ORIGINAL PAPERS

Comparative study of overweight and obese patients with nonalcoholic fatty liver disease

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ABSTRACT

Background and aims: non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in the western world. Although NAFLD prevalence is higher in patients with a BMI > 25 kg /m², it is unclear if there are differences between overweight and obese patients. The associated biochemical, dietary and genetic parameters were compared between overweight and obese patients with NAFLD.

Methods: patients with biopsy-proven NAFLD (n = 203) were enrolled in a cross-sectional study. The MEDAS questionnaire was used to assess adherence to the Mediterranean diet. Biochemical, anthropometrical parameters and the I148M variant (rs738409) of the PNPLA3 gene and rs180069 of the TNF-α gene were evaluated.

Results: overweight patients had higher serum adiponectin levels (22.5 ± 21.9 vs 11.2 ± 18.1 ng/ml; p < 0.05) and lower resistin (3.3 ± 1.7 vs 8.1 ± 8 ng/ml; p < 0.001) and leptin concentrations (22.9 ± 21.9 vs 55.8 ± 45 ng/ml; p < 0.001) than obese patients. Non-alcoholic steatohepatitis (NASH) was more frequent in the obese group (59.3% vs 41.3%; p = 0.02). The multivariate analysis showed adherence to the Mediterranean diet to be an independent protective factor for NASH and liver fibrosis in overweight patients (OR 0.7, 95% CI 0.5-0.8).

Conclusions: NASH was more prevalent in obese patients than in overweight subjects. HOMA-IR and adherence to the Mediterranean diet provided protection against fibrosis in overweight patients. Adherence to the Mediterranean diet was the only independent factor associated with NASH in these patients.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in the western world (1,2). Even though it is often related with obesity, NAFLD can also affect non-obese individuals (3). It encompasses a broad spectrum ranging from simple steatosis to NASH, advanced fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (4), and is accepted as the hepatic manifestation of metabolic syndrome (5,6). NAFLD increases the risk of subclinical atherosclerosis and coronary artery disease. The correct management of these patients could modify the natural history of both liver and cardiovascular disease (7). Epidemiological data suggest that 10-30% of all non-obese subjects have NAFLD (8,9). Zhang et al. reported a 5-fold lower prevalence of NAFLD in non-obese versus obese subjects. The triglyceride glucose-body mass index has been shown to be an effective marker for detecting NAFLD in non-obese individuals (3). NAFLD is characterized by fat accumulation in the liver (steatosis), in the absence of significant alcohol consumption (10,11). On the other hand, non-obese subjects with NAFLD may constitute a subgroup of metabolically obese individuals but with a normal body weight index (BMI) that present metabolic abnormalities similar to those that characterize the obesity-related metabolic profile (12). Non-obese individuals with NAFLD undoubtedly have a genetic predisposition for the disease and different genetic polymorphisms

involved in the regulation of lipid metabolism have been shown to play a role in the development and progression of NAFLD in these individuals (13). Although NAFLD is more frequent in patients with BMI > 25 kg/m², there is no available evidence on the histological, genetic predisposition and clinical differences between overweight or obese NAFLD patients. The present study comparatively analyzed the following parameters in overweight patients with NAFLD *versus* obese subjects: clinical features, laboratory parameters, liver histological characteristics, dietary habits (Mediterranean diet adherence), circulating adipocytokine profiles and two single nucleotide polymorphisms (SNPs) including the genetic variant I148M of the patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene and variant G308G of the TNF-α gene.

SUBJECTS AND METHODS

Subjects

The study group consisted of 203 Caucasian subjects with biopsy-proven NAFLD, due to persistently elevated liver enzyme levels. The following exclusion criteria were applied: significant alcohol consumption (> 30 g/day in men and > 20 g/day in women), hepatitis B, hepatitis C, cytomegalovirus or Epstein-Barr virus infection, positive non-organ-specific autoantibodies, type 1 diabetes mellitus, antihypertensive drug or statin therapy and primary metabolic diseases (iron and copper storage diseases and alpha 1-antitrypsin deficiency). All participants signed the corresponding informed consent form. The study was conducted according to the guidelines of the Declaration of Helsinki, the local Ethics Committee (HCUV) approved all procedures involving patients and patient data were coded in order to guarantee anonymity.

The diagnosis of NAFLD was established via a liver biopsy. Basal glucose, C-reactive protein (CRP), insulin, homeostasis model assessment-insulin resistance (HOMA-IR), total cholesterol, low-density lipoprotein cholesterol (LDL-chol), high-density lipoprotein cholesterol (HDL-chol), triglycerides, leptin, adiponectin and resistin were determined. With regard to the genetic polymorphisms, the variants I148M (*rs738409)* of the PNPLA3 gene and the *rs180069* variant of the TNF gene were evaluated.

Liver histology

The diagnosis of NAFLD was confirmed via a percutaneous liver biopsy using a Menghini-type biopsy needle in all patients. Liver samples were sectioned and stained with hematoxylin-eosin and the Masson trichrome stain. NAFLD was histologically defined by the presence of a minimum of 5% steatosis in the liver biopsy. The degree of steatosis in turn was scored as 1 (5-33%), 2 (34-66%) or 3 (> 66%). Fibrosis was scored as 0 (no fibrosis), 1 (peri-sinusoidal or periportal fibrosis), 2 (peri-sinusoidal and portal/periportal fibrosis), 3 (bridging fibrosis) or 4 (cirrhosis). Lobular inflammation was scored as 0 (no inflammation), 1 (< 2 foci per 200x field), 2 (2-4 foci per 200x field) or 3 (> 4 foci per 200x field). Ballooning was scored as 0 (no balloon cells), 1 (few ballooned cells) and 2 (many cells/prominent ballooned cells). A case

that presented with at least grade 1 of each of the three abovementioned features (steatosis, ballooning and lobular inflammation) was classified as non-alcoholic steatohepatitis (NASH) (14). In order to minimize inter-observer variability, the liver biopsy specimens were interpreted by the same pathologist using the SAF score (steatosis, activity and fibrosis). This assesses the grade of steatosis (S) from S0 to S3, the grade of activity (A) from A0 to A4 by addition of the grades of ballooning and lobular inflammation from 0 to 2 and the fibrosis stage (F) from F0 to F4 (11).

Genotyping and biochemical parameters (supplementary material)

Biochemical parameters

Blood samples were collected in Na-EDTA tubes after 12 hours of fasting. All samples were frozen at -80 °C until laboratory testing. Insulin was measured by radioimmunoassay (RIA Diagnostic Corporation, Los Angeles, CA, USA) with a sensitivity of 0.5 mIU/l (normal range 0.5-30 mIU/l) (15) and the homeostasis model assessment. Insulin resistance (HOMA-IR) was calculated using the following formula: fasting insulin x fasting glucose concentrations / 22.5. The LDL-cholesterol levels were calculated using the Friedewald formula (16).

Metabolic syndrome

Metabolic syndrome was defined according to the criteria proposed by the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III (5). The panel specifies that a diagnosis of metabolic syndrome should be based on the presence of any three of the following five components: waist circumference ≥ 90 cm in males or ≥ 80 cm in females; blood pressure ≥ 130/85 mmHg or the current use of antihypertensive drugs; triglycerides ≥ 150 mg/dl; HDL-cholesterol < 40 mg/dl in males or < 50 mg/dl in females; and fasting blood glucose ≥ 100 mg/dl or the current use of glucose-lowering drugs.

Adipocytokines

Resistin was measured by an enzyme-linked immunosorbent assay (ELISA) (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml and a normal range of 4-12 ng/ml (17). Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., TX, USA) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml (18). Adiponectin was also measured by ELISA (R&D Systems, Inc., Minneapolis, USA) with a sensitivity of 0.246 ng/ml and a normal range of 8.6521.43 ng/ml (19).

Anthropometric measurements

Body weight and height, respectively, were measured with an electrical scale (Omrom, Los Angeles, CA, USA) and a telescopic height-measuring instrument (Omrom, Los Angeles, CA, USA). Body mass index (BMI) was calculated as body weight in kg divided by height in m². Obesity was defined as a BMI ≥ 30 kg/m². Dyslipidemia was defined

based on the NCEP guidelines (20) as follows: total cholesterol ≥ 200 mg/dl; triglycerides ≥ 150 mg/dl; LDL-cholesterol > 100 mg/dl and HDL-cholesterol < 40 mg/dl in males or < 50 mg/dl in females. Waist circumference was measured using a Seca® instrument (Seca, Birmingham, UK).

Statistical analysis

All statistical analyses were performed using the SPSS statistical package, version 20.0. Normality testing was performed and data were expressed as the mean ± standard deviation or as frequencies for categorical variables. Categorical variables were analyzed using the Chi-square test, with Yates correction as required as well as the Fisher's exact test. Continuous variables with a normal distribution were analyzed using a two-tailed Student's t-test. Variables with a non-normal distribution were analyzed using the nonparametric Mann-Whitney U-test. The statistical analysis was performed for the combined GC and GG genotypes as one group and the CC genotype as a second group for the *rs738409* variant of the PNPLA3 gene. With regard to the *rs180069* variant of the TNFα gene, the GA and AA genotype were combined as one group and the GG genotype as a second group. Data was analyzed using a dominant model.

Logistic regression analysis (backward: Wald, cut-off point for entry: 0.05 and cut-off point for removal: 0.10) was performed to assess the risk factors for NASH and fibrosis. The adjusted variables were derived from the significant results in the univariate analysis. Statistical significance was considered as p < 0.05 (two-tailed testing).

RESULTS

The study sample consisted of 203 patients with biopsy-proven NAFLD. The mean age was 47.4 ± 37.2 years and 115 patients were male (56.7%). The patients were divided into two groups according to BMI; there were 63 overweight (BMI > 25 and < 30 kg/m²) (31%) and 140 obese patients (BMI ≥ 30 kg/m²) (69%).

A comparison of the clinical characteristics of the groups (Table 1) showed that there were more males in the overweight group (46% *vs* 47.9%; p < 0.01) and a better compliance with the Mediterranean diet than in obese subjects (9 *vs* 7 items; p < 0.05). There were no statistically significant differences between the groups in terms of the proportion of patients carrying the G allele of the PNPLA3 gene and the A allele of the TNF-α gene. On the other hand, overweight patients had higher adiponectin levels (22.5 ± 21.9 *vs* 11.2 ± 18.1 ng/ml; p < 0.05) and lower resistin (3.3 ± 1.7 *vs* 8.1 ± 8 ng/ml; p < 0.001) and leptin concentrations (22.9 ± 21.9 *vs* 55.8 ± 45 ng/ml; p < 0.001) than obese patients (Table 2).

With regard to the severity of liver damage, the proportion of patients with NASH was higher in the obese group (59.3% *vs* 41.3%; p = 0.02) and there were no significant differences in any other histopathological parameters. Likewise, there were no significant differences with regard to the presence of fibrosis defined as F > 1 (41.3% *vs* 50.7%; ns) or advanced fibrosis defined as F ≥ 2 (7.9% *vs* 4.3%; p = ns) (Table 3).

Table 1. *Clinical characteristics of overweight* versus *obese patients with NAFLD*

Variable	Overweight (n = 63)	Obese (n = 140)	p-value
Age, years	44.9 ± 12.8	48.6 ± 44.3	ns
Male sex, n (%)	48 (76.2)	67 (47.9)	< 0.001
Body mass index (kg/m²)	27.4 ± 2.5	41.2 ± 9.1	ns
Waist circumference (cm)	94.1 ± 8.3	112.2 ± 13.3	ns
Systolic blood pressure (mmHg)	124.3 ± 23.4	138.6 ± 90.8	ns
Diastolic blood pressure (mmHg)	75.7 ± 17	82 ± 20.1	ns
Total cholesterol, mg/dl	201.9 ± 51	181 ± 47.2	ns
Triglycerides, mg/dl	153.9 ± 85.5	153.2 ± 76.6	ns
LDL cholesterol, mg/dl	123.4 ± 37.2	110.7 ± 40.5	ns
HDL cholesterol, mg/dl	49.6 ± 14.8	42.2 ± 17.4	ns
Fasting glucose, mg/dl	103.4 ± 24.3	113.1 ± 37.6	ns
HOMA-IR	3.6 ± 2.3	4.7 ± 4.1	ns
Total bilirubin, mg/dl	0.8 ± 0.7	0.7 ± 0.7	ns
Creatinine, mg/dl	0.9 ± 0.2	0.8 ± 0.2	ns
AST, IU/l	49.1 ± 32.2	38.9 ± 31.9	ns
ALT, IU/l	86.5 ± 55.2	57.1 ± 37.4	ns
γ-GT, IU/l	91.7 ± 64.2	88 ± 77	ns
Platelet count, ×10³/µl	270.4 ± 63.7	264.3 ± 73	ns
Metabolic syndrome, n (%)	25 (39.7)	78 (55.7)	ns
MEDAS (median)	9 (8-10)	7 (6-8)	0.04
PNPLA3 rs738409			
CC n (%)	19 (42.2)	47 (48)	ns
CG or GG n (%)	26 (57.8)	51 (52)	
TNF-α			
GG n (%)	39 (81.3)	90 (80.4)	ns
GA or AA n (%)	9 (18.8)	22 (19.6)	

Data are presented as the mean ± SD or median (interquartile range) unless otherwise indicated. LDL: low-density lipoprotein; HDL: high-density lipoprotein; HOMA-IR: homeostasis model assessment of insulin resistance; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyltransferase; PNPLA3: patatin-like phospholipase domain containing protein 3; TNF-α: tumor necrosis factor-alpha.

Table 2. *Adipocytokines in overweight* versus *obese patients with NAFLD*

Variable	Overweight (n = 63)	Obese (n = 140)	p-value
Adiponectin (ng/ml)	22.5± 6.7	11.2 ± 8	< 0.001
Resistin (ng/ml)	3.3 ± 1.2	8.1 ± 3.1	< 0.001
Leptin (ng/ml)	22.9 ± 8	55.8 ± 15	< 0.001

p < 0.05 in each group with basal values.

Overweight patients (BMI > 25 and < 30 kg/m²)

The univariate analysis identified gender (male), advanced age, elevated systolic and diastolic blood pressure, fasting

Table 3. *Histological parameters in overweight* versus *obese patients with NAFLD*

Variable	Overweight (n = 63)	Obese (n = 140)	p-value
Simple steatosis, n (%)	16 (25.4)	25 (17.9)	ns
Ballooning, n (%)	35 (55.6)	92 (65.7)	ns
Lobular inflammation, n (%)	42 (66.7)	110 (71.4)	ns
Steatohepatitis, n (%)	26 (41.3)	83 (59.3)	0.01
Fibrosis (F > 1), n (%)	26 (41.3)	71 (50.7)	ns
Advanced fibrosis (F ≥ 2), n (%)	5 (7.9)	6 (4.3)	ns

Chi-square test. $p < 0.05$ (%) frequencies in each genotype group.

blood glucose, HOMA-IR and the presence of type 2 diabetes mellitus or arterial hypertension as risk factors for the development of NASH in overweight patients with NAFLD. In contrast, adherence to the Mediterranean diet (MEDAS questionnaire > 7 items) was shown to be a protective factor (Table 4). This adherence was identified as an independent protective factor in the multivariate analysis (OR 0.7; 95%CI 0.5-0.8).

The univariate analysis of factors associated with fibrosis showed that the presence of fibrosis was significantly more common in older, male, overweight patients with higher transaminase levels and HOMA-IR scores (Table 5). In contrast, adherence to the Mediterranean diet and the A allele of variant *rs180069* of the TNF-α gene protected against fibrosis in these patients. According to the multivariate analysis, only a higher HOMA-IR index was independently associated with fibrosis in non-obese patients (OR 1.8, 95% CI 1.12.8). Adherence to the Mediterranean diet was the only factor that protected against fibrosis in this group of overweight patients (OR 0.7, 95% CI 0.5-0.8).

DISCUSSION

A number of epidemiological studies have reported a close relationship between obesity and NAFLD (3). However, few studies have addressed factors associated with NAFLD in non-obese overweight patients (BMI < 30 kg/m²) (21). The proportion of patients with NASH was significantly higher in the obese group than in the overweight group, with a trend towards less inflammation and ballooning in overweight subjects. However, these differences did not reach

Table 4. *Factors associated with non-alcoholic steatohepatitis in overweight patients with NAFLD*

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p-value	OR (95% CI)	p value
Age (years)	1.067 (1.019-1.117)	0.005		
Male sex	0.250 (0.073-0.855)	0.027		
Body mass index (kg/m²)	1.184 (0.942-1.488)	ns		
Waist circumference, cm	1.018 (0.955-1.084)	ns		
Systolic blood pressure, mmHg	1.073 (1.032-1.116)	0.001		
Diastolic blood pressure, mmHg	1.073 (1.032-1.115)	0.001		
Total cholesterol, mg/dl	0.997 (0.987-1.007)	ns		
Triglycerides, mg/dl	1.005 (0.999-1.012)	ns		
ALT, IU/l	1.008 (0.998-1.019)	ns		
AST, IU/l	1.016 (0.998-1.035)	ns		
GGT, IU/l	0.994 (0.986-1.003)	ns		
Fasting plasma glucose, mg/dl	1.038 (1.004-1.074)	0.03		
HOMA-IR	2.036 (1.305-3.176)	0.002		
LDL-chol, mg/dl	1.001 (0.987-1.015)	ns		
HDL-chol, mg/dl	0.975 (0.939-1.013)	ns		
Platelet count, ×10³/µl	0.992 (0.984-1.001)	ns		
PNPLA3 G carrier	1.810 (0.511-6.403)	ns		
TNFA-α A carrier	0.483 (0.105-2.220)	ns		
History of diabetes	8.571 (0.937-78.405)	0.057		
History of hypertension	8.156 (2.601-15.577)	0.001		
Metabolic syndrome	0.201 (0.068-0.598)	0.004		
MEDAS	0.667 (0.501-0.890)	0.006	0.7 (0.5-0.8)	0.002

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; HOMA-IR: homeostasis model assessment of insulin resistance; LDL-chol: low-density lipoprotein cholesterol; HD-chol: high-density lipoprotein cholesterol; PNPLA3: patatin-like phospholipase domain containing protein 3; TNF-α: tumor necrosis factor-alpha.

Table 5. *Factors associated with liver fibrosis in overweight patients with NAFLD*

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p-value	OR (95% CI)	p value
Age (years)	1.049 (1.005-1.094)	0.028		
Male sex	4 (1.169-13.683)	0.027		
Body mass index (kg/m²)	0.951 (0.767-1.179)	ns		
Waist circumference, cm	0.97 (0.91-1.035)	ns		
Systolic blood pressure (mmHg)	1.029 (0.999-1.060)	ns		
Diastolic blood pressure (mmHg)	1.018 (0.988-1.049)	ns		
Creatinine, mg/dl	0.157 (0.006-3.874)	ns		
Total bilirubin, mg/dl	1.229 (0.657-2.297)	ns		
ALT, IU/l	1.016 (1.003-1.030)	0.019		
AST, IU/l	1.031 (1.004-1.059)	0.022		
GGT, IU/l	1.002 (0.994-1.010)	ns		
Fasting plasma glucose, mg/dl	1.022 (0.994-1.049)	ns		
HOMA-IR	1.423 (1.057-1.916)	0.020	1.8 (1.1-2.8)	0.007
Total cholesterol, mg/dl	0.992 (0.981-1.002)	ns		
LDL-chol, mg/dl	0.996 (0.982-1.010)	ns		
HDL-chol, mg/dl	0.978 (0.942-1.015)	ns		
Triglycerides, mg/dl	1.001 (0.995-1.007)	ns		
Platelet count, ×10³/μl	0.996 (0.988-1.004)	ns		
PNPLA3 rs 738409 carrier	0.857 (0.263-2.792)	ns		
TNF-α	0.179 (0.032-0.996)	0.049		
History of diabetes	8.571 (0.937-18.405)	ns		
History of hypertension	3.223 (1.128-9.21)	0.029		
Metabolic syndrome	0.632 (0.227-1.761)	ns		
MEDAS	0.695 (0.528-0.915)	0.010	0.7 (0.5-0.8)	0.001

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; HOMA-IR: homeostasis model assessment of insulin resistance; LDL: low-density lipoprotein; HDL: high-density lipoprotein; PNPLA3: patatin-like phospholipase domain containing protein 3; TNF-α: tumor necrosis factor-alpha.

significance. Likewise, no differences with regard to the presence of fibrosis or advanced fibrosis were observed between the two groups.

Few histological studies involving non-obese patients with NAFLD have been published to date and the results are inconclusive. The study of Leung et al. (21) with a cohort of 307 patients that included 72 non-obese cases found a lower NASH frequency in these individuals. This was attributed, in part, to a lower degree of steatosis and ballooning. However, unlike our study, the authors did not observe significant differences between the groups with regard to the presence of NASH. On the other hand, they reported a lower degree of fibrosis in the non-obese group, although there were no differences with regard to the presence of advanced fibrosis between the groups. We measured the levels of three adipocytokines (adiponectin, leptin and resistin) in peripheral blood. Adiponectin is a protein that protects against excess fat accumulation in the liver, inflammation and fibrosis. Adiponectin levels are decreased in patients with obesity, steatosis and NASH (22,23), which is consistent with the results of our study. Adiponectin levels

were significantly lower in the obese patient group, which also had a greater number of NASH cases. Different studies have reported conflicting results with regard to the role of leptin in the pathogenesis of NAFLD. In our study, leptin levels remained significantly higher in obese patients compared with overweight subjects. These results are consistent with the results of other studies, which suggest that leptin levels are directly related to the severity of hepatic steatosis and elevated levels may lead to higher rates of liver steatosis and NASH (22,24). Finally, resistin levels were significantly higher in the obese patients than in the overweight subjects. The role of resistin in the pathogenesis of NAFLD has not been fully clarified to date and further research is therefore needed. Some studies have reported higher resistin levels in patients with liver inflammation and severe fibrosis (25).

In our study, the multivariate analysis showed an improved adherence to the Mediterranean diet as a protective factor in the overweight group (OR 0.7, 95% CI 0.5-0.8; p < 0.005). We can speculate that the Mediterranean diet may not exert sufficient protection against NAFLD in a more

advanced predisposing scenario such as obesity. No studies have directly verified this association. Metabolic risk factors included arterial hypertension, diabetes mellitus, low HDL-cholesterol levels and hypertriglyceridemia. In this study, being overweight and the presence of at least one of these factors (indistinctly) was independently associated with the presence of NASH.

Insulin resistance plays a key role in the pathogenesis of NAFLD (25). Hashiba et al. (26) observed a gradual increase in HOMA-IR score as liver fibrosis progressed. Angulo et al. (27) also performed a study of 88 patients diagnosed with NAFLD on the basis of liver biopsy findings. Sixty-one of these individuals were obese (69.3%), 69 had non-advanced fibrosis (F 02) (78.4%) and 19 had advanced fibrosis (F 34) (21.6%). The HOMA-IR index was significantly associated with the presence of advanced fibrosis. García-Monzon et al. (28) observed only two factors (increased HOMA score and fatty liver on ultrasound) that were independent predictors of biopsy-proven NASH. However, a subgroup analysis of only non-obese patients was not performed to confirm this association.

Adherence to the Mediterranean diet was identified to exert a protective effect against the development of both NASH and fibrosis in our cohort (OR 0.7, 95% CI 0.50.8). Many studies warrant the beneficial impact of the Mediterranean diet on NALFD. A review published by Sofi et al. (29) demonstrated the effectiveness of this diet to facilitate weight loss, based on the different nutrients afforded, such as monounsaturated fatty acids and vitamins. Olive oil was the main source of fat. This diet exerts a beneficial effect in terms of the control of risk factors for both metabolic syndrome and NAFLD (9). Aller et al. (30) analyzed the adherence to the Mediterranean diet in 82 patients diagnosed with NAFLD using the 14-item Mediterranean Diet Assessment Tool. Their study showed that an increased diet adherence was associated with a lower probability of steatosis and steatohepatitis.

The beneficial effect of the Mediterranean diet upon the liver enzyme profile was subsequently confirmed by the ATTICA study, which evaluated the prevalence of metabolic syndrome among more than 3,000 adult Greeks (31). Further evidence of the beneficial role of the Mediterranean diet in relation to fatty liver was provided by the recent study of Ryan et al. in a randomized, cross-over dietary intervention study in 12 biopsy-proven NAFLD diabetic subjects (32). All patients with NAFLD were randomized to either the Mediterranean diet or to a control diet during a period of six weeks, with a washout period. The mean weight loss at the end of the intervention peri-

od was similar in the two groups of patients, although a significant reduction in liver fat content was observed via magnetic resonance imaging after the Mediterranean diet. In another study, a negative correlation was observed between the Mediterranean Diet Score and transaminases and insulin levels, fibrosis stage and the severity of steatosis in patients with NAFLD (32).

Recent genomic and observational studies have identified PNPLA3 (33) and TNF-α gene polymorphisms (34) as factors associated with NAFLD and/or its severity. Moreover, in our study, a significant association was only found between the A allele of TNF-α rs238 and fibrosis in overweight subjects, although this association was not observed in the multivariate analysis. This observation may be attributed to the low number of overweight patients with advanced disease.

TNF-α is as a biomarker of systemic inflammation that is involved in the physiopathology of metabolic syndrome, including NAFLD (35). Crespo et al. performed a study in 52 obese patients and concluded that the intrahepatic expression of TNF-α mRNA was increased in patients with NAFLD and was even higher in patients with liver fibrosis (36). Previous studies related TNF-α overexpression to the development of NAFLD, atherosclerosis and coronary artery disease via lipid metabolism affects and increased insulin resistance (37). Valenti et al. showed that the presence of the A allele of TNF-α 238 was associated with higher HOMA-IR scores in 99 patients with NAFLD diagnosed by ultrasound and confirmed by biopsy (38).

Several limitations need to be highlighted in the present study. Firstly, many uncontrolled non-genetic factors could influence the relationship of our design such as exercise and hormones, among others. Secondly, other cytokines related to glucose and lipid control such as TNF-α or IL-6 were not measured; these cytokines could help explain our findings and would have enriched the results obtained. Thirdly, we only analyzed one SNP of the TNF and PNPLA3 genes and, in this regard, other genetic variants could be related to metabolic parameters. Lastly, the lack of a control group without the Mediterranean diet could constitute a source of bias.

In conclusion, adherence to the Mediterranean diet was an independent protective factor against both NASH and fibrosis in overweight patients with a high systolic blood pressure as an independent factor associated with the presence of NASH. Furthermore, HOMA-IR index was the only independent factor associated with fibrosis. The proportion of patients with NASH was significantly higher in obese patients than in overweight subjects.

SUPPLEMENTARY MATERIAL

Determination of the patatin-like phospholipase domain-containing protein 3 (PNPLA3 or adiponutrin) variant rs738409

Oligonucleotide primers and probes were designed using Beacon Designer 4.0 (Premier Biosoft International, Los Angeles, CA, USA). The polymerase chain reaction (PCR) was performed with 250 ng of genomic DNA and 0.5 µl of each oligonucleotide primer in a final volume of 25 µl (iCycler IQ thermocycler; Bio-Rad, Hercules, CA, USA). The DNA was denatured at 95 °C for three minutes, followed by 50 denaturation cycles at 95 °C for 15 seconds and annealing at 59.3 °C for 45 seconds. The PCR was performed in a final volume of 25 µl with 12.5 µl of IQTM Supermix (Bio-Rad®, Hercules, CA, USA) containing hot-start Taq DNA polymerase. The *PNPLA3* gene variant was in Hardy-Weinberg equilibrium (p = 0.49).

Genotyping of G308A gene polymorphism of TNF-α variant rs180069

Oligonucleotide primers and probes were designed using Beacon Designer 4.0 (Premier Biosoft International, Los Angeles, CA, USA). The PCR was performed with 50 ng of genomic DNA and 0.5 µl of each oligonucleotide primer in a final volume of 25 µl (iCycler IQ thermocycler (Bio-Rad®, Hercules, CA, USA). The DNA was denatured at 95 °C for three minutes, followed by 50 denaturation cycles at 95 °C for 15 seconds and annealing at 59.3 °C for 45 seconds. The PCR was performed in a final volume of 25 µl with 12.5 µl of IQTM Supermix (Bio-Rad®, Hercules, CA, USA) containing hot-start Taq DNA polymerase. The TNF-α gene variant was in Hardy-Weinberg equilibrium (p = 0.49).

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