Non-alcoholic fatty liver disease, cytokeratin-18, and risk of type 2 diabetes mellitus: A cohort study

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Research article

Abstract

Background & Aims: Although many studies have shown that non-alcoholic fatty liver disease (NAFLD) is associated with type 2 diabetes mellitus (T2DM), no cohort study has explored the relationship between the histopathological grade of NAFLD and the risk of T2DM in NAFLD patients. We aimed to explore whether a higher concentration of cytokeratin-18 (CK-18), as a reliable marker of hepatic fibrosis, was associated with a greater risk of T2DM in patients with NAFLD. Methods: The population-based cohort study was based on China National Diabetes and Metabolic Disorders Survey with a follow-up of five years. NAFLD was determined by ultrasonography. T2DM were diagnosed based on oral glucose tolerance test. Serum CK-8 was measured using the M30 Apoptosense ELISA kit. Results: 457 subjects were enrolled and three groups were analyzed: a non-NAFLD group (n=363), a low-CK-18 NAFLD group (n=46), and a high-CK-18 NAFLD group (n=48). 20 (3.9%) developed diabetes during follow-up. The incidence of T2DM was 2.5%, 8.7%, and 12.5% in the non-NAFLD, low-CK-18 NAFLD, and high-CK-18 NAFLD groups, respectively. Cox proportional hazard regression showed that, compared with the non-NAFLD group, the adjusted relative risks of T2DM were 3.37 (95% CI: 1.05-10.86, P =0.042) and 4.71 (95% CI: 1.71-12.99, P =0.003), respectively, in the low-CK-18 NAFLD and high-CK-18 NAFLD groups. Conclusions: Higher CK-18 level in ultrasound-diagnosed NAFLD patients is associated with higher risk of T2DM. We recommend screening for NAFLD using ultrasound in the first instance, with, if possible, CK-18 assay being subsequently used to screen individuals at higher risk of diabetes.

Background

Non-alcoholic fatty liver disease (NAFLD) has become one of the most important diseases affecting public health in the twenty-first century, with an estimated worldwide prevalence of 25% [1]. It is characterized by significant lipid deposition in the hepatocytes of patients in the absence of other etiologies, including excessive alcohol use, poisoning, or viral infection [1–3]. Although many studies have shown that NAFLD is associated with type 2 diabetes mellitus (T2DM), and some studies suggest that insulin resistance is an important mechanism [4,5], no cohort study has yet explored the relationship between the histopathological grade of NAFLD and the risk of T2DM in NAFLD patients.

The level of cytokeratin-18 (CK-18), an intermediate product of protein metabolism in the liver, is thought to reflect the degree of hepatocyte apoptosis that occurs during the development of non-alcoholic steatohepatitis (NASH) and to show a close relationship with the degree of NASH inflammation and hepatic fibrosis. However, the correlation between serum levels of CK18, which is a biomarker of hepatic cell damage, and T2DM is less investigated. In a 2015 pilot study that covered the relationship between CK-18 level and T2DM [6], NAFLD patients who developed T2DM had significantly higher baseline CK-18 levels than those who did not develop T2DM. This suggests that measurement of CK-18 could be a useful marker for the development of T2DM in the NAFLD population. However, a relatively small patient sample was studied, so the findings may have been affected by selection bias. Another study supported the idea of an independent relationship between T2DM and cytokeratin-18 by establishing a significant positive association between fasting plasma glucose and serum cytokeratin-18 concentration, and this

correlationship was not found to be related to the existence of NAFLD. However, the study was conducted in patients with T2DM, with fewer samples and only 244 cases. The relationship between T2DM and CK18 is not strong enough [7]. Large-scale population studies in NAFLD patients are warranted to validate the findings of these two experiments.

The purpose of this study was to determine whether a higher concentration of circulating CK-18 in patients with NAFLD is associated with a greater risk of T2DM.

Methods

Study population

We conducted a cohort study that enrolled subjects for a period of at least five years, and was based on the findings of the China National Diabetes and Metabolic Disorders Survey (CNDMDS) (2007-2008). The baseline study was completed between August 2007 and June 2008, and the follow-up study was completed between June 2012 and October 2013. On each occasion, the subjects completed the same questionnaire. One thousand nine hundred and fifteen subjects in the CNDMDS from the Xi'an area (including the city, counties, townships, and villages) of Shaanxi Province were recruited, and 806 subjects (42.1%) agreed to participate in the study and completed the follow-up questionnaire. Of these, subjects with T2DM diagnosed by oral glucose tolerance test (OGTT) or self-reported T2DM (n=75), pre-diabetes (n=172), excessive alcohol use (n=39), those missing key data (n=10), and those suspected to be secondary to liver cirrhosis or suspicion of malignancy (n=2) were excluded. In addition, individuals with missing CK-18 values (n=35) or those with outlying serum CK-18 values (n=16) were also excluded. Thus, data from 457 subjects were analyzed. The research protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Ethics Committee of Xijing hospital, Fourth Military Medical University. All subjects signed their informed consent prior to the start of the study.

Data collection

Data regarding demographics, personal history, family history, and lifestyle were collected using a standardized questionnaire by doctors or nurses who had received uniform training. Current smokers were defined as persons who reported smoking at least 100 cigarettes during their lifetime; alcohol use was defined as an intake of least 30 g of alcohol monthly within a year; and regular physical exercise was defined as undertaking physical activities at least three times per week, for at least 30 min on each occasion. In addition, data concerning social status, educational level, occupation, and income were also collected.

Anthropometric measurements were performed by specially assigned personnel. Subjects removed their shoes and socks and wore light clothing for the measurement of their height and weight. Body mass index (BMI) was calculated as body weight (kg)/ height (m)^2. For the measurement of waist circumference, subjects were asked to stand with their body weight equally distributed, and it was

undertaken using a tape measure placed horizontally around the abdomen at the midpoint of the line between anterior superior iliac spine and the lower edge of the twelfth rib. The waist circumference measurement obtained was accurate to 0.1 cm. A mercury desk sphygmomanometer was used to measure blood pressure twice in the right upper arm after at least 5 min rest. All subjects were fasted for at least 8 h, then subjects without a history of diabetes underwent a 75-g oral glucose tolerance test, whereas those with a history of diabetes underwent a steamed bread meal test, for safety reasons. Fasting blood samples were collected for the measurement of triglyceride, high-density lipoprotein, low-density lipoprotein, total cholesterol, and insulin.

CK-18 measurement

Serum CK-18 was measured in 806 subjects using the M30 Apoptosense ELISA (Apoptosis Biomarker Assay) kit (Peviva, Stockholm, Sweden) and a Molecular Devices Tecan Spectra Max 190 Microplate Devices (Tecan Trading AG, Switzerland).

Definitions

Diabetes mellitus (DM): The 1999 World Health Organization (WHO) criteria for diabetes and pre-diabetes were applied [8]: a) Diabetes: fasting blood glucose (FBG) $\geq$ 7.0 mmol/L, OGTT 2-hour postprandial glucose (PPG) $\geq$ 11.1 mmol/L, or self-reported use of hypoglycemic medications. b) Pre-diabetes: impaired fasting glucose (IFG): 6.1 $\leq$ FBG < 7.0 mmol/L; and impaired glucose tolerance (IGT): 7.8 $\leq$ 2h-PPG < 11.1 mmol/L. Patients with IFG or IGT were collectively referred to as having impaired glucose regulation, also known as pre-diabetes. 3) Normal blood sugar: diabetes was ruled out if FBG < 5.6 mmol/L and OGTT 2h-PPG < 7.8 mmol/L.

NAFLD: NAFLD was diagnosed if it met two of the following three criteria, after ruling out excessive alcohol use or the presence of other etiological factors: a) ultrasound examination showed diffuse enhancement of near-field echo in the hepatic region ("bright liver"), with the echo being stronger than that of the kidneys; b) the intrahepatic ductal structures were not clearly visible; and c) the far-field echo in the hepatic region gradually became attenuated [9].

Statistical analysis

Data were analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (SD) or median (interquartile ranges). Comparisons between two groups were conducted using Student's t test or the rank sum test. Count data are presented using rates or percentages, and inter-group comparisons were made using the chi-square test. Serum CK-18 was not normally distributed and is therefore presented as median (interquartile range); however, the data became normally distributed after logarithmic transformation.

Based on their median CK-18 values, the NAFLD subjects were divided into two groups: a high-CK-18 NAFLD group and a low-CK-18 NAFLD group. The risks of NAFLD and T2DM were calculated using a Cox proportional hazards regression model; the relative risk and 95% confidence intervals were calculated using the stepwise regression method. The covariates used in the multivariate analysis were baseline age (continuous variable), gender, educational level, smoking, drinking, sports history, family history of diabetes, BMI (continuous variable), blood pressure (continuous variable), FBG (continuous variable), 2h-PPG (continuous variable), serum TG (continuous variable), and HDL cholesterol (continuous variable). A two-sided test was used, with P < 0.05 being considered statistically significant.

Results

Of the 1915 subjects who participated in the baseline study, 806 subjects (42.1%) agreed to participate in and completed the follow-up study five years later. Based on the above inclusion criteria, 457 subjects were enrolled in this cohort study. Subjects were divided into a NAFLD group and a non-NAFLD group, based on ultrasound diagnosis of fatty liver. According to their median CK-18 levels, subjects in the NAFLD group were further divided into high-CK-18 NAFLD and low-CK-18 NAFLD groups. Thus, three groups were analyzed: a non-NAFLD group (n=363), a low-CK-18 NAFLD group (n=46), and a high-CK-18 NAFLD group (n=48). These three groups showed no significant differences in age, gender, smoking history, sports history, and family history of diabetes. However, the NAFLD groups had significantly higher BMI, systolic blood pressure, diastolic blood pressure, FBG, TG, and HDL cholesterol levels than the non-NAFLD group (all P<0.001) (**Table 1**).

Serum CK-18 level at baseline was 125.3 U/L (95% CI: 57.3-476.6 U/L), and the data distribution is shown in **Figure 1**. Serum CK-18 was 235.9 U/L (167.3-392.5 U/L) in the high-CK-18 NAFLD group, which was significantly higher than in the non-NAFLD group (120.6 U/L; 87.6-186.3 U/L) (P < 0.001). However, serum CK-18 was not significantly different between the low-CK-18 NAFLD (105.2 U/L; 75.6-116.9 U/L) and non-NAFLD groups (P=0.068) (**Figure 2**).

After 5 years of follow-up, 19 of the 457 subjects had developed diabetes. The incidence of T2DM was 2.5%, 8.7%, and 12.5% in the non-NAFLD, low-CK-18 NAFLD, and high-CK-18 NAFLD groups, respectively (**Figure 3**).

The Cox proportional hazard regression model showed that, compared with the non-NAFLD group, the relative risks (RRs) of T2DM, after adjustment with multiple variables, were 3.37 (95% CI: 1.05-10.86, P=0.042) and 4.71 (95% CI: 1.71-12.99, P=0.003), respectively, in the low-CK-18 NAFLD and high-CK-18 NAFLD groups (**Table 2**).

Discussion

In this population-based cohort study, the 5-year risk of T2DM was higher in patients with NAFLD. In addition, among subjects with ultrasound-diagnosed NAFLD, higher serum CK-18 was associated with a

higher risk of T2DM, with RRs of 3.37 (95% CI: 1.05-10.86) and 4.712 (95% CI: 1.71-12.99) in the low-CK-18 NAFLD and high-CK-18 NAFLD groups, respectively.

This study represented a follow-up to our previous study [10], in which the risk of T2DM was shown to be significantly increased in a Chinese population with NAFLD, and NAFLD was shown to be an independent predictor of T2DM [10]. The findings of this earlier study were consistent with other previous studies [11-23], demonstrating the association between NAFLD and the risk of T2DM. However, all the previous studies (including our own) failed to investigate whether a higher pathological grade of NAFLD is associated with a higher risk of T2DM in NAFLD patients.

In the present study, we have shown that higher CK-18 levels in ultrasound-diagnosed NAFLD patients are associated with a relatively higher risk of T2DM. Nagpal *et al.* generated similar findings in a pilot study to assess whether baseline CK-18 levels in NAFLD patients diagnosed by hepatic biopsy could predict the future development of diabetes [6]. In their study, the American Diabetes Association (ADA) criteria were used for the diagnosis of diabetes and baseline CK-18 levels were measured using an apoptosis assay kit (ELISA M30-Apoptosense). Thirty-nine patients who met the inclusion criteria were enrolled, among whom 32 (82.1%) were Caucasians and 20 (51.3%) were female. During the baseline examination, the average age of the study population was 51.3 ± 11.2 years, the average BMI was 33.8 ± 5.6 kg/m^2, and there was an average follow-up period of 62.4 (38.8, 81.0) months. Fourteen patients were diagnosed with diabetes at baseline, and among the remaining 25 patients, 6 (24%) developed diabetes during the follow-up period. The baseline CK-18 level in subjects who developed diabetes was significantly higher than that of the 19 subjects who did not develop diabetes [(768.8 ± 821.5) U/L vs. (285.1 ± 166.5) U/L, *P*=0.019] [6].

However, the sample size of this pilot study was small (only 25 patients were followed). As a result, the authors were not able to calculate the relative risk of higher CK-18 for diabetes and potential confounding factors could not be adjusted for in their analysis. Furthermore, selection bias might exist in the study population, because the subjects who underwent a liver biopsy might have had more severe NAFLD. In addition, a healthy population without any evidence of NAFLD should ideally also have been included as a control group [24]. Nevertheless, this pilot study was still of interest, because it suggested that baseline serum CK-18 could be used to predict the development of diabetes in patients with NAFLD. In contrast, our current study was a population-based cohort study with a relatively large sample size, but its findings in general corroborate the above assertion.

Our findings also suggest some uncertainty in the pathologic grading of NAFLD provided by ultrasound examination. Although liver biopsy is the gold standard for the diagnosis of NAFLD [25], it is not suitable for large-scale epidemiological studies. Ultrasound is widely used in clinical practice because of its low cost, safety, and high feasibility, and thus it was used in our study for the diagnosis of NAFLD. However, there are some drawbacks to the use of this method. For example, its sensitivity decreases significantly when the liver fat content is less than 33% or when there is obvious fat accumulation in the abdomen [26-28]. More importantly, it does not permit accurate pathological grading and thus cannot provide information on the degree of NAFLD fibrosis. Previous studies have reported that most NAFLD (especially

mild to moderate NAFLD) cases did not have hepatic fibrosis or serious adverse outcomes [29]. Therefore, it is difficult to identify patients with higher pathologic grades among ultrasound-diagnosed NAFLD cases.

CK-18 is an intermediate fragment generated by protein metabolism in liver that is released in greater quantities by apoptotic cells because of the loss of cell matrix integrity [29]. Because CK-18 is mainly produced by caspase 3, which has been found to be active in NASH liver [30], serum CK-18 may be elevated in patients with NASH. This suggests that CK-18 could be used as a biomarker that reflects the degree of NAFLD fibrosis [31-36]. Here, serum CK-18 in the low-CK-18 NAFLD group was not significantly different from that of the non-NAFLD group in a population diagnosed using ultrasound, suggesting that ultrasound may not be ideal for the pathological grading of fibrosis in NAFLD patients.

Unlike in the previous study by *Nagpal et al.* [6], our study did not use liver biopsy to confirm NAFLD, which means that our conclusions must be tentative. The risk of T2DM is significantly higher in NAFLD patients, suggesting that more accurate and non-invasive methods should be found to identify populations at higher risk. Due to its practical advantages, ultrasound has been widely used in clinical settings to screen for fatty liver. CK-18 measurement, as another non-invasive investigation, may be able to provide additional information to aid the differentiation of NASH patients from the rest of the NAFLD population. Therefore, we recommend screening for NAFLD in the first instance using ultrasound, with CK-18 testing being subsequently used to screen individuals at higher risk of diabetes if possible, so that proactive management may be provided at an earlier stage of the disease. Insulin resistance may be one of the important causes which lead to the higher incidence of DM in NAFLD patients[4,37,38]. Hepatic insulin resistance, which is caused by diacylglycerol-mediated activation of protein kinase C epsilon (PKC+), may be an important factor in the interaction between the NAFLD and T2DM[37]. Therefore, CK-18 has a high predictive value for DM, which may be related to insulin resistance. Our research did not focus on it, and this need further study.

Our study also had onther limitations. First, as described above, although a great deal of effort was expended, the follow-up rate was still relatively low (42.1%), which is a common problem faced by all population-based follow-up studies in China. Second, as a population-based study, most of our subjects had no evidence of liver fibrosis, and the number of subjects was relatively small in the high-CK-18 NAFLD group. Third, since we did not undertake liver biopsies, our study could not define a suitable CK-18 cutoff level to clearly distinguish between NASH and simple NAFLD; thus, we could only use a median value to define the study groups.

In summary, our current study suggests that higher CK-18 level in ultrasound-diagnosed NAFLD patients is associated with higher risk of T2DM. Because both ultrasound and CK-18 determination are non-invasive examinations, we recommend screening for NAFLD using ultrasound in the first instance, with, if possible, CK-18 assay being subsequently used to screen individuals at higher risk of diabetes. This would facilitate proactive management during the early stages of the disease, if required.

Abbreviations

NAFLD: non-alcoholic fatty liver disease; T2DM: type 2 diabetes mellitus; RR: relative risk; CI: confidence interval; CNDMDS: China National Diabetes and Metabolic Disorders Survey; OGTT: oral glucose tolerance test; OR: odds ratio; HDL: high-density lipoprotein; SD: standard deviation; CK-18: cytokeratin-18.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

We thank all of the physicians and participants of the study for their co-operation and generous participation.

Authors' contribution: RH, SX and HS contributed equally to the study. QJ, SX and JM conceived and designed the study. RH and SX contributed to the data extraction, performed the analysis and interpreted the results. RH and HS wrote the first draft; AJ, JM, YX and SLcontributed to the revision of the final report. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments (Ethical code: KY20162099-1).

Consent for publication

Not Applicable

Competing interests

The authors declare that they have no competing interests

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Tables

Table 1. Baseline data for the study population

Variables	Non-NAFLD group	NAFLD groups		P value
		Low-CK-18	High-CK-18	
n	363	46	48	
Demographic data				
Age (years)	45.2±12.6	48.9±12.8	46.8±12.5	0.147
Gender (Male) [n(%)]	155 (42.7)	21 (45.7)	20 (41.7)	0.915
Body mass index (kg/m^2)	23.2±3.1	26.0±2.8	25.7±4.0*	<0.001
Education level, n (%)				
High school and above	305 (85.5)	38 (82.6)	37 (84.3)*	0.016
Family history of diabetes, n(%)	37 (10.2)	8 (17.4)	7 (14.6)	0.267
History of smoking, n(%)	64 (17.6)	6 (13.3)	10 (20.8)	0.634
History of alcohol use, n (%)	55 (15.3)	2 (4.4)	3 (6.3)	0.041
Sport history, n (%)	168 (46.4)	26 (56.5)	17 (35.4)	0.121
Clinical data				
Systolic blood pressure (mmHg)	118.8±19.2	122.0±25.1	129.6±21.3*	0.002
Diastolic blood pressure (mmHg)	74.9±11.1	76.3±12.1	80.4±11.9*	0.006
Fasting blood glucose (mmol/L)	4.9±0.5	5.1±0.6	5.2±0.6*	0.003
2-hour postprandial blood sugar (mmol/L)	5.5±1.2	5.8±1.1	5.8±1.2	0.099
Total cholesterol (mmol/L)	4.7±0.9	4.6±1.0	4.8±0.9	0.444
Triglycerides (mmol / L)	1.3±0.9	1.9±1.2	1.9±1.7*	<0.001
High-density lipoprotein (mmol/L)	1.4±0.3	1.1±0.3	1.2±0.3*	<0.001

* P<0.05, non-NAFLD group as ref. NAFLD, non-alcoholic fatty liver disease; CK-18, cytokeratin-18

Table 2. Regression analysis of the relationship between NAFLD and the risk of T2DM, based on the median level of CK-18

Variables	Not adjusted		Adjusted*	
	RR (95% CI)	*P* value	RR (95% CI)	*P* value
Non-NAFLD group	1.000		1.000	
Non-NAFLD group				
Low-CK-18 NAFLD group	3.431 (1.076-10.940)	0.037	3.371 (1.047-10.858)	0.042
High-CK-18 NAFLD group	4.825 (1.754-13.276)	0.002	4.712 (1.710-12.987)	0.003

NAFLD: non-alcoholic fatty liver disease; RR: relative risk; CI: confidence interval.

*The relative risk and 95% confidence intervals were calculated using the backward stepwise regression method. The covariates included age, gender, educational level, history of smoking, history of alcohol use, sports history, family history of diabetes, body mass index, blood pressure, blood glucose, triglyceride, and high-density lipoprotein.
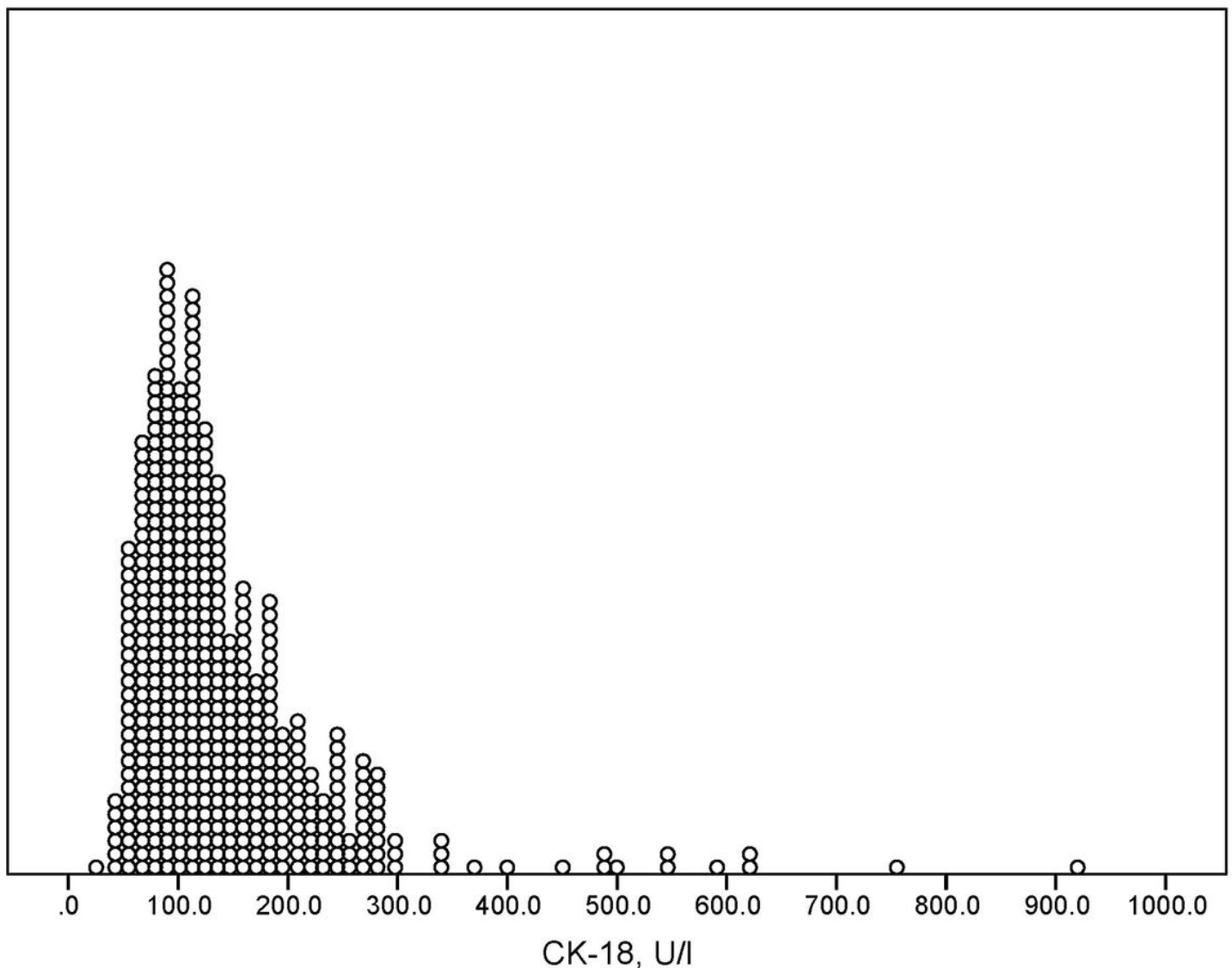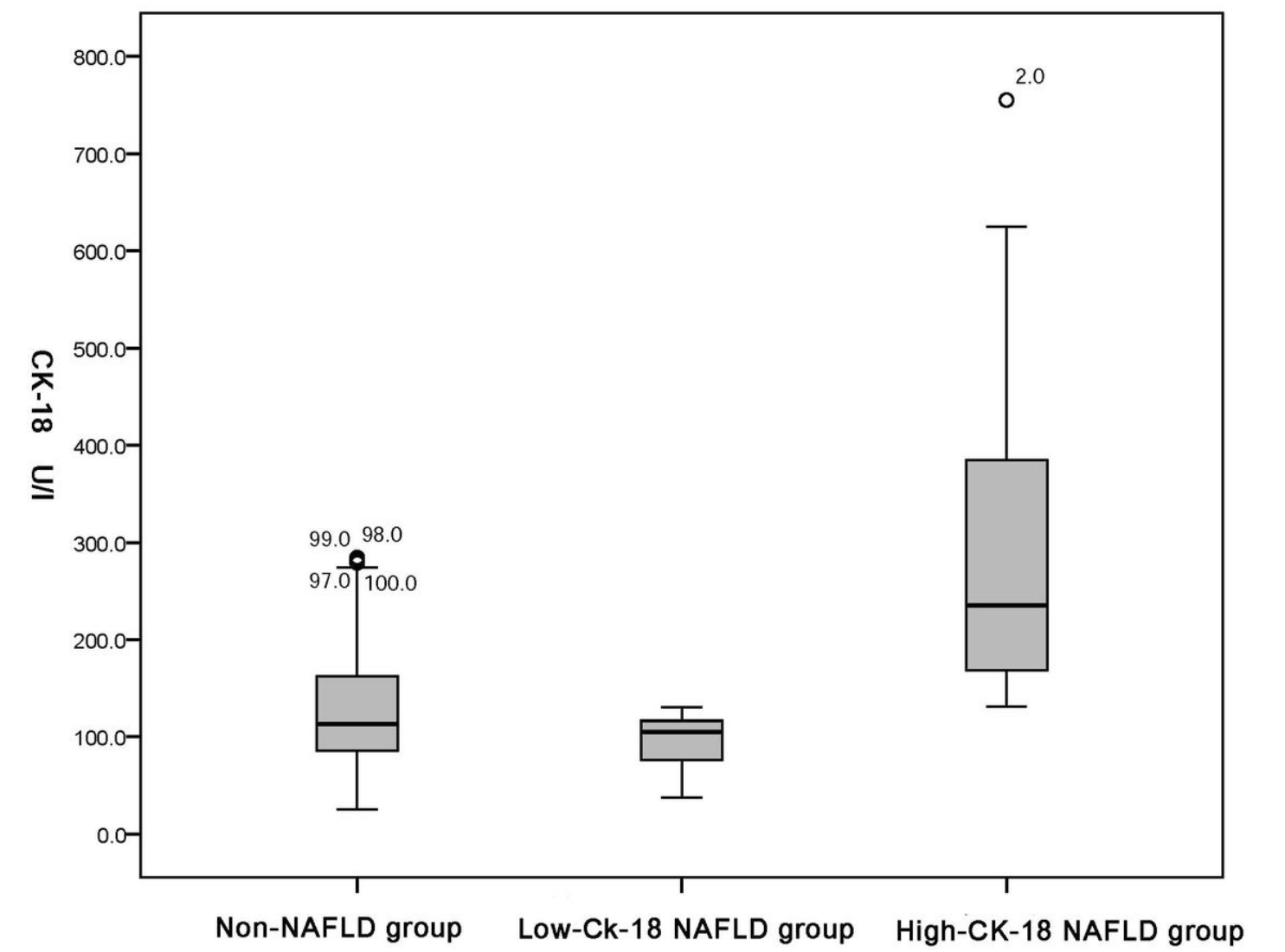
Figures

Figure 1

Distribution of baseline CK-18 concentrations in the study population CK-18: cytokeratin-18

Figure 2

Comparison of CK-18 levels between NAFLD and non-NAFLD groups NAFLD: non-alcoholic fatty liver disease; CK-18: cytokeratin-18
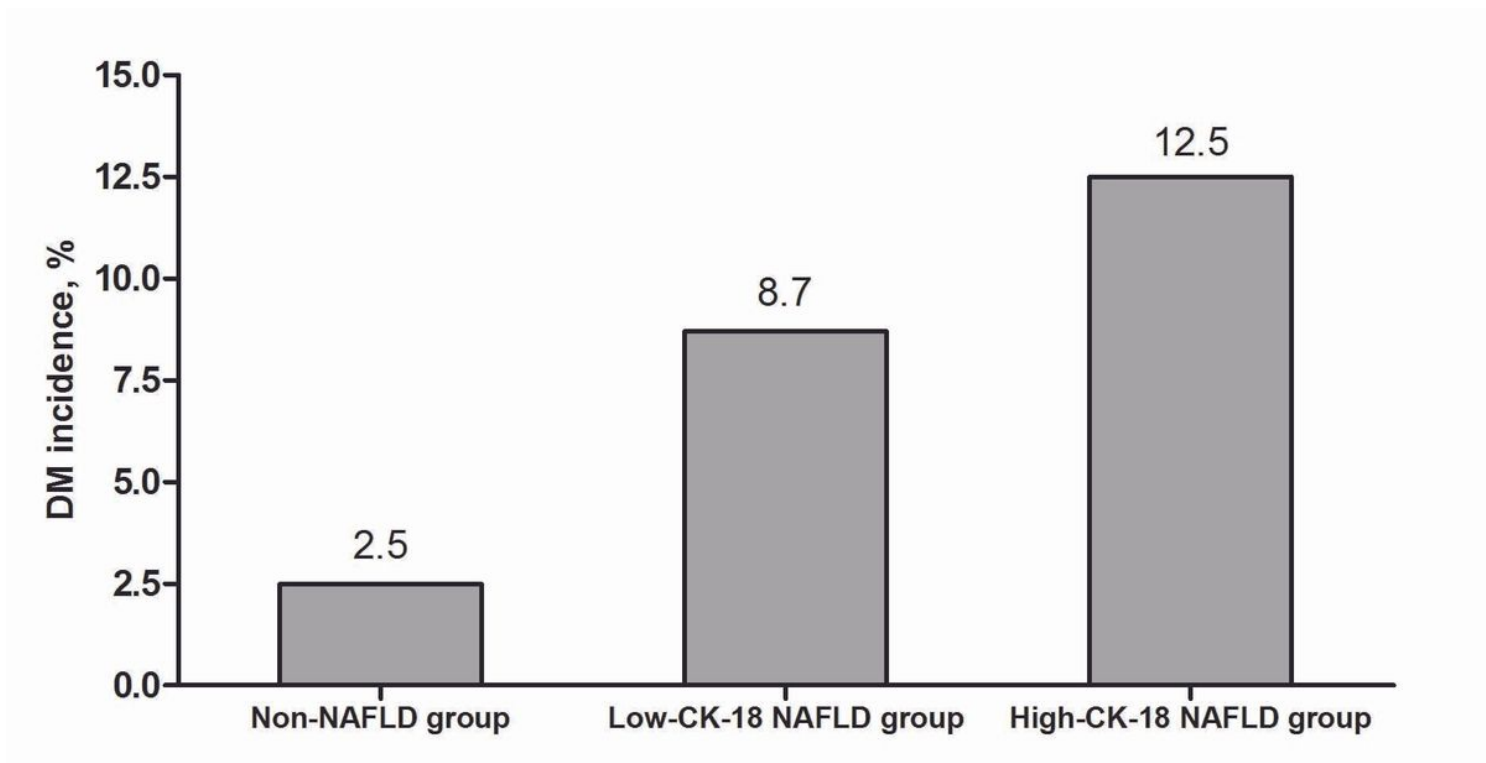
Figure 3

The 5-year incidence of diabetes in each patient group NAFLD: non-alcoholic fatty liver disease; CK-18: cytokeratin-18