Histological Characteristics of Non-alcoholic Steatohepatitis in NAFLD Patients With Low Degree of Hepatocyte Apoptosis

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ABSTRACT

Background: *Caspase-cleaved K18 (cK18) may accurately reflect hepatocyte apoptosis in patients with non-alcoholic steatohepatitis (NASH). However, NASH can also exist within the normal range of cK18. The aim of this study was to investigate the risk factors and characteristics of NASH within the normal serum levels of cK18.*

Methods: *In the study, 227 histopathologically confirmed non-alcoholic fatty liver disease (NAFLD) patients with normal cK18 levels (≤200 U/L), measured in serum using ELISA kits, were enrolled. The Rs738409 allele, coding patatin-like phospholipase domain-containing protein 3 (PNPLA3), was detected by MALDI-TOF mass spectrometry. Non-alcoholic steatohepatitis was defined as an NAFLD activity score (NAS) ≥5 with each part >0.*

Results: *The prevalence of NASH was 31.7% among NAFLD patients with normal serum cK18 levels. Compared with non-NASH, NASH had a higher possibility of occurrence with central obesity, insulin resistance, and the G allele of PNPLA3. The mean serum levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were higher in NASH patients. Moreover, ALT, AST, TC, LDL-C, central obesity, and the PNPLA3 G allele were risk factors for NASH in NAFLD patients with normal serum cK18 levels, with odds ratios of 1.01 (95% CI: 1.00, 1.02), 1.03 (95% CI: 1.01, 1.05), 1.33 (95% CI: 1.04, 1.68), 1.41 (95% CI: 1.03, 1.92), 2.19 (95% CI: 1.15, 4.18), and 2.48 (95% CI: 1.15, 5.36), respectively; all P < .05.*

Conclusions: *The major risk factors for NASH were central obesity, AST, and the PNPLA3 G allele, in NAFLD with low hepatocyte apoptosis.*

Keywords: *Caspase-cleaved K18, hepatocyte apoptosis, non-alcoholic steatohepatitis, non-alcoholic fatty liver disease, central obesity, aspartate aminotransferase, patatin-like phospholipase domain-containing protein 3*

INTRODUCTION

Parallel to the increasing prevalence of metabolic syndrome and obesity, non-alcoholic fatty liver disease (NAFLD) has gradually become the most common reason for chronic liver disease, affecting more than a quarter of Asia's population.[1-4] Unchallenged, NAFLD creates a potentially enormous cost to society and patients.[5,6] Non-alcoholic steatohepatitis (NASH), which is the aggressive form of NAFLD, can progress to cirrhosis and hepatocellular cancer and is rapidly becoming the leading cause for end-stage liver disease or liver transplantation.[7]

Globally, the mortality rate due to liver disease among NASH patients is approximately 11.8%.[8]

Increasing evidence suggests that hepatocyte apoptosis plays a significant role in the progression of NAFLD. Meanwhile, serum caspase-cleaved K18 (cK18) levels (i.e., a serum biomarker, also recommended by the United States practice guidelines) have shown promising results for diagnosing NASH.[9-11] Moreover, serum cK18 is predominantly found in hepatocytes and is a specific marker for early apoptosis, with a specificity of more than 80% for detecting NASH.[12-15] Therefore, patients with persistently increased levels of cK18 are considered to be at high risk for NASH, making it one of the indications for liver biopsy.[16] Serum cK18 levels within the normal range can be perceived as indicating low apoptotic activities in the liver and a low risk for NASH. However, patients with

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serum cK18 levels below the abnormal range may still progress to NASH from simple steatosis.

We aim to characterize the clinical features of NAFLD patients with normal serum levels of cK18, and among them, the risk factors for NASH progression.

METHODS

Patients

Patients with suspected NAFLD were prospectively enrolled at our hospital over the course of 3 years (December 2016 to December 2019). The inclusion criteria were as follows: (1) aged 18 years or older; (2) provided written informed consent; (3) suspected as having fatty liver disease by ultrasound, CT, MRI, or FibroScan® and subsequently confirmed by biopsy; and (4) had available data for serum cK18. Patients who met these conditions were excluded: (1) excessive alcohol consumption (more than 140 g per week for males or 70 g per week for females); (2) diagnosed with viral hepatitis; and (3) serum level of cK18 >200 U/L.

The study protocol was approved by the ethics committee of the local hospital and the cohort was registered in the clinical trial registry of China.

Measurement of Serum Caspase-Cleaved K18

Serum samples were collected from patients in an overnight (8-hour) fasting condition on the day of liver biopsy and stored at –80°C prior to testing. We measured cK18 in serum by using a commercially available ELISA kit provided by Herui Biomed Company (Suzhou, China). The inter-assay and intra-assay variations were both <15%. The normal range of cK18 was defined as ≤200 U/L, according to the manufacturer's instruction. It should also be noted that there is currently no consensus on the threshold of normal cK18 levels.[15]

MAIN POINTS

- *The study focused on investigating the clinical characteristics of patients with non-alcoholic steatohepatitis (NASH) with a normal apoptotic marker (caspase-cleaved K18 (cK18)).*
- *The incidence of steatohepatitis was 37% in non-alcoholic fatty liver disease (NAFLD) patients with a low degree of liver apoptotic activity.*
- *The independent risk factors for NASH in NAFLD patients who had normal levels of the apoptotic marker were central obesity, AST, and the G allele of PNPLA3-I148M.*

Anthropometry

Blood pressure, weight, height, and waist circumference were measured on the same day as the liver biopsy. Blood pressure was measured with patients seated in a quiet environment for at least 10 minutes. The calculation of BMI (kg/m^2) was weight (kg) divided by height (m^2). The circle through the point between the iliac crest and the lower ribs was measured as waist circumference.

Comorbidity Definition

Diabetes was diagnosed as fasting glucose more than 7 mmol/L, hemoglobin A1c (HbA1c) more than 6.5%, or the use of anti-hyperglycemic agents. Hypertension was diagnosed as systolic pressure more than 140 mmHG, diastolic pressure more than 90 mmHG, or a history of use of anti-hypertensive agents. Dyslipidemia was defined as any of the following criteria: high-density lipoprotein cholesterol (HDL-C) less than 1.0 mmol/L for females and 1.3 mmol/L for males; low-density lipoprotein cholesterol (LDL-C) more than 3.4 mmol/L; total cholesterol more than 5.2 mmol/L and triglycerides more than 1.7 mmol/L, or the use of lipid-lowering drugs. The waist circumference of more than 90 cm for males and more than 80 cm for females was considered as central obesity.

Clinical and Laboratory Data

All blood samples were collected from patients in a fasting condition. The laboratory data included levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), HDL-C, LDL-C, total cholesterol, triglycerides, fasting glucose, HbA1c, and fasting insulin. The measurements were obtained through an automated analyzer (Abbott AxSYM), which met with standard methods. The formula for homeostasis model assessment insulin resistance (HOMA-IR) was fasting glucose (mmol/L) * fasting insulin (mU/ml)/22.5.

Genetic Analysis

The DNA sample extracted from whole blood was approximately 20 ng for each patient. The I148M patatin-like phospholipase domain-containing protein 3 (PNPLA3), that is related with NAFLD, was evaluated. The method of measuring PNPLA3-I148M (rs738409) has been reported previously.[17] In brief, the allele type of PNPLA3-I148M (rs738409) was detected by MALDI-TOF mass spectrometry.

Liver Histology

In brief, liver specimen was collected from all patients by percutaneous liver biopsy, guided by ultrasound.

Histology assessment was undertaken by an experienced liver pathologist according to the NASH-Clinical Research Network Scoring System.[18] The pathologist was blinded to patients'clinical and biochemical details. Non-alcoholic steatohepatitis was defined as a non-alcoholic fatty liver disease activity score (NAS) ≥5 with at least 1 score for each of the 3 histologic components (steatosis, ballooning, and lobular inflammation). Fibrosis ≥F2 was considered as significant fibrosis.

Statistical Analysis

Continuous variables were expressed as mean ± SD and compared by the Student's *t*-test. Categorical variables were expressed as frequency (%) and compared by the chi-square test. Independent risk factors for NASH were measured by the odds ratio (OR), which was calculated by multivariate regression analysis. Statistical analysis was performed using R software (version 3.5.2, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline Characteristics of the Study Population

We enrolled 489 biopsy-proven patients from 2016 to 2019. The number of patients excluded for excessive alcohol intake, infected with viral hepatitis, and abnormal CK-18 M30 were 48, 30, and 184, respectively. A total of 227 NAFLD patients were included for the final analysis. Table 1 shows the main clinical, biochemical, and genetic characteristics of biopsy-proven NAFLD patients with a normal range of cK18 (i.e., <200 U/L). Among these patients, 70.5% were male, 90.6% had dyslipidemia, and 66.4% were centrally obese. Genetic profiling by PNPLA3-I148M (rs738409) polymorphism revealed that these patients were predominantly carriers of the wild-type (CC), while only 50 (25.6%) were carriers of the risk G allele. Interestingly, among these NAFLD patients with normal serum levels of cK18, we observed 72 (31.7%) patients diagnosed with NASH and 44 (19.4%) patients with significant fibrosis.

Characteristics of Non-alcoholic Fatty Liver Disease Patients With Normal Serum Caspase-Cleaved K18 Levels Stratified by Non-alcoholic Steatohepatitis Status

As summarized in Table 2, there was a significantly lower proportion of male patients (66.1% vs. 74.8%, $P = .04$) and a markedly higher proportion of centrally obese patients (77.5% vs. 61.1%, $P = .02$) in the NASH as compared to the non-NASH group. NASH patients, compared with the non-NASH, had a more severe biological profile in that they had a higher levels of ALT (71 U/L vs. 44 U/L, $P < .01$), AST (40 U/L vs. 30 U/L, $P < .01$), total

Table 1. Baseline Characteristics of Histopathologically Confirmed NAFLD Patients with Normal Range of Caspase-Cleaved K18

	Total Population
Variables	n = 227
Demographics	
Age (years)	43 ± 11
Male (%)	160 (70.5)
Anthropometry	
Waist circumference (cm)	91 ± 9
BMI (kg/m^2)	26 ± 3
Concomitant diseases	
Diabetes (%)	65 (28.8)
Hypertension (%)	65 (28.6)
Dyslipidemia (%)	203 (90.6)
Central obesity (%)	146 (66.4)
Laboratory parameters	
ALT (U/L)	52 ± 69
AST (U/L)	33 ± 22
Total cholesterol (mmol/L)	5.08 ± 1.22
Triglycerides (mmol/L)	2.19 ± 1.33
HDL (mmol/L)	1.05 ± 0.25
LDL (mmol/L)	3.01 ± 0.93
Fasting glucose (mmol/L)	5.7 ± 1.5
Hemoglobin A1c (%)	6.20 ± 1.51
HOMA-IR	5.1 ± 7.6
Cytokeratin 18 M30 (U/L)	100 ± 50
Genetic variables	
PNPLA3 I148M polymorphism	
GG+CG	50 (25.6)
CC	145 (74.4)
Histological characteristics	
Steatosis (%)	
1	125 (55.1)
2	45 (19.8)
3	57 (25.1)
Ballooning (%)	
0	30 (13.2)
1	129 (56.8)
2	68 (30.0)
Lobular inflammation (%)	
0	21 (9.3)
1	180 (79.3)
2	26 (11.5)
3	0 (0.0)
NAS score	3.9 ± 1.4
NASH (%)	72 (31.7)
Significant fibrosis (%)	44 (19.4)

Categorical values are shown as n (%). Continuous variables are shown as mean ± SD. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance.

Table 2. Baseline Characteristics of Histopathologically Confirmed NAFLD Patients with Normal Range of Caspase-Cleaved K18, Stratified by NASH Status

	Non-NASH	NASH	*P*
Variables	n = 155	n = 72	
Demographics			
Age (years)	44 ± 11	42 ± 12	.30
Male (%)	116 (74.8)	44 (61.1)	.04
Anthropometry			
Waist circumference (cm)	26.0 ± 3.1	26.7 ± 3.1	.09
BMI (kg/m^2)	90 ± 9	92 ± 9	.36
Concomitant diseases			
Diabetes (%)	41 (26.6)	24 (33.3)	.30
Hypertension (%)	40 (25.8)	25 (34.7)	.17
Dyslipidemia (%)	136 (88.3)	67 (95.7)	.08
Central obesity (%)	91 (61.1)	55 (77.5)	.02
Laboratory parameters			
ALT (U/L)	44 ± 57	71 ± 87	<.01
AST (U/L)	30 ± 16	40 ± 31	<.01
Total cholesterol (mmol/L)	4.95 ± 1.21	5.36 ± 1.19	<.01
Triglycerides (mmol/L)	2.21 ± 1.40	2.16 ± 1.18	.92
HDL (mmol/L)	1.02 ± 0.22	1.12 ± 0.29	<.01
LDL (mmol/L)	2.92 ± 0.94	3.21 ± 0.88	.02
Fasting glucose (mmol/L)	5.6 ± 1.5	6.0 ± 1.7	.09
Hemoglobin A1c (%)	6.17 ± 1.56	6.26 ± 1.39	.70
HOMA-IR	4.4 ± 6.6	6.4 ± 9.3	<.01
Cytokeratin 18 M30 (U/L)	97 ± 48	108 ± 53	.11
Genetic variables			
PNPLA3 rs738409			.02
GG+CG	90 (67.7)	52 (83.9)	
CC	43 (32.3%)	10 (16.1)	

Significant *P* values are marked in bold.
Categorical values are shown as n (%). Continuous variables are shown as mean ± SD. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance.

cholesterol (5.36 mmol/L vs. 4.95 mmol/L, $P < .01$), HDL (1.12 mmol/L vs. 1.02 mmol/L, $P < .01$), LDL (3.21 mmol/L vs. 2.92 mmol/L, $P < .01$), and HOMA-IR (6.4 vs. 4.4, $P < .01$); while the serum levels of cK18 were not significantly different (108 U/L vs. 97 U/L, $P = .11$). Significantly higher proportions of NASH patients were carriers of the risk G allele of PNPLA3-I148M (rs738409) when compared to non-NASH patients (83.9% vs. 67.7%, $P = .02$).

Table 3. Independent Risk Factors for Steatohepatitis in Histopathologically Confirmed NAFLD in Patients With Normal Range of Caspase-Cleaved K18

Risk Factors	Odds Ratio (95% CI)	*P*
Central obesity	2.19 (1.15, 4.18)	.02
Alanine aminotransferase	1.01 (1.00, 1.02)	.04
Aspartate aminotransferase	1.03 (1.01, 1.05)	.01
Total cholesterol	1.33 (1.04, 1.68)	.02
LDL-cholesterol	1.41 (1.03, 1.92)	.03
PNPLA3 rs738409 (GG+CG)	2.48 (1.15, 5.36)	.02

Data are expressed as odds ratio (OR) and 95% CI as tested by multivariable logistic regression analyses. In these regression models, the dependent variable was the presence of NASH on histology.

Independent Risk Factors for Non-alcoholic Steatohepatitis Among Patients With Normal Serum Caspase-Cleaved K18 Levels

In Table 3, using the multivariate logistic regression analysis, central obesity, ALT, AST, total cholesterol, LDL, and the PNPLA3-I148M risk G allele were independently and significantly related with the incidence of NASH, with ORs (OR) of 2.19 ($P = .02$), 1.01 ($P = .04$), 1.03, ($P = .01$), 1.33, ($P = .02$), 1.41($P = .03$), and 2.48 ($P = .02$), respectively.

Subgroup Analysis of Patients With Caspase-Cleaved K18 Normal Serum Levels and With Normal ALT Levels

ALT is traditionally recognized as a specific biomarker for liver injury. However, NASH patients may present with lower liver apoptotic activity, and may test normal for serum ALT.[19] We analyzed a subgroup of 121 NAFLD patients with normal levels of serum cK18 and ALT. The baseline of this subgroup, divided as NASH and non-NASH, is shown in Supplementary Table 1. Compared with non-NASH patients, there was a lower proportion of males (29.2% vs. 71.1%, $P < .01$) and a higher proportion of central obesity (87.0% vs. 59.6%, $P < .01$) among NASH patients. Moreover, NASH patients had a higher mean value of HOMA-IR (8.8 vs. 4.6, $P = .01$) when compared with non-NASH patients; whereas the levels of HDL, LDL, total cholesterol, and cK18 were not significantly different. The NASH patients of this subgroup also had a higher proportion of the risk G allele as compared with the non-NASH patients (86.4% vs. 61.2%, $P = .03$). Using multiple logistic regression (Supplementary Table 2), we found that the independent risk factors for NASH were central obesity (OR = 4.52, $P = .02$), AST (OR = 1.11, $P = .02$), and the G allele of PNPLA3-I148M (rs738409) (OR = 4.02, $P = .04$).

DISCUSSION

As far as we know, this is a novel investigation to explore NASH patients with low degree of apoptotic activity in the liver. Among NAFLD patients with normal serum levels of cK18, there was a 31.7% prevalence of NASH, which may be suggestive of missed diagnoses in those who opt out of liver biopsy. We found that NAFLD patients who also had central obesity, higher LDL, ALT, AST, or were carriers of the PNPLA3 G allele would have a higher risk for NASH. Similarly, in the subgroup of NAFLD patients with normal levels of both ALT and cK18, considered to indicate a low degree of hepatocyte injury as well as apoptotic activity, we found that central obesity, AST, and the risk G allele of PNPLA3-I148M (rs738409) were independent risk factors for NASH.

The relationship between PNPLA3-I148M polymorphism and NAFLD has already been demonstrated by various studies. Koo et al. suggested that carriers of the risk G allele of PNPLA3 were more likely to develop NAFLD and progress to NASH and fibrosis.[20,21] While one study reported the association of PNPLA3 and decreased kidney function among NAFLD patients, another reported that the risk G allele is found to be associated with early glomerular and tubular damage in NAFLD patients who had persistently normal ALT levels.[22,23] Similarly, in our study, we found a high prevalence NASH in carriers of the PNPLA3-I148M risk G allele, among patients with normal serum levels of cK18.

In both NASH and non-NASH groups with lower levels of cK18, we observed abnormal liver biochemistry, such as ALT level. This suggested that liver injury may occur even with lower apoptotic activity and that alternative pathways exist for liver injury. Although we found that LDL-C and total cholesterol were higher in NASH patients compared with non-NASH patients, a similar increase was not discovered in the subgroup. Therefore, excess accumulation of LDL-C and total cholesterol in the liver may be associated with liver injury.[24] Other studies have reported that insulin resistance may contribute to NASH in NAFLD patients with normal ALT, while cK18 was closely related to insulin resistance.[25-27] Our findings were similar to the others in that HOMA-IR was significantly increased in NASH patients, both in those with lower levels of apoptotic activity (i.e., low cK18 levels) and in those with combined lower apoptotic activity and normal ALT levels. However, HOMA-IR was not found to be an independent risk factor for NASH, while obesity and elevated AST were independent risk factors for NASH in both patients of the main analysis and the subgroup analysis. Therefore, in NAFLD patients with normal cK18 levels, clinicians should pay close attention to risk factors such as central obesity, increased serum AST levels, and presence of the risk G allele of PNPLA3-I148M (rs738409) for NASH diagnosis and histological assessment.

Current non-invasive algorithms (e.g., FIB-4 and NFS) may be useful to exclude advanced fibrosis among NAFLD patients with normal transaminase levels.[28] However, NASH is often difficult to assess non-invasively. Yilmaz et al.[29,30] reported that cK18 levels are associated with the presence of NASH among NAFLD patients with a normal range of ALT. Compared with NASH, non-NASH was associated with higher levels of cK18 in the patients with normal ALT levels.[29] In addition, we previously reported that cK18 was an independent predictor of NASH in NAFLD patients with normal ALT levels.[31] This evidence suggests that cK18 assessment might be a reliable method to predict NASH, as an alternative to liver biopsy.

The strength of our study includes the use of a well-characterized prospective cohort of patients with histopathologically confirmed NAFLD. However, we also acknowledge some important limitations. While there is no definite threshold value to distinguish NASH from simple steatosis, the normal range of cK18 we used was ≤200 U/L, according to the manufacturer's instruction. The inclusion of only patients of Chinese Han ethnicity may limit the extrapolation and generalization of our results to other ethnic groups; also, the sample size of those with normal serum cK18 is relatively small.

Our study suggested that central obesity, AST, and the risk G allele of PNPLA3-I148M (rs738409) were independent risk factors for NASH in NAFLD patients with lower levels of hepatocyte apoptotic activity and liver injury.

Ethics Committee Approval: Ethics committee of the First Affiliated Hospital of Wenzhou Medical University (2016-246, December 1, 2016).

Informed Consent: Chinese Clinical Trial Registry (ChiCTR-EOC-17013562).

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Supplementary Table 1. Baseline Characteristics of Histopathologically Confirmed NAFLD Patients With Normal Range of Both Caspase-Cleaved K18 and Alanine Aminotransferase, Stratified by NASH Status

Variables	Non-NASH	NASH	Total	
	n = 97	n = 24	n = 121	*P*
Demographics				
Age (years)	46 ± 11	48 ± 12	46 ± 11	.26
Male (%)	69 (71.1)	7 (29.2)	76 (62.8)	<.01
Anthropometry				
Waist circumference (cm)	90 ± 9	90 ± 7	90 ± 8	.76
BMI (kg/m^2)	26.0 ± 3.1	26.3 ± 2.9	26.1 ± 3.1	.42
Concomitant diseases				
Diabetes (%)	31 (32.3)	11 (45.8)	42 (35.0)	.24
Hypertension (%)	24 (24.7)	6 (25.0)	30 (24.8)	.98
Dyslipidemia (%)	88 (90.7)	23 (100.0)	111 (92.5)	.24
Central obesity (%)	56 (59.6)	20 (87.0)	76 (65.0)	.02
Laboratory parameters				
ALT (U/L)	27 ± 8	28 ± 7	28 ± 8	.72
AST (U/L)	24 ± 4	27 ± 7	24 ± 5	.11
Total cholesterol (mmol/L)	4.85 ± 1.16	5.28 ± 1.40	4.93 ± 1.21	.16
Triglycerides (mmol/L)	2.23 ± 1.57	2.17 ± 1.15	2.22 ± 1.49	.63
HDL (mmol/L)	1.01 ± 0.22	1.15 ± 0.34	1.04 ± 0.25	.06
LDL (mmol/L)	2.83 ± 0.92	3.12 ± 0.97	2.89 ± 0.93	.16
Fasting glucose (mmol/L)	5.8 ± 1.6	6.6 ± 2.2	5.9 ± 1.8	.11
Hemoglobin A1c (%)	6.40 ± 1.83	6.54 ± 1.43	6.4 ± 1.8	.62
HOMA-IR	4.6 ± 7.6	8.8 ± 12.8	5.4 ± 8.9	.01
Cytokeratin 18 M30 (U/L)	87 ± 46	90 ± 60	88 ± 49	.90
Genetic variables				
PNPLA3 rs738409				.03
GG+CG	52 (61.2)	19 (86.4)	23 (21.5)	
CC	33 (38.8)	3 (13.6)	84 (78.5)	

Significant *P* values are marked in bold.
Categorical values are shown as n (%). Continuous variables are shown as mean ± SD. BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance.

Supplementary Table 2. Independent Risk Factors for Steatohepatitis in Histopathologically Confirmed NAFLD in Patients with Normal Range of Both Caspase-Cleaved K18 and Alanine Aminotransferase

Risk Factors	Odds Ratio (95% CI)	*P*
Central obesity	4.52 (1.26, 16.30)	.02
Aspartate aminotransferase	1.11 (1.01, 1.21)	.02
PNPLA3 rs738409 (GG+CG)	4.02 (1.10, 14.65)	.04

Data are expressed as odds ratio (OR) and 95% CI as tested by multivariable logistic regression analyses. In these regression models, the dependent variable was the presence of NASH on histology.