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ORIGINAL ARTICLE

Non-obese histologically confirmed NASH patients with abnormal liver biochemistry have more advanced fibrosis

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Abstract

Background and aims Non-alcoholic fatty liver disease (NAFLD) commonly affects subjects with obesity, yet non-obese NAFLD is increasingly being recognized. We aimed to investigate the clinicopathological and genetic characteristics of non-obese NAFLD patients.

Methods The clinical, histological and genetic data of 84 NAFLD patients with biopsy for abnormal liver function test were reviewed. Both NAS-CRN and SAF scoring systems were applied for histopathological evaluation. *PNPLA3* and *TMS6F2* genotyping were also performed.

Results All of the 84 patients were histologically diagnosed with non-alcoholic steatohepatitis (NASH), with 36 of them (42.9%) being non-obese (BMI < 25 kg/m^2). Compared with the obese group, non-obese group were predominantly females (88.9% vs 52.1%, $p < 0.001$), tended to have higher prevalence of diabetes ($p = 0.068$). More importantly non-obese patients had a significant higher prevalence of advanced fibrosis ($F \geq 3$) (58.3% vs 29.2%, $p = 0.013$), and a trend of higher degree of ballooning ($p = 0.061$). In addition, values of liver stiffness measurement were also significantly higher in non-obese group (12.1 kPa vs 8.1 kPa, $p = 0.032$). There was also a trend of higher prevalence of TM6SF2 T allele in non-obese group ($p = 0.085$), while the prevalence of PNPLA3 risk allele did not differ between two groups. Multivariate analysis showed that higher fasting glucose ($p = 0.038$) and lower serum platelets ($p = 0.040$) were two independent predictors for advanced fibrosis in non-obese patients.

Conclusions Non-obese NASH patients have a female predominance and more advanced fibrosis. Liver biopsy is crucial to evaluate the severity of disease in non-obese patients especially those with abnormal liver biochemistry.

Clinical trial number NCT03386890.

Keywords Non-alcoholic fatty liver disease · Non-obese · Non-alcoholic steatohepatitis · Hepatic fibrosis

Abbreviations

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
MetS	Metabolic syndrome
BMI	Body mass index
PNPLA3	Polymorphisms of patatin-like-phospholipase domain-containing protein 3
TM6SF2	Transmembrane 6 superfamily antigen 2
WC	Waist circumference
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
GGT	γ-Glutamyltransferase
CHE	Cholinesterase
LDL-C	Low-density lipoprotein cholesterol

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HDL-C	High-density lipoprotein cholesterol
TG	Triglycerides
UA	Uric acid
PTA	Prothrombin activity
HOMA-IR	Homeostatic model assessment insulin resistance
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
FPG	Fasting plasma glucose
LSM	Liver stiffness measurements
CAP	Controlled attenuation parameter
H&E	Hematoxylin and eosin
NASH CRN	NASH Clinical Research Network
SD	Standard deviation
IQR	Interquartile range
TE	Transient elastography

Introduction

Non-alcoholic fatty liver disease (NAFLD) associated with obesity and the metabolic syndrome (MetS) has emerged as a global epidemic affecting both Western and Asian countries [1]. A relatively novel paradigm is the presence of NAFLD in individuals with a relatively normal body mass index (BMI), which is being referred to as lean or non-obese NAFLD. In fact, variating with BMI cut-off values to define overweight (23–25 kg/m^2) and obesity (25–30 kg/m^2) in the West and East, the prevalence of non-obese NAFLD is between 10 and 30% [2], making this group of patients a significant proportion of those with NAFLD. The available data suggest that non-obese NAFLD occurs more commonly in Asians [2]. As with conventional NASH associated with obesity, the risk factors for the disease may include visceral obesity, high fructose and high cholesterol intake [3, 4], and genetic risk factors such as polymorphisms of patatin-like-phospholipase domain-containing protein 3 (*PNPLA3*) and transmembrane 6 superfamily antigen 2 (*TM6SF2)* [5]. Since insulin resistance is also a significant feature of non-obese NAFLD patients, suggesting this is a phenotypic subset of NAFLD.

Due to the relatively recent recognition of non-obese NAFLD, data on clinical characteristics, pathogenetic mechanisms and histological features of this group remain sparse. In particular, the histopathological severity of non-obese NAFLD is still a matter of debate as most studies on non-obese NAFLD are based on noninvasive diagnostic means. Some studies showed less severe inflammation and fibrosis in non-obese NAFLD patients compared with obese patients [6, 7], whereas other studies demonstrated that non-obese NAFLD patients had similar or even more severe inflammation and fibrosis [8, 9].

Therefore, we conducted the current study to compare the clinical, histological and genetic characteristics of non-obese NAFLD with a group of conventional obese NAFLD in a specialized liver center, with an aim to identify risk factors associated with disease severity.

Materials and methods

Study population

This cross-sectional study consisted of consecutive adult patients with histologically proven NAFLD diagnosed at the Liver Research Centre in Beijing Friendship Hospital, Capital Medical University from January 2014 to May 2018. Unexplained liver diseases including elevation of liver enzymes were the main indications for liver biopsy.

The exclusion criteria were: (1) age less than 18 years; (2) positive serologic markers for hepatitis B or C virus; (3) alcohol consumption of more than 20 g/day for men and more than 10 g/day for women; (4) other concomitant liver disease (autoimmune hepatitis, primary biliary cholangitis, sclerosing cholangitis, drug-induced liver injury, hemochromatosis, Wilson's disease); (5) fatty liver disease secondary to inherited metabolic liver diseases or systemic use of steroids; (6) any malignant tumor, and (7) any complications of severe heart, lung, kidney, brain, blood diseases or other systemic diseases.

Clinical data

Demographic data and medical history including hypertension, diabetes mellitus, alcohol consumption, smoking, drug history, and family history were recorded. Physical examination, anthropometric and laboratory measurements were performed before liver biopsy. Body weight, height, waist circumference (WC, measured from the bottom edge of the last rib and the iliac crest) and blood pressure were measured.

BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2). In accordance with the WHO criteria for the Asian BMI, the patients were defined as non-obese if they had a BMI < 25.0 kg/m^2, whereas those were defined as obese if they had a BMI ≥ 25.0 kg/m^2 [10].

Hematological, biochemical and genetic investigations

Blood samples were obtained after a minimum of 12 h fasting, and liver biochemistries, fasting glucose, insulin, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), uric acid (UA), creatinine, ferritin and hemoglobin A1c were measured according to standard procedures.

Homeostatic model assessment insulin resistance (HOMA-IR) was used as the index of insulin resistance based on the following formula:

HOMA-IR = (fasting serum insulin [mIU/ml] × fasting serum glucose [mmol/l]/22.5) [11].

Polymorphisms of *PNPLA3* and *TM6SF2* were identified using the TaqMan SNP Genotyping Assay (Life Technologies, Grand Island, NY, USA).

The diagnosis of MetS was based on the ATP III criteria [12], with the presence of three or more of the following conditions: (1) central obesity (defined as a waist circumference > 90 cm for Chinese men and > 80 cm for Chinese women); BMI > 25 kg/m^2; (2) raised circulating TG levels (defined as TGs ≥ 1.7 mmol/l) or specific treatment for this lipid abnormality; reduced HDL-C levels (defined as HDL-C < 1.03 mmol/l in male patients and < 1.29 mmol/l in female patients); (3) raised systolic blood pressure (SBP) or diastolic blood pressure (DBP) (defined as SBP ≥ 130 mm Hg or DBP ≥ 85 mm Hg) or treatment for previously diagnosed hypertension; and (4) raised fasting plasma glucose (FPG) (defined as FPG ≥ 5.6 mmol/l) or previously diagnosed type 2 diabetes.

Measurements of liver stiffness and controlled attenuation parameter (CAP)

Liver stiffness measurements (LSM) by transient elastography (Fibroscan; Echosens, Paris, France) were performed by experienced operators according to previously described methods [13] on the same day, or 1 day before or after liver biopsy. The result was considered reliable when at least 10 valid measurements were obtained, with success rate exceeding 60% and the interquartile range-to-liver stiffness ratio ≤ 30%. The liver stiffness was expressed as kPa. CAP values were expressed as dB/m.

Histological evaluations

All patients underwent a percutaneous ultrasound-guided liver biopsy. The specimens were routinely formalin fixed, paraffin embedded, and stained with hematoxylin and eosin (H&E), reticulin and Masson's trichrome stain.

All liver biopsy samples were assessed by both two specialized liver pathologists (RS, ASHG) who were blinded to the clinical information. They discussed together to give the final scores and diagnosis. Histological scoring was performed according to two systems, the NASH Clinical Research Network (NASH CRN) system [14] and the SAF/FLIP algorithm [15]. In our study, advanced fibrosis was defined as F3 and F4.

In addition, portal inflammation and steatosis location were also assessed in this study. Portal inflammation was graded from 0 to 3 according to the number of inflammatory cells in the portal tract (0: none, 1: few to minimal, 2: mild, 3: moderate). In non-cirrhotic patients, the predominant distribution pattern of steatosis was defined as 0: zone 3, 1: zone 1, 2: azonal and 3: panacinar, and in cirrhotic patients the location of steatosis was recorded as "can't define".

Statistical analysis

Continuous variables were expressed in mean ± standard deviation (SD) or median (interquartile range; IQR). Categorical variables were summarized by counts and percentages. Chi-squared test and Fisher's exact test were used for categorical variables. Independent variables associated with advanced liver fibrosis ($F \geq 3$) were identified using the binary logistic regression model. Statistical significance was considered when $p < 0.05$. Statistical analyses were performed using SPSS 20.0 software (IBM Corp, Armonk, NY, USA).

Results

Demographic, clinical and histological characteristics of the patients

Demographic characteristics

There were 124 patients with histologically proven NAFLD between January 2014 and May 2018 in our department, of whom a total of 116 patients fulfilled the inclusion criteria. After a re-review of the histology, 84 patients with histologically confirmed NASH were included in the final analysis. The flowchart is shown in Fig. 1.

All patients are ethnic Chinese, from different provinces all over China, with 57.1% (48/84) patients from Beijing. The mean age of the patients was 47.2 ± 14.2 years, and 67.9% (57/84) were female, 44 (77.2%) of them were postmenopausal, and none of the remaining 13 females took contraceptives. The mean BMI was 25.8 ± 3.5 kg/m^2. A total of 30 patients (35.7%) had type 2 diabetes, with average fasting glucose level of 6.9 ± 1.5 mmol/l, the median time of diagnosis was 27 months (range 1–240), 19 (63.3%) diabetic patients had received treatment, with median treatment duration of 60 months (range 1–240). Twenty-eight patients (33.3%) had hypertension and the median time of diagnosis was 102 months (range 1–360). Thirty-nine patients (46.4%) had MetS, which were all diagnosed at the time of liver biopsy. A total of 59 (70.2%) patients had hypertriglyceridemia, with average TG level of 2.5 ± 0.8 mmol/l, only 8 patients had received anti-hyperlipidemia therapy. All the 57 female patients denied history of smoking. In 27 male patients, there were 6 who had the history of smoking, two

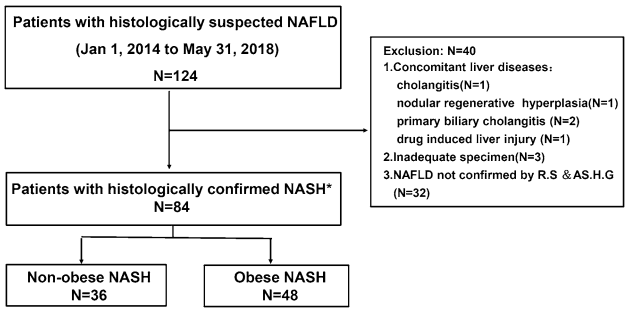

Fig. 1 Flowchart of the study. *Asterisk* All the 84 patients were diagnosed as NASH using the SAF score and the FLIP algorithm after a re-review of the histology

of them had already quitted. The demographic and clinical characteristics of the 84 patients are shown in Table 1.

Hematological, biochemical and genetic characteristics

The biochemical and genetic characteristics are shown in Table 2. There were 27.4% of the patients had elevated fasting glucose, 70.2% had elevated triglyceride. The *PNPLA3* rs738409, *PNPLA3* rs6006460 and *TM6SF2* rs58542926 polymorphism were genotyped in 42 patients. Genotype frequencies of CC, CG and GG in *PNPLA3* rs738409 were 23.8% (10/42), 45.2% (19/42) and 31.0% (13/42), respectively. Genotype frequencies of CC, CT and TT in *TM6SF2* rs58542926 were 83.3% (35/42), 11.9% (5/42) and 4.8% (2/42), respectively. All patients were homozygous wild-type (GG) in *PNPLA3* rs6006460 in our study.

Histological characteristics

On histological examination, all patients had varying degrees of steatosis and lobular inflammation. Steatosis was predominantly localized to acinar zone 3 (50/84, 59.5%). According to the NAS score, 97.6% of patients (82/84) had ballooning, and 53.6% of the patients (45/84) had NAS score ≥ 5. Using the SAF score and the FLIP algorithm, all the patients had the grade of ballooning ≥ 1, thus could be diagnosed as NASH.

Remarkably, liver fibrosis was discovered in a considerable proportion of patients (76/84, 90.5%). The prevalence of advanced fibrosis ($F \geq 3$) was 41.7%. Sixteen patients (19.0%) had a histological diagnosis of cirrhosis. Fibrosis stage 1C was not observed. Portal inflammation could be observed in 90.5% of patients, and its grade was significantly associated with the stage of fibrosis ($r = 0.798$, $p < 0.001$). The histological characteristics are shown in Table 3.

Comparison of the clinical characteristics between non-obese and obese patients with NASH

Based on the WHO criteria, we divided the patients into 2 groups: the non-obese NASH group of 36 patients (42.9%) with a BMI < 25 kg/m^2 and the obese NASH group of 48 patients (57.1%) with BMI ≥ 25 kg/m^2. The average age did not differ between the two groups, but a female preponderance was found in the non-obese NASH group (88.9% vs 52.1%, $p < 0.001$). There was no significant difference in the proportion of patients with hypertension or MetS. However, there was a trend to have higher prevalence of diabetes ($p = 0.068$) in non-obese group. There was no significant

Table 1 Demographic and clinical characteristics of the NASH patients

Variable	Total ($N = 84$)	BMI < 25 ($N = 36$)	BMI ≥ 25 ($N = 48$)	p
Age, years	47.2 ± 14.2	50.4 ± 13.2	44.8 ± 14.6	0.069
Sex				
Female	57 (67.9%)	32 (88.9%)	25 (52.1%)	< 0.001
Male	27 (32.1%)	4 (11.1%)	23 (47.9%)	
Body mass index, kg/m^2	25.8 ± 3.5	22.8 ± 1.4	28.1 ± 2.8	< 0.001
Waist circumference, cm				
Female	87.7 ± 8.7	83.1 ± 5.8	93.5 ± 8.3	< 0.001
Male	97.7 ± 10.7	87.8 ± 3.1	99.4 ± 10.6	< 0.001
Waist-to-hip ratio				
Female	0.93 ± 0.03	0.93 ± 0.03	0.93 ± 0.02	0.847
Male	0.96 ± 0.05	0.96 ± 0.02	0.96 ± 0.05	0.917
Diabetes	30 (35.7%)	17 (47.2%)	13 (27.1%)	0.068
Hypertension	28 (33.3%)	10 (27.8%)	18 (37.5%)	0.483
MetS	39 (46.4%)	13 (36.1%)	26 (54.2%)	0.124
CAP, dB/m	286.9 ± 50.3	271.8 ± 54.9	297.5 ± 44.2	0.021
LSM, kPa	10.4 (6.6, 16.7)	12.1 (7.0, 20.5)	8.1 (5.9, 14.1)	0.032

MetS metabolic syndrome, *CAP* controlled attenuation parameter, *LSM* liver stiffness measurements

Table 2 Biochemical and genetic characteristics of the NASH patients

Variable	Total ($N=84$)	BMI < 25 ($N=36$)	BMI ≥ 25 ($N=48$)	p
White blood cells, $\times 10^9$/l	5.6 ± 2.0	5.2 ± 1.5	5.9 ± 2.3	0.125
Hemoglobin, g/l	135.3 ± 17.0	132.8 ± 17.2	137.2 ± 16.8	0.247
Platelets, $\times 10^9$/l	190.7 ± 67.2	178.5 ± 70.7	199.9 ± 63.7	0.150
PTA, %	96.8 ± 18.9	93.7 ± 20.2	99.0 ± 17.7	0.206
ALT level, IU/l	76.0 (42.0, 120.3)	57.0 (32.5, 101.0)	94.0 (45.5, 135.3)	0.085
AST level, IU/l	55.7 (38.9, 77.8)	56.8 (37.4, 73.5)	53.9 (39.4, 79.5)	0.820
ALP level, IU/l	100.1 ± 35.3	101.5 ± 37.6	98.9 ± 33.9	0.749
GGT level, IU/l	66.0 (39.0, 105.5)	64.0 (39.0, 119.0)	68.0 (39.5, 102.5)	0.721
Total bilirubin, μmmol/l	13.9 ± 6.0	14.0 ± 6.2	13.9 ± 5.9	0.942
Albumin, g/l	41.1 ± 4.4	41.5 ± 3.7	41.8 ± 4.8	0.486
Globulin, g/l	30.4 ± 4.4	31.1 ± 4.4	29.9 ± 4.4	0.261
CHE, KU/l	8.4 ± 2.1	8.2 ± 2.5	8.5 ± 1.7	0.483
Fasting glucose, mmol/l	5.7 ± 1.4	5.9 ± 1.3	5.7 ± 1.4	0.547
Triglyceride, mmol/l	2.1 ± 0.9	2.3 ± 0.8	1.9 ± 1.0	0.071
Total cholesterol, mmol/l	4.7 ± 1.5	4.9 ± 1.3	4.7 ± 1.5	0.648
HDL-C, mmol/l	1.2 ± 0.3	1.2 ± 0.3	1.1 ± 0.3	0.262
LDL-C, mmol/l	2.8 ± 0.7	2.9 ± 0.7	2.8 ± 0.7	0.789
Ferritin, ng/ml	164.1 (81.1, 428.3)	163.0 (91.9, 471.3)	172.5 (75.4, 428.3)	0.518
Hemoglobin A1c, %	6.8 ± 1.6	7.3 ± 1.5	7.3 ± 1.5	0.689
HOMA-IR	3.9 (2.7,6.0)	4.8 ± 2.2	3.8 ± 1.8	0.526
PNPLA3 rs738409				
CC, *n* (%)	10/42 (23.8%)	2/16 (12.5%)	8/26 (28.8%)	
CG + GG, *n* (%)	32/42 (76.2%)	14/16 (87.5%)	18/26 (69.2%)	0.270
TM6SF2 rs58542926				
CC, *n* (%)	35/42 (83.3%)	11/16 (68.8%)	24/26 (92.3%)	
CT + TT, *n* (%)	7/42 (16.7%)	5/16 (31.2%)	2/26 (7.7%)	0.085

PTA prothrombin time activity, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *GGT* γ-glutamyltransferase, *CHE* cholinesterase, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *HOMA-IR* homeostatic model assessment insulin resistance

difference in the duration time of type 2 diabetes and hypertension between the two group ($p=0.326$ and 0.226 relatively). The prevalence of smoking did not differ between two groups ($p=0.131$). Compared with the obese NASH patients, the non-obese NASH patients had smaller WC but similar waist-to-hip ratios, both for females and males. Apart from a trend of higher level of triglyceride ($p=0.087$) and lower level of ALT ($p=0.085$) in non-obese NASH patients, the rest biochemical test values did not show significant differences between the two groups (Tables 1, 2).

Genetic analysis showed no significant difference in the proportion of patients carrying G in *PNPLA3* rs738409 (87.5% vs 69.2%, $p=0.27$) between two groups. However, there was a trend that the non-obese NASH patients were more likely to carry T in *TM6SF2* rs58542926 (Table 2).

Comparison of the histological characteristics between non-obese and obese patients with NASH

There were no statistically significant differences between the two groups with regard to the grade or location of hepatic steatosis, grade of lobular/portal inflammation and proportion in the diagnostic category of NASH. According to NASH CRN system, the grade of ballooning did not differ between two groups ($p=0.159$). However, when using the SAF system, non-obese NASH patients had a trend to have higher degree of ballooning ($p=0.061$). The degree and prevalence of fibrosis in non-obese NASH was comparable with the obese group, however, there was a significant higher prevalence of advanced fibrosis in the non-obese group ($p=0.013$) (Table 3, Fig. 2). Two representative cases from non-obese and obese NASH groups, respectively, are shown in Fig. 3. When using different cut-off values of BMI (24 kg/m^2 and 23 kg/m^2, respectively), the same trend could be seen (Fig. 4).

Table 3 Histological characteristics of the NASH patients

Variable	Total ($N=84$)	BMI < 25 kg/m^2 ($N=36$)	BMI ≥ 25 kg/m^2 ($N=48$)	p value
Steatosis				
Grade				0.109
1	57 (67.9%)	29 (80.6%)	28 (58.3%)	
2	20 (23.8%)	5 (13.9%)	15 (31.3%)	
3	7 (8.3%)	2 (5.6%)	5 (10.4%)	
Location				0.564
Zone 3	50 (59.5%)	19 (52.8%)	31 (64.6%)	
Zone 1	1 (1.2%)	1 (2.8%)	0	
Azonal	7 (8.3%)	4 (11.1%)	3 (6.3%)	
Panacinar	10 (11.9%)	4 (11.1%)	6 (12.5%)	
Can't define	16 (20.8%)	8 (22.2%)	8 (16.6%)	
Lobular inflammation				
NAS-CRN				0.476
1	40 (47.6%)	16 (44.4%)	24 (50.0%)	
2	34 (40.5%)	17 (47.3%)	17 (35.4%)	
3	10 (11.9%)	3 (8.3%)	7 (14.6%)	
SAF				0.663
1	40 (47.6%)	16 (44.4%)	24 (50.0%)	
2	44 (52.4%)	20 (55.6%)	24 (50.0%)	
Ballooning				
NAS-CRN				0.159
0	2 (2.4%)	0	2 (4.2%)	
1	25 (29.8%)	8 (28.6%)	17 (35.4%)	
2	57 (67.9%)	28 (71.4%)	29 (60.4%)	
SAF				0.061
0	0	0	0	
1	18 (21.4%)	4 (11.1%)	14 (29.2%)	
2	66 (78.6%)	32 (88.9%)	34 (70.8%)	
Activity (SAF)				0.349
2	10 (11.9%)	2 (5.6%)	8 (16.7%)	
3	34 (40.5%)	16 (44.4%)	18 (37.5%)	
4	40 (47.6%)	18 (50.0%)	22 (45.8%)	
≥ 3	74 (88.1%)	34 (94.4%)	40 (83.3%)	0.177
NAFLD activity score (NAS)				0.327
1–2	1 (1.2%)	0	1 (2.1%)	
3–4	38 (45.2%)	16 (44.4%)	22 (45.8%)	
5–8	45 (53.6%)	20 (55.6%)	25 (52.1%)	
Portal inflammation				0.278
0	8 (15.1%)	4 (11.2%)	7 (14.6%)	
1	11 (20.8%)	8 (22.2%)	19 (39.6%)	
2	22 (41.5%)	16 (44.4%)	16 (33.3%)	
3	12 (22.6%)	8 (22.2%)	6 (12.5%)	
≥ 2	34 (64.2%)	24 (66.7%)	22 (45.8%)	0.077
Fibrosis stage				0.478
0	8 (9.5%)	2 (5.6%)	6 (12.5%)	
1A	11 (13.1%)	2 (5.6%)	9 (18.8%)	
1B	17 (20.2%)	6 (16.7%)	11 (22.9%)	
1C	0	0	0	
2	13 (15.5%)	5 (13.8%)	8 (16.7%)	
3	19 (22.6%)	13 (36.1%)	6 (12.5%)	
4	16 (19%)	8 (22.2%)	8 (16.7%)	
Presence of fibrosis[a]	76 (90.5%)	34 (94.4%)	42 (87.5%)	
Advanced fibrosis[b]	35 (41.7%)	21 (58.3%)	14 (29.2%)	**0.013**

Bold value indicates statistically significant

[a]Includes patients with fibrosis stage ≥ 1

Table 3 (continued)

[b]Includes patients with F3 or F4

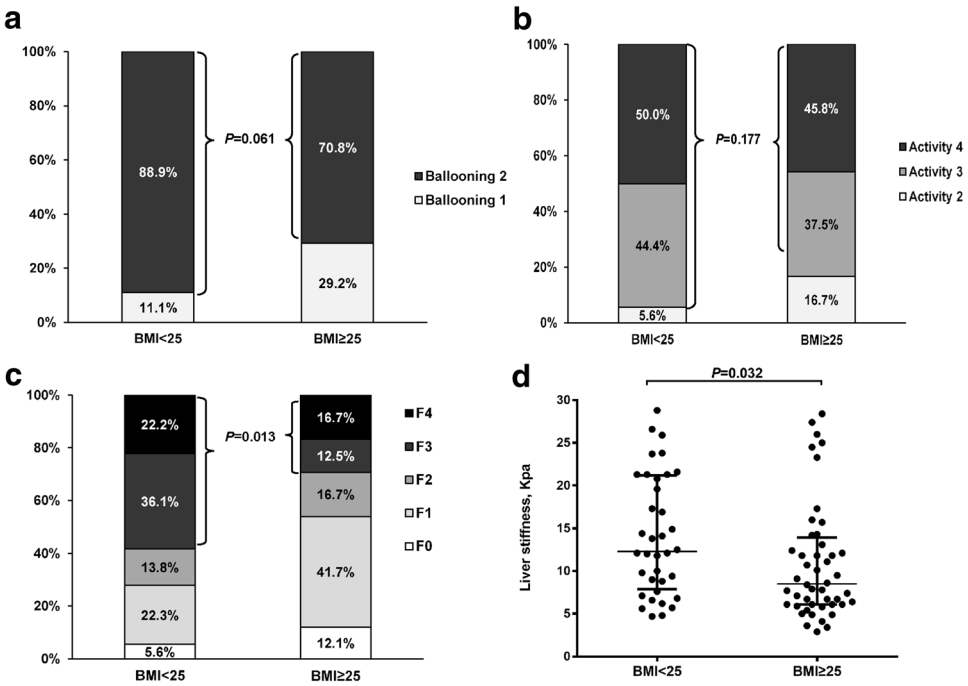

Fig. 2 Comparison of histological features and values of liver stiffness measurement between the obese and non-obese groups. **a** Comparison of grades of ballooning (SAF scoring system) between the obese and non-obese groups. **b** Comparison of activity grades (SAF scoring system) between the obese and non-obese groups. **c** Comparison of fibrosis between the obese and non-obese groups. **d** Comparison of LSM values between obese and non-obese groups

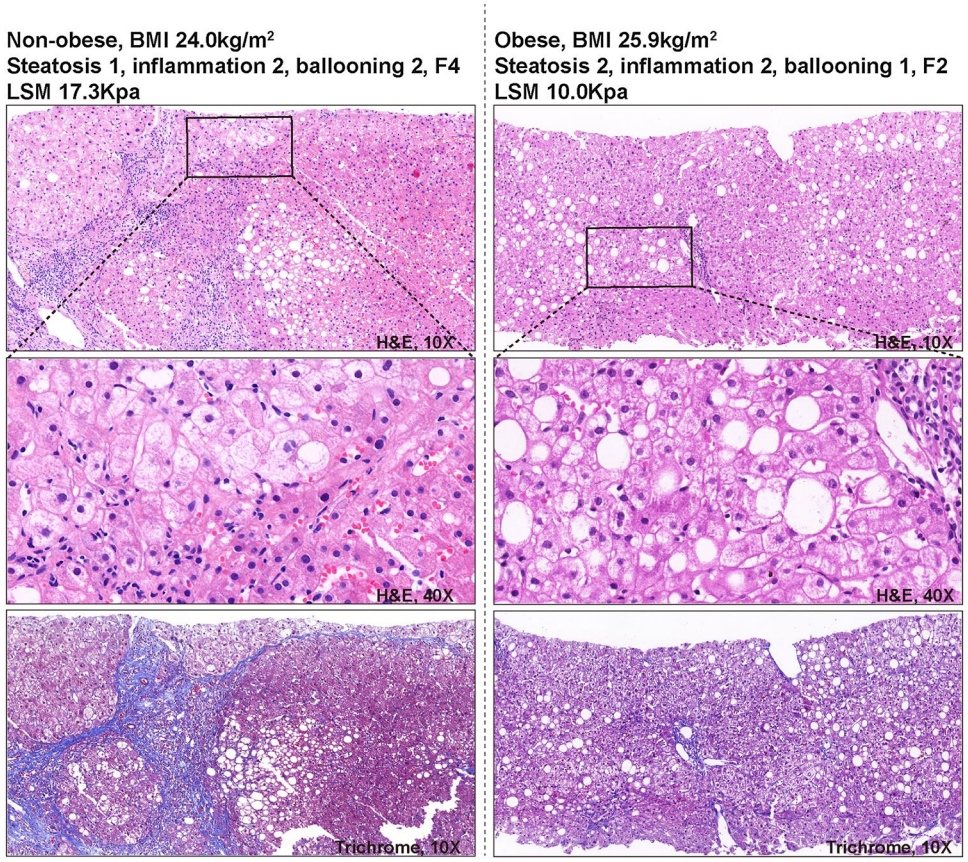

Fig. 3 Liver biopsy samples of non-obese and obese NASH patients. Ballooning, inflammation and fibrosis are more severe in the non-obese patient

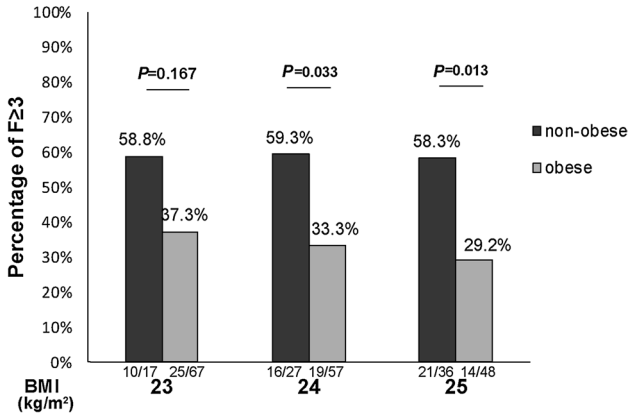

Fig. 4 Prevalence of advanced fibrosis ($F \geq 3$) in obese and non-obese groups using different BMI cut-off values

Accordingly, similar results were obtained using a non-invasive diagnostic method. In non-obese group, LSM value was significantly higher than that of the obese group (12.1 kPa vs 8.1 kPa, $p = 0.032$), and the CAP value was significantly lower (Table 2, Fig. 2).

Factors associated with advanced fibrosis in NASH patients of both groups and non-obese group

By univariate analysis, there were ten factors associated with the presence of advanced fibrosis in NAFLD patients of both groups. Four factors (creatinine, prothrombin time activity and white blood cells) were excluded due to multicollinearity, so multivariate analysis showed that BMI < 25 kg/m^2 (OR 2.97; 95% CI 1.00–8.79; $p = 0.049$), fasting glucose (OR 1.66; 95% CI 1.08–2.55; $p = 0.021$) and platelets (OR 0.98; 95% CI 0.97–0.99; $P < 0.001$) were the only three factors (Supplementary Table S1).

Further on, factors associated with advanced fibrosis in non-obese sub-group were analyzed. By univariate analysis, a history of diabetes, serum fasting glucose, total bilirubin, prothrombin time activity, white blood cells and platelets are the six factors associated with the presence of advanced fibrosis in non-obese NAFLD patients. Multivariate analysis showed that only higher fasting glucose (OR 2.62; 95% CI 1.05–6.53; $p = 0.038$) and lower serum platelets level (OR 0.99; 95% CI 0.98–1.00; $p = 0.040$) were the two independent predictors of advanced fibrosis (Table 4).

Discussion

In the current study, we demonstrated that non-obese NASH group had a significant higher prevalence of advanced fibrosis histologically. The non-obese NASH patients had a predominance of female, an increased prevalence of diabetes, and a trend of higher level of triglycerides compared with their obese counterparts.

We found a higher prevalence of advanced fibrosis in non-obese NASH patients, even after using different cut-off values of BMI (25 kg/m^2, 24 kg/m^2 and 23 kg/m^2, respectively), which was the most intriguing part in the present study. In fact, data on the histology of non-obese NAFLD patients are limited and results are inconsistent. Most of the published data showed lower degree of steatosis, inflammation and fibrosis in lean/non-obese NAFLD patients [6, 16, 17]. A prospective study from Hong Kong that included 307 patients with a histological diagnosis of NAFLD demonstrated that non-obese patients had lower fibrosis stage and may have better prognosis than obese patients on long-term follow-up [7]. However, a study from India also provided evidence that the proportion of steatohepatitis and advanced fibrosis were similar in obese and non-obese NAFLD patients [18]. On the contrary, the most recent study showed higher risk for development of severe liver disease in lean NAFLD (BMI < 25 kg/m^2) despite lower stages of fibrosis [19].

Our results showed a trend towards more disease activity and significant higher prevalence of advanced fibrosis in non-obese group, which was further supported by the value of LSM. The inconsistent results from different studies may be due to the heterogeneity of subjects. In our study, on all the patients were performed liver biopsy for persistent abnormal liver biochemistry, or even suspected cirrhosis, with more than half of the patients that had NAS ≥ 5, 90.5% of the patients had varying degrees of liver fibrosis, and all of them could be diagnosed with NASH according to the SAF/FLIP algorithm. Therefore, our results suggest non-obese NAFLD may not be a simple benign condition.

There was an increased prevalence of diabetes in non-obese NASH patients in our study, and higher fasting glucose was found to be an important factor associated with the presence of advanced fibrosis. In fact, several studies have shown that metabolic risk factors associated with insulin resistance are relevant for non-obese NAFLD as they are for obese NAFLD [20, 21]. Non-obese NAFLD patients can have higher levels of fasting blood sugar, and higher prevalence of MetS in comparison with healthy controls. In line with our study, recent research suggested that the association with components of MetS was even stronger for non-obese NAFLD than for obese NAFLD [22, 23]. Non-obese NAFLD patients had higher adjusted prevalence ratios for certain components of MetS such as high triglyceride levels, high blood pressure, and impaired fasting glucose. Thus, non-obese NAFLD patients with diabetes or MetS should be regularly screened during their follow-up.

Compared with obese group, non-obese NASH patients had lower BMI, lower waist circumference and hip

Table 4 Factors associated with advanced fibrosis in non-obese NASH patients

	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Sex	0.43	0.04–4.58	0.483			
Age	0.98	0.94–1.04	0.591			
History of diabetes	4.47	1.05–18.94	0.042	1.14	0.14–9.55	0.906
History of hypertension	0.35	0.08–1.58	0.174			
Metabolic syndrome	2.06	0.49–8.65	0.322			
ALT level, IU/l	1.00	0.99–1.01	0.670			
AST level, IU/l	1.01	0.99–1.02	0.416			
ALP level, IU/l	1.01	0.99–1.03	0.427			
GGT level, IU/l	1.00	0.99–1.00	0.794			
Albumin, g/l	0.97	0.81–1.17	0.762			
Total bilirubin, μmmol/l	1.21	1.02–1.42	0.027	1.14	0.97–1.33	0.106
CHE, KU/l	0.87	0.65–1.16	0.349			
Total cholesterol, mmol/l	0.78	0.45–1.35	0.376			
Triglyceride, mmol/l	0.85	0.38–1.93	0.704			
LDL-C, mmol/l	0.78	0.31–2.01	0.610			
HDL-C, mmol/l	0.52	0.06–4.28	0.542			
Ferritin, ng/ml	1.00	1.00–1.01	0.350			
Prothrombin time activity, %	0.94	0.90–0.99	0.013			
White blood cells, $\times 10^9$/l	0.61	0.36–1.02	0.057			
Hemoglobin, g/l	0.97	0.93–1.01	0.168			
Fasting glucose, mmol/l	2.38	1.07–5.28	0.033	2.62	1.05–6.53	0.038
Platelets, $\times 10^9$/l	0.99	0.98–0.99	0.035	0.99	0.98–1.00	0.040
PNPLA3 rs738409 G carrier	0.40	0.02–8.07	0.550			
TM6SF2 rs58542926 T carrier	0.56	0.06–5.22	0.613			

Advanced fibrosis includes patients with F3 or F4. Factors are determined by ordinal logistic regression; factors that reach a significance level of $p < 0.1$ on univariate analysis are entered into multivariate analysis

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *ALP* alkaline phosphatase, *GGT* γ-glutamyltransferase, *CHE* cholinesterase, *LDL-C* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol

circumference, so such patients may not be screened or be aware that they were at risk for more progressive disease. However, waist-to-hip ratio did not differ between two groups and most of non-obese NASH patients still had visceral fat obesity. Researches have showed that visceral adipose tissue area rather than the total body fat content is more relevant for the development of non-obese NAFLD [24]. Since BMI does not have the ability to evaluate the body fat distribution, new anthropometric measurements for visceral adiposity are greatly needed.

We found a very high prevalence of non-obese NASH (42.9%) in our study, especially in female (over 50% of female and less than 15% of male patients were non-obese). Notably, 73.7% of female patients in non-obese group were postmenopausal, which suggest that besides metabolic risk factors, sex hormone such as estrogens may play a role in fibrogenesis. One study showed that postmenopausal women were at greater risk of having more advanced fibrosis compared to premenopausal women at a given degree of hepatocyte ballooning and portal inflammation [25]. Thus, decrease of the estrogen levels may explain the higher proportion of advanced liver fibrosis.

Genetic factors may be important in the development of non-obese NAFLD. Recent studies have shown that *PNPLA3* and *TM6SF2* polymorphisms are associated with NAFLD susceptibility and progression. A population-based study from Hong Kong revealed that the G allele at the *PNPLA3* rs738409 was more common in non-obese than obese NAFLD patients (78.4% vs 59.8%), and it was considered one of independent factors associated with NAFLD in non-obese subjects [5]. A recent research from Japan also demonstrated that *PNPLA3* rs738409 was strongly associated with the development and progression of NAFLD in non-obese patients [17]. The *TM6SF2* polymorphism affected hepatocytic triglyceride content and was found to be associated with hepatic fibrosis independent of *PNPLA3* genotype [26]. However, data on the association of *TM6SF2* polymorphism and non-obese NAFLD are lacking. In our study, we found a trend that the non-obese

NAFLD patients were more likely to carry T in *TM6SF2* rs58542926. Due to the small number of cases in both the groups, further research will be needed to elucidate the genetic impact of *TM6SF2* on non-obese NAFLD patients.

In our study, the following limitations should be considered. Firstly, this was a cross-sectional study, therefore, information on natural course is not available, and it is impossible to prove a causal link. Secondly, although the diagnosis is histologically confirmed in all patients, the case number remains relatively small, so statistically significant differences could not be easily elucidated. Thirdly, we were not able to record the information on the diet and the physical activity in the current study, which may be the risk factors associated with disease progression of non-obese NAFLD patients. Therefore, further studies with larger number of patients, prospective design and long-term follow-up are needed.

In conclusion, non-obese NASH patients with abnormal liver function tests tend to present with severe histological fibrosis in the absence of other significant clinical abnormalities. Our study underscores the necessity to screen and monitor the liver fibrosis in non-obese NASH patients with abnormal liver biochemistry. This is especially important for females with diabetes and elevated serum triglycerides. Liver biopsy is warranted to establish correct diagnosis and evaluate the severity of disease if there is evidence of disease progression, such as decreased level of platelets.

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Author contributions Study design: JJ, RS, ASHG, HY. Data collection: QW, MW, XO, YW, WD, XW. Liver biopsy assessment: XZ, RS, ASHG. Statistical analysis: QW, YS, YK, SW. Manuscript writing: QW. Genotype analysis: PW, QW, MW. Critical revision of the manuscript: HY, JJ, RS, ASHG.

Compliance with ethical standards

Conflict of interest Qianyi Wang, Hong You, Xiaojuan Ou, Xinyan Zhao, Yameng Sun, Min Wang, Ping Wang, Yu Wang, Weijia Duan, Xiaoming Wang, Shanshan Wu, Yuanyuan Kong, Romil Saxena, Annette S. H. Gouw, Jidong Jia declare that they have no conflicts of interest.

Ethical approval The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the institutional Ethics Committee (Ethical approval no. 2015-P2-070-01). All patients signed informed consent for liver biopsy and blood sample storage.

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