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Low Density Lipoprotein Cholesterol May Be a Key Independent Risk Factor for Asymptomatic Gallbladder Stone Disease in Non-Alcoholic Fatty Liver Disease Patients in Northwest China: A Case-Control Study

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Keywords:

Non-alcoholic fatty liver disease; Gallbladder stone disease; Low density lipoprotein cholesterol; Epidemiology

Abbreviations:

GSD: Gallbladder stone disease; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; BMI: Body mass index; WC: Waist circumference; TC: Total cholesterol; TG: Triglyceride; HDL-c: High density lipoprotein cholesterol; LDL-c: Low density lipoprotein cholesterol; NAFLD: Nonalcoholic fatty liver disease; HC: Hypercholesterolemia; ROS: Reactive oxygen stress.

1. Abstract

1.1. Background: Previous studies reported that Non-Alcoholic Fatty Liver Disease (NAFLD) is associated with gallbladder stone disease (GSD). The study aimed to detect the key factors influencing the formation of new-onset GSD among the NAFLD patients.

1.2. Method: A retrospective analysis included 610 patients with new-onset asymptomatic GSD and 610 healthy subjects, aged 30 to 80 years undergoing routine health check-ups annually. The height, weight, blood pressure, serum lipids indexes, fasting blood glucose of the participants were measured before and after experiment. The hazard factor of GSD was compared between individuals with NAFLD and not, and further the relationships between body mass index (BMI), blood lipid and gallbladder stone hazards were examined by Logistic multivariate regression models. **1.3. Results:** A significantly higher morbidity with GSD in NAFLD versus non-NAFLD subjects was found (P<0.001). Of NAFLD, BMI, waist circumference, low density lipoprotein cholesterol (LDL-c) and triglyceride (TG) levels, were significantly positive correlated with GSD (P<0.01, all), while high density lipoprotein (HDL-c) (P<0.001) was significantly negative correlated with GSD in univariate analysis. Multivariate logistic regression showed that there was positive correlation between serum LDL-c and hazard of GSD (P<0.01), while significantly negative correlation between BMI, serum TG, HDL-c levels and hazard of GSD (P<0.01, all).

1.4. Conclusions: NAFLD played an essential link to trigger off this domino effect on GSD, which was related to obesity and dyslipidemia. Interestingly, we first found that serum LDL-c levels may be a key risk factor for GSD in NAFLD patients, probably because sharp low TG or improper drug therapy may cause imbalance of cholesterol homeostasis and fatty acid in hypertriglyceridemia with NAFLD.

2. Introduction

Gallbladder Stone Disease (GSD), a common gastrointestinal disease, is prone to develop into severe cholecystitis. Approximately 10%-20% of the national adult populations are currently with GSD, and the prevalence of gallstone is continuously rising [1]. Notably, GSD is also strongly associated with gallbladder, pancreatic and colorectal cancer occurrence, and the National Institutes of Health estimated that almost 3,000 deaths (0.12% of all deaths) per year were attributed to the complications of GSD [1]. Due to the westernization of the diet of Chinese people, the incidence of GSD is increasing rapidly and has become a major health problem in China [2]. Especially, asymptomatic GSD has become prevalent in the gen-

eral population and imposed a heavy economic burden due to the soaring expense on diagnosis, treatment, and indirect healthcare [3]. The prevalence of asymptomatic GSD is 12.1% in China and greatly increasing [4]. Therefore, it is of great significance to explore the molecular mechanisms of gallstone development and then to prevent it from further progressing into more advanced hepatobiliary disease.

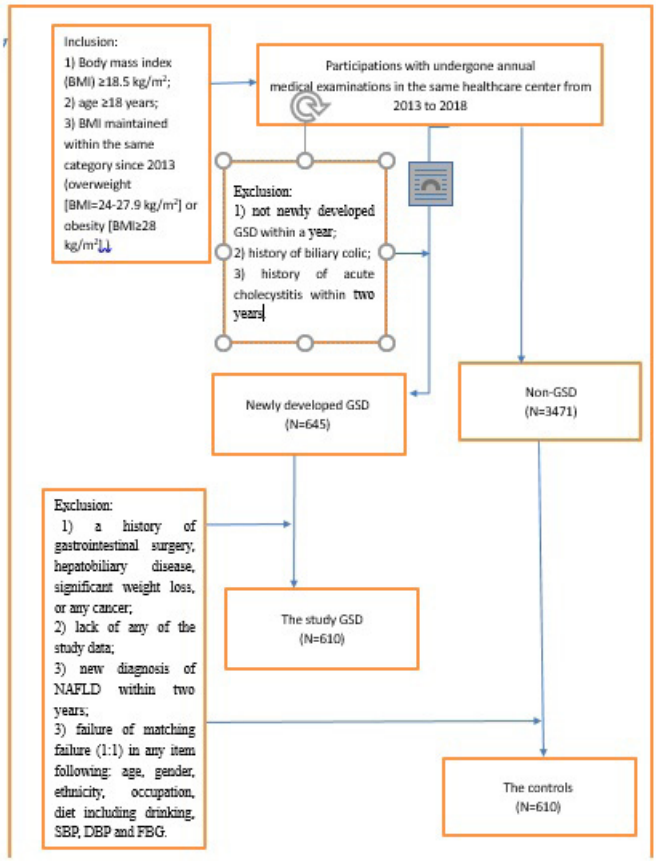
Epidemiological knowledge of GSD is essential for formulating preventive plans and determining optimal treatment strategies for this disease. The epidemiological data confirmed that genetic factors were estimated to account for approximately 25% of the overall risk of gallstones [5], while other risk factors for asymptomatic gallstones included obesity, age, diabetes, hyperlipidemia, high caloric intake, hepatitis C, and metabolic syndrome (MetS) [4]. Thus, to control these risk factors of metabolism would be promising strategies to prevent the development of GSD. Non-Alcoholic Fatty Liver Disease (NAFLD) occurs more frequently in the MetS status, and is an increasingly common chronic liver disease worldly [6]. More importantly it is the common risk factor of asymptomatic gallstones. On the other hand, studies have revealed that asymptomatic GSD patients were more prone to develop NAFLD due to impaired gallbladder motility and increased bile lysogenicity [7]. It was known that NAFLD and obesity, dyslipidemia was correlated with each other [8]. However, it was not clear whether NAFLD affected the formation of gallbladder stone disease (GSD) in patients with obesity and hyperlipidemia. Although a few studies showed that NAFLD was associated with gallstones in Pakistani, Turkey, Singapore and Taiwan and the southern of Chinese populations, while this correlation was demonstrated to be shortage in the US and Korean populations [7, 9]. Furthermore, few studies have clearly clarified the causal relationship between NAFLD and GSD.

NAFLD is usually overlooked by carriers but might be controlled and even reversed by dietary or lifestyle interventions. It may also of potential to prevent or cure NAFLD related GSD. Therefore, the first step would be revealed the relationship between NAFLD and GSD and then may provide potential preventive strategies for GSD, and further for gallbladder cancer. In China, northwestern people feed on domestic foods with high lipid and sugar, and epidemiological investigations reported that these people were more inclined to develop NAFLD and GSD, gallbladder cancer patients. Thus, individuals in this typical area were recruited in this study. Specifically, this study reviewed the clinical records of patients with new-onset asymptomatic gallbladder stone during a 5-year period, so as to reveal whether NAFLD played key roles in the formation of GSD. It may shed light on the molecular mechanism of GSD development and provide therapeutic approaches for further GSD treatment.

3. Methods

3.1. Subjects Selection and Data Eligibility

In this retrospective analysis, 645 Chinese new patients aged 30 to 80 years who had undergone routine health check-ups annually in the healthcare center of the Affiliated Hospital of Medical School: Xi'an Jiaotong University, Xi'an, China, from August 2013 to July 2018. 35 patients were excluded from the analysis. Diagnosis was completed by senior associate chief physicians with more than 10 years of experience in ultrasound diagnosis. These final data were selected from the mean value of 5-year multiple check-up results so as to reduce the selection bias caused by difference in observation time between the control group and GS group. The inclusion and exclusion criteria are shown in (Figure 1). We conducted this study in accordance with the Institutional Ethics Committee requirements of the above-mentioned hospital (No: XJTU1AF2019LSK-017).



Step 1: It shows that 645 patients with GSD and 3471 healthy controls aged 30 to 80 years, undergone annual medical examinations in the same healthcare center from August 2013 to July 2018. Inclusion criteria were: 1) age ≥18 years; 2) Body mass index (BMI) ≥18.5 kg/m^2; 3) BMI maintained within the same category (overweight [BMI=24-27.9 kg/m^2] or obesity [BMI≥28 kg/m^2]) from 2013 to 2018. Exclusion criteria were: 1) a history of gastrointestinal surgery, hepatobiliary disease, significant weight loss, or any cancer; 2) history of hypertension or diabetes; 3) new diagnosis of nonalcoholic fatty liver disease (NAFLD) within two years; 4) lack of any of the data such as age, height, weight, waist circumference (WC), systolic pressure (SBP), diastolic pressure (DBP), serum total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), fasting blood glucose (FBG), and ultrasonographic examination for gallbladder. In additional, 645 patients with GSD were diagnosed with new-onset asymptomatic GSD by abdominal ultrasonography examination in 2018 were included. Exclusion criteria were: 1) no previous diagnosis of

gallbladder stone in annual check-ups before 2018 (2013-2017); 2) history of biliary colic; 3) history of acute cholecystitis within two years.

Step 2: It shows that 35 patients with GSD and 2781 healthy controls were excluded by: 1) a history of gastrointestinal surgery, hepatobiliary disease, significant weight loss, or any cancer; 2) lack of any of the study data; 3) failure of matching failure (1:1) in any item following: age, gender, ethnicity, occupation, diet including drinking, SBP, DBP and FBG.

Step 3: There were 610 patients with GSD and 610 healthy controls aged 30 to 80 years.

3.2. Data Questionnaires and Determination of Clinical Parameters

Each participant needed to fill a questionnaire alone containing questions about race; ID number; occupation; history of medication, diabetes mellitus, gastrointestinal surgery, hepatabiliary disease, weight loss and any cancer. A nurse validated their age using their identity cards and confirmed the information and a full-time clinician measured the height and weight during the medical examination.

A screening panel including common tumor mark, hypertension and transabdominal ultrasonography were performed. Blood samples were collected after a 10-h fast from an antecubital vein. Serum TG, LDL-c, HDL-c, TC, FBG were assayed. TG and T-Chol analyses were done using GPO-PAP, COD-PAP method respectively, and LDL-c, HDL-c analyses were done using homogenous method. Glucose oxidase method was used to analyses FBG. All measurements were done at the same central laboratory in a blind fashion according to the manufacturer's instructions. Blood pressure was measured three times after sitting or rest for 30 min by a blood pressure measuring instrument as our previously reported [10].

3.3. Type-B Ultrasonic Examination for Diagnosing GSD and NAFLD

A Color Doppler ultrasonic instrument (Toshiba, SSA-510A, Japan) was used to perform to find gallbladder and hepatic diseases. The subjects were fasting and in the supine position. The liver, gallbladder, pancreas and spleen were examined in turn. GSD and NAFLD were diagnosed as our previously reported [11].

3.4. Determination of Obesity Factors and Hypercholesterolemia

A height and weight scale of our previous report was used to measure patient height and weight. BMI was determined as weight (kg) by squared height (m^2) [11]. Patients were grouped by BMI according to expert consensus for medical nutrition therapy of overweight / obesity in China [12], where patients with BMI <23.9 kg/m^2 were considered as normal, 24–27.9 kg/m^2 were considered as overweight, and≥28 kg/m^2 were considered as obese. WC was measured four times using a flexible, tension sensitive, non-stretching measuring tape placed directly on the skin, at the end of normal expiration by a trained researcher as described previously [10]. Blood TC ≥ 6.22 mmol/L or LDL-C ≥ 4.14 mmol/L were diagnosed as hypercholesterolemia, which was based on blood lipoprotein profiles by jointing Committee for developing Chinese guidelines on prevention and treatment of dyslipidemia in adults [13].

4. Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (SD), and categorical variables as percentage (%). There were trending to continuous data with normal distribution as a sufficient number of participants so Student's t-test was applied to compare the above variables to reduce the statistical error, respectively. Chi square test were applied to categorical variables. The relationships between BMI, blood lipid and gallbladder stone hazards were examined by using logistic multivariate regression models, and stratified by age, respectively. P<0.05 was considered statistically significant. All statistical analyses were performed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA).

5. Results

5.1. Analysis of General Characteristics and Serum Index Between Gallbladder Stone Group and The Control Group

The different characteristics in general characteristics and serum indexes between gallbladder stone and the control group are summarized in (Table 1). There were 610 of 645 patients with GSD and 610 subjects of 3471 healthy controls aged 30 to 80 years including 231 (37.9%) and 253 (41.1%) female participants in GSD and the control group respectively. There were significantly higher TC, TG, LDL-c levels and lower HDL-c levels in GSD group versus the controls (P<0.05, respectively), see table 1. Of them, there were 334 (54.7 %) normal BMI, 294 (48.1%) overweight and 77 (12.6 %) obesity patients with GSD, while 334 (54.7 %) normal BMI, 211 (34.5 %) overweight and 65 (10.6 %) obese in the controls. There were 197 (32.3%) and 142(23.2 %) participants with NAFLD, and 74 (12.1 %) and 46 (7.5 %) participants with hypercholesterolemia in both GSD and the control group, respectively. There was a significantly higher morbidity in hypercholesterolemia than not subjects (χ^2=7.307, P=0.007, OR=1.693, 95%CI: 1.150~2.491). Similar result was observed in patients NAFLD than not (χ^2=12.399, P<0.001, OR=1.572, 95%CI: 1.221~2.025).

5.2. The Effects of NAFLD on GSD

As shown in (Table 2), the effects of NAFLD and overweight/obese on hazard rate of GSD were determined. Significant higher morbidity with GSD was found in NAFLD subjects than non-NAFLD ones in overweight/obese (χ^2=28.703, P<0.001). Similar result was observed in normal BMI (χ^2=4.203, P=0.04).

As shown in (Table 3), the hazard rate between hypercholesterolemia and GSD with or without NAFLD was determined. It was not significantly higher in NAFLD than non-NAFLD subjects (χ^2=1.227, P=0.268) with hypercholesterolemia, while significantly higher morbidity of GSD was revealed in NAFLD than non-NAFLD subjects without hypercholesterolemia (χ^2=12.668, P<0.001).

Table 1: Demographic and clinical data of the patients before and after matching

	Before matching				After matching			
	Control group (N=3471)	GS group (N=645)	χ^2 or T value	P value	Control group (N=610)	GS Group (N=610)	χ^2 or T value	P value
Female (%)	1713 (49.4%)	291 (45.1%)	3.913*	0.048	251 (41.1 %)	231(37.9 %)	1.372*	0.241
Age (Years)	56.32±13.7	59.66±12.2	6.259	<0.001	59.51±12.4	59.86± 11.7	0.505	0.614
Han (%)	2982 (85.9%)	580 (89.9%)	8.013*	0.005	530 (86.9 %)	533 (87.3%)	0.066*	0.798
Occupation (%)			49.425*	<0.001			1.583*	0.812
Worker	218 (6.3%)	42 (6.5%)	0.049*	0.825	35 (5.7%)	38 (6.2%)	0.131*	0.717
Professional technician	316 (9.1%)	78 (12.1%)	5.298*	0.021	77 (12.6%)	75 (12.3%)	0.03*	0.862
Manager	193 (5.6%)	56 (8.7%)	8.484*	0.004	58 (9.5%)	53 (8.7%)	0.248*	0.619
Retiree	2062 (59.4%)	407 (63.1%)	3.117*	0.078	410 (67.2%)	405 (66.4%)	0.011*	0.915
Others	682 (19.6%)	62 (9.6%)	41.996*	<0.001	30 (4.9%)	39 (6.4%)	1,248*	0.264
SBP (mmHg)	120.99±17.9	127.58±19.5	7.972	<0.001	126.65±18.5	125.74±17.5	-0.884	0.377
DBP (mmHg)	76.67±10.6	83.02±12.1	12.263	<0.001	80.06±7.5	79.82±9.1	-0.493	0.622
FBG (mmol/L)	5.27±1.3	5.18±1.4	-1.417	0.135	5.15±1.5	5.19±1.1	0.436	0.663
Height (cm)	165.26±8.6	165.24±8.5	-0.068	0.946	165.75±8.2	166.41±8.0	1.424	0.155
Weight (kg)	65.82±11.4	67.29±11.5	2.98	0.003	66.30±11.5	68.50±10.6	3.473	0.001
BMI (kg/m^2)	24.00±3.1	24.55±3.0	4.232	<0.001	24.04±3.1	24.65±2.7	3.631	<0.001
Normal (%)	1472 (42.4%)	217 (33.6%)	17.594*	<0.001	334 (54.7%)	239 (39.1%)	29.823*	<0.001
Overweight (%)	750 (21.6%)	144 (22.3%)	0.164*	0.685	211 (34.5%)	294 (48.1%)	23.360*	<0.001
Obesity (%)	1249 (36.0%)	284 (44.0%)	14.817*	<0.001	65 (10.6%)	77(12.6%)	1.149*	0.284
WC(cm)	82.41±7.0	83.53±7.8	20.304	<0.001	85.07±8.0	86.92±7.2	4.232	<0.001
TC (mmol/L)	4.88±0.9	4.91±1.1	0.593	0.505	4.78±0.9	5.00±1.0	3.967	<0.001
TG (mmol/L)	1.49±0.8	1.57±0.9	1.972	0.027	1.33±0.3	1.73±1.0	9.123	<0.001
HDL-c (mmol/L)	2.53±1.0	2.19±1.1	-6.865	<0.001	2.92±0.8	1.26±0.2	-47.361	<0.001
LDL-c (mmol/L)	1.99±1.3	2.24±1.3	4.271	<0.001	1.63±1.2	3.02±0.8	23.09	<0.001
Drink (%)	506 (146%)	87 (13.5%)	0.531*	0.466	68 (11.1%)	72 (11.8%)	0.129*	0.719
Eating habits (%)			86.277*	<0.001			0.165*	0.983
Vegan	127 (3.7%)	56 (8.7%)	26.916*	<0.001	51 (8.4%)	48 (7.9%)	0.099*	0.753
Vegetarian	508 (14.6%)	78 (12.1%)	2.987*	0.084	70 (11.5%)	72 (11.8%)	0.032*	0.858
Meat & vegetarians	2730 (78.7%)	382 (59.2%)	101.648*	<0.001	380 (62.3%)	378 (62%)	0.014*	0.906
Carnivorous diet	376 (10.1%)	129 (20.0%)	37.764*	<0.001	109 (17.9%)	112 (18.4%)	0.05*	0.824
NAFLD (%)	744 (21.4%)	190 (29.5%)	18.986*	<0.001	142 (20.8%)	197 (28.1%)	12.399*	<0.001
HC	327 (9.4%)	106 (11.5%)	0.531*	0.068	46 (7.5%)	74 (12.1%)	7.307*	0.007

GS: gallbladder stone; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; BMI: body mass index; T-Chol: total cholesterol; TG: triglyceride; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; NAFLD: nonalcoholic fatty liver disease; HC: hypercholesterolemia.

* χ2 for making a difference with student test.

Table 2: The differences of hazard rate of GSD between NAFLD and not stratified by overweight/obesity or normal BMI

	Overweight/obesity[&]		Normal BMI[&&]	
	GSD	The controls	GSD	The controls
NAFLD	122*	52	75[#]	79
Non-NAFLD	194**	225	164[##]	255
total	316	277	239	334

Note：GDS: gallbladder stone diseases; BMI: Body mass index; NAFLD: nonalcoholic fatty liver disease

* vs **: χ^2=28.703, P<0.001, OR=2.721, 95%CI: 1.866~3.968

[#] vs [##]: χ^2=4.203, P=0.04, OR=1.476, 95%CI: 1.018~2.141

Table 3: The differences of hazard rate of GSD between NAFLD and not stratified by HC or not

	HC		Non-HC	
	GSD	Control	GSD	Control
NAFLD	13*	12	159#	115
Non-NAFLD	61**	34	377##	449
Total	74	46	536	564

Note：GDS: gallbladder stone disease；NAFLD: nonalcoholic fatty liver disease; HC: hypercholesterolemia.

* vs **: $\chi 2$=1.227, P=0.268, OR=0.604, 95%CI: 0.248~1.470

vs ##: χ 2=12.668, P<0.001, OR=1.647, 95%CI: 1.249~2.171

5.3. Relationships Between the Indexes of Obesity, Hyperlipidemia, NAFLD and Hazard Rate of GSD

(Table 4) shows the risk factors for developing GSD based on univariate and multivariate logistic regression. Univariate logistic regression showed significantly positive correlation between weight, BMI, WC, NAFLD, hypercholesterolemia, serum TC, TG and LDL-c levels, and hazard of GSD (P<0.05, all), while significantly negative correlation between serum HDL-c levels and hazard of GSD (P<0.001). Multivariate logistic regression including BMI showed that there was significantly positive correlation between NAFLD, hypercholesterolemia and hazard of GSD (P<0.05, all), while significantly negative correlation between serum HDL-c levels and hazard of GSD (P<0.05, all). Similar result was observed by multivariate logistic regression including weight.

To investigate the possible reason that caused above result, we further analyzed data using univariate and multivariate logistic regression analysis (as Shown in Table 5). The difference between GSD and control group was studied by stratifying with NAFLD. Univariate logistic regression showed significantly positive correlation between BMI, WC, serum TG, LDL-c levels, and hazard of GSD (P<0.01, all), drink was weak risk for GSD (P=0.087), while significantly negative correlation between serum HDL-c levels and hazard of GSD (P<0.001). Multivariate logistic regression showed that there was positive correlation between serum LDL-c and hazard of GSD (P<0.01), while significantly negative correlation between BMI, serum TG, HDL-c levels and hazard of GSD (P<0.01, all).

Table 4: Univariate and multivariate analysis of gallbladder stone and BMI, WHtR, blood lipid, NAFLD and HTC

Factors	Univariate				Multivariate1				Multivariate2			
	β	OR	95%CI	*P*	β	OR	95%CI	*P*	β	OR	95%CI	*P*
Weight	0.017	1.017	1.007~1.028	0.001	*	*	*	*	-0.02	0.98	0.958~1.003	0.092
BMI	0.065	1.067	1.026~1.109	0.001	-0.06	0.942	0.866~1.024	0.16	*	*	*	*
WC	0.031	1.031	1.016~1.047	<0.001	0.028	1.029	0.996~1.063	0.088	0.028	1.028	0.995~1.062	0.092
TC	0.239	1.27	1.128~1.431	<0.001	*	*	*	*	*	*	*	*
HDL-c	-5.13	0.006	0.003~0.012	<0.001	-5.441	0.004	0.002~0.009	<0.001	-5.468	0.004	0.002~0.09	<0.001
LDL-c	1.494	4.454	3.766~5.268	<0.001	*	*	*	*	*	*	*	*
TG	0.888	2.429	1.966~3.003	<0.001	-0.02	0.98	0.684~1.405	0.913	-0.031	0.97	0.678~1.387	0.867
NAFLD	0.491	1.634	1.269~2.104	<0.001	1.022	2.779	1.516~5.096	0.001	1.004	2.73	1.487~5.015	0.001
HC	0.571	1.771	1.205~2.603	0.004	1.634	5.123	1.768~14.848	0.003	1.627	5.087	1.753~14.762	0.003

BMI: body mass index; WC: waist circumference; TG: triglyceride; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; NAFLD: nonalcoholic fatty liver disease; FBG: fasting blood glucose; HC: hypercholesterolemia; SBP: systolic blood pressure; DBP: diastolic blood pressure.

*eliminated for the definition of BMI or hyperlipidemia in multivariate regression.

- eliminated for no statistical significance in univariate regression.

Table 5: Univariate and multivariate analysis of gallbladder stone and BMI, WC, blood lipid, HC stratified by NAFLD

Factors		Univariate				Multivariate		
	β	OR	95%CI	*P*	β	OR	95%CI	*P*
Gender (M)	0.208	1.231	0.798~1.899	0.346	-	-	-	-
Age	0.014	1.014	0.996~1.037	0.123	-	-	-	-
Weight	0.015	1.015	0.995~1.036	0.134	-	-	-	-
BMI	0.079	1.082	1.005~1.166	**0.036**	-0.307	0.736	0.590~0.916	0.006
WC	0.046	1.047	1.014~1.081	**0.005**	0.019	1.02	0.925~1.123	0.695
SBP	0.002	1.002	0.989~1.014	0.794	-	-	-	-
DBP	-0.012	0.988	0.963-1.013	0.332	-	-	-	-

FBG	-0.057	0.944	0.733-1.217	0.658	-	-	-	-
TC	0.197	1.218	0.963~1.541	0.101	-	-	-	-
TG	0.676	1.965	1.329~2.907	**0.001**	-1.285	0.277	0.122~0.630	0.002
HDL-c	-5.832	0.003	0.001~0.012	**<0.001**	-7.545	0.001	0.000~0.008	<0.001
LDL-c	1.896	6.657	4.547~9.747	**<0.001**	2.158	8.652	3.464~21.611	<0.001
HC	0.61	1.84	0.847~3.997	0.123	-	-	-	-
Drink	0.647	1.911	0.911~4.007	**0.087**	1.069	2.912	0.078~108.202	0.562

BMI: body mass index; TG: triglyceride; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; NAFLD: nonalcoholic fatty liver disease; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; HC: hypercholesterolemia.

- eliminated for no statistical significance in univariate regression

6. Discussion

Gallbladder Stone Disease (GSD), a digestive disorder, originates from multifactorial factors including genetic, metabolic and environmental ones. Among metabolic factors, obesity, dyslipidemia and non-alcoholic fatty liver disease (NAFLD) have been gradually revealed as the risks of GSD development. In this clinical based, age-, occupation-, race-, diet- blood pressure-, FBG- and gender-matched case-controlled study of northwestern individuals of China, we found that NAFLD demonstrated as a key factor for GSD development, which was consistent with the recent study [14]. Otherwise, the present study revealed that NAFLD was associated with occurrence of GSD based on the synergistic effects of overweight/obesity and dyslipidemia after further adjustment for metabolic risk factors. This study showed that significantly higher risk of morbidity of GSD was detected in patients with NAFLD than those without NAFLD. Although publications indicated the homeostatic imbalance of parameters such as increased serum leptin concentrations and hyperlipidemia (hypercholesterolemia or hypertriglyceridemia) were associated with canine cholelithiasis and that might affect the pathogenesis of gallstones [15], the recent studies have revealed that NAFLD was significantly associated with gallstone formation [16], and three potential explanations were reported to be responsible for why gallstones were always accompanied by NAFLD, for instance a chance co-occurrence, the shared common risk factors, including obesity, insulin resistance, and type 2 diabetes mellitus [17]. Moreover, one hand, central obesity was a strong risk factor for GSD without consideration of abdominal fat distribution [18], the meta-analysis confirmed that an almost two-fold increase in the risk of GSD was revealed even within the "normal" BMI range, suggesting that even moderate increases in BMI would be enough to induce the increase risk of GSD [19]. Subjects with obesity showed a higher incidence of GSD by comparison with lean controls [20], and obesity has been well recognized as strong association with GSD [21]. Meanwhile, as this study showed that It was not significantly higher in NAFLD than non-NAFLD subjects with hypercholesterolemia, emerging studies supported the notion that hypercholesterolemia most likely led to cholesterol gallstone and is an independent risk factor for GSD [22]. Constant high concentrations of TC may be related to the hypersecretion of cholesterol in the bile and the subsequent supersaturation of bile, and then contributed to the formation of biliary sludge [23]. Besides, hypertriglyceridemia consequently reduced gallbladder motility with decreased sensitivity to cholecystokinin [23]. In addition, this study raised HDL-c levels are inversely associated with gallstone prevalence because it might not only increase primary bile acid formation which solubilizes cholesterol and reduces biliary cholesterol saturation [24], but also mediate the transport of excess cholesterol from lipid-laden macrophages within the vascular wall back to the liver for excretion into the bile [25]. However, in NAFLD patients our logistic regression further penetrated that HC is not significant correlation to GSD, and multivariate analysis detected that WC is not significant correlation while BMI is significant negative correlation to GSD. It seemed that NAFLD would pay a direct role in lithogenesis following de-novo lipogenesis increasing in hepatocytes following obesity, hypercholesterolemia and hypertriglyceridemia, among which the latter would show synergistic effects on GSD.

Further, it was further supported by the finding that NAFLD was typically characterized by atherogenic dyslipidemia, featuring larger triglyceride over enriched circulating very-low-density lipoproteins (VLDLs), small dense LDL-c and low and dysfunctional HDL-c [26]. It was generally known that NAFLD, an acquired metabolic disease of the liver, occurred in two phases: triglycerides accumulated in liver without hepatic damage in first phase, then hepatic damaged with necrobiotic inflammatory reaction, fibrosis and cirrhosis took place in the second phase [27]. And it was recently supported that hypercholesterolemia was a major risk factor for the initiation and development of NAFLD [28], arising from high-cholesterol diet, lipase deficiencies and liver inflammation [29]. Secondly, NAFLD, which affected approximately 12% to 40% of the general population, up to 95% of obese people and nearly 70% of the overweight, especially among middle-aged populations with the developments of obesity, type 2 diabetes, dyslipidemia, and other metabolic syndromes. Obesity can cause mitochondrial reactive oxygen species (ROS) production and antioxidant depletion in the liver due to higher saturated fatty acid availability and oxidation. Prolonged oxidative stress might fa-

vour n-3 long-chain polyunsaturated fatty acid depletion and IR. Liver oxidative stress and insulin resistance (IR) are considered primary abnormalities leading to alterations in hepatic metabolism and the onset of steatosis [30], and it was further revealed that NAFLD was observed in 96.5% ($p<0.001$) of subjects with body overweight, hypertriglyceridemia, and hypercholesterolemia [31]. Thirdly, NAFLD was strongly associated with obesity which might trigger reduction of leptin receptor expressions in liver tissue [32], and the both of which promoted the occurrence and development of fatty liver [33]. The prevalence of obesity and NAFLD is undoubtedly closely related to the change of diet structure characterized by an increase in energy intake and the consumption of added sugar and fats. The use of high fructose as sweeteners in beverages has increased significantly. Fructose intake increases the accumulation of fats in the liver and this high fructose intake is considered as one of the components dietary responsible for promoting NAFLD [34]. Fat quantity and quality is in relation to the development of hepatic steatosis. High fat diet and saturated fat may cause hepatic steatosis. Saturated fat changes mitochondrial structure and function and leads to the development and progression of NAFLD [35].

Note of, NAFLD may trigger gallbladder stone disease following obesity and dyslipidemia. First, insulin resistance was a key phenomenon in NAFLD development and progression [36]. Hepatic insulin resistance directly increased biliary secretion of cholesterol, whereas reduced the synthesis of bile, leading to promotion of cholesterol crystals nucleation, which has been pointed as a key determinant for cholesterol gallstones formation by itself [37]. Secondly, recent studies suggested that broad primarily histologic categories of NAFLD showed macrovascular steatosis in more than 5% of hepatocytes [38], while oxidative stress and lipid peroxidation would contribute to hepatic noxious pathologies in the above progress [39]. Due to fatty degeneration and lipid accumulation of liver cells in NAFLD tissue, liver X receptor (LXR), a member of the heterodimeric nuclear receptors superfamily that was activated by oxidative stress and lipid peroxidation, would induce the expression of the ATP binding cassette transporters ABCG5/ABCG8, which was very likely to trigger cholesterol stone formation by increasing canalicular excretion of cholesterol into the bile [40]. Finally, nonalcoholic steatohepatitis (NASH) showed additional histologic characteristics of liver injury including ballooned, hepatocytes, inflammatory foci, and fibrosis (38). The farnesios X receptor (FXR), a bile acids nuclear receptor highly expressed in liver and gut, monitored the expression of the canalicular transporters ABCB11 and ABCB4 that respectively transported bile acids and phosphatidylcholine into bile with impact in biliary cholesterol solubilization. NASH induced FXR-deficiency would spontaneously bring about the supersaturation of bile with cholesterol, precipitation of cholesterol crystals in the gallbladder, leading to bile salt hydrophobicity and gallbladder inflammation [41]. Additionally, hepatocyte dysfunction may trigger abnormal bile secretion and dysfunction of gallbladder motility, which would then induce gallbladder stone production. Clearly, NAFLD is an independent risk factor for asymptomatic GSD.

Interestingly, this multivariate logistic regression showed that there was positive correlation between serum LDL-c and hazard of GSD, while significantly negative correlation between BMI, serum TG, HDL-c levels and hazard of GSD. We trend to consider that LDL-c maybe an independent risk factor of GSD in NAFLD patients, and reducing HTG treatment might improve the risk of GSD by increasing bile cholesterol saturation and bile acid synthesis disorder. First, it is clear that bile composition (supersaturation with cholesterol), gallbladder dysmotility, inflammation, hypersecretion of mucin gel in the gallbladder and slow large intestinal motility and increased intestinal cholesterol absorption may contribute to the pathogenesis of cholesterol gallstones [23, 42]. LDL is the most cholesterol and cholesterol ester containing lipoprotein [43]. The recent study found that LDL-c was increased in gallbladder cholesterosis, which was strongly associated with the formation of calculi [44]. Furthermore, the modified minor particles of LDL would more rapidly penetrate than other LDL fractions into the gallbladder tissue, where the gallbladder wall was intensively captured by macrophages, and participated in the formation of foamy cells [45]. Second, NAFLD is a risk factor for dyslipidemia in hypercholesterolemia. It is known that because the liver does not serve as a storage depot for fat, there is a precise balance between acquisition by uptake of non-esterified fatty acids from the plasma and by de novo lipogenesis, versus triglyceride disposal by fatty acid oxidation and by the secretion of triglyceride-rich lipoproteins under physiological conditions. The hallmark of NAFLD is triglyceride accumulation in the cytoplasm of hepatocytes since the low steady-state triglyceride concentrations in the liver is destroyed between lipid acquisition (i.e., fatty acid uptake and de novo lipogenesis) and removal (i.e., mitochondrial fatty acid oxidation and export as a component of VLDL particles) [46, 47]. Moreover, LDL in plasma is transformed from very low density lipoprotein cholesterol (VLDL) with lipolysed by LPL after entering the blood stream, resulting in the formation of IDL. This particle is either taken up by the liver, mediated by apoE, or converted to LDL by Hepatic Lipase (HL). The process of VLDL synthesis ending with LDL clearance is called the "endogenous lipid pathway" [43]. As result, we guess that intensive lowing serum TG might be accompanied by higher serum LDL. Third, NAFLD is a hepatic manifestation of metabolic syndrome and secondary to hyperinsulinemia, the latter leads to hepatic steatosis by multiple mechanisms including greater uptake rates of plasma non-esterified fatty acids resulting from diminished insulin responsiveness, the transcriptional upregulation of genes with promoting de novo lipogenesis in the liver. increased release from an expanded mass of adipose tissue and then increased hepatic lipid accumulation is not offset by fatty acid oxidation or by increased secretion rates of triglyceride-rich lipoproteins [43]. As above-mention,

NAFLD might be complicated by hypertriglyceridemia. However, Fibrates or fish-oil is a common drug of TG lowering therapy [42]: the fore might increase the risk for cholelithiasis by increasing biliary cholesterol saturation and by reduction of bile acid synthesis. the latter has been shown to increase cholesterol secretion into bile, to increase bile acid pool size and synthesis rate, and to produce a great quantity of peroxidation, so as to still result in gallstone formation in HTG patients [48, 49].

7. Study Strengths and Limitations

A large number of same race participants, a retrospective design, and complete follow up for morbidity are strengths of our study. The information comes from participants who take regular annual physical screening, long-term follow-up. We reviewed the participants' case history with more than five years, made sure that the patients had been new-noted in the case group and the matched participants come from same occupation, similar environment. However, the present study has several limitations. First, this is not a multicenter and cohort study, statistics may be biased. Second, we main diagnosed the participant by B-mode ultrasonography and laboratory parameters so there is a hazard of the misdiagnosis and missed diagnosis rate. Third, the limited ethnic population may affect the generalizability of these findings. In particular, the association between BMI and gallbladder stone may differ among ethnic and regional groups with varying dietary structures and genetic diversity. Forth, this study lacked data such as chemical composition analysis, cholesterol gallbladder stone-related risk factors, bile acid levels, and gastrointestinal dysfunction, because imbalance between bile salts and cholesterol in bile fluid turns bile fluid into sludge, crystals and eventually gallstones. Finally, although our study matched FBG, the absence of insulin results might reduce our research efforts, which need to be confirmed in the future studies.

Taken together, we found that NAFLD associated to new-onset GSD in northwest population of China, whereas NAFLD played an essential link to trigger off this domino effect in the lithogenous process of GSD, which was related to obesity and dyslipidemia. in NAFLD patients, Notably, this study first finds that serum LDL-c levels was a key risk factor for GSD in NAFLD patients probably because sharp low TG or improper drug therapy may cause imbalance of cholesterol homeostasis and fatty acid. The results of this study suggest that for NAFLD the first step to be taken in the prevention of GSD is dietary intervention with special attention to limitation of alcohol and carbohydrate intake, and to weight reduction in case of overweight, rather than management of HTG. However, further studies were warranted to elucidate the molecular mechanism regarding how NAFLD induces the development of GSD both in vitro and in vivo.

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