

# A case-control study on the relationship between HDL2b and non-alcoholic fatty liver disease in Chinese type 2 diabetic patients

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## Abstract

**Background:** It has been shown that low concentrations of high-density lipoprotein cholesterol (HDL-C) are associated with non-alcoholic fatty liver disease (NAFLD) in patients with type 2 diabetes mellitus (T2DM). HDL2b, a major subfraction of high-density lipoprotein (HDL), is more significantly correlated with coronary heart disease (CHD) compared with total HDL. In this study, we analysed HDL2b in a cohort of Chinese T2DM subjects with or without NAFLD.

**Methods:** A highly sensitive and reliable microfluidic chip method was adopted to measure HDL2b. In total, 48 T2DM patients with NAFLD diagnosed by a B-ultrasound were enrolled from our Beijing Community Pre-Diabetes (BCPD) study cohort. A total of 48 age and gender matched diabetic controls without NAFLD were selected from the same population.

**Results:** Clinical characteristics and serum biochemical analyses demonstrated a significantly increased body mass index (BMI), waist circumference, homeostasis model assessment-insulin resistant index (HOMA-IR), total cholesterol (TC), and triglyceride (TG) concentrations in the NAFLD group. However, the concentrations of HDL2b and its ratio to total HDL in NAFLD patients was decreased, compared with controls ( $p < 0.01$ ). Significantly increased concentrations of high sensitive C-reactive protein (hs-CRP) ( $p < 0.01$ ) were also found. Multifactor logistic regression analysis showed that BMI and TG were the predominant risk factors for fatty liver, while HDL2b was a protective factor.

**Conclusions:** T2DM patients with NAFLD have characteristics including obesity, marked insulin resistance, high TG, high hs-CRP, low HDL2b and a low HDL2b ratio to total HDL. These factors may increase the incidence of atherosclerosis as well as the risk of CHD.

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**Keywords:** fatty liver; high-density lipoprotein sub-fractions; microfluidic chip; type 2 diabetes.

## Introduction

Patients with type 2 diabetes mellitus (T2DM) who have non-alcoholic fatty liver disease (NAFLD) often have an atherogenic lipoprotein profile of high triglycerides (TG), and lower high-density lipoprotein cholesterol (HDL-C) (1). T2DM is associated with a marked increase in morbidity and mortality from coronary heart disease (CHD) (2). HDL-C is an important factor associated with an increased risk of future ischaemic heart disease (3). There is a significant inverse correlation between the large and low-density subfractions of HDL (HDL2a, HDL2b and HDL3a) and CHD, and a significant positive correlation between the small but high-density subfraction of HDL (HDL3b and HDL3c) and CHD (4, 5). Moreover, it has been shown that HDL2b is a more significant factor correlated with CHD compared with total HDL (6, 7). Among these alterations in dyslipidaemia, changes in HDL subfractions have not been thoroughly investigated due to limitations in the quantitative methods used to measure HDL subfractions. These methods include sequential or gradient ultracentrifugation, gradient gel electrophoresis (8, 9), ion mobility measurements, capillary isotachopheresis (10) and high performance liquid chromatography (HPLC) (11). Some of these methods are labour intensive, expensive and time consuming. Others require expert technical personnel for proper performance. Although lipoprotein subfractions can be detected by nuclear magnetic resonance (NMR), this technology has only been developed in recent years and has not been widely applied due to complex calculations and instrument requirements (12). We analysed HDL2b and other cardiovascular risk factors in Chinese T2DM patients with and without NAFLD using a novel microfluidic chip-based technique. This technique is fast, highly reproducible, easy to operate and has been successfully used to measure HDL2b in subjects from the Prospective Cardiovascular Munster (PROCAM) study (7). Our study revealed the characteristics and

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significance of dyslipidaemia in T2DM patients with NAFLD.

## Materials and methods

### Study population

The study subjects were selected from an ongoing large-scale population-based cohort research study, the Beijing Community Pre-Diabetes (BCPD) study. The BCPD study was designed to facilitate the conduct of genetic epidemiology investigations and clinical trials. It was conducted in the community of Nanfaxin, a rural suburb of Beijing. With the approval of the medical Ethical Committee of Beijing Tongren Hospital, Capital Medical University, the BCPD study was begun in May 2007. We mailed screening requests to all inhabitants aged from 35 to 74 years ( $n=9033$ ). We received responses from 3457 and obtained written informed consent. Of the 3457 responders, 3294 individuals finished diabetes screening, 1063 were male (32.3%) and 2231 female (67.7%). A total of 499 T2DM subjects were identified, of whom 285 subjects had previously been diagnosed. The other 214 T2DM subjects were identified using the oral glucose tolerance test.

### Study subjects

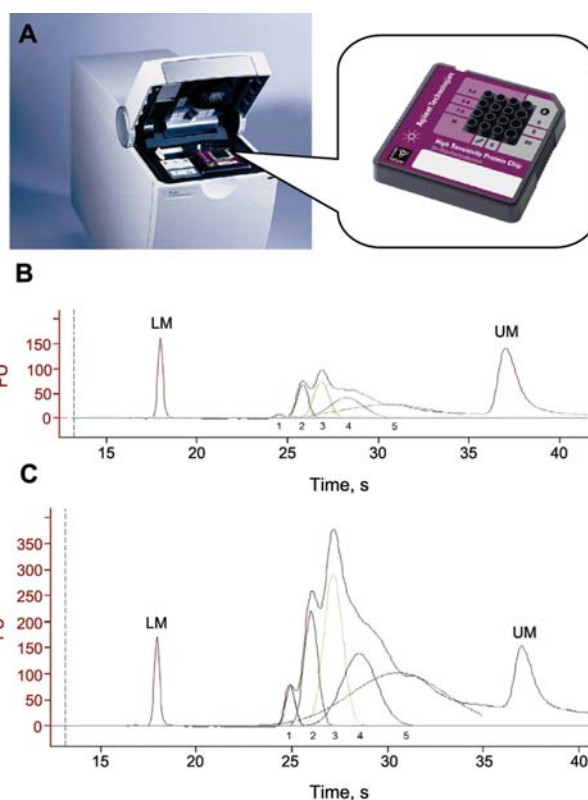
Of the 499 subjects with T2DM, 131 subjects were identified as having fatty liver. Based on the criteria of inclusion and exclusion, 48 T2DM subjects with NAFLD were randomly selected, and 48 gender and age matched T2DM subjects without NAFLD were chosen for the control group. Criteria for participation included confirmed diagnosis of type 2 diabetes in accordance with the 1999 World Health Organization (WHO) diagnostic criteria (13), duration  $<5$  years, and a confirmed diagnosis of fatty liver in accordance with guidelines for the assessment and management of NAFLD in the Asia-Pacific region (14). Fatty liver can be diagnosed by the presence of at least 2 of 3 abnormal findings on abdominal ultrasonography: [1] diffusely increased echogenicity ("bright liver"), with liver echogenicity greater than the kidney [2] blurring of hepatic vessels [3] deep attenuation of the ultrasound signal.

Patients were excluded if they had a known history of CHD or hepatic disease, serum TG  $>4.52$  mmol/L (400 mg/dL), treatment with insulin, statins or fibrates, treatment with sulphonylureas, repaglinide, or metformin at submaximal doses (alone or in combination) within the past 6 months, substance abuse, or those whose weekly consumption of more than 40 g of alcohol.

Physical examinations (measurements of waist circumference, height, weight and blood pressure) were performed by a trained nurse. Body mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters. Fasting sera and EDTA anticoagulant blood samples were obtained and stored at  $-80^{\circ}\text{C}$  prior to biochemical analysis. Fasting blood glucose (FBG), total cholesterol (TC), TG, low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), creatinine, high sensitive C-reactive protein (hs-CRP), total bilirubin (TBIL), and direct bilirubin (DBIL) were measured at the Beijing Tongren Hospital Laboratory using the Beckman Coulter Unicel DX800 (Beckman Coulter Inc, Fullerton, CA, USA). Plasma insulin was measured with an ELISA kit from Linco (Linco Research, St. Charles, MO, USA). Haemoglobin A1c (HbA1c) was measured using HPLC with the Bio Rad Variant (Bio Rad Laboratories Inc, California, USA).

### LipoChip analysis

The concentrations of total HDL and HDL2b were measured using two quality control (QC) materials at 0.62 mmol/L and 1.50 mmol/L of HDL-C obtained from Solomon Park Research Institute (Kirkland, WA, USA). All tests were performed using the Agilent 2100 Bioanalyser (Agilent, Waldbronn, Germany) with a newly developed HDL subfraction assay (Figure 1A). In brief, a linear polymer solution of poly N,N-dimethyl acrylamide (PDMA; Polysciences, Warrington, PA, USA) is used as the separation matrix. Serum samples, calibrator and QC materials (Solomon Park Research Institute, Kirkland, WA, USA) were diluted 1:50 in the sample buffer (250 mM TAPS, pH 7.5) in the presence of a lipophilic fluorescent dye (DY-676, Dyomics, Jena, Germany). These were then incubated for 15 min prior to analysis. Buffer wells of the microfluidic chips (Caliper Life Sciences, Hopkinton, MA, USA) were filled with 10 mL of the polymer. The diluted calibrators and QC materials were placed into the appropriate wells on the microfluidic chips, and the patient samples were added to the remaining nine wells. Separation was performed by starting the chip which executed a software script that applied current and voltage in a predefined manner. Fluorescent stained lipoproteins were detected with laser-induced fluorescence at 680 nm. Upon completion of the



**Figure 1** Electrophoretic trace of microfluidic separation. (A) Agilent 2100 bioanalyzer Agilent and the high sensitivity protein assay chip. (B) Lipid profile of a patient with fatty liver. (C) Lipid profile of a control patient. The electrophoretic trace consists of the lower marker (LM), high-density lipoprotein (HDL) region and upper marker (UM). Both markers are part of the sample dilution buffer and are used for sample alignment. A deconvolution algorithm identifies five individual peaks in the HDL region. Peak 1 represents the HDL3 component; peak 2 represents HDL3 and HDL2a; peak 3 is HDL2b; peak 4 has somehow fallen to the density range of HDL3; peak 5 is a systematic peak – a baseline of the system across all subfractions.

run, the chip was discarded and the electrodes cleaned with a designated cleaning chip. The entire procedure was performed in <1 h.

### Statistical analysis

Normal distributed data were expressed as means  $\pm$  standard deviations (SDs). Non-normal distributed values were expressed as medians and quartile ranges [M(QR)]. Non-normal distributed values were logarithmically transformed for parametric statistical analysis. Significant differences in parameters between patients with fatty liver and controls were assessed with Student's t-test. The risk of developing fatty liver was analysed by single factor logistic regression. Multivariate analysis was then performed by binary logistic regression using forward stepwise and Wald statistical criteria. The statistical analysis was calculated in SPSS 11.5 (SPSS Inc, Chicago, IL, USA). Statistical significance was defined as  $p < 0.05$ , and all hypotheses were tested using two-sided analysis.

### Results

Figure 1A shows the Agilent 2100 Bioanalyser and the high sensitivity HDL subfraction assay chip. Figure 1B, C shows two groups representing the electrophotogram of serum total HDL separated by the size-to-charge ratio with microfluidics gel electrophoresis.

The electrophoretic trace shows the lower marker (LM), HDL region and upper marker (UM), as shown in Figure 1B. Both markers are part of the sample dilution buffer and are used for sample alignment. A deconvolution algorithm identified five individual peaks in the HDL region. Peak 1 represents the HDL3 component; peak 2 represents HDL3 and HDL2a; peak 3 is HDL2b; peak 4 has unexpectedly fallen into the density range of HDL3; peak 5 is a systematic peak representing a baseline of the system across all sub-fractions. It shows that all components of HDL are decreased in patients with T2DM and NAFLD (Figure 1B, C).

Table 1 and Figure 2 show the comparisons of patient demographics and blood testing results between patients with fatty liver and controls.

Comparison of patient demographics demonstrated no statistical differences in age, sex, body height and blood pressure, but significantly increased BMI, waist circumference, hip circumference and waist-to-hip ratio (WHR) ( $p < 0.05$ ) in patients with T2DM and NAFLD compared with the control group.

Comparison of blood test data showed no statistical differences in FBG, HbA1c, TBIL, DBIL, LDL-C, TC and creatinine, but showed significantly increased concentrations of fasting insulin, homeostasis model assessment-insulin resistant index (HOMA-IR), ALT, TG and hs-CRP ( $p < 0.01$ ). There were also decreased concentrations of HDL-C, HDL2b ( $p < 0.01$ ) and decreased HDL2b to total HDL ( $p < 0.01$ ) in T2DM patients with NAFLD when compared with the control group.

Table 2 shows the logistic regression analysis of the fatty liver group and the control group. Logistic regression analyses demonstrated that BMI, waist cir-

cumference, hip circumference, WHR, fasting insulin, HOMA-IR, ALT, TG and hs-CRP were independent risk factors which may affect T2DM patients with NAFLD. However, HDL-C, HDL2b and the HDL2b ratio to total HDL were protective factors.

Table 3 shows the multifactor logistic regression analysis of fatty liver patients and the control group. It shows that BMI and TG were the predominant risk factors, while HDL2b is a protective factor against fatty liver.

### Discussion

NAFLD is a condition that is becoming increasingly common, and in some cases can progress to end-stage liver disease (15). The prevalence of NAFLD in the general population ranges from 13% to 15% (16), while the prevalence of NAFLD in subjects with T2DM has been reported to range from 21% to 55% (17). The results of this study show that T2DM patients with NAFLD are more likely to have obesity, insulin resistance and disorders of lipid metabolism.

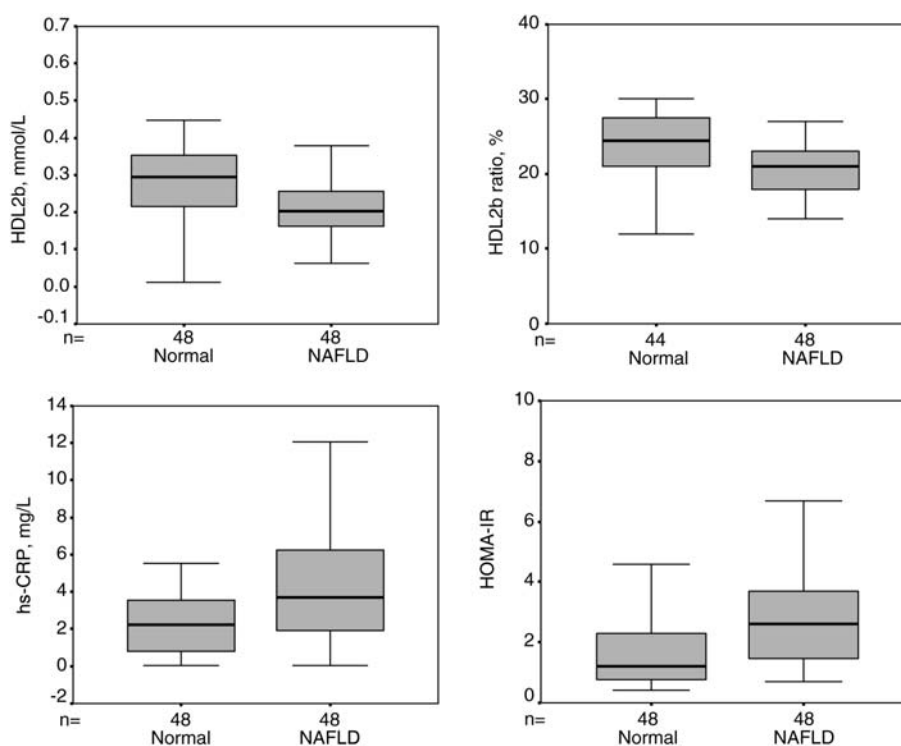
HDL-C plays a key role in reverse cholesterol transportation. Its functions include antiatherogenic, antioxidant, anti-inflammatory, anti-apoptosis and anti-thrombotic effects (18). HDL is comprised of a heterogeneous group of lipoproteins that may be classified by decreasing size as HDL2b, HDL2a, HDL3a, HDL3b, HDL3c (19). These HDL subclasses have different antiatherogenic characteristics, such as their different capacity to promote cholesterol efflux from peripheral tissues (20). HDL2b plays a significant role of protection against atherosclerosis and coronary artery disease (21). In humans, HDL subclasses may be influenced by several factors including ethnicity (22), TG concentrations (23), antidiabetic or lipid regulating drugs (24, 25), blood glucose (26, 27) and CHD (5, 7). In this matched case-control study using a representative sample population with strict inclusion criteria to exclude the impact of factors such as FBG and HbA1c, we found significantly decreased HDL2b ( $p < 0.01$ ) and a decreased HDL2b to total HDL ratio ( $p < 0.01$ ) in T2DM patients with NAFLD as compared with the control group. This difference may result in an increased risk of atherosclerosis and CHD in patients with T2DM and NAFLD. The logistic regression analyses demonstrated that BMI, waist circumference, hip circumference, WHR, fasting insulin, HOMA-IR, ALT, TG and hs-CRP were independent risk factors for NAFLD, while HDL-C, HDL2b and the ratio of HDL2b to total HDL were protective factors. Multifactorial logistic regression analysis showed that BMI and TG are the predominant risk factors, and HDL2b is a protective factor against fatty liver.

C-reactive protein (CRP) is one of the major acute phase proteins and is a marker of systemic inflammation. In contrast to standard CRP assays, the hs-CRP assay allows for measurement of low CRP concentrations seen with even low-grade inflammation. Recently, increased serum hs-CRP was reported to be a strong predictor for future cardiovascular

**Table 1** Comparisons of patient demographics and results of blood test analysis in patients with fatty liver and controls.

Characteristics	Fatty liver patients	Controls
Subjects, n	48	48
Female, %	52.08	52.08
Age, years	54.90±8.66	54.92±9.19
Body height, cm	162.15±8.51	160.94±7.68
Body weight, kg	76.81±10.32 <sup>b</sup>	67.82±12.10
BMI, kg/m <sup>2</sup>	29.19±3.03 <sup>b</sup>	26.07±3.43
Waist circumference, cm	96.20±7.59 <sup>b</sup>	88.93±10.90
Hip circumference, cm	104.14±6.47 <sup>b</sup>	98.67±7.08
WHR, %	92.35±4.98 <sup>a</sup>	89.92±6.48
Systolic blood pressure, mm Hg	138.85±17.83	139.60±19.72
Diastolic blood pressure, mm Hg	83.50 (12.50)	85.00 (13.00)
TBIL, μmol/L	12.59±3.92	13.30±6.92
DBIL, μmol/L	2.85±0.84	2.91±1.44
HbA1c, %	8.26±1.29	8.03±1.91
Fasting glucose, mmol/L	7.41 (1.89)	6.69 (2.56)
Fasting insulin, pmol/L	7.61 (7.51) <sup>b</sup>	3.66 (4.96)
HOMA-IR	2.60 (2.28) <sup>b</sup>	1.20 (1.57)
hs-CRP, mg/L	4.27±3.16 <sup>b</sup>	2.41±1.84
ALT, IU/L	29.0 (15.5) <sup>b</sup>	23.00 (8.50)
Serum creatinine, μmol/L	58.00 (23.25)	52.50 (28.75)
LDL-C, mmol/L	3.37±0.99	3.28±0.69
TC, mmol/L	4.93±0.90	4.97±0.81
TG, mmol/L	2.39±0.96 <sup>b</sup>	1.55±0.75
HDL-C, mmol/L	1.04±0.22 <sup>b</sup>	1.27±0.26
HDL2b, mmol/L	0.21±0.07 <sup>b</sup>	0.28±0.11
HDL2b ratio, %	20.35±3.91 <sup>b</sup>	23.43±5.32

Means±SD, n (%), M(QR), <sup>a</sup>p<0.05, <sup>b</sup>p<0.01 compare with controls.

**Figure 2** Comparison of HDL2b, HDL2b ratio (%), high sensitive C-reactive protein (hs-CRP) and homesostasis model assessment-insulin resistant index (HOMA-IR) between patients with fatty liver and controls.

events. The increased hs-CRP degree is related to the severity of CHD in CHD patients undergoing coronary angiography (28). Multiple prospective studies have demonstrated that hs-CRP is an independent and

good predictor of future cardiovascular events (29, 30). Inflammatory processes play a pivotal role in the pathogenesis of atherosclerosis and mediate many of the stages of atheroma development from initial leu-

**Table 2** Logistic regression analyses of fatty liver patients and controls.

	Regression Exp (b)	Exp (B)	p-Value	95% CI for Exp (B)
BMI	0.30±0.08	1.35	0.000	1.16~1.56
Waist circumference, cm	0.08±0.03	1.09	0.001	1.04~1.14
Hip circumference, cm	0.12±0.03	1.13	0.000	1.05~1.20
WHR, %	0.07±0.04	1.08	0.046	1.00~1.16
Fasting insulin, pmol/L	0.23±0.06	1.25	0.000	1.11~1.42
HOMA-IR	0.56±0.18	1.76	0.002	1.24~2.49
hs-CRP, mg/L	0.30±0.10	1.35	0.002	1.12~1.62
ALT, IU/L	0.04±0.15	1.04	0.021	1.01~1.07
TG, mmol/L	1.18±0.30	3.26	0.000	1.80~5.91
HDL-C, mmol/L	-0.12±0.03	0.89	0.000	0.84~0.94
HDL2b, mmol/L	-0.34±0.08	0.71	0.000	0.61~0.84
HDL2b ratio, %	-0.15±0.05	0.86	0.004	0.78~0.95

**Table 3** The multifactor logistic regression analyses of fatty liver patients and controls.

	Regression Exp (b)	Exp (B)	p-Value	95% CI for Exp (B)
BMI	0.31±0.09	1.37	0.001	1.15~1.63
TG, mmol/L	1.03±0.38	2.79	0.007	1.33~5.85
HDL2b, mmol/L	-0.24±0.10	0.79	0.016	0.65~0.96

The multivariate logistic regression with the independent variable (HOMA-IR, ALT, TG, hs-CRP, HDL2b, BMI, waist circumference and hip circumference) and the dependent variable (fatty liver) were carried out. The probability to enter the model was set at 0.05 and the probability to remove from the model was set at 0.10.

kocyte recruitment to eventual rupture of an unstable atherosclerotic plaque. Pathogen infection may be an important cause of CHD. A variety of inflammatory markers such as interleukin-6 (IL-6), and tumour necrosis factors (TNF) are closely related to the development of CHD (31). hs-CRP has been regarded as the most powerful marker of inflammation for predicting future cardiovascular disease (32). In this study, hs-CRP concentrations ( $p < 0.01$ ) in patients with T2DM and NAFLD were significantly increased compared with the control group.

In conclusion, we found that patients with T2DM and NAFLD had characteristics including obesity, hyperinsulinaemia, marked insulin resistance, high TG, high hs-CRP, low HDL2b and a low HDL2b to total HDL ratio. These factors may increase the incidence of atherosclerosis as well as the risk of CHD.

## Statement

The authors have not accepted any funding or support from an organisation that may in any way gain or lose financially from the results of the study. The authors are not employees of any organisation that may in any way gain or lose financially from the results of the study. We have no other conflict of interest.

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