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Association of Serum Homocysteine Levels with Histological Severity of NAFLD

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ABSTRACT

Background & Aims: Studies on the association between homocysteine and non-alcoholic fatty liver disease (NAFLD) have shown inconsistent results. Our study concerns the association of homocysteine with the histological severity of NAFLD, especially non-alcoholic steatohepatitis (NASH) and significant fibrosis (SF) after adjusting for other well-identified risk factors.

Methods: This study enrolled 289 patients with biopsy proven NAFLD. The association of homocysteine with the severe histological features was examined using multivariable logistic regression analysis and subgroup analysis. The area under curves (AUC) and Hosmer-Lemeshow goodness-of-fit test for the adjusted logistic regression models was analyzed.

Results: After multivariable regression analysis, homocysteine showed significant correlation with NASH (OR 0.79 95%CI: 0.69-0.89), p<0.001) and SF (OR 0.83 95%CI: 0.72-0.95, p=0.009). Spearman's correlation analysis showed homocysteine levels were inversely correlated with the grade of hepatocellular ballooning and the stage of liver fibrosis (Spearman's ρ=-0.13, p=0.033; Spearman's ρ=-0.16, p=0.007), but had no correlation with the severity of steatosis and lobular inflammation. The subgroup analyses showed that homocysteine was strongly associated with NASH in females but was weaker in males (female OR: 0.61 95%CI: 0.45-0.84; male 0.86 95%CI: 0.75-0.99), and on SF showed no significant differences in the subgroups. The models showed good discrimination for NASH (AUC 0.789, 95% CI: 0.736-0.843) and for SF (0.784 95%CI: 0.719-0.848) and calibration (Hosmer-Lemeshow goodness-of-fit test, p=0.346 for NASH; p=0.908 for SF).

Conclusion: Elevated serum homocysteine levels are negatively associated with NASH and SF in subjects with NAFLD.

Key words: NAFLD-NASH – significant fibrosis – homocysteine.

Abbreviations: ALP: alkaline phosphatase; ALT: aspartate aminotransferase; AST: alanine aminotransferase; γGT: gamma-glutamyl transferase; HCC: hepatocellular carcinoma; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; NAFLD: non-alcoholic fatty liver disease; NASH: non- alcoholic steatohepatitis; NAS: NAFLD Active Score; PLT: platelet; ROS: reactive oxygen species; SF: significant fibrosis; TB: total bilirubin; UA: uric acid.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease worldwide [1]. The prevalence of NAFLD in the global population is about 25% and is still rising parallel to western diet, sedentary lifestyle, and increased prevalence of obesity [2]. NAFLD is defined as the presence of steatosis in at least 5% of hepatocytes, in the exclusion of other liver disease, such as alcoholic liver disease, chronic viral hepatitis, use of medications that induce steatosis and other chronic liver diseases, such as autoimmune hepatitis, Wilson's disease, etc. [3]. It includes a spectrum of liver diseases that ranges from simple fat accumulation in hepatocytes to liver necroinflammation, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [4]. Non-alcoholic steatohepatitis (NASH), which is a more progressive type of NAFLD, is characterized by the presence of steatosis, lobular inflammation and hepatocyte ballooning [5]. The patient with steatohepatitis can progress to liver fibrosis more rapidly than without it (7 years per fibrosis stage versus 14 years) [6]. Fibrosis is considered to be the only histological feature that is independently associated with an increased likelihood of liver-related events and all-cause (i.e., cardiovascular disease)

mortality [7], increasing with the increased stage of fibrosis [8]. Liver biopsy is considered the golden standard for distinguishing NASH from NAFL and staging fibrosis.

In the United States, NAFLD has become the second leading indication for liver transplantation and the third leading cause of HCC, resulting in severe economic burden [9, 10]. Therefore, it is extremely important to fully understand the mechanistic pathways of NAFLD and disease progression. "The multiple-hit" hypothesis that includes insulin resistance, oxidative stress, nutritional factors largely explain the pathogenesis, but knowledge on the mechanisms of NAFLD still remains incomplete [11].

Homocysteine, formed as an intermediary in methionine metabolism, is a sulfur-containing amino acid [12]. It can be remethylated to methionine or catabolized in the transsulfuration pathway to cysteine [13]. The liver is central for the synthesis and catabolism of homocysteine, as it metabolizes the majority of dietary methionine [14]. Thus, it is plausible that changes in homocysteine levels may occur in the event of liver injury. In recent years, a few original studies have explored the association between homocysteine and NAFLD [15-19]. However, the conclusions are still controversial, and the data based on biopsy proven NAFLD are lacking.

For this reason, we studied in biopsy proven NAFLD the posible independent association between serum homocysteine levels and NASH and significant fibrosis (SF) after adjusting for other well-identified risk factors (e.g. metabolic risk factors and insulin resistance).

METHODS

Patients and study design

We performed a retrospective, cross-sectional study searching the liver biopsy database from The First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China. The clinical records of 1371 subjects during January 2016 and July 2019 were reviewed. We excluded 1082 subjects because of the following: (1) ≤ 18-year-old; (2) the steatosis ≤5% of liver cells at histology; (3) excessive alcohol consumption (>140 g/week for men and >70 g/week for women) evaluated by a questionnaire; (4) history of malignancy; (5) history of viral hepatitis, autoimmune hepatitis, or other forms of chronic liver disease; (6) insufficient clinical data. Finally, 289 strictly screened subjects were enrolled in this study. No informed consent was required because all the data were anonymized. The protocol was in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the Medical University.

Data collection and measurements

Data was collected from the time of liver biopsy. Standing height and body weight of the subjects were measured, when they were barefoot and wearing light clothing. Body mass index (BMI) was categorized as normal: <24 kg/m^2 and overweight: (based on Chinese criteria): overweight: ≥ 24. Venous blood sampling was collected after overnight fasting for at least 8-12 hours and measured at the hospital Clinical Sample Test Room. Homocysteine (μmol/L), triglyceride (TG) (mmol/L), total cholesterol (TC) (mmol/L), low-density lipoprotein cholesterol (LDL-c) (mmol/L), high-density lipoprotein cholesterol (HDL-c) (mmol/L), albumin (g/L), aspartate aminotransferase (ALT) (U/L), alanine aminotransferase (AST) (U/L), alkaline phosphatase (ALP) (U/L), gamma-glutamyl transferase (γGT) (U/L), glucose (mmol/L), insulin (mU/l), platelet (PLT)(*10^9), total bilirubin (TB) (μmol/L) and uric acid (UA) (μmol/L) were measured with standard clinical chemistry laboratory techniques. As for the measuring serum homocysteine levels, after about 2ml venous blood was extracted by the nurse, it was transported to the test room at low temperature and the serum was centrifuged within 1 hour. The serum was stored at 2-8^{0}C for testing within 48 hours. The serum homocysteine levels were performed by standard methods using automated techniques (Beckman Coulter AU5811, USA). The test kit was made by Zhejiang Kuake Biotechnology Co., Ltd. Insulin resistance was evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR): fasting blood glucose (mmol/l)*insulin (mU/l) / 22.5 [20]. Diabetes mellitus was diagnosed by fasting blood glucose ≥7.0 mmol/L and/or treatment with antidiabetic drugs. Hypertension was defined as systolic blood pressure ≥140 mmHg/diastolic blood pressure ≥90 mmHg and/or the current use of anti-hypertensive medication. Smokers were defined as those who had smoked at least one cigarette per day during the previous year.

Liver biopsy

Liver biopsy was performed by senior operators using 16G x 16cm biopsy needles under ultrasonography positioning. The liver specimens were fixed in 10% formalin and were scored by experienced hepatologists who were blinded to the clinical data, treatment allocation, and imaging findings. A scoring system published by Kleiner et al. [21] was used. The histological NAFLD Active Score (NAS) consists of 0 to 8 points that represent the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2). Subjects with scores of 5 or greater were diagnosed as NASH. Fibrosis was staged as follows: stage 0 = no fibrosis; stage 1 = perisinusoidal or periportal fibrosis with 3 different patterns: 1a = mild, zone 3, perisinusoidal; 1b= moderate, zone 3, perisinusoidal fibrosis, and 1c = portal/periportal fibrosis; stage 2 = perisinusoidal and portal/periportal fibrosis; stage 3 = bridging fibrosis; stage 4 = cirrhosis. In this study, we pooled the subtype 1a, 1b, 1c of fibrosis into a single F1 score. Significant fibrosis (SF) was defined as stage 2 or greater (≥2).

Statistical analysis

Continuous data were presented as mean ± standard deviation (normal distribution) or median (1st quartile, 3rd quartile) (skewed distribution), and categorical variables were expressed as frequency or percentage. First, The One-Way ANOVA (normal distribution), Kruskal-Wallis *H* (skewed distribution) test and chi-square tests (categorical variables) were used to determine any statistical differences between the means and proportions of the groups. Second, the Univariate linear regression model was used to evaluate the associations between variables and NASH and SF, respectively. Third, according to the recommendation of the STROBE statement [22], we simultaneously showed the results from unadjusted,

minimally adjusted analysis and those from fully adjusted analyses. The covariates, when added to this model, changed the matched odds ratio by at least 10% and were adjusted. The continuous variables that were entered in the models were all checked for the functional form-the log-linearity assumption. The multicollinearity between variables was checked after multivariable analysis. Fourth, Spearman's correlation analysis was performed to assess the relationship between serum homocysteine levels and histological features. Fifth, subgroup analyses were performed using stratified linear regression models. The modifications and interactions of subgroups were inspected by likelihood ration tests. Lastly, the area under curves (AUC) and Hosmer-Lemeshow goodness-of-fit test for the adjusted logistic regression models was analyzed. All of the analyses were performed with the statistical software packages R (http://www.R-project.org, The R Foundation) and EmpowerStats (http://www.empowerstats.com, X&Y Solutions, Inc., Boston, MA). A p value less than 0.05 (two-sided) was considered statistically significant.

RESULTS

A total of 289 people were enrolled in this study, with an average age of 41.90±12.29 years old, among which 72.66% were males. Biopsy proven NASH was found in 116 patients (men: 83, 71.6%). Significant fibrosis was found in 62 subjects (men: 42, 67.7%). Data were divided into tertiles according to serum homocysteine levels (T1: 7-10, T2: 11-12, T3: 13-36). Baseline characteristics were listed in Table I.

The results of univariate analysis are shown in Table II. BMI, HOMA-IR, serum level of TC, LDL-c, AST, ALT, ALP, γGT, TB and UA were positively correlated with NASH. Age was negatively associated with NASH. BMI, HOMA-IR and serum level of TC, AST, ALT and ALP were positively correlated with SF.

Multiple regression analysis evaluated the independent associations between homocysteine and NASH, and homocysteine and SF, respectively. Table III shows the non-adjusted and adjusted models. The variable AST did not conform to the linear assumption, so it was not included in the regression analysis. For NASH, in the crude model (not adjusted covariates), homocysteine showed significant correlation with NASH (OR: 0.90, 95%CI: 0.82-0.98, p=0.018). In the minimally adjusted model I (adjusted for age, BMI), the effect size showed no obvious change (OR: 0.83, 95%CI: 0.75-0.93, p=0.001). After adjusting for model II (adjusted for age, BMI, LDL-c, ALT, γGT, HOMA-IR and UA), these associations were consistently maintained (OR: 0.79, 95%CI: 0.69-0.89, p<0.001). As for SF, homocysteine showed no correlation with SF in the crude model (OR: 0.90, 95%CI: 0.80-1.01, p=0.063), but when we adjusted more covariates (model I (adjusted for age, BMI), model II(adjusted for age, BMI, LDL-c, ALT and HOMA-IR)), homocysteine showed a significant correlation with SF (OR: 0.87, 95CI%: 0.77-0.99, p=0.030; OR: 0.83, 95%CI: 0.72-0.95, p=0.009). For the purpose of sensitivity analysis, we

Table I. Baseline characteristics of subjects (n =289)

Homocysteine	T1	T2	T3	p
Number	89	91	109	
Male, number (%)	42 (47.19%)	71 (78.02%)	97 (88.99%)	<0.001
Diabetes (yes)	37 (41.57%)	27 (29.67%)	22 (20.18%)	0.005
Hypertension (yes)	26 (29.21%)	18 (19.78%)	19 (17.43%)	0.116
Smoke (yes)	9 (10.11%)	20 (21.98%)	26 (23.85%)	0.034
Age (years)	45 (35-54)	42 (33-51.5)	38 (28-48)	0.003
BMI (kg/m²)	26.13 (24.01-27.83)	26.64 (25.22-28.84)	26.63 (24.23-29.27)	0.075
TG (mmol/L)	1.67 (1.23-2.45)	1.95 (1.44-2.62)	1.98 (1.31-2.82)	0.165
TC (mmol/L)	5.00 (4.14-5.79)	5.30 (4.42-5.98)	5.04 (4.43-5.83)	0.375
LDL-c (mmol/L)	2.87 (2.28-3.54)	3.05 (2.40-3.79)	3.17 (2.47-3.65)	0.128
HDL-c (mmol/L)	1.02 (0.88-1.15)	1.02 (0.88-1.12)	0.96 (0.88-1.09)	0.604
Albumin (g/L)	45.4 (42.4-47.3)	46.60 (44.80-48.95)	47.6 (45.4-50)	<0.001
ALT (U/L)	41 (25- 88)	59 (30- 92)	53 (33-101)	0.188
AST (U/L)	28 (22-54)	36 (24.5-55.5)	34 (26-58)	0.802
ALP (U/L)	79 (63-97)	83 (69-96.5)	79 (66-94)	0.971
γGT (U/L)	36 (25-73)	53 (36-85)	55 (36-91)	0.135
Glucose (mmol/L)	5.70 (5.00-6.70)	5.40 (4.90-6.75)	5.10 (4.70-5.60)	0.003
HOMA-IR	3.60 (1.98-5.06)	3.86 (2.59-6.43)	3.88 (2.41-5.29)	0.488
PLT (*10^9)	235 (200-293)	224 (187-254.5)	243 (213-284)	0.016
TB (μmol/L)	12 (9-16)	13 (10-17)	14 (11-17)	0121
UA (μmol/L)	349 (276-393)	384 (309-445)	407 (349-474)	<0.001

ALT: aspartate aminotransferase; AST: alanine aminotransferase; ALP: alkaline phosphatase; BMI: Body mass index; γGT: gamma-glutamyl transferase; HDL-c: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; LDL-c: low-density lipoprotein cholesterol; PLT: platelet; TB: total bilirubin; TC: total cholesterol; TG: triglyceride; UA: uric acid.

Table II. The results of univariate analysis

	Statistics	OR (95%CI) for NASH	p	OR (95%CI) for SF	p
Sex					
Male	210 (72.66%)	Reference		Reference	
Female	79 (27.34%)	1.10 (0.65, 1.86)	0.728	1.36 (0.74, 2.49)	0.328
Diabetes					
No	203 (70.24%)	Refrence		Refrence	
Yes	86 (29.76%)	0.73 (0.43, 1.23)	0.236	0.87 (0.46, 1.62)	0.650
Hypertension					
No	226 (78.20%)	Reference		Reference	
Yes	63 (21.80%)	0.63 (0.35, 1.14)	0.126	0.94 (0.47, 1.87)	0.858
Smoke					
No	234 (80.97%)	Reference		Reference	
Yes	55 (19.03%)	1.09 (0.60, 1.98)	0.778	1.49 (0.76, 2.93)	0.245
Age (years)	41.90 ± 12.29	0.97 (0.95, 0.99)	0.005	1.00 (0.98, 1.02)	0.907
BMI (kg/m²)	26.93 ± 3.46	1.16 (1.08, 1.24)	<0.001	1.15 (1.06, 1.24)	0.001
TG (mmol/L)	2.13 ± 1.11	1.21 (0.98, 1.50)	0.077	1.17 (0.92, 1.48)	0.206
TC (mmol/L)	5.13 ± 1.19	1.37 (1.11, 1.68)	0.003	1.31 (1.04, 1.66)	0.024
LDL-c (mmol/L)	3.05 ± 0.97	1.38 (1.08, 1.77)	0.011	1.30 (0.98, 1.74)	0.069
HDL-c (mmol/L)	1.01 ± 0.20	1.27 (0.40, 4.09)	0.687	1.63 (0.41, 6.43)	0.487
Albumin (g/L)	46.30 ± 3.80	1.05 (0.98, 1.11)	0.157	0.95 (0.89, 1.03)	0.194
ALT (U/L)	71.03 ± 60.65	1.02 (1.01, 1.02)	<0.001	1.01 (1.01, 1.01)	<0.001
AST (U/L)	45.25 ± 33.14	1.04 (1.03, 1.05)	<0.001	1.02 (1.01, 1.03)	<0.001
ALP (U/L)	83.52 ± 24.00	1.01 (1.00, 1.02)	0.048	1.02 (1.00, 1.03)	0.009
γGT (U/L)	67.18 ± 53.58	1.01 (1.00, 1.01)	0.012	1.00 (1.00, 1.01)	0.215
Glucose (mmol/L)	5.76 ± 1.38	1.00 (0.84, 1.19)	0.985	1.20 (0.99, 1.44)	0.064
HOMA-IR	4.98 ± 4.96	1.15 (1.07, 1.24)	<0.001	1.13 (1.06, 1.20)	<0.001
PLT (*10^9)	241.49 ± 61.21	1.00 (1.00, 1.01)	0.455	1.00 (1.00, 1.01)	0.305
TB (μmol/L)	14.63 ± 7.38	1.04 (1.00, 1.07)	0.032	1.00 (0.97, 1.04)	0.889
UA (μmol/L)	388.62 ± 108.83	1.00 (1.00, 1.01)	0.001	1.00 (1.00, 1.00)	0.164

CI: confidence interval; NASH: non-alcoholic steatohepatitis, OR: odds ratio; SF: significant fibrosis; (For abbreviations see Table I).

also handled homocysteine as a categorical variable (tertiles, T1-T3) and found the same results. Using T1 as a reference, the relationship between homocysteine and NASH, homocysteine and SF respectively remained statistically significant in T3 versus T1 (OR: 0.43, 95%CI: 0.22-0.85, p=0.015; OR: 0.38, 95%CI: 0.17-0.86, p=0.020, respectively) after adjusting for model II, and significant linear trend was observed (p for trend=0.016; 0.019, respectively). Spearman's correlation analysis showed that the serum level of homocysteine was inversely correlated with the grade of hepatocellular ballooning and the stage of liver fibrosis (Spearman's ρ=-0.13, p=0.033; =-0.16, p=0.007), but had no correlation with the severity of steatosis and lobular inflammation (Fig. 1).

To determine the effect of potential confounders, the relationship between homocysteine and NASH, homocysteine and SF respectively was further investigated in subgroups (Table IV). The subgroup analysis by gender showed that homocysteine was strongly associated with NASH in females but was weaker in males (female OR: 0.61, 95%CI: 0.45 to 0.84; male OR: 0.86, 95%CI: 0.75 to 0.99).

The AUC and Hosmer-Lemeshow goodness-of-fit test for the adjusted logistic regression models was analyzed. For NASH, the model demonstrated good discrimination (the AUC was 0.789, 95CI%: 0.736 to 0.843) (Fig. 2) and calibration (Hosmer-Lemeshow goodness-of-fit test, p=0.346). For SF, the model also showed good discrimination (the AUC is 0.784, 95CI%: 0.719 to 0.848) (Fig. 3) and calibration (Hosmer-Lemeshow goodness-of-fit test, p=0.908).

DISCUSSION

Over the past decade, the relationship between homocysteine and NAFLD has attracted attention, but no unified conclusion has been reached due to different study designs, diagnostic methods of NAFLD, and different sample sizes. To our knowledge, our study is the largest analysis to date that evaluated the association between homocysteine and biopsy proven NAFLD in adults. In our cohort elevated serum homocysteine levels were negatively associated with NASH and SF in subjects with biopsy-proven NAFLD. Homocysteine was

Table III. Relationship between homocysteine and NASH or SF in different models

Variable	Crude Model		Model I		Model II	
	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Homocysteine as a continuous variable						
NASH	0.90 (0.82, 0.98)	0,018	0.83 (0.75, 0.93)	0,001	0.79 (0.69, 0.89)	<0.001
SF	0.90 (0.80, 1.01)	0.063	0.87 (0.77, 0.99)	0,030	0.83 (0.72, 0.95)	0.009
Homocysteine as a categorical variable (tertile)						
NASH						
T1 (7-10)	Reference		Reference		Reference	
T2 (11-12)	0.84 (0.46, 1.52)	0.562	0.66 (0.36, 1.24)	0.196	0.55 (0.28, 1.08)	0.082
T3 (13-36)	0.68 (0.38, 1.21)	0.191	0.51 (0.28, 0.95)	0.034	0.43 (0.22, 0.85)	0.015
Trend test		0.190		0.035		0.016
SF						
T1 (7-10)	Reference		Reference		Reference	
T2 (11-12)	0.91 (0.47, 1.80)	0.796	0.77 (0.38, 1.55)	0.467	0.66 (0.31, 1.41)	0.285
T3 (13-36)	0.53 (0.26, 1.07)	0.077	0.47 (0.22, 0.98)	0.045	0.38 (0.17, 0.86)	0.020
Trend test		0.075		0.044		0.019

CI: confidence interval; NASH: non-alcoholic steatohepatitis; OR: odds ratio; SF: significant fibrosis. (For abbreviations see Table I). Crude model did not adjust for other covariates; Model I adjusted for age and BMI: Model II adjusted for age, BMI, LDL-c, ALT, γGT, HOMA-IR and UA to NASH; age, BMI, LDL-c, ALT and HOMA-IR to SF, respectively

strongly associated with NASH in females. In addition, lower homocysteine levels were correlated with progression of liver fibrosis and hepatocellular ballooning [5]. Hepatic steatosis could induce oxidative fat injury, endoplasmic reticulum dysfunction and abnormalities of the cytoskeleton resulting in hepatocellular ballooning. Therefore, hepatocellular ballooning, the hallmark to distinguish steatohepatitis from steatosis with inflammation, is characterized by cellular swelling, rarefaction of the hepatocytic cytoplasm and clumped strands of intermediate filaments [23].

These results were similar a previous study that matched for gender, age and BMI 39 patients with biopsy proven

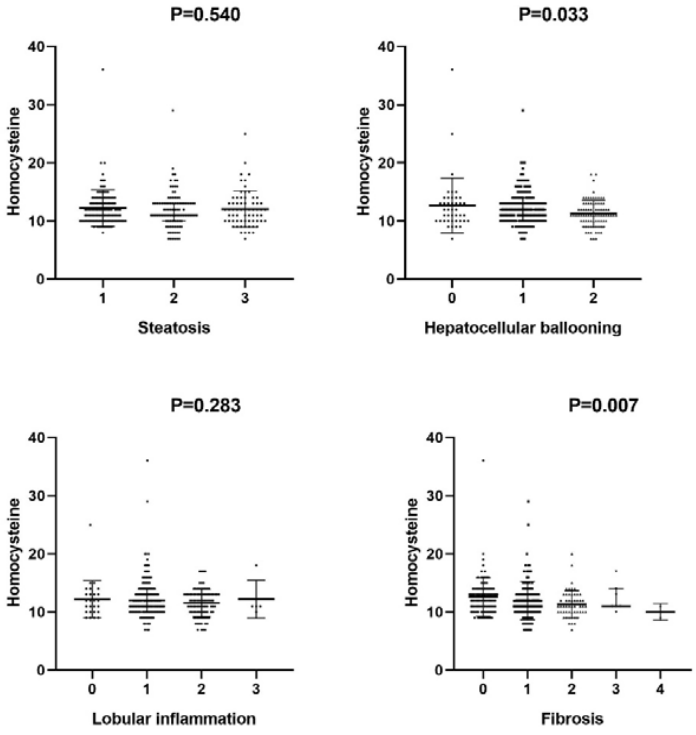

Fig. 1. Correlation analysis between serum homocysteine levels and histological features of NAFLD. The homocysteine showed a strong negative relationship with hepatocellular ballooning and liver fibrosis stage (all $p < 0.05$), and no association with steatosis and lobular inflammation.

Table IV. Effect size of homocysteine on NASH or SF in prespecified and exploratory subgroups

Characteristic	N	OR (95%CI) for NASH	p for interaction	OR (95%CI) for SF	p for interaction
Gender			0.04		0.78
Male	210	0.86 (0.75, 0.99)		0.83 (0.69, 0.99)	
Female	79	0.61 (0.45, 0.84)		0.87 (0.65, 1.15)	
Age (years)			0.60		0.27
< 50	205	0.79 (0.68, 0.91)		0.86 (0.75, 1.00)	
≥ 50	84	0.72 (0.53, 0.98)		1.00 (0.81, 1.22)	
Smoke			0.99		0.51
Yes	55	0.78 (0.58, 1.04)		0.79 (0.67, 0.93)	
No	234	0.78 (0.68, 0.90)		0.88 (0.67, 1.14)	
Hypertension			0.96		0.48
Yes	63	0.77 (0.55, 1.07)		0.92 (0.66, 1.29)	
No	226	0.78 (0.67, 0.89)		0.81 (0.68, 0.95)	
Diabetes			0.55		0.67
Yes	86	0.73 (0.57, 0.95)		0.86 (0.64, 1.16)	
No	203	0.80 (0.69, 0.92)		0.80 (0.68, 0.95)	
HOMA-IR			0.53		0.59
< 2.5	80	0.90 (0.66, 1.23)		0.77 (0.51, 1.15)	
≥ 2.5	209	0.80 (0.70, 0.92)		0.86 (0.74, 1.00)	
BMI (kg/m^2)			0.34		0.96
< 24	61	0.70 (0.53, 0.94)		0.87 (0.57, 1.31)	
≥ 24	228	0.81 (0.71, 0.93)		0.86 (0.74, 1.00)	

CI: confidence interval; NASH: non-alcoholic steatohepatitis; OR: odds ratio; SF: significant fibrosis. (For abbreviations see Table I).

NAFLD and 22 healthy controls [19]. Inconsistent with our conclusion, two studies in adult Chinese patients found that serum homocysteine level was positively associated with the prevalence of NAFLD, but the diagnosis of hepatic steatosis was made by ultrasonography [15, 16]. Polyzos et al. [17] concluded that homocysteine was unrelated to NAFLD biopsy proven, but their conclusions were limited by the small sample size (30 patients). Therefore, we hypothesized that the serum homocysteine levels may have a threshold effect on NAFLD. A study in pediatric NAFLD that included 128 age-matched subjects (NAFLD: 64, healthy controls: 64) found similar results with ours; plasma homocysteine levels were significantly increased in NAFLD children as compared to the controls, but significantly decreased in NASH compared to non-NASH children [18]. However, our study design lacked a healthy control group to test our hypothesis, representing a limit of the present study.

The pathophysiological mechanisms between homocysteine and NAFLD are multifactorial and not fully understood. Deminice et al. [24] suggested that low plasma homocysteine levels may result in decreased glutathione formation leading to increased susceptibility of hepatic cells to reactive oxygen species (ROS). Oxidative stress causes changes in mitochondrial function, depletion of ATP, DNA damage, lipid peroxidation and consequently hepatic inflammation and fibrosis [11, 25]. Some studies found significantly lower homocysteine levels in patients with insulin resistance syndrome or high glucose levels compared to control groups [26, 27]. Increased homocysteine

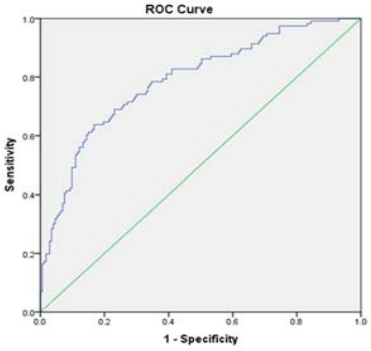

Fig. 2. The area under the curve of the adjusted logistic regression models for NASH.

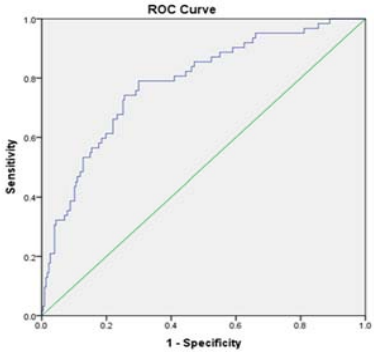

Fig. 3. The area under the curve of the adjusted logistic regression models for SF..

levels were associated with weight loss (independent of folate deficiency) in patients after vertical gastroplasty for severe obesity [28]. All of the above-mentioned risk factors, ROS, BMI and insulin resistance, contribute to the progression of NAFLD [11]. In addition, low levels of homocysteine may lead to decreased production of methionine through remethylating pathway. Methionine is the precursor of S-adenosylmethionine (S-AdoMet), the key methyl donor for phosphatidylcholine synthesis. Low methyl group availability reduces the synthesis of phosphatidylcholine, a major phospholipid required for the assembly and the export of very low-density lipoprotein from the liver, which may lead to hepatic lipid accumulation resulting in hepatic steatosis [19, 29]. However, some experimental studies have suggested that high plasma homocysteine levels induced hepatocytes in endoplasmic reticulum stress activating both the unfolded protein response and the sterol regulatory element–binding proteins (SREBPs), which causes significant increases in intra-hepatic cholesterol and triglycerides levels leading to the progression of hepatic steatosis [30, 31].

Although our cross-sectional study did not reveal an in-depth mechanism, our finding illustrates the necessity to actively assess serum homocysteine levels in patients with NAFLD. From a clinical point of view, our results suggests that serum homocysteine should be included in the multidisciplinary baseline assessment of patients with NAFLD. Changing the serum levels of homocysteine might be one of the promising potential therapy for NAFLD cure or for preventing progression.

This study had some limitations. Firstly, the cross-sectional design of the study made it possible to investigate associations but not causalities. Secondly, we did not measure folate and vitamin B12 in our study. Finally, this study does not reflect institutional and regional diversities because the cohorts of this study were composed of Chinese patients who were recruited at a single hospital.

CONCLUSION

Elevated serum homocysteine levels are negatively associated with NASH and SF in subjects with NAFLD. Further prospective cohort studies should establish a causal relationship between homocysteine and NAFLD (NAFL, NASH and SF) and elucidate the associated mechanisms.

Conflicts of interest: None to declare.

Authors' contribution: Y.X., J.W.: study concept; Y.X., Y.G., X.Y., Z.X.: data-collection; Y.X., Y.G.: data analysis and interpretation; Y.X., Y.G., X.Y., Z.X.: drafting of the manuscript; Y.X., J.W.: critical revision of the manuscript for important intellectual content.

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