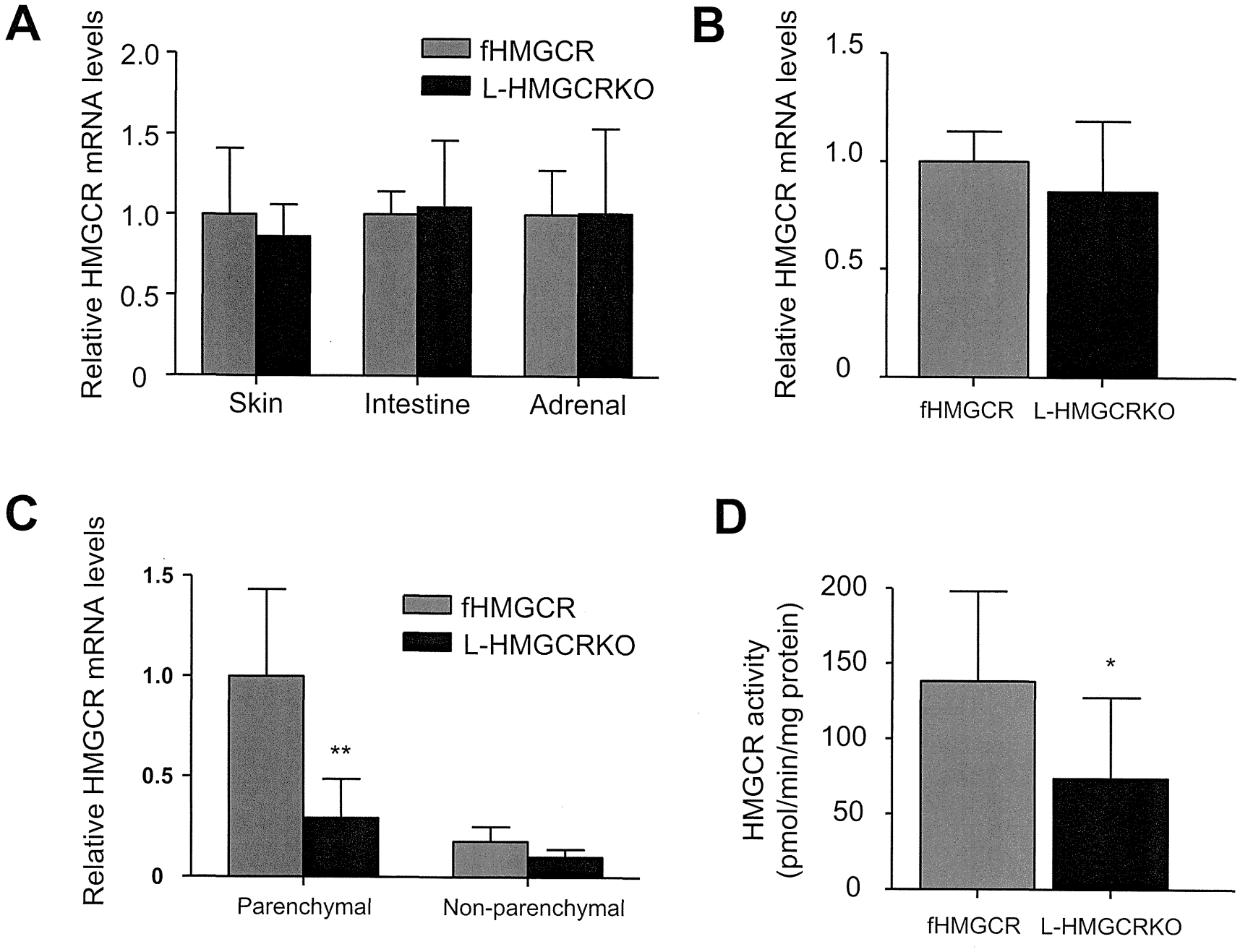
Supplementary Figure II



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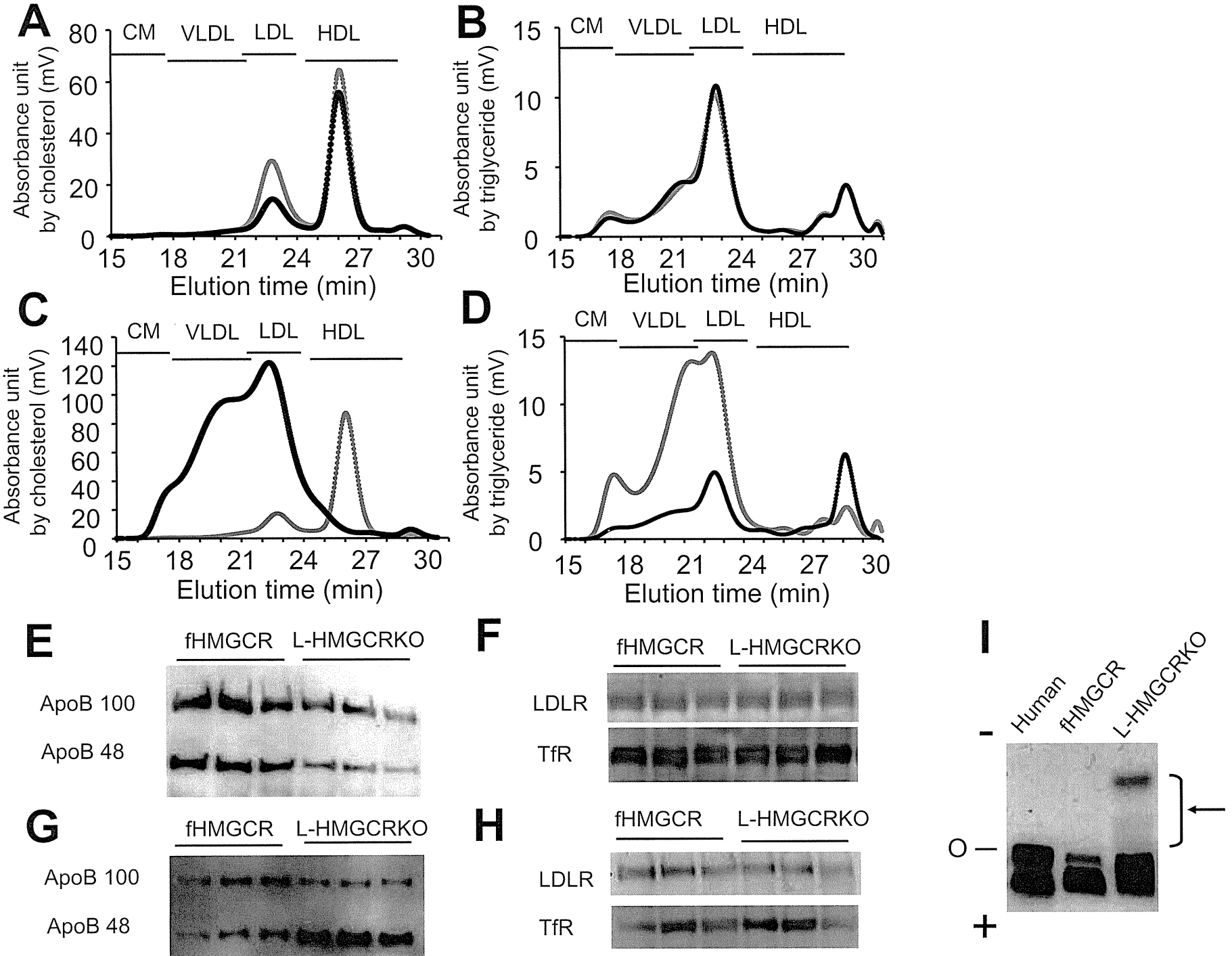
Supplementary Figure III



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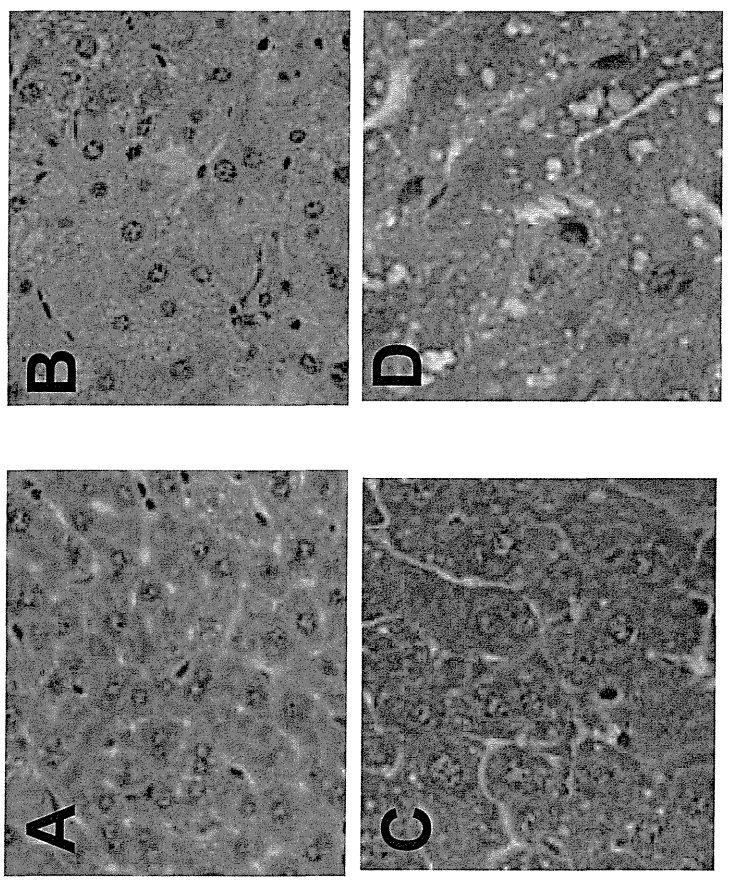
Supplementary Figure IV



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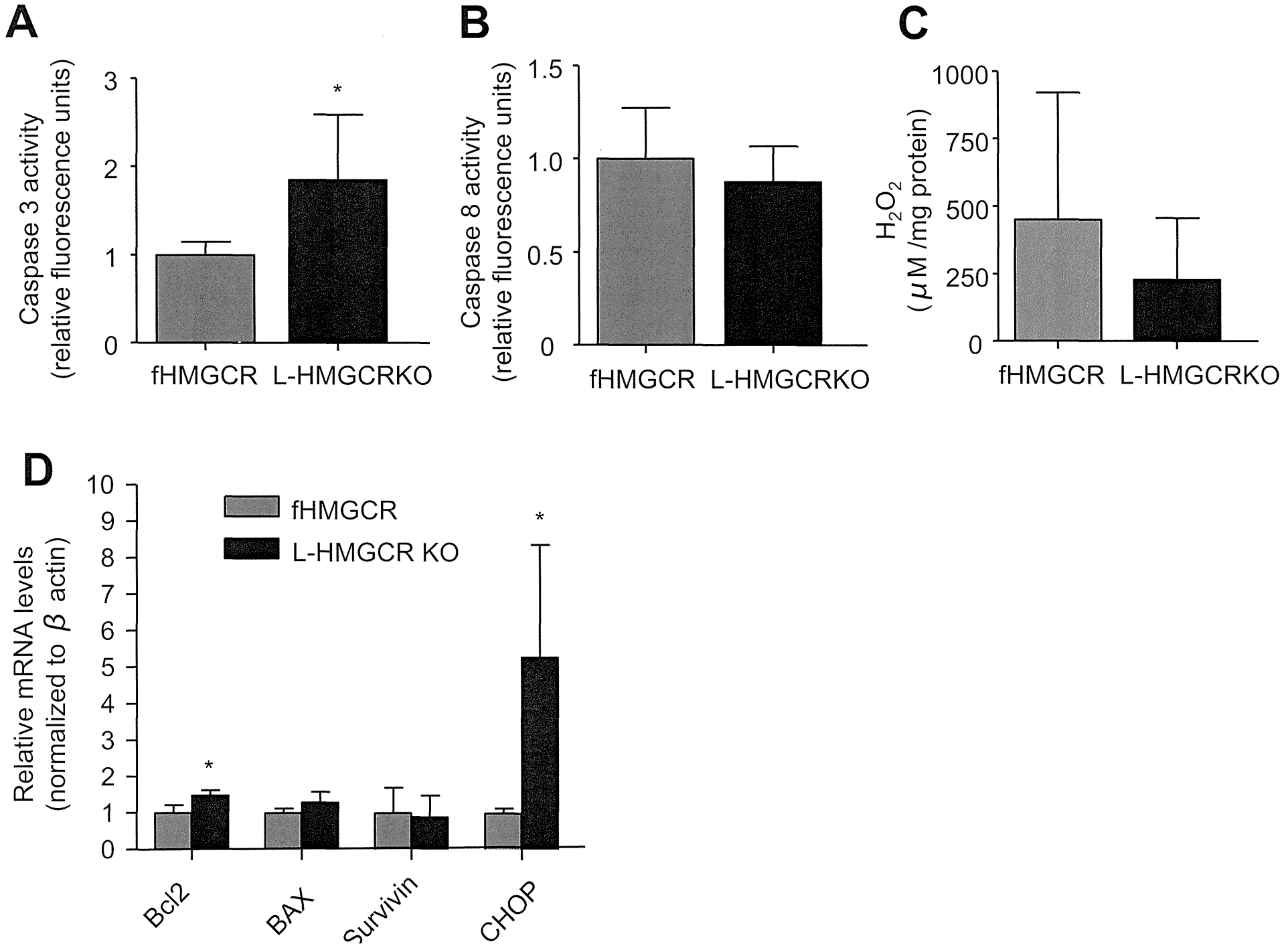
Supplementary Figure V



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Supplementary Figure VI

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Comparison of Various Lipid Variables as Predictors of Coronary Heart Disease in Japanese Men and Women With Type 2 Diabetes

Subanalysis of the Japan Diabetes Complications Study

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OBJECTIVE—To determine the best lipid variable to predict coronary heart disease (CHD) in Japanese patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS—Eligible Japanese men and women (1,771) aged 40–70 years with type 2 diabetes from 59 institutes nationwide were followed for a planned 8-year period. The performance of eight conventional lipid variables, i.e., total cholesterol (TC), LDL-cholesterol (LDLC), HDL-cholesterol (HDLC), triglycerides (TGs), non-HDLC, TC/HDLC ratio, LDLC/HDLC ratio, and TG/HDLC ratio, as predictors of incident CHD were evaluated by four methods: hazard ratio (HR) per one SD increment by multivariate Cox analysis, χ^2 likelihood ratio test, area under the receiver operating characteristic curve (AUC), and tertile analysis.

RESULTS—Although all variables significantly predicted CHD events in men, non-HDLC (HR per one SD 1.78 [95% CI 1.43–2.21]; AUC 0.726) and TC/HDLC (HR 1.63 [1.36–1.95]; AUC 0.718) had the better predictive performances among the variables, including LDLC. In women, TGs (log-transformed; HR 1.72 [1.21–2.43]; AUC 0.708) were the best predictor according to results of tertile analysis (HR of the top tertile versus the bottom tertile 4.31 [1.53–12.16]). The associations with incident CHD were linear and continuous.

CONCLUSIONS—For Japanese diabetic men, non-HDLC and TC/HDLC were the best predictors, whereas TGs were most predictive for women. These findings, which included prominent sex differences, should be considered among clinical approaches to risk reduction among East Asians with diabetes.

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Type 2 diabetes is characterized by an excessive incidence of coronary heart disease (CHD), and serum lipid values are among the strongest predictors of CHD (1,2). Although serum LDL-cholesterol (LDLC) has been conventionally used as a therapeutic marker and/or target in many guidelines based on trials using statins (1,2), characteristic features of diabetic dyslipidemia, which are closely associated with insulin resistance, are elevated levels of triglycerides (TGs) and small, dense LDLC (independent of LDLC level) as well as decreased levels of HDL-cholesterol (HDLC) (1,2). The use of LDLC alone for assessment of cardiovascular risk would ignore these TG-rich lipoproteins (TRLs, i.e., VLDL and intermediate-density lipoprotein) and low HDLC, all of which affect the risk of a CHD event independently of LDLC (1–4). Moreover, LDLC values, as estimated by the Friedewald formula, become progressively less accurate as the TG level increases.

Based on this background, it has been established that other lipid parameters, typically non-HDLC (determined by subtracting the HDLC concentration from the total cholesterol [TC] concentration in plasma) or apolipoprotein B (apoB), both of which reflect TRLs and small, dense LDLC, can be considered better predictors of CHD than LDLC and have been introduced into some guidelines as a secondary target for therapy (5–7). Furthermore, the ratios of TC to HDLC (TC/HDLC), which has clinical significance equivalent to non-HDLC/HDLC, LDLC to HDLC (LDLC/HDLC), and TGs to HDLC (TG/HDLC) are also used for assessing cardiovascular risk (3,4). It should be mentioned that non-HDL/HDL is always one unit lower than TC/HDLC.

Despite these considerations, these fundamental lipid measures (TC, HDLC, and TGs) and their calculated indices (LDLC, non-HDLC, TC/HDLC, LDLC/HDLC, and TG/HDLC) have not been completely and directly compared as predictors of CHD by

multiple analytical methods in past prospective studies in diabetic subjects (8–19). Results obtained have been inconsistent, and only one study (19) analyzed men and women separately. Therefore, whether LDLC performs better than the other indices or, if not, which variable is the best predictor of a CHD event has not been fully determined in diabetic subjects. Furthermore, all previous examinations of the performance of lipid variables as predictors of CHD in diabetic subjects (8–19) were performed in Western countries or in Caucasians. It is uncertain whether their results can be extrapolated to East Asian diabetic subjects, who have substantially different profiles regarding CHD and its risk factors, including a much lower incidence of CHD and degree of obesity (20–22).

In this analysis of data from a long-term follow-up of Japanese patients with type 2 diabetes, we compared eight conventional lipid variables, all of which are routinely measured or can be easily calculated in clinical care settings, as predictors of CHD events. To directly and quantitatively compare variables having different average values as well as variations in quantities and ratios, we used four different analytical methods to determine the best predictor of CHD. These were the multivariate-adjusted hazard ratio (HR) per one SD increment in the Cox hazard model, χ^2 likelihood ratio test, area under the receiver operating characteristic (ROC) curve (AUC), and tertile analysis.

RESEARCH DESIGN AND METHODS

Recruitment of patients

The present analysis was conducted as part of the Japan Diabetes Complications Study, a multicenter prospective study of the incidence of and risk factors for macro- and microvascular complications among 2,033 Japanese patients with type 2 diabetes aged 40–70 years with HbA_{1c} levels >6.5% who were registered from January 1995 to March 1996 from outpatient clinics in 59 university and general hospitals nationwide that specialize in diabetes care. For this analysis of macrovascular complications, of those 2,033 individuals, 940 men (mean age 57.8 ± 7.1 years) and 831 women (mean age 58.7 ± 6.8 years) were selected for the current study after consideration of the exclusion criteria prespecified in the study protocol (23). Excluded were patients with impaired glucose tolerance, a history of angina pectoris, myocardial infarction, stroke, peripheral artery disease, familial hypercholesterolemia, type III hyperlipidemia (diagnosed by broad β band on electrophoresis), nephrotic syndrome (urine protein >3.5 g per day and serum total protein <6.0 mg/dL), and serum creatinine levels >1.3 mg/dL (120 μmol/L). In the 8-year planned observation period, the median follow-up for the 1,771 patients was 7.86 years (final follow-up rate was 75%; 1,332/1,771 patients). The total person-years studied was 11,743 (6,106 for men and 5,637 for women). Diabetes and impaired glucose tolerance were diagnosed according to the Report of the Committee of the Japan Diabetes Society on the Classification and Diagnostic Criteria of Diabetes Mellitus, which is almost identical in terms of thresholds for glucose levels to those of the World Health Organization. The study protocol, which is in accordance with the Declaration of Helsinki and the Ethical Guidelines for Clinical/Epidemiological Studies of the Japanese Ministry of Health, Labor, and Welfare, received ethical approval from the institutional review boards of all participating institutes. All enrolled patients provided written informed consent.

Clinical and laboratory measurements

Patients were assessed yearly after the baseline evaluation. Mean values of at least two measurements each year were obtained for HbA_{1c}, fasting plasma glucose, and fasting serum lipids. HbA_{1c} assays were performed according to procedures outlined by the Laboratory Test Committee of the Japan Diabetes Society (JDS), which is known to be converted by the formula HbA_{1c} (JDS)(%) = HbA_{1c} (National Glycohemoglobin Standardization Program [NGSP])(%) − 0.4%. All other laboratory tests were performed at each participating institute. Serum LDLC was calculated using the Friedewald equation, except where TGs exceeded 400 mg/dL, in which case LDLC data were treated as "missing". This was applicable to 20 subjects. All other measurements, including those for body weight, blood pressure, and a 12-lead electrocardiogram, were performed at least once yearly. A baseline dietary survey, which was validated and is widely used in Japan (24) and was comprised of food records and a food frequency questionnaire that included alcohol consumption, was undertaken. Information on cigarette smoking was collected using a self-administered questionnaire. Smoking status was classified into one of three categories: current smokers, ex-smokers, and never smokers (25).

Outcome measures

The outcomes analyzed were a fatal or first nonfatal manifestation of CHD comprised of angina pectoris and myocardial infarction, both of which were diagnosed according to criteria defined by the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA; World Health Organization) project. A patient with a first percutaneous coronary intervention or coronary artery bypass graft was also counted as having a CHD event. Information regarding primary outcome and other clinical variables for each subject was collected through an annual report that included detailed findings at the time of the event from each participating diabetologist who was providing care to those patients. The adjudication of end points was performed by central committees comprised of experts who were masked to risk factor status and was based on additional data such as a detailed history, sequential changes in electrocardiogram and serum cardiac biomarkers, and results of coronary angiography. The rate of concordance in diagnosis between participating diabetologists and committee experts was 93%.

Statistical analysis

All statistical analyses and data management were conducted at the central data center. Patient characteristics were described as mean ± SD, median and interquartile range, or percentage. We compared a CHD group with a no-CHD group by Student *t* test and Fisher exact test for numerical and categorical variables, respectively. Multivariate Cox regression analysis was used to calculate the adjusted HRs and 95% CIs for risk factors. The strength of associations of each lipid variable was assessed using the χ^2 likelihood ratio test, and the corresponding *P* values were estimated from the regression coefficient based on the Cox proportional hazards model. In addition, the relationships between tertiles of each baseline lipid variable and HR for CHD risks were assessed by the Cox proportional hazards model using the first tertile of each variable as the reference group. The discriminatory powers for CHD of the lipid variables were also compared by ROC curve analysis with application of various thresholds to the predicted probability obtained from the logistic regression model. The AUC was calculated by integrating the area between the ROC curve and the diagonal line where sensitivity

is equal to one specificity based on the trapezoidal rule. Multivariate-adjusted generalized additive models with a spline function of three degrees of freedom were used to explore potential nonlinear relationships. All *P* values are two sided and the significance level is 0.05. All statistical analyses were conducted using SAS packages version 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Baseline clinical variables according to occurrence of CHD events

Table 1 summarizes clinical baseline variables for men and women who had or had not experienced a CHD event during the follow-up period. In comparison with men without CHD, those with CHD had significantly higher levels of all lipid variables (but lower HDLC values) determined except for TGs, which was higher with borderline significance. Women with CHD had significantly higher systolic blood pressure and significantly higher levels of lipid variables with the exception of LDLC/HDLC, which was of borderline significance, and HDLC. In addition, women with, rather than without, CHD were significantly more likely to use an insulin sensitizer and agents for hypertension and dyslipidemia.

Relationships between various lipid variables and CHD outcome

Multivariate-adjusted HRs per one SD, χ^2 values, and AUCs for CHD events for each lipid variable at baseline are shown in Table 2. In men, all lipid variables significantly predicted a CHD event with HRs per one SD ranging between 1.42 and 1.78. The largest HR value per one SD, χ^2 statistics, and AUCs were found for non-HDLC followed by TC/HDLC, which had findings very close to non-HDLC results.

In women, the largest HR per one SD was found for TGs (log-transformed) followed by non-HDLC and TC. These three indices had substantially larger χ^2 values and slightly larger AUCs than the other indices, whereas non-HDLC had the largest χ^2 value and TC had the largest AUC value (Table 2). Since subjects with elevated TGs are likely have higher glycemic or weight levels, we performed stratified analysis to categorize women according to values equal to or above or below the median of HbA_{1c} or BMI, which were 7.6% and 22.8 kg/m^2, respectively. As a result, a significantly larger multivariate-adjusted HR per one SD of log-transformed TGs was observed only in those whose HbA_{1c} or BMI level was equal to or greater than the median, i.e., HbA_{1c} ≥7.6%, HR 1.78 (95% CI 1.21–2.63), and *P* = 0.005 versus HbA_{1c} <7.6%, 1.37 (0.76–2.47), and *P* = 0.27 (Supplementary Table 1); BMI ≥22.8, 1.75 (1.17–2.62), and *P* = 0.008 versus BMI <22.8, 1.51 (0.86–2.65), and *P* = 0.14 (Supplementary Table 2).

In the combined analysis of men and women, non-HDLC identified patients at greater risk of CHD than the other lipid variables and had an HR of 1.69 (95% CI 1.41–2.01), χ^2 statistic of 29.4 (*P* < 0.001), and AUC of 0.713 (95% CI 0.663–0.762) followed by TC/HDLC, for which results were 1.55 (1.33–1.81), 23.9 (*P* < 0.001), and 0.703 (0.651–0.754), respectively. These were better predictors than LDLC, for which results were 1.51 (1.26–1.80), 18.2 (*P* < 0.001), and 0.690 (0.641–0.738), respectively.

Table 3 shows HRs for CHD according to tertiles of lipid variables. In men, HRs were significantly elevated in the top compared with the bottom tertile (bottom compared with the top in case of HDLC) in all variables determined. Subjects in the top tertile of TC/HDLC and LDLC/HDLC had a four times or greater risk of CHD than those in the respective bottom tertile, followed by non-HDLC and LDLC, both of which had relatively high HRs of ~3.5 between extreme tertiles. In women, significantly elevated HRs in the top tertile compared with the bottom tertile were observed only for TGs, TG/HDLC ratio, and LDLC. Among those, the highest HR was noted for TGs, and was 4.31, which was considerably higher than that for the other lipid variables. Even subjects in the middle tertile for TGs, which indicated the normal level of 0.90–1.36 mmol/L, had a significantly higher risk of CHD than those in the bottom tertile. On the other hand, the HR for the TG/HDLC ratio was not higher than that for TGs alone either in men or women. If we again stratified women with values below and equal to or above the median for HbA_{1c} or BMI, which were 7.6% and 22.8 kg/m^2, respectively, significantly elevated HRs for TGs in the top tertile compared with the bottom tertile were observed only in those whose HbA_{1c} or BMI was at or greater than the median, i.e., HbA_{1c} ≥7.6%, HR 6.74 (95% CI 1.43–31.67), and *P* = 0.016 versus HbA_{1c} <7.6%, 2.95 (0.65–13.47), and *P* = 0.163 (Supplementary Table 3); BMI ≥22.8, 3.95 (1.08–14.54), and *P* = 0.039 versus BMI <22.8, 5.13 (0.90–29.30), and *P* = 0.066 (Supplementary Table 4).

Dynamic change in risk association of important lipid variables

To explore dynamic changes in risk association, including possible thresholds for lipid variables that were found to be good predictors, sex-stratified spline analysis was performed for non-HDLC, TC/HDLC, and TGs (Fig. 1). In each variable, the relationship was on a continuum, indicating difficulty in determining a clear cutoff value. When risks for men and women whose non-HDLC was 3.88 mmol/L (150 mg/dL) were set as a reference, risks of those with a non-HDLC value of ~4.3 mmol/L (170 mg/dL) became significant with HRs of ~1.5 in both men and women. When the TC/HDLC level of 5.0 was set for reference, risks in those whose TC/HDLC levels were ~6.3 became significant in both men and women but the HR was greater in women (~2.0) than in men (~1.5).

CONCLUSIONS—The current analysis of our Japanese subjects with type 2 diabetes revealed distinct sex differences in lipid variables that predict a CHD event. Although large sex differences in incidence and risk profiles (such as smoking) of CHD are well known, most previous studies on lipid variables as predictors of CHD (8–15,17,18) did not separately analyze men and women with diabetes. Our previous investigation to clarify risk factors (involving nonlipid parameters) for cardiovascular complications in Japanese diabetic subjects, which also analyzed men and women together, demonstrated that the serum TG level was a potent risk factor, unlike findings for Western diabetic subjects (23). Our current results further clarified that this effect of TGs was exclusively derived from its effect in women (23).

In our Japanese men with diabetes, non-HDLC and TC/HDLC, which are calculated from TC and HDLC, were the two best predictors of CHD and were superior to LDLC. These results confirmed the validity in Japanese diabetic men of the previously reported superiority of non-HDLC (9–11,13) or TC/HDLC (or non-HDLC/HDLC) (9,10,12,17,18) over LDLC as CHD predictors among Western diabetic populations. Also supported is that lipoproteins other than LDL, such as VLDL and chylomicron remnants, provide predictive power in addition to that of LDLC and could

Table 1—*Patient characteristics at baseline*

	Men			Women		
	No-CHD	CHD	*P*	No-CHD	CHD	*P*
n	870	70		786	45	
Age (years)	57.9 ± 7.1	60.0 ± 6.3	0.027	58.8 ± 6.8	59.9 ± 6.7	0.28
Diabetes duration (years)	11.4 ± 7.6	12.2 ± 7.7	0.35	10.2 ± 6.6	11.2 ± 4.9	0.053
BMI (kg/m^2)	22.8 ± 2.7	22.7 ± 2.4	0.90	23.2 ± 3.4	24.2 ± 3.1	0.060
	131 ± 16/	134 ± 16/		132 ± 17/	139 ± 15/	
Blood pressure (mmHg)	77 ± 10	79 ± 9	0.40/0.19	76 ± 10	78 ± 8	0.004/0.16
Fasting plasma glucose (mmol/L)	8.5 ± 2.6	8.4 ± 3.4	0.33	8.6 ± 2.8	9.2 ± 3.1	0.23
HbA_{1c} (%)	7.7 ± 1.2	8.0 ± 1.5	0.17	8.1 ± 1.4	8.2 ± 1.3	0.36
Serum lipid variables						
TC (mmol/L)	5.00 ± 0.89	5.37 ± 0.77	<0.001	5.38 ± 0.86	5.81 ± 0.93	0.004
HDLC (mmol/L)	1.36 ± 0.42	1.25 ± 0.38	0.008	1.49 ± 0.46	1.43 ± 0.49	0.29
TGs (mmol/L)*	1.19 (0.82)	1.35 (0.91)	0.076	1.10 (0.81)	1.45 (0.51)	<0.001
LDLC (mmol/L)	2.99 ± 0.84	3.40 ± 0.81	<0.001	3.31 ± 0.79	3.64 ± 0.79	0.014
Non-HDLC (mmol/L)	3.64 ± 0.92	4.12 ± 0.85	<0.001	3.88 ± 0.89	4.39 ± 0.97	0.002
TC/HDLC ratio	3.97 ± 1.30	4.63 ± 1.36	<0.001	3.89 ± 1.19	4.49 ± 1.59	0.023
LDLC/HDLC ratio	2.41 ± 1.07	2.96 ± 1.07	<0.001	2.43 ± 0.95	2.91 ± 1.34	0.056
Therapeutic measures						
Diabetes						
Diet only (%)	21	17	0.54	16	9	0.29
Insulin (%)	20	23	0.65	23	33	0.15
Sulfonylureas (%)	55	61	0.32	60	60	1.00
α-Glucosidase inhibitors (%)	21	21	0.88	20	20	1.00
Biguanides (%)	6	2	0.72	5	4	1.00
Insulin sensitizer (%)	2	1	1.00	2	9	0.014
Others						
Antihypertensive agents (%)	21	21	0.88	30	58	<0.001
Agents for dyslipidemia (%)	14	16	0.72	34	53	0.010
Diet						
Energy intake (kJ/day)*	1,776 (567)	1,703 (508)	0.82	1,597 (491)	1,568 (394)	0.94
Fat intake (g/day)*	53 (22)	53 (17)	0.45	50 (21)	49 (16)	0.94
Exercise (kJ/day)*	140 (302)	145 (264)	0.73	118 (229)	95 (254)	0.35
Current/past smoker (%)	44/39	54/36	0.20	9/6	7/5	1.00
Alcohol intake: never, three drinks or less, more than three drinks (%)**	40/48/12	45/46/9	0.61	87/13/0	87/13/0	1.00

Data are mean ± SD or *median (interquartile range). **One drink is equivalent to 12.6 g of ethanol based on the U.S. Department of Agriculture definition.

explain part of the residual cardiovascular risk characterized by the LDLC level alone (3,4). It also has been suggested that non-HDLC is superior as a predictor to LDL-C because non-HDLC is an indirect estimate of LDL particle number, and LDL particle number relates more closely to risk than LDL-C (6). Although studies have attempted to determine whether non-HDLC or TC/HDLC best identifies patients at greater risk of CHD, the statistical differences between the two were relatively small (10,12). For example, in the UK Prospective Diabetes Study (12), although TC/HDLC was a significantly stronger predictor of CHD than non-HDLC, HRs per one SD increment for those two variables were very close (1.36 and 1.35, respectively), and differences in results of ROC analysis were not clinically important, which was supported by the results of another study (10).

Although our results for men were quite close to those in Western studies that analyzed men and women together, our findings in female subjects differed from those findings or results in Japanese men with diabetes. Among our female subjects, TGs, TC, and non-HDLC were the best predictors of CHD risk as assessed by HRs for one SD increment, χ^2 statistics, or AUCs. However, tertile analysis indicated that TGs were the best variable examined, and that it was a significant predictor beginning at values as low as 0.90 mmol/L. That value was lower than reported in Western countries (14,17) but was close to the optimal upper limit in the newest U.S. guidelines (4).

Although the role of TGs in CHD is known to be influenced by ethnicity, especially in Asians (26), the specific reasons why TGs were a leading predictor of CHD in Japanese diabetic women but not in men have yet to be clarified. However, our results in women are similar to those in other studies of East Asian diabetic subjects (27–29), which showed that TGs had stronger associations with cardiovascular morbidity (27,29) and mortality (28) than LDLC, although these studies were either cross-sectional (27,29) or relatively small-scale and short-term (28). In particular, a cross-sectional study in Hong Kong (27) revealed that TGs were strongly associated with ischemic heart disease in women but not in men with type 2 diabetes. A meta-analysis of cohort studies in Asian-Pacific general populations also revealed that TGs were the best predictor of CHD death among single lipid variables, although

Table 2—*Multivariate-adjusted HRs per one SD increment with 95% CI, χ^2 (likelihood ratio test) statistics, and the AUC*

	Men			Women		
	HR (95% CI)	χ^2 (*P* value)	AUC (95% CI)	HR (95% CI)	χ^2 (*P* value)	AUC (95% CI)
TC	1.57 (1.25–1.99)	13.4 (<0.001)	0.697 (0.636–0.758)	1.58 (1.20–2.06)	9.6 (0.002)	0.721 (0.644–0.798)
LDLC	1.59 (1.28–1.98)	14.8 (<0.001)	0.694 (0.629–0.758)	1.41 (1.06–1.86)	5.3 (0.021)	0.705 (0.626–0.784)
HDLC	1.47 (1.09–1.98)	6.9 (0.009)	0.669 (0.604–0.734)	1.03 (0.72–1.48)	0.03 (0.85)	0.667 (0.577–0.756)
TGs (log-transformed)	1.42 (1.08–1.85)	6.4 (0.011)	0.664 (0.595–0.733)	1.72 (1.21–2.43)	9.2 (0.002)	0.708 (0.630–0.786)
Non-HDLC	1.78 (1.43–2.21)	22.0 (<0.001)	0.726 (0.664–0.787)	1.60 (1.21–2.12)	9.7 (0.002)	0.715 (0.634–0.796)
TC/HDLC ratio	1.63 (1.36–1.95)	19.7 (<0.001)	0.718 (0.656–0.780)	1.48 (1.11–1.95)	6.8 (0.009)	0.696 (0.609–0.782)
LDLC/HDLC ratio	1.52 (1.29–1.79)	16.1 (<0.001)	0.709 (0.646–0.772)	1.44 (1.09–1.91)	6.2 (0.013)	0.695 (0.608–0.781)
TG/HDLC ratio	1.49 (1.20–1.85)	10.4 (0.001)	0.680 (0.615–0.746)	1.36 (1.01–1.85)	3.4 (0.066)	0.683 (0.597–0.769)

Each lipid variable for CHD events at baseline adjusted by age, diabetes duration, BMI, systolic blood pressure, HbA_{1c}, smoking, and alcohol intake.

men and women were not separately analyzed (30). Interestingly, in our female subjects, TC was a better predictor than LDLC by all four analytical methods, suggesting that TLRs involving remnant or small, dense LDL strongly affect the etiology of CHD in this population.

It is well known that the serum level of TGs, which is closely associated with insulin resistance, is influenced by a number of metabolic factors, typically including glycemic and weight status. Insulin resistance is believed to contribute to the atherogenic dyslipidemia seen in diabetes by increasing the hepatic secretion of VLDL and other apoB-containing lipoprotein particles as a result of increased free fatty acid flux to the liver (31). This raises the long-standing debate as to whether the association of the TG level to CHD is a direct effect of the TRLs themselves or is a biomarker of accompanying disorders (32). Our results in stratified, multivariate-adjusted analysis suggested that at least the serum level of TGs is a significant and independent predictor in women whose HbA_{1c} or BMI was equal to or above the median. Although the precise mechanisms of these phenomena cannot be derived from epidemiological observations, improving glycemic and weight status could be beneficial to avoid the harmful influence of hypertriglyceridemia. Conversely, HDLC was not a significant predictor of CHD in women although it was moderately predictive in

Table 3—*HRs with 95% CIs for each lipid variable according to tertiles*

	Men			Women		
	Ranges	HR (95% CI)	*P*	Ranges	HR (95% CI)	*P*
TC (mmol/L)	4.63–5.40	1.81 (0.95–3.44)	0.069	5.02–5.69	1.23 (0.45–3.38)	0.687
	5.41–	2.98 (1.61–5.51)	0.001	5.70–	2.23 (0.90–5.56)	0.084
LDLC (mmol/L)	2.66–3.33	1.81 (0.93–3.52)	0.081	2.97–3.62	2.31 (0.82–6.54)	0.114
	3.34–	3.45 (1.83–6.48)	0.0001	3.63–	3.02 (1.12–8.12)	0.029
HDLC (mmol/L)	1.14–1.40	1.74 (0.82–3.67)	0.147	1.27–1.55	0.83 (0.38–1.84)	0.652
	–1.13	2.48 (1.23–5.00)	0.011	–1.26	1.31 (0.61–2.79)	0.487
TGs (mmol/L)	0.94–1.48	1.09 (0.55–2.13)	0.810	0.90–1.36	3.35 (1.21–9.23)	0.020
	1.49–	2.01 (1.07–3.78)	0.031	1.37–	4.31 (1.53–12.16)	0.006
Non-HDLC (mmol/L)	3.25–3.98	1.42 (0.70–2.86)	0.328	3.49–4.19	1.14 (0.44–2.94)	0.791
	3.99–	3.67 (1.97–6.83)	<0.0001	4.20–	2.02 (0.84–4.86)	0.118
TC/HDLC ratio	3.4–4.3	1.95 (0.91–4.19)	0.088	3.3–4.2	1.17 (0.50–2.73)	0.724
	4.4–	4.13 (2.05–8.33)	<0.0001	4.3–	1.50 (0.67–3.35)	0.329
LDLC/HDLC ratio	1.9–2.7	1.66 (0.78–3.53)	0.185	2.0–2.7	1.11 (0.48–2.58)	0.810
	2.8–	4.11 (2.09–8.08)	<0.0001	2.8–	1.57 (0.71–3.48)	0.265
TG/HDLC ratio	0.70–1.26	1.38 (0.66–2.90)	0.399	0.56–1.05	2.60 (1.04–6.46)	0.041
	1.27–	2.86 (1.44–5.69)	0.003	1.06–	3.27 (1.30–8.25)	0.012

HRs with 95% CIs for each lipid variable according to tertiles (HRs for the lowest tertile as a reference are shown except for HDLC where the top tertile is the reference) for CHD risk analyzed by Cox multivariate models adjusted by age, sex, diabetes duration, BMI, HbA_{1c}, systolic blood pressure, smoking status, and alcohol intake.

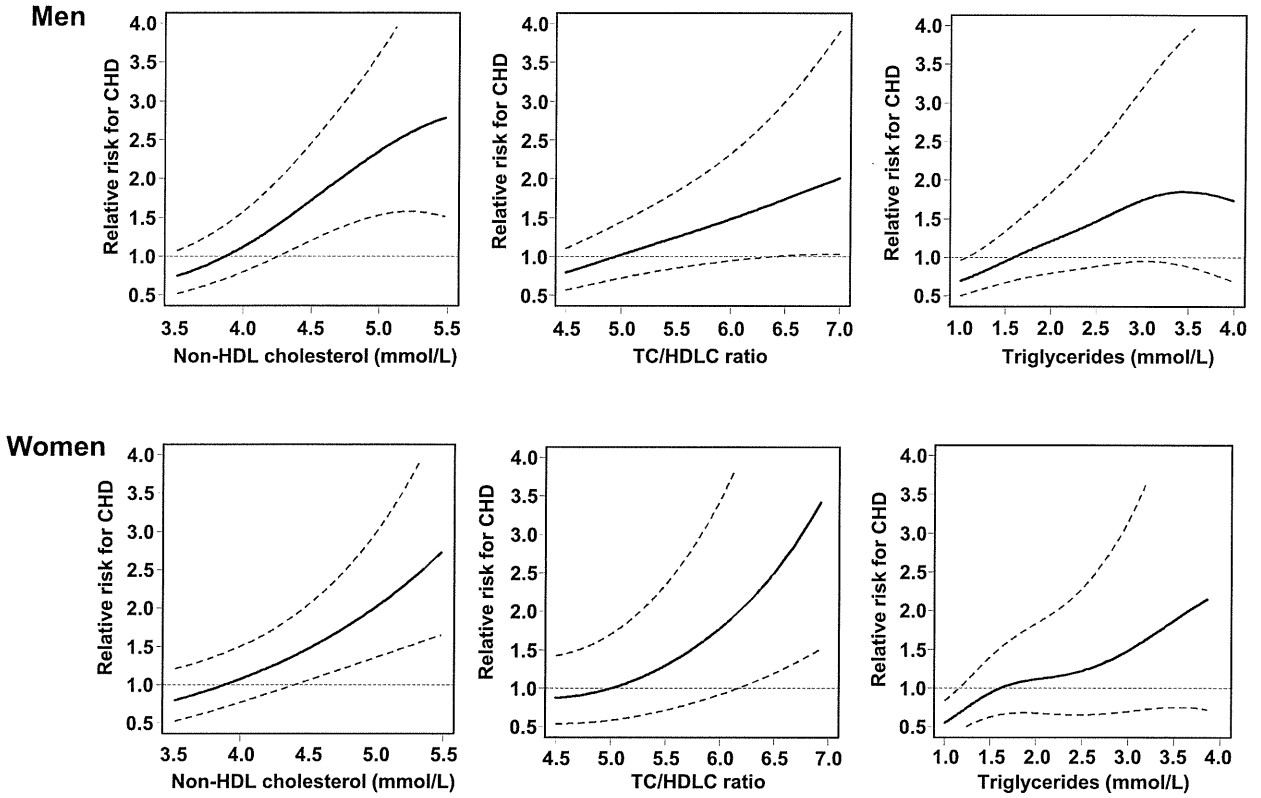

Figure 1—*Relative risk (solid line) and 95% CIs (broken line) of the incidence of CHD in relation to non-HDLC, TC/HDLC ratio, and TGs estimated by generalized additive models.*

men. The serum level of HDLC is naturally higher in East Asians than in Western populations, especially women (33,34), as in our cohort. Therefore, it is possible that the clinical impact of low HDLC was not apparent and, instead, that of TGs was enhanced in East Asians. Accordingly, TG/HDLC did not add useful information to that provided by TGs alone either in men or women. TG/HDLC was also reportedly not superior to non-HDLC in Spanish patients with type 2 diabetes (35).

This investigation has several strengths, including the nationwide sampling from nearly 60 institutes. We also used four different analytical methods and analyzed men and women separately, which was not done in past studies. Nevertheless, some limitations of our study deserve consideration. Variability in laboratory measurements could be present among participating hospitals (36). However, such an influence is virtually negligible because laboratory testing in Japan is well standardized. In fact, a nationwide precision control survey (37) demonstrated that coefficients of variation of tests of TC, HDLC, and TGs were <5%. Only baseline data were used for this analysis; therefore, therapeutic management during the follow-up period could have influenced results. Baseline proportions of women receiving therapy with insulin sensitizers or agents for hypertension or dyslipidemia were higher in the CHD group than in the no-CHD group, probably because of treatment selection bias. The large difference in the proportion of subjects taking agents for dyslipidemia (mainly statins) between men and women also might have influenced the results.

That we did not measure apolipoproteins in this study was another limitation. Although some studies of subjects with (14,15) and without (38,39) diabetes have provided relatively small support for replacement of conventional variables with measurements of apolipoproteins, recent meta-analysis (7) demonstrated that the use of apoB, a measure of the number of atherogenic lipid particles, could be more beneficial to prevent cardiovascular events than that of non-HDLC in clinical settings because there might be substantial discordance between apoB and non-HDLC levels depending on individual differences in composition of the apoB lipoproteins. In addition, apoB is a better predictor of cardiovascular risk especially when cholesterol-enriched remnants or cholesterol-enriched LDL is present; therefore, apoB is not necessarily interchangeable with non-HDLC for evaluation of individual patients in clinical settings (40). Finally, in this analysis, we did not use detailed dietary data, including data on saturated fat, carbohydrates, and the ratio of energy requirements to ingested calories, which could influence serum lipid profiles. This should be clarified in a future study.

In conclusion, the present analysis shows that for Japanese subjects with diabetes, non-HDLC and TC/HDLC for men and TGs for women were the best predictors of CHD. These findings should be considered in the clinical approach to risk reduction among East Asians with diabetes, and using these variables as management markers for dyslipidemia among this population has potential value.

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ORIGINAL

Ezetimibe, an inhibitor of Niemann-Pick C1-like 1 protein, decreases cholesteryl ester transfer protein in type 2 diabetes mellitus

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Abstract. To address the effects of ezetimibe on high-density lipoprotein (HDL) metabolism, the HDL subclasses, cholesteryl ester transfer protein (CETP), and lecithin-cholesterol acyltransferase (LCAT) were measured in patients with type 2 diabetes mellitus (T2DM). Twenty-three hypercholesterolemic patients with T2DM were treated with 10 mg of ezetimibe daily for 12 weeks. Plasma total cholesterol (TC), low-density lipoprotein (LDL)-cholesterol (C), HDL-C, HDL_2-C, HDL_3-C, CETP mass, and LCAT activity were measured. HDL-C and HDL_2-C increased by 5% ($p<0.05$) and 12% ($p<0.01$), respectively, in response to ezetimibe. Of the 23 patients, 21 had decreased CETP mass, which led to an average reduction of 20% ($p<0.0001$). LCAT activity also decreased by 6% ($p<0.01$). A significant positive correlation was found in the changes from baseline between HDL_2-C and CETP mass, whereas a significant inverse relationship was observed between HDL_3-C and CETP mass. Furthermore, the change in HDL-C was positively correlated with the change in LCAT activity. In conclusion, ezetimibe may affect HDL metabolism and reverse cholesterol transport, especially CETP, in T2DM. These observations may provide some insights into how ezetimibe prevents atherosclerosis.

Key words: Ezetimibe, Type 2 diabetes, High-density lipoproteins, Lecithin-cholesterol acyltransferase, Cholesteryl ester transfer protein

EZETIMIBE is a drug that lowers plasma low-density lipoprotein (LDL)-cholesterol (C) and non-high-density lipoprotein (HDL)-C by inhibiting intestinal cholesterol absorption [1, 2]. It has been proposed that ezetimibe inhibits Niemann-Pick C1-like 1 (NPC1L1) protein [3], which is highly expressed in the brush border membrane of the enterocyte, where it facilitates intestinal cholesterol absorption. Furthermore, humans, but not mice, also express NPC1L1 in the liver as much as in the small intestine [3, 4], suggesting the essential role of hepatic NPC1L1 in hepatic and plasma lipid metabolism. In fact, ezetimibe ameliorates non-alcoholic steatohepatitis in humans [5].

Elevated LDL-C and decreased HDL-C levels are conventional risk factors for cardiovascular disease in patients with type 2 diabetes mellitus (T2DM). Ezetimibe has been shown to be effective in reducing plasma LDL-C and non-HDL-C levels in hypercholesterolemic patients with T2DM [6-8]. In addition, a meta-analysis of randomized, controlled trials in which diabetic patients accounted for 2-8% of the patients also showed that ezetimibe monotherapy significantly increased plasma HDL-C levels by 3.0% in patients with primary hypercholesterolemia [9], though the precise mechanism has not yet been determined.

Lecithin-cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) are key enzymes in the reverse cholesterol transport system and are involved in HDL metabolism [10, 11]. LCAT converts free cholesterol into cholesteryl esters (CEs), which are then sequestered into the core of lipoprotein particles, making the spherical HDL_3 particles that are converted into HDL_2 particles by the phospholipid transfer protein (PLTP)-activated fusion of smaller

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HDL particles. CETP mediates neutral transport of CEs and triglycerides (TGs) between lipoproteins, resulting in the transfer of CEs in exchange for TGs from HDL to atherogenic lipoproteins such as LDL and very low-density lipoprotein (VLDL). TGs accumulating in HDL are then hydrolyzed by hepatic lipase (HL). Moreover, CETP transfers CEs between HDL particles and generates HDL_2 and pre-β HDL from HDL_3 particles.

We recently reported that pitavastatin decreases CETP mass and LCAT activity in hypercholesterolemic patients [12]. Furthermore, genome-wide association studies involving >100,000 individuals of European ancestry identified LCAT and CETP as significantly associated loci with plasma HDL-C [13]. In the present study, the effects of ezetimibe on the HDL subclasses, CETP mass, and LCAT activity were assessed in T2DM, and their relationships were examined.

Subjects and Methods

Hypercholesterolemic patients with T2DM (n = 23; men/women = 13/10, age = 59.1 ± 9.5 years) were enrolled in the study (Table 1). They received sulfonylureas (n=11), glinides (n=3), pioglitazone (n=3), biguanides (n=6), α-glucosidase inhibitors (n=2), and dipeptidyl peptidase-4 inhibitors (n=1). According to the plasma LDL-C levels as recommended by the guidelines of the Japan Atherosclerosis Society [14], patients with plasma LDL-C >120 mg/dL were the subjects of this study. None of the patients were taking any lipid-lowering drugs such as statins and fibrates. The patients were given 10 mg of ezetimibe daily for 12 weeks, while other drugs for other diseases, such as diabetes and hypertension, were left unchanged. The study was approved by the Ethics Committee of Jichi Medical University, and all patients gave their written, informed consent.

At baseline and after 12 weeks of treatment, blood samples were collected after a 12-h fasting period, and the following parameters were determined in plasma: total cholesterol (TC), TGs, HDL-C, HDL_2-C, HDL_3-C, LCAT activity, CETP mass, sitosterol, campesterol, cholestanol, lathosterol, high-sensitivity C-reactive protein (hs-CRP), glucose, and hemoglobin A1c (HbA1c).

Plasma TC, TGs, and HDL-C were measured by automated enzymatic assays. LDL-C levels were calculated by the Friedewald formula, since all patients had TG levels <400 mg/dL. HDL_2 and HDL_3 were isolated by density gradient ultracentrifugation, and the cholesterol levels in these lipoproteins were measured enzymatically. CETP mass was measured by ELISA assay (CETP ELIZA-DAIICHI, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). LCAT activity was measured using dipalmitoyl lecithin as the substrate [15]. Sitosterol, campesterol, cholestanol, and lathosterol were measured by gas-liquid chromatography [16]. The hs-CRP level was determined using an

Table 1 Patients' characteristic at baseline and after 12 weeks of ezetimibe treatment

parameter	at baseline	after 12 weeks
Sex (Men/women)	10/13	-
Age	59.1 ± 9.5	-
TC (mg/dL)	232.7 ± 33.9	201.6 ± 28.1***
TG (mg/dL)	127.8 ± 54.3	118.65 ± 67.3
LDL-C (mg/dL)	152.8 ± 26.8	121.0 ± 20.0***
HDL-C (mg/dL)	53.7 ± 13.4	56.4 ± 17.1*
HDL_2-C (mg/dL)	33.1 ± 11.0	37.1 ± 14.4**
HDL_3-C (mg/dL)	19.8 ± 4.1	20.0 ± 3.3
LCAT (U)	116.3 ± 15.5	108.8 ± 14.0**
CETP (μg/mL)	2.5 ± 0.5	2.0 ± 0.5***
Sitosterol (μg/mL)	3.9 ± 1.5	2.0 ± 0.8***
Campesterol (μg/mL)	6.8 ± 2.8	3.2 ± 1.3***
Cholestanol (μg/mL)	3.0 ± 0.7	2.7 ± 0.5*
Lathosterol (μg/mL)	4.4 ± 2.4	4.9 ± 2.1
Glucose (mg/mL)	128.0 ± 27.4	136.6 ± 35.4
HbA1c (%)	7.1 ± 0.8	7.2 ± 0.9
Hs-CRP (ng/mL)	100.0 ± 277.8	58.4 ± 99.2

Results were expressed as mean ± S.D. ***$p < 0.0001$, *$p < 0.05$, **$p < 0.01$ vs. baseline

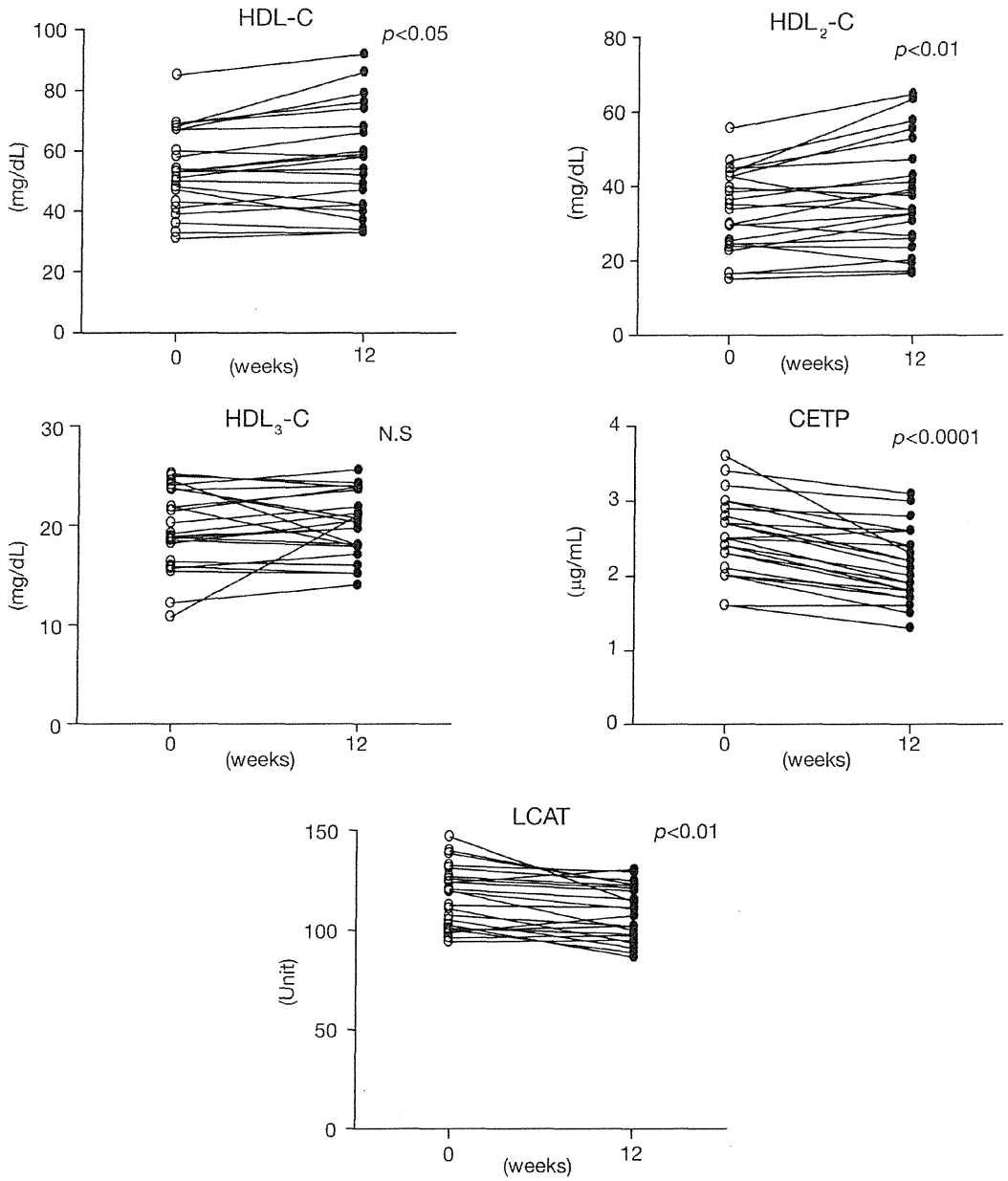

Fig. 1 HDL subclasses, CETP mass, and LCAT activity at baseline and at 12 weeks of ezetimibe treatment. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. Plasma HDL-C, HDL_2-C, HDL_3-C, CETP mass, and LCAT activity were measured and compared before and after treatment. Data are expressed as means ± S.D. N.S: not significant.

ultra-high-sensitivity latex turbidimetric immunoassay (Bering Nephelometry, Tokyo, Japan).

Student's paired *t*-test was used for statistical analyses. Univariate Pearson's correlation coefficient analysis was performed to estimate the relationship between two variables. A p value of <0.05 was considered significant.

Results

Plasma TC and LDL-C levels decreased by 13% ($p<0.0001$) and 21% ($p<0.0001$), respectively, when patients were treated with ezetimibe for 12 weeks (Table 1, Fig. 1). Ezetimibe did not affect TG levels. HDL-C and HDL_2-C levels were marginally but significantly

increased after treatment. The cholesterol absorption markers, sitosterol, campesterol, and cholestanol, were significantly decreased by 49%, 53%, and 10%, respectively (Table 1). In contrast, no significant change was observed in lathosterol, a marker of cholesterol synthesis. There was no significant relationship in the changes from baseline between each cholesterol synthesis or absorption marker and LDL-C levels. The changes in HDL-C and HDL_2-C also did not have a significant relationship with the changes in any cholesterol absorption markers. The change in HDL_3-C had a significant positive correlation with the change in campesterol ($p<0.01$), but not sitosterol and cholestanol (data not shown).

After 12 weeks of treatment, 21 of the 23 patients had decreased CETP mass, which led to an average reduction of 20% ($p<0.0001$) (Table 1, Fig. 1). A significant positive correlation was found in the changes from baseline between HDL_2-C levels and CETP mass, whereas a significant inverse relationship was observed in the changes from baseline between HDL_3-C levels and CETP mass (Fig. 2). The change in HDL-C levels was not associated with that in CETP mass. LCAT activity was also decreased by 6% ($p<0.01$) with ezetimibe (Table 1, Fig. 1). A significant positive correlation was observed in the change from baseline between LCAT activity and HDL-C, but not HDL_2-C or HDL_3-C levels (Fig. 3). The change in LDL-C was not associated with the change in CETP mass or LCAT activity (data not shown).

Plasma hs-CRP, glucose, and HbA1c levels were not affected by ezetimibe therapy.

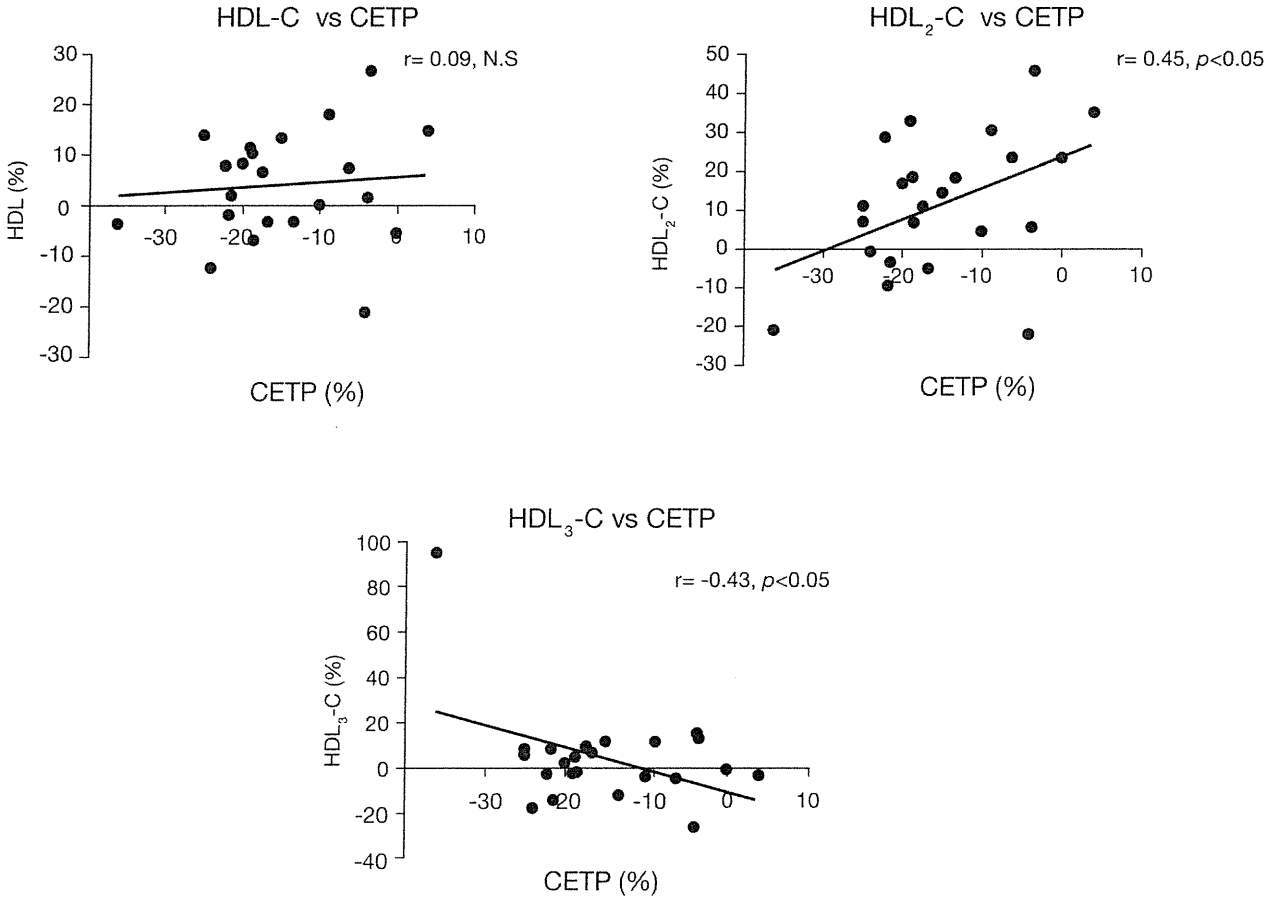

Fig. 2 Relationships between HDL subclasses and CETP mass. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. The percent changes in HDL subclasses and CETP mass in response to ezetimibe treatment were calculated, and their relationships were examined. Data are expressed as means ± S.D. N.S: not significant.

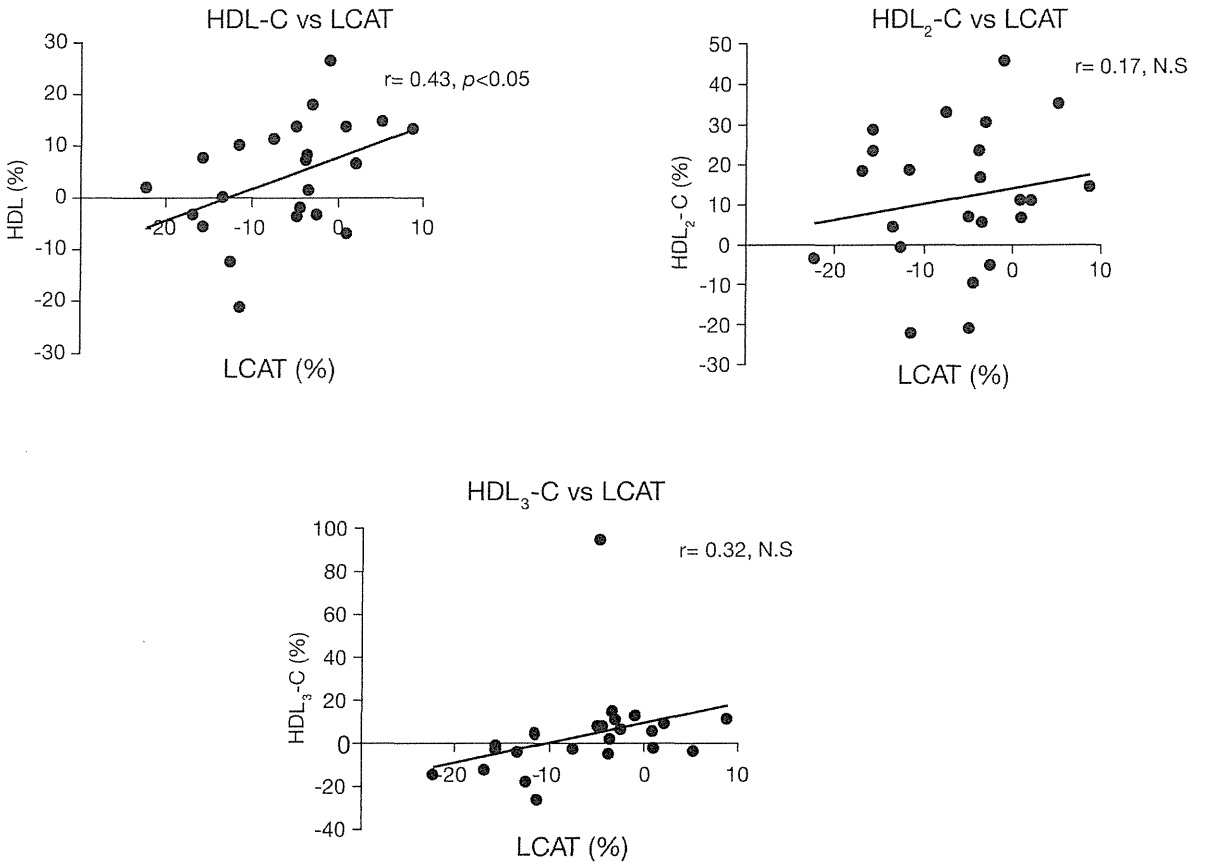

Fig. 3 Relationships between HDL subclasses and LCAT activity. Ezetimibe (10 mg/daily) was given for 12 weeks, and blood was collected before and after ezetimibe treatment. The percent changes in HDL subclasses and LCAT activity in response to ezetimibe treatment were calculated, and their relationships were compared. Data are expressed as means ± S.D. N.S: not significant.

Discussion

This is the first study to demonstrate the effects of ezetimibe on HDL subclasses, CETP mass, and LCAT activity in hypercholesterolemic patients with T2DM. Because these patients had not used any other drugs for hyperlipidemia, the observations in the present study simply reflect the effects of ezetimibe.

As expected, ezetimibe significantly reduced plasma TC and LDL-C levels. Markers for cholesterol absorption were also significantly decreased, but lathosterol, a marker of cholesterol synthesis, was not affected by ezetimibe therapy. In contrast to a study in which there was a positive correlation in the changes between a cholesterol absorption/synthesis marker and LDL-C in response to ezetimibe treatment [7], such a correlation was not observed in the present study. The reason for this discrepancy is unclear, but the results of the present study imply that the LDL-lowering effect of ezetimibe is not simply determined by the prevention of cholesterol absorption in T2DM. The reduction of CETP and LCAT by ezetimibe may also affect LDL metabolism, though a significant correlation was not found in the changes from baseline between CETP mass or LCAT activity and LDL-C.

In the present study, it was demonstrated that HDL-C and HDL_2-C, but not HDL_3-C, were significantly increased at 12 weeks of ezetimibe administration. Because HDL-C is 10-20% lower in T2DM [17], which is mainly the result of a decrease in HDL_2 and to some extent in HDL_3, ezetimibe is the drug to improve the decreased HDL levels in T2DM. Why ezetimibe modulates HDL metabolism remains unclear. It has been reported that plasma HDL-C levels are positively correlated with markers of cholesterol absorption in metabolic syndrome cases and healthy individuals [18, 19]. In the present study, the change in HDL_3-C was significantly associated with the change in campesterol.

However, the change in HDL_3-C was not associated with the changes in other cholesterol absorption markers such as sitosterol and cholestanol. Furthermore, the changes in HDL-C and HDL_2-C were not associated with changes in any cholesterol synthesis markers. Taken together with the observation that the changes in LDL-C was not associated with the changes in any cholesterol absorption markers, ezetimibe may be involved in plasma lipoprotein metabolism by means of not only intestinal but also hepatic lipid metabolism, because humans express NPC1L1, a target of ezetimibe, in the liver as much as in the small intestine.

Interestingly, the change in HDL_2-C levels was positively correlated with that in plasma CETP mass, whereas an inverse relationship was observed in the change between HDL_3-C levels and CETP mass (Fig. 2). Moreover, the change in HDL-C was positively correlated with the change in LCAT activity (Fig. 3). With regard to the relationship between CETP and HDL_2-C or HDL_3-C in the present study, these observations are contrary to the function of CETP, in which inhibition of CETP causes an imbalance between PLTP and CETP and results in the generation of larger HDL particles such as HDL_2, whereas smaller HDL particles such as HDL_3 are diminished [10]. Thus, these observations must be interpreted carefully. Indeed, the significant relationships in the changes between CETP and HDL_2-C or HDL_3-C disappeared after exclusion of one patient who had extremely high HDL_3-C elevations with ezetimibe. Moreover, in addition to HDL-C, the change in HDL_3-C came to have a significant positive correlation with the change in LCAT activity ($p<0.01$) after exclusion of the patient who had extremely high HDL_3-C elevations with ezetimibe. Because many other proteins, e.g., HL and PLTP, are also involved in reverse cholesterol transport and HDL metabolism [11, 17], ezetimibe may affect these proteins, which acted in concert in HDL_2 and HDL_3 remodeling in the present study.

It should be noted that 91% of the patients had decreased CETP mass by an average of 20% in response to ezetimibe. This may simply reflect the effects of lipid-lowering drugs, because statins also decrease CETP mass in hypercholesterolemic patients [12]. Alternatively, ezetimibe may regulate the CETP gene at transcriptional levels. The CETP gene has sterol regulatory elements (SREs) in the promoter where sterol regulatory element-binding proteins (SREBPs) bind and then activate the CETP gene [20, 21]. The CETP gene has been shown to be activated by SREBP-1a rather than SREBP-2 in the liver and human liposarcoma cells, an adipocytic cell line [20, 21]. Furthermore, ezetimibe upregulates hepatic SREBP-2 and downregulates hepatic SREBP-1c in wild-type mice fed a high-fat diet for 10 weeks [22]. Taken together, this suggests that ezetimibe suppresses CETP gene expression by modulating hepatic SREBPs. The mechanism by which ezetimibe decreased plasma LCAT activity in the present study is still unknown. This is simply secondary to the reduction of CETP by ezetimibe. Alternatively, ezetimibe may affect the gene expression levels of hepatic LCAT. Indeed, fibrates, which are drugs for the treatment of hyperlipidemia, have been shown to decrease hepatic LCAT mRNA at the transcriptional level, thereby reducing plasma LCAT activity [23]. Further study will be needed to determine whether ezetimibe decreases hepatic LCAT mRNA.

The role of ezetimibe in the prevention of atherosclerosis is still a matter of debate [24-26]. In the SEAS and ENHANCE trials, ezetimibe did not reduce the composite outcome of combined aortic valve events and ischemic events or carotid-artery intima-media thickness in patients with familial hypercholesterolemia [25, 26]. In contrast, reduction of plasma LDL-C levels by the combination of simvastatin and ezetimibe decreased the incidence of major atherosclerotic events in patients with advanced chronic disease in the SHARP trial [24]. Taken together with the fact that a CETP inhibitor has attracted attention as an anti-atherosclerotic agent [27, 28], ezetimibe may suppress atherosclerosis by means of its inhibitory effect on CETP.

In conclusion, ezetimibe modulates HDL metabolism and reverse cholesterol transport, especially CETP, in T2DM. These effects of ezetimibe, beyond the well-known effects on LDL, may provide some insights into how ezetimibe prevents atherosclerosis in humans.

Conflict of Interest

The authors have no further conflicts of interest to disclose.

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