



# Roles of hepatic lipase and cholesteryl ester transfer protein in determining low density lipoprotein subfraction distribution in Chinese patients with non-insulin-dependent diabetes mellitus

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## Abstract

Patients with non-insulin-dependent diabetes mellitus (NIDDM) are known to have abnormalities in their low density lipoprotein (LDL) subclass pattern with a preponderance of small dense LDL. The present study was performed to define the roles of lipolytic enzymes (hepatic and lipoprotein lipase) and cholesteryl ester transfer protein (CETP) in determining the distribution of LDL subfractions in these patients. LDL subfractions were measured by density gradient ultracentrifugation in 137 patients with NIDDM (75 male, 62 female) and 140 matched controls (80 male, 60 female). The male diabetic patients had a lower concentration of LDL-I ( $P < 0.01$ ) and a higher concentration of LDL-III than the controls ( $P < 0.01$ ). In the female diabetic patients, both LDL-I ( $P < 0.001$ ) and LDL-II concentrations ( $P < 0.05$ ) were significantly lower than the controls whereas LDL-III was increased ( $P < 0.001$ ). Hepatic lipase (HL) was significantly increased in both the male and female diabetic patients ( $P < 0.01$ ,  $P < 0.05$ , respectively) compared to their controls. No significant changes were seen in plasma lipoprotein lipase (LPL) and CETP activity. On multivariate analysis, plasma triglyceride (TG), CETP and HL accounted for 10, 5 and 3% of the variability in LDL-III, respectively, in the diabetic patients (adjusted  $R^2 = 0.18$ ,  $P = 0.0003$ ). Our findings would support the hypothesis that plasma triglyceride influences LDL particles through a cycle of lipid exchange via the action of CETP. LDL become enriched in triglyceride and are then acted on by HL to produce a population of small dense lipid-poor LDL. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Non-insulin-dependent diabetes mellitus; Small dense low density lipoprotein; Hepatic lipase; Cholesteryl ester transfer protein

## 1. Introduction

It is well recognised that plasma low density lipoprotein (LDL) is not homogenous and is composed of a number of distinct subspecies which vary in size, density and lipid content. LDL heterogeneity is linked to coronary risk status and there is substantial evidence indicating that individuals with predominantly small dense LDL particles have an increased risk of developing coronary heart disease (CHD) [1–4]. A number of studies have examined the origins of LDL heterogeneity and the close relationship between triglyceride and small dense LDL has been consistently demonstrated [5–7]. It has been postulated that high plasma triglyceride

influences LDL through a cycle of lipid exchange. Through the action of plasma cholesteryl ester transfer protein (CETP), LDL become enriched in triglyceride and are then acted on by the lipases to produce a population of small dense lipid-poor LDL [8]. In normolipidaemic subjects, two studies have shown that hepatic lipase (HL) was an important determinant of LDL subfraction distribution and in the study by Watson et al., CETP was also measured but did not appear to have a significant effect on LDL subclass pattern [9,10]. In patients with CHD, the role of HL has also been evaluated. Zamboni et al. had shown that HL was an important determinant of LDL subclass pattern but Jansen et al. did not find a similar relationship in their study [11,12]. Several cross-sectional studies have shown that patients with non-insulin-dependent diabetes mellitus (NIDDM) have abnormalities in their

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LDL subclass pattern with a preponderance of small dense LDL and it has been suggested that the increase in small dense LDL in these patients contributes to their high cardiovascular risk [7,13–15]. Since abnormalities in HL activity have been described in patients with NIDDM [16], the present study was performed to evaluate the roles of lipolytic enzymes and CETP activities in determining LDL subfraction distribution in these patients.

## 2. Method

A total of 137 patients with NIDDM (75 male, 62 female) with stable glycaemic control (no change in therapy for diabetes in the preceding 3 months), normal renal and liver function and proteinuria <1 g/day, were recruited from the diabetic clinics at Queen Mary Hospital. Patients on lipid lowering agents were excluded from the study. The clinical characteristics of the patients are shown in Table 1. Evidence of coronary heart disease was based on clinical history of angina, myocardial infarction or coronary revascularisation and case-note review. The majority of the hypertensive patients were on angiotensin converting enzyme inhibitors and/or calcium antagonists. There were only seven patients on beta-blockers (three male and four female). A total of 140 controls (80 male, 60 female) of similar age and BMI were recruited from hospital personnel and from the local community. There was a similar proportion of post-menopausal women in the female diabetic and control groups (54.8 vs. 51.7%, respectively) and none of the post-menopausal women were on hormonal replacement therapy. In all subjects, fasting blood was taken for the measurement of lipids and lipoproteins and CETP activities. Plasma was separated immediately and stored at 4°C until lipoprotein preparations were commenced. A second fasting blood sample was taken 15 min after 100 U/kg heparin was administered intravenously for the measurement of HL and lipoprotein lipase (LPL) activities. Plasma aliquots (pre- and post-heparin) were stored at –70°C for subsequent assays of CETP and lipase activities. All subjects gave informed consent and the protocol was approved by the Ethics Committee of the University of Hong Kong.

Plasma total cholesterol and triglyceride were determined enzymatically (Boehringer Mannheim, Germany) on an Hitachi 717 analyzer (Boehringer Mannheim, Germany). High density lipoprotein (HDL)-cholesterol was measured by the same method after precipitation of apo B-containing lipoproteins with PEG 6000. LDL-cholesterol was calculated by the Friedewald equation. Apolipoproteins AI and B were measured by rate nephelometry using the Beckman Array System (Beckman Instruments). HbA<sub>1c</sub> was measured in whole blood

using ion-exchange high performance liquid chromatography by Bio-Rad Variant Analyser System (Bio-Rad Laboratories, CA, USA).

To isolate LDL and obtain total LDL mass, plasma was adjusted to a density of 1.21 g/ml with KBr and overlaid with 1.006 g/ml salt solution. Centrifugation was carried out at 65 000 rpm for 90 min in a Beckman VTi65.2 rotor. LDL was collected by aspiration and the content of cholesterol, triglyceride, phospholipid and protein of LDL was determined. Cholesterol and triglyceride were measured enzymatically as described above. Phospholipid was measured using an enzymatic colorimetric assay (Boehringer Mannheim, cat. No. 691844). Total LDL protein was measured by the method of Lowry et al. [17].

LDL subfractionation was achieved by density gradient ultracentrifugation using a six-step discontinuous salt gradient as described by Griffin et al. [18]. Fresh plasma was fractionated into three distinct LDL subfractions after 24-h centrifugation in a Beckman SW40 rotor (40 000 rpm, 23°C). The gradient containing the separated LDL fractions was displaced from the tube by upward displacement and identified by absorbance at 280 nm. The elution times of the first, least dense, LDL fraction and the appearance of plasma proteins were reproducible and provided references for the identification of LDL subfractions. Major LDL subfractions were identified by peak maxima that occurred between hydrated density intervals of 1.025–1.034 g/ml (LDL-I), 1.034–1.044 g/ml (LDL-II), or 1.044–1.060 g/ml (LDL-III). The individual subfraction areas beneath the LDL profiles were quantified and the total LDL mass (all protein and lipid components) was then subdivided in proportion to the percentage area. This gave rise to concentration values for each LDL subfraction in mg of lipoprotein per 100 ml of plasma.

Total lipolytic activity in post-heparin plasma was measured using an emulsion of triolein and gum arabic as substrate [19]. Hepatic lipase activity was determined as the activity of the salt-resistant lipase in the presence

Table 1  
Clinical characteristics of diabetic patients

	Male DM (n = 75)	Female DM (n = 62)
Age (years)	49.2 ± 9.3	50.6 ± 9.4
BMI (kg/m <sup>2</sup> )	25.0 ± 3.0	25.5 ± 3.6
Duration of diabetes (years)	8.4 ± 5.2	8.4 ± 6.0
% Diet (n)	4.0 (3)	3.2 (2)
% Oral hypoglycaemic drug (n)	85.3 (64)	83.9 (52)
% Insulin (n)	10.7 (8)	12.9 (8)
% Hypertension (n)	34.7 (26)	40.3 (25)
% CHD (n)	2.7 (2)	6.5 (4)
% Smoker (n)	22.7 (17)	1.6 (1)

of 1 M NaCl. Lipoprotein lipase activity was obtained as the difference between total post-heparin plasma lipase activity and HL activity [20]. The inter-assay coefficient of variations of HL and LPL were 7.4 and 8.2%, respectively. Plasma CETP activity was measured by an isotopic method as previously described [21]. In brief, HDL isolated from pooled normal plasma labelled with [<sup>3</sup>H]cholesteryl oleate was used as donor and a combined VLDL/LDL fraction was used as acceptor. A total of 5 µl of plasma was incubated with [<sup>3</sup>H]HDL (25 µg of protein) and VLDL/LDL (40 µg of protein) for 4 h at 42°C. The assay was stopped by placing on ice and HDL was separated by precipitation with heparin/MnCl<sub>2</sub>. The precipitate was separated by centrifugation and radioactivity of the supernatant measured. Cholesteryl ester transfer activity was expressed as % transferred from supernatant to pellet. The inter-assay coefficient of variation of CETP was 6.6%.

Results in this study were expressed as the means and standard deviations, or as median and range if the distribution of the data was skewed. Triglyceride was logarithmically transformed before analyses were made because of the skewed distribution. Differences between the two groups was tested by *t*-test. Associations between different parameters were determined by Pearson correlation coefficients. Multiple stepwise linear regression analysis was used to assess the relationships between LDL subfraction and various variables simultaneously. The statistical package RS/1 (Bolt Beranek and Newman, Cambridge, MA, USA) was used for data analysis.

### 3. Results

The age and BMI of the male patients were similar to the controls (Table 2). The patients had significantly higher fasting triglyceride ( $P < 0.05$ ) and lower HDL ( $P < 0.05$ ) typical of diabetic dyslipidemia (Table 2). As expected, they also had higher fasting glucose ( $P < 0.001$ ) and HbA<sub>1c</sub> ( $P < 0.001$ ) than the controls. Results of LDL subfractions are shown in Table 3. The diabetic patients had a lower concentration of LDL-I ( $P < 0.01$ ) and a higher concentration of LDL-III ( $P < 0.01$ ) than the controls. Hepatic lipase activity was significantly higher than the controls ( $P < 0.01$ ) but no changes were seen in plasma LPL or CETP activities (Table 3). To avoid the confounding effects of smoking and beta-blocker usage, analyses were also performed after excluding patients on beta-blockers and smokers in both the patient and control groups. Each diabetic patient ( $n = 56$ ) was specifically matched with a control and the differences in LDL-I, LDL-III and HL remained highly significant (data not shown).

Table 2  
Fasting lipid profiles and glycaemic control in male subjects<sup>a</sup>

	Male DM ( <i>n</i> = 75)	Male controls ( <i>n</i> = 80)
Age (years)	49.2 ± 9.3	48.3 ± 8.9
BMI (kg/m <sup>2</sup> )	25.0 ± 3.0	25.2 ± 3.8
% Smoker ( <i>n</i> )	22.7 (17)	17.1 (14)
Fasting glucose (mmol/l)	8.41 ± 2.00***	5.12 ± 0.36
HbA <sub>1c</sub> (%)	7.59 ± 1.24***	5.73 ± 0.43
TC (mmol/l)	5.31 ± 0.97	5.52 ± 0.83
TG (mmol/l) <sup>b</sup>	1.60 (0.50–4.40)*	1.30 (0.50–2.80)
LDL-C (mmol/l) <sup>c</sup>	3.46 ± 0.82	3.69 ± 0.74
HDL-C (mmol/l)	1.04 ± 0.24*	1.13 ± 0.22
Apo AI (g/l)	1.31 ± 0.24**	1.44 ± 0.23
Apo B (g/l)	1.06 ± 0.28	1.09 ± 0.23

<sup>a</sup> Values are means (S.D.).

<sup>b</sup> Expressed as median (range).

<sup>c</sup> LDL-C calculated by Friedewald equation.

\*  $P < 0.05$ ,

\*\*  $P < 0.01$ ,

\*\*\*  $P < 0.001$  versus controls.

The results of the female patients and controls are shown in Tables 4 and 5. Diabetic women also had a higher fasting triglyceride ( $P < 0.01$ ) and lower HDL ( $P < 0.001$ ) than their counterparts. Both LDL-I ( $P < 0.001$ ) and LDL-II ( $P < 0.05$ ) were significantly lower in the diabetic patients whereas LDL-III was increased ( $P < 0.001$ ). Excluding smokers and patients on beta-blockers did not alter our results. A sex difference in LDL-III was seen, with the female controls having significantly lower LDL-III than the male controls ( $P < 0.05$ ). However, this sex difference was not seen in the diabetic patients, and the female diabetic patients had a similar concentration of LDL-III to the male diabetic patients. The female diabetic patients had significantly higher HL than the female controls ( $P < 0.05$ ). A sex difference in HL was seen both in the

Table 3  
LDL subfractions, lipolytic enzymes and CETP activities in male subjects<sup>a</sup>

	Male DM ( <i>n</i> = 75)	Male controls ( <i>n</i> = 80)
LDL-I (mg/dl)	45.8 ± 24.4**	56.8 ± 29.5
LDL-II (mg/dl)	150.0 ± 44.1	155.6 ± 43.1
LDL-III (mg/dl)	101.1 ± 44.5**	84.3 ± 38.3
CETP (% transferred/5 µl per 4 h)	23.74 ± 8.31	26.16 ± 9.10
HL (µmol FFA released/ml per h)	28.76 ± 9.63**	23.05 ± 8.51
LPL (µmol FFA released/ml per h)	6.18 ± 2.45	7.37 ± 4.80

<sup>a</sup> Values are means (S.D.).

\*\*  $P < 0.01$  versus controls.

Table 4  
Fasting lipid profiles and glycaemic control in female subjects<sup>a</sup>

	Female DM (n = 62)	Female controls (n = 60)
Age (years)	50.6 ± 9.4	50.0 ± 5.8
BMI (kg/m <sup>2</sup> )	25.5 ± 3.6	24.4 ± 3.1
% Smoker (n)	1.6 (1)	1.7 (1)
Fasting glucose (mmol/l)	8.32 ± 2.16***	5.01 ± 0.40
HbA <sub>1c</sub> (%)	7.79 ± 1.66***	5.65 ± 0.45
TC (mmol/l)	5.51 ± 0.91	5.46 ± 0.74
TG (mmol/l) <sup>b</sup>	1.40 (0.50–4.20)**	1.00 (0.40–2.70)
LDL-C (mmol/l) <sup>c</sup>	3.58 ± 0.77	3.49 ± 0.59
HDL-C (mmol/l)	1.19 ± 0.29***	1.45 ± 0.35
Apo AI (g/l)	1.34 ± 0.19***	1.65 ± 0.30
Apo B (g/l)	1.07 ± 0.26*	0.94 ± 0.20

<sup>a</sup> Values are means (S.D.).

<sup>b</sup> Expressed as median (range).

<sup>c</sup> LDL-C calculated by Friedewald equation.

\*  $P < 0.05$ ,

\*\*  $P < 0.01$ ,

\*\*\*  $P < 0.001$  versus controls.

controls (male versus female,  $P < 0.001$ ) and also in the diabetic patients (male versus female,  $P < 0.05$ ). LPL and CETP were similar in the female diabetic patients and the female controls.

Univariate analyses were performed to determine whether LDL subfractions correlated with any of the following parameters in the diabetic patients and in the control group: namely age, BMI, duration of diabetes, HbA<sub>1c</sub>, fasting TG, HDL, HL, LPL, and CETP. In the diabetic patients (data from male and female patients were combined), LDL-I correlated with TG ( $r = -0.26$ ,  $P < 0.01$ ) and with HDL ( $r = 0.36$ ,  $P < 0.01$ ). LDL-II did not correlate with any of the parameters. LDL-III correlated with TG ( $r = 0.28$ ,  $P < 0.01$ ), HDL ( $r = -0.24$ ,  $P < 0.05$ ), HL ( $r = 0.23$ ,  $P < 0.05$ ) and CETP ( $r = 0.21$ ,  $P < 0.05$ ). In the control subjects, LDL-I correlated with TG ( $r = -0.38$ ,  $P < 0.01$ ), HDL ( $r =$

Table 5  
LDL subfractions, lipolytic enzymes and CETP activities in female subjects<sup>a</sup>

	Female DM (n = 62)	Female controls (n = 60)
LDL-I (mg/dl)	44.2 ± 26.1***	70.0 ± 37.4
LDL-II (mg/dl)	126.4 ± 32.3*	139.5 ± 34.7
LDL-III (mg/dl)	90.2 ± 33.4***	69.0 ± 34.3
CETP (% transferred/5 µl per 4 h)	26.56 ± 7.41	28.06 ± 8.03
HL (µmol FFA released/ml per h)	21.49 ± 7.20*	18.10 ± 8.62
LPL (µmol FFA released/ml per h)	4.26 ± 2.67	5.13 ± 3.37

<sup>a</sup> Values are means (S.D.).

\*  $P < 0.05$ ,

\*\*\*  $P < 0.001$  versus controls.

0.46,  $P < 0.01$ ), and HL ( $r = -0.29$ ,  $P < 0.01$ ). LDL-II correlated with HL ( $r = 0.28$ ,  $P < 0.01$ ). LDL-III correlated with TG ( $r = 0.34$ ,  $P < 0.01$ ), HDL ( $r = -0.27$ ,  $P < 0.01$ ) and HL ( $r = 0.30$ ,  $P < 0.01$ ).

To find out which factors were important in determining the concentration of LDL-III, multivariate analysis was performed by entering parameters that were significant on univariate analyses. In the diabetic patients, the major determinants of LDL-III were TG, CETP and HL, accounting for 10, 5 and 3% of the variability in LDL-III, respectively (adjusted  $R^2 = 0.18$ ,  $P = 0.0003$ ). In the control subjects, the major determinants of LDL-III were TG and HL, accounting for 18 and 5% of the variability in LDL-III (adjusted  $R^2 = 0.23$ ,  $P = 0.00001$ ). As there were previous studies suggesting that age, sex, BMI and smoking might also be important determinants, multivariate analyses performed with the inclusion of these additional variables did not further improve the predictive power of the model in either the diabetic patients or the controls.

#### 4. Discussion

Using the method of gradient gel electrophoresis to determine LDL size, several large cross-sectional studies have shown that NIDDM is associated with small LDL [7,13–15]. Based on the LDL size distribution, two phenotypes have been described: a normal pattern A characterised by large, less dense LDL and an abnormal pattern B consisting of small dense LDL [1]. Feingold et al. had shown that in a sample of normolipidaemic men, a more than twofold increase in the prevalence of LDL subclass phenotype B was seen in the diabetics compared with age-matched controls with similar lipid levels [7]. In the Kaiser Permanente Women Twins Study, 67% of women with NIDDM had phenotype B [13]. In the San Antonio Heart Study, LDL peak particle size was significantly lower in diabetic men and women than non-diabetics and similar findings were seen in the Framingham Offspring Study [14,15]. As classification of LDL heterogeneity based on subclass patterns does not take into account of the relative proportion and absolute concentration of LDL subfractions, we have measured LDL subfractions by a density gradient ultracentrifugation method described by Griffin et al. which gives a quantitative measurement of individual LDL subfractions. Using this method, we have previously shown in a small study of Caucasian male patients with NIDDM, that LDL-III is increased in those with hypertriglyceridaemia [22]. In the present study, we have measured LDL subfraction in a large number of Chinese subjects with and without NIDDM and investigated the roles played by plasma lipids, duration of diabetes, glycaemic control, lipolytic enzymes and CETP in determining LDL subfraction con-

centrations. In both male and female diabetic patients, LDL-III was significantly increased compared to their matched non-diabetic controls. The mean concentration of LDL-III in our female diabetic patients (90.2 mg/dl) was similar to that of male diabetic patients (101.1 mg/dl). The sex difference in LDL-III that was seen in the control subjects (with female controls having significantly lower LDL-III than male controls) was lost in the diabetic patients. This might partly explain the loss of protection in female diabetic patients from CHD conferred by their gender. Although our study was not designed to study the cardiovascular risk associated with LDL-III concentration, a recent case control study by Griffin et al. had suggested that a plasma concentration of LDL-III greater than 100 mg/dl was associated with a relative risk estimate of 4.5 for CHD [4].

Enhanced HL activity is associated with insulin resistance and increased HL activity has been described in patients with NIDDM [16,23]. HL was significantly increased in both the male and female diabetic patients in our study and no significant changes were seen in plasma LPL activity. It is of interest that the female diabetic patients had HL activity approaching that of the male non-diabetic controls and it has previously been demonstrated that an HL activity in the male range is required to lipolyse triglyceride-rich LDL and generate a concentration of small dense LDL-III that exceeds the risk limit of 100 mg/dl [10]. Plasma CETP activity was normal in our diabetic patients, which confirms previous data [24,25]. The increase in cholesteryl ester transfer rate in plasma of patients with NIDDM, previously described, has been shown to be mainly due to alterations in the composition and concentration of endogenous lipoproteins and no significant differences have been noted in plasma CETP activity or concentration between NIDDM and controls [24,25].

Similar to previous studies in Caucasian subjects, we have found a strong positive correlation between LDL-III and plasma triglyceride and an inverse relationship with HDL in both the diabetic patients and in the controls. The relationship with HDL was no longer significant after controlling for plasma triglyceride in multivariate analysis. In the diabetic patients, duration of diabetes and glycaemic control did not appear to affect LDL-III concentration. However, it must be noted that the range of HbA<sub>1c</sub> was relatively narrow in our study and few of our patients had poorly controlled diabetes. We can also not exclude any potential confounding effects from dietary factors. Plasma TG, CETP and HL were the important determinants of LDL-III concentration in the diabetic patients whereas in the controls, only plasma TG and HL appeared to be important. The finding in our control group is similar to that of Watson et al. [9] and would support the hypothesis that small dense LDL is formed as a result

of lipolytic action of HL on triglyceride-enriched LDL. The concentration of triglyceride-enriched LDL is in turn influenced by plasma levels of triglyceride-rich lipoproteins and CETP and lipid exchange is mainly substrate and not enzyme driven. We postulate that CETP activity appears to affect LDL-III concentration only in the diabetic subjects and not in the controls for the following reason. In the non-diabetic control subjects with normal HL activity, lipolysis by HL is probably one of the major rate-limiting steps in the formation of LDL-III. However, in the diabetic patients, HL activity is significantly increased. Lipolysis by HL thus becomes less of a limiting factor and the rate of lipid exchange by CETP now also plays a role in determining the concentration of LDL-III. Plasma TG, CETP and HL account for approximately 20% of the variability in LDL-III in diabetes and factors that may account for the unexplained variance include genetic and dietary factors. Twin studies have indicated that about a third to a half of the variation seen in the LDL subfraction profile is due to genetic influences with the remainder being attributed to nutritional, hormonal and environmental influences [26,27]. Variations in dietary fat and carbohydrate have also been shown to influence LDL particle size distribution [28,29].

In summary, it has been suggested that the expression of small dense LDL phenotype is a result of the interaction of multiple genetic, metabolic and environmental factors [26,30]. We have demonstrated in the present study that plasma triglyceride, CETP and HL activities contribute significantly to the metabolic heterogeneity of LDL in patients with NIDDM.

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