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High-density lipoprotein subspecies between patients with type 1 diabetes and type 2 diabetes without / with intensive insulin therapy

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Abstract. The reduced levels of high-density lipoprotein (HDL) 2-cholesterol (C) in diabetes and other metabolic disorders associated with a high risk of cardiovascular disease are well established. Few studies, however, have compared the HDL subspecies in type 1 diabetes (T1D) with those in type 2 diabetes (T2D) with or without insulin. We examined HDL subspecies in 27 T1D with insulin, 33 T2D with insulin or insulin plus oral-anti-diabetic drugs (OADs), 36 T2D with OADs or diet/exercise, and 25 non-diabetic controls. Insulin was injected four times daily in a basal-bolus manner for both T1D and T2D. Plasma levels of C, apolipoprotein (apo) AI, and AII were determined in HDL2 and HDL3 by the single precipitation method. HDL-C levels were significantly higher in T1D and lower in T2D, compared with the controls. Insulin-treated T2D had higher HDL-C than non-insulin-treated T2D. T1D had higher HDL2-C and HDL2-apo AI levels than T2D. Insulin-treated T2D had higher HDL2-C and HDL2-apo AI levels than non-insulin-treated T2D. All of these differences were more pronounced for men than for women. HDL3 levels were comparable among controls, T1D and T2D. HDL2-C levels were inversely associated with BMI, HbA1c, triglyceride, small dense LDL-C, and LDL-C. Multiple regression analysis revealed that HDL2-C was independently associated with triglyceride, LDL-C, and intensive insulin therapy but not with HbA1c. In conclusion, these results suggest that intensive insulin therapy is associated with alterations of HDL subspecies, irrespective of the type of diabetes.

Key words: High-density lipoprotein subspecies, Type 1 diabetes, Type 2 diabetes, Intensive insulin therapy

HIGH-DENSITY LIPOPROTEIN (HDL)-cholesterol (C) is a negative risk factor for coronary heart disease (CHD), and low levels of HDL-C increase the risk of CHD just as powerfully as high levels of low-density lipoprotein (LDL)-C [1]. Patients with diabetes usually have lower HDL-C and higher triglyceride (TG) levels than healthy subjects [2]. The reduced levels of HDL-C in diabetes also have an established association with a high risk of CHD [3]. HDL consists of two major sub-fractions, large buoyant HDL₂ (d=1.063-1.125 g/mL) and small dense HDL₃ (d=1.125-1.210 g/mL) [4, 5]. Though studies have yet to determine which of the two is more predictive of CHD events, most evidence suggests that the HDL2-C concentration better reflects the

strong protective effect of HDL than the concentration of total-HDL-C or HDL3-C [6-8]. While several studies have demonstrated significantly reduced HDL2-C levels in patients with type 2 diabetes (T2D) [6, 9], few reports have been published on the difference of treatment on HDL subspecies. To our knowledge, there are no reports directly comparing HDL subspecies between insulin-treated T1D and T2D.

We found that HDL2-C levels are higher than HDL3-C in healthy Japanese individuals [10]. In Western populations, in contrast, HDL3-C is usually higher than HDL2-C [6, 11]. This conspicuous difference in the composition of HDL subspecies between Japanese and Western populations underlines the importance of examining HDL subspecies in Japanese patients with T1D and T2D. In the present study we sought to elucidate the characteristics of HDL subspecies in Japanese diabetic populations by measuring the concentrations of HDL2 and HDL3 in T1D treated with insulin, in T2D treated with or without insulin, and in

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non-diabetic controls.

Subjects and Methods

We studied 27 T1D treated with insulin, 33 T2D treated with insulin alone (n=18) or insulin plus OADs (n=15) (insulin-treated group), 36 T2D treated with diet and exercise therapy, either alone or in combination with OADs (non-insulin-treated group), and 25 non-diabetic subjects (controls). The non-diabetic controls were defined as individuals with fasting plasma glucose levels below 110 mg/dL and non-fasting plasma glucose levels below 140 mg/dL. Insulin was injected four times daily in a basal-bolus manner for both T1D and T2D. Subjects with apparent histories of atherosclerotic events (stroke, CHD, or peripheral artery diseases), severe kidney dysfunction, liver disease, thyroid dysfunction, infectious disease, or malignancy were excluded. A half of the insulin-treated T2D and 22% of the non-insulin-treated T2D were statin-users (Table 1). A half of the T2D without insulin therapy were treated with sulfonylureas, whereas none of

the insulin-treated T2D used OADs to stimulate insulin secretion (Table 1). Subjects treated with fibrates or niacin were excluded. Blood was taken in a non-fasting state, and serum samples were quickly stored at minus 80 °C before the measurements. Informed consent was obtained from all subjects, and the study was approved by the ethics committee of the Showa University School of Medicine.

HDL subfractions

We established a simple method for assaying HDL2 and HDL3 by a single precipitation [10]. Briefly, a precipitation reagent (0.06 mL) containing 1,071 units/mL heparin, 500 mmol/L MnCl₂, and 12 mg/mL dextran sulfate (DS) was added to a serum (0.3 mL). The sample was incubated and centrifuged at 10,000 rpm for 10 min. All of the lipoproteins but HDL3 were precipitated with heparin-Mn-DS under this condition, yielding a supernatant containing HDL3 alone. The HDL3-C in the supernatant was then measured by a homogenous HDL-C assay (HDL-EX, Denka Seiken, Tokyo, Japan), and HDL2-C was estimated by sub-

Table 1 General profiles and clinical parameters of controls and diabetes mellitus group

	Controls	T1D	T2D with insulin	T2D without insulin
Number (Male/Female)	25(11/14)	27(17/10)	33(19/14)	36(21/15)
Age (year)	41.0±6.9	47.2±16	63.3±11.4 ^b	60.2±14.7
Boby mass index (kg/m ²)	23.3±4.4	21.3±3.5	23.9±3.4	25.8±4.7 ^{ab}
HbA1c (%)		7.6±2.0	8.0±1.2	8.9±1.6 ^{bc}
Triglyceride (mg/dL)	94.1±34.3	92.2±76.9	142.4±71.8	197.6±148.8 ^{ab}
LDL-C (mg/dL)	112.2±32.0	101.2±18.9	110.3±24.5	123.6±28.9 ^b
Small dense LDL-C (mg/dL)	23.8±7.2	24.5±8.7	31.2±17	34.5±15.2 ^{ab}
HDL-C (mg/dL)	63.0±14.9	76.6±21.1 ^a	58.5±16.6 ^b	43.2±10.1 ^{abc}
Apo-A I (mg/dL)	170.8±27.6	181.4±40.8	171.6±30.9	146±27.3 ^{abc}
Apo-A II (mg/dL)	32.2±4.6	30.9±8	32.7±8.1	30.3±5.4
Proliferative retinopathy		11%	30%	10%
Diabetic nephropathy		4%	22%	6%
Taking statin		0%	48%	22% ^c
Smoking		33%	13%	28%
Oral anti-diabetic agents				
Sulfonylureas		0%	0%	50% ^c
Meglitinides		0%	0%	3%
Metformin		0%	15%	25%
Thiazolidinediones		0%	3%	14%
Alpha-glucosidase inhibitors		0%	33%	25%
Diet and exercise therapy only				31%

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; C, cholesterol; Apo, apolipoprotein

Data is expressed as mean ± standard deviation. Significance ($p < 0.05$): a, vs. Controls; b, vs. T1D; c, vs. T2D with insulin

tracting the HDL3-C from the direct HDL-C. The HDL3-C and HDL2-C values determined by the precipitation method were identical to those determined by ultracentrifugation, and there were excellent correlations ($r > 0.93$) between these methods [10]. The precipitation method and ultracentrifugation also proved to be highly correlated ($r > 0.91$) in the measurement of apo AI and AII in HDL subspecies [10]. Total HDL was isolated by the classical precipitation method with heparin-Mn [12].

Measurements

Apo AI, AII, and B were measured by an immunoturbidometric assay (Sekisui Chemical Co., Tokyo, Japan). LDL-C and HDL-C were measured by the homogenous direct assay (LDL-EX and HDL-EX, Denka Seiken, Tokyo, Japan). Small dense LDL-C was directly measured by a homogenous method recently established [13]. In this manuscript, the value for HbA1c(%) is estimated as an NGSP (National Glycohemoglobin Standardization Program) equivalent value(%) as calculated by the formula $\text{HbA1c (\%)} = \text{HbA1c (Japan Diabetes Society [JDS]) (\%)} + 0.4\%$, considering the relational expression of HbA1c (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [14].

Data were expressed as means \pm standard deviations. The significance of differences between groups was tested by analysis of variance (ANOVA) followed by post hoc Tukey-Kramer's multiple comparison. Relationships between two variables were tested by Pearson's correlation coefficient, and multiple regression analyses were performed to determine independent variables for HDL2-C. Statistical significance was accepted at $p < 0.05$.

Results

The general patient profiles are listed in Table 1. The prevalences of diabetic retinopathy and nephropathy were higher in insulin-treated T2D than in the other diabetic groups. T1D were comparable to controls for age and BMI, while T2D were older and had higher BMI, compared to T1D and controls. HbA1c levels were higher in T2D than in T1D, but there was no significant difference between insulin-treated T2D and T1D for HbA1c. The total dose of injected insulin was higher in T1D than in T2D (42 ± 17 vs 32 ± 12 , $p < 0.05$). Table 1 shows the lipid profiles of the four groups. The

levels of TG, LDL-C, and sdLDL-C in T1D were comparable to the control levels, but the HDL-C levels in T1D were significantly higher. HDL-C and HDL2-C levels in insulin-treated T2D with statin were lower than those in insulin-treated T2D without statin (55 ± 15 vs 63 ± 17 and 29 ± 12 vs 37 ± 13 , respectively). Serum apo AI and apo AII levels in T1D were comparable to those of controls. Serum TG and sdLDL-C levels were significantly higher in T2D without insulin than in controls or in T1D. TG and sdLDL-C levels tended to be higher in insulin-treated T2D than in controls and T1D, but not to a statistically significant extent. HDL-C levels were significantly lower in T2D than in T1D (50 ± 16 vs 7 ± 21 , $p < 0.001$). Importantly, the HDL-C levels in the non-insulin-treated T2D were the lowest among the four groups. The Apo AI levels were also the lowest in the non-insulin-treated T2D, while no differences between groups were found in the levels of Apo AII.

Fig. 1A depicts HDL2 and HDL3 concentrations in the four groups. T1D had remarkably higher HDL2-C levels (51 ± 20) than controls (37 ± 15) or T2D treated with insulin (33 ± 13) or without insulin (18 ± 8). HDL2-C was lowest in T2D treated without insulin, reaching no more than about half of the control level. HDL2-apo AI levels were comparable between controls (88 ± 28), T1D (105 ± 34), and insulin-treated T2D (85 ± 25), but the values were lower in non-insulin-treated T2D (54 ± 23) than in the other groups. The HDL2-C and HDL2-apo AI levels in the insulin-treated T2D were lower than those in T1D but higher than those in the non-insulin-treated T2D. HDL2-apo AII levels were slightly decreased in the non-insulin-treated T2D. HDL3-C, HDL3-Apo AI, and HDL3-Apo AII levels were comparable among the four groups. Fig. 1B depicts HDL2/HDL3 ratio in the four groups. The HDL2-C/HDL3-C ratio was the highest in T1D and the lowest in non-insulin-treated T2D, among the four groups, and the difference in this ratio between T1D and non-insulin-treated T2D was significant. A similar tendency was seen in the HDL2-Apo AI/HDL3-Apo AI ratio. The HDL2-Apo AII/HDL3-Apo AII ratio did not differ among the groups.

Table 2 lists various measurements in the male subjects. The general characteristics and serum lipid profiles in the male subjects were similar to those in the total study population. Type 1 diabetic males had higher HDL-C levels, whereas non-insulin-treated type 2 diabetic males had elevated TG and lower HDL-C

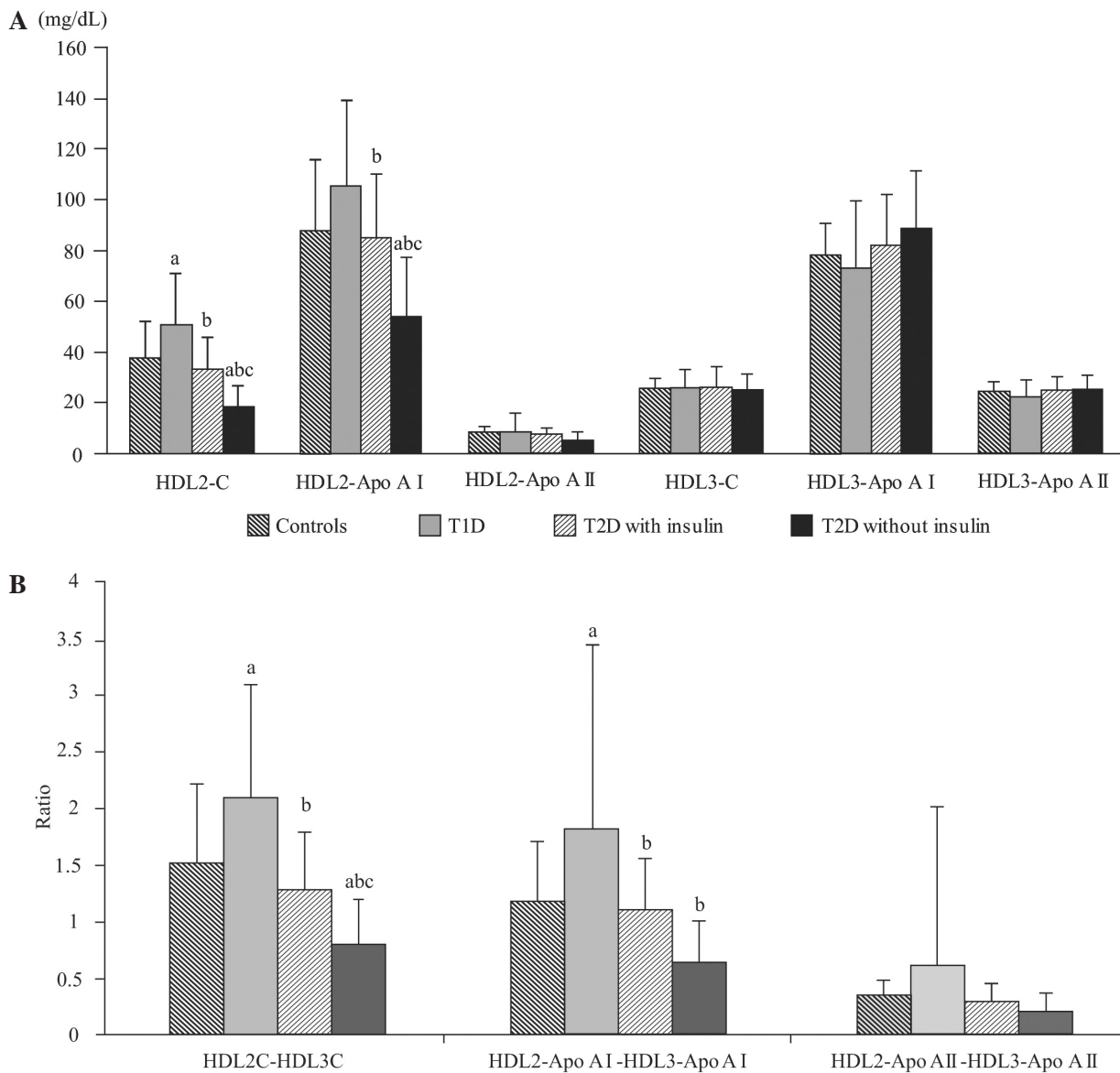


Fig. 1 HDL subspecies levels (A) and their ratios (B) in controls, T1D, T2D with insulin, and T2D without insulin
Abbreviations: HDL, high-density lipoprotein; C, cholesterol; Apo, apolipoprotein; T1D, type 1 diabetes; T2D, type 2 diabetes
Significance ($p < 0.05$): a, vs. Controls; b, vs. T1D; c, vs. T2D with insulin

levels, compared with the control males. Likewise, the HDL subspecies in the male subjects were similar to those in the total study population. HDL2-C levels were significantly higher in type 1 diabetic males and significantly lower in non-insulin-treated type 2 diabetic males, compared with the control males. HDL2-C levels in insulin-treated type 2 diabetic males were comparable to those in control males. HDL2-apo AI levels were significantly higher in type 1 diabetic males than in controls and type 2 diabetic males. The

HDL2-apo AII levels did not differ among the groups. The HDL2-C/HDL3-C ratio was the highest in type 1 diabetic males and the lowest in non-insulin-treated type 2 diabetic males among the four groups. A similar tendency was seen in the HDL2-apo AI/HDL3-apo AI ratio. The HDL2-apo AII/HDL3-apo AII ratio did not differ among the groups.

Table 3 lists various measurements in the female subjects. The non-insulin-treated type 2 diabetic females had a higher BMI than the other groups, while

Table 2 Clinical parameters, and HDL subspecies of control males and diabetic males

	Controls	T1D	T2D with insulin	T2D without insulin
Number (Male)	11	17	19	21
Age (year)	37.6±7.1	46.6±12.6	62.6±13.2 ^b	57.4±16.0 ^a
Boby mass index (kg/m ²)	24.4±4.7	21.3±2.2	24.5±4.0	25.6±4.7 ^b
HbA1c (%)		7.4±2.3	7.9±1.1	9.0±1.6 ^b
Triglyceride (mg/dL)	108±39.1	97.6±93.2	141.2±61.0	222.3±187.3 ^b
LDL-C (mg/dL)	115.7±38.8	101.8±21.7	107.9±28.5	119.0±21.3
Small dense LDL-C (mg/dL)	25.9±8.8	26.4±9.2	33.1±19.6	36.7±17.5
HDL-C (mg/dL)	56.7±14.2	80.0±20.1 ^a	55.8±13.7 ^b	41.4±8.6 ^{abc}
Apo-A I (mg/dL)	159.9±25.4	188.5±33.8	168.4±28.2	142.8±27.8 ^{bc}
Apo-A II (mg/dL)	33.8±4.0	32.9±5.1	33.1±9.7	30.2±4.9
HDL2-C (mg/dL)	29.4±11.8	52.5±21.4 ^a	32.0±12.0 ^b	16.7±8.6 ^{bc}
HDL2-Apo A I (mg/dL)	71.2±21.7	106.8±33.5 ^a	85.0±26.2	56.2±25.9 ^{bc}
HDL2-Apo A II (mg/dL)	6.7±1.8	9.6±8.1	7.8±3.2	5.1±3.3
HDL3-C (mg/dL)	27.3±4.2	27.5±6.0	23.8±4.9	24.5±6.9
HDL3-Apo A I (mg/dL)	84.9±10.7	78.5±23.5	76.8±14.2	88.6±24.2
HDL3-Apo A II (mg/dL)	27.1±3.5	23.1±6.3	24.4±5.5	25.5±5.6
HDL2-C-HDL3-C ratio	1.07±0.41	2.06±1.19 ^a	1.38±0.58 ^b	0.75±0.4 ^{bc}
HDL2-Apo A I-HDL3-Apo A I ratio	0.85±0.29	1.6±1.0	1.16±0.49	0.62±0.37 ^{bc}
HDL2-Apo A II-HDL3-Apo A II ratio	0.25±0.07	0.77±1.77	0.33±0.17	0.21±0.17

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; C, cholesterol ; Apo, apolipoprotein
Data is expressed as mean ± standard deviation. Significance ($p<0.05$): a, vs. Controls; b, vs. T1D; c, vs. T2D with insulin

Table 3 Clinical parameters, and HDL subspecies of control females and diabetic females

	Controls	T1D	T2D with insulin	T2D without insulin
Number (Female)	14	10	14	15
Age (year)	43.8±5.5	48.2±21.2	64.3±9.1 ^b	64.1±12.1 ^{ab}
Boby mass index (kg/m ²)	20.8±2.6	21.5±5.1	23.1±2.4	26.0±4.8 ^{ab}
HbA1c (%)		8.1±1.4	8.1±1.3	8.8±1.5
Triglyceride (mg/dL)	83.2±26.6	82.9±38.5	147.3±89.5	165.3±66.0 ^{ab}
LDL-C (mg/dL)	109.4±26.8	100.3±14.0	113.7±18.9	129.3±36.2 ^b
Small dense LDL-C (mg/dL)	22.0±5.4	21.1±7.0	28.7±13.1	31.7±11.6
HDL-C (mg/dL)	68.1±13.8	70.7±23.0	62.1±19.9	45.5±11.7 ^{abc}
Apo-A I (mg/dL)	179.4±27	169.4±50.2	172.2±33.4	150.0±26.8
Apo-A II (mg/dL)	31.0±5.0	27.5±10.7	31.4±4.7	30.4±6.2
HDL2-C (mg/dL)	44.06±13.3	47.7±17.9	33.8±14.4	20.4±7.9 ^{abc}
HDL2-Apo A I (mg/dL)	100.7±26.5	102.8±37.3	84.5±24.4	56.4±20.0 ^{abc}
HDL2-Apo A II (mg/dL)	9±2.5	7.5±3.1	6.7±1.8	5.3±2.3 ^a
HDL3-C (mg/dL)	23.9±3.4	23.0±8.2	27.4±11.3	25.1±6.6
HDL3-Apo A I (mg/dL)	72.77±11.2	63.5±29.1	86.9±27.0	88.1±21.5
HDL3-Apo A II (mg/dL)	21.7±3.5	19.9±8.4	25.3±3.9	24.9±5.8
HDL2-C-HDL3-C ratio	1.88±0.68	2.15±0.70	1.26±0.52 ^b	0.85±0.36 ^{ab}
HDL2-Apo A I-HDL3-Apo A I ratio	1.43±0.55	2.20±2.38	1.03±0.40	0.70±0.34 ^b
HDL2-Apo A II-HDL3-Apo A II ratio	0.42±0.14	0.38±0.13	0.27±0.08	0.23±0.12 ^{ab}

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; C, cholesterol ; Apo, apolipoprotein
Data is expressed as mean ± standard deviation. Significance ($p<0.05$): a, vs. Controls; b, vs. T1D; c, vs. T2D with insulin

Table 4 HDL subspecies in the total subjects stratified by use of statins

	T2D with insulin		T2D without insulin	
	with statin	without statin	with statin	without statin
HDL-C (mg/dL)	54.5±15.2	62.9±16.5	38.6±5.8	44.8±11.0
HDL2-C (mg/dL)	28.7±11.8	36.6±13	17.1±4.9	18.4±9.3
HDL2- Apo A I (mg/dL)	78.7±22.8	90.5±26.4	47.0±13.9	56±25.6
HDL2- Apo A II (mg/dL)	6.8±2.1	7.9±3.2	5.3±2.4	5.2±3
HDL3-C (mg/dL)	25.1±6	26.2±10.4	21.5±3.3	25.8±7.3
HDL3-Apo A I (mg/dL)	83.2±14.3	80.1±25.6	80.2±14.9	91.5±24.3
HDL3-Apo A II (mg/dL)	25.6±5.3	23.5±4.2	24.4±3.6	25.7±6.1

Abbreviations: HDL, high-density lipoprotein; C, cholesterol ; Apo, apolipoprotein

Data is expressed as mean ± standard deviation.

Table 5 Correlation between HDL2-C and other clinical parameters in total subjects**A) Simple regression for HDL2-C**

	<i>r</i>	<i>p</i>
Age	-0.164	0.1096
Boby mass index	-0.375	<0.0001
HbA1c	-0.272	0.0068
Triglyceride	-0.464	<0.0001
LDL-C	-0.406	<0.0001
Small dense LDL-C	-0.354	0.0004
Insulin injected dose	0.161	0.2199

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; C, cholesterol Significance: *r*=correlation coefficient

B) Multiple regression for HDL2-C

	β	<i>p</i>
Boby mass index	-0.017	0.8519
HbA1c	-0.077	0.3739
Triglyceride	-0.300	0.0015
LDL-C	-0.210	0.0149
Intensive insulin therapy	0.389	<0.0001

$R^2=0.43$

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; C, cholesterol

the HbA1c levels were comparable among the three diabetic groups. The female controls had significantly higher HDL-C levels than the male controls (68 ± 14 vs 57 ± 14 , $p < 0.05$). Thus, the HDL-C levels in type 1 diabetic females were comparable to those in the female controls. As seen in the males, the non-insulin-treated type 2 diabetic females had higher TG and lower HDL-C than the control or type 1 diabetic females. Serum lipids in insulin-treated type 2 diabetic females were comparable to those in control females and type 1 diabetic females. HDL2-C and HDL2-apo AI levels in type 1 diabetic females were comparable to those in control females. HDL2-C, HDL2-apo AI, and HDL2-apo AII levels were lower in non-insulin-treated type 2 diabetic females than in the other groups. HDL3-C, HDL3-apoAI, and HDL3-apoAII levels were comparable among the four groups. The HDL2-C/HDL3-C ratio was lower in type 2 diabetic females than in the control females and type 1 diabetic females, irrespective of insulin therapy. A similar tendency was seen in the HDL2-apo AI/HDL3-apo AI ratio. The HDL2-apo AII/HDL3-apo AII ratio was lower in non-insulin-treated type 2 diabetic females than in the control

females and type 1 diabetic females.

Table 4 lists HDL subspecies in the total subjects stratified by use of statins. We did not find any significant differences between statin-user and non-statin user for HDL subspecies.

Table 5A lists simple correlation coefficients in comparisons of HDL2-C with other variables in the total study population. HDL2-C was inversely correlated with BMI, HbA1c, TG, LDL-C, and sdLDL-C, but not with the dose of injected insulin. Multiple regression analysis (Table 5B) revealed that HDL2-C was independently associated with TG, LDL-C, and intensive insulin therapy but not with BMI or HbA1c. HDL3-C was not associated with BMI, HbA1c, TG, LDL-C, sdLDL-C, or the insulin dose (data not shown).

Discussion

The present study has shown that T1D and T2D treated with insulin had higher HDL-C levels than non-insulin-treated T2D. Other studies have demonstrated that HDL-C levels are normal or slightly increased in well-controlled T1D treated with insulin[15-18], and

lower in T2D treated with OADs than in T2D treated with insulin [19]. To the best of our knowledge, however, there have been no earlier reports on the measurement of cholesterol and apoAI/AII in HDL subspecies in T1D and T2D simultaneously. Helen M. Colhoun *et al.* [20] reported findings concordant with the results we report here, namely, that small HDL (i.e., HDL3) was reduced and large HDL (i.e., HDL2) was increased in type 1 diabetic patients, compared with non-diabetic controls. Indeed, several reports have demonstrated that insulin therapy increases HDL2-C in T1D [21] and T2D [22]. These previous reports strongly support our result that insulin treatment exclusively increases HDL2 levels, irrespective of the type of diabetes. Several possible mechanisms may explain the HDL2 elevation in response to insulin therapy. First, insulin activates lipoprotein lipase (LPL), a limiting enzyme of TG-rich lipoprotein catabolism. Taskinen *et al.* [22] reported that insulin therapy induced a significant reduction of very low density lipoprotein (VLDL)-TG and an increase of HDL2-C in association with increased LPL activity. Patsch *et al.* [23] proposed that low HDL2-C strongly reflects the degree of postprandial hypertriglyceridemia induced by reduced LPL activity. Thus, insulin-induced LPL activation could be associated with reduced VLDL and chylomicrons, and through this association could promote increased HDL2 formation. Our present findings also revealed a close inverse association between plasma TG and HDL2-C. A second possible explanation for the insulin-induced HDL2 elevation could be exogenous insulin's effect in suppressing hepatic lipase (HL) activity [24], an important contributor to the conversion from HDL2 to HDL3 *via* enhanced phospholipase activity. A third potential mechanism, albeit one still open to controversy, could be an insulin-induced suppression of cholesteryl ester transfer protein (CETP) [25]. The association between CETP deficiency and elevated levels of cholesterol-enriched large HDL particles is well recognized. Our present findings revealed no correlation between the dose of injected insulin and the HDL2-C level in our diabetic cases. This suggests that endogenous insulin production differs significantly from one diabetes patient to the next, and hence that the exogenous (injected) insulin dose is not reflective of the total insulin action on lipid metabolism. Apart from the presence or absence of insulin therapy, insulin resistance may also regulate the HDL subspecies. We once again performed multivariate analysis, removing TG, which

was strongly correlated with BMI. However, multivariate analysis revealed that BMI reflecting insulin resistance lost an independent association with HDL2-C when insulin therapy was entered into as a dependent variable (data not shown). Nevertheless, it remains to be elucidated whether insulin resistance is associated with HDL2 levels independently upon insulin/OAD treatment by prospective study.

Similar to our previous report [10], the present study demonstrated that HDL2-C levels were higher than HDL3-C levels in non-diabetic controls, especially in women. Our literature review on HDL-C subspecies indicated that HDL3-C levels are much higher than HDL2-C levels in non-diabetic populations [6, 11], and that HDL2-C levels are substantially reduced in diabetic populations [6, 9] in Western countries. The relatively low ratio of HDL2-C to HDL3-C might explain the higher incidence of CHD in Western populations compared to that in the Japanese population.

Results have been in conflict as to whether HDL2-C or HDL3-C constitutes a stronger negative predictor of CHD events [26-30]. HDL can also be immuno-separated, on the basis of apolipoprotein composition, into particles containing only apoAI (LpAI) and particles containing both apoAI and apoAII (LpAI/A-II) [31]. ApoAI is distributed approximately equally between LpAI and LpAI/AII, whereas virtually all apoAII is to be found in LpAI/A-II. The HDL2 particle is more abundant in apoAI than in apoAII, whereas apoAI and apoAII are equally distributed in the HDL3 particle [4, 31]. Thus, we know that LpAI is more strongly associated with HDL2-apoAI than with HDL3-apoAI. Most clinical and clinical studies have agreed that the levels of LpAI are inversely associated with the risk of CHD [31, 32]. As such, we know that HDL2-apoAI is probably superior to total apoAI or HDL3-apoAI as a negative risk marker for CHD events. Our current finding on HDL2-apo AI, specifically, the higher levels of HDL2-apo AI in insulin-treated T2D *versus* that in non-insulin-treated T2D, suggests that insulin therapy may serve favorably in suppressing the prevalence of CHD events. Indeed, the UKPDS 80 study revealed that early intervention of glycemic control with sulfonylurea and/or insulin injection suppresses the prevalence of CHD events and related mortalities [33]. Meanwhile, we await large-scale clinical trials to determine whether the elevation of HDL2 attained through insulin therapy is associated with the suppression of CHD events. Weakness of this study is cross-

sectional study and the cause-effect relationship is not clear. To confirm our conclusion, HDL subspecies should be measured before and after insulin treatment in the same sets of T2D.

In conclusion, T1D had remarkably higher HDL2 levels, and insulin-treated T2D had significantly higher HDL2 levels, compared to T2D without insulin ther-

apy. These results suggest that intensive insulin therapy is associated with alterations of HDL subspecies, irrespective of the type of diabetes

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References

1. N E Miller, F Hammett, S Saltissi, et al. (1981) Relation of angiographically defined coronary artery disease to plasma lipoprotein subfractions and apolipoproteins. *Br Med J* 282:1741-1744.
2. Syväne M, Ahola M, Lahdenperä S, et al. (1995) High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *J Lipid Res* 36:573-582.
3. Laakso M (1997) Dyslipidemia, morbidity, and mortality in non-insulin-dependent diabetes mellitus. Lipoproteins and coronary heart disease in non-insulin-dependent diabetes mellitus. *J Diabetes Complications* 11:137-141.
4. Kontush A, Chapman MJ (2006) Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol Rev* 58:342-374.
5. Chapman MJ, Goldstein S, Lagrange D, et al. (1981) A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res* 22: 339-358.
6. M. Chr. Bakogianni, Chr. A. Kalofoutis, K. I. Skenderi, et al. (2001) Clinical evaluation of plasma high-density lipoprotein subfractions (HDL2, HDL3) in non-insulin-dependent diabetics with coronary artery disease. *J Diabetes Complications* 15:265-269.
7. Drexel H, Amann FW, Rentsch K, et al. (1992) Relation of the level of high-density lipoprotein subfractions to the presence and extent of coronary artery disease. *Am J Cardiol* 70:436-440.
8. Lamarche B, Moorjani S, Cantin B, et al. (1997) Associations of HDL2 and HDL3 subfractions with ischemic heart disease in men. Prospective results from the Québec Cardiovascular Study. *Arterioscler Thromb Vasc Biol* 17:1098-1105.
9. Pérez-Méndez O, Torres-Tamayo M, Posadas-Romero C, et al. (2007) Abnormal HDL subclasses distribution in overweight children with insulin resistance or type 2 diabetes mellitus. *Clin Chim Acta* 376:17-22.
10. Hirano T, Nohtomi K, Koba S, et al. (2008) A simple and precise method for measuring HDL-cholesterol subfractions by a single precipitation followed by homogenous HDL-cholesterol assay. *J Lipid Res* 49:1130-1136.
11. Kantartzis K, Rittig K, Cegan A, et al. (2008) Fatty liver is independently associated with alterations in circulating HDL2 and HDL3 subfractions. *Diabetes Care* 31:366-368.
12. Warnick GR, and Albers JJ (1978) A comprehensive evaluation of the heparin-manganese precipitation procedure for estimating high density lipoprotein cholesterol. *J Lipid Res* 19:65-76.
13. Ito Y, Fujimura M, Ohta M, et al. (2011) Development of a homogeneous assay for measurement of small dense LDL cholesterol. *Clin Chem* 57:57-65.
14. The Committee of Japan Diabetes Society on the Diagnostic Criteria of Diabetes Mellitus (2010) Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. *J Japan Dia Soc* 53:450-467 (In Japanese).
15. Mattock MB, Salter AM, Fuller JH, et al. (1982) High density lipoprotein subfractions in insulin-dependent diabetic and normal subjects. *Atherosclerosis* 45:67-79.
16. Dullaart RP (1995) Plasma lipoprotein abnormalities in type 1 (insulin-dependent) diabetes mellitus. *Neth J Med* 46:44-54.
17. Eckel RH, Albers JJ, Cheung MC, et al. (1981) High density lipoprotein composition in insulin-dependent diabetes mellitus. *Diabetes* 30:132-138.
18. Kahri J, Groop PH, Viberti G, et al. (1993) Regulation of apolipoprotein A-I-containing lipoproteins in IDDM. *Diabetes* 42:1281-1288.
19. G.D. Calvert, T. Mannik, J.J. Graham, et al. (1978) Effects of therapy on plasma-high-density-lipoprotein-cholesterol concentration in diabetes mellitus. *The Lancet* 312:66-68.
20. Helen M. Colhoun, James D. Otvos, Mike B. Rubens, et al. (2002) Lipoprotein subclasses and particle sizes and their relationship with coronary artery calcification in men and women with and without type 1 diabetes. *Diabetes* 51:1949-1956.
21. Pacifico A, Cherchi GM, Baggi MA, et al. (1983) Changes in HDL subfractions in patients with type I diabetes mellitus before and after metabolic control. *Boll Soc Ital Biol Sper* 59:1618-1624.
22. Taskinen MR, Kuusi T, Helve E, et al. (1988) Insulin therapy induces antiatherogenic changes of serum lipopro-

- teins in noninsulin-dependent diabetes. *Arteriosclerosis* 8:168-177.
23. J R Patsch, S Prasad, A M Gotto, Jr., et al. (1987) High density lipoprotein2. Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. *J Clin Invest* 80: 341-347.
 24. Geremia Romano, Lidia Patti, Francesca Innelli, et al. (1997) Iulin and sulfonylurea therapy in NIDDM patients. Are the effects on lipoprotein metabolism different even with similar blood glucose control? *Diabetes* 46:1601-1606.
 25. Kaoru Arii, Tadashi Suehiro, Michiya Yamamoto, et al. (1997) Suppression of plasma cholesteryl ester transfer protein activity in acute hyperinsulinemia and effect of plasma nonesterified fatty acid. *Metabolism* 46:1166-1170.
 26. Johansson J, Carlson LA, Landou C, et al. (1991) High density lipoproteins and coronary atherosclerosis. A strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler Thromb* 11:174-182.
 27. Drexel H, Amann FW, Rentsch K, et al. (1992) Relation of the level of high-density lipoprotein subfractions to the presence and extent of coronary artery disease. *Am J Cardiol* 70: 436-440.
 28. Skinner ER (1994) High-density lipoprotein subclasses. *Curr Opin Lipidol* 5: 241-247.
 29. Barter P, Kastelein J, Nunn A, et al. (2003) High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions. *Atherosclerosis* 168:195-211.
 30. Desai MY, Rodriguez A, Wasserman BA, et al. (2005) Association of cholesterol subfractions and carotid lipid core measured by MRI. *Arterioscler Thromb Vasc Biol* 25: e110-e111.
 31. Ohta T, Saku K, Takata K, et al. (1995) Different effects of subclasses of HDL containing apoA-I but not apoA-II (LpA-I) on cholesterol esterification in plasma and net cholesterol efflux from foam cells. *Arterioscler Thromb Vasc Biol* 15: 956-962.
 32. Duriez P and Fruchart JC (1999) High-density lipoprotein subclasses and apolipoprotein A-I. *Clin Chim Acta* 286: 97-114.
 33. Holman RR, Paul SK, Bethel MA, et al. (2008) 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med* 359:1577-1589.