Association between plasma HDL-cholesterol concentration and Taq1B CETP gene polymorphism in non-insulin-dependent diabetes mellitus

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Abstract The effects of CETP gene Taq1B polymorphism on plasma lipoproteins were investigated in 176 patients with non-insulin-dependent diabetes mellitus. The distribution of CETP genotypes was similar to that previously described in the general population. A significant association was found between CETP genotype and both CETP and HDL cholesterol (HDL-c) concentrations. B1B1 had the highest CETP and the lowest HDL-c whereas B2B2 had the lowest CETP and the highest HDL-c. However, HDL-c was not correlated with CETP concentration, even when genetic groups were separately considered. By multivariate analysis, the determinants of HDL were body mass index, triglycerides concentration, net mass CE transfer, and CETP genotype. No association was found between CETP genetic groups and HDL or LDL size distribution. In contrast, net mass CET was positively and HDL and LDL sizes were negatively correlated with plasma triglyceride concentration. Overall, our work demonstrates that, in a population of diabetic patients where lipoprotein-related parameters vary over a large range, the association of CETP gene polymorphism with HDL-c is independent of plasma CETP concentration.—**Bernard, S., P. Moulin, L. Lagrost, S. Picard, M. Elchebly, G. Ponsin, F. Chapuis, and F. Berthezène.** Association between plasma HDL-cholesterol concentration and Taq1B CETP gene polymorphism in non-insulin-dependent diabetes mellitus. *J. Lipid Res.* 1998. **39:** 59–65.

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Plasma lipoproteins are continuously remodelled through the actions of enzymes and lipid transfer proteins. In particular, the transfers of cholesteryl esters (CE) and triglycerides (TG) are facilitated by a specialized protein known as the cholesteryl ester transfer protein (CETP) (1). CETP catalyzes the net mass transfer of CE from CE-rich lipoproteins (HDL and LDL) to CE-poor lipoproteins (VLDL). Thus an increase of net mass CE transfer (CET) might favor the lowering of the plasma HDL-cholesterol concentration (HDL-c) and the formation of small-sized LDL (1–3). As both the decrease of HDL-c and the increase of small LDL have been demonstrated to elevate the atheromatous risk, the determination of criteria that regulate the net mass CET is of critical importance with respect to our overall understanding of the atherosclerotic process (4). Previous studies in humans have shown that in mild hypertriglyceridemia, the elevation of net mass CET essentially resulted from the accumulation of VLDL, while in more severe hypertriglyceridemia the CETP concentration might become rate-limiting (2). This interaction between hypertriglyceridemia and CETP was confirmed in the transgenic mouse model (5). In a normolipidemic population, the plasma CETP concentration varies mostly over a 3-fold range (6) and is influenced by environmental factors. Plasma CETP has been shown to be elevated in smokers (7) and to be decreased by heavy alcohol drinking (8) and physical training (9). In addition, several works have been undertaken to examine the putative consequences of CETP gene polymorphisms on CETP concentrations and plasma lipid parameters. Several studies have reported that a Taq1B polymorphism occurring in the first intron of CETP gene was associated with variations in HDL-c concentration (10–15). One could therefore speculate that Taq1B polymorphism induced variations

Abbreviations: apo, apolipoprotein; BMI, body mass index; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; RFLP, restriction fragment length polymorphism; VLDL, very low density lipoprotein.

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in CETP concentrations which were in turn able to modify net mass CET, ultimately leading to the modulation of HDL-c concentration. In fact, such an expected mechanism has never been demonstrated. Several of the studies mentioned above have shown that, besides HDL-c concentration, Taq1B polymorphism was also associated with variations of CETP concentration and/or activity. However, no direct correlation between HDL-c and CETP was established (13–15). Moreover, the association between Taq1B polymorphism and HDL-c concentration was found to depend upon the environmental factors that modulate HDL-c. It was blunted by obesity and smoking (13, 15), while it was found conserved and even improved in alcohol drinkers (14). Additionally, the association of Taq1B polymorphism with HDL-c was recently considered as independent of that with CETP concentrations (14). However, in none of these studies was the net mass CET measured, and only HDL-c concentration was examined as a direct consequence of the CETP-mediated reaction. We have therefore re-investigated the effects of Taq1B polymorphism on plasma CETP and lipid parameters, including the determinations of net mass CET and of LDL size. This study was carried out in a large population of patients with non-insulin-dependent diabetes mellitus (NIDDM). This specific population was chosen on the basis of two considerations. First, HDL-c is lowered in NIDDM and we and others have shown that the net mass CET is increased (16, 17). Both these parameters exhibit a broad range of variation, thereby permitting us to study their putative relationship. Second, in these patients, the variation of plasma TG concentration from normoglyceridemia to relatively severe hypertriglyceridemia constitutes a situation where the relative effects of CETP and TG concentrations on net mass CET can be investigated.

MATERIALS AND METHODS

Subjects and blood samples

NIDDM patients (176), recruited in the department of endocrinology and metabolic diseases, were included in the present study. All were between 30 and 70 years old. They had to have either two fasting glycemia above 7.7 mmol/L, or two glycemia above 11 mmol/L 2 h after an oral glucose tolerance test. They were not ketonuric and had a conserved insulin secretion. Exclusion criteria were: previous myocardial infarction, angina pectoris or abnormal resting ECG, peripheral arteriopathy (intermittent claudication or absence of pulses confirmed by ultrasonography), severe degenerative complications (proliferative retinopathy, creatininemia >200 μmol/L), concomitant illness (hepatic insufficiency, neoplasia), insulin therapy, or severe hypertriglyceridemia (>5 mmol/L). All patients were informed volunteers. The study had the approval of our local ethics committee. Blood samples were drawn, after subjects had fasted overnight, into tubes containing EDTA and plasma was immediately separated by low-speed centrifugation.

Taq1B polymorphism of CETP gene

After DNA extraction from blood leukocytes, amplification of a fragment encompassing 535 bp in the first intron of CETP gene was obtained by polymerase chain reaction (PCR), according to the method of Fumeron et al. (14). Then, the PCR products were digested for 2 h at 65°C in the presence of one unit of Taq1B. After separation by electrophoresis on 2% agarose gels, the digestion products were revealed by ethydium bromide staining. CETP genotypes were determined after analysis of restriction fragment length polymorphism.

Serum lipids and apolipoproteins

HDL-containing fractions were obtained after precipitation of apoB-containing lipoproteins by heparin/$MnCl_2$ at 4°C (18). Total cholesterol (TC) and free cholesterol (FC) as well as triglycerides (TG) were determined using commercial kits (BioMérieux). Cholesteryl ester (CE) was calculated from the difference between TC and FC. The plasma concentrations of apolipoproteins A-I and B were determined by radioimmunoassay.

Determinations of CETP concentration and of net mass CET in plasma

The mass concentration of CETP was determined by ELISA according to Guyard-Dangremont et al. (19). The net mass CET from HDL to apoB-containing lipoproteins was measured in a subgroup of 109 NIDDM patients during incubations of plasma at 37°C, as previously described (16). This subgroup included the 105 patients whose net mass CET was already measured for another study (16).

Polyacrylamide gradient gel electrophoresis of lipoproteins

Plasma samples were adjusted at a density of 1.21 g/mL with NaBr and ultracentrifuged for 6 h at 100,000 rpm in a Beckman TL 100 table-top ultracentrifuge, using a TLA 100.2 fixed-angle rotor. After dialysis, aliquots of the resulting lipoprotein fractions were submitted to polyacrylamide gradient gel electrophoresis in nondenaturing conditions. After staining with Coomassie blue, the gels were scanned using a computer-assisted scanning densitometer (Pharmacia). LDL and

TABLE 1. Clinical characteristics, plasma lipid and apolipoprotein concentrations in NIDDM patients according to CETP genotype

Variable	B1B1	B1B2	B2B2	*P*
Sex (M/F)	47/20	54/23	21/11	ns
Age, years	54.0 ± 1.0	53.7 ± 1.0	57.5 ± 1.1	ns
Smoking status (%)	14 (20.9)	16 (20.8)	8 (25.0)	ns
Hypolipemic drugs (%)	11 (16.4)	6 (7.8)	3 (9.4)	ns
BMI, kg/m^2	27.4 ± 0.6	28.9 ± 0.5	28.8 ± 0.8	ns
HbAlc, %	9.0 ± 0.2	8.7 ± 0.2	8.7 ± 0.3	ns
TC, mmol/l	5.00 ± 0.10	5.10 ± 0.11	5.10 ± 0.14	ns
TG, mmol/l	1.62 ± 0.09	1.75 ± 0.12	1.69 ± 0.15	ns
LDL-c, mmol/l	3.35 ± 0.09	3.30 ± 0.10	3.28 ± 0.15	ns
ApoB, g/l	1.16 ± 0.03	1.15 ± 0.04	1.10 ± 0.04	ns
ApoA-I, g/l	1.31 ± 0.02	1.34 ± 0.03	1.36 ± 0.04	ns

Analysis by ANOVA or contingence test (for sex and hypolipemic drugs). Values are expressed as mean ± SEM.

HDL profiles were analyzed using 2/16 and 4/30% gradient gels (Isolab), respectively. The particle diameter of the main LDL peak was determined by reference to the mobilities of high molecular weight standards (Pharmacia) and of calibrated latex beads (Interfacial Dynamics Corporation, Portland, OR). LDL was considered as small-sized when the particle diameter was smaller than 25.5 nm (4, 20). HDL profiles were analyzed according to Blanche et al. (21). For the sake of clarity, only the proportion of large HDL has been explicitly indicated in the present report. It was calculated by dividing the HDL_{2b} peak surface by the total surface of HDL zone.

Statistical analysis

Data were analyzed using the statistical softwares STATVIEW 4.0 and SPSS (SPSS, Inc.). Comparisons between genetic subgroups were made by one-way variance analysis. The PLSD Fisher test was used for post-hoc analysis. When appropriate, the univariate correlation coefficients (*r*) were calculated assuming a linear relationship between variables. The respective effects of various factors on HDL-c concentrations were assessed by performing a multivariate analysis that used a general linear model with a backward step.

RESULTS

Analysis of Taq1B RFLP showed that the frequencies of B1 (presence of cutting site) and B2 alleles were 60 and 40%, respectively. The genotype frequencies were in Hardy-Weinberg equilibrium (B1B1: 38.1%; B1B2: 43.8%; B2B2: 18.1%). No statistically significant differences in sex distribution, age, or BMI were observed among the three genetic groups (**Table 1**). The plasma concentrations in total cholesterol and triglycerides, as well as in apolipoproteins B and A-I were similar in all genotypes. In contrast, CETP genotypes were significantly associated with the plasma concentrations of both CETP and HDL-c (**Table 2**). B1B1 had the highest CETP and the lowest HDL-c concentrations whereas B2B2 had the lowest CETP and the highest HDL-c concentrations. There was no association between genetic groups and either net mass CET or the percent of large HDL (Table 2).

The putative relationship between HDL-c and CETP concentrations was first analyzed by univariate linear regression (**Fig. 1**). No correlation was found between these two parameters, even when the three genetic groups were separately considered. In a second step, we submitted HDL-c, as the dependent variable, to a multivariate analysis in the subgroup of 109 NIDDM patients who had measurements of net mass CET. Sex, smoking, non-HDL-cholesterol, and CETP concentration had no effect on HDL-c. In contrast, four variables were found to independently influence HDL-c concentration. These were TG, net mass CET, CETP genotype, and BMI (**Table 3**). Net mass CET is mediated by CETP, but is also largely influenced by TG concentration in NIDDM

TABLE 2. Plasma lipoprotein parameters, CETP concentration, and CE net transfer according to CETP genotype

Variable	B1B1	B1B2	B2B2	*P*
n	67	77	32	
CETP concentration, mg/l	2.35 ± 0.08	2.12 ± 0.07	1.95 ± 0.11	<0.005
Net mass CET, μmol/l/5 h	103.3 ± 4.9	92.5 ± 4.2	96.8 ± 6.0	ns
HDL-cholesterol, mmol/l	0.94 ± 0.03	1.01 ± 0.03	1.08 ± 0.05	0.03
HDL_2 subfraction, %	12.2 ± 2.4		13.2 ± 2.2	ns
Mean LDL peak, nm	25.0 ± 1.0		25.0 ± 1.1	ns
Small LDL, %	57.6		57.7	ns

Values are expressed as mean ± SEM. Net mass CET was determined in a subgroup of 109 subjects. HDL_2 subfraction and LDL size were determined in a subset of 60 patients with B1B1 or B2B2 genotypes. *P* ANOVA between subgroups: for CETP concentration, B1B1 vs. B1B2, $P = 0.02$; B1B1 vs. B2B2, $P = 0.02$; B1B2 vs. B2B2, $P = 0.19$; for HDL-c concentration, B1B1 vs. B2B2, $P = 0.01$; B1B1 vs. B1B2, B1B2 vs. B2B2, $P =$ ns.

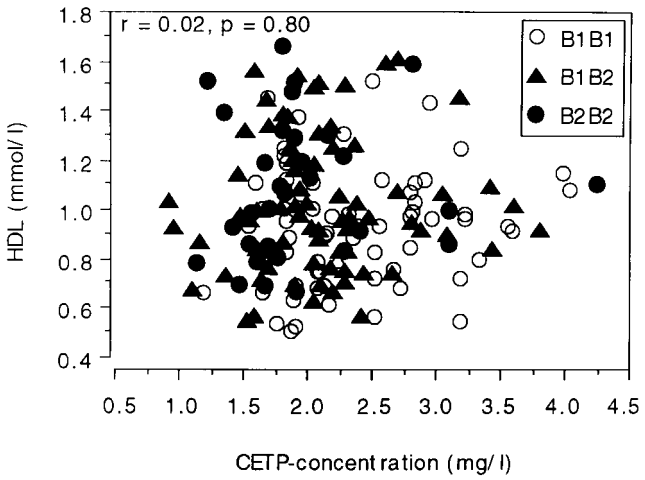

Fig. 1. Relationship between plasma HDL-c and CETP concentrations in NIDDM patients according to CETP genotype (○ B1B1; ▲ B1B2; ● B2B2).

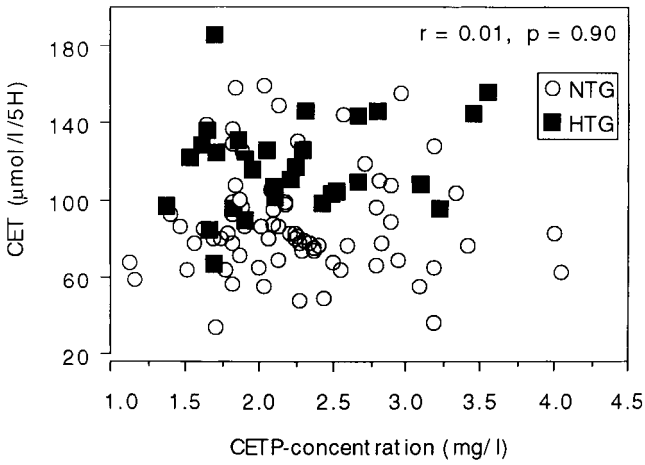

Fig. 2. Relationship between net mass transfer of CE (CET) after 5 h of incubation and plasma CETP concentration according to plasma TG concentration (○ NTG: normotriglyceridemia: TG < 2 mmol/l; ■ HTG: hypertriglyceridemia: TG ≥ 2 mmol/l).

(16, 17); in our group of patients a strong positive correlation between CET and log-TG was observed ($r = 0.56$, $P = 0.0001$). Consequently, we studied the direct relationship between net mass CET and CETP concentration after separating the patients into two groups according to their triglyceridemia (**Fig. 2**). A cut-off of 2 mmol/L was arbitrarily chosen on the basis of the previous work of Mann et al. (2). Whether the data were separately considered in each group or analyzed together, no correlation was found between net mass CET and CETP concentrations. Moreover, the associations between genetic groups and either HDL-c or CETP concentrations were independent of triglyceridemia as they remained significant when only the normotriglyceridemic group was considered (data not shown, ANOVA, $P = 0.04$ and $P = 0.03$, respectively).

As HDL and/or LDL particle size may be affected by CETP, we analyzed the size distribution of lipoproteins according to the CETP genotype and concentration. The percent of HDL_2 subfraction was not correlated with CETP concentration (**Fig. 3A**) but negatively with that of TG (Fig. 3B). The relative proportion of patients having small-sized LDL was similar in genotype subgroups (57.6 and 57.7% for B1B1 and B2B2, respectively) (Table 2). There was no association between CETP genotypes and LDL particle size (Table 2), and the latter was not correlated with CETP concentration (Fig. 3C). In contrast, it exhibited a strong negative correlation with TG concentration (Fig. 3D).

TABLE 3. Multivariate analysis for parameters influencing HDL-c using a general linear model procedure with backward step

Parameter	Estimate	T for H0	*P*
Sex	0.049	0.96	0.337
Smoking	−0.040	−0.82	0.414
CETP genotype	0.066	2.21	0.030
TG	−0.131	−4.61	<0.001
BMI	−0.010	−2.10	0.038
Net mass CET	0.002	2.10	0.038
CETP concentration	0.028	0.65	0.518
Non-HDL-c concentration	−0.016	−0.55	0.582

DISCUSSION

To our knowledge, this work describes the first study of the effects of Taq1B CETP gene polymorphism on CETP and HDL-c concentrations, including net mass CET as a putative causal intermediate. In this large population of NIDDM patients, the distribution of CETP genotypes was similar to that previously reported in a control population (10–15), which does not suggest the occurrence of any linkage disequilibrium between CETP gene and those that might determine NIDDM. As in the general population, the Taq1B polymorphism of CETP gene was associated with variations of both CETP and HDL-c concentrations, the B2B2 group having the highest HDL-c and the lowest CETP concentrations (14). Thus, from these viewpoints, NIDDM patients had no specific characteristics, thereby permitting us to extend the relevance of our data to the general population.

No correlation was observed between HDL-c and CETP concentrations either when the patients were considered together or when the three genetic groups were separately analyzed (Fig. 1). However, HDL-c concentration was logically influenced by the magnitude of

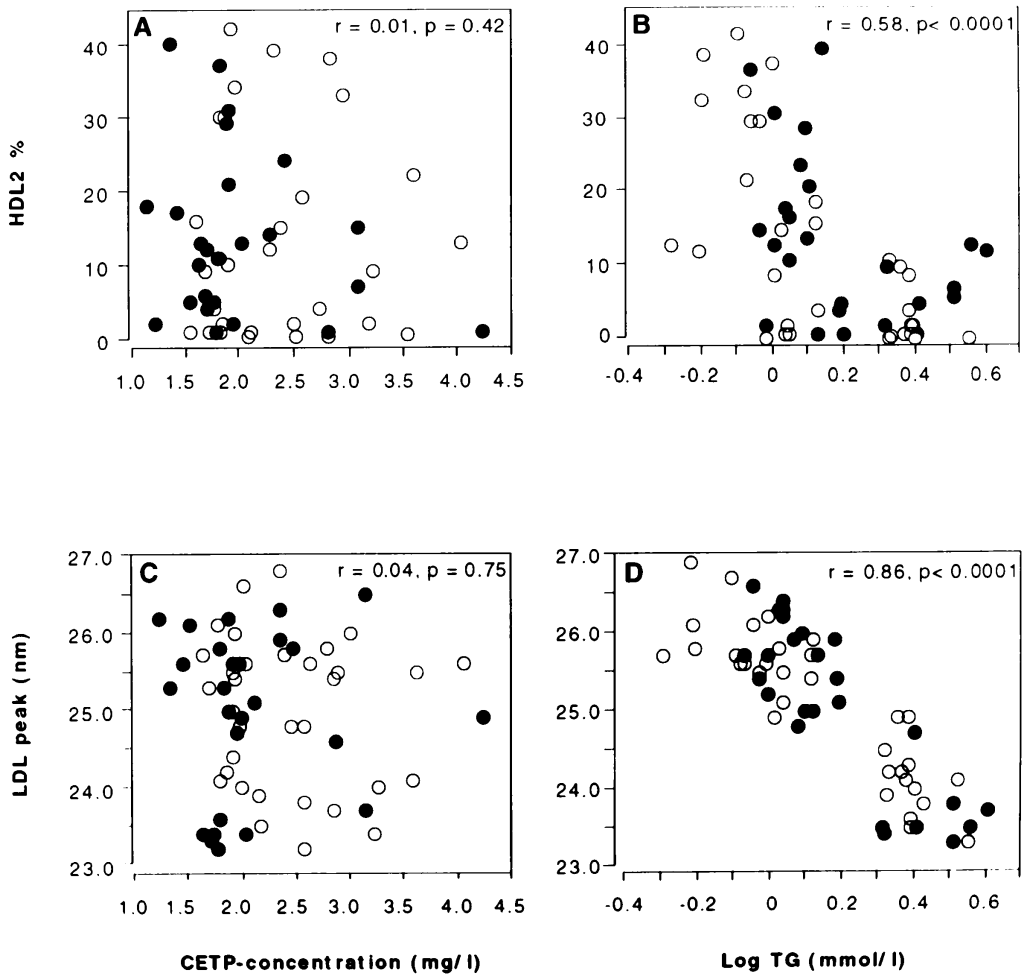

Fig. 3. Relationship between plasma CETP concentration (left panel) and log of TG concentration (right panel) and percentage of HDL_2 (upper panel) and LDL size (lower panel), according to CETP genotype (○ B1B1; ● B2B2).

net mass CET, which suggests that the in vitro measurements of the latter have some physiological relevance. In spite of this, net mass CET exhibited no correlation with CETP concentration. As our data confirmed that net mass CET was largely dependent upon triglyceridemia (2, 16), we re-examined the putative correlation between net mass CET and CETP after separating the patients into two groups according to their plasma TG concentration. The only new information emerging from these conditions of analysis was that the correlation coefficient observed in the group with the highest TG, albeit far from reaching statistical significance, was slightly more elevated than in the group with the lowest TG ($r = 0.26$ vs. $r = 0.06$). Thus, the lack of correlation between CETP and HDL-c concentrations cannot be convincingly explained on the basis of the effect of triglyceridemia on net mass CET. Moreover, this is confirmed by our observation that the association between genetic groups and HDL-c concentration does not depend upon TG levels. Thus, no data from our work suggest the occurrence of any consistent relationship between CETP concentration and net mass CET. As to the LDL particle size, which is considered to be affected by the net mass CET from LDL to VLDL, it was not correlated with CETP concentration (3, 20). In contrast, as was observed for HDL-c, LDL particle size appeared to be largely dependent upon plasma TG concentrations, probably through the magnitude of net mass CET. Thus, collective consideration of our data shows that two different parameters, i.e., HDL-c concentration and LDL particle size, which could be in principle affected by CETP, were not correlated with the latter, even when the CETP genetic groups were separately considered. Our finding that LDL particle size did not vary according to CETP genetic group and was not correlated with CETP concentration does not support the

previous speculation by Ladhenpera et al. (20) that CETP contributes to the occurrence of small-sized LDL in NIDDM.

Overall, our work confirms and extends previous studies by demonstrating that even in a NIDDM population, where lipoprotein-related parameters vary over a large range, the association of CETP genotypes with HDL-c is independent of that with plasma CETP concentration (14, 22). Moreover, there is no doubt that CETP concentration must influence that of HDL-c. One could argue that our failure to identify the missing link between these two parameters might result from a lack of precision of our study due to a too small sample size. In fact, the need for larger samples would mean that the contribution of CETP variation to that of HDL-c would be at least apparently too small to explain the association of the latter with genetic groups.

Two main speculations have been made by others to explain this situation. First, a CETP-mediated variation in HDL-c concentration might take place either in the postprandial state (1) and/or in a particular tissue such as the adipose tissue (23). Second, the Taq1B restriction site in CETP gene might be in linkage disequilibrium with another critical allele independently involved in HDL metabolism (14). Our data indicate that this presently unknown critical allele, if any, is not likely to affect HDL-c concentration by modulating net mass CET. Indeed, in this situation, net mass CET would have been expected to vary in association with the CETP gene polymorphism, which was not observed.

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