Hepat Mon. 2017 June; 17(6):e13570. doi: 10.5812/hepatmon.13570.

Published online 2017 May 17. **Research Article**

The Association of Nicotinamide Phosphoribosyltransferase Polymorphism with Markers of Hepatic Injury and De Novo Lipogenesis in Nonalcoholic Fatty Liver Disease

Bahareh Amirkalali,[1] Masoud Reza Sohrabi,[1] Parvaneh Rahimi-Moghaddam,[2] Ali Esrafily,[3] Ali Gholami,[4,5] Payam Hosseinzadeh,[1] Hossein Keyvani,[6] Farzad Shidfar,[7,*] and Farhad Zamani[1,*]

[1]Gastrointestinal and Liver Disease Research Center (GILDRC), Iran University of Medical Sciences, Tehran, Iran
[2]Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[3]Department of Environmental Health Engineering, School of Public Health, Iran University of Medical Sciences, Tehran, Iran
[4]Department of Public Health, School of Public Health, Neyshabur University of Medical Sciences, Neyshabur, Iran
[5]Department of Epidemiology, School of Public Health, Iran University of Medical Sciences, Tehran, Iran
[6]School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[7]Department of Nutrition, School of Public Health, Iran University of Medical Sciences, Tehran, Iran

[*]*Corresponding authors*: Farzad Shidfar, Shahid Hemmat Highway, Iran University of Medical Sciences. Tel: +98-2188622721, E-mail: f-shidfar@tums.ac.ir; Farhad Zamani, Karim Khan Blv, Behafarin Street, Firoozgar Hospital, Gastrointestinal and Liver Disease Research Center, Tehran, Iran. Tel: +98-2188940489, E-mail: zamani.farhad@gmail.com

Received 2016 October 29; **Revised** 2017 March 11; **Accepted** 2017 April 30.

Abstract

Background: De novo lipogenesis (DNL) increases in NAFLD and nicotinamide phosphoribosyltransferase (NAMPT) up regulates two essential enzymes in this pathway. On the other hand, NAMPT function could be affected by the promoter region polymorphism and sex hormones.

Objectives: This study explored the association of -4689 G/T polymorphism in the promoter region of NAMPT gene with markers of hepatic injury and DNL in patients with NAFLD in order to see whether or not these associations are the same for both sexes.

Methods: In this cross-sectional study, 62 consecutive patients (32 men and 30 women) with NAFLD were recruited. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to identify -4689 G/T polymorphism. DNL index of erythrocyte membrane as the marker of hepatic DNL was analyzed by gas chromatography. Fasting serum NAMPT, Caspase-cleaved cytokeratin 18 (cCK18), total soluble cytokeratin 18 (CK18), liver enzymes (AST, ALT, ALKP, GGT), and lipid-glucose profile were measured. Anthropometric measurements, Fibroscan, assessment of dietary intake and physical activity were also performed. Two-independent sample t test, chi-square test, one-way analysis of variance, and multiple linear regression were used to analyze the data.

Results: Serum NAMPT and erythrocyte membrane DNL index were not significantly different among the three genotypes in both sexes. In men, serum AST ($P = 0.04$) and ALT ($P = 0.03$) were significantly higher in GT genotype than GG genotype. Serum CK18, cCK18, and CAP also had the highest levels in GT genotype but not statistically significant. In women, the markers of hepatic injury were not significantly different between GG and GT genotypes. Serum AST ($P = 0.01$), ALT ($P = 0.01$) and cCK18 ($P = 0.001$) levels were significantly higher in TT genotype. Serum GGT, CK18, and CAP also had the highest level in TT genotype but not statistically significant. These associations remained significant even after adjustment for confounding variables in multiple linear regression.

Conclusions: -4689 G/T polymorphism was not associated with hepatic DNL index but T allele in this polymorphism was associated with increased biomarkers of hepatic inflammation, apoptosis and necrosis in patients with NAFLD especially in men, as one T allele (GT genotype) was enough for increased biomarkers of hepatic injury in men but not in women.

Keywords: NAMPT, Lipogenesis, Non-Alcoholic Fatty Liver

1. Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide (1) and increased unhealthy life styles have resulted in the increased prevalence of the disease (2, 3). The disease may progress from simple steatosis to non-alcoholic steatohepatitis (NASH) (4), hepatic fibrosis, cirrhosis (5), and ultimately to hepatocellular carcinoma (6).

De novo lipogenesis (DNL), which contributes to 26% of liver lipids, increases in patients with NAFLD (7, 8). In this metabolic pathway, fatty acid synthase (FAS) catalyzes the synthesis of palmitic acid from malonyl-CoA and acetyl-CoA (9). Acetyl-CoA (the precursor of palmitic acid) is synthesized by Acetyl-coenzyme a synthetase (ACS) (10).

Previous studies have shown that both of these enzymes are up regulated by nicotinamide phosphoribosyltransferase (NAMPT). In 1994, NAMPT was first described as

a molecule with cytokine functions (11) but later it was revealed that this molecule has an important enzymatic role in the production of nicotinamide adenine dinucleotide (NAD) and is a regulatory factor for NAD-consuming enzymes such as sirtuins (SIRT) (11, 12). NAMPT contributes to the formation of acetyl coenzyme A by providing NAD for SIRT3 which activates ACS (9). Incubation of differentiated adipocytes with NAMPT has resulted in FAS up regulation (12) and NAMPT inhibition has significantly reduced the level of de novo synthesized myristic and palmitic acid in prostate cancer cells (12, 13).

As hepatocytes are among the main sources of NAMPT in the body (12), it can be suggested that in patients with NAFLD, NAMPT may have an important role in hepatic DNL and the progress of the disease.

The studies on the association between NAMPT and NAFLD have reported contradictory results (13) and the role of this adipokine in NAFLD pathogenesis has remained unclear but NAMPT function could be affected by many factors. Previous studies have shown that the presence of polymorphism in the promoter region of the gene has an important effect on its expression (14). -4689 G/T or rs2110385 is a common single nucleotide polymorphism (SNP) in the promoter region of NAMPT gene (15). Previous studies indicate that serum NAMPT level is different between the three genotypes of this SNP (GG, GT, and TT) and is significantly increased TT genotype (16). This SNP is also in association with serum lipid profile and insulin resistance in patients with type 2 diabetes (17) which is a metabolic disorder just like NAFLD. But as far as we know, there is no study on the association of this SNP with hepatic injury and DNL in NAFLD.

Another factor which has an important effect on NAMPT expression is sex hormones. Studies indicate that testosterone down regulates NAMPT expression more than two folds than progesterone in pre-adipocytes (18) and estrogen increases NAMPT expression in adipocytes (19).

2. Objectives

The aim of this study was to explore the association between -4689 G/T polymorphism in the promoter region of NAMPT gene and markers of hepatic injury and DNL in patients with NAFLD in order to see whether or not these associations are the same for both sexes.

3. Methods

3.1. Design and Sample

In this cross-sectional study, 62 patients with NAFLD, who referred to the liver disease clinic at Firoozgar hospital in Tehran, Iran, were recruited consecutively. A related cross-sectional formula was used to calculate sample size with a power of 80%. The inclusion criteria were: 18 years of age or older with a diagnosis of steatosis based on ultrasonographic findings and controlled attenuation parameter (CAP) on fibroscan (20), a stable body weight ($\pm$ 2%), and physical activity for at least 3 months.

The exclusion criteria were: liver stiffness measurement (LSM) above 10 in the fibroscan (LSM is a marker of liver fibrosis, and advanced liver fibrosis may affect markers of hepatic fat infiltration and inflammation) (21), having diabetes (fasting blood sugar $\geq$ 126 mg/dL or use of blood glucose lowering drugs), drug abuse, exposure to chemical pollutants, use of lipid lowering drugs, use of steatogenic or hepatotoxic drugs (amiodarone, calcium channel blockers, perhexiline maleate, tamoxifen, chloroquine, methotrexate, corticosteroids, synthetic estrogens), use of drugs that affect weight (antidepressants, antipsychotics or hormone therapy), antioxidants and polyunsaturated fatty acid supplements in the 6 months prior to the study, endocrine disease which affects weight (such as hyperprolactinemia, Cushing's syndrome, thyroid disorders, congenital adrenal hyperplasia), kidney or heart disease, other acute or chronic liver diseases such as viral hepatitis or cirrhosis or a history of alcohol intake (> 20 g/day).

This study was approved by the ethics committee of Iran University of Medical Sciences and carried out in accordance with the Helsinki declaration (1975). All participants gave written informed consent.

3.2. Diagnosis of Fatty Liver in Sonography

Fatty liver was diagnosed using sonography as an increase in hepatic echogenicity using renal echogenicity as a reference, enlargement of the liver and a lack of differentiation of the periportal and bile duct wall reinforcement because of advanced hyper echogenicity of the parenchyma (22).

3.3. Fibroscan

Fibroscan (Echosens; France) is an ultrasound-based vibration-controlled transient elastography device used to assess liver stiffness (correlated to fibrosis) as a non-invasive method. 10 measurements of stiffness were performed by pressing a probe between the ribs. The median of the 10 measurements (in kPa) was compared with the designated values from the fibroscan scoring card. It quantifies steatosis at the same time using the controlled attenuation parameter (CAP), a measurement of ultrasound attenuation correlated to the decrease in amplitude of ultrasound waves as they spread through the liver. Fat affects ultrasound broadcasts; therefore, an increase in steatosis will result in a higher CAP value. The final CAP value was

the median of individual measurements and ranged from 100 to 400 decibels per meter (dB/m) (21).

3.4. Medical History, Dietary and Alcohol Intake

Each participant was given a medical history questionnaire to complete. As dietary macronutrient composition affects DNL (23), the habitual dietary intake of the participants was assessed using the semi-quantitative food frequency questionnaire consisting of 168 items that has been validated on a sample of healthy population (24). Physical activity level was assessed using the long form of international physical activity questionnaire (IPAQ) (25). Alcohol intake was estimated by recording the type of alcohol consumed, its volume and the frequency of consumption. A fatty liver was considered non-alcoholic if the patient consumed less than 20 g of alcohol per day.

3.5. Anthropometric Measurements

Weight, height, waist and hip circumferences were measured according to standard protocols (26). Body mass index (BMI) was calculated as body weight (kg) divided by the square of height (m) as kg/m^2.

3.6. Biochemical Measurements

A 12 hours fasting venous blood sample was taken from each participant to measure biochemical parameters. Liver enzymes as aspartate aminotransferase (AST), alanine aminotransferase (ALT), Alkaline phosphatase (ALKP) and Gamma-glutamyl transpeptidase (GGT), fasting blood glucose (FBS), triglyceride (TG) and cholesterol levels were determined by auto analyzer alpha classic (Tehran, Iran) and Pars Azmoon reagent kits (Tehran, Iran). Insulin resistance was assessed using the Homeostasis model assessment (HOMA) index as follows:

HOMA index = (serum glucose (mg/dL) $\times$ serum insulin (mU/L))/405.

Serum insulin (Diaplus; Canada), NAMPT (BioVendor; Czech Republic), Caspase-cleaved cytokeratin 18 (cCK18, marker of hepatic apoptosis) (M30 kit, PEVIVA, Sweden) and total soluble cytokeratin 18 (CK18, marker of hepatic apoptosis and necrosis) (M65 kit, PEVIVA, Sweden) (27) were measured by ELIZA method. Hepatitis B surface antigens (HbsAg), hepatitis B surface antibodies (HbsAb) hepatitis B core antibodies (HBcAb), hepatitis C virus antibodies (HCVAb), and antinuclear antibodies (ANA) were evaluated using third-generation ELISA kits (Acon; USA).

3.7. DNL Index and Fatty Acid Measurement in Erythrocyte Membrane

Palmitic to linoleic acid ratio (DNL index) of erythrocyte membranes was used as the marker of hepatic DNL (28). To measure erythrocyte membrane fatty acids, venous blood samples were centrifuged at 3000 g for 10 min at 4°C and the erythrocytes were then separated. An equal volume of physiological saline (sodium chloride 0.9%) was added to the erythrocytes, shaken and then centrifuged at 3000 g for 10 minutes at 4°C. The erythrocytes were washed three times using this procedure.

The washed erythrocytes were aliquoted and stored at -80°C. 200 μL of washed erythrocytes were evaporated to dryness under nitrogen gas. Boron trifluoride-methanol solution 14% (2 cc) and methanol (1 cc) were added to the dried erythrocytes and heated in bain-marie for 10 minutes at 60°C. Next, 2 cc of n-hexane was added to each tube and shaken for 2 minutes. After settling, the n-hexane layer containing methylated fatty acids was transferred into another tube and the solvent was removed by evaporation. The residue was redissolved in 50 μL n-hexane, mixed thoroughly and then, 1 μL of this solution was injected into the gas chromatograph. A gas chromatograph (YL6500; Young Lin; Korea) equipped with a 60 m $\times$ 0.25 mm (film thickness = 0.2 μm) capillary column (TR-CN100; Teknokroma) and a flame ionization detector were used to measure the erythrocyte membrane fatty acid profiles. The YL Autochro3000 chromatograph data system version 2.0.15 was used for quantification and identification of peaks.

3.8. Genotyping -4689 G/T Polymorphism

Total genomic DNA was extracted from the Buffy coat using a DNA extraction kit (high pure PCR template preparation Kit, Roche, Switzerland), according to the producer's protocol. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used to identify the polymorphism. The primers (Macrogen Inc., Korea) used for amplification were as follows:

The forward primer: 5'- GGTGGGCACTCAGACTGGT -3'

The reverse primer: 5'- CAAGAAGTTTCCTCAGACCTGC -3'

The amplification was performed in a volume of 40 μL, containing 8 μL genomic DNA, 20 μL PCR Master Mix (2X) (Thermo Inc., USA), 2 μL of each prepared primers, and 8 μL distilled water.

The PCR initial denaturation was done at 94°C for 5 minutes. 35 cycles of 94°C for 45 s, 60°C for 1 minutes, 72°C for 1 minutes and a final elongation step at 72°C for 10 minutes. 26 μL of the PCR products was digested with RapidDigest Alu1 (Cinnagen Co., Iran) for 1 huour at 37°C. The digestion products were analyzed by electrophoresis on 3.5% agarose gels stained with DNA safe stain (Cinnagen Co., Iran).

3.9. Statistical Analysis

The Shapiro-Wilks test was used to check the normality of the continuous variables and arithmetic transforma-

tions were performed if necessary. Two independent sample t-test and chi-square test were used to compare continuous and categorical variables between sexes, respectively.

One-way analysis of variance (one-way ANOVA) was used to compare hepatic injury and DNL markers (Serum AST, ALT, ALKP, GGT, CK18, cCK18, CAP, LSM and RBC membrane DNL Index) between the three genotypes of -4689 G/T SNP. Baseline characteristics that could affect hepatic injury and DNL marker [25] (age, BMI, HOMA index, waist to hip ratio, blood lipid and glucose profile, serum insulin, dietary energy, carbohydrate and protein intake and physical activity level) were also analyzed between the three genotypes by one-way ANOVA. Each baseline characteristic with $P < 0.2$ in one-way ANOVA was entered into multiple linear regression analysis (backward method) to confirm the independent association between -4689 G/T SNP and markers of hepatic injury. P value less than 0.05 was considered statistically significant. SPSS software version 23 was used to analyze the data.

4. Results

The baseline characteristics of the participants are shown in Table 1. This study included 62 patients with NAFLD of which, 32 were men and 30 were women. The participants were between 18 and 67 years of age. Women were significantly older (47.83 $\pm$ 81.10 vs. 39.84 $\pm$ 12.10, P = 0.008), had higher BMI (32.31 $\pm$ 4.56 vs. 29.42 $\pm$ 4.89, P = 0.02) and serum HDL levels (50.31 $\pm$ 11.05 vs. 43.03 $\pm$ 7.41, P = 0.004) but lower waist to hip (W/H) ratio (0.87 $\pm$ 0.05 vs. 0.9 $\pm$ 0.06, P = 0.022) (Table 1).

Serum NAMPT, -4689 G/T genotype distribution, dietary protein, and carbohydrate intake, physical activity level, serum total cholesterol, LDL, triglyceride, FBS, insulin and HOMA index were not significantly different between men and women (Table 1).

4.1. Baseline Characteristics, Hepatic Injury, and DNL Markers Among the Three Genotypes of -4689 G/T Polymorphism in Men with NAFLD

Age, anthropometric parameters, serum lipid and glucose profile, dietary energy, carbohydrate and protein intakes and physical activity level were not significantly different between the three genotypes in men. Serum NAMPT level was not significantly different among the three genotypes either (Table 2).

Serum AST level was significantly higher in GT genotype than GG genotype (53.33 $\pm$ 26.07 vs. 38.81 $\pm$ 9.98, P = 0.04) and TT (53.33 $\pm$ 26.07 vs. 27.33 $\pm$ 2.08, P = 0.03) but it was not significantly different between GG and TT genotypes.

Serum ALT level was also significantly higher in GT genotype compared to GG (71.17 $\pm$ 40.84 vs. 44.18 $\pm$ 25.17, P = 0.03) but it was not significantly different between GT and TT genotypes.

CAP level was significantly higher in GT genotype compared to TT (302.75 $\pm$ 44.31 vs. 210.33 $\pm$ 9.50, P = 0.02) but it was not significantly different between GG and GT genotypes.

Serum CK18 and cCK18 also had the highest levels in GT genotype compared to GG and TT genotypes but not statistically significant.

Serum ALKP, GGT, LSM, and hepatic DNL index were not significantly different between the three genotypes (Table 3).

4.2. Baseline Characteristics, Hepatic Injury, and DNL Markers Among the Three Genotypes of -4689 G/T Polymorphism in Women with NAFLD

Age, anthropometric parameters, serum lipid and glucose profile, dietary energy, carbohydrate and protein intakes, and physical activity level were not significantly different among the three genotypes in women. Serum NAMPT level was not significantly different among the three genotypes either (Table 4).

Serum AST level was significantly higher in TT genotype compared to GG (73 $\pm$ 35.35 vs. 31.81 $\pm$ 10.48, P = 0.01) and GT (73 $\pm$ 35.35 vs. 40.36 $\pm$ 26.68, P = 0.04) but it was not significantly different between GG and GT genotypes.

Serum ALT level was also significantly higher in TT genotype compared to GG (76 $\pm$ 11.31 vs. 28.93 $\pm$ 15.88, P = 0.01) and GT (76 $\pm$ 11.31 vs. 40.18 $\pm$ 30.38, P = 0.048) but it was not significantly different between GG and GT genotypes.

Serum cCK18 level was also significantly higher in TT genotype compared to GG (526.62 $\pm$ 442.04 vs. 154.38 $\pm$ 83.82, P = 0.001) and GT (526.62 $\pm$ 442.04 vs. 157.75 $\pm$ 108.91, P = 0.001) but it was not significantly different between GG and GT genotypes.

Serum GGT, CK18, and CAP also had the highest level in TT genotype and the lowest level in GG genotype although the differences were not statistically significant.

Serum ALKP, LSM, and hepatic DNL index were not significantly different between the three genotypes (Table 5).

4.3. Multiple Linear Regression Analysis of -4689 G/T Polymorphism with Markers of Hepatic Injury in Patients with NAFLD

After adjustment for confounding variables (baseline characteristics with $P < 0.2$ in one-way ANOVA) in multiple linear regression, GT genotype still had its significant associations with serum AST ($\beta = 0.40$, P = 0.04) and ALT ($\beta = 0.41$, P = 0.02) in men. Besides, GT genotype also showed a significant association with serum CK18 ($\beta = 0.48$, P = 0.01).

Table 1. Baseline Characteristics of Participants[a]

Variables	Male	Female	P Value
Age, y	39.84 ± 12.10	47.83 ±10.62	0.008[b]
BMI, kg/m²	29.42 ± 4.89	32.31 ± 4.56	0.02[c]
W/H ratio	0.9 ± 0.06	0.87 ± 0.05	0.022[c]
FBS, mg/dL	105.37 ± 22.37	112.86 ± 25.94	0.23
Serum Insulin, micIU/mL	12.77 ± 9.47	11.95 ± 5.22	0.70
HOMA Index	3.44 ± 2.76	3.31 ± 1.62	0.84
Serum total cholesterol, mg/dL	186.58 ± 41.31	196.91 ± 37.92	0.31
Serum LDL-cholesterol, mg/dL	115.90 ± 41.20	112.20 ± 34.13	0.71
Serum HDL-cholesterol, mg/dL	43.03 ± 7.41	50.31 ± 11.05	0.004[b]
Serum triglyceride, mg/dL	137.35 ± 74.13	173.06 ± 94.11	0.11
Serum NAMPT, ng/mL	2.44 ±1.07	2.45 ±1.17	0.98
-4689 G/T genotype (GG/GT/TT), %	53.1 - 37.5 - 9.4	56.7 - 36.7 - 6.7	0.91
Dietary energy intake, kcal/d	2367.19 ± 521.60	2311.37 ± 434.01	0.68
Dietary carbohydrate, %	53.16 ± 5.02	50.31 ± 6.05	0.065
Dietary protein, %	13.51 ± 2.04	12.45 ± 2.16	0.07
Physical activity, METs-min/w	4419.54 ± 7018.28	1723.97 ± 2841.17	0.067

Abbreviations: BMI, Body Mass Index; FBS, Fasting Blood Sugar; HOMA, Homeostasis Model Assessment; MET, Metabolic Equivalent of Task; NAMPT, Nicotinamide Phosphoribosyltransferase; W/H ratio, Waist to Hip Ratio.
[a] Data presented as Mean ± SD.
[b] Significant difference with P value < 0.05.
[c] Significant difference with P value < 0.01.

In women, TT genotype still had its significant associations with serum AST (β = 0.45, P = 0.01), ALT (β = 0.44, P = 0.01), and cCK18 (β = 0.55, P < 0.001).

5. Discussion

According to our results there was not any significant association between -4689 G/T polymorphism and hepatic DNL index (palmitic to linoleic acid of erythrocyte membrane) in patients with NAFLD. This polymorphism had no association with serum NAMPT either. These results were the same in both sexes. A previous study on a group of healthy sedentary individuals also reported that there were no significant differences in serum NAMT level among the genotypes of this polymorphism (29). But another study reported higher serum NAMPT level in TT genotype in a group of patients with type 2 diabetes (16). Perhaps, the measurement of hepatic tissue visfatin level and its interpretation according to sex would help better understand the relationship between this polymorphism and visfatin gene expression. We could not find any study regarding the association between -4689 G/T polymorphism and hepatic DNL in NAFLD or any metabolic disorder to compare the results.

In our study, there was a significant association between the presence of T allele in -4689 G/T polymorphism and increased serum level of hepatic injury biomarkers. In men, GT genotype was significantly associated with increased serum AST, ALT, and CAP levels. Serum CK18 and cCK18 also had the highest levels in GT genotype but not statistically significant. In women, TT genotype was significantly associated with increased serum AST, ALT, and cCK18 compared to GT and GG genotypes while these markers had no significant differences between GG and GT. Serum CK18 and CAP also had the highest level in TT genotype and the lowest level in GG genotype although the differences were near significant but not reached the level. These associations remained significant even after adjustment for confounding variables in multiple linear regression.

The results indicate that the presence of T allele in -4689 G/T polymorphism is associated with increased biomarkers of hepatic inflammation, cell apoptosis, and necrosis in both sexes and these associations are independent of serum glucose, lipid profile, insulin resistance, dietary intake or physical activity level. A previous study on patients with type 2 diabetes also showed that the frequency of T allele in this polymorphism was higher in pa-

Table 2. Comparison of Baseline Characteristics Among Three Genotypes of -4689 G/T Polymorphism in Men with NAFLD[a]

Variables	GG	GT	TT
Age, y	40.18 ± 13.01	40.25 ± 11.14	36.33 ± 14.64
BMI, kg/m²	30.26 ± 4.63[b]	29.30 ± 5.44	25.28 ± 2.20
W/H ratio	0.90 ± 0.06[b]	0.90 ± 0.06[b]	0.84 ± 0.05
FBS, mg/dL	112.29 ± 27.97[c]	98.16 ± 10.55	95 ± 3
Serum Insulin, micIU/mL	14.84 ± 12.02[b]	11.97 ± 5.62	5.57 ± 1.23
HOMA Index	4.26 ± 3.45[b]	2.94 ± 1.56	1.30 ± 0.27
Serum total cholesterol, mg/dL	185.45 ± 28.20	186.92 ± 57.76	191.67 ± 41.20
Serum LDL-cholesterol, mg/dL	114 ± 32.22	113.67 ± 52.46	135 ± 37.59
Serum HDL-cholesterol, mg/dL	41.37 ± 4.97[c]	46 ± 9.30	40 ± 8.72
Serum triglyceride, mg/dL	148.18 ± 85.7	136.33 ± 61.88[b]	83.67 ± 30.43
Serum NAMPT, ng/mL	2.45 ± 0.80	2.45 ± 1.35	2.34 ± 1.04
Dietary energy intake, kcal/d	2223.52 ± 589.70[c]	2532.03 ± 440.73	2388.78 ± 332.48
Dietary carbohydrate, %	53.85 ± 5.27	51.84 ± 4.94	54.71 ± 4.22
Dietary protein, %	13.60 ± 1.80	13.25 ± 2.33	13.88 ± 2.46
Physical activity, METs-min/w	5045.21 ± 8563.03	3240.28 ± 4249.60	6177.00 ± 8735.59

Abbreviations: BMI, Body Mass Index; FBS, Fasting Blood Sugar; HOMA, Homeostasis Model Assessment; MET, Metabolic Equivalent of Task; NAMPT, Nicotinamide Phosphoribosyltransferase; W/H ratio, Waist to Hip Ratio.
[a] Data presented as Mean ± SD.
[b] Difference with P < 0.2 compared to TT genotype.
[c] Difference with P < 0.2 compared to GT genotype.

Table 3. Comparison of Hepatic Injury and DNL Marker Among Three Genotypes of -4689 G/T Polymorphism in Men with NAFLD

Variables	GG	GT	TT	P Value, GG vs. GT	P Value, GG vs. TT	P Value, GT vs. TT
Serum AST , IU/L	38.81 ± 9.98	53.33 ± 26.07	27.33 ± 2.08	0.04[a]	0.31	0.03[a]
Serum ALT , IU/L	44.18 ± 25.17	71.17 ± 40.84	33± 6.56	0.03[a]	0.57	0.07
Serum ALKP , IU/L	213.56 ± 67.78	185.08 ± 43.88	168.33 ± 62.52	0.21	0.23	0.66
Serum GGT , IU/L	36.31 ± 16.20	43.67 ± 21.59	41.33 ± 18.04	0.31	0.67	0.84
Serum CK 18 , U/L	421.44 ± 256.55	659.69 ± 428.98	341.89 ± 215.73	0.07	0.70	0.15
Serum cCK18 , U/L	189.45 ± 182.02	313.90 ± 299.36	85.18 ± 34.50	0.16	0.47	0.13
CAP , dB/m	288.23 ± 56.40	302.75 ± 44.31	210.33 ± 9.50	0.44	0.02[a]	0.008[b]
LSM , kpa	7.63 ± 4.06	6.82 ± 2.98	4.67 ± 0.57	0.54	0.19	0.35
RBC membrane DNL Index	3.25 ± 0.99	3.25 ± 0.85	2.77 ± 0.72	0.99	0.42	0.43

Abbreviations: ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; CAP, Controlled Attenuation Parameter,; cCK18, Caspase-Cleaved Cytokeratin-18; CK 18, Cytokeratin 18; GGT, Gamma-Glutamyl Transpeptidase; LSM, Liver Stiffness Measurement; DNL Index, De Novo Lipogenesis Index.
[a] Significant difference with P value < 0.05.
[b] Significant difference with P value < 0.01.

tients with type 2 diabetes compared to the control group (30). In contrast to these findings, McKenzie reported that GG genotype was associated with a significantly higher insulin AUC value than TT and GT genotypes. However, it must be considered that in this research, the study population consisted of individuals with sedentary lifestyles, without diabetes, cardiovascular, or hepatic diseases (29).

Another finding of this study was that men were more susceptible to T allele. In men, one T-allele (GT genotype) was enough for increased serum level of hepatic injury biomarkers; but in women two T-alleles (TT genotype) were required to have this outcome. This is in accordance with

Table 4. Comparison of Baseline Characteristics Among Three Genotypes of -4689 G/T Polymorphism in Women with NAFLD[a]

Variables	GG	GT	TT
Age, y	47.12 ± 13.40	49.82 ± 6.44	44 ± 4.24
BMI, kg/m²	31.55 ± 5.15	33.07 ± 3.84	34.07 ± 1.42
W/H ratio	0.87 ± 0.05	0.86 ± 0.04	0.89 ± 0.00
FBS, mg/dL	104.94 ± 16.99[b]	122.09 ± 32.57	129.50 ± 41.72
Serum Insulin, micIU/mL	13.22 ± 5.47[b]	9.99 ± 4.66	12.50 ± 0.09
HOMA Index	3.44 ± 1.50	3.15 ± 1.86	3.97 ± 1.25
Serum total cholesterol, mg/dL	199.21 ± 37.56	194.18 ± 39.34	192.50 ± 58.69
Serum LDL-cholesterol, mg/dL	116.87 ± 33.35	107.18 ± 34.41	102.50 ± 57.27
Serum HDL-cholesterol, mg/dL	49.68 ± 7.81	52.36 ± 15.09	44 ± 9.90
Serum triglyceride, mg/dL	165.93 ± 96.51	173 ± 99.45	230.50 ± 44.54
Serum NAMPT, ng/mL	2.14 ± 1.25	2.93 ± 1.04	2.48 ± 0.36
Dietary energy intake, kcal/d	2249.28 ± 445.90	2385.35 ± 478.52	2487.07 ± 176.72
Dietary carbohydrate, %	50.29 ± 6.90	51.64 ± 4.43	45.81 ± 3.80
Dietary protein, %	12.69 ± 2.26	11.89 ± 2.27	12.66 ± 1.39
Physical activity, METs-min/w	1364.81 ± 1573.70	2532.25 ± 4500.28	960.0 ± 1264.30

Abbreviations: BMI, Body Mass Index; FBS, Fasting Blood Sugar; HOMA, Homeostasis Model Assessment; MET, Metabolic Equivalent of Task; NAMPT, Nicotinamide Phosphoribosyltransferase; W/H ratio, Waist to Hip Ratio.
[a] Data presented as Mean ± SD.
[b] Difference with P < 0.2 compared to GT genotype.

Table 5. Comparison of Hepatic Injury and DNL Marker Among Three Genotypes of -4689 G/T Polymorphism in Women with NAFLD

Variables	GG	GT	TT	P Value, GG vs. GT	P Value, GG vs. TT	P Value, GT vs. TT
Serum AST, IU/L	31.81 ± 10.48	40.36 ± 26.68	73 ± 35.35	0.27	0.01[a]	0.04[a]
Serum ALT, IU/L	28.93 ± 15.88	40.18 ± 30.38	76 ± 11.31	0.21	0.01[a]	0.048[a]
Serum ALKP, IU/L	231.93 ± 93.81	220.36 ± 65.85	190.50 ± 105.36	0.73	0.52	0.65
Serum GGT, IU/L	23.75 ± 10.81	34.45 ± 21.70	37 ± 5.65	0.09	0.27	0.83
Serum CK 18, U/L	424.75 ± 183.69	514.31 ± 363.83	819.01 ± 624.50	0.44	0.08	0.18
Serum cCK18, U/L	154.38 ± 83.82	157.75 ± 108.91	526.62 ± 442.04	0.94	0.001[b]	0.001[b]
CAP, dB/m	310.41 ± 39.31	333.27 ± 22.22	357.50 ± 45.96	0.09	0.07	0.36
LSM, kpa	8.34 ± 3.60	6.92 ± 1.55	6.30 ± 1.70	0.19	0.34	0.78
RBC membrane DNL Index	3.33 ± 1.38	3.19 ± 1.19	2.88 ± 0.41	0.80	0.64	0.75

Abbreviations: ALKP, Alkaline Phosphatase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; CAP, Controlled Attenuation Parameter; cCK18, Caspase-Cleaved Cytokeratin-18; CK 18, Cytokeratin 18; GGT, Gamma-Glutamyl Transpeptidase; LSM, Liver Stiffness Measurement; DNL index, De Novo Lipogenesis Index.
[a] Significant difference with P value < 0.05.
[b] Significant difference with P value < 0.01.

a previous study which showed that TT genotype is associated with higher lipid profile and there is a stronger association between serum hs-CRP and serum NAMPT level in TT genotype of -4689 G/T polymorphism among type 2 diabetic patients. It must be considered that 83.9% of the participants in this study were women (31). McKenzie also reported that there was a significant gender-genotype interaction for the association between -4689 G/T polymorphism and markers of insulin sensitivity (29). These findings may explain the higher prevalence of NAFLD in men which has been reported in recent studies (32, 33). Because, according to our findings, GT genotype is more prevalent than TT genotype in both sexes (Table 1) and this genotype (GT) is associated with increased markers of hepatic injury

in men not in women (including CAP, the marker of hepatic fat infiltration), therefore it may end up in a higher prevalence of NAFLD in men.

On the other hand, if we accept the negative effect of T allele (in -4689 G/T polymorphism) on NAFLD, we expect a higher level of hepatic injury in TT genotype compared to GT genotype in men. But on the contrary, CAP and serum AST level were significantly lower in TT genotype compared to GT genotype. Although, this finding should be viewed with caution because of the low sample size in TT genotype, but two possible interpretations of this issue can be raised. According to previous studies, increased hepatic injury might be associated with reduced steatosis and serum inflammatory markers (34, 35). Thus, the significantly lower CAP and serum AST in TT genotype compared to GT genotype in men could be due to higher hepatic injury in TT genotype. Another interpretation is that, GT genotype in men is located in a haplotype with significant impact on hepatic injury while in women TT genotype is located in such a haplotype.

The human NAMPT gene is located on the long arm of chromosome from 7q22.1 to 7q31.33 and according to previous reports this region is in association with metabolic syndrome, BMI, and lipid profile (36, 37). -4689 G/T polymorphism is located in the promoter region of NAMPT gene so it can affect its transcriptional activity, but to our knowledge, no study has investigated the functional effect of this polymorphism.

The present study is the first investigation on the association of -4689 G/T polymorphism with hepatic DNL and biomarkers of hepatic injury with a focus on sex-genotype interaction for these associations. In this study other variables that could stimulate DNL (38) (age, BMI, HOMA index, waist to hip ratio, blood lipid and glucose profile, serum insulin, dietary energy, carbohydrate and protein intake and physical activity level) were all evaluated and the associations between -4689 G/T polymorphism and the markers of hepatic injury were all adjusted for these variables, so that the independence of these associations could be confirmed.

This study also had some limitations. The low sample size in TT genotype prevents confirmation of a definite relationship for this genotype; therefore, this study can be considered as a hypothesis-generating study. More detailed studies with larger sample sizes are needed to completely understand the association between -4689 polymorphism, hepatic DNL, and hepatic injuries in patients with NAFLD and the influence of sex-genotype interaction on these associations. For instance, direct measurement of DNL activity in hepatic tissue and investigating biopsy-proven pathologic findings may further clarify these associations in NAFLD. Although baseline characteristics of participants in this study were not statistically significant between the genotypes in both sexes, we also suggest that men and women to be carefully matched for confounding variables in future studies.

5.1. Conclusion

-4689 G/T polymorphism in NAMPT gene was not associated with hepatic DNL index but the presence of T allele in this polymorphism was associated with increased biomarkers of hepatic inflammation, apoptosis and necrosis in patients with NAFLD especially in men, as one T allele (GT genotype) was enough for increased biomarkers of hepatic injury in men but not in women.

Acknowledgments

We would like to show our gratitude to Dr. Shabnam Razavi (Microbiology depatment, School of Medicine, Iran University of Medical Sciences) and Ms. Zohre Atai (Genetic depatment, school of Medicine, Iran University of Medical Sciences) who provided insight and expertise that greatly assisted the research.

Footnotes

Authors' Contribution: Concept and design, Bahareh Amirkalali, Farhad Zamani; acquisition of data, Bahareh Amirkalali; analysis and interpretation of data, Ali Gholami; drafting of the manuscript, Bahareh Amirkalali; critical revision of the manuscript for important intellectual content, Farhad Zamani, Farzad Shidfar, Masoud Reza Sohrabi; statistical analysis, Ali Gholami; administrative, technical, and material support, Masoud Reza Sohrabi, Ali Esrafily, Parvaneh Rahimi-Moghaddam, Payam Hosseinzadeh, Hossein Keyvani; study supervision, Farhad Zamani, Farzad Shidfar; Role of the sponsor, Iran University of medical sciences scientifically and ethically assessed the proposal of this study and financially supported this research.

Financial Disclosure: Authors of this manuscript declare that they have no financial interests related to the material in the manuscript.

Implication for Health Policy Makers/Practice/Research/Medical Education: The results of this study could help have a better understanding of NAMPT function in the progress of NAFLD and screen patients who are at higher risk of having advanced stages of this disease.

Funding/Support: This study was supported by Iran University of Medical Sciences.

References

1. Younossi ZM, Stepanova M, Afendy M, Fang Y, Younossi Y, Mir H, et al. Changes in the prevalence of the most common causes of chronic liver diseases in the United States from 1988 to 2008. *Clin Gastroenterol Hepatol.* 2011;**9**(6):524–530 e1. doi: 10.1016/j.cgh.2011.03.020. [PubMed: 21440669] quiz e60.
2. Fan JG. Epidemiology of alcoholic and nonalcoholic fatty liver disease in China. *J Gastroenterol Hepatol.* 2013;**28 Suppl 1**:11–7. doi: 10.1111/jgh.12036. [PubMed: 23855290].
3. Ostovaneh MR, Zamani F, Sharafkhah M, Ansari-Moghaddam A, Akhavan Khaleghi N, Saeedian FS, et al. Prevalence of metabolic syndrome in Amol and Zahedan, Iran: a population based study. *Arch Iran Med.* 2014;**17**(7):477–82. [PubMed: 24979559].
4. Liu Q, Bengmark S, Qu S. The role of hepatic fat accumulation in pathogenesis of non-alcoholic fatty liver disease (NAFLD). *Lipids Health Dis.* 2010;**9**:42. doi: 10.1186/1476-511X-9-42. [PubMed: 20426802].
5. Malik SM, deVera ME, Fontes P, Shaikh O, Ahmad J. Outcome after liver transplantation for NASH cirrhosis. *Am J Transplant.* 2009;**9**(4):782–93. doi: 10.1111/j.1600-6143.2009.02590.x. [PubMed: 19344467].
6. Turati F, Talamini R, Pelucchi C, Polesel J, Franceschi S, Crispo A, et al. Metabolic syndrome and hepatocellular carcinoma risk. *Br J Cancer.* 2013;**108**(1):222–8. doi: 10.1038/bjc.2012.492. [PubMed: 23169288].
7. Lee JJ, Lambert JE, Hovhannisyan Y, Ramos-Roman MA, Trombold JR, Wagner DA, et al. Palmitoleic acid is elevated in fatty liver disease and reflects hepatic lipogenesis. *Am J Clin Nutr.* 2015;**101**(1):34–43. doi: 10.3945/ajcn.114.092262. [PubMed: 25527748].
8. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest.* 2005;**115**(5):1343–51. doi: 10.1172/JCI23621. [PubMed: 15864352].
9. Berlanga A, Guiu-Jurado E, Porras JA, Auguet T. Molecular pathways in non-alcoholic fatty liver disease. *Clin Exp Gastroenterol.* 2014;**7**:221–39. doi: 10.2147/CEG.S62831. [PubMed: 25045276].
10. Hallows WC, Lee S, Denu JM. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc Natl Acad Sci U S A.* 2006;**103**(27):10230–5. doi: 10.1073/pnas.0604392103. [PubMed: 16790548].
11. Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol.* 1994;**14**(2):1431–7. doi: 10.1128/MCB.14.2.1431. [PubMed: 8289818].
12. Genc H, Dogru T, Kara M, Tapan S, Ercin CN, Acikel C, et al. Association of plasma visfatin with hepatic and systemic inflammation in nonalcoholic fatty liver disease. *Ann Hepatol.* 2013;**12**(4):548–55. [PubMed: 23813132].
13. Bekaert M, Verhelst X, Geerts A, Lapauw B, Calders P. Association of recently described adipokines with liver histology in biopsy-proven non-alcoholic fatty liver disease: a systematic review. *Obes Rev.* 2016;**17**(1):68–80. doi: 10.1111/obr.12333. [PubMed: 26597657].
14. Yan JJ, Tang NP, Tang JJ, Jia EZ, Wang MW, Wang QM, et al. Genetic variant in visfatin gene promoter is associated with decreased risk of coronary artery disease in a Chinese population. *Clin Chim Acta.* 2010;**411**(1-2):26–30. doi: 10.1016/j.cca.2009.09.033. [PubMed: 19804767].
15. Bailey SD, Loredo-Osti JC, Lepage P, Faith J, Fontaine J, Desbiens KM, et al. Common polymorphisms in the promoter of the visfatin gene (PBEF1) influence plasma insulin levels in a French-Canadian population. *Diabetes.* 2006;**55**(10):2896–902. doi: 10.2337/db06-0189. [PubMed: 17003359].
16. Hossein-nezhad A, Hosseinzadeh-Attar M, Najmafshar A, Jafari N, Rahmani M, Larijani B. Relationship between genotype and serum levels of adipokines and bone mineral density in type 2 diabetes mellitus patients. *J Diabetes Metab Disord.* 2009;**8**:77–86.
17. Mirzaei K, Hossein-Nezhad A, Javad Hosseinzadeh-Attar M, Jafari N, Najmafshar A, Mohammadzadeh N, et al. Visfatin genotype may modify the insulin resistance and lipid profile in type 2 diabetes patients. *Minerva Endocrinol.* 2009;**34**(4):273–9. [PubMed: 20046156].
18. MacLaren R, Cui W, Cianflone K. Visfatin expression is hormonally regulated by metabolic and sex hormones in 3T3-L1 pre-adipocytes and adipocytes. *Diabetes Obes Metab.* 2007;**9**(4):490–7. doi: 10.1111/j.1463-1326.2006.00625.x. [PubMed: 17587391].
19. Zhou J, Seidel ER. Estrogens induce visfatin expression in 3T3-L1 cells. *Peptides.* 2010;**31**(2):271–4. doi: 10.1016/j.peptides.2009.12.004. [PubMed: 20004694].
20. Sasso M, Tengher-Barna I, Ziol M, Miette V, Fournier C, Sandrin L, et al. Novel controlled attenuation parameter for noninvasive assessment of steatosis using Fibroscan((R)): validation in chronic hepatitis C. *J Viral Hepat.* 2012;**19**(4):244–53. doi: 10.1111/j.1365-2893.2011.01534.x. [PubMed: 22404722].
21. Sandrin L, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol.* 2003;**29**(12):1705–13. doi: 10.1016/j.ultrasmedbio.2003.07.001. [PubMed: 14698338].
22. Sanyal AJ, American Gastroenterological A. AGA technical review on nonalcoholic fatty liver disease. *Gastroenterology.* 2002;**123**(5):1705–25. doi: 10.1053/gast.2002.36572. [PubMed: 12404245].
23. Hudgins LC, Hellerstein M, Seidman C, Neese R, Diakun J, Hirsch J. Human fatty acid synthesis is stimulated by a eucaloric low fat, high carbohydrate diet. *J Clin Invest.* 1996;**97**(9):2081–91. doi: 10.1172/JCI118645. [PubMed: 8621798].
24. Esmaillzadeh A, Mirmiran P, Azizi F. Whole-grain intake and the prevalence of hypertriglyceridemic waist phenotype in Tehranian adults. *Am J Clin Nutr.* 2005;**81**(1):55–63. [PubMed: 15640460].
25. Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc.* 2003;**35**(8):1381–95. doi: 10.1249/01.MSS.0000078924.61453.FB. [PubMed: 12900694].
26. McDowell MA, Fryar CD, Hirsch R, Ogden CL. Anthropometric reference data for children and adults: US population, 1999-2002. *Adv Data.* 2005;**361**(361):1–5.
27. Yilmaz Y. Systematic review: caspase-cleaved fragments of cytokeratin 18 - the promises and challenges of a biomarker for chronic liver disease. *Aliment Pharmacol Ther.* 2009;**30**(11-12):1103–9. doi: 10.1111/j.1365-2036.2009.04148.x. [PubMed: 19769633].
28. Chong MF, Hodson L, Bickerton AS, Roberts R, Neville M, Karpe F, et al. Parallel activation of de novo lipogenesis and stearoyl-CoA desaturase activity after 3 d of high-carbohydrate feeding. *Am J Clin Nutr.* 2008;**87**(4):817–23. [PubMed: 18400702].
29. McKenzie JA. The influence of visfatin and visfatin gene polymorphisms on glucose and obesity-related variables and their responses to aerobic exercise training. 2008.
30. Wang Y, Yan LL, WU JJ, Dong P, Zhang CH, Wang JH. Relationship between single nucleotide polymorphisms of visfatin and type 2 diabetes mellitus. *Acad J Xian Jiaotong Univ.* 2011;**4**.
31. Mirzaei K, Hosseinzadeh MJ, Hossein-Nezhad A, Jafari N, Rahmani M. The association between diabetes evaluation criteria and promoter polymorphism on visfatin gen. *Iran J Diab Metab.* 2009;**8**(3):237–46.
32. Pan JJ, Fallon J, Michael B. Gender and racial differences in nonalcoholic fatty liver disease. *World J Hepatol.* 2014;**6**(5):274–83.
33. Lazo M, Hernaez R, Eberhardt MS, Bonekamp S, Kamel I, Guallar E, et al. Prevalence of nonalcoholic fatty liver disease in the United States: the Third National Health and Nutrition Examination Survey, 1988-1994. *Am J Epidemiol.* 2013;**178**(1):38–45. doi: 10.1093/aje/kws448. [PubMed: 23703888].
34. Tiniakos DG. Nonalcoholic fatty liver disease/nonalcoholic steatohepatitis: histological diagnostic criteria and scoring systems. *Eur J Gastroenterol Hepatol.* 2010;**22**(6):643–50. doi: 10.1097/MEG.0b013e32832ca0cb. [PubMed: 19478676].
35. Caldwell SH, Lee VD, Kleiner DE, Al-Osaimi AM, Argo CK, Northup PG, et al. NASH and cryptogenic cirrhosis: a histological analysis. *Ann Hep-*

atol. 2009;**8**(4):346–52. [PubMed: 20009134].
36. Stumvoll M, Tschritter O, Fritsche A, Staiger H, Renn W, Weisser M, et al. Association of the T-G polymorphism in adiponectin (exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes. *Diabetes.* 2002;**51**(1):37–41. doi: 10.2337/diabetes.51.1.37. [PubMed: 11756320].
37. Wu X, Cooper RS, Borecki I, Hanis C, Bray M, Lewis CE, et al. A combined analysis of genomewide linkage scans for body mass index from the National Heart, Lung, and Blood Institute Family Blood Pressure Program. *Am J Hum Genet.* 2002;**70**(5):1247–56. doi: 10.1086/340362. [PubMed: 11923912].
38. Hellerstein MK, Schwarz JM, Neese RA. Regulation of hepatic de novo lipogenesis in humans. *Annu Rev Nutr.* 1996;**16**:523–57. doi: 10.1146/annurev.nu.16.070196.002515. [PubMed: 8839937].