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Abstract
BACKGROUND: Non-alcoholic fatty liver disease (NAFLD) development is linked to insulin resistance and influenced by environmental factors, but it also underlined a genetic predisposition. The aim of this research was 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.
METHODS: A 6-months nutritional intervention was conducted in 86 overweight/obese volunteers with NAFLD randomly assigned to 2 energy-restricted diets: the American Heart Association (AHA) diet and the Fatty Liver in Obesity (FLiO) diet. Individuals were genotyped using a pre-designed panel of 95 genetic variants. A genetic Risk Score (GRS) for each diet was computed using statistically relevant SNPs for the change on Fatty Liver Index (FLI) after 6-months of nutritional intervention. Body composition, liver injury and insulin resistance markers, as well as physical activity and dietary intake were also assessed.
RESULTS: Under energy restriction, both the AHA and FLiO diets induced similar significant improvements on body composition, insulin resistance markers, hepatic health and dietary and lifestyle outcomes. The calculated score included in the linear mixed regression model was able to predict the change of FLI adjusted by diet, age and sex. This model allowed to personalize the most suitable diet for 72% of the volunteers. Similar models were also able to predict the changes on Triglycerides and Glucose (TyG) index and Retinol-binding protein 4 (RBP4) levels depending on diet.
CONCLUSIONS: Models integrating genetic screening and insulin resistance markers can be useful for the personalization of weight loss NAFLD treatments.

Key words: NAFLD, genetic risk score, obesity, insulin resistance, precision nutrition

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the leading cause of liver diseases in high-income countries, where alarming prevalence is increasing worldwide, usually in parallel to obesity (1,2). This syndrome encompasses a spectrum of progressive conditions including, simple steatosis, steatohepatitis (NASH), fibrosis (with variable severity degrees), cirrhosis and hepatocellular carcinoma (HCC), according to accepted paradigms (3). However, the risk of developing each condition is influenced by numerous complex factors (1). Indeed, inherited genetic variants have also been proposed as one of the contributing factors to the pathogenesis of NAFLD (3).

Emerging data from genome-wide association studies (GWAS) have uncovered strong and reproducible associations between genetic variants in PNPLA3, TM6SF2, MBOAT7, GCKR, HSB17B13 genes, and the development and progression of NAFLD (4).

However, according to available evidence, the effect of the aforenamed gene variants explains just a small proportion of the disease variance, suggesting an influence of less prevalent genetic variants on the pathogenesis of this disease (5). For example, genes implicated in NAFLD related traits, such as the SH2B1 gene has also been correlated with a higher risk of developing a severe stage of NAFLD (6).

Concerning diagnosis, liver biopsy remains the reference for NAFLD, but the presence of some limitations, has limited its use (7). Indeed, imaging techniques such as

magnetic resonance imaging (MRI) has been suggested to be a great alternative in the detection and quantification of liver fat content, even if limited availability and technologized make this method challenging and inappropriate for screening purposes (1). Therefore, earlier diagnostic and less-invasive methods have been proposed as first-line tools to identified and stratified NAFLD patients (7,8). In this sense, predictive models and algorithms for predicting or grading steatosis have been developed, such as the Fatty Liver Index (FLI), which was initially designed to predict hepatic steatosis in the general population (9). Moreover, several guidelines have recommended the Fatty Liver Index (FLI) as one of the best minimal invasive tools for the diagnosis of steatosis (10,11). Also, the consideration of recognized risk factors such as insulin resistance measured by HOMA-IR, triglycerides and glucose (TyG) index or biomarkers such as retinol-binding protein 4 (RBP4) levels might help for a better feature of liver disease and to implement more precision treatments (12,13).

To date, no pharmacological therapies have been unanimously accepted for the treatment of NAFLD (14). However, there are several novel drugs that are being revising for safety and security such as glucagon-like peptide 1 receptor agonist (GLP-1 RA) (7,15). Therefore, the primary approach recommended for the treatment of NAFLD is lifestyle modification (14). Furthermore, it has been demonstrated that individual response to a nutritional treatment differs depending on genetic background and other personalized variables such as sex or age, which needs to be accounted for precision nutrition understanding (3).

In this sense, a large number of single nucleotide polymorphism (SNPs) and other genetic variants associated with lipid metabolism, body weight regulation, inflammation, insulin resistance and adipogenesis have been identified to interact with nutrients intake (5). Additionally, the design of genetic risk scores in combination with clinical risk factors has demonstrated to improve the predictability of NAFLD responses and evolution (16). Recently, a study based on three different genetic risk scores (GRS), has shown a variance of 53% in hepatic functionality (FLI), 16% in lipidomic metabolism (OWLiver®-test) and 34% in liver fat content (MRI), in overweight/obese subjects with NAFLD after a 6-months nutritional treatment (17). Therefore, the implement of prototypes by integrating potential biomarkers, genetic/epigenetic information as well as age, gender, physiopathological status and environmental issues, including lifestyle could be useful to personalize NAFLD management (3). In this context, the aim of this research was to personalize NAFLD management by the evaluation of hepatic improvement (measured by FLI) of participants depending on two energy-restricted nutritional treatments and genetic. Furthermore, a predictive model analyzing the potential interaction between genetic and hepatic information, as well as inflammation and insulin resistance markers, was

performed in order to individualized dietary treatment. We present the following article in accordance with the CONSORT reporting checklist.

Materials and methods

Study participants

This randomized controlled clinical trial (FLiO: Fatty Liver in Obesity study; NCT03183193) enrolled 98 men and women between 40–80 years old, who presented overweight or obesity (body mass index (BMI) ≥ 27.5 kg/m$_2$ to <40 kg/m$_2$) and hepatic steatosis confirmed by abdominal ultrasonography (14). Volunteers were recruited at the Center for Nutrition Research of the University of Navarra in the city of Pamplona, Navarra, Spain. Major exclusion criteria included: the presence of known liver disease other than NAFLD; individuals reporting weight change (≥3 kg) within the 3 months before the study; excessive alcohol consumption (>21 standard drinks per week in men and >14 standard drinks per week for women) (18), 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), severe psychiatric disorders, active autoimmune disease or requiring pharmacological treatment, the use of weight modifiers, and the lack of autonomy or inability to follow the diet, as well as the difficulties in following the scheduled visits. All research procedures were implemented in agreement with the ethical principles of the Declaration of Helsinki and the study was conducted following the CONSORT 2010 guidelines. Each participant voluntarily provided written informed consent before inclusion in the study. The study protocol was specifically approved by the Research Ethics Committee of the University of Navarra, Spain on 24 April, 2015 (ref. 54/2015).

Nutritional intervention

A 6-months nutritional intervention was conducted involving 2 well-defined diets with a 30% energy restriction with the objective to achieve a loss of at least 3%-5% of the initial body weight, in accordance with the recommendations of the American Association for the Study of Liver Diseases (AASLD) (19) with different macronutrient distribution. The American Heart Association (AHA) diet was based on the guidelines of the American Heart Association (20), which propose 3-5 meals/day and provide a 50-55% of the total energy (E) intake from carbohydrates, 15% E from proteins, and 30% E from lipids with a healthy fatty acid profile. On the other hand, the Fatty Liver in Obesity (FLiO) diet was designed with a higher meal frequency (7 meals/day). The macronutrient distribution according to the total energy value was: 40%-45% carbohydrates (preferring those with low glycemic index), 25% proteins (predominantly from vegetable sources), and 30%-35% from lipids (favoring extra virgin olive oil and omega-3 polyunsaturated fatty acids to the detriment of saturated and trans fats), as well as a high adherence to the MedDiet, involving an increased quantity of natural antioxidants. Moreover, a 7 days menu plan was provided to the participants in both groups. Participants were randomly assigned to 1 of the 2 dietary interventions and the professionals in charge of hepatic assessment and other determinations were blinded to the dietary treatment, clinical information, and laboratory data.

Anthropometry, body composition and biochemical determinations

Anthropometric measurements including body weight, height and waist circumference and body composition (Lunar iDXA, Encore 14.5, Madison, WI, USA) were assessed in fasting conditions by trained nutritionists at the Metabolic Unit of the University of Navarra following standardized procedures (20). BMI was calculated as the body weight divided by the squared height (kg/m_2). Blood samples for biochemical determinations were careful 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 and triglycerides (TG) concentrations were determined on an autoanalyzer with specific commercial kits and following the instructions of the company (Cobas 8000, Roche Diagnostics). Insulin and Retinol binding protein-4 (RBP4), concentrations were measured with specific ELISA kits (Demeditec; Kiel-Wellsee, Germany) in a Triturus autoanalyzer (Grifols, Barcelona, Spain). The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was computed using the following formula: HOMA-IR = (insulin (μU/mL) x glucose (mmol/L))/22.5. The Triglycerides/Glucose index (TyG) (ln[triglycerides (mg/dL) x glucose(mg/dL)/2)]) was also computed as a surrogate of glucose tolerance (21).

Hepatic assessment

The presence of hepatic steatosis was determined by ultrasonography (Siemens ACUSON S2000 and S3000, Erlangen, Germany) in accordance with previously described methodology (14). 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 (22). 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. The whole liver assessment was performed under fasting conditions at the University of Navarra Clinic.
The Fatty liver index (FLI) was computed using serum triglycerides, BMI, waist circumference and GGT concentrations using the formula described elsewhere (9). 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).

Habitual dietary intake and physical activity assessments

Habitual dietary intake at baseline and after 6 months of the study was assessed with a validated semiquantitative food frequency questionnaire (FFQ) of 137 food items (23). The composition of the food items was derived from accepted Spanish food composition tables and appropriate software. The adherence to the MedDiet was evaluated 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 Mediterranean Diet Score (MedDiet Score). Physical activity was assessed using the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire.

SNP selection and genotyping

For the DNA genotyping assay, a total of 86 oral epithelium samples were collected with a liquid-based kit (ORAcol- lect-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, which were selected after an exhaustive bibliographic review (24), was applied and analyzed. Genotyping was performed by targeted next generation sequencing on Ion Torrent PGM equipment (Thermo Fisher Scientific Inc., Waltham, MA, USA). Overall, the amplicon mean size was 185 bp. 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).

GRS Calculation

The construction of the two different scores (one for each diet, AHA and FLiO) was approached by using a previous detailed pre-designed panel of 95 SNPs (24). First, Kruskal–Wallis test was to perform to a variable selection procedure for identifying SNPs statistically or marginally associated with the change in FLI (absence of allele, presence of one allele or presence of two alleles). As a result, a total of 22 different SNPs with a p-value lower than 0.20 (11 SNPs for each diet) were obtained in our sample. 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 22 SNPs. A risk genotype was defined as the one that was associated with a lower change of FLI. 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, a GRS for each diet was calculated by summing the number of risk alleles at each locus (GRS_{AHA} and GRS_{FLiO}). Finally, a different genetic risk score was obtained by calculating the difference of the genetic risk score of each type of diet.

Statistical analyses

The primary outcome of the study was the change in body weight in accordance to the current recommendations of the AASLD to ameliorate NAFLD features (19). Therefore, in order to detect a minimum difference of weight loss of approximately 1.0 kg ± 1.5 kg between dietary groups, the required sample size was estimated at 36 individuals in each arm of the study ($\alpha = 0.05$ and statistical power of 80%. Considering a potential dropout rate of 20-30%, 50 subjects were included in each group, 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 for suitable analyses. Moreover, after 6 months, a total of 70 participants had complete information and epithelium buccal cells to carry out the study.

Continuous variables were expressed as means and 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 analyzed variables was screened with the Shapiro-Wilk test. The differences at baseline and after the 6-month dietary intervention within each group were estimated using Student's t-tests of independent samples and Wilcoxon-matched-pairs signed ranks test when appropriate. Categorical variables were compared using the Chi-squared test at different timepoints of the study. Significant changes after 6-months of nutritional intervention in each diet were assessed by Student's t-test or Mann-Whitney test according to the distribution of data. Linear mixed models were implemented to predict FLI decrease according to the total
genetic risk score and the interactions with the diet. An interaction term between the total genetic score with diet was intentionally sought in order to personalize the diet. Age and sex were used as adjusting variables. Subjects were used as random effects. All p-values presented are two-tailed and a $p<0.05$ was considered statistically significant. Statistical analyses and graph designs were carried out using Stata 15 (StataCorp LLC, College Station, TX, USA; http://www.stata.com).

Data availability

The data associated with the paper are not publicly available but are available from the corresponding author on reasonable request.

Results

The participants of the study were recruited between June 2016 and June 2017 in Navarra, Spain. Baseline and 6-month phenotypic characteristics of individuals according to dietary group have been described (Table 1). Noteworthy, no differences were observed between the two dietary groups at baseline concerning anthropometry and body composition and insulin resistance markers. After 6 months of nutritional intervention, a total of 70 participants were assessed. Significant improvements in body composition and biochemical parameters were observed throughout the study in both dietary groups. Notably, both groups showed significant reductions in triglycerides, glucose, insulin, HOMA-IR, TyG index and RBP4 levels during the follow-up of the study, without significant differences between the changes in AHA and FLiO diets. Moreover, a total of 6 (6.98%) participants had type 2 diabetes mellitus at the baseline evaluation without significant differences between groups (AHA group n = 3 and FLiO group n = 3).

Regarding hepatic assessment, both groups showed significant improvements in the follow-up evaluations of the trial. The decrease reported in the Fatty Liver Index was significant in both groups even if the change was greater in the FLiO compared with AHA group (-41.94% vs. -30.64%). Interestingly, significant differences were reached between dietary groups in the change of Fatty Liver Index (p=0.033). Also, a higher and significant decrease in the hepatic fat content measured by MRI was also observed in FLiO group.

Although the targets of macronutrient intake (%E) were not fully achieved in the AHA (carbohydrates: 45.7 (6.9); protein: 17.5 (16.4; 19.4); 34.4 (29.2; 38.2) and the FLiO diet (carbohydrates: 39.2 (7.2); protein: 21.9 (19.2; 23.2); fat: 34.7 (31.4; 42.4), the reported dietary intake confirmed that participants modified their intakes of macronutrients in the direction of the intervention. Moreover, significant differences
between diets were observed for the intake of carbohydrates and protein, following the

expected trends. The MedDiet adherence significantly increased in both groups after the nutritional intervention and this change was significantly greater in the FLiO group compared to the AHA group. On the other hand, both groups increased their physical activity but only the AHA group showed significant increases at 6 months ($p<0.001$). However, the changes in physical activity did not reveal statistical differences between the groups. Lastly, noticeable different response in carbohydrates and MedDiet Score between dietary groups was observed after the nutritional treatment ($p=0.019$ and $p<0.001$, respectively).

A total of 22 SNPs were statistically or marginally associated with percentage FLI decrease, all of which were different between the AHA and FLiO diets (Table 2). Therefore, the SNPs rs1801133 (methylenetetrahydrofolate reductase, MTHFR), rs10838738 (Mitochondrial carrier 2, MTCH2), rs11091046 (Angiotensin II receptor type 2; AGTR2); rs1801131 (Methylenetetrahydrofolate reductase, MTHFR); rs17817449, rs8050136, rs3751812, rs9939609, rs1558902, rs1121980 (Fat mass and obesity associated, FTO); rs2605100 (lysophospholipase like 1, LYPLAL1), accounted for the differences in FLI decrease in the AHA diet. However, FLI decrease diverged among genotypes of rs2075577 (Uncoupling protein 3, UCP3); rs2419621 (Acyl-CoA synthetase long chain family member 5, ACSL5); rs3123554 (CNR2FUCA1); rs7903146 (Transcription factor 7 like 2, TCF7L2); rs660339 (uncoupling protein 2, UCP2); rs2959272 (Peroxisome proliferator activated receptor gamma, PPARG); rs10830963 (Melatonin receptor 1B , MTNR1B); rs6123837 (GNAS complex locus, GNAS); rs12502572 (Uncoupling protein 1, UCP1); rs1440581 (Protein phosphatase, $Mg2+/Mn2+$ dependent 1K, PPM1K) and rs571312 (Melanocortin 4 receptor, MC4R), accounted for the differences in FLI decrease in the FLiO diet.

The equation for prediction FLI decreases by diet was evaluated using a linear mixed regression model (Table 3) including the generic score and the interaction term with diet as fixed effects, and the subjects as random effects. Age and sex were used as adjusting variables. Furthermore, Figure 1 depicts a decision algorithm for a personalized management of NAFLD by the measured of the change on FLI using genetic, phenotypic, and environmental characteristics. At each patient's visit, conventional data such as age, sex and dietary intake are obtained through clinical and nutritional evaluations. After buccal sampling, genotyping of relevant SNPs influencing FLI decrease is performed and the GRS is computed. Once genetic, phenotypic, and environmental information is registered, FLI decrease depending on diet. Therefore, nutritional advice is given based on the prescription of a specific diet (if statistically significant differences between predictive values for the AHA and FLiO diets are detected) or, taking into account individual food preferences, to follow either diet (if no statistically significant differences are identified between predictive values concerning nutritional and FLI decrease for the AHA and FLiO diets), as shown in Figure 1.

Statistical differences between the predictions of FLI percentage loss with both diets were analyzed using a Z test involving the standard errors of each estimation in order to prescribe the most suitable diet to each individual. Thus, a total of 72% volunteers were significantly assigned to a most successful weight loss diet following this model. Hence, when the GRS was ≤13, volunteers following to the FLiO diet showed a higher decreased compared to AHA diet ($p<0.001$) (Figure 2). However, these association was opposite when the GRS was ≥16 points ($p=0.052$). Moreover, when the GRS was between 14-15, both diets demonstrated beneficial effects with no significant differences among them ($p=0.341$).

Additionally, RBP4 levels and TyG index were also predicted by using the same genetic

risk score and linear mixed regression model (Table 4). In both RBP4 and TyG index models, the interaction Diet X genetic score was statistically significant ($p<0.005$ and 0.009, respectively) (Figure 3A and 3B). Interestingly, the same interaction remained significant when selecting participants with a liver fat content (by MRI) ≥ 5% at baseline ($p=0.001$) (Table S1 and Figure S1).

Discussion

There is no doubt that NAFLD is the result of a combination of genetic background and environmental factors, and where complex nutrigenetic interactions between them needs to be accounted (5,25). Unhealthy lifestyles play a role in the development and progression of NAFLD, which makes lifestyle modification mandatory in these patients (14). Moreover, increasingly evidence indicates that lifestyle can also differ on the impact of genetic variations on this liver disease (3). However, the mechanism of these interactions, as well as the different approaches for the management of liver steatosis need further investigations (3). To this end, we focus on the implementations of a nutrigenetic model based on 22 different SNPs that were previously associated with the percentage of FLI decrease and able to differentiate the best dietary approach for a non-invasive management of overweight and obese subjects with NAFLD.
Liver biopsy, although an invasive procedure, remains the standard for the diagnosis of NAFLD (1). Nonetheless, patients undergoing liver biopsy are highly selected and several non-invasive procedures and algorithms have been proposed (7). One of the best validated and accepted biomarkers is the Fatty Liver Index, which has often been used in epidemiological studies, but also in clinical interventions being easy to implement in individual NAFLD patients despite some drawbacks for accuracy (26). One of the best accepted and validated biomarkers is the Fatty Liver Index, which has often been used in epidemiological studies, but also in clinical interventions and is easy to implement, despite some drawbacks for accuracy, in individual NAFLD patients. Moreover, a recent metanalyses evaluating 27,221 subjects without secondary causes of fatty liver disease, showed that FLI was an adequate instrument in stratifying the risk of NAFLD, even if it showed only weak evidence of a discriminatory performance in excluding or diagnosing this disorder (27). In terms of treatment, lifestyle assessment encompasses diet and regime changes is the only effective therapeutic modality (14). However, long-term adherence to a diet is very complicated, being one of the most important challenges in the management of this liver disease to offer dietary alternatives to encourage adherence and compliance to diets. Thus, several pharmacological approaches using diverse drugs such as metformin, pioglitazone, glucagon-like peptide-1 or vitamin E have been prescribed or therapeutically considered in the management of NAFLD and NASH (19,28). It has also recently been proposed that the use of new methodologies for NAFLD-NASH approaches in preclinical or early-stage research could accelerate the drug development process, where nutrigenetic interactions may have a role (28). Thus, in this study, after 6-months of nutritional treatment, most of the measured variables were improved in both AHA and FLiO diet. However, no differences between the interventions groups, diets were observed concerning most of the evaluated variables (29). Nonetheless, NAFLD is considered a complex disease trait where interindividual variability and environmental features interact determining the specific phenotype and progression of the disease (3,5). Multiple genome-wide association and large candidate gene studies have been
witnessed in the recent years, which have increased our understanding of the genetic

background of NAFLD (4). In this sense, the change on FLI after 6-months of nutritional intervention was chosen in this trial as a non-invasive method of NAFLD and for the construction of the genetic risk score Therefore, 11 SNPs involved in process such as regulating homocysteine metabolism (MTHFR), mitochondrial metabolism (MTCH2), pressor hormone processes (AGTR2), as well as different variants in FTO and in LYPLAL1 genes, were included in the AHA genetic risk score. On the other hand, genetic variants associated with insulin resistance (UCP3, UCP2 and TCF7L2), fatty acid metabolism (ACSL5 and PPARG), fasting plasma glucose (MTNR1B), endocrine/enzymatic regulation of lipid metabolism (GNAS), thermogenesis (UCP1), mitochondrial permeability (PPM1K) and a variant in CNR2FUCA1 gene were associated with FLiO GRS (30). Although the SNP MC4R did not show a significant statistical trend when analyzed individually, it contributed to %FLI decrease variance in the FLiO diet. Thus, it was also included. As a consequence, different response to diet was found depending on genetic background. Importantly, the model was also able to predict liver fat content at baseline by diet and according to the designed GRS when participants with a liver fat content (by MRI) ≥ 5% at baseline were selected. In this context, studies have suggested that MRI proton density fat fraction may be the method of choice in the clinical trial setting, as it has been shown to be more sensitive than liver biopsy for assessing changes in liver fat (7). However, in this trial, due to the greater number of associations between polymorphisms and changes in FLI, as well as its advantages in clinical practice, this non-invasive obesity index was selected to assess improvement in NAFLD. Unfortunately, the knowledge of the genetic X environment interaction in the context of NAFLD and NASH remains largely unexplored (3,31). Nonetheless, these results are in line with previous studies that support the novel and potentially role of genetic in regulating the response to diet (17,32). In the 2-year Preventing Overweight Using Novel Dietary Strategies (POUNDS Lost) trial, authors showed that genetic variation in lean body mass may be differentially associated with appetite changes, and may be therefore, related to changes in body weight and waist circumference, according to dietary fat intake (33). In the same cohort, the minor C allele of the PCSK7 rs236918 genetic variant was found significantly correlated on changes in fasting insulin levels and HOMA-IR in response to high-carbohydrate diet consumption (34). Indeed, a study carried out in healthy obese adults showed that adherence to both the Dietary Approach to Stop Hypertension (DASH) score and the Mediterranean Dietary Score (MDS) modified the association between FADS2 rs174583 polymorphism and cardio-metabolic risk factors (35).

Moreover, numerous studies have shown the association between FLI and diabetes, metabolic syndrome components, as well as being recognized as a predictor of cardiovascular disease (36,37). In this sense, the association between insulin resistance (IR) and NAFLD have been widely studied (38). According to this, the triglycerides and glucose (TyG) index has shown promising results in predicting high risk for incident of NAFLD, as well as for detecting insulin resistance and diabetes related manifestations (39,40). Other authors have focused on the role of adipokines in the development of NAFLD (41). An example of this, is the retinol-binding protein 4 (RBP4), which has been associated with the development and regression of NAFLD, as well as it has been described as a marker and causative factor of chronic vascular injury (13,42). High levels of this adipokine have been shown in obesity and causes insulin resistance (13). In this trial, the change on TyG index and RBP4 levels was also associated with different response to diet depending on the previous design genetic risk score. In this

sense, an association between carriers of the PNPLA3 148 M allele with both fatty liver and obesity or obesity alone have lower fasting circulating retinol concentrations was also reported (43). Therefore, these results highlighted that the combination of specific biomarkers, environmental and genetic data might help to develop a more precise and personalized diagnostic method of NAFLD (3,6).

To the best of our knowledge, this is the first study showing the cumulative effects of GRS built from obese related genetic variant selected according to FLI decrease and related to weight-loss and adiposity regulation in response to different hypocaloric diets in NAFLD patients. According to this, previous articles have reported the influence of SNPs involve in lipid metabolism such as PNPLA3 in the carbohydrate and sugar intake (5). Besides, a recent research concerning this gene has also reported an association between high dietary intakes of n-3 PUFAs, total isoflavones, methionine, and total choline, suggesting an attenuation of liver fibrosis in individual carrying the PNPLA3 rs734809 G-allele (44). Indeed, mutated patients for the TM6SF2 and MBOAT7 genes were also affected by diet showing reduction of circulating atherogenic lipoproteins and synthesis and hepatic deposition of saturated and mono-unsaturated triglycerides, respectively (45,46). Nonetheless, because of the small percentage of inherited variability explained by the common studied variants in NAFLD, the study of less prevalent genetic variants, as well as the impact of nutritional environment and inherited factors is crucial (47). In this sense, a recent study has reported an association between an obese-related genetic variants (SH2B1) and NAFLD, showing that subjects carrying the risk allele of this gene, may benefit more in terms of hepatic health and liver status when prescribing an energy restricted treatment (2). Hence, the consideration of adiposity and genotype have been reported to improved prediction of individuals at higher risk of developing advanced stages of NAFLD (48). Based on our results, we have shown that the use of these integrative models may help to personalize NAFLD management by using non-invasive methods, such as FLI after following a nutritional treatment. As a result, the use of these prototypes allowed to prescribe the most suitable diet to 72% of our volunteers. Subjects who could not be significantly assigned to one of the two diets for a better improvement of hepatic health, might choose the type of diet according to individual cultural and food preferences, since both diets would result in similar hepatic improvement (measured by FLI). Therefore, this approach may lead to personalized dietary advice using precision nutrition and even though we have focused on FLI decrease as outcome of interest, it could be applied to any continuous variable from a randomized control intervention trial. In fact, this statistical approach, was previously used to personalized diet on weight loss by introducing more fiber and whole grain depending on pre-treatment fasting plasma glucose and fasting insulin levels (49).

The main strength of this investigation is the new conceptual modeling for personalized prescription of two energy-restricted diets (AHA and FLiO) with different macronutrient distribution using genetic information. Also, robust statistical approaches were applied to select the best multiple linear regression models explaining FLI, RBP4 and TyG index decrease difference in each type of diet. On the other hand, some limitations of this research include the lack a non-NAFLD liver disease control group with genetic, dietary, and liver histology data. However, the selection of the participants was performed by ultrasound and a complete evaluation of the liver status using imaging techniques, blood biomarkers and hepatic indexes. In addition, the control AHA diet is a well- recognised healthy dietary pattern that was used as a reference to corroborate the hypothesis, which evidenced the positive results obtained with the FLiO

diet, suggesting this approach as an alternative for lifestyle management of NAFLD. Second, the sample size and enrollment of subjects are not very large, but enough to find plausible results despite that type I and II errors cannot be discarded. Thus, these findings must be taken with caution before applying them to other ethnic groups, as well as models may be further validated in different populations. Thirdly, dietary intake was evaluated using self-reported information of the participants, which may produce some bias on the evaluation of the results given the subjective nature and memory lapses, but they were based on validated tools. Furthermore, in our results, the questionnaires used to assess dietary adherence showed a different trend. However, both dietary groups significantly improved body composition and biochemical parameters, as well as in liver health after 6 months of nutritional intervention. Lastly, the construction of the GRS using specific obesity-related SNPs is also an important issue that may have influenced the prediction of the model. 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 in order to increase genetic knowledge around NAFLD. In this sense, further studies might also confirm whether this GRS-diet interaction seen in patients with NAFLD are reproducible in the context of other chronic liver diseases. Overall, the design of this experiment should be considered as a proof of concept in order to evaluate if combining information from genetic variants may be useful to personalize NAFLD treatments. It is important that future studies utilize outcome research, not only considering the effects of a nutritional intervention on surrogate parameters in different genetic groups, but also looking at effects on disease development, survival and quality of life.

Conclusions

This work presents a novel tool for the personalization of weight loss treatments for NAFLD based on genetic screening and insulin resistance markers as factors for the selection of the most suitable energy-restricted diet. These insights and models may help to optimize individualized nutritional strategies for modeling the prevention and management of non-alcoholic fatty liver disease through precision nutrition approaches.

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between 14-15, both diets demonstrated beneficial effects with no significant differences among them (p=0.341).
Additionally, RBP4 levels and TyG index were also predicted by using the same genetic risk score and linear mixed regression model (Table 4). In both RBP4 and TyG index models, the interaction Diet X genetic score was statistically significant ($p<0.005$ and 0.009, respectively) (Figure 3A and 3B). Interestingly, the same interaction remained significant when selecting participants with a liver fat content (by MRI) ≥ 5% at baseline (p=0.001) (Table S1 and Figure S1)
Discussion
There is no doubt that NAFLD is the result of a combination of genetic background and environmental factors, and where complex nutrigenetic interactions between them needs to be accounted (5,25). Unhealthy lifestyles play a role in the development and progression of NAFLD, which makes lifestyle modification mandatory in these patients (14). Moreover, increasingly evidence indicates that lifestyle can also differ on the impact of genetic variations on this liver disease (3). However, the mechanism of these interactions, as well as the different approaches for the management of liver steatosis need further investigations (3). To this end, we focus on the implementations of a nutrigenetic model based on 22 different SNPs that were previously associated with the

percentage of FLI decrease and able to differentiate the best dietary approach for a non-invasive management of overweight and obese subjects with NAFLD.

Liver biopsy, although an invasive procedure, remains the standard for the diagnosis of NAFLD (1). Nonetheless, patients undergoing liver biopsy are highly selected and several non-invasive procedures and algorithms have been proposed (7). One of the best
validated and accepted biomarkers is the Fatty Liver Index, which has often been used in epidemiological studies, but also in clinical interventions being easy to implement in individual NAFLD patients despite some drawbacks for accuracy (26). One of the best accepted and validated biomarkers is the Fatty Liver Index, which has often been used in epidemiological studies, but also in clinical interventions and is easy to implement, despite some drawbacks for accuracy, in individual NAFLD patients. Moreover, a recent metanalyses evaluating 27,221 subjects without secondary causes of fatty liver disease, showed that FLI was an adequate instrument in stratifying the risk of NAFLD, even if it showed only weak evidence of a discriminatory performance in excluding or diagnosing this disorder (27). In terms of treatment, lifestyle assessment encompasses diet and regime changes is the only effective therapeutic modality (14). However, long-term adherence to a diet is very complicated, being one of the most important challenges in the management of this liver disease to offer dietary alternatives to encourage adherence and compliance to diets. Thus, several pharmacological approaches using diverse drugs such as metformin, pioglitazone, glucagon-like peptide-1 or vitamin E have been prescribed or therapeutically considered in the management of NAFLD and NASH (19,28). It has also recently been proposed that the use of new methodologies for NAFLD-NASH approaches in preclinical or early-stage research could accelerate the drug development process, where nutrigenetic interactions may have a role (28). Thus, in this study, after 6-months of nutritional treatment, most of the measured variables were improved in both AHA and FLiO diet. However, no differences between the interventions groups, diets were observed concerning most of the evaluated variables (29). Nonetheless, NAFLD is considered a complex disease trait where interindividual variability and environmental features interact determining the specific phenotype and progression of the disease (3,5).
Multiple genome-wide association and large candidate gene studies have been witnessed in the recent years, which have increased our understanding of the genetic background of NAFLD (4). In this sense, the change on FLI after 6-months of nutritional intervention was chosen in this trial as a non-invasive method of NAFLD and for the construction of the genetic risk score Therefore, 11 SNPs involved in process such as regulating homocysteine metabolism (MTHFR), mitochondrial metabolism (MTCH2), pressor hormone processes (AGTR2), as well as different variants in FTO and in LYPLAL1 genes, were included in the AHA genetic risk score. On the other hand, genetic variants associated with insulin resistance (UCP3, UCP2 and TCF7L2), fatty acid metabolism (ACSL5 and PPARG), fasting plasma glucose (MTNR1B), endocrine/enzymatic regulation of lipid metabolism (GNAS), thermogenesis (UCP1), mitochondrial permeability (PPM1K) and a variant in CNR2FUCA1 gene were

associated with FLiO GRS (30). Although the SNP MC4R did not show a significant statistical trend when analyzed individually, it contributed to %FLI decrease variance in the FLiO diet. Thus, it was also included. As a consequence, different response to diet was found depending on genetic background. Importantly, the model was also able to predict liver fat content at baseline by diet and according to the designed GRS when participants with a liver fat content (by MRI) ≥ 5% at baseline were selected. In this context, studies have suggested that MRI proton density fat fraction may be the method of choice in the clinical trial setting, as it has been shown to be more sensitive than liver biopsy for assessing changes in liver fat (7). However, in this trial, due to the greater number of associations between polymorphisms and changes in FLI, as well as its advantages in clinical practice, this non-invasive obesity index was selected to assess improvement in NAFLD. Unfortunately, the knowledge of the genetic X environment interaction in the context of NAFLD and NASH remains largely unexplored (3,31).
Nonetheless, these results are in line with previous studies that support the novel and potentially role of genetic in regulating the response to diet (17,32). In the 2-year Preventing Overweight Using Novel Dietary Strategies (POUNDS Lost) trial, authors showed that genetic variation in lean body mass may be differentially associated with appetite changes, and may be therefore, related to changes in body weight and waist circumference, according to dietary fat intake (33). In the same cohort, the minor C allele of the PCSK7 rs236918 genetic variant was found significantly correlated on changes in fasting insulin levels and HOMA-IR in response to high-carbohydrate diet consumption (34). Indeed, a study carried out in healthy obese adults showed that adherence to both the Dietary Approach to Stop Hypertension (DASH) score and the Mediterranean Dietary Score (MDS) modified the association between FADS2 rs174583 polymorphism and cardio-metabolic risk factors (35).

Moreover, numerous studies have shown the association between FLI and diabetes, metabolic syndrome components, as well as being recognized as a predictor of cardiovascular disease (36,37). In this sense, the association between insulin resistance (IR) and NAFLD have been widely studied (38). According to this, the triglycerides and glucose (TyG) index has shown promising results in predicting high risk for incident of NAFLD, as well as for detecting insulin resistance and diabetes related manifestations (39,40). Other authors have focused on the role of adipokines in the development of NAFLD (41). An example of this, is the retinol-binding protein 4 (RBP4), which has been associated with the development and regression of NAFLD, as well as it has been described as a marker and causative factor of chronic vascular injury (13,42). High levels of this adipokine have been shown in obesity and causes insulin resistance (13). In this trial, the change on TyG index and RBP4 levels was also associated with different response to diet depending on the previous design genetic risk score. In this
sense, an association between carriers of the PNPLA3 148 M allele with both fatty liver and obesity or obesity alone have lower fasting circulating retinol concentrations was also reported (43). Therefore, these results highlighted that the combination of specific

biomarkers, environmental and genetic data might help to develop a more precise and personalized diagnostic method of NAFLD (3,6).

To the best of our knowledge, this is the first study showing the cumulative effects of GRS built from obese related genetic variant selected according to FLI decrease and related to weight-loss and adiposity regulation in response to different hypocaloric diets in NAFLD patients. According to this, previous articles have reported the influence of SNPs involve in lipid metabolism such as PNPLA3 in the carbohydrate and sugar intake (5). Besides, a recent research concerning this gene has also reported an association between high dietary intakes of n-3 PUFAs, total isoflavones, methionine, and total choline, suggesting an attenuation of liver fibrosis in individual carrying the PNPLA3 rs734809 G-allele (44). Indeed, mutated patients for the TM6SF2 and MBOAT7 genes were also affected by diet showing reduction of circulating atherogenic lipoproteins and synthesis and hepatic deposition of saturated and mono-unsaturated triglycerides, respectively (45,46). Nonetheless, because of the small percentage of inherited variability explained by the common studied variants in NAFLD, the study of less prevalent genetic variants, as well as the impact of nutritional environment and inherited factors is crucial (47). In this sense, a recent study has reported an association between an obese-related genetic variants (SH2B1) and NAFLD, showing that subjects carrying the risk allele of this gene, may benefit more in terms of hepatic health and liver status when prescribing an energy restricted treatment (2). Hence, the consideration of adiposity and genotype have been reported to improved prediction of individuals at higher risk of developing advanced stages of NAFLD (48).

Based on our results, we have shown that the use of these integrative models may help to personalize NAFLD management by using non-invasive methods, such as FLI after following a nutritional treatment. As a result, the use of these prototypes allowed to prescribe the most suitable diet to 72% of our volunteers. Subjects who could not be significantly assigned to one of the two diets for a better improvement of hepatic health, might choose the type of diet according to individual cultural and food preferences, since both diets would result in similar hepatic improvement (measured by FLI). Therefore, this approach may lead to personalized dietary advice using precision nutrition and even though we have focused on FLI decrease as outcome of interest, it could be applied to any continuous variable from a randomized control intervention trial. In fact, this statistical approach, was previously used to personalized diet on weight loss by introducing more fiber and whole grain depending on pre-treatment fasting plasma glucose and fasting insulin levels (49).

The main strength of this investigation is the new conceptual modeling for personalized prescription of two energy-restricted diets (AHA and FLiO) with different macronutrient distribution using genetic information. Also, robust statistical approaches were applied to select the best multiple linear regression models explaining FLI, RBP4 and TyG index decrease difference in each type of diet. On the other hand, some limitations of this research include the lack a non-NAFLD liver disease control group with genetic, dietary, and liver histology data. However, the selection of the participants was performed by ultrasound and a complete evaluation of the liver status using imaging techniques, blood biomarkers and hepatic indexes. In addition, the control AHA diet is a well- recognised healthy dietary pattern that was used as a reference to corroborate the hypothesis, which evidenced the positive results obtained with the FLiO diet, suggesting this approach as an alternative for lifestyle management of NAFLD.

Second, the sample size and enrollment of subjects are not very large, but enough to find plausible results despite that type I and II errors cannot be discarded. Thus, these findings must be taken with caution before applying them to other ethnic groups, as well as models may be further validated in different populations. Thirdly, dietary intake was evaluated using self-reported information of the participants, which may produce some bias on the evaluation of the results given the subjective nature and memory lapses, but they were based on validated tools. Furthermore, in our results, the questionnaires used to assess dietary adherence showed a different trend. However, both dietary groups significantly improved body composition and biochemical parameters, as well as in liver health after 6 months of nutritional intervention. Lastly, the construction of the GRS using specific obesity-related SNPs is also an important issue that may have influenced the prediction of the model. 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 in order to increase genetic knowledge around NAFLD. In this sense, further studies might also confirm whether this GRS-diet interaction seen in patients with NAFLD are reproducible in the context of other chronic liver diseases. Overall, the design of this experiment should be considered as a proof of concept in order to evaluate if combining information from genetic variants may be useful to personalize NAFLD treatments. It is important that future studies utilize outcome research, not only considering the effects of a nutritional intervention on surrogate parameters in different genetic groups, but also looking at effects on disease development, survival and quality of life.

Conclusions

This work presents a novel tool for the personalization of weight loss treatments for NAFLD based on genetic screening and insulin resistance markers as factors for the

selection of the most suitable energy-restricted diet. These insights and models may help to optimize individualized nutritional strategies for modeling the prevention and management of non-alcoholic fatty liver disease through precision nutrition approaches.

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TABLES

Table I. Baseline and 6-months characteristics of the study participants according to assigned dietary intervention.

Table II. Genotype codifications of SNPs selected to construct the genetic risk score concerning FLI changes by dietary groups.

Table III. Linear mixed model of changes in Fatty Liver Index after 6-months nutritional intervention.

Table IV. Linear mixed model of changes in RBP4 levels and TyG index after 6-months nutritional intervention.

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Figure 1. Difference in the change of FLI according to a Genetic Risk Score between AHA and FLiO diet. AHA, American Heart Association; FLI, Fatty Liver Index; FLiO, Fatty Liver in Obesiy.

Figure 2. Comparisons of the change of FLI after 6-months following AHA or FLiO diet and according to a Genetic Risk Score. Data are expressed as means ± standard deviations. AHA, American Heart association; Fatty Liver Index; FLiO, Fatty Liver in Obesity; GRS, Genetic Risk Score.

Figures 3A and 3B. Estimated values of RBP-4 and TyG index decrease for each type of diet according to the genetic risk score values. AHA, American Heart association; Fatty Liver Index; FLiO, Fatty Liver in Obesity; TyG index: Triglycerides and glucose index; RBP4, Retinol-binding protein 4