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RESEARCH PAPER

A case–control study on the effect of metabolic gene polymorphisms, nutrition, and their interaction on the risk of non-alcoholic fatty liver disease

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Abstract The oxidative stress is a key issue in the etiology of non-alcoholic fatty liver disease (NAFLD). The aim of our study was to evaluate the effect of metabolic gene polymorphisms involved in the oxidative stress (*GSTT1*, *GSTM1*, *SULT1A1*, *CYP2E1,* and *1A1*), lifestyle and nutrition aspects, and their interaction, on the risk of NAFLD. We enrolled 294 cases and 359 controls, and collected demographics, anthropometric, lifestyle, and nutrition data. A subgroup of NAFLD provided additional data on nutrients and on physical activity engagement. Each patient provided a blood sample for DNA extraction and genotyping. Clinical and laboratory data were collected from cases. Multivariable analysis shows a significant protective effect of age, gender, and moderate drinking habits on the risk of NAFLD, while an increased risk for greater consumption of fruit and grilled meat or fish. Significant interactions were reported between alcohol consumption, fruit intake, grilled meat and fish, and selected genetic variants. From the subgroup analysis, a moderate/high consumption of fat and/or grilled meat/fish, and a high consumption of white meat increase the risk of NAFLD. Engaging any physical activity at least 1 time/week halves the risk of NAFLD. Besides confirming the beneficial effect of moderate alcohol intake and regular physical activity, and the increased risk associated with high fruit and fat intake, for the first time, we report a detrimental effect of grilled food on NAFLD risk. An effect modification by selected gene variants increases the risk in combination with fruit and grilled food intake.

Keywords Non-alcoholic fatty liver disease · Metabolic genes · Polymorphism · Nutrition · Gene-environment interaction

Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the most common cause of transaminases elevation in Western countries, where it has been recognized as a major public health burden (Loomba and Sanyal 2013). NAFLD is considered as the hepatic side of metabolic syndrome and is extremely common in subjects with visceral obesity, type 2 diabetes mellitus, dyslipidemia, or insulin resistance (Chalasani et al. 2012). Evidences of high prevalence and incidence of NAFLD in the general population require joint efforts to identify the modifiable risk factors for the disease prevention.

The onset of non-alcoholic steatohepatitis (NASH) on a background of fatty liver is believed to be due to an interplay between genetic and environmental factors, with a major role played by the oxidative stress (Sumida et al.

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2013). The "multiple parallel hit" has recently replaced the classical "two-hit" theory to explain the liver injury, even though the mechanisms involved in the pathogenesis of NAFLD are not yet clarified (Hijona et al. 2010; Tilg and Moschen 2010; Miele et al. 2005). As the natural course of NAFLD and its progression to NASH is highly variable even with the same risk factors, it is reasonable that single nucleotide polymorphisms (SNPs) in genes potentially involved in oxidative stress could play a role in the disease onset and progression as reported by recent studies (Hashemi et al. 2012; Hori et al. 2009; Hardwick et al. 2013; Bell et al. 2011; Fisher et al. 2009). A meta-analysis pointed out at the role of Patatin-like phospholipase domain-containing protein 3 gene to confer either increased risk of NAFLD or its progression (Sookoian and Pirola 2011). Among the lifestyle factors, poor dietary habits and lack of physical activity engagement have been recently reported as risk factors for NAFLD onset (Loomba and Sanyal 2013). High carbohydrate and fructose consumption, as well as high red meat and low fish intake, have been associated with an increased risk of NAFLD and overall inflammation at liver biopsy (Solga et al. 2004; Zelber-Sagi et al. 2011; Tappy and Le 2012). Few reports, however, have examined the interaction between nutritional factors and genes involved in the oxidative stress in the pathogenesis of NAFLD so far (Bell et al. 2011; Daly et al. 2011; Hardwick et al. 2013). This might lead to identification of subjects at higher risk of NAFLD progression to NASH, thus suggesting a potential target of subjects that might benefit from tailored prevention programs.

The aim of our hospital-based case–control study was to evaluate the effect of selected polymorphisms in glutathione *S*-transferase (*GSTM1*, *GSTT1*), sulfotransferase (*SULT1A1*), and cytochrome (*CYP2E1* and *1A1*) genes, their mutual combination, and their interaction with nutritional factors on the risk of NAFLD. In a subgroup of patients with information on additional nutritional factors and recreational physical activity assessment, we also assessed the association between these variables and the risk of NAFLD.

Materials and methods

Study population

Study participants were recruited among patients admitted to the teaching hospital "Agostino Gemelli" of the Università Cattolica del Sacro Cuore (Rome, Italy) from January 2005 until July 2011, and eligibility was restricted to Caucasian individuals born in Italy. Cases were recruited among subjects referred to the Outpatient Liver Unit of the hospital (De Feo et al. 2012). According to the current recommendations (Loria et al. 2010), the diagnosis of NAFLD was based on the presence of sonographic features of hepatic steatosis based on the presence of the bright liver pattern as recommended by the American Gastroenterology Association (Sanyal 2002) and the absence of all the following factors: significant ethanol intake (>20 g/day for females and >30 g/day for males); drug-induced liver disease within the last 5 years; autoimmune liver disease (manifested by positive serum antinuclear, liver/kidney microsomal, mitochondrial, smooth-muscle, and/or neutrophil cytoplasmic antibody titers); seropositivity for hepatitis B (HBs-Ag) or C infection and autoantibodies (anti-HCV IgG); fasting transferrin saturation >45 %; low serum alpha1-antitrypsin levels; and ceruloplasmin levels indicative of Wilson's disease. Ultrasound determinations were performed by the same identical operator (GR) during the entire study period.

The control group was selected among patients without steatosis admitted to the same hospital during the same time period with a broad range of diagnoses without fatty liver and metabolic disease and no alcohol abuse. In closer details, around 50 % of our control population were blood donors and the remaining were patients undergoing surgical interventions (laparoscopic cholecystectomy, appendicitis, inguinal hernia) and patients affected by chronic disease as hypertension, or chronic obstructive pulmonary disease undergoing routine physical examinations.

With a response rate of 98 and 93 %, respectively, for cases and controls, we finally recruited 294 NAFLD cases and 359 controls. Written informed consent was obtained from all study subjects. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Informed consent was obtained from all patients for being included in the study.

Data collection

Demographics, anthropometric, clinical and laboratory data

Information on age and gender was collected from the study participants. Cases and controls underwent anthropometric investigation, with height (m) and weight (kg) taken at the recruitment day. Clinical and laboratory data were collected only from NAFLD patients. Waist circumference was measured in a standing position at the level of the umbilicus. The presence of diabetes mellitus (fasting glucose level >7.1 mmol/l or treatment with antidiabetic drugs) and hypertension (blood pressure >130/85 mm Hg or current treatment for hypertension) were recorded. The laboratory evaluation included liver biochemistry, blood count, total and high-density lipoprotein cholesterol and

total triglyceride level, fasting glucose, and insulin level. Definition of hypercholesterolemia was performed according the third Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, or ATP III) presents the National Cholesterol Education Program (NCEP) (http://www.nhlbi.nih.gov/guidelines/cholesterol/atp3full.pdf). The degree of insulin resistance was determined by the homeostatic model assessment (Matthews et al. 1985).

Lifestyle and nutrition data

Cases and controls were interviewed by trained physicians using a structured questionnaire to collect information on lifestyle habits including smoking and drinking history, fruit and grilled meat or fish intake, and salt addition to meals. The response rate for interview completion was 94.8 % among cases and 93.8 % among controls.

In a subgroup ($N = 280$) of NAFLD patients ($N = 82$) and controls ($N = 198$), information were retrieved using an additional questionnaire detailing on specific nutrients, namely fresh vegetables, pulses, red meat, processed meat, white meat and fish consumption, and recreational physical activity (walking, competitive sport, housework, gardening, cycling, and gymnastics).

Questions about lifestyle habits all focused on the time period ending 1 year prior to diagnosis for cases and on the year prior to the interview date for controls.

DNA extraction and genotyping

DNA was extracted from the peripheral blood lymphocytes of each participating subject. *GSTM1* and *GSTT1* null alleles were identified using a multiplex polymerase chain reaction (PCR)-based method (Arand et al. 1996). The polymorphic site at nucleotide 638 in exon 7 (Arg213His (**2* allele), rs9282861) of the *SULT1A1* gene was genotyped by PCR–restriction fragment length polymorphisms (RFLP) analysis as described by Coughtrie et al. (Coughtrie et al. 1999), *CYP1A1* 3′—flanking region *Msp*I polymorphism (*CYP1A1*2A* allele, rs4646903), *CYP2E1 Pst*I polymorphism [*CYP2E1*5B* allele, rs3813867 (*Pst*I)] and *CYP2E1 Dra*I (**5A* or **6* alleles, rs6413432) were also determined by PCR–RFLP analyses. Quality control for each genotyping was performed in each experiment, and 10 % of the total samples were randomly selected and reanalyzed with 100 % concordance. All laboratory procedures were carried out blind to case–control status.

Statistical methods

The body mass index (BMI) was calculated as the weight divided by the height squared [weight (kg)/height $(m)^2$]. Demographic characteristics, lifestyle, and the genetic polymorphisms of the two groups were compared by means of univariate and bivariate statistical tests. In particular, the Pearson's Chi-square test for categorical variables (or Fisher's Exact test for expected cell frequencies <5) and the Student's *t* test for continuous variables were used.

A logistic regression analysis was implemented to assess the risk of NAFLD. Odds ratio (OR) and 95 % confidence intervals (CI) were calculated. The factors associated with the disease were selected in a forward-stepwise fashion, via the likelihood ratio test. Two-way interactions between the variables of the so derived statistical model and the candidate genes, and two-way gene–gene interactions, were fitted.

A logistic regression analysis was also implemented to assess the risk of NAFLD in the subgroup of 280 subjects with the aim of exploring the role of dietary patterns and physical activity. Strongly associated variables were combined in class variables taking into account the estimated pure effect of each variables when assigning the order of the classes (see results section). The association between pairs of variables was formerly tested with the Chi-square test (or Fisher's exact test for cell frequencies <5); subsequently, the pure effect of each variable was estimated by fitting a simple logistic regression.

Hardy–Weinberg equilibrium (HWE) was verified for all candidate genes using the tool accessible at the following website: http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. The low frequency of subjects with double mutation (<7 % for all genes) suggested the use of a dominant model for all genes (Cordell and Clayton 2005). Statistical significance was set at the 5 % level. Statistical analyses were implemented in STATA/SE V10 (Stata Corporation, College 162 Station, TX).

Results

Our study included 653 subjects, comprising 294 cases and 359 controls. Table 1 reports the clinical features of NAFLDs, with 34 % of cases being obese and 14 % diabetics. Around 45 % of patients had hypercholesterolemia and 22 % had metabolic syndrome (MS). Table 2 details demographic and anthropometric data, together with lifestyle factors and genetic polymorphisms in the entire population. Mean age was lower among cases than controls ($p < 0.001$), while BMI was higher in cases than controls ($p = 0.008$). Gender, smoking, and drinking habits were balanced across the groups, with 30–40 % females, around 55 % of never smokers, and approximately 50 % of never drinkers (Table 2).With the exception of *SULT1A1* ($p = 0.017$), we did not observe significant differences in distribution of the studied SNPs (Table 2). From the

Table 1 Clinical features of 234 cases with non-alcoholic fatty liver disease (NAFLD)

Clinical parameters	Cases (mean ± SD[a])
Weight (kg)	84.11 ± 16.96
Height (m)	1.70 ± 0.09
Waist length (cm)	100.87 ± 11.44
Obesity (BMI >30 kg/m^2)	98 (34 %)
Diabetes [*n* (%)]	41 (14 %)
Hypertension [*n* (%)]	93 (32 %)
Glucose level (mg/dl)	97.73 ± 32.43
Fasting insulin level (mmol/l)	15.85 ± 10.87
HOMA score	3.88 ± 3.34
HOMA score >2.5 [*n* (%)]	117 (40 %)
Total cholesterol (mg/dl)	196.25 ± 47.18
HDL (mg/dl)	46.96 ± 11.51
Hypercholesterolemia [*n* (%)]	124 (43 %)
Triglyceride level (mg/dl)	145.68 ± 82.94
Hypertriglyceridemia [*n* (%)]	101 (35 %)
MS ATP III[b] [*n* (%)]	64 (22 %)
AST (IU/l)	38.65 ± 33.49
ALT (IU/l)	55.91 ± 40.58
AST/ALT ratio	0.85 ± 0.68
AST/ALT ratio ≥1.0 [*n* (%)]	48 (17 %)
GGT (IU/l)	67.33 ± 88.06
Alkaline phosphatase (IU/l)	174.58 ± 83.45
Total bilirubin (μmol/l)	1.11 ± 1.64
Albumin (g/dl)	4.49 ± 0.45
INR	1.08 ± 0.31
PLT (×10^3/ml)	232.79 ± 72.24

[a] Standard deviation

[b] Metabolic syndrome—Adult Treatment Panel III (ATP III) criteria: abdominal obesity, waist circumference >102 cm (men) or >88 cm (women); hyperglycemia, fasting blood glucose ≥110 mg/dl or previously diagnosed type 2 diabetes; hypertriglyceridemia, triglycerides ≥150 mg/dl or current treatment for this abnormality; hypertension, blood pressure ≥130/≥85 mmHg or treatment for previously diagnosed hypertension); and low HDL cholesterol, <40 mg/dl (men) or <50 mg/dl (women) or specific treatment for this abnormality). Patients meeting >3 of these criteria were considered to have the metabolic syndrome

Table 2 Demographic and anthropometric data, and lifestyle and candidate gene factors among 234 NAFLD cases and 349 controls

	Controls	Cases	*p* value
Demographics and lifestyle			
Age	62.06 ± 13.98	44.94 ± 14.11	<0.001
Sex			
Male	220 (61 %)	196 (67 %)	0.15
Female	139 (39 %)	98 (33 %)	
BMI (kg/m^2)	26.91 ± 5.91	28.89 ± 5.25	0.008
Smoker			
Never	194 (54 %)	165 (56 %)	0.66
Ever	159 (44 %)	126 (43 %)	
Alcohol consumption			
Never drinkers	170 (47 %)	160 (54 %)	0.07
Males: <30 g/females: <20 g	182 (51 %)	129 (44 %)	
Fruit intake			
0–2 per day	208 (58 %)	120 (41 %)	<0.001
>2 per day	135 (38 %)	161 (55 %)	
Grilled meat or fish intake			
0–1 per week	142 (40 %)	88 (30 %)	<0.001
>1 per week	165 (46 %)	192 (65 %)	
Candidate genes			
GSTM1			
Present	172 (48 %)	147 (50 %)	0.54
Null	187 (52 %)	145 (49 %)	
GSTT1			
Present	276 (77 %)	217 (74 %)	0.45
Null	83 (23 %)	75 (26 %)	
CYP1A1			
*CYP1A1*1/*1*	278 (77 %)	231 (79 %)	0.67
*CYP1A1*2A carriers*	81 (23 %)	62 (21 %)	
*CYP2E1Pst*I			
*CYP2E1*1A/1A*	331 (92 %)	277 (94 %)	0.17
*CYP2E1*5B carriers*	28 (8 %)	15 (5 %)	
*CYP2E1Dra*I			
*CYP2E1*1A/1A*	323 (90 %)	271 (92 %)	0.52
*CYP2E1*6 carriers*	29 (8 %)	20 (7 %)	
SULT1A1			
*SULT1A1*1/*1*	224 (62 %)	155 (53 %)	0.02
*SULT1A1*2 carriers*	134 (37 %)	136 (46 %)	

univariate analysis, cases and controls, however, differed on their eating habits, as greater proportion of cases reported to consume more than 2 portions/day of fruit ($p < 0.001$) and more than once/week portion of grilled meat or fish ($p < 0.001$) (Table 2).

Results of the multivariable analysis including the entire population under study are reported in Table 3. We observed protective effect of age (OR 0.92; 95 % CI 0.91–0.94), gender (OR for females = 0.51; 95 % CI 0.31–0.83), and moderate drinking habits (OR 0.59 for less than 30 g/day among males and 20/day among females with respect to never drinkers; 95 % CI 0.37–0.94) toward the risk of NAFLD. Increased odds of NAFLD were observed for greater consumption of fruit (>2/day: OR 3.75; 95 % CI 2.34–6.03) and consumption of grilled meat or fish >1/week (OR 2.38; 95 % CI 1.49–3.81) (Table 3). From the multivariable analysis, the SNPs of the selected genes were not found to be associated with the risk of NAFLD (Table 3).

Table 3 Adjusted [a]OR and 95 % CI for NAFLD risk according to selected variables (234 cases and 349 controls)

	OR [95 % CI]	*p* value
Main predictors		
Age	0.92 [0.91; 0.94]	<0.001
Sex		
Male	1.00	0.007
Female	0.51 [0.31; 0.83]	
Alcohol drinkers		
Never drinkers	1.00	0.028
Males: <30 g/females: <20 g	0.59 [0.37; 0.94]	
Fruit intake		
0–2 per day	1.00	<0.001
>2 per day	3.75 [2.34; 6.03]	
Grilled meat or fish intake		
0–1 per week	1.00	<0.001
>1 per week	2.38 [1.49; 3.81]	
Additional factors		
Smoker		
Never	1.00	0.14
Ever	0.7 [0.44; 1.12]	
GSTM1		
Present	1.00	0.81
Null	0.95 [0.61; 1.48]	
GSTT1		
Present	1.00	0.16
Null	1.45 [0.86; 2.43]	
CYP1A1		
*CYP1A1*1/*1*	1.00	0.74
*CYP1A1*2A carriers*	1.09 [0.65; 1.85]	
CYP2E1PstI		
*CYP2E1*1A/1A*	1.00	0.31
*CYP2E1*5B carriers*	0.63 [0.26; 1.53]	
CYP2E1DraI		
*CYP2E1*1A/1A*	1.00	0.58
*CYP2E1*6 carriers*	1.28 [0.53; 3.06]	
SULT1A1		
*SULT1A1*1/*1*	1.00	0.54
*SULT1A1*2 carriers*	1.15 [0.74; 1.80]	

[a] Estimates adjusted for age, sex, drinking habits, fruit, and grilled meat and fish

The result of two-way interactions between alcohol consumption, fruit intake, grilled meat and fish, and candidate genes in the entire population is reported in Table 4. Statistically significant interactions were reported for fruit intake and *GSTM1*, *GSTT1*, *CYP1A1* and $CYP2E1_{DraI}$, and *SULT1A1* genes, as the risky effect of high fruit intake on NAFLD was additionally increased among subjects carriers of one of the aforementioned gene variants (Table 4). Lastly, significant interaction was also reported for *CYP2E15B* and alcohol intake, and *GSTM1*, *GSTT1*, *CYP1A1*, and *SULTI1A1* and grilled meat or fish intake. In testing for the effects of gene–gene interaction, a significant interaction was reported for $CYP2E1_{PstI}$ and *GSTM1*, with a reduced risk of NAFLD (OR 0.21; 95 % CI 0.05–0.93, data not shown) for those contemporarily carrying the two unfavorable gene variants.

Table 5 reports the results of the multivariable analysis conducted in the subgroup of 82 cases and 198 controls. Beside confirming the effects of age and alcohol on the risk of NAFLD, the subgroup analysis showed that a moderate/high consumption of fat and/or grilled food and a high consumption of white meat increase the risk of NAFLD (OR 3.25; 95 % CI 1.64–6.42, and OR 2.37; 95 % CI 1.23–4.58, respectively) (Table 5). Concerning physical activity, performing at least one activity per week halves the risk of NAFLD, with an OR of 0.45 for those walking or performing competitive sport or house working or gardening, and an OR of 0.36 for those cycling or gymnastics (Table 5).

Discussion

Our hospital-based case control study including 294 NAFLD cases and 359 controls evaluated the effect of selected polymorphisms in *GSTM1*, *GSTT1*, *SULT1A1*, *CYP2E1* and *1A1* genes, as well as their interaction with lifestyle and dietary habits in NAFLD etiology in an Italian population. Results show that young adults and males are at higher risk of NAFLD, as well as those with a high consumption of fruit and grilled meat/fish. A moderate alcohol consumption, however, is a protective factor toward the risk of NAFLD. Subgroup analysis showed that a combination of moderate/high fat intake and/or grilled food intake is associated with increased risk of NAFLD, as well as high white meat intake. Additionally, the subgroup analysis reported that performing any physical activity at least once per week halves the risk of NAFLD.

A recent review (Zelber-Sagi et al. 2011) summarized the effect of nutrition and physical activity in the etiology of NAFLD. The reports show that most of the observational studies conducted support an increased risk of disease associated with high fat and sugar intake (the so called "Western" dietary pattern) (Oddy et al. 2013) and a decreased risk associated with physical activity engagement (Zelber-Sagi et al. 2011).

Our study shows an increased odd of NAFLD among males and young adults which is supporting some recent findings (Adams et al. 2005; Oddy et al. 2013), while the protective effect of moderate alcohol drinking is still under debate. Moderate alcohol drinking might have a protective role on the risk of developing NAFLD in view of the

Table 4 Adjusted [a]ORs and 95 % CI for the effect of the interaction of candidate genes with lifestyle factors on the development of NAFLD (234 cases and 349 controls)

	Alcohol intake		Fruit intake		Grilled meat or fish intake	
	Never drinkers	Males: <30 g Females: <20 g	0–2/day	>2/day	0–1/week	>1/week
GSTM1						
Present	1.00	0.56 [0.29; 1.07]	1.00	3.06 [1.60; 5.84]	1.00	1.83 [0.96; 3.46]
Null	0.89 [0.48; 1.66]	0.56 [0.29; 1.07] $p^b = 0.081$	0.77 [0.41; 1.45]	3.55 [1.88; 6.68] $p < 0.001$	0.68 [0.34; 1.36]	2.18 [1.18; 4.03] $p = 0.013$
GSTT1						
Present	1.00	0.59 [0.35; 1.01]	1.00	4.23 [2.46; 7.29]	1.00	2.65 [1.54; 4.58]
Null	1.47 [0.71; 3.06]	0.85 [0.41; 1.76] $p = 0.655$	1.83 [0.89; 3.76] [−0.829; 0.732]	4.82 [2.26;10.32] $p < 0.001$	1.82 [0.82; 4.03] [−0.856;0.726]	3.26 [1.57; 6.79] $p = 0.002$
CYP1A1						
*CYP1A1*1/*1*	1.00	0.55 [0.32; 0.94]	1.00	3.06 [1.80; 5.20]	1.00	2.37 [1.39; 4.04]
*CYP1A1*2A carriers*	0.92 [0.43; 1.99]	0.70 [0.34; 1.43] $p = 0.326$	0.72 [0.34; 1.53]	5.17 [2.37;11.27] $p < 0.001$	1.10 [0.48; 2.51] [−0.686; 0.779]	2.59 [1.25; 5.36] $p = 0.011$
$CYP2E1_{PstI}$						
*CYP2E1*1A/1A*	1.00	0.62 [0.38; 1.00]	1.00	3.82 [2.34; 6.25]	1.00	2.23 [1.37; 3.62]
*CYP2E1*5B carriers*	0.92 [0.28; 3.08]	0.25 [0.06; 0.98] $p = 0.046$	0.68 [0.18; 2.58]	2.31 [0.71; 7.47] $p = 0.163$	0.41 [0.10; 1.71]	1.93 [0.59; 6.28] $p = 0.273$
$CYP2E1_{DraI}$						
*CYP2E1*1A/1A*	1.00	0.56 [0.34; 0.92]	1.00	3.53 [2.14; 5.82]	1.00	2.38 [1.45; 3.91]
*CYP2E1*6 carriers*	1.09 [0.36; 3.32]	0.92 [0.22; 3.80] $p = 0.911$	0.52 [0.13; 2.05]	11.34 [2.70;47.55] $p = 0.001$	2.32 [0.37; 14.67] [−2.547; 1.682]	2.58 [0.95; 7.04] $p = 0.063$
SULT1A1						
*SULT1A1*1/*1*	1.00	0.55 [0.30; 1.01]	1.00	4.05 [2.17; 7.53]	1.00	2.07 [1.12; 3.83]
*SULT1A1*2carriers*	1.04 [0.56; 1.94]	0.71 [0.36; 1.38] $p = 0.314$	1.28 [0.68; 2.42] [−0.697; 0.624]	4.18 [2.20; 7.94] $p < 0.001$	0.97 [0.48; 1.95]	2.69 [1.42; 5.10] $p = 0.002$

[a] Estimates adjusted for age, sex, drinking habits, fruit, and grilled meat and fish

[b] p for the interaction from logistic regression analysis

associated insulin-sensitizing effect (Dunn et al. 2008) or through the beneficial effect of high-density lipoprotein (HDL) cholesterol increased levels as for cardiovascular disease (CVD) (Djousse et al. 2009). Additional studies, however (Abdelmalek et al. 2010; Abid et al. 2009), suggested that a high intake of soft drinks which are rich of fructose, might have a detrimental effect on the risk of NAFLD (Miele et al. 2009). In our study, the information on alcohol, however, was limited to wine and spirits consumption, thus did not include information on soft drinks consumption. This might partly explain the absence of any risky effect associated with moderate alcohol intake, in

Table 5 Adjusted [a]OR and 95 % CI for the risk of NAFLD—a subgroup analysis (82 cases and 198 controls)

	Cases	Controls	OR [95 % CI]	*p* value
Main predictors				
Age	57.07 ± 15.45	45.85 ± 14.09	0.95 [0.93; 0.98]	<0.001
Alcohol consumption				
Never drinkers	29 (35 %)	104 (52 %)	1	
Males: <30 g/females: <20 g	47 (57 %)	90 (45 %)	0.39 [0.2; 0.77]	0.006
Sausages–red meat–grilled meat/fish[b]				
Low-grilled food and low–moderate fat intake	51 (62 %)	69 (35 %)	1	
Moderate–high fat intake and/or high grilled food	24 (29 %)	123 (62 %)	3.25 [1.64; 6.42]	0.001
White meat				
<8 times/month	43 (52 %)	63 (32 %)	1	
≥8 times/month	34 (41 %)	133 (67 %)	2.37 [1.23; 4.58]	0.010
Walking–competitive sport–housework–gardening				
≤1 activity	41 (50 %)	141 (71 %)	1	
>1 activity/week	34 (41 %)	51 (26 %)	0.45 [0.23; 0.88]	0.02
Cycling–gymnastics				
No activity	58 (71 %)	155 (78 %)	1	
≥1 activity/week	17 (21 %)	41 (21 %)	0.36 [0.15; 0.85]	0.019
Additional factors				
Sex				
Female	30 (37 %)	63 (32 %)	1	
Male	52 (63 %)	135 (68 %)	1.47 [0.69; 3.16]	0.320
Smoker				
Never	31 (38 %)	108 (54 %)	1	
Ever	46 (56 %)	88 (44 %)	0.61 [0.3; 1.24]	0.174
Fruit–vegetables–pulses[c]				
Low–moderate intake	39 (48 %)	137 (69 %)	1	
High intake	38 (46 %)	56 (28 %)	0.58 [0.29; 1.15]	0.120
Eat between meals				
No	58 (71 %)	123 (62 %)	1	
Yes	19 (23 %)	70 (35 %)	1.16 [0.57; 2.35]	0.690

[a] Estimates adjusted for age, drinking habits, additional use of salt, meat intake, and physical activity

[b] Sausage, red meat and grilled meat/fish (low-grilled food: ≤1 portions/month; high grilled food: >1 portions/month; low fat from sausage and red meat: <3 portions of sausages/month and ≤13 portions of red meat/month (≤4 portions/week); moderate fat intake: ≥3 portions of sausages/month or >13 portions of red meat/month (>4 portions/week); high fat intake: ≥3 portions of sausages/month and >13 portions of red meat/month (>4 portions/week))

[c] Fruit, vegetables and pulses (low intake: <5 portions of pulses/month and ≤9 portions of fruit/week and ≤9 portions of vegetables/week, or <5 portions of pulses/month and >9 portions of fruit/week and >9 portions of vegetables/week; moderate intake: <5 portions of pulses/month and >9 portions of fruit/week and >9 portions of vegetables/week, or ≥5 portions of pulses/month and ≤9 portions of fruit/week and ≤9 portions of vegetables/week; high intake: ≥5 portions of pulses/month and >9 portions of fruit/week and/or >9 portions of vegetables/week (or vice versa))

view of the different fructose concentration among the aforementioned beverages.

Concerning nutrients intake, our data show that high fruit intake increases the risk of NAFLD, which has already been reported, and as already mentioned likely due to the potential role played by fructose in liver *de novo* lipogenesis and hepatic inflammation (Shi et al. 2012; Lim et al. 2010). Additionally, we report that a high consumption of grilled meat/fish alone, or in combination with moderate/high fat consumption, and a high consumption of white meat increases the risk of NAFLD. However, we were unable to demonstrate any beneficial effect of vegetable consumption on disease risk. As mentioned, several reports summarized in a recent review (Zelber-Sagi et al. 2011) agree on the effect of unhealthy dietary pattern rich in fat and poor in vegetables, on the risk of NAFLD. Our results confirm such observation, though we were unable to estimate the effect of vegetables and pulses independently of

the effect of fruit. This might have led to an underestimation of the true beneficial effect of such nutrients on disease risk. As for the high consumption of grilled meat/fish associated with an increased odd of disease, to our knowledge, there are no data currently available correlating the presence of aromatic hydrocarbons generated by grilled meat and fish and the risk of NAFLD.

Our study reports that performing any kind of physical activity at least one time per week exerts a protective role on the risk of NAFLD. The effect is stronger for those engaging cycling and gymnastic. The beneficial effects of physical activity in the treatment for NAFLD are already documented, as regular physical activity improves liver enzyme activities and reduces the overall oxidative stress (Zelber-Sagi et al. 2008, 2011; Church et al. 2006).

We were unable to demonstrate a direct effect on the risk of NAFLD related to SNPs of oxidative-stress-associated genes. The candidate genes were selected on the background hypothesis that the onset and progression of fatty liver may be due to a contribution of oxidative stress (Sumida et al. 2013). Namely, glutathione-*S*-transferases (GSTs) have a pivotal role as antioxidant defense mechanisms, and in conjunction with *SULT* and *CYP2E*1, products act by inactivating xenobiotics and products of oxidative stress (Bolt and Thier 2006). Moreover, *GSTM 1* and *GSTT*1 null genotypes are associated with type 2 diabetes and its complications which (Doney et al. 2005) are closely related to NAFLD with several reports underlining the role of *GSTT* on liver damage (Targher et al. 2010; Huang et al. 2007; Ueda et al. 2007; Oniki et al. 2007). No one study so far ever addressed the effect of *SULT1A1* and *CYP1A1* polymorphisms on NAFLD, while *GSTT1* but not *GSTT*1 genetic polymorphisms have been associated with NAFLD in a sample of the Iranian population (Hashemi et al. 2012). Polymorphisms of *CYP2E1* and insulin resistance are associated with higher levels and activity of the enzyme in obese patients with NASH, which have been related to increased risk of liver damage. Therefore, the occurrence of genetic polymorphisms increasing *CYP2E1* expression in the etiology of NASH can in principle represent susceptibility factors to acquire insulin resistance mediated by oxidative stress (Varela et al. 2008). In our population, however, we were unable to show association between the aforementioned genetic variants and the risk of NAFLD.

From the gene, lifestyle, and nutrient interaction analysis, we report that significant interactions occurred between fruit intake and *GSTM1*, *GSTT1*, *CYP1A1* and $CYP2E1_{DraI}$, and *SULT1A1* genes, as the risky effect of high fruit intake on NAFLD was additionally increased among subjects carriers of one of the aforementioned gene variants. Additionally, a significant interaction was also reported for *CYP2E15B* and alcohol intake, and *GSTM1*, *GSTT1*, *CYP1A1*, and *SULTI1A1* and grilled meat or fish intake. It is reasonable to postulate that in subjects with enzyme null variants of *GST* or polymorphisms associated with an increased activity of the cytochromes, in the presence of exposure to aromatic hydrocarbons, there is an imbalance in the liver function which favors the development of damage.

To our knowledge, this study is the first study testing the effects of the selected gene–gene interaction on the risk of NAFLD. The results show a significant interaction between $CYP2E1_{PstI}$ and *GSTM1* genes, with a 80 % reduced risk of NAFLD for those contemporarily carrying the two unfavorable gene variants. No one reported a protective effect of the two unfavorable gene variants on the risk of developing a disease so far, while their combination has been related to increased susceptibility to alcoholic liver cirrhosis (Khan et al. 2009) and Head and Neck Cancer (Ruwali et al. 2009). As we may have been underpowered due to sample size in the interaction analysis, our result requires confirmation from independent and larger studies.

Some limitations of the study should be considered in interpreting our results. Our sample size though large limits the possibility to detect significant gene–lifestyle interaction, thus we need to increase the sample size in order to confirm our results, also because the absence of liver biopsy for all the participating subjects cannot allow us to draw any conclusion of the interaction on liver histological liver damage. Secondly, as in all case–control studies, information bias may exist, leading to biased ORs due to exposure misclassification of the lifestyle and nutrients exposure. Thirdly, we were unable to explore the effect of certain dietary patterns in the entire sample size and unable to assess the independent effect of vegetables and pulses on the risk of NAFLD. Lastly, even though the measurement of oxidative stress biomarkers was beyond the aim of this study, it would be worth to measure such levels in future investigations.

Besides the acknowledged limitations, however, our report is the first examining the effect of genetic variants involved in the oxidative stress and nutrition on NAFLD etiology. Even though replication is demanded, our results show that the genetic background among genes involved in metabolism of liver detoxification might modify the effect of fruit and grilled meat/fish intake that already affect *per se* the risk of NAFLD. Additionally, our report confirms the excess risk of NAFLD associated with high fruit and fat intake, while for the first time, we reported a significant association with high intake of grilled food and disease risk. Lastly, the beneficial effect of any kind of physical activity is confirmatory of previous evidences and strengthens the knowledge of the benefits of physical activity on NAFLD beyond encouraging weight reduction.

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Conflict of interest Luca Miele, Valentina Dall'Armi, Consuelo Cefalo, Bojan Nedovic, Dario Arzani, Rosarita Amore, Gianlodovico Rapaccini, Antonio Gasbarrini, Walter Ricciardi, Antonio Grieco, and Stefania Boccia declare that they have no conflict of interest.

Ethical Standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Informed consent was obtained from all patients for being included in the study.

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