso (FLI), la resonancia magnética (MRI) y la lipidómica (OWLiver®-test) para una manejo nutrigenético personalizado de la EHGNA tras un tratamiento nutricional de pérdida de peso de 6 meses. (Capítulo 3); y 4) Construir un modelo predictivo basado en información genética y de salud hepática, que considere marcadores de resistencia insulinica para personalizar el tratamiento dietético en sujetos con sobrepeso/obesidad y con EHGNA (Capítulo 4). En cuanto al primer objetivo, los resultados sugirieron que el genotipo de riesgo de la variante genética *SH2B1* rs7359397 se asoció con un mayor modelo homeostático de resistencia a la insulina, FLI e ingesta proteica, mientras que también se observó una menor ingesta de ácidos grasos monoinsuaturados y fibra. Además, los sujetos portadores del alelo minoritario mostraron una mayor susceptibilidad de desarrollar estadios más avanzados de EHGNA. Con relación al segundo objetivo, los portadores del alelo minoritario de la variante genética *SH2B1* rs7359397 mostraron una mejor respuesta a la intervención dietética de pérdida de peso en términos de salud hepática y estado del hígado. Además, la adherencia a un patrón dietético mediterráneo, rico en fibra y otros componentes como los ácidos grasos omega-3 podría potenciar estos beneficios. En cuanto al tercer objetivo, tres GRSs basados en diferentes instrumentos diagnósticos para la detección de EHGNA fueron capaces de predecir la mejora de la salud hepática tras un tratamiento nutricional de restricción energética de 6 meses. Estas asociaciones fueron especialmente influenciadas por factores como la resistencia insulinica, biomarcadores inflamatorios y nutrientes específicos. En lo relativo al cuarto objetivo, el GRS diseñado fue capaz de predecir el cambio en el FLI ajustado por dieta, edad y sexo, permitiendo así, personalizar la dieta más adecuada para el 72% de los voluntarios. Modelos similares fueron igualmente capaces de predecir los cambios en las variables relacionadas con la resistencia a la insulina en función de la dieta. En conclusión, nuevos enfoques diagnósticos y de intervención personalizada basados en instrumentos de nutrigenética podrían ayudar a mejorar el manejo nutricional de precisión en pacientes con EHGNA, reduciendo así la gravedad, comorbilidades asociadas y el impacto en los sistemas sanitarios de esta enfermedad, así como explicar los beneficios de los patrones dietéticos prescritos individualmente.

Table of contents

INTRODUCTION

1. Non-alcoholic fatty liver disease (NAFLD)

1.1. Definition

Non-alcoholic fatty liver disease (NAFLD) is defined on liver biopsy as an excessive accumulation of fat content in the liver arising in the absence of significant alcohol intake ≥21 units/week for men and ≥14 units/week for women or long-term use of a steatogenic medication, or monogenic hereditary disorders (Chalasani *et al.,* 2018; Roeb, 2021).

NAFLD encompasses a spectrum of liver abnormalities characterized by the presence of hepatic steatosis (Brunt *et al*., 2020). Histologically, NAFLD can be categorized into non-alcoholic fatty liver (NAFL) or non-alcoholic steatohepatitis (NASH) (Lonardo *et al*., 2020). NAFL is the presence of at least 5% steatosis without sufficient inflammation and hepatocellular injury that can progress to NASH (Stefan *et al*., 2019), which is a more active form of hepatic steatosis accompanied by lobular inflammation with hepatocyte ballooning (Petroni *et al*., 2021). NASH has been identified as having the potential for progression to advanced fibrosis and clinically evident cirrhosis, and eventually to hepatocellular carcinoma (HCC) (Younes *et al*., 2019).

Following pioneer attempts and in order to overcome the negative attributed to NAFLD, it has been proposed to change the term NAFLD to MAFLD (metabolic (dysfunction) associated fatty liver disease), assigning the disease a name linked with its pathogenesis (Shiha *et al*., 2021; Xian *et al*., 2020). More recently, an alternative nomenclature has also been suggested: dysmetabolism-associated fatty liver disease (DAFLD), instead of NAFLD or MAFLD, in view of the robust evidence that a dysfunction of metabolic factors (namely dysmetabolism) is the main factor able to develop NAFLD and its complications (Polyzos *et al*., 2020). However, a debate is presently ongoing, and an international consensus is still need (Ratziu *et al*., 2020).

1.2. Prevalence and natural history

Non-alcoholic fatty liver disease has become the most prominent cause of chronic liver disease worldwide and is treated as a public health priority (Liu Y. *et al*., 2019). One of the most important caveats of NAFLD prevalence is its heterogeneity depending on the method of ascertainment (Bullón-Vela M.V. *et al*., 2018). The overall prevalence has been estimated to be around 24-25% of the general adult population and it is expected to be 33.5% in 2030 among individuals aged ≥ 15 and 25.8% among ages. The highest prevalence has been

evidenced in South America (31%) and the Middle East (32%), followed by Asia (27%), the United States of America (24%) and Europe (23%), whereas Africa shows lower rates (14%) (Younossi, Anstee, *et al.*, 2018).

Concerning NASH, the prevalence and incidence in the general population is unknown due to practical cost and ethical considerations (Schattenberg *et al.*, 2021). However, some studies have estimated a prevalence of NASH between 1.5% and 6.45% in the general population, which is one in four to five patients with NAFLD (Younossi *et al.*, 2016). Moreover, available data indicated that the number of NASH cases is projected to increase 63% from 16.52 million cases in 2015 to 27.00 million in 2030 (Estes, Razavi, *et al.*, 2018). All these complications of NASH can result in significant health, economic, and experiential burden on patients, their families and the society (Schattenberg *et al.*, 2021). Assuming that this trend of annually increasing NAFLD-related costs is added to the annual growth in obesity prevalence, it is estimated that in 10 years the burden of NAFLD will cost $1.005 trillion in the United States and 334 billion euros in Europe (Younossi, Anstee, *et al.*, 2018).

Thus, although shared environmental risk factors may contribute to the NAFLD phenotype, the broad spectrum of disease severity and variability across it implies a strong heritable component (Buzzetti *et al.*, 2016; Chalasani *et al.*, 2018). In fact, the progression from NAFL to NASH is quite dynamic and even fibrosis can progress, regress, or remain stable over time (Loomba *et al.*, 2021). Notably, among 5-10% of patients with NAFLD diagnosis will develop NASH, from which almost 30% will progress to cirrhosis, while 1-2% will develop HCC (**Figure 1**) (Buzzetti *et al.*, 2016).

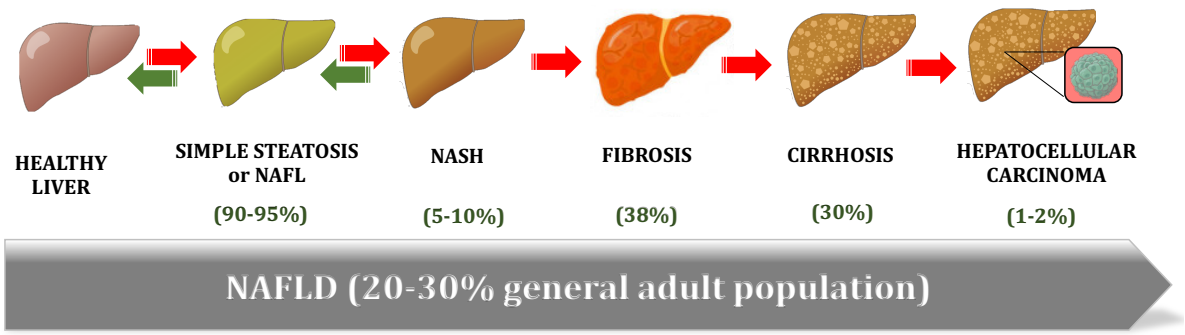

Figure 1. Spectrum and natural history of non-alcoholic fatty liver disease. Adapted from Buzzetti *et al.*, 2016; Chalasani *et al.*, 2018; and Loomba *et al.*, 2021. Abbreviations: NAFLD, Non-alcoholic Fatty Liver Disease; NAFL, Non-alcoholic Fatty Liver; NASH, Non-alcoholic Steatohepatitis.

In addition, the increasing number of components of metabolic syndrome appears to increase the risk of progression to NASH (Younossi, 2019). Importantly, individual variations in the progression of the disease have been reported depending on the presence and the diversity of risk factors such as obesity, diabetes, metabolic syndrome, as well as the genetic component and gut microbiota (Huang D.Q. *et al*., 2021).

Moreover, variations in the prevalence and severity of NAFLD depend not only on different geographic regions and ethnicities, but also on ages and sexes (Estes, Razavi, *et al*., 2018). As age increases so does the prevalence of NAFLD and NAFLD-related fibrosis (Younossi, 2019). The peak prevalence has been reported to be in the 50-65 age group, where up to 54% of subjects had NAFLD. In this sense, numerous studies have reported that inherited and acquire genomic and epigenomic changes could have a cumulative effect on the ageing phenotype (Stefan *et al*., 2019). Moreover, age related skeletal muscle disorders, as well as the age-related decline in sex hormones are thought to contribute to the pathogenesis of NAFLD (González-Muniesa *et al*., 2019; Stefan *et al*., 2019). In addition to age, it has been suggested that female sex was associated with an increased risk of NAFLD (Younossi, 2019). In contrast, data consistently indicate that the overall prevalence of NAFLD is higher in men than in women in young adulthood (Lonardo *et al*., 2019; Stefan *et al*., 2019). However, this tendency seems to change, as it has been observed that in menopause women the decrease of estrogen levels produced an increased visceral adipose tissue (VAT) and ectopic fat accumulation (Bullón-Vela V., Abete, Tur, Konieczna, *et al*., 2020; Lonardo *et al*., 2019).

Indeed, due to longer exposure to metabolic risk factors, the prevalence of advanced forms of NAFLD is expected to increase significantly in both pediatric and ageing population (Koehler *et al*., 2012; Nobili *et al*., 2019). Furthermore, a recent NHANES-III database study of 12,253 estimated that in the United States, 8% of all-causes mortality and more than one third of specific deaths from liver disease and diabetes are related to NAFLD (Alvarez *et al*., 2020). However, cardiovascular disease remained the most common cause of death among this population (Tana *et al*., 2019)

1.3. Etiology and Physiopathology

The mechanism underlying the development and progression of NAFLD, as well as the prediction of hepatic and extrahepatic risk in this phenomenon, is a complex and not yet fully understood area (Sivell, 2019). In this sense, different theories have been proposed trying to explain the complex and multifactorial mechanism leading to the "*two hits hypothesis*" (Day *et al*., 1998). This hypothesis stated that insulin resistance leads to hepatic

steatosis (*first hit*), which will render the liver more susceptible to the action of oxidative stress, adenosine triphosphate (ATP) depletion and endotoxins, ending with inflammation, fibrosis and cancer (*second hit*). However, this theory becomes rapidly too simplistic to explain the complexity of NAFLD pathogenesis (Friedman *et al*., 2018).

Nowadays, this theory has been replaced by a *multiple-hit hypothesis* (Buzzetti *et al*., 2016; Juanola *et al*., 2021). This hypothesis considers that on genetically predisposed subjects, multiple etiopathogenic factors such as obesity, environmental factors, insulin resistance and changes in the gut microbiota, act parallele or sequentially causing NAFLD (Marchisello *et al*., 2019) (**Figure 2**). Concretely, some subjects will develop NAFL which could lead to NASH, but others, will directly present inflammation and fibrosis, probably because of the influence and interaction of environmental, metabolism and demography, gut microbiome, genetic and epigenetic factors (Marchisello *et al*., 2019).

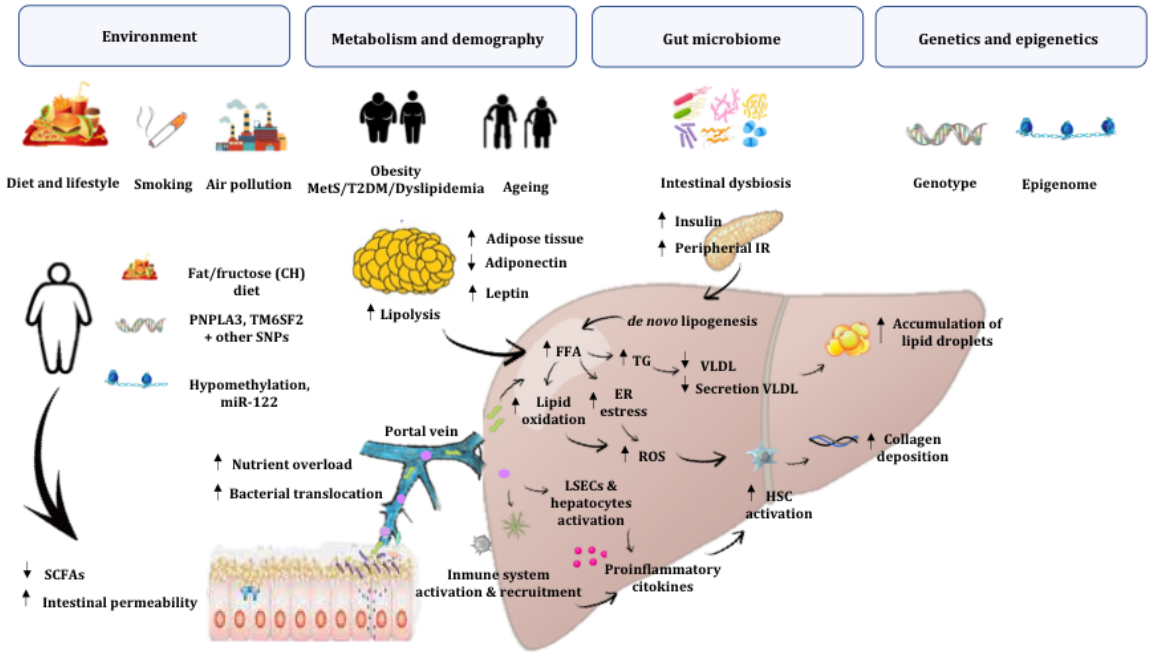

Figure 2. Multiple factors contributing to the pathophysiology of NAFLD. Adapted from: Juanola *et al*., 2021 and Buzzeti *et al*., 2016. Abbreviations: CH, Carbohydrates; ER, Endothelial Reticulum; FFAs, Free Fatty Acids; HSC, Hepatic Stellate Cells; IR, Insulin Resistance; LSECs, Liver Sinusoidal Endothelial Cells; MetS, Metabolic Syndrome; *PNPLA3*, Patatin-like phospholipase domain-containing 3; ROS, Reactive Oxygen Species; SCFAs, Short-Chain Fatty Acids; TG, Triglycerides; *TM6SF2*, Transmembrane 6 superfamily 2; T2DM, Type 2 Diabetes Mellitus; VLDL, Very Low-Density Lipoprotein.

Insulin resistance is considered a key factor in the development of steatosis/NASH, acting on adipose tissue and worsening adipocyte dysfunction, lipolysis and the release of adipokines and proinflammatory cytokines (Bullón-Vela M.V. *et al*., 2018). The excess of carbohydrate, especially glucose and fructose resulted in increased hepatic *de novo* lipogenesis (DNL) by increasing the flux of hepatic free fatty acids (Softic *et al*., 2016). This

state activates transcription factors such as the sterol regulatory element-binding protein (SREBP), carbohydrate response element binding protein (ChREBP) and peroxisome proliferator-activated receptor-α (PPAR-α) (Buzzetti *et al*., 2016; Softic *et al*., 2016).

When liver mechanisms are overwhelmed by the excessive of carbohydrates and fatty acids, the accumulation of triglycerides, free cholesterol and other lipid metabolites causes hepatic lipotoxicity (Bullón-Vela M.V. *et al*., 2018). This leads to mitochondrial dysfunction with oxidative stress, inflammation and the production of reactive oxygen species provoking the endoplasmic reticulum (ER) perturbation (Friedman *et al*., 2018). ER dysfunction, lack of ATP or increased protein synthesis can ultimately trigger to the "unfolded protein response" (UPR) which leads into the activation of c-Jun terminal kinase (JNK) and Sterol receptor-binding protein 1-c (SREBP-1c) pathways, which are related to inflammation, apoptosis and maintenance of liver fat accumulation and aggravation of ER stress, respectively (Sumida *et al*., 2018). Moreover, it has been suggested an association between UPR/ER stress and inflammatory and insulin pathways and abnormality in VLDL assembly in hepatic steatosis (Choi *et al*., 2014; Wei *et al*., 2008). In NAFLD, factors that induce UPR include hyperglycemia, hypercholesterolemia, mitochondrial injury and oxidative stress, among others (Buzzetti *et al*., 2016).

In addition, increased hepatic iron concentration has been observed in about one third of adult NAFLD patients (Marmur *et al*., 2018) being involved in oxidation-reduction process, leading to the production of reactive oxygen species (Ma B. *et al*., 2021) and the depletion of long-chain polyunsaturated fatty acids and fat accumulation (Barrera *et al*., 2020). However, in the Iron on Insulin Resistance and Liver Histology in Non-alcoholic Steatohepatitis (IIRON2) study, a positive correlation was observed between hepatic iron concentration, serum adiponectin and insulin sensitivity (Britton *et al*., 2018). Therefore, the data should be interpreted with caution, as the role of iron in NAFLD is multidimensional and may differ between NAFLD cases (Bloomer *et al*., 2019; Mehta *et al*., 2019).

Finally, complex interactions between genetic background, gut microbiota, diet and the risk of developing obesity and metabolic syndrome features such as NAFLD has been devoted (Cuevas-Sierra *et al*., 2019; Poeta *et al*., 2017). In this regard, current knowledge indicates that not only inter-ethnic variation and familial aggregation are sufficient to explain the complexity and heterogeneity of NAFLD, but also the strong genetic component plays a key role (Lonardo *et al*., 2021). Therefore, subjects genetically predisposed to adverse environmental conditions, such as smoking, air pollution and diet or other lifestyle factors

will be more prone to developing NAFLD (Juanola *et al*., 2021). In this context, genetic variants in *PNPLA3*, *TM6SF2*, *GCKR*, *MBOAT7*, and *HSD17B13* and epigenetics factors such as hypomethylation or miR-122 are the most studied factors affecting NAFLD.

On the other hand, the gut microbiota is specific to an individual (Jia *et al*., 2018). However, humans share similar functional gene profiles implying a core functional microbiome (Jiang X. *et al*., 2020). Moreover, increasing evidence points out an important role of the gut-liver axis (GLA) dysfunction and NAFLD (Kwong *et al*., 2021; Poeta *et al*., 2017). In general, diet and other environmental factors may induce intestinal dysbiosis with reduced production of short-chain fatty acids (SCFAs) and increased intestinal permeability (Juanola *et al*., 2021; Radziejewska *et al*., 2020). Bacterial translocation to the portal circulation, along with the interaction with toll-like receptors promotes gut dysmotility, resulting in activation of immune cells, hepatocytes and LSECs, and release of systematic inflammation and the consequent liver damage (Bajaj *et al*., 2014; Milosevic *et al*., 2019). Moreover, it has been established a link between a higher prevalence of Firmicutes and intestinal dysbiosis and obesity/NAFLD and a lower prevalence of Bacteroidetes when comparing NASH patients *vs*. obese patients without NASH (Doulberis *et al*., 2017).

1.4. NAFLD as a multi-system disease

The clinical burden of NAFLD is not limited to liver-related morbidity and mortality, but it involves a wide range of disorders that can increase its prevalence (Adams *et al*., 2017; Mantovani *et al*., 2020) (**Figure 3**).

In particular, extrahepatic complications such as insulin resistance and type 2 diabetes mellitus, metabolic and cardiovascular disorders, especially obesity, or chronic kidney disease, have been strongly associated with NAFLD (Godoy-Matos *et al*., 2020; Rosato *et al*., 2019). Also, recent studies have linked NAFLD to extrahepatic cancers, polycystic ovarian syndrome, psoriasis, obstructive apnea and a higher prevalence of hyperlipidemia/dyslipidemia and hypertension in these subjects, among others (Adams *et al*., 2017).

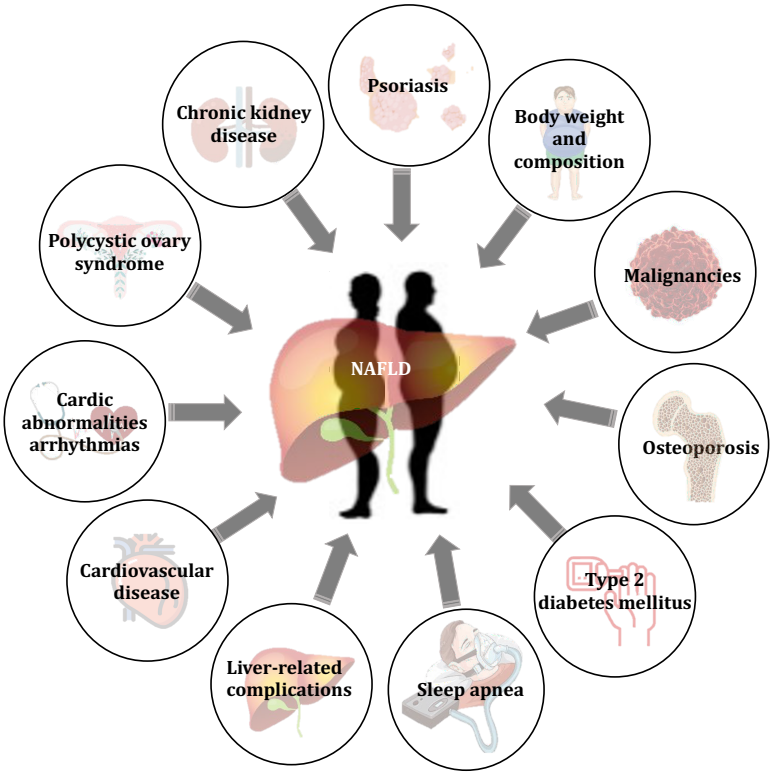

Figure 3. Comorbidities associated to non-alcoholic fatty liver disease. Adapted from Adams *et al.*, 2017 and Mantovani *et al.*, 2020. Abbreviations: NAFLD, Non-alcoholic Fatty Liver Disease.

Body weight and composition

The epidemic of obesity is continually increasing worldwide, as well as the obesity-related complications, including NAFLD (Estes, Anstee, *et al.*, 2018). The obesity is a recognized and well documented risk factor for NAFLD (Chalasani *et al.*, 2012). Overweight has been defined by the World Health Organization (WHO) as a body mass index (BMI) greater than or equal to 25 and obesity is defined as a BMI greater than or equal to 30. In fact, the prevalence of NAFLD is proportional to the increase in BMI, being 4.6-fold higher in the obese population and increasing to over 90% for very obese individuals undergoing weight reduction procedures and surgery (Abd El-Kader *et al.*, 2015; Younossi, 2019). Furthermore, despite differences in estimates, a large-scale European study found NAFL in 91% of obese patients (≥30 kg/m^2), 67% of overweight patients (BMI≥25 kg/m^2) and 24.5 % of normal weight patients (Bellentani *et al.*, 2004).

However, although most patients with NAFLD are overweight or obese, some of them may have a normal BMI (<25 kg/m^2 or <23 kg/m^2 in Asians) which is considered lean (Chen F. *et al.*, 2020; Semmler *et al.*, 2021). The prevalence of lean NAFLD in the United States was reported to be 7%, while in rural areas of some Asian countries it ranges from 25-30%, although it varies greatly depending on the used criteria (Chen F. *et al.*, 2020; Fan *et al.*,

2017; Younossi, 2018). Lean NAFLD patients can develop the full spectrum of liver damage, being therefore essential to understand the phenotype of this population (Younes *et al*., 2019; Younossi, 2019). Compared to healthy individuals, lean NAFLD is associated with metabolically obese and normal weight phenotypes, with increased visceral obesity, insulin resistance and the presence of metabolic dysfunction, and also with environmental factors, such as high fructose and fat intake, and with genetic risk factors, including congenital defects of metabolism (Chen F. *et al*., 2020; Younossi, Anstee, *et al*., 2018). Besides, lean NAFLD subjects are more prone to developing advanced stages of the disease, probably as a consequence of a more dysfunctional adipose tissue and increased insulin resistance (Lang *et al*., 2020; Pais *et al*., 2021).

In this sense, recent research has point out that the genetic background may play a central role on the observed differences in metabolism among normal-weight and overweight or obese subjects (Stefan *et al*., 2017; Younes *et al*., 2019). However, more studies are urgently needed to understand the natural history of this disease, but also to promote an accurate definition and therapeutic options for lean NAFLD (Younossi, Anstee, *et al*., 2018).

Insulin resistance and type 2 diabetes mellitus

Insulin resistance (IR) is defined as the inability of exogenous or endogenous insulin to increase glucose uptake and utilization (Lebovitz, 2001). The key hypothetical mechanism that linked IR and NAFLD implicated an increase in free fatty acids (FFAs) availability involving different adipokine-controlled pathways such as adiponectin, leptin, and increased mitochondrial β-oxidation in response not only to the increased lipogenesis but also to hyperglycemia (Armandi, Rosso, *et al*., 2021). In addition, the adipose tissue inflammation promoted by cytokines (interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α)), generates toxic lipid intermediators and leads to impaired insulin signaling (Khan *et al*., 2019).

Type 2 diabetes mellitus (T2DM) is an insulin-resistant condition which is also increasing in prevalence worldwide (Goyal *et al*., 2016; Younossi, 2019). The prevalence of NAFLD in patients with T2DM is almost 75% (Adams *et al*., 2017), whereas 25% of patients with NAFLD have T2DM (Kwok *et al*., 2016). Moreover, multiple large cohort studies with a median follow-up period of at least 5 years have evidenced an association between NAFLD and 1.5–2-fold increased risk of new-onset T2DM, typically higher in men (Adams *et al*., 2017; European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO), 2016).

Additionally, diabetic patients have almost 3 to 5-fold higher risk to be hospitalized or die due to chronic liver disease related to NAFLD (Pais *et al*., 2021). This association is clinically relevant since T2DM represented an important risk factor for progression to NASH, cirrhosis, and mortality (Estes, Razavi, *et al*., 2018; Younossi *et al*., 2020).

Metabolic syndrome features

Metabolic syndrome (MetS) is a clinical entity of substantial heterogeneity, commonly represented by the combination of obesity, hyperglycemia with insulin resistance, dyslipidemia and/or hypertension with increased risk of cardiovascular disease (Jarvis *et al*., 2020). Indeed, in individuals without a genetic predisposition, NAFLD is increasingly recognized as the liver disease component of metabolic syndrome (Kang *et al*., 2006). Given the growing evidence supporting a strong and bidirectional association between NAFLD and MetS, several studies have proposed that visceral obesity, insulin resistance and subclinical inflammation appear to play an important role in the pathogenesis of both conditions (Bugianesi *et al*., 2010; Yang *et al*., 2016).

The estimated prevalence of MetS among subjects with NAFLD and NASH was 42.54% and 70.65%, respectively (Younossi *et al*., 2016). Furthermore, a cohort of 271,906 patients showed that the addition of metabolic components increased the risk of cirrhosis and HCC in patients with NAFLD, revealing that the clinical and economic burden of NAFLD could be enormous (Kanwal *et al*., 2020).

Cardiovascular disease

The association of cardiovascular disease (CVD) and NAFLD is well establish, even though the underlying mechanism remains incomplete and speculative (Muzurović *et al*., 2021). Common risk factors of CVD such as obesity, insulin resistance and/or type 2 diabetes, hypertriglyceridemia or hypertension, among others, often characterize NAFLD patients (Younossi *et al*., 2016). Moreover, there is growing evidence that having NAFLD/NASH can increase the risk of CVD, through the release of pro-atherogenic factors (Niederseer *et al*., 2021; Targher *et al*., 2008). Indeed, while liver-related complications are a major cause of mortality in NAFLD, CVD counts for at least 40% of all deaths, especially heart-related deaths, which are a leading cause of death in NAFLD (Muzurović *et al*., 2021; Przybyszewski *et al*., 2021). In this regard, screening for NAFLD in patients with CVD has been proposed (Francque *et al*., 2016). However, long-term data are needed to confirm whether improving liver condition will have an impact on the occurrence of incident cardiovascular events (Pais *et al*., 2021).

Chronic kidney disease

NAFLD and chronic kidney disease (CKD) share multiple cardiometabolic risk factors, existing an independent link among both diseases (Abbate *et al*., 2021). CKD is defined by abnormalities of kidney structure or function present for ≥3 months, with a complex and progressive chronic condition and serious implications for health (Andrassy, 2013). CKD is characterized by the presence of an estimated glomerular filtration rate (eGRF) <60 ml/min/1.73 m^2, albumin-to-creatinine ratio ≥ 30mg/g, as well as the presence of other markers of kidney damage. In this sense, NAFLD patients are likely to have elevated albuminuria levels, with a higher prevalence of CKD in patients with NAFLD (20-50%), compared to patients without NAFLD (5-30%) (Abbate *et al*., 2021; Targher *et al*., 2017). Moreover, data of a meta-analysis involving nearly 30,000 individuals showed that NAFLD was associated with a 2-fold increased prevalence of CKD (Musso *et al*., 2014). Also, it has been recently published that participants with magnetic resonance imaging-proven NAFLD had a worse metabolic profile and higher levels of the estimated glomerular filtration rate than those without NAFLD (Abbate *et al*., 2021), providing new insights for earlier identification of patients at increased risk of CKD.

1.5. Diagnosis

The majority of subjects with NAFLD are asymptomatic and are diagnosed incidentally (Younossi, Loomba, *et al*., 2018). It is important that coexisting causes of chronic liver disease or other etiologies for steatosis are excluded before initiating the diagnostic evaluation of NAFLD. Once other causes of steatosis have been ruled out, NAFLD should be considered.

According to the American Association for the Study of Liver Diseases (AASLD), the diagnosis of NAFLD requires the following conditions (Chalasani *et al*., 2018):

a) The presence of hepatic steatosis by imaging or histology
b) There is no significant alcohol consumption (>21 standard units per week in men and >14 standard units per week in women)
c) Absence of competing etiologies for hepatic steatosis
d) There are no coexisting causes of chronic liver disease

Moreover, the presence of metabolic syndrome is a strong predictor for the presence of steatohepatitis in patients with NAFLD and may be used to best identify patients (Yang *et al*., 2016). In this sense, the initial evaluation of patients should carefully consider the presence of commonly associated comorbidities such as obesity, dyslipidemia, insulin resistance or diabetes or chronic kidney disease (Adams *et al*., 2017).

Liver biopsy is currently the *gold standard* for the diagnosis of NAFLD (Chalasani *et al*., 2018; Tsai *et al*., 2018), being able to identify not only the presence of liver fat stored, but also differentiate simple steatosis from NASH (Merriman *et al*., 2006). The diagnosis of NAFLD by liver biopsy includes the analysis of distinctive histopathological characteristic features including the presence of fat shown as hepatocellular triglyceride accumulation, hepatocellular injury in the centrilobular location, cytoskeletal damage presented as hepatocellular ballooning, parenchymal inflammation with predominance of lymphocytes and macrophages, even though neutrophils may be present in advance stages and perisinusoidal fibrosis shown as collagen deposition in the space of Disse (Younossi, Loomba, *et al*., 2018).

However, the used of this technique is limited by its low acceptability among patients and its invasive nature with potential risk of complications such as high morbidity, pain and mortality (Kogachi *et al*., 2021). Other limitations include cost, sampling error and differences in variability (Isabela Andronescu *et al*., 2018).

Because of the limitations of liver biopsy there is an urgent and growing interest in the development of alternative methods for the diagnosis of NAFLD (Lonardo *et al*., 2021; Zhou J.-H.H. *et al*., 2019). In clinical practice, a number of serum markers, scores and imaging techniques are currently under investigation and are widely applied instead of biopsy (Neuman *et al*., 2014; Younossi *et al*., 2021). Non-invasive markers of NAFLD should aim (Chalasani *et al*., 2018; European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO), 2016):

a) To identify the risk of NAFLD among individuals with increased metabolic risk, in primary care settings
b) To identify subjects with worse prognosis in secondary and tertiary care settings
c) To monitor disease progression
d) To predict the future response to therapeutic interventions

Imaging techniques

Conventional ultrasound imaging technique is the first-line modality employed when hepatic steatosis is suspected where a qualitative analysis is performed (Lee S.S. *et al*., 2014). The severity of the liver steatosis is based on the echogenicity of the liver parenchyma, the distinction of intrahepatic vessels and the visibility of the right diaphragm and is classified into mild, moderate, and severe or using ordinal ultrasonography scores (Lee S.S. *et al*., 2014; Stefan *et al*., 2019). However, it is not effective in detecting the early stages of steatosis, must be interpreted by a professional and has limited use in morbidly obese individuals (Zhou J.-H.H. *et al*., 2019).

In this context, magnetic resonance imaging (MRI) techniques have proven to be a better reference standard for the amount of fat in the liver than histological evaluation, due to the high accuracy and reproducibility of these techniques (Tsai *et al*., 2018; Zhou J.-H.H. *et al*., 2019). Magnetic Resonance Imaging derived proton density fat fraction (MRI-PDFF) is the preferred analytical method for quantification of liver fat and iron content (Kogachi *et al*., 2021; Trujillo *et al*., 2021). However, it is not a routinely test due to the high cost and time involved (Younossi, Loomba, *et al*., 2018). Indeed, this technique is unable to assess liver inflammation, ballooning, or resolution of NASH or improvement in fibrosis (Thiagarajan *et al*., 2021). Magnetic resonance spectroscopy (MRS) is another magnetic resonance-based technique that directly measures the chemical compositions of the liver, even though its usage is limited because of its complexity and potentially sampling error (Zhou J.-H.H. *et al*., 2019). Other techniques include the controlled attenuation parameter (CAP), which is measured through Fibroscan® and the computerized tomography (CT) that has been used in clinics to evaluate the severity of hepatic fat content since 1970, even if it is also limited by insufficient accuracy for mild-to-moderate hepatic steatosis and it is not recommended to use in a "routine manner" because of the radiation exposure (Trujillo *et al*., 2021; Zhou J.-H.H. *et al*., 2019).

Importantly, hepatic steatosis can progress to fibrosis and cirrhosis and increases the risk of liver-related mortality (Younossi *et al*., 2017). Therefore, it is clinically important to diagnose the development of NASH in patients with NAFLD (Ramai *et al*., 2021). One of the most accurate non-invasive methods to assess the liver stiffness is transient elastography (TE), through Fibroscan®, which consisted in the measured of the propagation speed of elastic waves through hepatic tissue, allowing to determinate fibrosis degree (Tsai *et al*., 2018; Younossi, Loomba, *et al*., 2018). TE is not a difficult procedure to learn and can be performed by a nurse or a technician after minimal training and includes other advantages

such as a short procedure time with immediate results (Boursier *et al*., 2008). Furthermore, emerging techniques such as 2-dimensional shear wave elastography (2D-SWE) and acoustic radiation force impulse imaging (ARFI) have demonstrated successful results in the identification of liver fibrosis (Castera *et al*., 2019; Park C.C. *et al*., 2017).

Serum biomarkers and scores

In addition to non-invasive test based on imaging modalities, there has been an increase in instruments using predictive algorithms or serum biomarkers (Marchisello *et al*., 2019). Practical advantages of these techniques include their high applicability for disease progression and/or determine its severity, their good interlaboratory reproducibility and their potential widespread availability (Lonardo *et al*., 2021). As such, a prognostic indicator could be used for risk stratification of the general population.

A first-level assessment will include common anthropometric indices such as BMI (weight (kg)/heigh $(m)^2$) or waist circumference assessment (R Rocha *et al*., 2005). Also, blood pressure should be recorded, given that hypertension is a risk factor for the progression of liver fibrosis (Oikonomou *et al*., 2018). Other common markers are alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST), especially in patients with NASH (Chalasani *et al*., 2018; Marti del Moral *et al*., 2018). However, liver enzymes are not considerate *per se* as a precise and accurate marker of NAFLD (Castera *et al*., 2019). In fact, a recent case-control study evidence that patients with T2DM and normal transaminase levels were diagnosed with NAFLD, grade 3 steatosis and advanced hepatic fibrosis (Makker *et al*., 2021). Moreover, almost 80% of subject with fatty liver in cohort studies have shown ALT levels within normal limits (Mofrad *et al*., 2003). Thus, the consideration of all these risk factors into algorithms could be a useful tool for a more precise and minimally invasive screening (Younossi, Loomba, *et al*., 2018).

Given the importance of an early detection of NAFLD, several scores have been proposed for the detection of both steatosis and fibrosis. On the one hand, some of the most used indexes and scores used for the detection of steatosis are: The Hepatic steatosis index (HSI), the Fatty liver index (FLI), the SteatoTest, and the NAFLD liver fat score (**Table 1**) (Kogachi *et al*., 2021; Zhou J.-H.H. *et al*., 2019).

Table 1: Description and accuracy of commonly used indexes and scores for diagnosing hepatic steatosis in subjects with non-alcoholic fatty liver disease

Index	Components	Accuracy
HSI	BMI, diabetes, AST/ALT	AUROC 0.81 HFC
FLI	Triglycerides, BMI, GGT, waist circumference	AUROC 0.84 HFC
SteatoTest	α2-MG, haptoglobin, apolipoprotein A1, total bilirubin, GGT, fastin glucose, triglycerides, colesterol, ALT, age, sex and BMI	AUROC 0.80 HFC
NAFLD LFS	Insulin, AST, AST/ALT, T2DM, MetS	AUROC 0.87 HFC

Adapted from Kogachi *et al.*, 2021. Abbreviations: α2-MG, Alpha-2 Macroglobin; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; AUROC, Area Under the Receiver-Operating Characteristics Curve; BMI, Body Mass Index; FLI, Fatty Liver Index; GGT, Gamma-Glutamyl Transferase; HFC, Hepatic Fat Content; HSI, Hepatic Steatosis Index; MetS, Metabolic Syndrome; NAFLD-LFS, Non-alcoholic Fatty Liver Disease-Liver Fat Score; T2DM, Type 2 Diabetes Mellitus; WC, Waist Circumference.

On the other hand, 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 score (BMI, aspartate aminotransferase/alanine aminotransferase ratio, diabetes), or the NAFLD fibrosis score (NFS) **(Table 2**) (Castera *et al.*, 2019; Kogachi *et al.*, 2021; Zhou J.-H.H. *et al.*, 2019).

Table 2: Description and accuracy of commonly used indexes and scores for diagnosing fibrosis in subjects with non-alcoholic fatty liver disease

Index	Components	Accuracy
FIB-4	Age, AST, ALT and platelet count	AUROC 0.75 for SF, 0.80 for AF, and 0.85 for cirrhosis
APRI	AST/platelet ratio index	AUROC 0.70 for SF, 0.75 for AF, and 0.75 for cirrhosis
BARD score	AST, ALT, BMI and diabetes	AUROC 0.64 for SF, 0.73 for AF, and 0.70 for cirrhosis
NFS	Age, BMI, impaired fasting glucose and/or diabetes, AST, ALT, platelet count and albumin	AUROC 0.72 for SF, 0.73 for AF, and 0.83 for cirrhosis

Adapted from Zhou *et al.*, 2019. Abbreviations: AF, Advanced Fibrosis; ALT, Alanine Aminotransferase; APRI, AST/Platelet Ratio Index; AST, Aspartate Aminotransferase; AUROC, Area Under the Receiver-Operating Characteristics Curve; BARD score, Body Mass Index, aspartate aminotransferase/alanine aminotransferase ratio, diabetes; BMI, Body Mass Index; FIB-4, Fibrosis-4; NFS, Non-alcoholic Fatty Liver Disease Fibrosis Score; SF, Significant Fibrosis.

Serum biomarkers have also been described for diagnosing NAFLD, grading steatosis or fibrosis, one of the most investigated markers of apoptosis is Cytokeratin 18 (CK-18), as well as predictive models such as FibroTest, Fibrometer and the Hepascore (Castera *et al*., 2019; Shen *et al*., 2012).

Other inflammatory markers, adipocytokines and hormones have also been studied as potential targets for NAFLD, including adiponectin, leptin, 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 (Caviglia *et al*., 2021; Meex *et al*., 2017). Other common inflammatory markers are TNF-α and Interleukin 8 (IL-8) (Kogachi *et al*., 2021).

Indeed, serum iron is a common protein associated with oxygen radicals, which contributes to necroinflammation and fibrosis, two important parameters of NAFL (Datz *et al*., 2017). Despite the absence of clear-cut data, given that hepatic iron overload is strictly associated with insulin sensitivity it may be logical to record the levels of ferritin in order to predict NASH (Yoneda *et al*., 2010). In this sense, an association of serum ferritin with liver health (ALT, liver fat content and hepatic iron), as well as with glucose and lipid metabolism markers was observed in subjects with NAFLD, suggesting that ferritin may be a potential biomarker of this liver disease (Galarregui, Marin-Alejandre, *et al*., 2020).

However, some of these modalities remain highly experimental and need further validation in future studies (Dongiovanni *et al*., 2021).

Omics-based markers and Genetic Risk Scores

There have been recent efforts in using "-omics" approaches for identifying novel biomarkers of NAFLD, NASH, and advanced fibrosis (Neuman *et al*., 2014). These techniques include proteomics, transcriptomics, metabolomics, genomics and other novel markers such as the bacterial microbiome (Castillo-Castro *et al*., 2021; Pirola *et al*., 2018).

Recent studies have shown that there are differences in protein expression between patients with NAFLD and healthy controls. For example, studies focusing on the bioavailability of circulating extracellular vesicles have proposed them as a potential biomarker for various disease such as cancer, cardiovascular disease, renal disease and liver disease (Castillo-Castro *et al*., 2021). Also, specific genetic markers such as circulating microRNAs (miR-122 and miR-34, among others) seem to be a potential and attractive biomarker under study for NAFLD severity (Castillo-Castro *et al*., 2021; Zhou J.-H.H. *et al*., 2019).

Moreover, technological advances in metabolomic analyses on feces, serum, plasma and urine have allowed the identification of specific metabolites in patients with NAFLD or advanced stages (Castillo-Castro *et al*., 2021). For example, an initial case-control studies on plasma metabolomics of NAFLD demonstrated that the level of 11-HETE, a nonenzymatic oxidation product of arachidonic acid, was significantly increased in NASH but not in NAFL patients (Puri *et al*., 2009). In this sense, the OWLiver®-test is a validated metabolomic method, for the evaluation of fatty liver and diagnosis of NAFLD, and is based on a prospective study, where subjects had previously been diagnosed by liver biopsy (Alonso *et al*., 2017).

Lastly, in the last decade, genome wide association studies (GWASs) have identified a large number of NAFLD susceptibility polymorphism. However, a great deal of the heritability remains unknown (Anstee *et al*., 2013; Sookoian *et al*., 2019). Several studies have indicated that this missing heritability is the interaction of genetic, epigenetic and environmental risk factors. In this context, genetic risk-allele scores (GRSs) are one of the most common approaches to assess the cumulative effect of many genetic factors with a small effect, with or without non-genetic clinical factors (Igo *et al*., 2019; Xian *et al*., 2020).

The simple way to calculate a GRS is by summing the number of accumulated risk alleles associated with the disease (Dongiovanni *et al*., 2015; Igo *et al*., 2019). Using this method, Nobili *et al*., 2014 showed that a genetic risk score was able to significantly predicts NASH in obese children with increased liver enzyme and biopsy proven NAFLD. Moreover, a GRS combining single nucleotide polymorphisms (SNPs) near/in *PNPLA3*, *LYPLAL1*, *PPP1R3B* and *GCKR* was associated with higher hepatic fat content, steatosis stage and higher ALT levels in obsess Mexican individuals (León-Mimila *et al*., 2015). Recently, a genetic risk score including variants in the *PNPLA3*, *TM6SF2*, *HSD17B13* and GCKR genes have found a significant association with steatosis, steatohepatitis and fibrosis in well-histologically characterized and large cohort of NAFLD subject (Anstee *et al*., 2021).

Another approach to calculate a GRS is performing a weighed genetic risk score, under which a sum of risk alleles is calculated from pre-selected number of SNPs reported by previous GWASs, in order to define a person´s individual genetic risk for the development of the disease (Dudbridge, 2013; Hüls *et al*., 2017). In this sense, a 4-SNPs (rs1260326 *GCKR*, rs58542926 *TM6SF2*, rs738409 *PNPLA3* and rs641738 *MBOAT7*) genetic risk score was associated with a 3-fold increased risk of NAFLD in a cohort of 218 NAFLD and 227 controls subjects (Di Costanzo *et al*., 2018). Also, a 11-SNP weighted genetic risk score (rs738409 (*PNPLA3*), rs58542926 (*TM6SF2*), rs1260326 (*GCKR*), rs2236212 (*ELOVL2*), rs116454156

(*GPR120*), rs1535 (F*ADS2*), rs13412852 (*LPIN1*), rs641738 (*MBOAT7*), rs1800591 (*MTTP*), rs3750861 (*KLF6*) and rs4880 (*SOD2*) combined with established risk factors improved risk prediction for NAFLD in obese children and adolescents (Zusi *et al*., 2019).

Indeed, mounting evidence indicates that epigenetic factors such as differential DNA methylation and circulating cell-free DNA methylation signatures in plasma may also potentially stratify patients with NAFLD (Hardy *et al*., 2017).

Therefore, the integration of analyses performed through OMICs tools represents a very interesting and useful approach to stratify the risk of disease progression in patients already diagnosed with NAFLD, as well as appropriate follow up (Castera *et al*., 2019; Pirola *et al*., 2018).

2. Heritability of NAFLD

NAFLD is a heritable and polygenic disease with complex traits. Data derived from epidemiological studies, familial clustering and twin studies have provided strong evidence for the heritability of NAFLD and NASH (Loomba *et al*., 2015; Sookoian *et al*., 2020). Depending on ethnicity, study design, environmental factors and the methodology used for NAFLD determinations, the estimate heritability ranges from 30 to 75% (Cui *et al*., 2016; Ratziu *et al*., 2020; Sookoian *et al*., 2020).

Similar ranges of heritability have been observed for other related factors such as BMI, T2DM and CVD, among others (Bouchard, 2021; Himanshu *et al*., 2020; Jansweijer *et al*., 2019). These findings, together with their close interrelationships, highlighted the importance of studying the genetic component of NAFLD (Sookoian *et al*., 2012). Two types of genetic studies are available in patients with NAFLD: the candidate gene studies and the genome wide-association studies (GWASs).

2.1. Candidate gene studies

The candidate gene studies are hypothesis-testing studies, which are done for a gene with known functions (Anstee *et al*., 2013; Pelusi *et al*., 2019). This type of studies looks for differences concerning a polymorphism between cases and controls with a small sample size (Lewis *et al*., 2012; Macaluso *et al*., 2015). Genetic associations based on these studies could be specific to NAFLD or non-specific related to inflammation, oxidative stress, insulin resistance or fibrosis as shown in **Table 3** (Choudhary *et al*., 2021; Dongiovanni *et al*., 2016; Eslam *et al*., 2018)**.**

Table 3. Examples of gene-candidate studies associated with the development and progression of NAFLD

Genes	Polymorphism	Function	Phenotype
Glucose metabolism and insulin resistance			
ENPP1	rs1044498 A>C	Insulin signaling inhibitor	↑ Fibrosis
IRS1	rs1801278 A>C	Insulin signaling	↑ Fibrosis
GCKR	rs780094 A>G and rs1260326 C>T	Regulation of de novo lipogenesis	↑ NAFLD, NASH and fibrosis
SLC2A1	Several	Promotes lipid accumulation and oxidative stress	↑ NAFLD
TCF7L2	rs7903146 C>T	Adipocyte metabolism and lipid homeostasis	↑ NAFLD
Lipid metabolism			
PNPLA3	rs738409 C>G	Lipid droplets remodeling	↑ NAFLD, NASH, fibrosis and HCC
TM6SF2	rs58542926 C>T	VLDL secretion	↑ NAFLD, NASH, fibrosis and HCC
MBOTAT	rs641738 C>T	Phosphatidylinositol remodeling	↑ NAFLD, NASH and fibrosis
LYPLAL1	rs12137855 C>T	Triglycerides catabolism	↑ NAFLD
LPIN1	rs13412852 C>T	Regulation of lipid metabolism	↓ NASH, fibrosis
NR1I2	rs7643645 A>G and rs2461823 C>T	Regulation of genes involved in xenobiotics metabolism	↑ NAFLD severity
PPAR α	rs1800234 T>C	TG accumulation by increasing fatty acid oxidation	↑ Steatosis, inflammation and fibrosis
PEMT	rs7946 G>A	Enzyme catalytic of de novo lipogenesis of choline	↑ NAFLD
HSD17B13	rs6834314 A>G	Lipid droplet remodeling. Retinol metabolism	↑ Steatosis ↓ Severity NAFLD
MTTP	Several	VLDL secretion	↑ NAFLD
APOB	Several	VLDL secretion	↑ NAFLD, NASH, fibrosis and HCC
Fibrosis			
AGTR1	rs3772622 A>G	Cholesterol handling	↑ NASH and fibrosis
Oxidative stress			
GCLC	rs17883901 G>A	Limiting enzyme in the formation of glutathione	↑ NASH
SOD2	rs4880 C>T	Mitochondrial antioxidant	↑ Fibrosis
UCP2	rs695366 G>A	Oxidative phosphorylation	↓ NASH
Immune response			
TNF	rs361525 G>A and rs1800629 G>A	Inmune response	↑ NAFLD, NASH
CD14	−159 C >T	Inmune response	↑ NAFLD
IL28B	rs12979860 C>T	Innate immunity	↓ Fibrosis
MERTK	rs4374383 G>A	Innate immunity Hepatic Stellate cells activation	↓ Fibrosis
Irisin	rs3480 A>G	Hepatic Stellate cells activation	↓ Fibrosis
Others			
CDKN1A	rs762623 G>A	Rate of disease progression	↑ NAFLD
KLF6	rs3750861 G>A	Regulation of de novo lipogenesis; fibrogenesis	↓ Fibrosis

Adapted from: Choudhary *et al*., 2021; Eslam *et al*., 2018 and Dongiovanni *et al*., 2016. Abbreviations: *AGTR1*, Type-1 angiotensin 2; dehydrogenase 13; *CD14*, Cluster of differentiation 14; *CDKN1A*, Cyclin-dependent kinase inhibitor 1A; *ENPP1*, Ectoenzyme nucleotide pyrophosphate phosphodiesterase 1; *FADS1*, Fatty acid desaturase 1; *GCKR*, Glucokinase regulatory protein; *GCLC*, Glutamate-cysteine ligase catalytic subunit; HCC, Hepatocellular Carcinoma; *HSD17B13*, 17-Beta hydroxysteroid dehydrogenase 13; *IRS1*, Insulin-receptor substrate 1; *KLF6*, Kruppel-like factor; *LPIN1*, Lipin 1; *LYPLAL1*, Lysophospholipase like 1; *MBOTAT*, Membrane-bound O-acyltransferase domain-containing 7; NAFLD, Non-alcoholic Fatty Liver Disease; NASH, Non-alcoholic Steatohepatitis; NR1I2, Nuclear receptor

subfamily 1 group I member 2; *PEMT*, Phosphatidylethanolamine N-methyltransferase; *PNPLA3*, Patatin-like phospholipase domain-containing protein 3; *PPAR alpha*, Peroxisome proliferator-activated receptor alpha; *SH2B1*, Src-homology-2 B adaptor protein 1; *SLC2A1*, Solute carrier family 2 member 1; *SOD2*, Superoxide dismutase 2; *TCF7L2*, Transcription factor 7-like 2; *TM6SF2*, Transmembrane 6 superfamily 2; *TNF*, Tumor necrosis factor; *UCP2*, Uncoupling protein 2.

The main association uncovered by these studies is between the Patatin-like phospholipase domain–containing 3 (*PNPLA3*) *I148* variant (rs738409) and NAFLD, which has been linked to increased liver fat content without a significant direct effect on body weight and insulin resistance (Krawczyk *et al*., 2020; Park S.L. *et al*., 2020; Romeo *et al*., 2008; Trépo *et al*., 2020). Moreover, it has also be unexpectedly associated with an apparent protection from cardiovascular disease (Santos *et al*., 2019; Stefan *et al*., 2019). Furthermore, in a human exome-wide association study (Kozlitina *et al*., 2014), the rs58542926 genetic variant in the transmembrane 6 superfamily member (*TM6SF2*) *E167K* was associated with steatosis development and progression (to NASH and cirrhosis), but also with defective in the hepatic very low-density lipoprotein secretion pathway and reduced low-density lipoprotein levels (Ko, 2019; Pirola *et al*., 2015).

Other discovery in variants of the *MBOAT7* (membrane bound O-acyltransferase domain-containing 7) and *GCKR* (Glucokinase regulatory protein) genes have been also linked to increased risk of NAFLD (Buch *et al*., 2015; Eslam *et al*., 2018; Ko, 2019). The rs641738 *MBOAT7* variant is involved in phospholipid remodeling, while the rs780094 *GCKR* variant may affect *de novo* lipogenesis by regulating the influx of glucose in hepatocytes (Ko, 2019; Santoro *et al*., 2012; Stefan *et al*., 2019; Umano *et al*., 2018). One of the latest additions to the genes that contribute to NAFLD is the Hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) gene, which has been identified as a lipid droplet-associated protein with retinol dehydrogenase activity (Carlsson *et al*., 2020; Martin *et al*., 2021; Trépo *et al*., 2020).

Other genes that have been proposed to be linked to specific pathways include: ecronucleotide pyrophophatase (*ENPP1*), insulin-receptor substrate 1 (*IRS1*), solute carrier family 2 member 1 (*SLC2A1*) and the transcription factor 7-like 2 (*TCF7L2*), which are involved in glucose metabolism and are related to insulin resistance, and also, genes involved in lipid metabolism, such as the lysophospholipase like 1 (*LYPLAL1*), lipin 1(*LPIN1*), nuclear receptor subfamily 1 group I member 2 (*NR1I2*), peroxisome proliferator-activated receptor alpha (*PPAR alpha*) and the 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*), suggesting a potential role of these genes in non-alcoholic fatty liver disease pathogenesis (Choudhary *et al*., 2021; Macaluso *et al*., 2015). Besides, genes associated with oxidative stress (Glutamate-Cysteine Ligase Catalytic Subunit *(GCLC)*, and uncoupling protein 2 (*UCP2*) and immune response (Tumor necrosis factor *(TNF)*, cluster of

differentiation 14 (*CD14*) have also been proposed to be considered in NAFLD pathology (Choudhary *et al*., 2021; Di Rosa *et al*., 2012).

Moreover, only a few genes have been associated with NAFLD through candidate-gene analysis and independently validated in large independent studies or through the use of transmission disequilibrium testing (Brunt *et al*., 2015). These genes included superoxide dismutase 2 (*SOD2*) (Al-Serri *et al*., 2012), phosphatidylethanolamine N-methyltransferase (*PEMT*) (Dong *et al*., 2007), fatty acid desaturase 1 (*FADS1*) and kruppel-like factor-6 (*KLF6*) (Miele *et al*., 2008), even though they were associated with progressive NAFLD rather than NAFLD *per se*. Although some drawbacks of this type of studies such as the small sample size or the possibility of find new genetic associations can limit the success of the research, candidate gene studies continue to contribute to our understanding of the genetic basis of NAFLD (Lewis *et al*., 2012).

2.2. Genome wide association studies

Owing to test genetic variants across the genomes of many individuals and in order to identify genotype-phenotype associations, GWASs have revolutionized the field of complex disease genetics over the past decade (Wang W.Y.S. *et al*., 2005). The GWASs approach is a high-throughput methodology for scanning and detecting a large number of SNP markers across the entire genome, where genotyping can be performed using SNP-arrays combined with imputation or shole genome sequency (Hindorff *et al*., 2009). Moreover, it allows to identify novel variant-trait associations leading to discovery novel biological mechanisms (Tam *et al*., 2019).

In this sense, several GWASs have consistently shown strong and reproducible associations between a set of genetic variants and NAFLD development and severity (**Table 4**). Specifically, this list includes a search in the GWAS Catalog (https://www.ebi.ac.uk/gwas/) using the "Non-alcoholic fatty liver disease" search string, revealing 13 meta-analyses of GWASs related to NAFLD carried out in children, adolescents and/or adults since 2010.

Table 4. NAFLD meta-analyses of GWASs

Reference	Population (n)	NAFLD phenotype	Association count
(Chalasani *et al*., 2010)	European (236)	NAFLD, serum ALT and cirrhosis	35
(Speliotes *et al*., 2011)	European (7,176)	NAFLD	1
(Kawaguchi *et al*., 2012)	East Asian (1,461)	NAFLD	5
(Adams *et al*., 2013)	European (928)	NAFLD	4
(Kitamoto *et al*., 2013)	East Asian (1,326)	NAFLD, cirrhosis	4
(Kozlitina *et al*., 2014)	Hispanic or Latin American; African American or Afro-Caribbean; European (4,708)	NAFLD	2
(Wattacheril *et al*., 2017)	Hispanic or Latin American (208)	NASH and hepatic fibrosis	7
(Chung G.E. *et al*., 2018)	East Asian (4,409)	NAFLD	2
(Kawaguchi *et al*., 2018)	East Asian (8,574)	NAFLD, NASH and HCC	10
(Namjou *et al*., 2019)	European (9,677)	NAFLD, hepatic fibrosis and serum AST	70
(Anstee *et al*., 2021)	European (19,264)	NAFLD, NASH and hepatic fibrosis	10
(Yoshida *et al*., 2020)	East Asian (1,686)	NAFLD	4
(Park S.L. *et al*., 2020)	African American or Afro-Caribbean; East Asian Hispanic or Latin American; Oceanian and European (1,529)	NAFLD	8

Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GWASs, Genome Wide Association Studies; NAFLD, Non-alcoholic Fatty Liver Disease; NASH, Non-alcoholic Steatohepatitis; HCC, Hepatocellular Carcinoma.

Patatin-like phospholipase domain-containing protein 3 was the first locus to be associated with ethnic and inter-individual differences in hepatic fat content and susceptibility to NAFLD (Romeo *et al*., 2008). Following the strong association of this variant joint to the SNP in *TM6SF2*, originally ascribed to the neurocan *(NCAN)* gene, investigators enhances GWASs in order to find new associations and increase the statistical power in the analysis of the polymorphisms (Anstee *et al*., 2021; Chalasani *et al*., 2010).

In this context, a genome wide association carried out in a European cohort was able to identify significant associations with histologic NAFLD in variants in or near *NCAN*, glucokinase regulatory protein (*GCKR*), *LYPLAL1* and patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) that have distinct effects on metabolic traits (Speliotes *et al*., 2011). In addition, a GWASs conducted in both adult and pediatric participants from the electronic medical records and genomics (eMERGE) network, reported novel loci for NAFLD disease severity including Interleukin 17 receptor A (*IL17RA*) and Zinc finger protein 90-Cadherin 1(*ZFP90-CDH1*) genes and more than 300 genes that were used for functional and pathway enrichment analysis (Namjou *et al*., 2019).

Although European ancestry is to date the most studied population in meta-analysis GWASs concerning NAFLD, there are some investigations that have been performed in Asian, Hispanic, African, African American or Native American populations. Results of a meta-analysis in a Japanese population confirmed the strong association between *PNPLA3* gene and the progression of NASH (Kawaguchi *et al*., 2012). Moreover, a different meta-analysis in Asian population found the previously identified loci in *PNPLA3*, as well as two new loci in Sorting and assembly machinery component 50 homolog (*SAMM50*) and parvin beta (*PARVB*) genes (Kitamoto *et al*., 2013).

Recently, it has been performed the largest histology-based NAFLD GWAS to date in a cohort of 1,483 European patients exhibiting the full spectrum of biopsy-proven NAFLD. This GWAS reported for the first time the combination of chromosome [chr] 2 *GCKR*/C2ORF16; chr4 *HSD17B13*; chr19 *TM6SF2*; chr22 *PNPLA3* as NAFLD risk modifiers (Anstee *et al*., 2021). Moreover, this study identified 2 other signals near leptin receptor (*LEPR*), Indoleamine 2,3-dioxygenase 2 (IDO2/TC1), phospholipase A2 group IVA (*PLA2G4A*) and Pygopus Family PHD Finger 1 (*PYGO1)* genes. Lastly, the latest GWASs meta-analysis, comprising older adults form five US racial/ethnic groups, added a new novel association between liver fat percentage and rs77249491 *LMBR1* domain containing 1 genetic variant (Park S.L. *et al*., 2020).

2.3. Genetic pleiotropy: NAFLD and obesity

The prevalence of NAFLD is increasing in line with obesity, with visceral adiposity being a major risk factor for NAFLD (Younossi, 2019). Generalizing, obesity is attributed to a chronic positive energy balance; arising when individuals consume more energy than they expend and is maintained over time (González-Muniesa *et al*., 2017; Martinez, 2000). However, not all individuals exposed to the same environmental risk factors will develop obesity, so several factors have been suggested to influence the equation (Bouchard, 2021; Silventoinen *et al*., 2017). Moreover, both environmental and genetic factors are involved in the development of increased fat in the liver, resulting in approximately 25–70% of body weight variability (Lonardo *et al*., 2017, 2021). Therefore, because of the close relationship of obesity and NAFLD, it is plausible that both pathologies share some of the genetic predisposition pathways (Eslam *et al*., 2016; Sookoian *et al*., 2019).

In this sense, authors of the candidate genes studies have identified several genetic variants involved in metabolic and inflammatory pathways that could be associated with NAFLD (Choudhary *et al*., 2021; Di Rosa *et al*., 2012). The effect of one gene on different phenotypes

is known as pleiotropy (Solovieff *et al*., 2013). There are two main forms of biological pleiotropy, the genic pleiotropy term refers to the altered function of a gene that influences multiple traits, while allelic pleiotropy refers to the effect of one variant influencing multiple traits (Eslam *et al*., 2020).

Thus, genome-wide association of complex diseases have demonstrated that a large number of SNPs are implicated in the susceptibility of multiple traits (Pirola *et al*., 2021). This implies the existence of "missing heritability" due to the existence of rare variants or common variants that do not reached genome wide significance levels (Boyle *et al*., 2017; Goldstein, 2009). Additionally, the modern obesogenic environment may expose a disease risk associated with genetic variants that interact with other environmental and dietary components (Martínez, 2014). For example, interaction with obesity have been reported for sequence variants in two genes (*TM6SF2* and *GCKR*) that contribute to NAFLD (Kozlitina *et al*., 2014; Speliotes *et al*., 2011). Therefore, the consideration of not only NAFLD-related genetic variants, but also of rare variants and other networks such as obesity or insulin resistance genetic variants, is essential for a complete understand of the genetic pathways involved in NAFLD (Sookoian *et al*., 2012).

These include variants involved in adipogenesis and lipid metabolism. Thus, peroxisome proliferator-activated nuclear receptor can be considered as a potential candidate (*PPAR*) (Dixon *et al*., 2021). *PPARα* is highly expressed in cells with high catabolic rates of fatty acids, such as the liver and the skeletal muscle, where under an increased hepatic fatty acid influx or decreased fatty acid efflux and its activation prevents the accumulation of triglycerides by increasing the expression of genes involved in catabolism (Tacke *et al*., 2021). On the other hand, *PPARγ* is highly expressed in adipose tissue and regulates adipocyte differentiation, FFA uptake, and storage. In fact, an abnormal expression of *PPARα* was closely related to inflammatory factors and with the occurrence and development of NAFLD (Dixon *et al*., 2021). Other genetic variant located in or near some genes related to lipid metabolism, is the apolipoprotein C3 (*APOC3*) which is a major constituent of VLDL, chylomicrons, and High-density lipoprotein cholesterol (HDL-c) (Saki *et al*., 2020), even if more studies to establish an association in the pathogenesis of NAFLD are needed (Niu *et al*., 2014). Other interesting candidates are the phosphatide phosphatase Lipin *1* (Kumari *et al*., 2012) and the *PPARG* coactivator 1 alpha, an important regulator of carbohydrates and fat metabolism and mitochondrial function (Gancheva *et al*., 2016). These genes have been linked to NAFLD, as well as to components of the metabolic syndrome, including body mass and insulin levels.

Concerning energy expenditure, there are some obesity-related genetic variants that could be potential targets on NAFLD such as the *β*-adrenergic receptor 3 (*ADRB3*) (Sakamoto *et al*., 2019) and Adiponectin (*ADIPOQ*) which has been reported to play a role in the onset and progression of NAFLD through *PPARγ* pathway (Saki *et al*., 2020). Interestingly, a genetic variant in the *UCP2* gene has also been strongly associated with severity of fibrosis in a study in a cohort of adolescents with biopsy proven (Hudert *et al*., 2019).

In the last years, genes involved in appetite control and food intake such as the polymorphism located in leptin receptor gene have focused much attention (Li X.-L. *et al*., 2016). Leptin acts through the leptin receptor which has been reported to be associated with NAFLD by influencing insulin and glucose metabolism (Lu *et al*., 2009). Hence, it has been suggested that the interaction between *LEPR* and *PNPLA3* genes increased the risk of NAFLD to either gene alone (Zain *et al*., 2013). The fat mass and obesity associated (*FTO*) gene, has also been proposed to be involved in NAFLD since it plays a role in the regulation of both weight and glucose metabolism (Mizuno, 2018). Besides, a study carried out in older Chinese Han population demonstrated and association between three different genetic variants of the gene and elevated risk of NAFLD (Gu *et al*., 2020). Furthermore, the most solid associations have been collected for inflammatory factors such as *TNF-α* and *IL-6*, which have been related to advanced stages of the disease (Zhang *et al*., 2018).

Other polymorphisms of interest are the transcription factors involved in the circadian rhythm (*CLOCK* transcription factor), that has been linked to obesity and NAFLD in humans (Atish Mukherji), the multidrug-resistance-associated protein gene (*ABCC2*) (Sookoian *et al*., 2009), the signal transducer and activator of transcription 3 *STAT3* (Sookoian *et al*., 2008) and the nuclear pregnane X receptor (*PXR*) (Sookoian *et al*., 2010). Also, a missense variant in serpin family A member 1 (*SERPINA1)* has been associated with the risk of cirrhosis in NAFLD and alcohol misuse (Sookoian *et al*., 2019).

Finally, a novel therapeutic target for NAFLD is bile acid regulation which may share genetics with other metabolic traits (Eslam *et al*., 2020). For instance, fibroblast growth factor 21 (FGF21) may also play an important role, since the *FGF21* rs838133 A allele has been linked to the development of severe stages of NAFLD (Tillman *et al*., 2020).

3. Management of NAFLD

The increased prevalence and heterogeneity of the population with NAFLD represent an important impediment to the discovery of highly effective drug treatments (Roeb, 2021). Nutritional status plays a key role in the development and progression of NAFLD (Donnelly *et al*., 2005). Indeed, epidemiological evidence suggests a close relationship between unhealthy lifestyle and NAFLD, making lifestyles correction a mandatory approach in these patients (Marchesini *et al*., 2016).

3.1. Diet

3.1.1. Weight loss

The usual diet of the NAFLD patient follow a Western dietary pattern and has been often associated with the development of this disease independently of physical activity (Hallsworth *et al*., 2019). This diet is high in saturated fat, trans-fat and high carbohydrate consumption, which have been shown to induce obesity, metabolic syndrome, NAFL and potentially NASH (Hosseini *et al*., 2016).

Thus, current management for NAFLD includes diet and lifestyle changes for achieving weight loss (Romero-Gómez *et al*., 2017). Calorie restriction and physical activity play a fundamental role in in the reduction of body weight, subcutaneous, visceral and hepatic fat, being consistently recommended in guidelines for the management of NAFLD (Eslamparast *et al*., 2017). The favorable effects of weight loss on surrogate biomarkers and imaging tests have been extensively demonstrated in several studies (Petroni *et al*., 2021). However, few randomized control trials are available with histological proven (Houttu *et al*., 2021).

Additionally, the guidance of the American Heart Association for the Study of Liver Diseases suggests that a weight loss of at least 3%-5% of body weight appears necessary to improve steatosis, but a greater weight loss (7%-10%) is needed to improve the majority of the histopathological features of NASH, including fibrosis (Chalasani *et al*., 2018). A recent systematic review and meta-analysis of forty-three studies with 2,809 participants evaluated the dose-response relationship between the magnitude of weight loss and improvements in NAFLD (Koutoukidis *et al*., 2021). The research results clinically demonstrated that modest weigh loss produced significant improvements, although the relationship was stronger with greater weight loss. Moreover, in the Fatty Liver in Obesity (FLiO) study the evaluation of 98 overweight and obese subjects with NAFLD, demonstrated that after 24-months of nutritional intervention, both energy-restricted diets were able to

reduce the body weight with significant improvements in body composition, biochemical, and liver determinations (Marin-Alejandre *et al*., 2021).

3.1.2. Dietary patterns and characteristics

Importantly, the European Association for the Study of the Liver (EASL) and the European Association for the Study of Diabetes (EASD) have recommended that the dietary approach in these patients, should be within the context and composition of the Mediterranean Diet (MedDiet) (European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO), 2016). In this sense, the Mediterranean diet has demonstrated beneficial effects on NAFLD patients due to its efficacy on hepatic health by improving the insulin resistance and lipid profile, as well as preventing metabolic-related diseases (Mirabelli *et al*., 2020; Munteanu *et al*., 2016). This dietary pattern is characterized by very low consumption of red meat, a high content of vegetables, fruits, whole grains, legumes, fish, nuts, and seeds and extra-virgin olive oil (Martínez-González, Gea, *et al*., 2019). Additionally, Mediterranean diet was shown to improved anthropometric parameters, lipid profile and to reduce hepatic fat accumulation and liver stiffness in overweight patients with NAFLD (Abenavoli *et al*., 2017). Indeed, a cross-sectional study in individuals with MetS, demonstrated that adherence to the Mediterranean diet, consumption of legumes and physical activity were inversely associated with a non-invasive marker of NAFLD (Bullón-Vela V., Abete, Tur, Pintó, *et al*., 2020).

On the other hand, alternative approaches for NAFLD management have also been suggested (Armandi & Schattenberg, 2021; Parra-Vargas *et al*., 2020). For example, the Dietary Approaches to Stop Hypertension (DASH) dietary pattern, designed in the 1990s to prevent and treat hypertension (Hekmatdoost *et al*., 2016; Sacks *et al*., 1995). The DASH dietary pattern emphasized the consumption of fruits, vegetables, low-fat dairy products, whole grains, poultry, fish, nuts, seeds, and legumes, while reducing the intake of fats, red meat, sweets, and sugar-containing drinks (Parra-Vargas *et al*., 2020). Adherence to this approach has shown a lower prevalence of NAFLD (Xiao *et al*., 2020) and beneficial effects on cardiometabolic risk factors such as insulin resistance and type-2 diabetes mellitus (Oliveira *et al*., 2016). Other diets, such as low and very low carbohydrates (ketogenic diet), vegetarian and vegan diets have also shown beneficial effects in the treatment of NAFLD, and recently, some studies have proposed intermittent fasting as an option to reduce liver steatosis and related metabolic disturbances (Drinda *et al*., 2019; Parra-Vargas *et al*., 2020).

However, in the management of NAFLD, it is important to consider not only the quantity of the diet, but also the qualitative nutritional features (Finelli *et al*., 2012).

In light of the difficulty in weight loss and maintaining the reduction in the long term, the macronutrient modification of the diets seems to be a key factor in the management of these individuals (Zelber-Sagi *et al*., 2011). Macronutrients components such as saturated fatty acids (SFA), trans fats, simple sugars (sucrose and fructose) and animal proteins are associated with liver damage (Dongiovanni *et al*., 2017; Juanola *et al*., 2021). On the other side, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3 fat, vegetable protein and dietary fiber appear to have a beneficial effect in the liver (Berná *et al*., 2020).

Most of the dietary intervention studies have suggested that high fat consumption plays a role in NAFLD´s pathogenesis (Chakravarthy *et al*., 2020; Malhotra *et al*., 2015). However, the effect of fat intake on NAFLD depends on the type of fat (Zelber-Sagi *et al*., 2011). In general, the diet of NAFLD patients is rich in saturated fat and cholesterol, while it is poorer in omega-3 PUFAs and higher Omega-6:Omega-3 ratio (Lujan *et al*., 2021). In fact, overfeeding with PUFA and SFAs has difference effects on liver and visceral fat accumulation in humans (Rosqvist *et al*., 2014). Excessive intake of SFAs promotes oxidative stress, mitochondrial dysfunction and inflammation, resulting a key mechanism in the pathophysiology of NASH and insulin resistance (Chakravarthy *et al*., 2020). In addition, trans fatty acids (TFAs) have been also linked to obesity, CVD, IR and also to liver damage, as they have been associated with the activation of systemic inflammatory responses, including substantially increased levels of IL-6, TNF-α, TNF receptors (Dhibi *et al*., 2011). Concerning unsaturated fatty acids, experimental studies have shown that diets enriched with omega-3 PUFAs, increase insulin sensitivity, reduce intrahepatic triglyceride content and ameliorate steatohepatitis (Yu *et al*., 2017). In this context, several meta-analyses of randomized controlled trials have conceded that omega-3 PUFAs supplementation (>3 g/day) is useful for the reduction of liver fat, hepatic enzymes, BMI, triglycerides and cholesterol (Lee C.-H. *et al*., 2020; Yan *et al*., 2018). Furthermore, it was shown that the adherence to weigh loss strategies led to changes in erythrocyte membrane omega-3 PUFA composition, which in turn were associated with an improve of liver health during the dietary treatment (Marin-Alejandre *et al*., 2020). However, the effects of supplementation with omega-3 PUFA still produce inconclusive evidence, possibly due to the use of differences in methodology, duration of trials, level and sources of PUFA, as well as the EPA/DHA ratios, in combination with the genetic background of the individuals, among other possible causes (Berná *et al*., 2020). Concerning MUFA, a randomized double-

bling clinical trial study in NAFLD subjects reported that the consumption of 20 g/day for 12 week of olive oil may alleviate the severity of fatty liver disease (Rezaei *et al*., 2019). Also, they have been associated with the improvement of plasma lipid levels, reducing central fat accumulation and decreasing the postprandial adiponectin gene expression (Paniagua González *et al*., 2007). In this sense, because of the different effect of the types of fat on NAFLD and NASH, a reduction in total fat intake is not the simple solution (Romero-Gómez *et al*., 2017). Moreover, the EASL-EASD-EASO Clinic Guidelines recommend the Mediterranean diet for NAFLD subjects due to its high content of MUFAs (European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO), 2016).

Regarding carbohydrate intake, there is evidence suggesting that lower carbohydrate intake (≤40 % of daily energy intake) may be beneficial for NAFLD patients (Estruch *et al*., 2018). Carbohydrates are classified as simple (fructose, glucose, galactosa) and complex (Lujan *et al*., 2021). Most of the studies are focused on the role of fructose intake and incidence of NAFLD (Lombardi *et al*., 2020). Fructose is involved in *de novo* lipogenesis inhibiting hepatic lipid oxidation via *PPAR* alpha and activating the c-Jun N-terminal kinase pathway leading to obesity, steatosis, insulin resistance, inflammation, hepatic fibrosis and leaky gut (Chakravarthy *et al*., 2020; Roglans *et al*., 2007). Although some studies claimed that fructose/sucrose and NAFLD relationship appear to be confounded, in some cases, by excessive energy intake (Chung M. *et al*., 2014), others suggested that excessive fructose intake, especially in genetically predisposed subjects and in the context of hypercaloric diets, is likely a major contributor of this disease (Lujan *et al*., 2021). Conversely to fructose, whole grains seem to have a protective role on cardiovascular risk (Lombardi *et al*., 2020). In this context, it has been shown that the type of dietary fiber differentially impacts liver health status in obese subjects under energy restriction (Cantero *et al*., 2017). Thus, cellulose, which is an insoluble no-fermentable fiber has exhibit protective anti-inflammatory effects (Kim Y. *et al*., 2020). On the other hand, soluble fiber such as inulin and *β*-glucan have also demonstrated a reduction in insulin resistance (Chambers *et al*., 2019; Jayachandran *et al*., 2018). In addition, β-glucan supplementation has been also linked to diminish hepatic steatosis and dislipemia jointly with alterations of gut microbiota (Jayachandran *et al*., 2018).

Expanding the issue of dietary considerations, the role of protein on NAFLD is controversial and not well completely understood, possibly because of the variability of the origin, food sources and composition that contained them, as well as the use of different methodologies in research (Berná *et al*., 2020). It seems that both insufficient (Ampong *et al*., 2020;

Dongiovanni *et al*., 2017) and excessive protein intake (Zelber-Sagi *et al*., 2007) might have effects on hepatic health. However, there is emerging evidence that dietary protein intake and specific amino acid patterns are relevant in the pathogenesis of NAFLD (Tricò *et al*., 2021). In the unhealthy Western diet, consumption of red meat has been associated with metabolic syndrome (Babio *et al*., 2012), and liver disease (Lang *et al*., 2020), as well as with higher liver iron content, which may contribute to the development of advance stages of the disease by increasing the oxidative stress (Recaredo *et al*., 2019). Moreover, the liver is a key site for the biosynthesis and catabolism of protein and amino acids (Sano *et al*., 2018). In this sense, a recent study has showed that a higher dietary intake of aromatic amino acids (AAA); branched-chain amino acids (BCAA) and sulfur amino acids (SAA) were positively associated with liver fat content in subjects with overweight/obesity and NAFLD (Galarregui, Cantero, *et al*., 2020). Nonetheless, studies performing weight loss intervention revealed that high protein diets are able to reduce the liver fat contain (Winters-van Eekelen *et al*., 2021). However, more studies for a better understanding of the mechanisms are needed (Lujan *et al*., 2021).

Additionally, the liver is the major iron storage organ playing a central role in the metabolism of this nutrient (Ferńandez-Real *et al*., 2015). One third of patients with NAFLD show signs of disturbed iron homeostasis, possibly reflecting an increased oxidative stress and an inflammation condition (Datz *et al*., 2017). In fact, increased ferritin levels are usually associated with NASH and the severity of liver damage, whereas in patients with mild iron overload, iron depletion may decrease insulin resistance and liver damage (Valenti *et al*., 2014). Consequently, iron metabolism-related parameters such as ferritin may be an adequate predictor of liver disease (Galarregui, Marin-Alejandre, *et al*., 2020).

On the other hand, the total antioxidant capacity (TAC) of foods is also considered a potential marker of diet quality and it has been associated with a lower risk of NAFLD (Galarregui *et al*., 2018). Therefore, consumption of food groups with higher content of components such as fruits, vegetables, legumes or tea, which have a high antioxidant content, could be a useful approach to prevent NAFLD (Salehi-Sahlabadi *et al*., 2020).

Finally, among micronutrients, vitamin E, vitamin C, vitamin D, several polyphenols (e.g., resveratrol, curcumin, caffeine, quercetin) and different methyl donors such as choline and betaine, have been also tested in clinical studies reporting beneficial effects in the management of NAFLD (Radziejewska *et al*., 2020; Tacke *et al*., 2021). Furthermore, in animal models, the SCFA butyrate has been associated with *PPAR*-alpha activation and upregulation of GLP-1R, contributing to the improvement of high-fat-diet induced NAFLD in mouse models (Sun *et al*., 2018; Zhou D. *et al*., 2018).

3.2. Physical activity and other behavioral factors

Everyday physical activity is associated with health (Romero-Gómez *et al*., 2017). Cross-sectional studies have suggested that people with NAFLD are more prone to fatigue (Newton *et al*., 2008), and have lower levels of physical activity than those without (Romero-Gómez *et al*., 2017; Zelber-Sagi *et al*., 2011). Moreover, patients with NAFLD usually exhibit the "triple-hit" behavioral phenotype which consisted in sedentary behaviors, low physical activity and poor diet (Romero-Gómez *et al*., 2017). In this sense, physical activity and increased energy expenditure have long been associated with amelioration of obesity and associated cardiometabolic risk factors (Campbell *et al*., 2021; Rinaldi *et al*., 2021). Notably, in NAFLD patients, exercise has been shown to reduce hepatic fat content, visceral adipose tissue, as well as the likelihood of having NASH and in the case of developing NASH, it also reduces the probability of having advanced fibrosis (Munteanu *et al*., 2016; Ratziu *et al*., 2015). In addition, the influence of exercise on the modulation of gut microbiome is also being investigated, even if the underling mechanism is poorly understood (Houttu *et al*., 2021). Exercise and its different types (aerobic exercise, resistance exercise, or high intensity intermittent exercise) considering other environmental factors, is therefore, an excellent approach to NAFLD treatment.

Other behavioral factors that have been related with NAFLD are major depressive disorders (MDD), among them, depression and anxiety are the most investigated in relationship to NAFLD/NASH (Macavei *et al*., 2016). In this sense, a case-control study showed that NASH subjects had increase lifetime rates of MDD being also associated with more advanced liver histological abnormalities (Elwing *et al*., 2006). Furthermore, sleep disturbances have been associated with NAFLD (Shetty *et al*., 2018). A recent study has also supported this association suggesting that sleep disruption may be contributing to the development and progression of NAFLD, as well as the alteration of the liver may be affecting sleep patterns (Marin-Alejandre *et al*., 2019). Thus, the influence of other putative less-known factors in the evaluation of NAFLD is needed.

3.3. Pharmacotherapy

Currently, there is no standard medical therapy with proven efficacy available for treating NAFLD (Ramai *et al*., 2021). The treatment of this disease is limited to lifestyle modifications, being therapeutic approaches focus on metabolic pathways connected to NAFLD (Friedman *et al*., 2018; Munteanu *et al*., 2016). All those therapies have discordant opinions and, up to now, the Food Drug Administration has not approved any pharmacological treatment for NAFLD (Shetty *et al*., 2019). However, the guidelines from

the United States, Europe and Japan recommended both pioglitazone and vitamin E for those patients with biopsy-proven NASH with and without diabetes, respectively (Sumida *et al*., 2020).

In this context, several potential targets have been widely investigated, including not only key factors of fibrogenesis such as fatty acids, insulin resistance, inflammatory cells caspases, oxidant stress, hepatic stellate cells, but also glucose and lipid homeostasis (Friedman *et al*., 2018; Polyzos *et al*., 2020). Also, glucose-lowering drugs, such as metformin, thiazolidines and glucagon-like peptide-1 receptor agonists (GLP-1 RA), or therapies related to lipid metabolism, as well as statins, are other interesting and promising candidates (David *et al*., 2021; Sumida *et al*., 2018). Interestingly, metformin has also been showed to be associated with changes of intestinal microbiota composition and lower translocation of bacterial endotoxins (Brandt *et al*., 2019; Mazza *et al*., 2012).

Moreover, there are several ongoing phase 2 and 3 studies focusing on promising and interesting agents such as the FXR agonist obeticholic acid, which has showed an improvement in fibrosis and key components of NASH disease activity among the subjects with NASH in a study conducted at 332 centers in 20 countries (Younossi *et al*., 2019). Also, other future candidates are the elafibranor, selonsertib, cenicriviroc (with autoinflammatory activiy) and resmetirom (Roeb, 2021). Therefore, while waiting for the approval of medications for NASH, given the high prevalence, heterogeneity and multifactorial pathogenesis of NAFLD, research and design of new techniques and personalized approaches are urgently needed (Polyzos *et al*., 2020).

3.4. Gene-environment interactions

Dietary patterns are associated with NAFLD risk and this association could be modified by genetic background (Meroni, Longo, *et al*., 2020). Thus, the different phenotypic manifestations and severity of NAFLD are the outcome of complex traits influenced by the interaction of genetic, nutrient intake/exposure and environment and behavioral factors (Younossi *et al*., 2021) (**Figure 4**).

Nutrigenetics is the science that investigates the combined effect of genetic variation and nutrition on health and performance needed for precision and personalized nutrition (Hesketh, 2012). However, the knowledge of these interaction in the biology of NAFLD and NASH remains scarcely understood (Juanola *et al*., 2021).

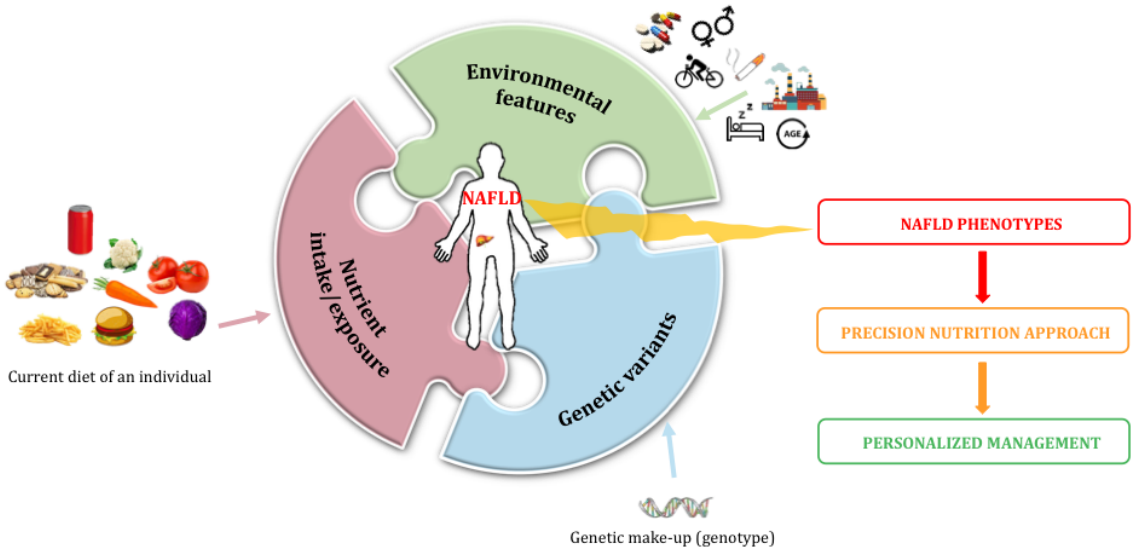

Figure 4: Gene-environment interactions on NAFLD**.** Adapted from: Dongiovanni *et al.*, 2017 and Mullins *et al.*, 2020. Abbreviations: NAFLD, Non-alcoholic Fatty Liver Disease.

In this context, *PNPLA3* is the most widely studied gene related to NAFLD which has been shown to interact with the environment (Albhaisi *et al.*, 2021). In this sense, in an Italian cohort report, a nutrigenetic analysis was carried out between the *PNPLA3 I148M* genotype and intake of sweetened beverages and of vegetables (Nobili *et al.*, 2014). Indeed, this gene is also influenced by dietary fatty acids, specifically by the omega-6/omega-3 PUFA ratio (Santoro *et al.*, 2012). *TM6SFT2* and *MBOT7* genetic variants have been also found to interact with fat ingestion and changes in phosphatidylinositol species, respectively (Meroni, Dongiovanni, *et al.*, 2020; Musso *et al.*, 2017). Besides, the LIPGENE study reported a statistically significant association between *GCKR* rs1260326 polymorphism and plasma omega-3 fatty acids modulating insulin resistance and inflammatory biomarkers (Lee H. *et al.*, 2015).

Overweight and obesity are risk factor for many chronic diseases such as NAFLD (Younossi, 2019). The *FTO* genetic variation is associated with adiposity (BMI and waist/hip circumference), metabolic biomarkers (total cholesterol, triglycerides, and fasting glucose), and adipokines (adiponectin and leptin) (Duicu *et al.*, 2016). In this sense, a recent study has also shown an association between different polymorphisms of the *FTO* gene and an increased risk of NAFLD (Gu *et al.*, 2020). Other genes that have demonstrated different response to diet depending on genetic background in NAFLD patients are *ADRB3*, tumor necrosis factor-α (*TNF-α*), *APOC3*, uncoupling protein type I (*UCP1*), peroxisome proliferator activated receptor *γ2 (PPAR-2)* and apolipoprotein E (*APOE*) (Stachowska *et al.*, 2016).

Also, data from the analyzed in the Preventing Overweight Using Novel Dietary Strategies (POUNDS LOST) trial, revealed that *PCSK7* rs236918 G allele was significantly correlated with a strong increase in fasting insulin levels and the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) in response to high-carbohydrate diet consumption (Huang T. *et al*., 2015), In addition to individual polymorphisms, more studies have focused on examine interactions with GRS (Zusi *et al*., 2019). In fact, in the Framingham Heath Study, a cohort of 1,521 participants were analyzed to demonstrated that improved diet quality was associated with reduction in liver fat, particularly in individuals with high genetic risk score for NAFLD (Ma J. *et al*., 2018).

Among lifestyle factors, physical activity is one of the most important factors involved in the risk of NAFLD (Semmler *et al*., 2021). In fact, an interaction between the *PNPLA3* polymorphism and physical activity or sedentary behavior on NAFLD was demonstrated (Wang S. *et al*., 2016). Moreover, other lifestyle behaviors may also interact with genetic factors in affecting NAFLD risk (Berná *et al*., 2020). In this sense, a recent study, in a Chinese Han population, identified interactions between the Adenylate Cyclase 3 (*ADCY3*), Paraoxonase 2 (*PON2*), and the Proprotein Convertase Subtilisin/Kexin Type 9 (*PCSK9*) gene variants and six environmental factors associated with NAFLD (Li Z. *et al*., 2020). Lastly, another important component of gene–diet interactions are epigenetic alterations (Meroni, Longo, *et al*., 2020). These epigenetic modifications are reversible processes being possible the design of diets based on specific micro/macro-nutrients modulating the epigenic pattern in order to ameliorate NAFLD and prevent its progression (Loomba *et al*., 2021). For example, an epigenome-wide association study conducted in two separate cohorts found an association between DNA methylation at SLC7A11 with reduced risk of hepatic steatosis in participants, as measured by ultrasound (Birerdinc *et al*., 2018).

Assuming the heterogenicity and complexity of this disease, NAFLD related disorders such as obesity and possible gene-diet interactions may be explored (Lombardi *et al*., 2020; Meroni, Longo, *et al*., 2020). Hence, modification of dietary pattern in genetically predisposed individuals by the influence of gene-diet interactions, could modulate specific clinical outcomes, so personalized nutrition therapy should be speculated in near future (Mullins *et al*., 2020).

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

NAFLD pathogenesis involves a myriad of causes and factors, including genetic susceptibility and predisposing comorbidities, such as obesity, type 2 diabetes mellitus, as well as environmental exposure and lifestyle. The heritability of NAFLD ranges from 20% to 70%, so genetic make-up plays a key role in the onset of the disease. Moreover, complex interactions among environmental factors, metabolism and social-economic features, genetic variants and gut microbiota are involved in the physiopathology of NAFLD. Thus, understanding the underlying mechanisms that cause the progression of NAFLD, as well as characterizing the shared genetic basis, is crucial to control and manage this disease. Liver biopsy continues to be the reference NAFLD diagnostic tool. However, due to the limitations of this instrument, non-invasive imaging techniques and biomarker panels are being devised and implemented. To date, there is no defined drug therapy for the treatment of NAFLD, with lifestyle modification, focusing on energy restriction and physical activity, being the main recommendations for the management of NAFLD. Therefore, we raised the following hypotheses: First: certain obese-related genetic variants may be associated with non-alcoholic fatty liver disease, whose knowledge can contribute to the prognosis and diagnosis of the disease. Second, genetic variability and nutrigenetic interactions may condition and facilitate nutritional management in subjects with overweight or obesity and NAFLD.

1. General objective

The main purpose of this research was to analyze the association between genetic variants and non-alcoholic fatty liver disease, both in terms of disease progression and associated response to personalized nutritional strategies for precision management.

2. Specific objectives

1) To analyze the association of the *SH2B1* rs7359397 gene polymorphism with steatosis severity in subjects with obesity and non-alcoholic fatty liver disease (**CHAPTER 1**).
2) To evaluate the influence of the *SH2B1* rs7359397 genetic variant on changes in body composition, metabolic status and liver health after a 6-month energy-restricted treatment in overweight/obese subjects with NAFLD **(CHAPTER 2).**
3) To assess three different genetic risk scores based on fatty liver index, magnetic resonance imaging and lipidomic (OWLiver®-test) for a nutrigenetic personalized management of NAFLD after a 6-months weight-loss nutritional treatment **(CHAPTER 3).**

4) To build a predictive model based on genetic and hepatic health information, deeming insulin resistance markers in order to personalize dietary treatment in overweight/obese subjects with NAFLD **(CHAPTER 4).**

SUBJECTS AND METHODS

The present work has been encompassed within the Fatty Liver in Obesity study. The proposed objectives have been addressed in different chapters (**Figure 5**), where each chapter corresponds to an article. A brief explanation of the design used in each chapter is presented next:

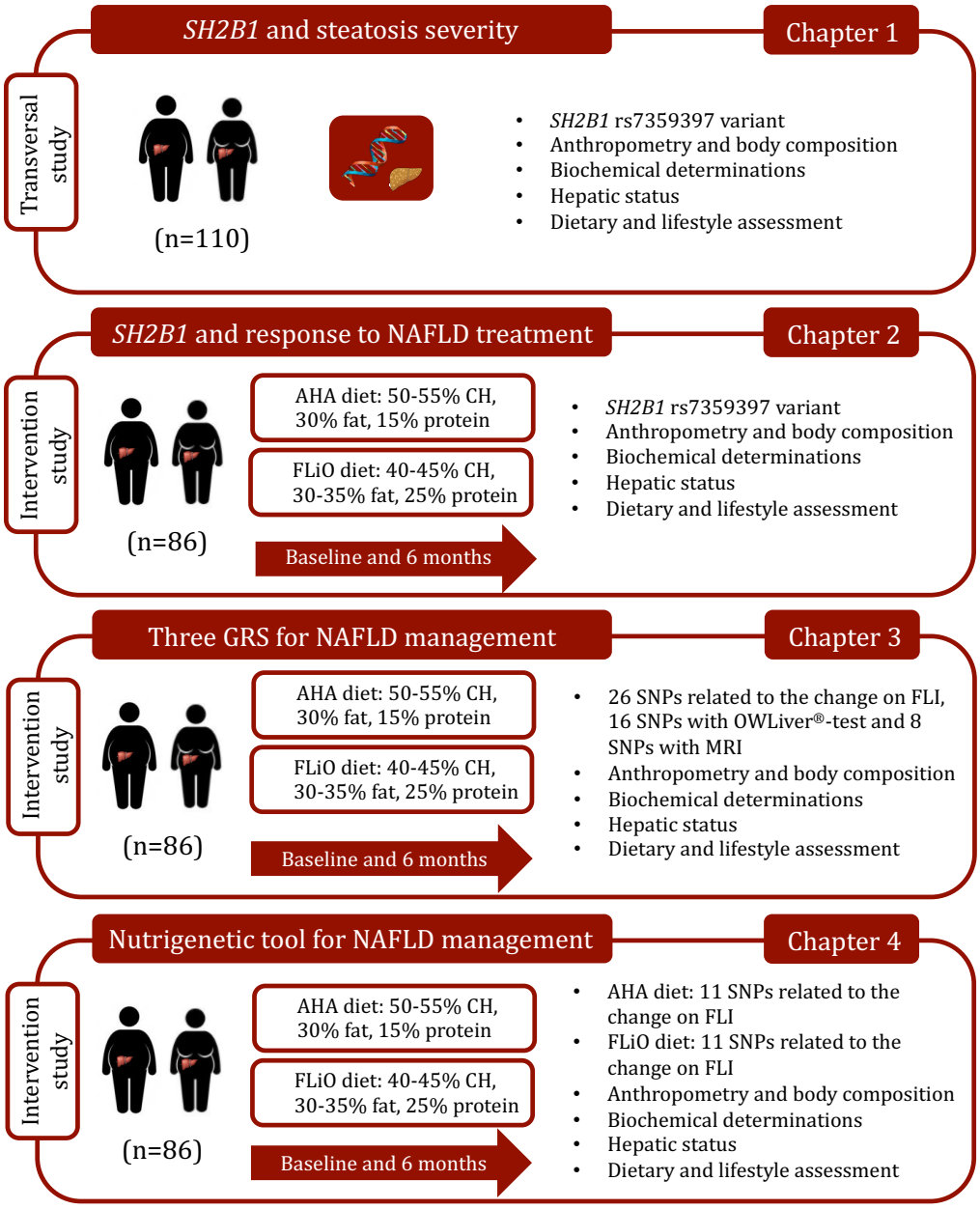

Figure 5. Overview of the experimental design conducted within each chapter. Abbreviations: AHA, American Heart Association; CH, Carbohydrates; FLI, Fatty Liver Index; FLiO, Fatty Liver in Obesity; GRS, Genetic Risk Score; MRI, Magnetic Resonance Imaging; OWLiver®-test, One Way Liver® S. L-test; *SH2B1*, Src-Homology-2 B adaptor protein 1; SNPs, Single Nucleotide Polymorphisms.

1. Study design

The FLiO study is a 24-months randomized, longitudinal and controlled intervention trial to compare the effects of two energy-restricted dietary strategies 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 lasted a total of two years where a complete evaluation of the participants was performed at baseline and after 6, 12 and 24 months. The participants were randomly assigned to the American Heart Association (AHA) or the Fatty Liver in Obesity (FLiO) group (Marin-Alejandre *et al*., 2021). The present work is focused on the analysis and evaluation of the results at baseline and after 6-months of nutritional intervention.

The FLiO study was approved by the Ethics Committee of the University of Navarra (54/2015) and was registered at Clinical Trials in www.clinicaltrials.gov (FLiO: Fatty Liver in Obesity study; NCT03183193). All procedures were performed in accordance with the ethical guidelines of the Declaration of Helsinki and the study current study was conducted following the CONSORT 2010 guidelines (Moher, D. *et al*., 2010). Each subject provided written informed consent after receiving an information sheet and an additional verbal explanation of the protocol.

2. Study population

The study population was recruited from June 2016 and June 2017 in the Metabolic Unit of the Centre for Nutrition Research of the University of Navarra, Spain. A group size of 228 subjects were evaluated to determine the presence of liver steatosis by abdominal ultrasonography, where 127 volunteers were selected. Consecutive, ninety-eight men and women with overweight or obesity (BMI ≥ 27.5 kg/m^2 to < 40 kg/m^2) between 40 and 80 years old and with NAFLD confirmed fulfilled the selection criteria and were enrolled in the study (Marin-alejandre *et al*., 2019). Two volunteers 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 inclusion and exclusion criteria were as follows:

Table 5. Inclusion and exclusion criteria of the FLiO study

Inclusion criteria
Adults: 40-80 y.o.
Overweight or obese (BMI: ≥27.5 kg/m^2 and <40 kg/m^2)
Diagnosis of NAFLD
Exclusion criteria
Presence of known hepatic disease other than NAFLD
Excessive alcohol consumption (>21 units of alcohol per week for men and >14 per women)
Weight-loss ≥ 3 kg in the last 3 months
Obesity known endocrine origin (except treated hypothyroidism)
Active cancer or history of malignancy in the last 5 years
Problems of massive edemas
Drug treatments: immunosuppressants, cytotoxic agents, systemic corticosteroids agents potentially causing liver steatosis or alteration in hepatic tests or weight modifiers
Surgical procedure for weight loss
Severe psychiatric disorders
Lack of autonomy or inability to follow the diet (including food allergies or intolerances or/and lifestyle recommendations, as well as to follow scheduled visits)
Consumption of any type of food supplements (antioxidants, prebiotics, probiotics, etc.)

Abbreviations: BMI, Body Mass Index; FLiO, Fatty Liver in Obesity; NAFLD, Non-alcoholic Fatty Liver Disease; y.o., years old.

3. Dietary interventions

Two different diets were prescribed and compared according to the allocation group (**Figure 6**). 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 control diet was a conventional and balanced diet based on American Heart Association (AHA) guidelines and lifestyle advice (De La Iglesia *et al*., 2014) which propose 3-5 meals/day and a conventional distribution of macronutrients according to the total energy intake: 50-55% from carbohydrates (adequate fiber 25-30 g/day), 15% from proteins and 30% from lipids with a healthy fatty acid profile. On the other hand, the experimental diet (FLiO) presented the following target macronutrients in relation to the total caloric value: 40-45% carbohydrates (preferring those with low glycemic index and fiber 30-35 g/day), 25% proteins (predominantly from vegetable sources), and 30-35% from lipids favoring extra virgin olive oil and omega-3 polyunsaturated fatty acid instead of saturated and trans fats (Marin-alejandre *et al*., 2019). The FLiO diet proposed a higher meal frequency of 7 meals/day and adherence to the Mediterranean diet including an increased quantity of

natural antioxidants and involving traditional foods with no additional economic cost. Besides, both dietary groups were provided with a 7 days menu plan.

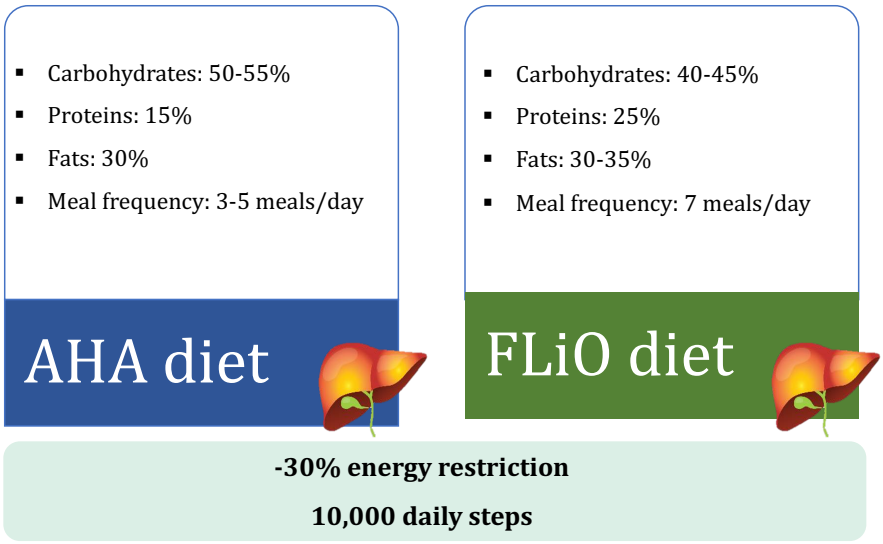

Figure 6. Composition of prescribed diets. Abbreviations: AHA, American Heart Association; FLiO, Fatty Liver in Obesity.

4. SNPs selection and genotyping

For genotyping, a total of 110 epithelial buccal cells sweeps from participants were collected using a liquid-based kit (ORAcollect-DNA, OCR-100, DNA genotek, Ottawa, Canada) (**Figure 7**). Genomic DNA was isolated using a Maxwell 16 Buccal Swab LEV DNA Purification Kit in the Maxwell 16 instrument (Promega, Madison, WI, USA) according to the instructions of the manufacturer. A customized panel of primers to amplify the regions containing the selected SNPs was designed using the "online" application of Thermo Fisher AmpliSeq Designer (https://www.ampliseq. com). This panel included the analysis of 97 SNPs in or near 59 genes that were related to body weight regulation, energy expenditure, appetite, adipogenesis, insulin resistance, and lipid metabolism (Goni *et al*., 2015; Ramos-Lopez *et al*., 2020). Overall, the amplicon average size was 185bp. The amplicon library for massive sequencing was constructed with the custom-designed panel and the Ion AmpliSeqTM Library Kit 2.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol.

Genotyping was performed by targeted next-generation sequencing in the Ion Torrent PGMTM equipment (Thermo Fisher Scientific Inc., Waltham, MA, USA). Raw data were processed in the Ion Torrent SuiteTM Server version 5.0.4 (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the Homo sapiens (HG19) as the reference genome for the alignment. A custom-designed Bed file was used to locate the SNPs of interest. Data were

analyzed with the Torrent Variant Caller 5.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA)) with a minimum coverage value of 20. The pilot validation tests resulted in the sequencing of 95 SNPs of the 97 designed. **Supplementary Table 1** reports the following genomic characteristic: gene name, gene symbol, SNP identifier, chromosome location and alleles.

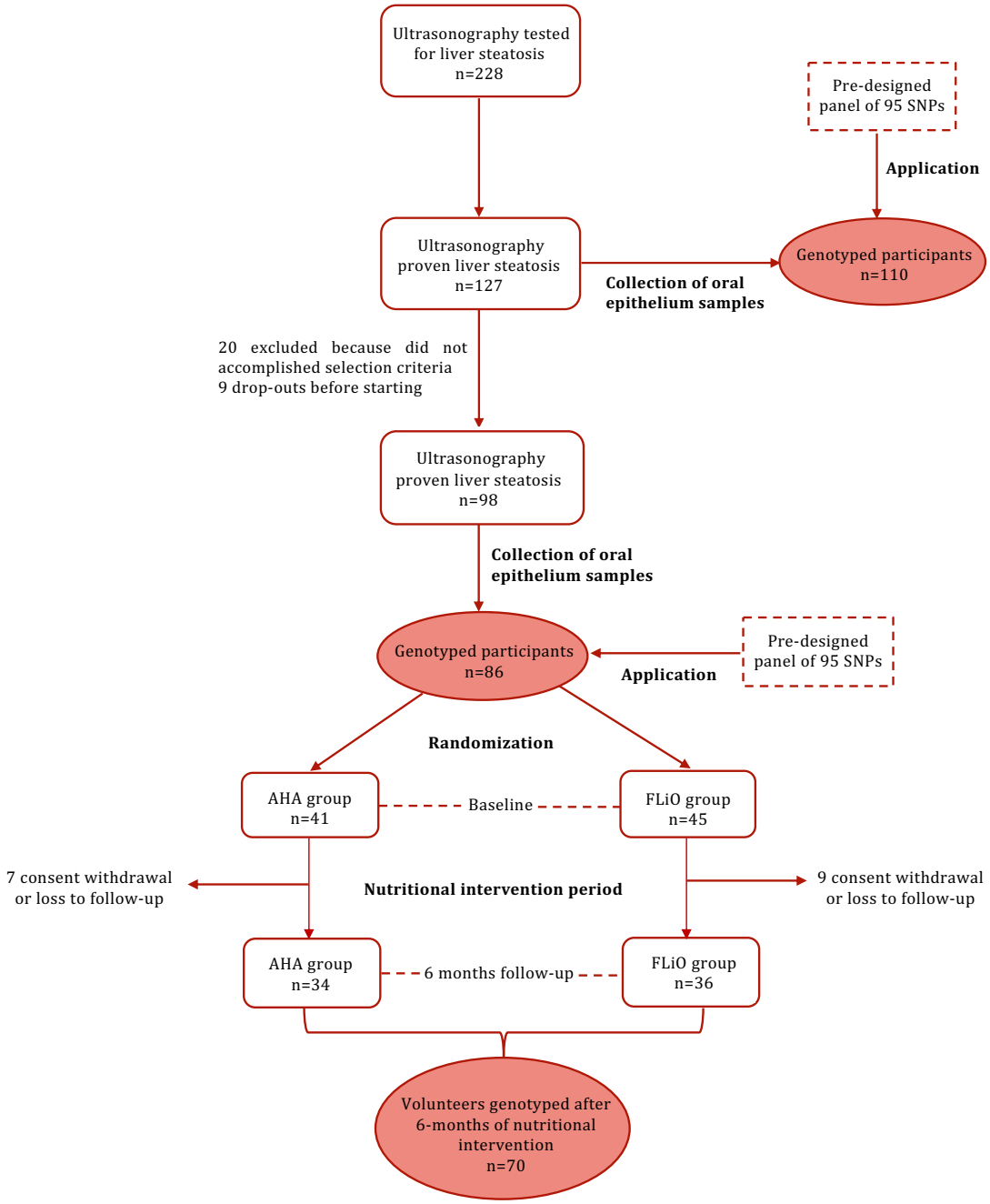

Figure 7. Flowchart of the participants in the FLiO study. Abbreviations: AHA, American Heart Association; FLiO, Fatty Liver in Obesity; SNPs, Single Nucleotide Polymorphisms.

5. Anthropometric, body composition and biochemical determinations

At the Metabolic Unit of the University of Navarra anthropometric measurements and body composition evaluation was carried out after overnight fasting. Body weight, height and waist circumference were assessed following previously described standardized procedures (De La Iglesia *et al*., 2014). Blood pressure (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, the Netherlands) and DXA body composition (Lunar iDXA, encore 14.5, Madison, WI, USA) were analyzed according to the instructions of the manufactures.

Blood samples for biochemical determinations were processed at the Laboratory of Biochemistry of the University of Navarra Clinic (Pamplona, Spain). The samples were properly collected after an overnight fasting of 8-10 hours. Fasting serum plasma glucose, glycosylated hemoglobin (HbA1c), homocysteine, total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) concentrations were determined on an autoanalyzer (Cobas 8000, Roche Diagnostics, Basel, Switzerland), following standardized protocols. The low-density lipoprotein cholesterol (LDL-c) levels were calculated using the Friedewald equation: LDL-c = TC − HDL-c − TG/5 (Friedewald, 1972).

Plasma concentrations of fibroblast growth factor 21 (FGF-21), C-reactive protein (CRP), insulin, retinol-binding protein 4 (RBP4), leptin and adiponectin were measured using specific ELISA kits (Demeditec; Kiel-Wellsee, Germany) in a Triturus autoanalyzer (Grifols, Barcelona, Spain) in accordance with the manufacturer´s instructions. Serum ferritin levels were analyzed by an external certified laboratory (Eurofins Megalab S.A, Madrid, Spain) using a Chemiluminescent Microparticle Immunoassay (CMIA) technology (Abbott Architect Ferritin Assay).

BMI was calculated as body weight divided by the squared height (kg/m^2) as reported by the World Health Organization and Spanish Society for the Study of Obesity (SEEDO) (Pérez-Rodrigo *et al*., 2006; Salas-Salvadó *et al*., 2007).

The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was computed as HOMA-IR = (insulin (μU/mL) x glucose (mmol/L))/22.5. 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(mg/dL)/HDL-c (mg/dL)] (Wang Q. *et al*., 2018).

6. 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 and 6 months). The dietary intake was registered with a validated semi-quantitative food frequency questionnaire (FFQ) of 137 items (Martin-Moreno *et al*., 1993). Each item in the questionnaire 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" (Fernández-Ballart *et al*., 2010), as previously described (Galarregui *et al*., 2018; Recaredo *et al*., 2019). The nutrient composition of the food items was derived from accepted Spanish food composition tables (Moreiras *et al*., 2009).

Glycemic Index (GI) values for single food items on the food frequency questionnaire were derived from the International Tables of Glycemic Index and Glycemic Load Values, as previously reported (Atkinson *et al*., 2008; Galarregui *et al*., 2018). Total dietary Glycemic Index was estimated by multiplying the amount of available carbohydrate (g) of each food item by its GI. The sum of these products was divided by the total carbohydrate intake. The amount of carbohydrates can vary in an overall diet and because of this the concept of Glycemic Load was also applied (Neuhouser *et al*., 2006).

The adherence to the MedDiet was assessed with a 17-point screening questionnaire (Martínez-González, Buil-Cosiales, *et al*., 2019), with a final score ranging from 0 to 17 and a higher score indicating a better adherence to the MedDiet (Galmes-Panades *et al*., 2019). Physical activity was evaluated applying a combination of methods: a validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire (Elosua *et al*., 1994, 2000) and a short 24-h physical activity questionnaire (NRC, 1989). In this last questionnaire, subjects were asked about the number of hours resting and practicing activities at work or at leisure time during a weekday and a weekend day. Activities were classified in four different categories according to intensity of effort: sedentary, mild, moderated or elevated. Individual daily physical activity level was calculated multiplying the average time spent on each group of activities during the week and the weekend and the multiples of physical activity levels. Moreover, a step-based physical activity recommendation of 10,000 steps/ day was given to the participants.

7. Assessment of liver status

Liver imaging techniques

The whole evaluation of the liver was performed under fasting at the University of Navarra Clinic. The radiologist was blinded to treatment allocation, clinical information and laboratory data. The presence of hepatic steatosis was determined by means of ultrasonography (Siemens ACUSON S2000 and S3000, Erlangen, Germany), which consisted in the evaluation of the steatosis status by visual quality of the liver echogenicity (Cantero *et al*., 2019; Lee S.S. *et al*., 2014). The qualitative clinical classification was done using a 4-point scale: less than 5% (grade 0), 5–33% (grade 1), 33–66% (grade 2), and greater than 66% (grade 3) as described elsewhere (Lee S.S. *et al*., 2014).

Transient elastography was performed through FibroScan® (Echosens, Paris, France), with the subject in the supine position and the right arm in maximum abduction. Depending on the obesity status, M and XL probes were selected under the professional criteria. Repeated shots were performed until obtaining 10 valid values of which the median was the selected value (Herrero *et al*., 2014). Hepatic fibrosis and cirrhosis were considered if the stiffness median was greater than 7 kPa or 12 kPa, respectively (Cantero *et al*., 2019).

Magnetic Resonance Imaging was performed through Siemens Aera 1.5 T and was used to determine the volume, fat and iron content of the liver by Dixon technique as described elsewhere (Cantero *et al*., 2019).

Hepatic index

The Fatty Liver Index (FLI), which has been validated in a large group of subjects with or without liver disease, was also assessed. It was computed using serum triglycerides, BMI, waist circumference and GGT concentrations using the following algorithm (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 0 to 100. An index <30 points indicates the absence of liver steatosis and ≥60 is a marker of liver steatosis with a good accuracy (Bedogni *et al*., 2006).

Lipidomic test

The OWLiver® test (One Way Liver S. L. Bilbao, Bizkaia, Spain) is a fasting blood probe that measures a panel of biomarkers that belong to the family of triacylglycerols, which are a reflection of the amount of fat and inflammation of the liver and therefore, a measure of disease development of NAFLD (Alonso *et al*., 2017). All TAGs are measured by high performance liquid chromatography and mass spectrometry (UHPLC-MS). The relative metabolite concentrations are analyzed together in an algorithm that generates the final OWLiver® score, which is able to distinguish between a normal liver, simple steatosis or NASH with high accuracy (Cantero *et al*., 2019). The test score is based on a prospective study, where subjects had previously been diagnosed by liver biopsy (Bril *et al*., 2018).

8. Statistical analyses

The calculation of the sample size was carried out taking into account the change in body weight as the principal variable, based on the current recommendations of the AASLD to ameliorate NAFLD features which are focused on the weight loss (Chalasani *et al*., 2018). In this sense, we aimed to detect a difference of 1.0 (1.5 kg) between both dietary groups (AHA *vs*. FLiO) in their reduction of weight, with a 95% confidence interval (α=0.05) and a statistical power of 80% (β=0.8). Considering all these points, a total of 36 subjects per study group was estimated, but 50 participants were included in each arm of the study, considering an estimated dropout rate of 20-30% according to the experience of the research group. However, two subjects were excluded from one of the dietary groups (AHA diet) due to important alterations in the initial assessment of biochemical parameters, which required medical treatment. Furthermore, this trail started with 98 subjects but only 86 epithelium buccal cells of them were collected. Moreover, after six months of weight loss intervention, a total of 70 participants had complete information and epithelium buccal cells to carry out the objective of this study.

Statistical analyses are specifically explained in each chapter. Briefly, normality was assessed by Shapiro-Wilk test and Kolmogorov–Smirnov test. Data normality and outliers were also checked using boxplots. Normally distributed variables are presented as means and standard deviations (SD), whereas continuous skewed variables as medians and interquartile ranges (IQR). Categorical variables are presented as absolute (n) and relative frequencies (%). Chi-square test was performed for comparison of qualitative variables across genotypes or GRS groups. Moreover, deviation from Hardy-Weinberg equilibrium (HWE) was tested by chi-squared test (Rohlfs *et al*., 2008).

For continuous variables, comparisons between two independent groups were performed using Student's t test of independent sample for normal distribution and Wilcoxon–Mann Whitney for non-normally distributed variables. Comparisons between two dependent groups were determined using a paired Student´s t-test or Wilcoxon-matched-pairs signed ranks test when appropriate. Additionally, ANOVA with repeated measures on time was performed to evaluate interactions analysis in continuous variables (e.g., *SH2B1* polymorphism x treatment). Categorical variables were evaluated by the McNemar's to test for marginal homogeneity. Also, significant testing was performed using 1000 Monte Carlo permutations when ANOVA with repeated measures was applied to ordinal variables.

Simple or multivariable linear regressions models were employed for two continuous variables in order to evaluate specific associations among them (e.g., FLI, liver fat content (by MRI) or OWLiver®-test changes). Indeed, logistic regression analysis were performed to evaluate associations among categorical and continuous variables (e.g., the steatosis degree with *SH2B1* genetic variant). Multinomial logistic regression models were also used to assess the influence of a genetic variant on the of risk of liver fat accumulation (by MRI) or to assess the risk of developing advanced stages of NAFLD (by OWL®). Diagnostic tests of the regression assumption for linearity and equal variance of residuals, and the variance inflation factor (VIF) for testing collinearity between independent variables, were conducted. Importantly, all models were adjusted for covariates and interactions terms (e.g., GRS x protein) were also included in order to evaluate gene-environment interactions, when appropriate. Lastly, linear mixed models were implemented to predict FLI decrease according to a genetic risk score and the interaction with diet. In this model, the interaction term between genetic score and diet was intentionally sought to personalize the diet, and subjects were used as random effects.

The GRSs were calculated assuming that each SNP acts independently and contributes equally to the risk of obesity in an additive manner, as has been previously reported (He, M. *et al*., 2010; Peterson *et al*., 2011). Genotypes were coded as 0, 1 or 2 according to the number or risk alleles for each variant. The GRSs were computed by summing the risk alleles across the selected SNPs for each individual.

All *p*-values presented are two-tailed and were considered statistically significant at $p < 0.05$. 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

Association of the SH2B1 rs7359397 gene polymorphism with steatosis severity in subjects with obesity and Non-Alcoholic Fatty Liver Disease

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Article

Association of the *SH2B1* rs7359397 Gene Polymorphism with Steatosis Severity in Subjects with Obesity and Non-Alcoholic Fatty Liver Disease

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver disease worldwide. Some genetic variants might be involved in the progression of this disease. The study hypothesized that individuals with the rs7359397 T allele have a higher risk of developing severe stages of NAFLD compared with non-carriers where dietary intake according to genotypes could have a key role on the pathogenesis of the disease. *SH2B1* genetic variant was genotyped in 110 overweight/obese subjects with NAFLD. Imaging techniques, lipidomic analysis and blood liver biomarkers were performed. Body composition, general biochemical and dietary variables were also determined. The *SH2B1* risk genotype was associated with higher HOMA-IR $p = 0.001$; and Fatty Liver Index (FLI) $p = 0.032$. Higher protein consumption ($p = 0.028$), less mono-unsaturated fatty acid and fiber intake ($p = 0.045$ and $p = 0.049$, respectively), was also referred to in risk allele genotype. Lipidomic analysis showed that T allele carriers presented a higher frequency of non-alcoholic steatohepatitis (NASH) (69.1% vs. 44.4%; $p = 0.006$). In the genotype risk group, adjusted logistic regression models indicated a higher risk of developing an advanced stage of NAFLD measured by FLI (OR 2.91) and ultrasonography (OR 4.15). Multinomial logistic regression models showed that risk allele carriers had higher liver fat accumulation risk (RRR 3.93) and an increased risk of NASH (RRR 7.88). Consequently, subjects carrying the T allele were associated with a higher risk of developing a severe stage of NAFLD. These results support the importance of considering genetic

predisposition in combination with a healthy dietary pattern in the personalized evaluation and management of NAFLD.

Keywords: NAFLD; obesity; steatosis; *SH2B1*; polymorphisms

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a frequent hepatic manifestation of metabolic syndrome with an estimated prevalence of 20–30% in the general population, whose rates rise with the increasing incidence of obesity [1,2]. NAFLD is described as an excessive hepatic fat deposition in the absence of history of alcohol abuse or other causes of secondary hepatic steatosis [3,4]. This disease encompasses a spectrum of clinical conditions, which can range from simple fat accumulation to non-alcoholic steatohepatitis (NASH) to advanced fibrosis leading to cirrhosis or hepatocellular carcinoma (HCC) and death [5,6]. The pathogenesis of NAFLD is multifactorial [7,8]. A sedentary lifestyle, obesity and related comorbidities such as diabetes, dyslipidemias, insulin resistance and other metabolic syndrome components are important risk factors associated with the development of NAFLD [9,10]. Besides, the current treatment of NAFLD is based on lifestyle interventions, such as changes in dietary patterns [6]. Thus, weight loss, exercise and healthy eating habits such as a Mediterranean lifestyle have been proposed as the main strategies in the reduction of NAFLD-associated comorbidities and to improve quality of life [2,3].

Not all subjects with similar sociodemographic and physical characteristics develop NAFLD and not all subjects with NAFLD develop more advanced stages of the disease, suggesting that important inter-individual differences concerning the mechanisms are involved in the pathogenesis and progression of NAFLD [11–14].

Research in this area has revealed that NAFLD development and progression towards more advanced stages have a genetic component [15–17]. The identification of genes that might confer a higher risk for the development of severe NAFLD as well as metabolic alterations directly related to the disease could be of special interest [1,17]. In order to improve and individualize the treatment, there are some identified genetic variants associated with increased liver fat accumulation, high risk for NAFLD and HCC development [18,19]. Advances in nutritional research and genomics may help to understand and improve the personalization of NAFLD treatment, taking into account genetic and gene–nutrient interactions [6,13]. Thus, there have been reported differences in cholesterol, adiposity and insulin resistance outcomes according to obesity-related variants in response to dietary interventions [20]. Moreover, the combination of environmental data and genetics was showed as an important predictor of blood lipid phenotypes [21].

Genetic variants more closely related to obesity might also be linked to NAFLD such as the Src homology (*SH2B*), among others. This gene family contains three members of adaptor proteins (*SH2B1*, *2* and *3*), being highly expressed in the liver [22]. Its potential mechanism may be as an adaptor protein, wherein *SH2B1* is implicated in several transduction processes such as enhancing JACK2 or the PI3-kinase pathway [23,24]. A genetic disruption of this gene has been associated with severe leptin resistance, energy imbalance, obesity and type 2 diabetes in humans, which are common comorbidities related to NAFLD [24]. In addition, it has been recently published that *SH2B1* can regulate the migration, proliferation and differentiation of cells, which could influence the development of some cancers [23]. In animal studies, an association has been drawn between *SH2B1* with an increasing hepatic lipid content and/or VLDL secretion, promoting hepatic steatosis in mice [22]. In humans, studies of this genetic variant linked to NAFLD have not been reported to date. However, among the *SH2B1* genetic variants identified as related to obesity traits, the polymorphism rs7359397 has been associated with glycosylated hemoglobin [25] and insulin sensitivity [24,25].

In this context, the aim was to analyze the effect of the *SH2B1* genetic variant related to NAFLD as well as possible associations between this polymorphism and diet, in order to identify possible gene–diet interactions that help clarify the role of this specific polymorphism in the pathogenesis of non-alcoholic fatty liver disease. Therefore, the study hypothesized that individuals with the rs7359397 T allele have a higher risk of developing severe stages of NAFLD compared with non-carriers where dietary intake according to genotypes could have a key role on the pathogenesis of the disease.

2. Materials and Methods

2.1. Study Population

The current study encompassed 127 men and women, overweight or obese (BMI ≥ 27.5 and <40 kg/m^2) with ultrasound-confirmed liver steatosis following accepted clinical criteria, as previously reported [10]. The analyses were conducted within the FLiO project (Fatty Liver in Obesity), a randomized controlled trial (www.clinicaltrials.gov; NCT03183193), which follows the Consort 2010 guidelines. The study was approved by the Ethics Committee of the University of Navarra (54/2015). All participants gave written informed consent for their participation in accordance with the Declaration of Helsinki. The exclusion criteria were endocrine disorders, (hyperthyroidism or uncontrolled hypothyroidism), known liver disease (other than NAFLD), alcohol abuse (>21 and >14 units of alcohol per week in men and women, respectively) and pharmacological treatments and a weight loss of ≥3 kg in the last 3 months, among others [14].

2.2. General Measurements

Anthropometric measurements (body weight, height and waist circumference) were assessed in fasting conditions following previously described standardized procedures [26]. The BMI was calculated as body weight divided by squared height (kg/m^2). The body composition was analyzed by dual-energy X-ray absorptiometry (DXA) according to the instructions of the manufacturer (Lunar iDXA, encore 14.5, Madison, WI, USA) [27].

Blood glucose, glycosylated hemoglobin (HbA1c), homocysteine, triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL-c), low density lipoprotein cholesterol (LDL-c), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT) were measured on a suitable autoanalyzer with routine validated procedures. On the other hand, insulin, C-reactive protein (CRP) and plasma concentrations of fibroblast growth factor 21 (FGF-21) values were quantified with specific ELISA kits (Demeditec, Kiel-Wellsee, Germany) in a Triturus auto-analyzer (Grifols, Barcelona, Spain), as described by the manufacturer. The Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), the TyG index (Ln[triglycerides (mg/dL)*glucose(mg/dL)]) and the Atherogenic Index of Plasma (log[triglycerides(mg/dL)/HDL-c(mg/dL)]) were also calculated as described elsewhere [4,10,26]. Physical activity was classified in 4 different categories depending on the level (sedentary, mild, moderated or elevated). Fatty Liver Index (FLI), which has been validated in a large group of subjects with or without liver disease, was also assessed. It is based on an algorithm including BMI, waist circumference, triglycerides and GGT. Accuracy was assessed by calculating the area (AUC) under the receiver operating curve (ROC) model of 0.84 with 95% confidence intervals (95% CI 0.810–0.87) in detecting fatty liver [12,28]. An index <30 points indicates the absence of fatty liver (negative likelihood ratio = 0.2) and ≥60 is a marker of fatty liver (positive likelihood ratio = 4.3) [28].

2.3. Dietary Assessment

The diet of the participants was assessed at baseline with a validated semiquantitative food frequency questionnaire (FFQ) of 137 items [9,11]. Each item in the questionnaire included a typical portion size. The nutrient composition of the food items was derived from accepted Spanish food

composition tables. 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.

Glycemic Index values for single food items on the food frequency questionnaire were derived from the International Tables of Glycemic Index and Glycemic Load Values, as previously reported [12]. Total dietary Glycemic Index was estimated by multiplying the amount of available carbohydrate (g) of each food item by its GI. The sum of these products was divided by the total carbohydrate intake. The amount of carbohydrate can vary in an overall diet and because of this the concept of Glycemic Load was also applied [12].

2.4. Hepatic Imaging Tests

The ultrasonography methodology consisted in the evaluation of the steatosis status by visual quality of the liver echogenicity, measurements of the difference between the kidneys and the liver in the amplitude of the echo and the determination of the clarity of the structures of the blood vessels in the liver. The clinical classification was defined using a 4-point scale: normal (less than 5%), mild steatosis (5–33%), moderate steatosis (33–66%) and severe steatosis (greater than 66%), as described elsewhere [12,29,30]. Transient elastography was performed through FibroScan® (Echosens, Paris, France), with the subject in the supine position and the right arm in maximum abduction. Depending on the obesity status, M and XL probes were selected under the professional criteria. Repeated shots were performed until obtaining 10 valid values of which the median was the selected value. Hepatic fibrosis and cirrhosis were considered if the stiffness median > 7 kPa or >12 kPa, respectively [12]. Magnetic Resonance Imaging (MRI) was performed through Siemens Aera 1.5 T and it was used to detect the volume, fat and iron content of the liver (Dixon technique) as reported by the manufactures [10].

2.5. Metabolomics

The OWLiver® test (One Way Liver S. L. Bilbao, Bizkaia, Spain) is a non-invasive and validated lipidomic serum able to distinguish between a normal liver, simple steatosis or NASH with high accuracy [31]. The metabolomic probe used was a fasting blood probe that measures a panel of biomarkers that belong to the family of triacylglycerols, which are a reflection of the amount of fat and inflammation of the liver and, therefore, a measure of disease development of NAFLD [12]. The relative metabolite concentrations are analyzed together in an algorithm that generates the final OWLiver® score, which discriminates between the three categories (No NAFLD, hepatic steatosis or NASH).

2.6. Genotyping

Genotype screen followed validated procedures [32,33]. A total of 110 epithelial buccal cells sweeps from participants were collected using a liquid-based kit (ORAcollect-DNA, OCR-100, DNA Genotek, Ottawa, ON, Canada). Genomic DNA was isolated using a Maxwell 16 Buccal Swab LEV DNA Purification Kit in the Maxwell 16 instrument (Promega, Madison, WI, USA) according to the instructions of the manufacturer. Genotyping of the *SH2B1* rs7359397 variant was performed by targeted next-generation sequencing using a pre-designed SNP panel (Ion AmpliSeq Custom NGS DNA Panels, Thermo Fisher Scientific Inc., Waltham, MA, USA), as previously described elsewhere [32]. Variants were identified with the Torrent Variant Caller 5.0 (Thermo Fisher Scientific) with a minimum coverage value of 20.

2.7. Statistical Analyses

The sample size for the main study (the FLiO study) was calculated based on the current recommendations of the AASLD on body weight to ameliorate NAFLD features [2]. Therefore, it was estimated to detect a difference of 1.0 ± 1.5 kg in body weight loss between dietary groups [10]. Furthermore, a previous study [12], where NAFLD participants were categorized considering liver fat content (MRI < 5% vs. ≥5%), enabled an "a posteriori" estimation, which revealed that to detect a difference of 3 ± 5 points on hepatic fat content between genotypes, with a 95% confidence

interval ($\alpha = 0.05$) and a statistical power of 80% ($\beta = 0.80$), the estimated sample size was n = 44 for each group. Continuous variables are expressed as means and standard deviation (SD) or as medians and interquartile ranges depending on its distribution, while qualitative categorical variables were analyzed with the chi-squared test and reported as absolute (n) and relative frequencies (%). Chi-squared test was also used to assess the Hardy–Weinberg equilibrium (HWE) concerning the alleles of risk. Distribution of variables was assessed through the Shapiro Wilk and Kolmogorov–Smirnov test. Data normality and outliers were also checked using boxplots. Those variables following a normal distribution were analyzed using parametric statistical tests while for those variables with a non-normal distribution, non-parametric statistics were applied. Descriptive statistics were used to compare baseline data of participants. For continuous variables, Student's *t*-tests (for parametric) of independent samples and Mann-Whitney U tests (for non-parametric) were applied.

The risk of developing severe stages of NAFLD was examined by categorizing the steatosis degree in two groups (mild steatosis vs. moderate steatosis plus severe steatosis), FLI according to the median (<80 vs. ≥80) and liver fat accumulation by MRI was categorized in tertiles. Logistic regression models were set up to evaluate the association of the steatosis degree (dependent variable) with *SH2B1* genetic variant (independent variable) and to assess the influence of the genetic variant on Fatty Liver Index (FLI). Data were expressed in Odds Ratio (OR) and confidence interval. A multinomial logistic regression analysis was also performed to assess the influence of *SH2B1* on the risk of liver fat accumulation (by MRI) and to assess the risk of developing advanced stages of NAFLD (by OWL®) such as NASH. Data were expressed in Relative Risk Ratio (RRR) and confidence interval. Regression models were adjusted for potential confounders, some of them linked to NAFLD such as age, sex and ALT concentrations and others related to lifestyle such as adherence to MedDiet, total energy intake and physical activity. Body Mass Index was used as a covariate in the logistic regression model between steatosis degree and *SH2B1* genetic variant and in the multinomial logistic regression analysis between liver fat content (by MRI) and the polymorphism. In the FLI and OWLiver®-test models, BMI was not included as a covariate due to the possibility of over fitting models, since both the FLI and OWLiver-test include the BMI in its calculation.

Analyses were performed using STATA 12.0 software (Stata Corp College Station, TX, USA). All calculated *p*-values were two-tailed. Values of $p < 0.05$ were considered to be statistically significant in the analyses.

3. Results

3.1. Characteristics of the Participants

A total of 110 participants with NAFLD were included in the study. The polymorphism was in Hardy–Weinberg Equilibrium ($p > 0.05$). The risk allele frequency (T allele) of the rs7359397_*SH2B1* genetic variant was present in about 51% of participants. No-risk genotype (CC) was present in 54 participants, while the heterozygous genotype (CT) was present in 46 subjects and the risk genotype (TT) in just 10 subjects. Because of the small sample of homozygotes for the risk allele, the sample was distributed and analyzed in two different groups: no-risk genotype (n = 54) and risk genotype, (including CT and TT subjects, n = 56). Baseline characteristics of the participants included in the present study were analyzed according to the *SH2B1* rs7359397 genetic variant. Main body composition and biochemical features are reported in Table 1. No significant differences were observed between groups in body composition variables. A marginal significant difference was observed in fat-free mass. However, when the analysis was repeated considering men and women separately, no differences were observed between carriers and non-carriers. Variables according to biochemical parameters showed higher insulin ($p = 0.002$) and lower HDL-c ($p = 0.003$) concentrations in the risk genotype group as compared to the non-risk. Likewise, risk allele carriers showed higher levels of HOMA-IR; Triglycerides/HDL-c ratio, waist*TyG index and atherogenic index ($p = 0.001$; $p = 0.021$; $p = 0.030$ and $p = 0.012$, respectively).

Table 1. Body composition and general biochemical parameters of participants according to genotype (risk and non-risk alleles).

	rs7359397_*SH2B1*		
	CC (No-Risk Genotype) n = 54	**CT/TT (Risk Genotype) n = 56**	***p*-Value**
Body composition			
Weight (kg)	94.2 (14.6)	97.3 (1.8)	0.133
BMI (kg/m2)	33.4 (4.1)	34.3 (3.6)	0.105
Age (y)	51 (47.0–57.0)	49.5 (45.0–56.5)	0.797
Sex n (%)			
Male	25 (46.3)	37 (66.1)	0.037
Female	29 (53.7)	19 (33.9)	
WC (cm)	108.3 (9.7)	111.1 (9.1)	0.119
DXA Body Fat Mass (kg)	38.0 (32.8–44.6)	38.6 (34.3–44.5)	0.935
DXA Lean Mass (kg)	51.9 (43.9–56.9)	55.5 (49.4–61.8)	0.059
DXA VAT (kg)	2.1 (1.4–3.0)	2.4 (1.8–3.1)	0.129
Biochemical parameters			
Glucose (mg/dL)	100 (91.0–111.0)	102.5 (92.5–102.5)	0.421
Insulin (U/mL)	14.1 (9.0–19.8)	20.1 (13.4–25.5)	0.002
TG (mg/dL)	106.5 (76.0–157.0)	127.5 (84.5–160.0)	0.066
TC (mg/dL)	197.5 (40.4)	191.3 (36.8)	0.346
HDL-c (mg/dL)	54.5 (47.0–64.0)	47.0 (40.0–55.5)	0.003
LDL-c (mg/dL)	117.1 (33.9)	115.3 (35.1)	0.788
HOMA-IR	3.5 (2.2–4.7)	4.9 (3.5–6.7)	0.001
HbA1C (%)	5.6 (5.4–5.9)	5.6 (5.4–5.9)	0.622
TyG index	8.5 (8.1–8.9)	8.7 (8.3–9.0)	0.051
Triglycerides/HDL-c (ratio)	1.8 (1.2–3.2)	2.7 (1.7–3.3)	0.021
Waist*TyG index	929.8 (862.3–1001.3)	971.2 (900.8–1033.8)	0.030
HCY (µmol/L)	14.5 (12.2–16.4)	15.1 (11.6–18.1)	0.627
AIP	0.5 (0.2–1.1)	0.9 (0.5–1.2)	0.012

Variables are shown as mean (SD) or as median (IQR) according to their distribution. Categorical variables are presented as absolute (n) and relative frequencies (%). Unpaired *t*-tests and Wilcoxon-Mann-Whitney were used. AIP, Atherogenic Index of Plasma; BMI, Body Mass Index; DXA, Dual-Energy X-ray Absorptiometry; HCY, Homocysteine; HbA1C, Hemoglobin A1c; HDL-c, High Density Lipoprotein-Cholesterol; HOMA-IR, Homeostasis Model Assessment Insulin Resistance; LDL-c, Low-Density Lipoprotein Cholesterol; TC, Total Cholesterol; TG, Triglycerides; TyG index, Triglycerides and Glucose Index; VAT, Visceral Adipose Tissue; WC, Waist Circumference.

Concerning dietary intake and lifestyle factors (Table 2), no significant difference was observed in total energy consumption ($p = 0.101$). Regarding macronutrient distribution, significant differences were found neither in carbohydrates ($p = 0.612$) nor in lipids ($p = 0.308$); but protein percentage was significantly different between genotypes ($p = 0.028$). In addition, the total ingestion of monounsaturated fatty acids ($p = 0.045$) and fiber ($p = 0.049$) were higher in the no-risk genotype. Finally, the adherence to the Mediterranean Diet ($p = 0.678$) and physical activity ($p = 0.685$) were not different between groups.

3.2. Hepatic Status According to Genetic Variant Alleles

Liver blood biomarkers results (Table 3) showed no significant differences between *SH2B1* alleles in most of the variables. Risk allele carriers showed a higher Fatty Liver Index, as well as steatosis degree assessed by ultrasonography. Specifically, significant differences were observed when comparing first vs second and third steatosis degree ($p < 0.001$ and $p = 0.049$, respectively). Risk genotype also showed a higher liver fat content by MRI ($p = 0.055$). The lipidomic analysis showed that some participants, in spite of having a positive result of hepatic steatosis by the ultrasonography, registered a negative result indicating no hepatic steatosis. The frequency of participants with no NAFLD was higher in no-risk genotypes (29.6%). On the other hand, the frequency of NASH was 69.1% in risk allele carriers compared with 44.4% in non-carriers. Concretely, significant differences were found between groups

when comparing No NAFLD (42.55%) vs Hepatic Steatosis (57.45%) and No NAFLD (24.39%) vs. NASH (75.61%) ($p = 0.047$ and $p = 0.001$, respectively).

Table 2. Daily nutrient intake and lifestyle factors of participants.

	rs7359397_*SH2B1*		
	CC (No-Risk Genotype) n = 54	**CT/TT (Risk Genotype) n = 56**	***p*-Value**
Energy and macronutrients			
Total energy (kcal/day)	2649.7 (2181.9–3257.9)	2369.4 (1952.7–2827.7)	0.101
Carbohydrates (%E)	42.3 (6.8)	43.0 (6.8)	0.612
Proteins (%E)	15.7 (14.9–18.1)	17.3 (15.4–20.4)	0.028
Fats (%E)	38.1 (6.14)	36.7 (7.3)	0.308
MUFA (%E)	18.6 (15.5–20.9)	16.2 (14.0–20.0)	0.045
PUFA (%E)	5.4 (4.4–6.7)	5.1 (4.4–6.4)	0.466
SFA (%E)	10.4 (9.3–12.1)	10.3 (9.21–1.8)	0.750
Dietary fiber (g/day)	25.2 (21.2–30.1)	21.3 (17.0–26.8)	0.049
Glycemic Index	53.3 (48.5–58.9)	54.9 (49.1–57.8)	0.988
Glycemic Load	158.6 (98.2–205.6)	139.1 (95.4–176.9)	0.449
Lifestyle factors			
Adherence to MedDiet	5.9 (2.1)	6.0 (2.0)	0.678
Physical activity n (%)			
Sedentary	21 (38.8)	24 (42.8)	0.685
Mild	16 (29.6)	13 (23.2)	
Moderated	9 (16.6)	13 (23.2)	
Elevated	8 (14.8)	6 (10.71)	

Variables are shown as mean (SD) or as median (IQR) according to their distribution. Unpaired *t*-tests were carried out. *p* value from paired *t*-test or from Wilcoxon-Mann-Whitney test. Categorical variables are presented as absolute (n) and relative frequencies (%). %E, Percentage of Energy; MUFA, Mono-Unsaturated Fatty Acid; PUFA, Poly-Unsaturated Fatty Acid; SFA, Saturated Fatty Acid.

3.3. Association between Genotype and Advanced Stages of the Disease

An adjusted logistic regression analysis was performed considering the median of the FLI (Table S1). Results showed that those subjects carrying the T allele presented a major risk (OR 2.91) of developing a higher punctuation in FLI than homozygous subjects for the C allele (Figure 1).

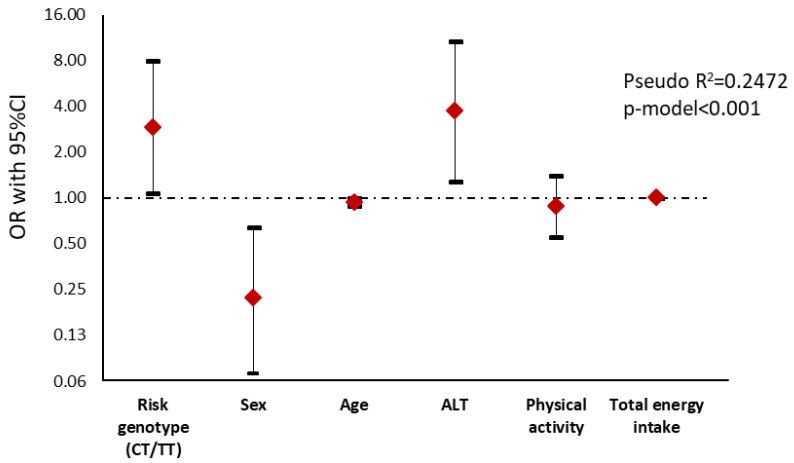

Figure 1. Graphical display of the Odds Ratio (OR) (95% confidence interval) of the logistic regression analysis between Fatty Liver Index and genotype in NAFLD subjects. Fatty Liver Index is the dependent variable and was dichotomized according to the median (0 = FLI < 80 vs. 1 = FLI ≥ 80). Notice that the *y*-axis is on a log scale. ALT, Alanine Aminotransferase.

Table 3. Liver status differences depending on the genotype.

	rs7359397_*SH2B1*		
	CC (No-Risk Genotype) n = 54	**CT/TT (Risk Genotype) n = 56**	***p*-Value**
Liver markers			
CRP (mg/dL)	0.2 (0.1–0.4)	0.2 (0.1–0.5)	0.959
FGF21 (pg/mL)	182.0 (96.6–302.0)	214.0 (122.0–478.0)	0.109
AST (U/L)	23.5 (18.0–28.0)	21.0 (18.0–29.0)	0.995
ALT (U/L)	26.0 (18.0–39.0)	30.0 (22.0–46.0)	0.266
Ratio AST/ALT	0.8 (0.6–1.0)	0.7 (0.6–0.9)	0.149
GGT (U/L)	26.0 (19.0–40.0)	32.0 (22.5–44.0)	0.109
FLI	79.8 (66.3–91.2)	87.5 (76.6–93.7)	0.032
Liver imaging techniques			
Grade of steatosis (ultrasonography) n (%)			
Mild steatosis	39 (72.2)	22 (39.29)	
Moderate steatosis	11 (20.37)	26 (46.43)	0.001
Severe steatosis	4 (7.41)	8 (14.29)	
TE liver stiffness (kPa)	4.5 (3.8–6.1)	4.5 (3.8–5.6)	0.738
MRI Hepatic Volume (mL)	1701.0 (1409.0–1998.0)	1843.0 (1589.0–2111.0)	0.150
MRI Liver fat—Dixon (%)	4.5 (2.9–8.9)	6.9 (4.4–12.4)	0.055
MRI Hepatic Iron—Dixon (%)	31.8 (28.2–44.2)	32.4 (29.2–38.0)	0.950
Lipidomic analysis (OWLiver®-test) n (%)			
No NAFLD	16 (29.6)	4 (7.3)	
Hepatic steatosis	14 (25.9)	13 (23.6)	0.006
NASH	24 (44.4)	38 (69.1)	

All variables are shown as median (IQR). Unpaired *t*-test was carried out. *p* value from Wilcoxon-Mann-Whitney test. Categorical variables are presented as absolute (n) and relative frequencies (%). ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; CRP, C-Reactive Protein; FLI, Fatty Liver Index; FGF21, Fibroblast Growth Factor 21; GGT, Gamma-Glutamyl Transferase; NAFLD, Non-Alcoholic Fatty Liver Disease; NASH, Non-Alcoholic Steatohepatitis; MRI, Magnetic Resonance Imaging; TE, Transient Elastography.

When the steatosis degree was assessed by ultrasonography (Table S2), subjects carrying the CT/TT genotype showed a significant association with a higher steatosis degree than non-carriers ($p = 0.004$). The genetic variant remained significant after adjusting for potential confounders such as sex, age and physical activity, indicating that those subjects carrying the risk allele had a 4.15 value risk of having a higher steatosis degree than non-carriers (Figure 2).

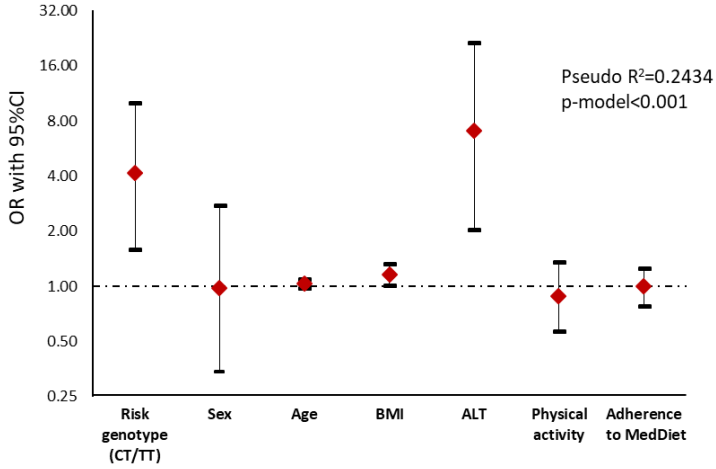

Figure 2. Graphical display of the Odds Ratio (OR) (95% confidence interval) of the logistic regression analysis showing the association between steatosis degree measured by ultrasonography (0 = no or mild steatosis vs. 1 = moderate and severe steatosis) and genotype risk in NAFLD subjects. Notice that the *y*-axis is on a log scale. ALT, Alanine Aminotransferase; BMI, Body Mass Index.

Multinomial logistic regression analyses were also performed to assess the influence of the genetic variant on liver fat accumulation by MRI as well as on the risk of developing NASH by the lipidomic test (Tables S3 and S4). The main results evidenced that risk allele carriers had an increased risk for liver fat accumulation (RRR 3.93) as well as for the development of NASH (RRR 7.88) in comparison with homozygous subjects (Figures 3 and 4).

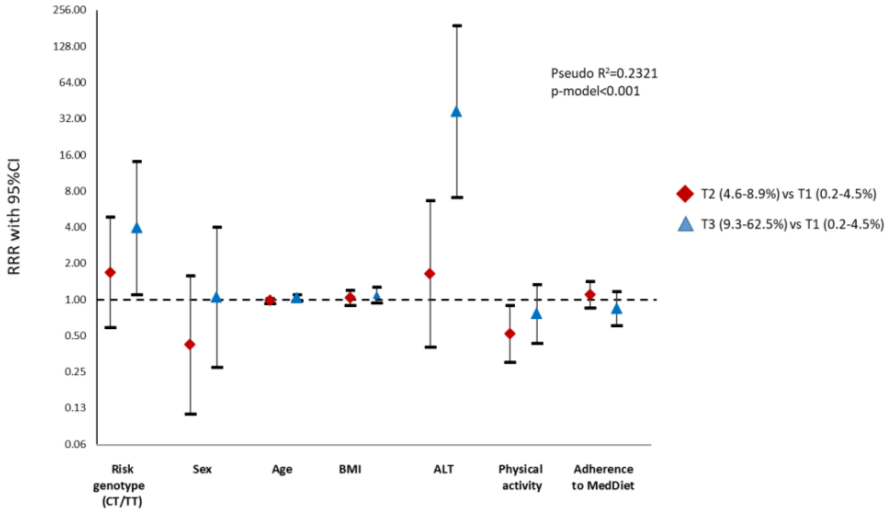

Figure 3. Graphical display of the Relative Risk Ratio (RRR) (95% confidence interval) of the multinomial logistic regression model with *SH2B1* genotype as independent variable and liver fat content (tertiles) assessed by Magnetic Resonance Imaging (MRI) as a dependent variable. Notice that the *y*-axis is on a log scale. ALT, Alanine Aminotransferase; BMI, Body Mass Index.

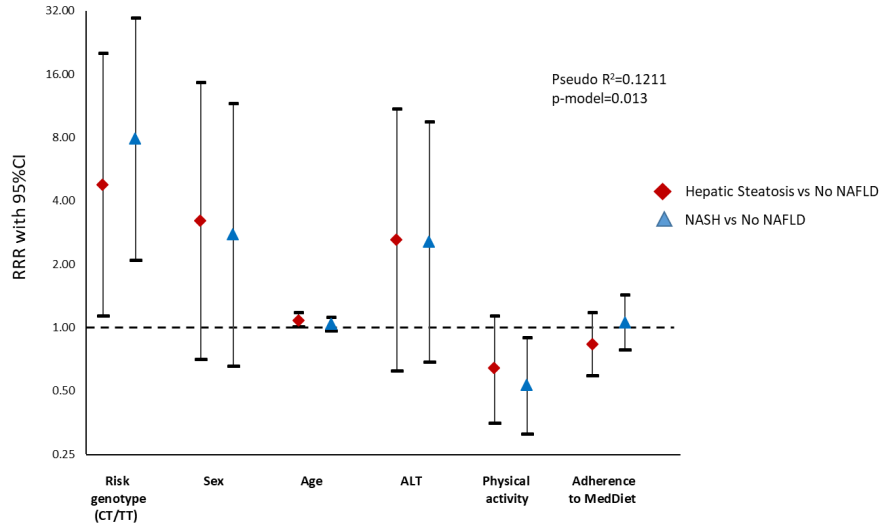

Figure 4. Graphical display of the Relative Risk Ratio (RRR) (95% confidence interval) of the multinomial logistic regression model with *SH2B1* genetic variant as an independent variable and the diagnostic of steatosis or steatohepatitis (by lipidomic analysis) as a dependent variable. Notice that the *y*-axis is on a log scale. ALT, Alanine Aminotransferase; NAFLD: Non-alcoholic Fatty Liver Disease; NASH, Non-alcoholic Steatohepatitis.

4. Discussion

This research project aimed to analyze the influence of a metabolism-related polymorphism on the development of advanced stages of the disease, as well as the influence of the diet in subjects with

NAFLD. Interestingly, the results yielded evidence that the *SH2B1* has an impact on the development and progression of this hepatic disease. Risk allele carriers showed high risk for higher liver fat accumulation as assessed by FLI, ultrasonography and MRI and higher risk for developing NASH as assessed by metabolomics in this cross-sectional analysis.

It should be taken into account that heritability is involved in the development of advanced phases, and NAFLD has a genetic component [34,35]. Some genetic variants in genes like the PNPLA3 I148M, the TM6SF2 and the MBOAT7, have been strongly associated with the development of NAFLD-HCC [18,36,37] and with hepatic fat accumulation [16]. However, the aforementioned variants are not separated enough themselves to identify patients at risk of developing severe stadiums, most likely due to the influence and interaction of other genetic and non-genetic factors [6,18,24].

Some investigations have demonstrated that patients with advanced stages of NAFLD also present a high frequency of less prevalent gene variants [18]. Because of this, a sequence variant located in the intergenic region between genes, *rs7359397 SH2B1,* was studied. This genetic variant, which is a member of the *SH2B* family of adapter proteins that also include *SH2B2* and *SH2B3* has been reported to be pathogenic for obesity [38–40]. *SH2B1* and *SH2B2* are abundantly expressed in the brain, liver, heart, skeletal muscle and adipose tissue [22]. By contrast, *SH2B3* expression is restricted to hematopoietic tissue [41]. Besides this, in another genome-wide association study (GWAS), the gene *SH2B1* was suggested as a physiological enhancer of insulin receptors and downstream signaling [42]. Being pathogenic for both obesity and insulin resistance, *SH2B1* is a strong candidate for involvement in NAFLD risk and severity. Because of this, we tested the hypothesis that individuals with the rs7359397 T allele have a higher risk of developing severe stages compared with the no-risk genotype, as well as the role of diet combined with genetics in the pathogenesis of the disease.

The *SH2B1* genetic variant has also been related to body composition [15]. In the study, we found a marginal association with DXA lean mass and the T allele ($p = 0.059$), which could be related to the differences observed in sex ($p = 0.037$). Due to the possible influence of sex in body composition and biochemical variables, such as HDL-c or waist circumference, an analysis stratifying by sex was performed and no statistical differences were found. Moreover, the risk genotype was correlated with a higher value of insulin, HOMA-IR, TyG-index and higher ratio of Triglycerides/HDL-c and Waist*TyG-index, all of them related to insulin resistance. In this sense, scientific evidence revealed that *SH2B1* knock-out mice develop obesity and hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance due to the central role of *SH2B1* in the regulation of glucose and lipid metabolism [39,42–44].

In relation to obesity, not only dietary intake and lifestyle, but also genetics have an impact on adiposity being involved in 25–70% in body weight variability [45]. Numerous genes and less frequent variants have been associated with the regulation of energy metabolism [18,39]. Investigations into gene–environment relationships have reported that genes related to nutrient metabolism and transport have a direct association with the requirements of specific nutrients [46]. For example, studies carried out in the Caucasian obese population, following up a hypocaloric diet, showed an association between obese genes and body weight loss, as well as changes in fasting insulin levels and HOMA-IR [47,48]. Furthermore, genetic interactions with environmental factors have been demonstrated to modulate the different responses to a dietary intervention [49,50]. Focusing on NAFLD, there is an increased interest on the study of possible interactions between nutritional factors and genes. An analysis on the Framingham Heart Study reported that increasing diet quality was associated with an improvement of the hepatic health, which is especially of benefit to individuals with a high genetic risk of NAFLD [51]. Moreover, it has been reported that there is a higher effect of I148M *PNPLA3* on steatosis severity in individuals consuming diets poor in vegetables [52]. Concerning the *SH2B* gene, researchers point out that it may be a key target in the regulation of energy balance and body weight [25,53]. A study in mice [39] revealed that neuronal *SH2B1* regulates body weight and nutrient metabolism, this being a genetic variant implicated in glucose and lipid metabolism [25,39]. Another study in children [54] was able to evidence the increased risk (>90%) of developing celiac disease by the genotype of

five candidate genes (*SH2B3, RGS1, TAGAP, cREL*, and *LPP*). Together, these findings can help to specifically establish personalized nutritional guidelines that complement the management of NAFLD [21,34]. In our results, dietary and lifestyle characteristics were also evaluated with a semiquantitative FFQ due to the reported association with NAFLD [34]. No significant differences were found in the majority of the nutrients. Besides, similar adherence to the MedDiet score was observed. However, risk allele genotypes referred to higher protein consumption and less MUFA and fiber intake. The higher percentage of dietary fiber could be associated with the lower liver damage presented in non-risk genotypes. This association has also been described previously in NAFLD subjects [3,9]. Second, even though no significant interaction was found between dietary variables and genotype, mechanisms underlying the modulation of macronutrient intake on the *SH2B1* genetic variant are not fully understood and further experimental studies are needed. Lastly, these results have been analyzed at baseline and at one particular moment, so further studies analyzing long-term dietary, lifestyle characteristics and possible gene interactions could be of interest.

Moreover, obesity and insulin resistance are risk factors for NAFLD, but liver biopsy remains the gold standard for the diagnosis [55]. However, it is a flawed and invasive procedure, which can lead to complications [56,57]. Effective screening is essential due to the high prevalence of NAFLD. There is an urgent need to develop a non-invasive and affordable method. In this sense, the study of the relationship of genetics and risk stratification of NAFLD could be useful to provide personalized information about the stage of the disease.

In this article, the *SH2B1* genetic variant was associated with the highest values of the FLI [28]. FLI is a tool for screening liver fat since it is a non-invasive method, inexpensive and is widely available and validated against ultrasonography, but not for diagnosis of NAFLD due to some limitations [58,59]. At the same time, rs7359397_*SH2B1* was associated with the steatosis degree, evidencing a higher risk of higher liver fat accumulation in subjects carrying the risk allele. In this context, Sheng et al. [22] showed that the deletion of *SH2B1* in peripheral tissues promoted hepatic steatosis with an accumulation of liver fat. The results of another article [24] showed defects in the *SH2B1* genetic variant in obese patients with diabetic problems, which are important risk factors for the development of steatosis. When FLI and ultrasonography logistic regression models were adjusted, their association with the polymorphism remained significant, evidencing the influence of the genetic variant in the development of severe stadiums of NAFLD.

On the other hand, one of the main findings of this research was the association between the genetic variant with the content of liver fat measured by MRI. Risk allele participants showed a higher risk for excessive liver fat deposition. Recent studies have indicated that higher levels of abdominal fat, particularly visceral fat, are closely related to NAFLD [60]. In addition, higher visceral fat content was observed in the risk allele group as compared with the other. However, a significant association between visceral fat and *SH2B1* genetic variants were not found in this cohort ($p = 0.129$).

Another relevant result concerned the lipidomic test, which has been used in liver examinations [61]. The OW-Liver Test® and *SH2B1* genetic variants were associated, showing that participants carrying the risk allele presented an increased risk for developing NASH than non-carriers. Therefore, carrying the polymorphism evidenced an important influence on the progression of the disease. This test is a valid, precise and non-invasive method. It has been previously related to more adverse liver markers and general metabolic status in subjects with higher liver damage, also revealing a higher association with steatosis gradation (ultrasonography), as described elsewhere [12]. However, it should be mentioned that even though it is a promising test, it is necessary to develop affordable non-invasive methods in clinical practice.

It is important to mention that most of the models increased their predictive value when adjusting for confounders (ALT, physical activity and diet characteristics). These results may indicate that lifestyle characteristics as well as sex and age should be specifically considered in the personalized management of NAFLD [62,63], along with the traditionally contemplated risk factors such as obesity or insulin resistance [2,13]. Furthermore, hepatic alterations may also be considered. In our case,

the prediction of the models was higher when including ALT, but not AST. According to the scientific bibliography, ALT seems to be a sensitive and accurate biomarker of NAFLD [12,64,65], even though in many other cases no associations have been found [12,66]. However, in our analysis, the association of the genetic variant and NAFLD was independent of the adjusting variables, and it is important to note that the prediction of the model was considerably raised when including this genetic variant. Therefore, these findings suggest that NAFLD is associated with this genetic obesity-related polymorphism.

Some limitations concerning this research should be mentioned: Firstly, due to the cross-sectional design of the study, causal inferences cannot be made. Secondly, liver biopsy results were not available to corroborate the precise diagnosis of patients, thus all the associations observed are related to the non-invasive markers of steatosis. Moreover, these data need to be confirmed by histology in future studies [67]. Thirdly, the screening of the participants, including information about competing causes of liver disease, was based on a clinical interview. Additionally, dietary evaluations were carried out using self-reported information of the participants. Thus, subjective measures could produce some biases. The relatively small sample size and the absence of a control group are other limitations. However, the participants included were all well-characterized by the evaluation of NAFLD using recognized techniques such as the MRI and the validated lipidomic test (OWL®). Likewise, the combination of TT and CT genotypes could have influenced the results. Despite this, analyses were repeated excluding TT participants and no differences were observed in the results (data not shown).

To our knowledge, a strong point of the study is the potential role of a *SH2B1* genetic variant in the promotion of NAFLD according to a nutritional assessment. Based on the findings reported here, further studies should contemplate the possibility of a risk stratification in accordance with the *SH2B1* genotyping once increasing the proportion of homozygous risk allele carriers in the study population in order to better assess the role of the polymorphism in NAFLD subjects. Moreover, the participants included presented early states of liver damage. Increasing the sample size and enrolling more advanced stages of the disease will also reveal interesting results regarding liver stiffness.

5. Conclusions

Carriers of the minor allele of *SH2B1* genotypes have been associated with a higher risk of developing NASH in overweight and obese individuals. These results support the importance of considering genetic predisposition in combination with a healthy dietary pattern in the personalized evaluation and management of NAFLD.

Little is known about the frequency of this risk allele in the general population, the non-obese population, and the non-NAFLD population. Besides, since multiple factors are involved in the pathogenesis of NAFLD, the likelihood of it developing in individuals without the variant should also be studied for the precision management of this disease. Future investigations regarding these issues as well as the possible influence of this genetic variant in the overweight or lean population with NAFLD could be of interest.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/5/1260/s1, Table S1: Logistic regression analysis between Fatty Liver Index and genotype in NAFLD subjects. Table S2: Logistic regression analysis showing the association between steatosis degree measured by ultrasonography and genotype risk in NAFLD subjects. Table S3: Multinomial logistic regression model with SH2B1 genotype as independent variable and Liver fat content assessed by Magnetic Resonance Imaging (MRI) as dependent variable. Table S4: Multinomial logistic regression model with SH2B1 genetic variant as an independent variable and the diagnostic of steatosis or steatohepatitis (by lipidomic analysis) as dependent variable.

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Conflicts of Interest: The authors declare no conflict of interest concerning this research.

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Table S1. Logistic regression analysis between Fatty Liver Index and genotype in NAFLD subjects.

	OR (95% CI)	**p-value**
rs7359397 *SH2B1*		
CC (No risk genotype)	Reference	
CT/TT (Risk genotype)	2.91 (1.07;7.91)	0.036
Sex	0.22 (0.07;0.64)	0.006
Age	0.94 (0.89;1.00)	0.060
ALT	3.72 (1.29;10.7)	0.015
Physical activity	0.88 (0.55;1.40)	0.602
Total energy intake	1.00 (0.99;1.00)	0.186
	Pseudo R^2 0.2472	**p-model <0.001**

p<0.05 was considered statistically significance. Odds Ratio (95% confidence interval) for hepatic steatosis were compared by logistic regression. ALT, Alanine aminotransferase. FLI was categorized according to the median (<80 vs. ≥80).

Table S2. Logistic regression analysis showing the association between steatosis degree measured by ultrasonography and genotype risk in NAFLD subjects.

	OR (95% CI)	**p-value**
rs7359397 *SH2B1*		
CC (No risk genotype)	Reference	
CT/TT (Risk genotype)	4.15 (1.59;10.8)	0.004
Sex	0.97 (0.34;2.76)	0.956
Age	1.03 (0.98;1.09)	0.183
BMI	1.16 (1.01;1.32)	0.027
ALT	7.01 (2.03;21.37)	<0.001
Physical activity	0.88 (0.57;1.36)	0.567
Adherence to MedDiet	0.99 (0.78;1.25)	0.935
	Pseudo R^2 0.2434	**p-model <0.001**

p<0.05 was considered statistically significance. Odds Ratio (95% confidence interval) for hepatic steatosis were compared by logistic regression. ALT, Alanine aminotransferase; BMI, Body Mass Index.

Table S3. Multinomial logistic regression model with *SH2B1* genotype as independent variable and Liver fat content assessed by Magnetic Resonance Imaging (MRI) as dependent variable.

	RRR (95% CI)	**p-value**
MRI Liver fat content		
T1 (0.2 – 4.5%)	Reference	
T2 (4.6 – 8.9%)		
CC (No-risk genotype)	Reference	
CT/TT (Risk genotype)	1.68 (0.58;4.88)	0.337
Sex	0.42 (0.11;1.58)	0.202
Age	0.98 (0.92;1.02)	0.649
BMI	1.03 (0.89;1.20)	0.635
ALT	1.64 (0.40;6.72)	0.490
Physical activity	0.52 (0.30; 0.90)	0.021
Adherence to MedDiet	1.10 (0.84;1.42)	0.470
T3 (9.3 – 62.5%)		
CC (No-risk genotype)	Reference	
CT/TT (Risk genotype)	3.93 (1.09;14.10)	0.036
Sex	1.04 (0.27;4.02)	0.948
Age	1.03 (0.96;1.10)	0.308
BMI	1.08 (0.93;1.27)	0.284
ALT	36.6 (7.03;191.22)	<0.001
Physical activity	0.76 (0.43;1.33)	0.346
Adherence to MedDiet	0.84 (0.60;1.17)	0.309
	Pseudo R^2 0.2321	**p-model <0.001**

Relative Risk Ratio (95% confidence interval) for Liver fat content assessed by Magnetic Resonance Imaging (MRI) were compared by multinomial logistic regression. p<0.05 was considered statistically significance. ALT, Alanine aminotransferase; BMI, Body Mass Index; MRI, Magnetic Resonance Imaging.

Table S4. Multinomial logistic regression model with *SH2B1* genetic variant as an independent variable and the diagnostic of steatosis or steatohepatitis (by lipidomic analysis) as dependent variable.

	RRR (95% CI)	**p-value**
OWLiver®-Test		
No NAFLD or inconclusive	Reference	
Hepatic steatosis		
CC (No-risk genotype)	Reference	
CT/TT (Risk genotype)	4.77 (1.13;20.09)	0.033
Sex	3.20 (0.70;14.64)	0.132
Age	1.08 (1.00;1.18)	0.047
ALT	2.61 (0.62;10.98)	0.191
Physical activity	0.64 (0.35;1.14)	0.131
Adherence to MedDiet	0.83 (0.59; 1.18)	0.313
NASH		
CC (No-risk genotype)	Reference	
CT/TT (Risk genotype)	7.88 (2.08;29.75)	0.002
Sex	2.76 (0.65;11.6)	0.164
Age	1.04 (0.96;1.12)	0.282
ALT	2.55 (0.68;9.55)	0.165
Physical activity	0.53 (0.31; 0.90)	0.020
Adherence to MedDiet	1.05 (0.78; 1.43)	0.712
	Pseudo R^2 0.1211	**p-model <0.013**

Relative Risk Ratio (95% confidence interval) for the diagnostic of steatosis or steatohepatitis (by lipidomic analysis) by multinomial logistic regression. p<0.05 was considered statistically significance. ALT, Alanine aminotransferase; NAFLD: Non-alcoholic Fatty Liver Disease; NASH, Non-alcoholic steatohepatitis.

Chapter 2

Differential response to a 6-month energy-restricted treatment depending on SH2B1 rs7359397 variant in NAFLD subjects: Fatty Liver in Obesity (FLiO) Study

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Chapter 3

Three different genetic risk scores based on Fatty Liver Index, Magnetic Resonance Imaging and Lipidomic for a nutrigenetic personalized management of NAFLD: The Fatty Liver in Obesity Study

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Article

Three Different Genetic Risk Scores Based on Fatty Liver Index, Magnetic Resonance Imaging and Lipidomic for a Nutrigenetic Personalized Management of NAFLD: The Fatty Liver in Obesity Study

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Abstract: Non-alcoholic fatty liver disease (NAFLD) affects 25% of the global population. The pathogenesis of NAFLD is complex; available data reveal that genetics and ascribed interactions with environmental factors may play an important role in the development of this morbid condition. The purpose of this investigation was to assess genetic and non-genetic determinants putatively involved in the onset and progression of NAFLD after a 6-month weight loss nutritional treatment. A group of 86 overweight/obese subjects with NAFLD from the Fatty Liver in Obesity (FLiO) study were enrolled and metabolically evaluated at baseline and after 6 months. A pre-designed panel of 95 genetic variants related to obesity and weight loss was applied and analyzed. Three genetic risk scores (GRS) concerning the improvement on hepatic health evaluated by minimally invasive methods such as the fatty liver index (FLI) (GRS_{FLI}), lipidomic-OWLiver®-test (GRS_{OWL}) and magnetic resonance imaging (MRI) (GRS_{MRI}), were derived by adding the risk alleles genotypes. Body composition, liver injury-related markers and dietary intake were also monitored. Overall, 23 SNPs were independently associated with the change in FLI, 16 SNPs with OWLiver®-test and 8 SNPs with MRI, which were specific for every diagnosis tool. After adjusting for gender, age and other related predictors (insulin resistance, inflammatory biomarkers and dietary intake at baseline) the calculated GRS_{FLI}, GRS_{OWL} and GRS_{MRI} were major contributors of the improvement in hepatic status. Thus, fitted linear regression models showed a variance of 53% (adj. $R^2 = 0.53$) in hepatic functionality (FLI), 16% (adj. $R^2 = 0.16$) in lipidomic metabolism (OWLiver®-test) and 34% (adj. $R^2 = 0.34$) in liver fat content (MRI). These results demonstrate that three different genetic scores can be useful for the personalized management of NAFLD, whose treatment must rely on specific dietary recommendations guided by the measurement of specific genetic biomarkers.

Keywords: NAFLD; genetic risk score; fatty liver index; lipidomic; magnetic resonance imaging

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver disease in high-income countries, affecting more than 25% of the population [1]. NAFLD includes a spectrum of liver disease conditions ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with variable degrees of fibrosis and cirrhosis [2,3], and it has become one of the most common causes of chronic liver diseases [3].

The NAFLD etiology is multifactorial and yet incompletely understood, but ultimately appears as determined by the combination of environmental factors, such as excessive adiposity or the presence of type 2 diabetes (T2D) as well as the accumulation of intrahepatic lipids, alterations of energy metabolism, insulin resistance and inflammatory processes, where the genetic make-up may emerge [1,4]. Other factors such as obesity and a sedentary lifestyle, together with metabolic syndrome features and ethnicity, influence the risk of NAFLD [5].

The gold standard for the diagnosis of NAFLD is liver biopsy [6]. However, the decision about when to perform this screening remains controversial [2], being necessary the search for less invasive methods for screening patients suspected of this disease [6–9]. To date, ultrasonography is recommended as the first-line diagnostic method in assessing steatosis [6]. Moreover, magnetic resonance imaging (MRI) has also shown a high accuracy for diagnosing liver fat content [10], as well as advances in the analysis of big data from lipidomic have provided novel insights [11]. Furthermore, non-invasive biomarkers and some validated algorithms such as the fatty liver index (FLI) have also become a useful tool for the diagnosis of simple steatosis and hepatic functionality [12,13].

Heritability and family history have a clinically relevant impact on fatty liver disease onset and progression [1,5,14]. In particular, the genetic variants in the genes *PNPLA3* (rs738409), *TM6SF2* (rs58542926), *GCKR* (rs1260326) and *MBOAT7* (rs641738) have been associated with the risk of NAFLD [15–17]. Not only these genes, but also other genetic variants related with obesity traits and loci have been associated with a higher risk of developing a severe stage of NAFLD [14,18].

Although these polymorphisms explain only a small fraction of the total heritability of NAFLD, it is possible that the combination of specific single nucleotide polymorphisms (SNPs) into a genetic risk score (GRS) could increase the detection and evolution of NAFLD [5,10,19]. Hence, some models have investigated the impact of the genetic predisposition to accumulate liver fat on NAFLD. In this context, some researchers have demonstrated an association between the combination of SNP in a GRS with de novo hepatocellular carcinoma (HCC) [16,20], but also with higher hepatic fat content, total cholesterol, steatosis degree and alanine aminotransferase (ALT) levels [5,15,17]. In this sense, an independent regulation of fat distribution from total adiposity has been suggested, where genes near loci regulating total body mass are enriched for expression in the Central Nervous System, while genes for fat distribution are enriched in adipose tissue itself [21].

Concerning treatment, there are no specific medications that directly treat NAFLD [22,23], being lifestyle modifications and weight control the most fundamental steps in the management of NAFLD [10]. In this context, the European Association for the Study of the Liver (EASL) recommends diet and physical activity as the best treatment for steatosis [24–26]. However, increasing evidence suggests that interindividual variability in weight loss also depends on interactions between genetic and environmental factors, including lifestyle [27–29]. Besides, the well and precise characterization of each gene and its different related pathways is essential in order to devise new therapies [6]. Indeed, a personalized treatment taking into account genetics, lifestyle and specific macronutrient recommendations is needed [26,30,31].

In this sense, the aim of the present study is to assess genetic and non-genetic factors putatively involved in the improvement of the hepatic health after a 6-month hypocaloric nutritional treatment. To test this hypothesis, we combined 47 obesity-related genetic variants associated with different NAFLD non-invasive methods based on the change of hepatic functionality (FLI), lipid metabolism (OWLiver®-test) and liver fat content (by MRI) into three differences scores, where the role of baseline status was assessed to predict outcomes from precise nutrition management.

2. Materials and Methods

2.1. Study Design

The current randomized controlled trial was designed to compare the effectiveness of two weight loss dietary strategies with different nutritional features, anthropometric measurements, body composition and biochemical markers on hepatic health in overweight or obese subjects with ultrasonography-proven liver steatosis, as described elsewhere [32,33]. The intervention had a duration of 24 months and the participants were randomly assigned to the American Heart Association (AHA) or the Fatty Liver in Obesity (FLiO) group [34]. However, the present study was performed concerning the results after 6 months of follow-up. The study was approved by the Research Ethics Committee of the University of Navarra, Spain on 24 April 2015 (ref. 54/2015) and accessed on www.clinicaltrials.gov (FLiO: Fatty Liver in Obesity study; NCT03183193). Each subject gave written informed consent prior to 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.

2.2. Study Participants

A total of 98 men and women with overweight or obesity (body mass index (BMI) $\geq$ 27.5 kg/m^2 to < 40 kg/m^2) between 40–80 years old and with hepatic steatosis confirmed by abdominal ultrasonography fulfilled the selection criteria and were enrolled in the study [35]. After 6 months, a total of 70 participants completed the evaluation. 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 standard drinks per week in men and >14 standard drinks per week for women) [36], endocrine disorders (hyperthyroidism or uncontrolled hypothyroidism), pharmacological treatments (immunosuppressants, cytotoxic agents, systemic corticosteroids or other drugs that could potentially cause hepatic steatosis or altering liver tests) [37], 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 the use of weight modifiers, severe psychiatric disorders and difficulties in following the scheduled visits.

2.3. Dietary and Lifestyle Intervention

Two energy-restricted diets, AHA (n = 41) and FLiO (n = 45), were prescribed [33]. Both diets applied an energy restriction of 30% of the total energy requirements of each participant in order 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 guidelines (AASLD) [37]. After 6 months of nutritional treatment, both AHA and FLiO groups (n = 34 and 36, respectively) achieved comparable results in the evaluated main variables and no significant differences in the changes between the intervention groups were found [33]. Therefore, participants were merged and compared together. The habitual dietary intake was registered with a validated semi-quantitative food frequency questionnaire (FFQ) of 137 items, both at baseline and after the 6-month intervention [38,39]. The composition of the food items was derived from accepted Spanish food composition tables as previously described [4,40]. The adherence to the Mediterranean Diet was assessed with a validated 17-point score questionnaire [41,42]. A physical activity prescription of

10,000 steps/day was given to the participants [43,44]. The physical activity level was evaluated using the validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire [44]. The volume of activity was indicated in metabolic equivalent of the task (METs), as described elsewhere [43].

2.4. Anthropometric, Body Composition and Biochemical Assessments

Anthropometric variables (body weight, height and waist circumference) and body composition (Lunar iDXA, Encore 14.5, Madison, WI, USA) were assessed in fasting conditions at the Metabolic Unit of the University of Navarra following standardized procedures [45]. BMI was calculated as the body weight divided by the squared height (kg/m^2). Blood samples were properly collected after overnight fasting of 8–10 h and processed at the Laboratory of Biochemistry of the University of Navarra Clinic (CUN, Pamplona, Spain). Blood glucose, triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) concentrations were determined on a Cobas 8000 autoanalyzer with specific commercial kits and following the instructions of the company (Roche Diagnostics, Basel, Switzerland). Insulin, fibroblast growth factor 21 (FGF-21), leptin and adiponectin concentrations were quantified with 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 computed as HOMA-IR = (insulin (μU/mL) × glucose (mmol/L))/22.5 [4]. The Triglycerides/Glucose index (TyG) (ln[triglycerides (mg/dL) × glucose(mg/dL)/2)]) was also calculated as a surrogate of glucose tolerance [46].

2.5. Imaging Techniques for the Assessment of Liver Status

The whole liver evaluation was performed under fasting conditions at the University of Navarra Clinic. Liver steatosis was determined by ultrasonography (Siemens ACUSON S2000 and S3000, Erlangen, Germany) in accordance with previously described methodology [47]. The clinical classification was established according to a 4-point scale: less than 5% (grade 0), 5–33% (grade 1), 33–66% (grade 2) and greater than 66% (grade 3), as described elsewhere [48]. Finally, magnetic resonance imaging (Siemens Aera 1.5 T) was used to determine the hepatic volume and the fat content of the liver (Dixon technique) as reported by the manufacturer [32].

Fatty liver index (FLI) was calculated using serum triglycerides, BMI, waist circumference and GGT concentrations using the formula described elsewhere [12].

2.6. Metabolomics

The metabolomic test OWLiver® (One Way Liver S.L., Bilbao, Spain) is a fasting blood probe able to measure the degree of NAFLD development [32]. The test score is based on a prospective study, where subjects had previously been diagnosed by liver biopsy [49]. The methodology of this test consisted of the measure of a panel of biomarkers that belong to the family of triacylglycerols (TGs), which are a reflection of the amount of fat and inflammation of the liver [32]. The final OWLiver® score is generated by the relative metabolite concentrations, which are analyzed together in a specific algorithm that gives the probabilities of normal liver, steatosis or NASH.

2.7. SNP Selection and Genotyping

A total of 86 oral epithelium samples were collected with a liquid-based kit (ORAcollect-DNA, OCR-100, DNA Genotek Inc, Ottawa, Canada). Genomic DNA was isolated using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit (Promega Corp, Madison, WI, USA). The quality characterization was carried out by dsDNA quantification (Qubit, Thermo Fisher, Waltham, MA, USA). A pre-designed panel of 95 genetic variants related to obesity and weight loss was applied and analyzed [10,50–52]. More information about these obesity-related SNPs can be found in a previous report [28]. Genotyping was performed by targeted next generation sequencing on Ion Torrent PGM equipment (Thermo Fisher

Scientific Inc., Waltham, MA, USA) [53], as previously published [50,54]. Overall, the amplicon mean size was 185 bp. As quality control of the sequencing process, the number of readings per amplifier and per sample were doubly checked, to make sure there are more than 50× (in fact, it is above about 400× even the worst). Library construction was carried out using a custom-designed panel and the Ion 198 AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) as per the manufacturer's protocol. The raw data were processed with the Ion Torrent Suite Server Version 5.0.4 (Thermo Fisher Scientific Inc, Waltham, MA, USA) using *Homo sapiens* (genome assembly Hg 19) as the reference genome for the alignment. A custom-designed Bed file was used to locate the SNPs of interest. Genetic variants were identified with the Torrent Variant Caller 5.0 (Thermo Fisher Scientific) with a minimum coverage value of 20. Hardy–Weinberg equilibrium, linkage disequilibrium and haplotype inferences were estimated using the Convert program (Version 1.31) and the Arlequin software (Version 3.0). Hardy–Weinberg equilibrium was calculated with a statistical test (Chi-square).

2.8. Genetic Risk Score (GRS)

Three individual GRS based on the pre-designed panel of 95 SNPs were calculated for the change of each non-invasive diagnostic method (FLI, MRI and OWLiver®-test) (Figure 1) according to the following steps. Firstly, Kruskal–Wallis tests were performed to identify SNPs statistically or marginally associated with the change in FLI, liver fat content by MRI and metabolomics assessed by OWLiver®-test (absence of allele, presence of one allele or presence of two alleles) in our samples, obtaining a total of 47 SNPs with a *p*-value lower than 0.20. Secondly, post-hoc tests (Mann–Whitney U-test pairwise) were run to define differences between genotypes in order to be differentially coded as risk and non-risk groups with these 47 SNPs. A risk genotype was defined as the one that was associated with a lower change of FLI, liver fat content (MRI) and OWLiver®-test. Genotypes with similar effects were clustered in a single category. In a third step, Mann–Whitney U-test was applied to confirm statistical differences between the categorized genotype groups (risk vs. non-risk), selecting those SNPs showing at least a marginal statistical trend ($p < 0.10$) and excluding those with a low sample (<10%) in either category or due to collinearity. To evaluate the combined effects of the previously selected SNPs on the change of FLI, fat liver content and OWLiver®-test, the three individual GRS were calculated by summing the number of risk alleles at each locus [55,56].

2.9. Statistical Analysis

The primary outcome of the study was the weight loss, according to the current recommendations of the AASLD to ameliorate NAFLD features [37]. The sample size was estimated assuming a mean difference of weight loss of 1.0 (1.5 kg) between both dietary groups (AHA vs. FLiO) with a 95% confidence interval ($\alpha = 0.05$) and a statistical power of 80% ($\beta = 0.80$). Considering a dropout rate of 20–30%, 50 subjects were included in each group of the study, even though two subjects were excluded from the AHA group due to the presence of important biochemical alterations in the initial assessment. This trial started with 98 participants but only 86 epithelium buccal cells from volunteers were available. Moreover, after 6 months, a total of 70 participants had complete information and epithelium buccal cells to carry out the study.

Results with normal distribution were expressed as means ± standard deviations (SD), whereas continuous skewed variables were presented as medians and interquartile ranges (IQR). Moreover, qualitative variables were expressed as number (n) and percentages (%). The normality of the distribution was checked through Shapiro–Wilk and Kolmogorov–Smirnov tests. Statistical differences for continuous variables at baseline (between men and women and according to age) and after the 6-month dietary intervention were estimated using Student's *t*-tests of independent samples and Wilcoxon–Mann–Whitney (for non-normally distributed variables). Categorical variables were compared using a Chi-squared test.

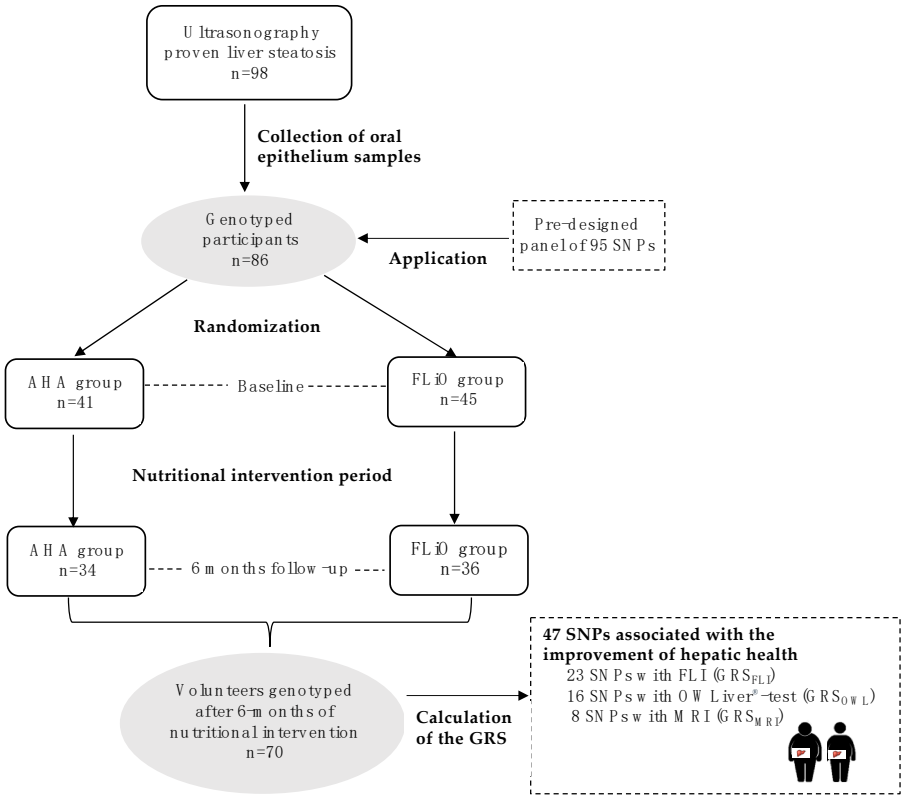

Figure 1. Design and flow chart of participants in the Fatty Liver in Obesity (FLiO) study. AHA, American Heart Association; FLI, fatty liver index; FLiO, Fatty Liver in Obesity; GRS, genetic risk score; GRSFLI, genetic risk score for FLI; GRSMRI, genetic risk score for magnetic resonance imaging; GRSOWL, genetic risk score for OWLiver®-test; MRI, magnetic resonance imaging; SNPs, Single Nucleotide Polymorphisms.

Diagnostic tests of the regression assumption for linearity and equal variance of residuals, and the variance inflation factor (VIF) for testing collinearity between independent variables, were conducted. Multiple linear regression models were used to predict FLI, liver fat content (by MRI) and OWLiver®-test changes. All the designed GRS were used as continuous variables in the multiple linear regression models. In addition to genetic variants, other conventional factors of personalization were evaluated, including age, sex and the following variables at baseline: insulin (U/mL), FGF-21 (pg/mL) and protein (%), as well as potential interaction introducing the corresponding interaction terms to the models.

All *p*-values presented are two-tailed and were considered statistically significant at $p < 0.05$. Analyses were carried out using Stata version 12.1 software (StataCorp 2011, College Station, TX, USA).

3. Results

Baseline characteristics of the participants, including body composition, biochemical and nutritional characteristics, are reported separated by sex and age (Table 1). Overall, 57% ($n = 49$) of subjects were men. The average values of weight and waist circumference followed expected trends depending on sex. Triglycerides and insulin resistance-related variables (HOMA-IR and TyG) showed statistical differences between genders, being

higher in men as compared to women. However, leptin concentration was significantly lower in males (20.1 ng/mL vs. 46.0 ng/mL in females). Analyzing variables associated with liver injury, statistical differences were observed in the fatty liver index (76.0 vs. 89.6) and in liver fat content measured by MRI (4.5% vs. 6.5%), showing worst hepatic health values in men in both measures. On the other hand, lipidomic analysis (OWLiver®-test) did not show significant differences. Concerning results according to age significant differences were only observed on weight, glucose and adiponectin concentrations and MedDiet Score. Regarding diet, the nutritional pattern of the study population was characterized by a relatively high consumption of energy derived from fat (37.4%), a concomitant low intake of carbohydrates (42.8%) and an average protein intake of 16.8%. Moreover, significant improvements in body composition, biochemical parameters, hepatic health variables, dietary intake and lifestyle factors were observed after the 6-month nutritional intervention, following the expected trends.

To study the genetic risk association with NAFLD, of a total of 95 SNPs related to obesity, 47 genetic variants were chosen because they were statistically or marginally associated with the amelioration of the hepatic health measured by non-invasive NAFLD diagnostic methods (FLI, MRI and OWLiver®-test). Of those, 1 SNP was common among all methods: rs2959272 (*PPARG*). On the other hand, 30 SNPs were exclusively related to a specific method—17 for FLI rs1801133 (*MTHFR*), rs1055144 (*NFE2L3*), rs17817449 (*FTO*), rs8050136 (*FTO*), rs3751812 (*FTO*), rs9939609 (*FTO*), rs2075577 (*UCP3*), rs324420 (*FAAH*), rs1121980 (*FTO*), rs2419621 (*ACSL5*), rs1558902 (*FTO*), rs3123554 (*CNR2/FUCA1*), rs6567160 (*MC4R*), rs660339 (*UCP2*), rs2605100 (*LYPLAL1*), rs1800629 (*TNF*APROMOTOR), rs4994 (*ADRB3*); 3 for MRI: rs6861681 (*CPEB4*), rs1440581 (*PPM1K*), rs1799883 (*FABP2*); and 10 for OWLiver® test: rs1175544 (*PPARG*), rs1797912 (*PPARG*), rs1386835 (*PPARG*), rs709158 (*PPARG*), rs1175540 (*PPARG*), rs1801260 (*CLOCK*), rs12502572 (*UCP1*), rs8179183 (*LEPR*), rs894160 (*PLIN1*), rs4731426 (*LEP*). In our population, we used the SNP associated with each non-invasive method for calculating the GRS (Figure S1).

The GRS, calculated as the number of risk alleles carried by each subject, was normally distributed. The sample was stratified, by the median, into a "low genetic risk group" (those with a $GRS_{FLI} \leq 9$, $GRS_{OWL} \leq 10$ and $GRS_{MRI} \leq 4$ risk alleles) and a "high genetic risk group" (those with a $GRS_{FLI} > 9$, $GRS_{OWL} > 10$ and $GRS_{MRI} > 4$ risk alleles). The results for the effect of each GRS on the change of different outcomes after the nutritional treatment are shown in Table 2. All groups exhibited a significant body weight loss, which was higher when the genetic risk was lower. Moreover, body composition variables including weight, BMI and waist circumference showed statistical differences when comparing GRS_{FLI} and GRS_{MRI} medians. Furthermore, general improvements in biochemical parameters were found. However, the amelioration was only statistically significant in TG, TyG and leptin concentrations, and TG and FGF-21 concentrations, when comparing GRS_{FLI} and GRS_{MRI} medians, respectively. On the other hand, no significant changes were found for dietary intake and lifestyle factors.

In order to evaluate the improvement of hepatic health depending on genetic and non-genetic risk factors, linear regression models were constructed (Table 3). These models were adjusted for sex, age, baseline protein intake, baseline FGF-21 and insulin concentration and the change in MedDiet Score. All the GRS included in the models showed an important association with the improvement on hepatic health. Moreover, a higher decrease in FLI was significantly associated with baseline insulin and protein and with the change in MedDietScore, showing an improvement in hepatic functionality. A high intake of protein at baseline also seemed to be important in the improvement of lipid metabolism, assessed by OWLiver®-test. Parallelly, the regression model established for the change in liver fat content (MRI) showed a significant interaction between the GRS_{MRI} and protein intake at baseline (p-value: 0.001). However, no statistically significant interactions between GRS_{FLI} and GRS_{OWL} were found. Overall, the change of FLI, OWLiver®-test and MRI variabilities were explained in approximately 52% (adj. $R^2 = 0.53$), 16% (adj. $R^2 = 0.16$) and 34% (adj. $R^2 = 0.34$), respectively.

Table 1. Baseline characteristics and after 6 months of dietary intervention and according to sex and age.

	Baseline					
	All Participants	**Women**	**Men**	**≤50 y**	**>50 y**	**6 Months [a]**
n	**86**	**37**	**49**	**43**	**43**	**70**
Body composition						
Weight (kg)	95.0 (13.9)	88.1 (13.0)	100.3 (12.3) ***	98.5 (13.8)	91.6 (13.3) *	84.4 (75.1; 92.1) ***
BMI (kg/m^2)	32.8 (30.6; 35.8)	32.9 (30.0; 36.2)	32.5 (30.9; 35.8)	33.8 (30.9; 36.6)	32.2 (30.2; 34.6)	28.7 (27.6; 32.6) ***
WC (cm)	109.0 (9.1)	103.8 (7.4)	112.9 (8.3) ***	109.2 (8.7)	108.7 (9.6)	99.9 (9.5) ***
DXA VAT (kg)	2.3 (1.6; 3.1)	2.2 (1.5; 3.1)	2.3 (1.7; 3.1)	2.3 (1.7; 3.2)	2.3 (1.6; 3.0)	1.5 (0.7) ***
Biochemical parameters						
TG (mg/dL)	121.0 (80.0; 155.0)	92.0 (72.0; 133.0)	127.0 (96.0; 170.0) **	126.0 (80.0; 167.0)	113.0 (72.0; 148.0)	83.0 (56.0; 114.0) ***
Glucose (mg/dL)	101.5 (91; 108)	97.0 (92.0; 105.0)	103.0 (91.0; 115.0)	97.0 (91.0; 104.0)	104.0 (95.0; 112.0) *	92.0 (87.0; 98.0) ***
Insulin (U/mL)	16.6 (7.9)	15.3 (7.8)	17.7 (7.9)	15.4 (7.1)	17.9 (8.5)	8.6 (6.5; 13.5) ***
HOMA-IR	4.1 (2.8; 5.7)	3.5 (2.2; 5.1)	4.4 (3.1; 6.0) *	3.9 (2.6; 5.1)	4.4 (2.9; 6.5)	1.9 (1.4; 3.3) ***
TyG index	8.6 (0.5)	8.4 (0.4)	8.8 (0.4) ***	8.6 (0.4)	8.6 (0.5)	8.2 (7.8; 8.6) ***
Adiponectin (µg/mL)	6.3 (5.0; 8.3)	6.6 (5.2; 8.3)	5.9 (5.0; 7.5)	5.8 (4.5; 7.9)	6.6 (5.7; 9.7)	8.0 (6.1; 9.9) ***
Leptin (ng/mL)	29.8 (17.6; 44.8)	46.0 (37.7; 69.3)	20.1 (14.0; 26.4) ***	32.1 (17.6; 46.09)	26.4 (15.9; 39.3) *	16.7 (7.5; 33.0) ***
FGF21 (pg/mL)	211.5 (108.0; 352.0)	190.0 (89.1; 387.0)	215.0 (124.0; 328.0)	182.0 (87.7; 302.0)	244.0 (130.0; 416.0)	187.5 (111.0; 355.0)
Liver injury						
FLI	83.1 (73.7; 92.3)	76.0 (60.7; 83.0)	89.6 (79.8; 94.1) ***	84.4 (74.2; 93.3)	79.8 (70.5; 91.7)	51.11 (23.6) ***
MRI Liver fat—Dixon (%)	5.6 (3.2; 9.6)	4.5 (2.9; 8.7)	6.5 (4.3; 10.1) *	5.9 (3.5; 12.4)	5.0 (3.0; 8.9)	2.0 (1.3; 3.8) ***
Lipidomic (OWLiver®-test) n (%)						
No NAFLD	17 (20.0)	7 (18.9)	10 (20.8)	7 (16.2)	10 (23.8)	23 (32.8)
Hepatic Steatosis	20 (23.5)	10 (27.0)	10 (20.8)	7 (16.2)	13 (30.9)	21 (30.0) *
NASH	48 (56.4)	20 (54.0)	28 (58.3)	29 (67.4)	19 (45.2)	26 (37.1)
Dietary intake per day						
Total energy (kcal/day)	2550 (1958; 2925)	2548 (2031; 3133)	2554 (1897; 2902)	2551 (2042; 3066)	2464 (1833; 2864)	2004 (576) ***
Carbohydrates (%E)	42.8 (37.6; 47.8)	43.0 (35.9; 48.4)	42.5 (39.2; 47.5)	40.8 (36.2; 46.8)	43.0 (39.9; 48.0)	42.3 (7.7)
Proteins (%E)	16.8 (15.1; 19.1)	16.9 (15.2; 20.9)	16.7 (15.1; 19.0)	16.7 (15.3; 18.8)	16.9 (14.6; 19.3)	19.4 (17.1; 22.8) ***
Fats (%E)	37.4 (6.8)	38.1 (7.5)	36.8 (6.2)	38.3 (6.8)	36.5 (6.7)	35.4 (7.8)
Lifestyle factors						
MedDiet Score	5.9 (1.9)	5.9 (2.3)	5.9 (1.6)	5.4 (1.7)	6.4 (2.0) *	12.0 (10.0; 14.0) ***
PA (METs-min/week)	2240 (1665; 4307)	2240 (1710; 4307)	2280 (1100; 4365)	2322 (1705; 4365)	2216 (1392; 4200)	3720 (2442; 5115) ***

Variables re shown as mean (SD) or as median (IQR) according to its distribution. Categorical variables are presented as absolute (*n*). Paired *t*-tests and Wilcoxon-matched-pairs signed ranks were carried out to compare baseline and 6 months participants characteristics. Independent samples *t*-tests and Wilcoxon–Mann–Whitney were carried out to compare changes between sex and age groups. Age was categorized according to the median. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. [a] Comparison within dietary groups (baseline and after 6 months). BMI, body mass index; DXA, dual-energy X-ray absorptiometry; %E, percentage of energy; FGF-21, fibroblast growth factor 21; FLI, fatty liver index; HOMA-IR, homeostasis model assessment insulin resistance; MRI, magnetic resonance imaging; OWL®; OWLiver®-test; PA, physical activity; TG, triglycerides; TyG index, triglycerides and glucose index; VAT, visceral adipose tissue; WC, waist circumference.

Table 2. Change in body composition, biochemical, dietary and lifestyle factors according to different genetic risk scores.

	GRS_{FLI}		GRS_{OWL}		GRS_{MRI}	
	<9	**≥9**	**<10**	**≥10**	**<4**	**≥4**
n	**30**	**40**	**29**	**41**	**31**	**39**
Body composition						
Mean	6.0 (1.6)	9.6 (3.9)	7.0 (1.5)	11.7 (1.4)	2.0 (0.8)	4.9 (1.0)
ΔWeight (kg)	−10.6 (−15.8; −6.9)	−8.4 (−10.7; −5.0) *	−9.5 (−12.4; −6.4)	−8.6 (−12; −6.4)	−9.6 (−16.9; −8.2)	−7.6 (−11.2; −4.9) *
ΔBMI (kg/m^2)	−3.6 (−5.1; −2.4)	−2.9 (−3.7; −1.6) *	−3.3 (−4.7; −2.2)	−3.2 (−4.1; −2.2)	−3.5 (−5.6; −2.9)	−2.8 (−3.8; −1.6) *
ΔWC (cm)	−11.7 (6.5)	−7.6 (5.6) **	−9.1 (6.4)	−9.5 (6.3)	−11.0 (5.2)	−8.0 (6.8) *
ΔDXA VAT (kg)	−0.8 (−1.2; −0.4)	−0.9 (−1.5; −0.4)	−1.0 (−1.5; −0.4)	−0.8 (−1.5; −3.2)	−1.0 (−1.5; −0.6)	−0.7 (−1.67; −0.2)
Biochemical parameters						
ΔTG (mg/dL)	−42.0 (−100.0; −18.0)	−15.0 (−56.0; 4.0) *	−32.0 (−68; 0)	−22.5 (−58.5; −0.5)	−46.0 (−102.0; −5.0)	−18.0 (−45.0; 1.0) *
ΔGlucose (mg/dL)	−8.6 (11.0)	−10.2 (12.4)	−9.8 (13.6)	−9.3 (10.4)	−9.2 (11.0)	−9.8 (12.5)
ΔInsulin (U/mL)	−7.4 (7.1)	−4.9 (8.0)	−6.2 (8.7)	−5.9 (7.0)	−6.7 (7.4)	−5.4 (8.0)
ΔHOMA-IR	−2.0 (−3.2; −0.2)	−1.6 (−3.3; −0.2)	−1.9 (−3.2; −0.2)	−1.8 (−3.2; −0.6)	−2.3 (−3.2; −1.0)	−1.5 (−3.2; −0.1)
ΔTyG index	−0.6 (0.4)	−0.2 (0.4) **	−0.4 (0.3)	−0.4 (0.5)	−0.5 (0.5)	−0.3 (0.3)
ΔAdiponectin (μg/mL)	1.5 (0.1; 4.7)	1.2 (−0.9; 3.2)	−0.1 (−0.6; 2.2)	1.8 (0.1; 3.4)	1.0 (−0.6; 3.4)	1.3 (−0.2; 4.1)
ΔLeptin (ng/mL)	−11.1 (−21.6; −7.2)	−7.5 (−14.5; −2.9) *	−9.5 (−15.8; −7.0)	−7.5 (−20.0; −3.0)	−9.1 (−15.8; −6.7)	−9.1 (−20.0; −3.0)
ΔFGF21 (pg/mL)	−9.1 (−123.0; 80.0)	−40.5 (−146.5; 95.5)	−41.7 (−132; 50)	−0.8 (−123; 88)	−55.4 (−217; 45)	12.0 (−64.2; 97) *
ΔFLI (%)	−54.5 (19.7)	−22.6 (17.9) ***	−33.7 (21.8)	−38.4 (26.2)	−41.1 (25.3)	−32.9 (23.5)
ΔMRI Liver fat—Dixon (%)	−2.7 (−6.8; −0.7)	−2.7 (−6.8; −1.2)	−4.3 (−8; −0.8)	−3.4 (−6.8; −1.2)	−4.5 (−7.8; −2.5)	−1.6 (−4.2; −0.2) ***
ΔLipidomic (OWLiver®-test) n (%)						
OWL® maintenance	19 (63.3)	28 (71.7)	13 (44.8)	34 (85.0) ***	17 (56.6)	30 (76.9)
OWL® reduction	11 (36.6)	11 (28.2)	16 (55.1)	6 (15.0)	13 (43.3)	9 (23.0)
Dietary intake per day						
ΔTotal energy (kcal)	−882 (−1261; −88)	−523 (−1099; −101)	−589 (−987; −132)	−603 (−1175; 44)	−881 (−1257; −308)	−479 (−1009; 66)
ΔCarbohydrates (%)	−1.3 (10.0)	−0.7 (8.7)	−1.9 (8.2)	−0.3 (9.9)	−2.0 (9.4)	−0.3 (9.1)
ΔProteins (%)	3.6 (4.3)	1.9 (5.7)	2.5 (3.5)	2.7 (6.1)	2.9 (6.0)	2.4 (4.6)
ΔFats (%)	−0.7 (−6.2; 4.5)	−2.1 (−9.1; 5.1)	−0.2 (−5.2; 5.2)	−1.8 (−10.1; 4.8)	−2.6 (−8.3; 4.1)	−1.4 (−9.8; 5.8)
Lifestyle factors						
ΔMedDiet Score	6.3 (3.1)	5.7 (3.4)	5.7 (3.2)	6.1 (3.4)	6.5 (3.3)	5.5 (3.3)
ΔPA (METs min/week)	758 (−217; 2405)	1215 (−120; 2798)	896 (73; 2798)	1111 (−753; 2405)	984 (−65; 2357)	1046 (−557; 2817)

Variables are shown as mean (SD) or as median (IQR) according to its distribution. Categorical variables are presented as absolute (*n*). Independent samples *t*-tests and Wilcoxon–Mann–Whitney were carried out to compare variables changes according to the median of $GRS_{FLI} < 9$ and $GRS_{FLI} \geq 9$, $GRS_{MRI} < 4$ and $GRS_{MRI} \geq 4$ and $GRS_{OWL} < 10$ and $GRS_{OWL} \geq 10$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. BMI, body mass index; DXA, dual-energy X-ray absorptiometry; %E, percentage of energy; FGF-21, fibroblast growth factor 21; FLI, fatty liver index; HOMA-IR, homeostasis model assessment insulin resistance; MRI, magnetic resonance imaging; OWL®; OWLiver®-test; PA, physical activity; TG, triglycerides; TyG index, triglycerides and glucose index; VAT, visceral adipose tissue; WC, waist circumference.

Table 3. Linear regression analyses of changes in fatty liver index, OWLiver®-test and liver fat content (MRI).

		β	*p*-Value	Adjusted R^2	*p*-Model
% Change in Fatty Liver Index (FLI)					
Model 1	GRS_{FLI}	3.75	<0.001	0.37	<0.001
Model 2	GRS_{FLI}	3.37	<0.001	0.39	<0.001
	Baseline protein	1.27	0.044		
Model 3	GRS_{FLI}	3.03	<0.001	0.45	<0.001
	Baseline protein	1.55	0.011		
	Baseline insulin	0.80	0.005		
Model 4	GRS_{FLI}	3.10	<0.001	0.53	<0.001
	Baseline protein	1.49	0.009		
	Baseline insulin	0.76	0.005		
	Change MedDiet Score	−1.99	0.002		
Change in OWLiver®-test					
Model 5	GRS_{OWL}	0.08	0.001	0.12	0.009
Model 6	GRS_{OWL}	0.07	0.011	0.16	0.005
	Baseline protein	0.04	0.022		
Change in liver fat content (MRI)					
Model 7	GRS_{MRI}	1.13	<0.001	0.23	<0.001
Model 8	GRS_{MRI}	1.28	<0.001	0.24	<0.001
	Baseline protein	0.04	0.741		
Model 9	GRS_{MRI}	1.17	<0.001	0.28	<0.001
	Baseline protein	0.09	0.448		
	Baseline FGF21	−0.004	0.051		
Model 10	GRS_{MRI}#baselineprotein	0.180	0.017	0.34	<0.001
	Baseline FGF21	−0.004	0.040		

All models were adjusted by age and sex. GRS_{FLI}, genetic risk score for FLI; GRS_{MRI}, genetic risk score for magnetic resonance imaging; GRS_{OWL}, genetic risk score for OWLiver®-test; MRI, magnetic resonance imaging.

In addition, Figure 2A–C plot simple linear regression analyses of statistically significant predictors of FLI, OWLiver®-test and liver fat content (MRI) decrease by diet. A lower change in FLI and OWLiver®-test was associated with a higher baseline protein intake (*p*-value: 0.009 and 0.022, respectively). Moreover, this association became more important when the genetic risk was higher (Figure 2A,B). Figure 2C shows that a higher baseline protein was associated with a lower change of liver fat content (by MRI) becoming the effect more evident when the genetic risk was higher (*p* interaction: 0.017).

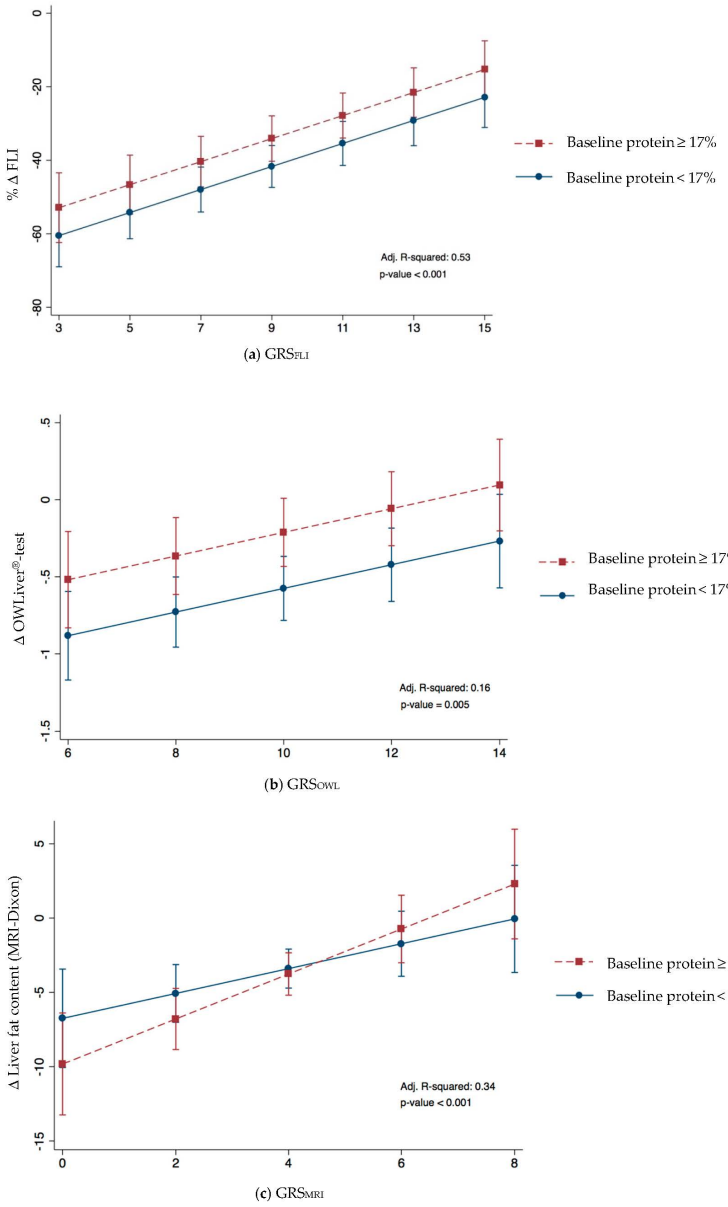

Figure 2. Effect of the changes in (**a**) % FLI and GRS_{FLI}, (**b**) OWLiver®-test and GRS_{OWL} and (**c**) MRI and GRS_{MRI} and baseline protein after 6-month nutritional treatment. Baseline protein was dichotomized according to median.

4. Discussion

NALFD has reached pandemic levels, being recognized as an important health burden with an urgent need for early diagnosis [57]. Genetic predisposition for NAFLD has been reported [18,20]. With this in mind, the objective of this research was to assess the impact of the interaction between genetic and non-genetic factors concerning the improvement

of the hepatic health using different diagnosis tools (FLI, MRI and OWLiver®-test) after a 6-month energy-restricted nutritional treatment for a more personalized management of this liver disease.

The high prevalence of NAFLD could be related to its strong link with obesity, which seems to play a role in both the initial simple steatosis and in its progression to NASH [11,58]. In this context, various genes and less frequent variants have been associated with the regulation of energy metabolism [18,59]. Moreover, the increasing of knowledge of the genetic component of NAFLD has promoted the development of noninvasive diagnosis methods based on Genome Wide Associations Studies (GWAS) [11,16,57,60], but few of them have examined the contribution of obesity-related variants linked to the evolution of this hepatic disease [37,61].

In order to better understand the contribution of genetics in the context of NAFLD, three different GRS were constructed based on the improvement of hepatic health after an energy-restricted treatment measured by three non-invasive diagnostic methods (FLI, magnetic resonance imaging and OWLiver®-test). On the one hand, fatty liver index has been highly correlated with measures of fatty liver disease showing an area under the curve of 0.84, predicting most cases of NAFLD [62]. Moreover, a recent study reported that the FLI joint to the waist circumference-to-height ratio could be one of the most accurate algorithms for the noninvasive diagnosis of NAFLD in both lean and overweight/obese population [63]. On the other hand, MRI can be considered the gold standard for steatosis measurement, being highly accurate and reproducible and superior in detecting and quantifying fat accumulation [61,64]. However, these two methods have limitations in detecting inflammation, ballooning and cellular injury, which are key components in NASH diagnosis [65]. Thus, in some cases, models based on "omics" sciences, such as the OWLiver®-test, could be of interest adding knowledge about diverse factors influencing weight loss variability among individuals. Due to the differences between methods in outcome measures, distinct genes and so pathways may be expected to be connected.

Therefore, a total of 47 polymorphisms were independently associated with differential responses to hepatic functionality (FLI), fat liver content (MRI) and lipid metabolism (OWLiver®-test). It is important to emphasize that each non-invasive diagnostic method has its specific SNPs. Only the rs2959272 (*PPARG*) genetic variant was the common element on the three GRS. In this sense, an intervention study indicated that the PPARG genotype was associated with success in body weight reduction [66]. Indeed, two common elements were also observed between GRS_{FLI} and GRS_{MRI}, SNPs were located in genes related to bile secretion (*ABCB11*) and the regulation of energy balance and body weight (*SH2B1*). Meanwhile, SNPs in genes implicated in weight loss (*SH2B1* and *STK33*) influenced both GRS_{OWL} and GRS_{MRI}. Instead, three common elements mapped to genes involved in endocrine/enzymatic regulation of lipid metabolism affecting macronutrient (*GNAS*) food intake and energy expenditure (*MC4R*) and thermogenesis (*UCP1*) were observed in GRS_{FLI} and GRS_{OWL}.

In this study, a greater change in most of the NAFLD-related variables was reported when the genetic risk was lower. According to these findings, it has been extensively debated the identification of the physiological pathways that control energy metabolism and body weight regulation [31,67]. A Genetic Investigation of ANtropometric Traits consortium (GIANT) metanalysis identified 97 loci for BMI where genes near these specific loci showed expression enrichment in the central nervous system, suggesting that BMI is mainly regulated by processes such as hypothalamic control of energy intake [68]. Similar results have been found in a recent study in a pediatric population, where the application of a GRS to established clinical risk factors significantly improved the discriminatory capability of the prediction of NAFLD risk [5]. Indeed, different genetic variants and interactions with environmental factors have been shown to modulate the differential individual responses to moderately high-protein and low-fat dietary interventions in a Caucasian population [55]. In this sense, genetic information could help to determine the

most appropriate dietary intervention for the prevention and treatment of NAFLD, as well as the development of associated comorbidities [69].

Moreover, for the purpose of explaining the variability on the improvement in hepatic functionality (FLI), liver fat content (by MRI) and lipidomic (OWLiver®-test), linear regression models were performed. The predictive accuracy of all models substantially improved when combining each of the previously mentioned SNPs in the multiple linear regression models, which is in line with previous studies [70]. In order to ameliorate these results, each regression model was fitted by sex, age and NAFLD-related variables such as inflammatory biomarkers or dietary compounds. Other variables such as the nutritional group of the participants was also considered, even though no significant differences were found. Factors related with proinflammatory and profibrogenetic pathways such as leptin, adiponectin or FGF-21, which appears to be elevated in patients with NAFLD, are therefore a promising target for the treatment [71,72]. Thus, GRS_{FLI}, GRS_{MRI} and GRS_{OWL} were major predictors of the change in FLI, liver fat content (MRI) and OWLiver®-test, respectively.

To the best of our knowledge, there are few studies showing the combined effects of GRS built from SNPs related weight and adiposity regulation in response to different energy-restricted diets [50]. Moreover, it has been reported that genetic background is an important factor explaining metabolically health and unhealthy phenotypes related to obesity, in addition to lifestyle variables [54].

In this sense, dietary factors seem to be of key importance and have been associated with weight gain, obesity and NAFLD development [73,74]. Interestingly, in this research, higher baseline protein was associated with worst hepatic health improvement measured by FLI and OWLiver®-test. Furthermore, an interaction between the liver fat content assessed by MRI and baseline protein was found. In the same line were the results obtained from the Nurse´s Health Study and the Health Professionals Follow-up Study, where an increased intake of sugar-sweetened beverages was found to amplify the association of a 32-SNP genetic risk score with BMI [75]. These findings suggest that not only genetic and dietary factor should be considered but also the interaction of both of them [76,77]. Hence, a combined analysis over 16,000 children and adolescent showed the *FTO* rs9939609 variant that confers a predisposition to higher BMI is associated with higher total energy intake and that lower dietary protein intake attenuates the association [78]. Among the macronutrient categories, protein is the main one that contributes to the satiety, therefore contributing to weight loss [79,80]. However, the effect of the high protein diet in patients with NAFLD remains controversial. On the one hand, it has been suggested that the consumption of specific dietary amino acids might negatively impact liver status and, to a lesser extent, glucose metabolism in subjects with overweight/obesity and NAFLD [81]. Moreover, high protein intake derived mainly from dairy products has been associated with higher risk to develop diabetes and also with NAFLD [82]. A recent study has also suggested that following a lower protein diet, particularly in genetically predisposed individuals, might be an effective approach for addressing cardiometabolic diseases among Southeast Asian women [83]. On the other hand, high protein diet has been reported to be a valid therapeutic approach to revert NAFLD, being of special importance the protein source and the functional status of the liver [84]. In addition, BCAA supplementation has been demonstrated to ameliorate liver fibrosis and suppress tumor growth in a rat model of HCC with liver cirrhosis, as well as alleviate hepatic steatosis and liver injury in NASH mice [85,86].

There are some drawbacks of this research. Firstly, liver biopsy results were not available to corroborate the precise diagnosis of patients [57]. Nonetheless, in this research, we carried out a complete evaluation of the liver status by means of validated and widely used techniques as well as blood biomarkers and hepatic indexes, which are affordable and practical methods to use in health assessment. Second, the sample size and the enrollment of subjects are not very large. For this reason, these models may be further validated in different populations to establish whether they might represent a reliable and accurate, "non-invasive alternative" to liver biopsy. In addition, the role of new SNPs associated

with excessive adiposity and accompanying metabolic alterations through a GRS approach needs to be re-explored, but our contribution re-states the value of the genetic make-up when prescribing personalized diets. Thirdly, type I and type II errors cannot be completely ruled out, especially those related to the selection of SNPs to be introduced into the GRS. However, due to the use of less stringent *p*-value thresholds compared to association studies of single variants, genomic profile risk scoring analyses can tolerate, at balance, some of these biases, as previously reviewed [87]. Fourthly, dietary intake was evaluated using self-reported information of the participants, which may produce some bias on the evaluation of the results. Lastly the constructions of the GRS using specific obesity-related SNP it is also an important limitation. However, the inclusion of these SNP on the evaluation of the genetic influence on NAFLD could be also considered an important strength of this investigation, as well as the use of different multiple linear regression models to test the contribution of genetics, baseline protein and inflammatory factors on the management of NAFLD. Finally, the study is a randomized controlled trial where each volunteer had an individual follow-up promoting the adoption of behavioral changes and a healthy lifestyle.

Overall, this experiment was designed as a proof of concept in order to evaluate if the genetic background linked to NAFLD-related factors may influence hepatic amelioration. In addition, examining new causes of disease and the underlying mechanism or alteration in specific pathways and clinical outcomes may be of interest.

5. Conclusions

Predicting the individual risk of NAFLD and determining the probability of disease progression is the basis for a precise diagnosis and treatment. These results demonstrate that three different genetic scores can be useful for the personalized management of NAFLD, whose treatment must rely on specific dietary recommendations guided by the measurement of specific genetic biomarkers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/diagnostics11061083/s1. Figure S1: Venn diagram showing the number of SNPs associated with each NAFLD non-invasive diagnostic methods. GRS, genetic risk score; MRI, magnetic resonance imaging; FLI, fatty liver index; OWL, OWLiver®-test.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Supplementary material

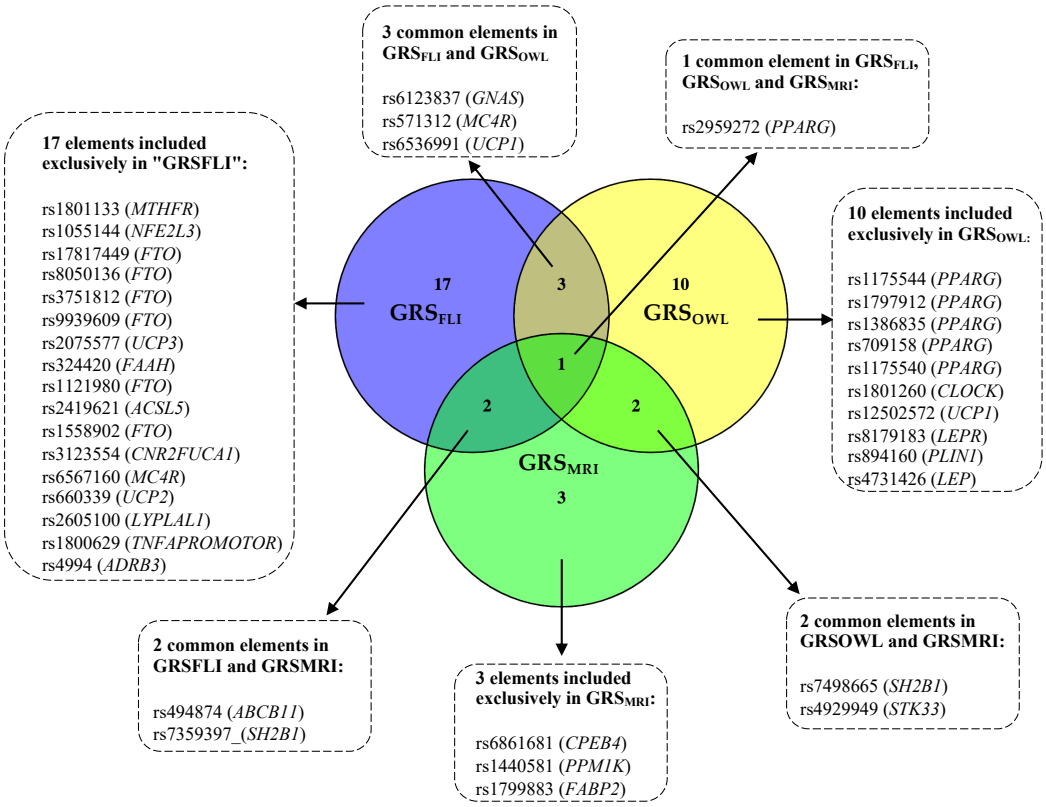

Figure S1. Venn diagram showing the number of SNPs associated with each NAFLD non-invasive diagnostic methods. GRS, Genetic Risk Score; MRI, Magnetic Resonance Imaging; FLI, Fatty Liver Index; OWL, OWLiver®-test.

Chapter 4

A nutrigenetic tool for precision dietary management of NAFLD deeming insulin resistance markers

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GENERAL DISCUSSION

1. Rationale of the study

NAFLD is the most common liver disorder in Western countries (Eslam *et al*., 2018). The prevalence of NAFLD is estimated to affect around 25% of the global population, which is usually increasing in parallel with the prevalence of obesity (Armandi & Schattenberg, 2021). As the clinical consequences of NAFLD grow, the economic consequences will also increase (Younossi *et al*., 2020). The economic burden of this liver disease is projected to be immense not only on morbidity and mortality, but also on health care utilization (Younossi, 2019). Thus, an effective national and global approach that incorporated social, behavioral and biological targets to deal with the epidemic of NAFLD relates-liver disease must be defined (Mitra *et al*., 2020).

The clinical phenotypes of NAFLD are very heterogeneous in association with the multiple disorders involved in disease progression (Trépo *et al*., 2020). According to scientific evidence, the main risk factor for NAFLD is insulin resistance associated with overweight, physical inactivity, and development of type 2 diabetes mellitus (Cariou *et al*., 2021). Furthermore, complex interactions among environmental and lifestyle factors, microbiota, epigenetic and genetic are involved (Juanola *et al*., 2021). In this sense, a strong heritability component of NAFLD is supported by converging evidence from epidemiological, familial and twin studies, and from clinical cases (Anstee *et al*., 2021; Choudhary *et al*., 2021). GWASs have identified vulnerable sites is genes loci such as *PNPLA3*, *MBOAT7* and *TM6SF2* leading to a major risk for developing advanced liver disease (Schattenberg *et al*., 2021). Nevertheless, genetic susceptibility for the development of the different stages might be shared with different metabolic traits (Cui *et al*., 2016; Sookoian *et al*., 2016). Thus, the combination of all these risk factors resulted in an accumulation increase fatty acid input/output balanced which led in an excessive hepatic fat (Juanola *et al*., 2021).

Regarding diagnosis, liver biopsy is considered the gold standard for NAFLD (Hagström *et al*., 2020), but is rarely achieved due to limitations and underlying complications, being diagnosis mostly made by the combination of radiology and exclusion of other liver diseases. Hence, other accurate non- or minimally invasive methods have been proposed, including abdominal ultrasound, MRI and different elastography techniques (Han, 2020). On the other hand, despite of the known bias determining the presence of NAFLD, the larger cohort studies have mostly classified NAFLD status using formulas and algorithms, such as the fatty liver index, which has shown good accuracy to estimate NAFLD, the NAFLD fibrosis score, or the fibrosis-4 index (Bedogni *et al*., 2006; Hagström *et al*., 2019; Han, 2020). Moreover, the used of genetic information from GWASs for the diagnosis of NAFLD through

GRS has also increased. In this context, a recent study has demonstrated that genetic variations amplified the health impact of NAFLD, especially on hepatic outcome in individuals with high GRS (Liu Z. *et al*., 2020). This finding was in accordance with a previous study where the addition of a 11-polymorphism GRS improved the discriminatory capability for prediction the risk of NAFLD in NAFLD obese children and adolescents (Zusi *et al*., 2019).

Despite our understanding of the mechanisms involved in the development and progression of NAFLD, to date, no drug therapy has been approved for the treatment of NAFLD (Chalasani *et al*., 2018). The management of NAFLD relies on lifestyle modifications being weight loss, exercise and healthy diet the basis for prevention and treatment of NAFLD (European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO), 2016). In this sense, several dietary factors, such as increased intake of refined carbohydrates, in particular fructose and sucrose and foods rich in saturated fat, such as processed meet or high fat dairy, have been associated with the development of NAFLD (Perdomo *et al*., 2019). On the contrary, dietary fiber and unsaturated fatty acids have been shown to reduce *de novo* lipogenesis, improve insulin sensitivity, increase satiety and modulate gut microbiota, attenuating the development or onset of NAFLD (Cantero *et al*., 2017; Parnell *et al*., 2012). However, there is still no consensus of the macronutrient composition for the dietary of NAFLD patients (Houttu *et al*., 2021). Lastly, in the management of NAFLD and metabolic syndrome features, gene-environment interactions may also plan an important role (Meroni, Longo, *et al*., 2020). In fact, scientific evidence has pointed out a key role of the diet on the association between a genetic variant and a phenotype (Li Z. *et al*., 2020).

Consequently, the present study was devised to assess possible genetic factors underlying NAFLD for a non- or minimally invasive assessment of subjects with overweight or obesity and NAFLD, under different nutritional interventions within a 6-month follow-up and evaluating also anthropometric measurements, body composition, general metabolic markers and hepatic status. Moreover, this work aimed to study the impact of dietary factors in the association between genetic and phenotypic features.

2. *SH2B1* genetic variant and NAFLD

The growing epidemic of obesity and metabolic syndrome is closely associated with the rising prevalence and impact of NAFLD (Godoy-Matos *et al*., 2020). However, even if obesity is a strong risk factor, by itself is not sufficient to produce NAFLD (Jain *et al*., 2021). Recent evidence on the genetics field of NAFLD is focused on missing heritability, suggesting that there may be a role of mitochondrial genetics, microribonucleic acids, long noncoding RNAs, epigenetics factors and SNPs (Jain *et al*., 2021; Pelusi *et al*., 2019). As expected from epidemiologic and genetic studies, genetic loci of NAFLD related disorders, such as obesity or T2DM or increased cardiovascular risk, have been suggested to influence NAFLD (Chandrasekharan *et al*., 2019; Eslam *et al*., 2020). Therefore, in the first and second chapters, based on a pre-designed panel of 95 SNPs, we tested the effect in overweight/obese subjects with NAFLD of *SH2B1* rs7359397 genetic variant, which is associated with severe leptin resistance, energy imbalance, obesity and T2DM in humans (Al-Hakeem, 2014; Rui, 2014). As an adaptor protein, *SH2B1* acts to assemble a multiprotein signaling complex, to couple upstream activators to downstream effectors and/or to enhance the catalytic activity of its bound tyrosine kinases (Cheng *et al*., 2020). *SH2B1* has been implicated in signal transduction processes for several receptor tyrosine kinases and for the Janus kinase (JAK) family of tyrosine kinases (Rui *et al*., 1997). Thus, *SH2B1* promotes leptin signaling by stimulating Janus kinase (JAK1 and JACK2) activity and by binding to JACK/insulin receptor substrate (*IRS1* and I*RS2*) complexes, which enhances insulin sensitivity (Bjørbaek *et al*., 2004; Morris *et al*., 2010). In addition, this genetic variant has been linked to increased hepatic lipid content and/or VLDL secretion, promoting hepatic steatosis in mice (Sheng *et al*., 2013). On the other hand, GWASs in humans, have identified several *SH2B1* polymorphisms linked to obesity, T2DM and cardiovascular disease (Doche *et al*., 2012; Thorleifsson *et al*., 2009). Specifically, in the first chapter we analyzed the effect of *SH2B1* rs7359397 genetic variant on NAFLD, as well as possible associations between this polymorphism and diet.

In the FLiO study, the analyzed results evidenced that the *SH2B1* has an impact on the development and progression of NAFLD showing a marginal association with DXA lean mass and the risk allele, as well as significant differences related to body composition and biochemical variables such as HOMA-IR or HDL-c. In accordance with this results, studies in animals have revealed that the genetic deletion of *SH2B1* results in severe obesity, leptin and insulin resistance and T2DM in mice (Duan *et al*., 2004; Minghua Li *et al*., 2006). Also, studies in Caucasian female twins have shown that the genetic variant rs7498665 of the

SH2B gene was associated with serum leptin, total fat, waist circumference and body weight (Jamshidi *et al*., 2007). However, a study in obese and lean Chinese children, concluded that rare non-synonymous mutations in *FTO* and *SH2B1* did not confer obesity risk (Zheng *et al*., 2013). Furthermore, in our study, higher protein consumption (p=0.028), less MUFA and fiber intake (p=0.045 and p=0.049, respectively), was referred to risk allele genotype. These results are in accordance with previous studies where the nutrient intake was different depending on genotype (Dallio *et al*., 2021). Carrying the risk allele of *PNPLA3* genetic variant showed an interaction with adolescent's hepatic fat, evidencing a decrease in fiber and vegetable protein intake and an increase in saturated fat (Cohen *et al*., 2021). Also, a study concerning 945 high-cardiovascular risk subjects demonstrated that adherence to the MedDiet modulated the effect of *GCKR* polymorphism on TAG (Sotos-Prieto *et al*., 2013). Thus, subjects with high genetic risk had lower TAG concentrations. In this regard, the *SH2B1* genetic variant has been proposed as a target in the control of energy balance and glucose homeostasis both *in vivo* and *in vitro* models (Flores *et al*., 2019). Moreover, it has been shown that the *SH2B1* expression in the brain was specifically increase > 20-fold in fed mice, suggesting a nutritional regulation of these obese gene (Yoganathan *et al*., 2012).

Important results were also obtained on the association between the *SH2B1* genotype and alternative methods of NAFLD diagnosis. On the one hand, *SH2B1* carriers of the T-allele were associated with an increased risk of liver fat accumulation, assessed by FLI and ultrasonography. In this sense, a study carried out in mice showed that LepR neuro-specific deletion of *SH2B1* in the hypothalamus resulted in liver steatosis, obesity and insulin resistance (Jiang L. *et al*., 2020). On the other hand, an association between the *SH2B1* and the liver fat content (by MRI), as well as higher risk of developing NASH (assessed by metabolomics), was demonstrated. In this context, a meta-analysis published similar results showing the association between genetics and NAFLD (Liu M. *et al*., 2019). In addition, the obesity related gene *ADIPOQ* −11377C>G was suggested as a NAFLD risk factor, while there was no association between *ADIPOQ* gene +276G>T polymorphism and NAFLD risk. Finally, a recent multicenter, double-blind, parallel design trial in obese subjects demonstrated beneficial effects of *LLF580* on serum lipids, liver fat and biomarkers of liver injury, suggesting it may be effective in the treatment of metabolic disorders such as NAFLD (Rader *et al*., 2021).

Based on these results, in the second chapter we aimed to evaluate the response of a 6-months energy restricted treatment depending on *SH2B1* genotype in NAFLD participants, as well as possible gene-treatment interactions. Lifestyle changes, including exercise and dietary interventions focused on weight loss, represent the first step in the management of

NAFLD (Armandi & Schattenberg, 2021; Marin-Alejandre *et al*., 2021). However, individual responses to body weight-loss interventions vary widely and several studies indicated that NAFLD develops as a result of complex interactions between genetic susceptibility and other environmental factors (Dongiovanni *et al*., 2013).

Surprisingly, our results indicate that both genotypes significantly improved their body composition (weight, BMI, or WC) and biochemical parameters (glucose, insulin, triglycerides, adiponectin) after following an energy-restricted diet. Previous studies have also shown the benefits of dietary interventions on NAFLD patients (Armandi & Schattenberg, 2021; Marin-Alejandre *et al*., 2021). In our results, carriers of the risk allele were associated with a greater increased in total fiber and omega-3 fatty acids, while a significant increase in MedDiet score was observed in both genotype groups. Moreover, no-risk genotype presented a relevant decrease in hepatic iron, as well as in MUFA intake after the 6-months nutritional intervention. According to these results, there are two plausible hypotheses. On the one hand, it should be considered that each subject could respond differently to food intake, being genetic variations the result of the adaptive evolution to specific dietary habits (Meroni, Longo, *et al*., 2020). In this sense, a study in mice evidenced an association between high-cholesterol diet and down-regulation genes involved in cholesterol metabolism such as *SREBF2* and *NR1H4* (*FXR*) genes, which reduces hepatic triglyceride in liver (Gil-Ramírez *et al*., 2015). Furthermore, fatty acids have been shown to inhibit *PNPLA3* protein degradation, which revealed a nutritional control of this gene (Huang Y. *et al*., 2010). On the other hand, it seems that the genetic predisposition affects the response to treatment. These results are in line with the "differential susceptibility hypothesis", which proposed that, in any case, those with vulnerability genes or risk alleles may function as plasticity genes and, therefore, these individuals will benefit the most as they are generally more affected by environmental exposures, including dietary factor (Belsky J. *et al*., 2009; Hartman *et al*., 2016). From this point of view, a previous study demonstrated that carriers of *PNPLA3 I148M* genetic variant evidenced a more effective reduction in liver fat levels after a lifestyle modification and bariatric surgery (Carlsson *et al*., 2020). Also, a study carried out in overweight/obese subjects showed that carrying the G allele of the rs10182181 *ADCY3* genetic variant may benefit more in terms of weight loss and improvement of body composition measurements, when undertaking a hypocaloric low-fat diet as compared to a moderately-high-protein diet (Goni *et al*., 2018).

Interestingly, our results also indicate that an increasing number of the T allele was associated with a greater decrease in liver fat content (− 44.3%, $p<0.001$), by MRI and in serum ferritin levels ($p<0.001$) after the nutritional intervention. Lipidomic analysis

revealed a higher improvement in liver status when comparing risk *vs*. no-risk genotype (p=0.006 *vs*. p=0.926, respectively). Although the mechanisms underlying the effect of *SH2B1* rs7359397 need clarification, there is evidence that it plays a crucial role on the control of energy balance and glucose homeostasis (Flores *et al*., 2019; Ren *et al*., 2007). Consequently, a plethora of genetic polymorphisms can contribute to the metabolic response by interfering with different mechanisms involved in several pathways (Taliento *et al*., 2019). This is consistent with previous studies in which the rs738409 C>G variant in *PNPLA3* has been showed to influence the response to PUFAs (particularly omega 9) (Simopoulos, 2016), carbohydrates (Davis *et al*., 2010) and also to specific nutrients intake and dietary habits (Dallio *et al*., 2021). Also, a previous systematic review and meta-analysis demonstrated that individuals with the *FTO* risk genotype and with the highest intakes of sugar, fat and sweet and snacks had the highest BMI (Livingstone *et al*., 2016). Indeed, in Italian patients with NAFLD-HCC, *APOB* variants were associated with lower circulating triglycerides and higher HDL-c (p<0.01) (Pelusi *et al*., 2019). Similar results have been found by other researches evidencing that in overweight and obese individuals the presence of dietary fat intake modified the effect of *MTNR1B* rs10830963 genetic variant on changes in total cholesterol and LDL cholesterol (Goni *et al*., 2019). Furthermore, concerning lipidomic analysis a significant interaction between the genetic variant and the OWLiver®-test -adjusted by the concentration of leptin- was found for the T-allele carriers. In this sense, *SH2B* has been described as an endogenous enhancer of both leptin and insulin sensitivity (Ren *et al*., 2005). Thus, the consideration of leptin in a personalized management of NAFLD may be of interest. As previously reported, it is important to take into account that the exposition to specific macro or micronutrients can influence the expression of these genes, as well as the activity of several enzymes involves in nutrient´s metabolism (Mullins *et al*., 2020). In this context, increased omega-3 fatty acids or fiber intake in the risk allele group, as well as adherence to the MedDiet, could have influenced the improvement of hepatic health in our results. In fact, the increase of omega-3 PUFAs was demonstrated to suppressed diet-induced steatosis and improved of insulin resistance in preclinical models (Jump *et al*., 2018; Levy *et al*., 2004). Moreover, it has also been demonstrated that the dietary monounsaturated to saturated fatty acid ratio may modulate the genetic effects of *GCKR* on serum lipid levels in children (Lee H. *et al*., 2015). In relation to MedDiet, it has been shown that it may not only reduce increased fasting glucose and lipids in risk alleles of *TCF7L2*-rs7903146 polymorphism, but also stroke incidence.

Overall, these results revealed the heterogeneity and complexity of NAFLD pathogenesis and highlighted the need to study and evaluate genetic variants of NAFLD related pathways

such as obesity and the interaction between diet and genetics (Dallio *et al*., 2021). In this sense, rs7359397 *SH2B1* genetic variant has been shown to be associated with the development of advanced stages of NAFLD. In addition, and apparently for the first time, we report a direct relationship between changes on hepatic health after a 6-month dietary intervention and this polymorphism. However, the mechanisms underlying the *SH2B1* genetic variant are not fully understood and further experimental and long-term studies are needed.

3. Genetic Risk Scores for a personalized management of NAFLD

GWASs have increased our knowledge of the genetic susceptibility of the NAFLD population, from the onset to the progression of this disease (Dallio *et al*., 2021). In this sense, identifying genetic variants involved in NAFLD related pathways such as inflammation, lipid metabolism and oxidation, which are associated with the development and progression of this liver disease, insulin resistance, T2DM, obesity and a higher risk of HCC, may be of interest (Fang *et al*., 2018). Therefore, approaches summarizing risk-associated variation across the genome by aggregating information from multiple risk SNPs into a GRS can be an efficient and effective tool (Belsky D.W. *et al*., 2013). Additionally, several algorithms have been proposed to diagnose NAFLD using prediction rules and blood biomarkers (Vilar-Gomez *et al*., 2018). Particularly, FLI has also been used in epidemiological studies and for general population screening as an alternative to ultrasonography and it has shown increasing values as the degree of hepatic steatosis worsens (Kim J.H. *et al*., 2020). Also, imaging techniques such as the MRI can detect changes in fat content during disease progression, being an excellent performance for diagnosing NAFLD (Zhou J.-H.H. *et al*., 2019). OMICS approaches are also one of the most novel minimal invasive strategies to detect patients at risk of NASH (Vilar-Gomez *et al*., 2018).

In this context, in the third chapter, 86 overweight/obese subjects with NAFLD from the FLiO study were evaluated with the aim to assess genetic and non-genetic determinants putatively involved in the onset and progression of NAFLD after a 6-month weight-loss nutritional treatment. Thus, we computed three different genetic risk score concerning the improvement on hepatic health evaluated by minimally invasive methods such as the FLI (GRS_{FLI}), lipidomic-OWLiver®-test (GRS_{OWL}) and MRI (GRS_{MRI}), by adding the risk alleles genotypes. Importantly, 23 SNPs were independently associated with the change in FLI, 16 SNPs with OWLiver®-test and 8 SNPs with MRI, which were specific for every diagnosis tool. In addition, when analyzing body composition and biochemical parameters, the improvement after 6-months of energy restriction was higher when the genetic risk was

lower. According to this, previous studies such as the POUNDS LOST trial, showed that distinct genetically determined adiposity subtypes may differentially modify the effect of weight-loss diet on improving glucose metabolism in overweight participants (Chen Y. *et al*., 2021). Also, an obesity-GRS was shown to modulate the relationship between MedDiet adherence, adiposity and MetS in European adolescents, with this interaction effect being stronger in females than in males (Seral-Cortes *et al*., 2020).

However, because of the complexity of NAFLD pathogenesis, the consideration of both genetic and non-genetic factors should be contemplated. Thus, in our study, three different regression models were generated. Importantly, the calculated GRS_{FLI}, GRS_{OWL} and GRS_{MRI} were major contributors of the improvement in hepatic status. Moreover, the prediction of our model improved when fitted by sex, age and other related factors such as insulin resistance, FGF-21 concentrations and dietary intake. Importantly, higher total protein intake was significantly associated with a lower hepatic improvement (measured by FLI, MRI and OWLiver®-test), when the genetic risk was higher. Regarding proteins, diverse theories have been proposed (Lujan *et al*., 2021; Perdomo *et al*., 2019). Hepatic fat mobility is independently associated with increased protein intake, which is modulated by BCAA and methionine content in the diet (Worm, 2020). In this sense, a six-week randomized controlled study using a eucaloric, protein-rich diet demonstrated a significant reduction in liver fat accumulation in patients with T2DM and NAFLD compared to their habitual diet (Markova *et al*., 2017). At the same time, an inverse association between dairy protein intake and the risk of incident of NAFLD was observed in men and women aged ≥50 years (Lee J.-H. *et al*., 2021). On the contrary, a recent study showed that a higher consumption of AAA, BCAA and SAA was associated with worse hepatic health in subjects with overweight/obesity and NAFLD (Galarregui, Cantero, *et al*., 2020). Also, it is well known that high meat intake, especially processed meats, is associated with insulin resistance (Perdomo *et al*., 2019). In this context, there are several hypotheses that may explain these controversies, such as the nature of the protein consumed (Recaredo *et al*., 2019). However, further mechanistic studies are required to fully elucidate this question.

In accordance with these results and considering the evidence implying that genetic background is associated with response to nutrient intakes (Dallio *et al*., 2021), in the chapter four of the present work, we aimed to build a predictive model based on genetic and hepatic health information, deeming insulin resistance markers in order to personalized dietary treatment in overweight/obese subjects with NAFLD.

After following 6 months of personalized energy-restricted dietary strategies, interesting results among insulin resistance were observed, with no statistical differences between the changes in the AHA and the FLiO group. Insulin resistance has been largely assessed as the main determinant of liver damage in NAFLD (Armandi, Rosso, *et al*., 2021). In this sense, a randomized crossover 6-week dietary intervention trial conducted in non-diabetic subjects with biopsy-proven NAFLD showed that Mediterranean diet, not only reduced liver steatosis, but also improved insulin sensitivity (Ryan *et al*., 2013). Another study conducted in adolescents with NAFLD following a moderately carbohydrates-restricted diet, found a significant decrease in hepatic lipid, as well as improvements in insulin resistance and body composition parameters (Goss *et al*., 2020). On the other hand, concerning liver injury, in our results, significant marginal differences between groups were observed in the FLI, while a higher decrease in liver fat content (by MRI) was observed in the FLiO group. In this regard, a study conducted on 293 patients with histologically proven NASH showed that: ≥5% weight loss was associated with NASH resolution and 2-point reduction in non-alcoholic fatty liver disease activity score (NAS) (Vilar-Gomez *et al*., 2015). Furthermore, all patients who lost ≥10% of their weight had NASH reductions, NASH resolution (90%) and regression of fibrosis (45%). In addition, in subjects with NAFLD following a 6-month dietary intervention based on the Mediterranean diet, significant improvements were shown in variables such as BMI, waist circumference, liver enzymes, lipid profile, HOMA-IR and NAFLD-related indices, including FLI (Gelli *et al*., 2017).

In this context, 22 different SNPs related to obesity, weight-loss and energy metabolism were associated with the percentage of FLI decrease and able to differentiate the best dietary approach. A statistical mixed model based on the construction of a GRS, previously associated with the percentage of FLI decrease, was used to differentiate the best dietary approach. This novel statistical method has been previously used to investigate the individual response to different treatment (Ritz C, Astrup A, Larsen TM, 2019). Thus, in this trial the improvement in liver health assessed by FLI revealed a different response depending on the GRS: a lower genetic risk score was related to a better response to the FLiO diet, while a higher genetic risk score was related to a better response to the AHA diet. These results fall within the fields of nutrigenetics, which at the same time may interfere with different mechanisms involved in the pathogenesis of NAFLD (Meroni, Longo, *et al*., 2020). Interestingly, the GRS designed in this trial was also able to predict the change in insulin resistance markers (RBP4 and TyG index) after 6-months of nutritional intervention. High insulin levels are therefore the results of multiple drivers, involving both environmental and genetic factors, of which balance determines the phenotype and the

natural history of liver disease (Bugianesi *et al*., 2010; Pais *et al*., 2021). In this context, similar statistical approaches involving various features such as microbiota, genetics, epigenetics and other relevant environmental markers and its interactions have been combined to personalize the management of multifactorial diseases. Specifically, in the study published by Ramos-Lopez *et al*., 2020, the incorporation of different genetic, phenotypic and environmental factors influencing BMI reduction, allowed the personalization of the prescription of two different energy-restricted diets with different macronutrient distribution. Also, a randomized controlled trial of 744 non-diabetic overweight or obese adults from the POUNDS LOST trial concluded that individuals with a lower genetic risk of diabetes may benefit more from consuming a low-protein weight loss diet to improve insulin resistance and β-cell function, while subjects with a higher genetic risk appear to benefit more from following a high-protein diet (Huang T. *et al*., 2015).

However, choosing the optimal diet approach for an individual with NAFLD is complicated because of the multifactorial complex of this disease. According to current scientific evidence, weight loss is considered the best therapeutic approach for NAFLD (Marin-Alejandre *et al*., 2021). Alternatively, several dietary patters including the low-fat diet, low-carbohydrate diet, ketogenic diet, the DASH pattern, the Mediterranean dietary pattern and more recently the intermittent fasting have been evaluated in the NAFLD setting (Parra-Vargas *et al*., 2020). Consequently, similar weight loss-oriented regimens could be suggested as possible dietary alternatives to NAFLD (Pugliese *et al*., 2021). On this basis, both the AHA diet and FLiO can be an optimal alternative in the management of NAFLD. However, so far, the Mediterranean Diet is the option recommended by the EASL guidelines as the best dietary treatment for NAFLD, supported by its effects on improving lipid profile and regression of NASH grade (Parra-Vargas *et al*., 2020).

Finally, these trials were designed as a proof-of-principle to display the applicability of different genetic risk score and their use in models aiming to predict the most suitable diet for each individual with NAFLD. However, given the limited scientific evidence to date, further research is needed to better understand the pathophysiological mechanism of these specific obesity-gene-diet interactions, as well as the role of other environmental variables that may modulate each individual´s response.

4. Strengths and limitations

The present research has successfully identified novel obesity-genetic variants and GRS associations, as well as gene-diet or GRS interactions based on non- or minimally invasive diagnostics methods such as the fatty liver index, ultrasound, magnetic resonance imaging and the OWLiver®-test. However, there are various considerations that should be mentioned as strengths or limitations:

Firstly, liver status of participants was evaluated by means of non-or minimally invasive techniques instead of histological results from liver biopsy, which were not available to corroborate the precise diagnosis of patients. Currently, liver biopsy is the gold-standard for the diagnosis of NAFLD, but its use is not always feasible and has potential risks and limitations including invasiveness, severe bleeding and sampling errors (Kogachi *et al*., 2021). Therefore, non-or minimally invasive techniques have been proposed as simple, first-line tools to stratify NAFLD patients (Younes *et al*., 2021). Thus, in this research a complete evaluation of the liver status including the assessment of liver steatosis and liver fibrosis was carried out by means of validated and widely used methods including imaging techniques such as ultrasonography, transient elastography and magnetic resonance imaging (Chalasani *et al*., 2018), as well as blood biomarkers and hepatic indexes which provide a more complete, precise and minimally invasive diagnostic of NAFLD (Kogachi *et al*., 2021). All the imaging tests were performed and evaluated by the same hepatologist within the medical team. Importantly, a validated lipidomic test (OWLiver®-test) which has shown promising results (Cantero *et al*., 2019), was also used for the evaluation of NAFLD.

Second, the relatively small sample size and enrollment of subjects might limit the power to detect the effect of the polymorphisms across the NAFLD phenotypes, gene-diet interactions and dietary changes across the study population. Also, the lack of a large sample could lead to increase the risk of type II errors (failing to detect real differences), especially those related to the selection of SNPs to be introduced into the genetic risk score. However, our approach is consistent with previous investigations that reported that due to the use of less stringent *p*-value thresholds compared to association studies of single variants, genomic profile risk scoring analyses can tolerate, at balance, some of these biases (Wray *et al*., 2014).

Third, because of the lack of gene expression data we could not confirm the mechanisms involved behind the observed associations between the polymorphisms and phenotypes, and neither how the diet could modify such association. Therefore, in the present study we

could only speculate about the underlying biological processes or based on the evidence reported by other authors. For example, a genetic disruption of the *SH2B1* rs7359397 genetic variant results in an energy imbalance, obesity, severe leptin resistance and type 2 diabetes in humans (Al-Hakeem, 2014).

Fourth, the used of obesity-related SNP for the constructions of the GRS it is also an important limitation. In addition, the lack of *PNPLA3* polymorphism, which is the best validated susceptibility modifier for steatosis and progressive hepatic injury is another important limitation (Dongiovanni *et al*., 2013). However, recently GWAS studies have revealed that other genetic variants might also contribute to steatosis and/or steatohepatitis, therefore, even if is still a need to explore this GRS approach, our contribution re-stated the value of the genetic make-up in personalized NAFLD management (Pirola *et al*., 2021).

Fifth, the used of a self-reported questionnaire in the assessment of dietary intake may produce some bias on the evaluation of the results. However, all the instruments used in this study have been previously validated in Spanish population and are commonly used in clinical and research evaluations (Moreiras *et al*., 2009). Finally, the included subjects live in a Mediterranean country, so the findings may not be extended to other ethnic groups. In addition, the absence of a non-NAFLD group and the inclusion of a cross-sectional study that may identify associations, but not causality are also weaknesses to consider. Therefore, further studies are required to validate and generalize these results in different populations.

On the other hand, there are various strengths to mention in this research. Firstly, the study is a randomized controlled trial in which both the AHA and FLiO strategies included a personalized diet trying to promote the adoption of behavioral and healthy lifestyle changes with individual follow-up of volunteers for 6 months. In addition, the AHA diet is a well-recognised healthy dietary pattern that was used as a reference to corroborate and evidence the positive results obtained with the FLiO diet, suggesting this approach as an alternative for lifestyle management of NAFLD.

Second, the inclusion and combination of obesity-related genetic variants demonstrate the existence of shared genetic components between NAFLD and obesity traits. Finally, a major strength is the potential applicability and translatability of SNPs identification and GRS construction to clinical care, including risk prediction, disease classification, drug development and drug toxicity (Manolio, 2013). In this context, in subjects with NAFLD, it has been recommended to consider PNPNL3 I148M and TM6SF2 E167K genotyping in

selected patients and in clinical studies (Marchesini *et al*., 2016). In addition, GRSs are being used to predict genetic predisposition to NAFLD-related disorders such as obesity or T2DM (Seral-Cortes *et al*., 2021; Udler *et al*., 2019). Therefore, given the current scenario, the importance of early detection, the availability of alternative treatments and the accessibility of genotyping, make these methods an excellent alternative for the management of NAFLD.

5. Corollary

Altogether the results presented in this thesis clearly show that dietary and obesity-related genetic factors, as well as their interaction, could modulate susceptibility to NAFLD. Notably, we reported that rs7359397 *SH2B1* genetic variant has an impact on the development and progression of NAFLD. Moreover, our research work also contributes to better understand not only the contribution of this obesity gene on NAFLD, but also the response to a nutritional treatment depending on *SH2B1* genotype, as well as possible gene-treatment interactions. Specifically, for the first time we reported a direct relationship between changes on hepatic health after an energy-restricted treatment and a genetic variant located in *SH2B1*.

In addition, individuals with a lower genetic predisposition to NAFLD, defined by a GRS for the change of each minimally invasive methods, such as the fatty liver index (GRS_{FLI}), lipidomic-OWLiver®-test (GRS_{OWL}) and magnetic resonance imaging (GRS_{MRI}), showed a greater improvement in most of the measured variables. Thus, in the present work we observed that three different genetic scores could be a useful tool for the personalized management of NAFLD. However, as suggested by scientific literature, genetic factors explain a small percentage of NAFLD heritability. Thus, the predictive accuracy of all models substantially improved when adjusted by phenotypic features such as age, sex and NAFLD-related variables such as insulin resistance and inflammatory biomarkers or dietary compounds.

Furthermore, given the differences in susceptibility among individuals, there is reason to believe that a similar differential response should characterize the effects of NAFLD treatment. In this context, a GRS based on 22 different SNPs previously associated with the decrease of FLI, was designed in order to predict the different response to 2 energy restricted diets. The designed model was able to personalize the most suitable diet for 72% of the volunteers. These results confirm that NAFLD should be considered as a multifactorial disease in which a large number of phenotypic and genotypic factors and its interactions are involved.

CONCLUSIONS

Conclusions

1) The risk genotype concerning the *SH2B1* rs7359397 genetic variant was associated with higher homeostatic model assessment of insulin resistance, fatty liver index and protein intake, while lower mono-unsaturated fatty acid and fiber intake was found. Moreover, individuals with the minor risk allele also showed a worse hepatic status and higher susceptibility of advanced stages of NAFLD. These results underline the importance of considering genetic predisposition in combination with dietary intake in the development of this liver disease.

2) Carriers of the minor allele of the *SH2B1* variant showed a better response to a weight loss dietary intervention in terms of hepatic health and liver status. Furthermore, adherence to a Mediterranean dietary pattern rich in fiber and other components such as omega-3 fatty acid might boost these benefits. These findings highlight the importance of the genetic background in combination with dietary intake in the response to NAFLD treatment.

3) Three genetic risk scores based on different diagnostic tools for detecting NAFLD and with specific polymorphisms for each method were able to predict the improvement in liver health after a 6-month energy-restricted nutritional treatment. These associations were particularly influenced by factors such as insulin resistance, inflammatory biomarkers and specific nutrients. These results reinforce the importance of using genetic risk scores, along with underlying mechanisms, in the assessment and management of NAFLD.

4) The designed genetic risk score was able to predict the change in fatty liver index adjusted by diet, age and sex, allowing to personalize the most suitable diet (AHA or FLiO) for 72% of the volunteers. Similar models were also able to predict the changes on variables related to insulin resistance (Triglycerides and Glucose index and RBP4 levels) depending on diet. These findings demonstrate that models involving genetic information and insulin resistance variables can determine differential individual responses to dietary interventions in subjects with NAFLD.

5) New diagnostic and personalized intervention approaches based on nutrigenetics instruments could help to improve precision nutrition management in subjects with NAFLD, reducing the severity, some associated comorbidities and impact on healthcare concerning this disease, as well as the benefits of individualized prescribed dietary patterns.

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APPENDICES

Supplementary material

Table S1. Genomic characteristics of the 95 SNPs related to energy homeostasis.

No.	Gene name	Gene symbol	SNP identifier	Chromosome location	Alleles
1	G protein subunit alpha transducin 2	*GNAT2*	rs17024393	Chr1:110154688	T/C
2	Methylenetetrahydrofolate reductase	*MTHFR*	rs1801131	Chr1:11854476	T/G
3	Methylenetetrahydrofolate reductase	*MTHFR*	rs1801133	Chr1:11856378	G/A
4	SEC16 homolog B, endoplasmic reticulum export factor	*SEC16B*	rs543874	Chr1:177889480	A/G
5	Lysophospholipase like 1	*LYPLAL1*	rs2605100	Chr1:219644224	A/G
6	Lysophospholipase like 1	*LYPLAL1*	rs4846567	Chr1:219750717	G/T
7	Cannabinoid receptor 2	*CNR2*	rs3123554	Chr1:24196401	A/G
8	Fatty acid amide hydrolase	*FAAH*	rs324420	Chr1:46870761	C/A
9	Leptin receptor	*LEPR*	rs8179183/rs1805094	Chr1:66075952	G/C
10	Neuronal growth regulator 1	*NEGR1*	rs2815752	Chr1:72812440	G/A
11	ATP binding cassette subfamily B member 11	*ABCB11*	rs519887	Chr2:169780885	T/C
12	ATP binding cassette subfamily B member 11	*ABCB11*	rs484066	Chr2:169782481	A/T
13	ATP binding cassette subfamily B member 11	*ABCB11*	rs569805	Chr2:169782880	A/T
14	ATP binding cassette subfamily B member 11	*ABCB11*	rs494874	Chr2:169789306	T/C
15	Insulin receptor substrate 1	*IRS1*	rs2943641	Chr2:227093745	T/C
16	Adenylate cyclase 3	*ADCY3*	rs10182181	Chr2:25150296	A/G
17	Adenylate cyclase 3	*ADCY3*	rs713586	Chr2:25158008	T/C
18	Transmembrane protein 18	*TMEM18*	rs2860323	Chr2:614210	A/G
19	Transmembrane protein 18	*TMEM18*	rs2867125	Chr2:622827	T/C
20	Transmembrane protein 18	*TMEM18*	rs13021737	Chr2:632348	A/G

21	Peroxisome proliferator activated receptor gamma	*PPARG*	rs1801282	Chr3:12393125	C/G
22	Peroxisome proliferator activated receptor gamma	*PPARG*	rs2959272	Chr3:12442833	T/G
23	Peroxisome proliferator activated receptor gamma	*PPARG*	rs1386835	Chr3:12450918	A/G
24	Peroxisome proliferator activated receptor gamma	*PPARG*	rs709158	Chr3:12463176	A/G
25	Peroxisome proliferator activated receptor gamma	*PPARG*	rs1175540	Chr3:12465243	C/A
26	Peroxisome proliferator activated receptor gamma	*PPARG*	rs1175544	Chr3:12467044	C/T
27	Peroxisome proliferator activated receptor gamma	*PPARG*	rs1797912	Chr3:12470239	A/C
28	ETS variant transcription factor 5	*ETV5*	rs1516725	Chr3:185824004	T/C
29	ETS variant transcription factor 5	*ETV5*	rs9816226	Chr3:185834499	A/T
30	Solute carrier family 39 member 8	*SLC39A8*	rs13107325	Chr4:103188709	C/A/T
31	Fatty acid binding protein 2	*FABP2*	rs1799883	Chr4:120241902	T/C
32	Uncoupling protein 1	*UCP1*	rs6536991	Chr4:141481581	T/C
33	Uncoupling protein 1	*UCP1*	rs12502572	Chr4:141485134	G/A
34	Uncoupling protein 1	*UCP1*	rs1800592	Chr4:141493961	T/C
35	PPARG coactivator 1 alpha	*PPARGC1A*	rs8192678	Chr4:23815662	C/T
36	Glucosamine-6-phosphate deaminase 2	*GNPDA2*	rs10938397	Chr4:45182527	A/G
37	Clock circadian regulator	*CLOCK*	rs1801260	Chr4:56301369	A/G
38	Protein phosphatase, Mg2+/Mn2+ dependent 1K	*PPM1K*	rs1440581	Chr4:89226422	T/C
39	Adrenoceptor beta 2	*ADRB2*	rs1042713	Chr5:148206440	G/A
40	Adrenoceptor beta 2	*ADRB2*	rs1042714	Chr5:148206473	G/C
41	Cytoplasmic polyadenylation element binding protein 4	*CPEB4*	rs6861681	Chr5:173362458	G/A

42	Tumor necrosis factor a	*TNFA*	rs1800629	Chr6:31543031	G/A
43	Nudix hydrolase 3	*NUDT3*	rs206936	Chr6:34302869	A/G
44	Transcription factor AP-2 beta	*TFAP2B*	rs987237	Chr6:50803050	A/G
45	Transcription factor AP-2 beta	*TFAP2B*	rs2207139	Chr6:50845490	A/G
46	Leptin	*LEP*	rs7799039	Chr7:127878783	G/A
47	Leptin	*LEP*	rs4731426	Chr7:127882070	G/C
48	Leptin	*LEP*	rs2071045	Chr7:127892980	T/C
49	Nuclear factor, erythroid 2 like 3	*NFE2L3*	rs1055144	Chr7:25871109	C/T
50	Adrenoceptor beta 3	*ADRB3*	rs4994	Chr8:37823798	A/G
51	Adrenoceptor alpha 2A	*ADRA2A*	rs1800544	Chr10:112836503	G/C
52	Acyl-CoA synthetase long chain family member 5	*ACSL5*	rs2419621	Chr10:114135013	C/T
53	Transcription factor 7 like 2	*TCF7L2*	rs7903146	Chr10:114758349	C/T
54	Transcription factor 7 like 2	*TCF7L2*	rs12255372	Chr10:114808902	G/T
55	Ankyrin repeat and kinase domain containing 1	*ANKK1*	rs1800497	Chr11:113270828	G/A
56	Apolipoprotein A5	*APOA5*	rs662799	Chr11:116663707	G/A
57	Brain derived neurotrophic factor	*BDNF*	rs6265	Chr11:27679916	C/T
58	Brain derived neurotrophic factor	*BDNF*	rs11030104	Chr11:27684517	A/G
59	Brain derived neurotrophic factor	*BDNF*	rs10767664	Chr11:27725986	T/A
60	Cryptochrome circadian regulator 2	*CRY2*	rs11605924	Chr11:45873091	A/C
61	Mitochondrial carrier 2	*MTCH2*	rs10838738	Chr11:47663049	A/G
62	Uncoupling protein 2	*UCP2*	rs660339	Chr11:73689104	G/A
63	Uncoupling protein 2	*UCP2*	rs659366	Chr11:73694754	C/T
64	Uncoupling protein 3	*UCP3*	rs2075577	Chr11:73715542	G/A

65	Uncoupling protein 3	*UCP3*	rs2734827	Chr11:73716277	G/A
66	Uncoupling protein 3	*UCP3*	rs1685325	Chr11:73717025	T/C
67	Uncoupling protein 3	*UCP3*	rs2075576	Chr11:73717121	C/T
68	Uncoupling protein 3	*UCP3*	rs1800006	Chr11:73717254	A/G
69	Uncoupling protein 3	*UCP3*	rs1800849	Chr11:73720165	G/A
70	Serine/threonine kinase 33	*STK33*	rs4929949	Chr11:8604593	T/C
71	Melatonin receptor 1B	*MTNR1B*	rs10830963	Chr11:92708710	C/G
72	Arachidonate 5-lipoxygenase activating protein	*ALOX5AP*	rs4769873	Chr13:31312689	C/T
73	Perilipin 1	*PLIN1*	rs1052700	Chr15:90208310	A/T
74	Perilipin 1	*PLIN1*	rs894160	Chr15:90211823	C/T
75	Perilipin 1	*PLIN1*	rs2289487	Chr15:90217096	C/T
76	SH2B adaptor protein 1	*SH2B1*	rs7498665	Chr16:28883241	A/G
77	SH2B adaptor protein 1	*SH2B1*	rs7359397	Chr16:28885659	C/T
78	Fat mass and obesity associated	*FTO*	rs1558902	Chr16:53803574	T/A
79	Fat mass and obesity associated	*FTO*	rs1121980	Chr16:53809247	G/A
80	Fat mass and obesity associated	*FTO*	rs17817449	Chr16:53813367	T/G
81	Fat mass and obesity associated	*FTO*	rs8050136	Chr16:53816275	C/A
82	Fat mass and obesity associated	*FTO*	rs3751812	chr16:53818460	G/T
83	Fat mass and obesity associated	*FTO*	rs9939609	Chr16:53820527	T/A
84	Aralkylamine N-acetyltransferase	*AANAT*	rs12452844	Chr17:74459243	G/A
85	NPC intracellular cholesterol transporter 1	*NPC1*	rs1805081	Chr18:21140432	T/C
86	Melanocortin 4 receptor	*MC4R*	rs6567160	Chr18:57829135	T/C
87	Melanocortin 4 receptor	*MC4R*	rs571312	Chr18:57839769	C/A

88	Melanocortin 4 receptor	*MC4R*	rs17782313	Chr18:57851097	T/C
89	Melanocortin 4 receptor	*MC4R*	rs17066866	Chr18:58055619	A/T
90	TNF receptor superfamily member 11a	*TNFRSF11A*	rs17069904	Chr18:60032949	G/A
91	Glutaminyl-peptide cyclotransferase like	*QPCTL*	rs2287019	Chr19:46202172	C/T
92	Catenin beta like 1	*CTNNBL1*	rs6013029	Chr20:36399580	G/T
93	GNAS complex locus	*GNAS*	rs6123837	Chr20:57465571	G/A
94	5-hydroxytryptamine receptor 2C	*HTR2C*	rs3813929	ChrX:113818520	C/T
95	Angiotensin II receptor type 2	*AGTR2*	rs11091046	ChrX:115305126	A/C

SNPs are sorted by chromosome location.

Other publications

1. **Title:** Effects of two personalized dietary strategies during a 2-year intervention in subjects with non-alcoholic fatty liver disease: a randomized trial

   Authors: Bertha Araceli Marin-Alejandre, Irene Cantero, Nuria Perez-Diaz-del-Campo, J. Ignacio Monreal, Mariana Elorz, José Ignacio Herrero, Alberto Benito-Boillos, Jorge Quiroga, Ana Martinez-Echeverria, Juan Isidro Uriz-Otano, María Pilar Huarte-Muniesa, Josep A. Tur, J. Alfredo Martinez, Itziar Abete and M. Angeles Zulet

   Journal: *Liver International*

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2. **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

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