Table 1
Effects of n-3 PUFA oil on plasma lipid profile, body weight gain and food intake in high fat diet-fed hamsters.

	Normal	2%		4%	
		Control	n-3 PUFA	Control	n-3 PUFA
TG (mg/dL)					
Day 7	269 ± 17	856 ± 76	536 ± 113*	932 ± 179	482 ± 159
Day 14	226 ± 43	1469 ± 224	664 ± 171*	2056 ± 191	539 ± 106**
Day 21	148 ± 22	1152 ± 225	818 ± 178	1651 ± 154	612 ± 187**
TC (mg/dL)					
Day 7	176 ± 8	294 ± 12	335 ± 29	290 ± 25	285 ± 50
Day 14	149 ± 9	344 ± 32	380 ± 40	358 ± 30	330 ± 35
Day 21	128 ± 7	221 ± 21	265 ± 20	208 ± 9	252 ± 18*
Non-HDL-C (mg/dL)					
Day 7	137 ± 7	221 ± 12	284 ± 28	214 ± 25	241 ± 48
Day 14	99 ± 8	251 ± 34	305 ± 40	261 ± 32	259 ± 34
Day 21	84 ± 5	149 ± 18	215 ± 19*	129 ± 8	201 ± 19**
HDL-C (mg/dL)					
Day 7	39 ± 2	74 ± 1	50 ± 1**	76 ± 3	43 ± 3**
Day 14	51 ± 4	93 ± 3	75 ± 2**	97 ± 4	71 ± 3**
Day 21	43 ± 2	72 ± 4	50 ± 3**	78 ± 3	51 ± 2**
Body weight gain (g)					
Day 21–Day 0	23.8 ± 3.0	31.4 ± 2.9	10.5 ± 3.3**	30.1 ± 2.0	12.3 ± 4.8**
Food intake					
(kcal/body weight/day)	0.23 ± 0.02	0.25 ± 0.01	0.18 ± 0.03	0.25 ± 0.00	0.23 ± 0.02

Plasma was prepared from hamsters fed a chow diet (normal) or a high fat diet supplemented with 2% or 4% (w/w) control (OA-E) or n-3 PUFA at day 7, 14 and 21. Body weight gain was determined by calculating body weight at day 21 – day 0. Food intake was determined as the mean of three administrations. Values are expressed as mean ± S.E.M. of individual samples (n=6–8).

* $P < 0.05$.

** $P < 0.01$ vs. control group.

3.3. Effects of EPA or DHA on plasma LDL-C

We examined the effects of EPA and DHA, the major components of n-3 PUFA, on plasma LDL-C. DHA at every concentration tested increased plasma LDL-C from day 7, with statistically significant differences observed on days 7 and 21 ($P < 0.05$ or $P < 0.01$). The maximum increase in plasma LDL-C was obtained with 2% (w/w) DHA. In contrast, EPA had neutral effects on plasma LDL-C at all concentrations compared with control (Fig. 1C–E).

3.4. Effects of EPA or DHA on plasma TG, TC, non-HDL-C and HDL-C

In contrast to DHA, EPA (1% and 2% (w/w)) decreased plasma TG level. All concentrations of DHA tested, but none of EPA, resulted in a significant increase in TC and non-HDL-C at day 21 ($P < 0.05$ or $P < 0.01$). Both EPA and DHA decreased plasma HDL-C (Table 2).

3.5. Effects of n-3 PUFA, EPA or DHA on body weight gain and food intake

Body weight gain was suppressed by n-3 PUFA oil and EPA, and partially by DHA; however, there was no significant difference in food intake compared with control (Tables 1 and 2).

3.6. Effects of EPA or DHA on plasma CETP activity

Plasma CETP activity in DHA-treated hamsters was higher than that of control 2% (w/w) oil-treated hamsters on days 7, 14 and 21, while in EPA-treated hamsters, CETP activity was lower; there were no statistical differences (Fig. 2A). A significant positive correlation between CETP activity and LDL-C in 2% (w/w) oil-treated hamsters was found ($P < 0.01$; Fig. 2B). DHA treatment resulted in a significant increase in CETP gene expression in white adipose tissue ($P < 0.05$), although an increase was also seen in the EPA group, albeit to a lesser extent (Fig. 2C).

3.7. Effects of EPA or DHA on the LDL-R and lipogenic enzyme expression in liver

DHA treatment, but not EPA, resulted in a significant decrease in LDL-R protein and mRNA expression ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2D–F). SREBP-2 gene expression was also significantly lowered by DHA ($P < 0.01$), but not by EPA (Fig. 2G). In contrast, both EPA and DHA treatment significantly decreased SREBP-1 and FAS mRNA levels to a similar extent ($P < 0.01$; Fig. 2G), along with mRNA levels of SCD1 and ACC to a similar extent (data not shown).

3.8. Effects of EPA or DHA on LDL-R and LDL uptake in HepG2 cells

Both EPA and DHA decreased LDL-R gene expression in HepG2 cells (Fig. 3A). In contrast, VLDL prepared from DHA-treated hamsters (DHA-VLDL), but not VLDL prepared from EPA-treated hamsters (EPA-VLDL), significantly decreased LDL-R gene expression ($P < 0.05$; Fig. 3B) and LDL uptake ($P < 0.05$; Fig. 3B).

4. Discussion

Although the LDL-C increasing effect of n-3 PUFA oil has already been reported, which fatty acid components of n-3 PUFA and what mechanisms contribute to this increase are unclear. This study attempted to clarify the above by investigating the effects of n-3 PUFA oil and its major components, EPA and DHA, on plasma LDL-C level in high fat diet-fed hamsters with different amounts and durations. Overall, the LDL-C increasing-effect of n-3 PUFA oil was found to be due to DHA alone, with no apparent contribution from EPA. In this study, n-3 PUFA oil, EPA and

Table 2
Effects of EPA-E or DHA-E on plasma lipid profile, body weight gain and food intake in high fat diet-fed hamsters.

	0.5%			1%			2%		
	Control	EPA	DHA	Control	EPA	DHA	Control	EPA	DHA
TG (mg/dL)									
Day 7	909 ± 135	926 ± 350	1482 ± 192*	1303 ± 502	799 ± 117	1644 ± 332	997 ± 77	836 ± 242	1714 ± 284
Day 14	1658 ± 283	1254 ± 399	1920 ± 506	1273 ± 302	333 ± 49**	1536 ± 329	1827 ± 372	551 ± 138*	3726 ± 841
Day 21	900 ± 125	1158 ± 459	1911 ± 530	1087 ± 172	398 ± 50**	1171 ± 237	2398 ± 1103	881 ± 236	5578 ± 1235*
TC (mg/dL)									
Day 7	329 ± 20	356 ± 62	533 ± 51**	398 ± 86	366 ± 28	531 ± 72	339 ± 23	361 ± 51	628 ± 24**
Day 14	473 ± 39	469 ± 53	614 ± 78	400 ± 53	306 ± 28	565 ± 64	419 ± 52	299 ± 33	825 ± 109**
Day 21	355 ± 22	460 ± 49	685 ± 88**	323 ± 21	299 ± 11	493 ± 35**	440 ± 111	347 ± 24	1025 ± 153*
Non-HDL-C (mg/dL)									
Day 7	217 ± 23	275 ± 59	450 ± 48**	307 ± 88	292 ± 29	471 ± 72	266 ± 22	307 ± 50	560 ± 22**
Day 14	385 ± 41	389 ± 59	540 ± 79	313 ± 55	232 ± 25	499 ± 64	350 ± 53	244 ± 32	762 ± 104**
Day 21	259 ± 23	372 ± 52	604 ± 91**	216 ± 23	212 ± 11	416 ± 36**	377 ± 109	294 ± 24	960 ± 151*
HDL-C (mg/dL)									
Day 7	112 ± 6	81 ± 7**	83 ± 3**	91 ± 4	74 ± 5**	60 ± 3**	72 ± 3	54 ± 3**	68 ± 7
Day 14	88 ± 4	80 ± 7	74 ± 4	87 ± 4	74 ± 3*	65 ± 3**	69 ± 2	55 ± 2**	64 ± 5
Day 21	96 ± 3	88 ± 5	81 ± 3*	107 ± 3	87 ± 3**	77 ± 4**	63 ± 3	54 ± 1	65 ± 3
Body weight gain (g)									
Day 21– Day 0	18.3 ± 1.5	7.7 ± 1.6**	10.6 ± 2.3*	19.5 ± 1.9	3.7 ± 1.8**	13.1 ± 2.2	25.1 ± 2.7	9.7 ± 2.9**	28.2 ± 2.5
Food intake									
(kcal/body weight/day)	0.24 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.22 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.25 ± 0.01	0.28 ± 0.01

Plasma was prepared from hamsters fed a high fat diet supplemented with 0.5%, 1% or 2% (w/w) control (OA-E), EPA-E or DHA-E at day 7, 14 and 21. Body weight gain was determined by calculating body weight at day 21 – day 0. Food intake was determined as the mean of three administrations. Values are expressed as mean ± S.E.M. of individual samples (n=7–10). *$P < 0.05$, **$P < 0.01$ vs. control group.

* $P < 0.05$.
** $P < 0.01$ vs. control group.

DHA suppressed body weight gain; however, the suppressive effect was more potent with EPA and relatively mild with DHA (Supplementary Fig. 1). Therefore one may consider that EPA does not increase LDL-C level because it is less affected by a high fat diet. However, the amount of food intake was not significantly different in the EPA-treated group compared with control and DHA-treated groups. Rather, EPA might have stronger body weight reduction than DHA possibly through increased energy expenditure. Considering that the data from n-3 PUFA containing EPA are consistent with a previous report [15], it could be concluded that the distinct effects of EPA and DHA observed here are basically due to their direct metabolic consequences.

Particularly in severe hypertriglyceridemia (500–2000 mg/dL under the fasting state), a notable increase in LDL-C caused by n-3 PUFA has been described [7]. Hamsters are known to possess LDL metabolism that is more readily comparable to that of humans than to rats and mice, and display hypertriglyceridemia and hypercholesterolemia when fed a high fat diet [12]. Therefore, we studied the effects of EPA and DHA on plasma LDL-C level using high fat diet-fed hamsters. In this study, administration of n-3 PUFA oil to hamsters was found to decrease plasma TG and to increase LDL-C, which is consistent with previous clinical studies [4–6]. HPLC analysis revealed that n-3 PUFA increased cholesterol levels in all LDL fractions, including small particles that have been suggested to be highly atherogenic [13]. Highly purified EPA and DHA were separately administered to high fat diet-fed hamsters in order to investigate the individual contributions of EPA and DHA on the LDL-C increasing-effect of n-3 PUFA oil. EPA had no effect on plasma LDL-C level, whereas a marked increase in LDL-C was seen with DHA alone. In one clinical study that administered fish oil, blood LDL-C level positively correlated with blood DHA level, but not with blood EPA level [16]. In addition, clinical studies from Mori et al. [17] and Geppert et al. [18] reported that highly purified DHA and DHA-rich oil increased LDL-C level. The effects of n-3 PUFA oil and highly purified DHA and EPA alone on plasma LDL-C level have not been studied simultaneously under the same experimental conditions; however, the results obtained in this study suggest that DHA is the primary contributor to the n-3 PUFA oil-induced increase in LDL-C in high fat diet-fed hamsters, whereas EPA contributes little or nothing to this increase.

CETP is known to be an important protein involved in lipid transfer between VLDL or LDL, and HDL, and CETP inhibitors have been reported to reduce LDL-C level [19]. We focused on CETP based on a report which showed that LDL-C level increased in fish oil-fed hamsters and monkeys [20,21], which have a CETP similar to humans, but not in rats and mice [20,22], which have little CETP. Importantly, we found a significant positive correlation between plasma CETP activity and LDL-C level. CETP activity therefore appears to be involved in the regulation of LDL-C level in this hamster model. Since CETP expression in adipose tissue is reported to be associated with blood CETP concentration [23], CETP gene expression in white adipose tissue was also examined. Hepatic CETP mRNA was not able to be detected in the present study. It has been reported that there is very little hepatic CETP expression in hamsters [24]. CETP activity in DHA-treated hamsters was higher than that of the control group and CETP mRNA expression in white adipose tissue was significantly elevated, whereas CETP activity in the EPA-treated group was lower than that of the control group. These results suggest that increased plasma CETP activity may be one of the factors that contributes to the DHA-induced increase in LDL-C level. Although CETP activity in the EPA-treated group decreased compared to control animals, mRNA expression in white adipose tissue tended to increase. The reason for this discrepancy is unknown. n-3 PUFA oil, EPA and DHA decreased plasma HDL-C in high fat diet fed hamsters. Small scale clinical and animal studies suggested that n-3 PUFA slightly decreased HDL-C [17,25], and it may be considered that CETP is, at least in part, associated with HDL-C regulation of DHA.

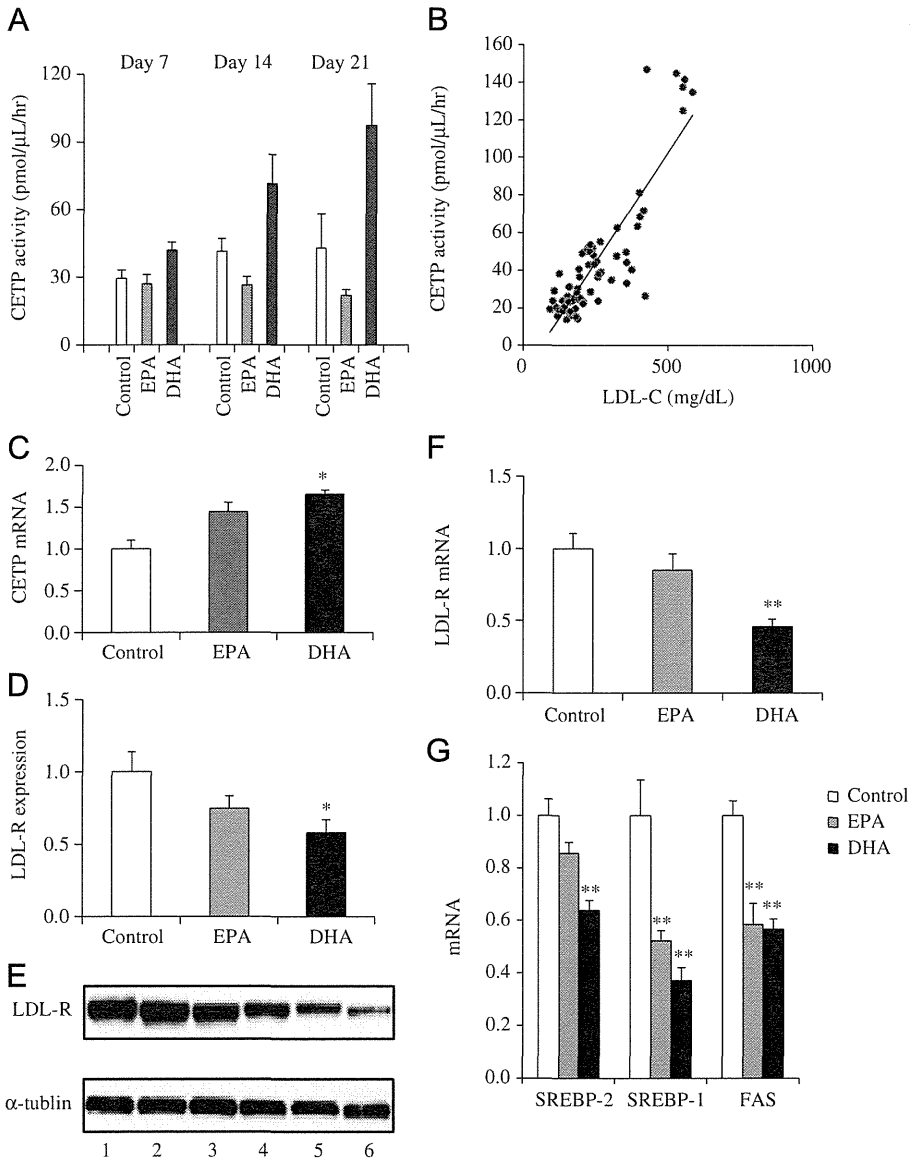

Fig. 2. Effects of EPA-E or DHA-E on CETP and hepatic LDL-R in high fat diet-fed hamsters. (A) Plasma was prepared from hamsters fed a high fat diet supplemented with 2% (w/w) control (OA-E), EPA-E or DHA-E at day 7, 14 and 21 and CETP activity was determined. Values are expressed as mean ± S.E.M. of individual samples (n=7–8). (B) Spearman's correlation test demonstrates a positive correlation between plasma CETP activity and LDL cholesterol at day 7, 14 and 21 in hamsters fed a high fat diet supplemented with 2% (w/w) fatty acids (r=0.86, $P<0.01$). (C) After 21 days of administration of each fatty acid (2% (w/w)), epididymal adipose CETP mRNA level was determined. Values, compared to the average control values, are expressed as mean ± S.E.M. of duplicate samples (n=7–8). After 21 days of fatty acid administration, LDL-R protein expression (D,E), LDL-R mRNA (F), SREBP-2, SREBP-1 and FAS mRNA (G) from the liver were determined. Representative western blots showing LDL-R and α-tublin expressions are shown (E). 1–2: Control, 3–4: EPA, 5–6: DHA. Values, compared to the average control values, are expressed as mean ± S.E.M. of individual samples (n=7–8). $^{*}P<0.05$, $^{**}P<0.01$ vs. control group.

The hepatic LDL-R is one of the most important factors that regulate blood LDL-C level. In hamsters fed with a high fat diet, administration of DHA significantly reduced expression of hepatic LDL-R mRNA and protein. DHA also significantly inhibited mRNA expression of SREBP-2, a transcription factor that regulates the LDL-R, whereas EPA had no effect. Reduced clearance via the LDL-R has been suggested as one mechanism for the LDL-C increasing effect of n-3 PUFA [25]. The differential effects of EPA and DHA on LDL-R expression observed in this study suggest the involvement of DHA-induced inhibition of the LDL-R in the n-3 PUFA oil-induced increase in LDL-C level. On the other hand, neither EPA nor DHA significantly affected NPC1L1 (Niemann-pick C1 like 1 protein), ABCG5, ABCG8 and ABCA1 mRNA in the small intestine, which are related to cholesterol absorption/excretion (Supplementary Fig. 2). Previous report suggested that n-3 PUFA had no effect on intestinal absorption of cholesterol [26].

EPA and DHA physiological activities are well known to inhibit lipogenesis in the liver. Gene expression of SREBP-1 and lipogenic enzymes such as FAS, ACC and SCD1 were similarly reduced by EPA and DHA. There is a report that SREBP-2 is more specific to cholesterogenic gene expression whereas SREBP-1 targets lipogenic genes [27]. According to the preferential suppression for SREBP-2 expression by DHA as seen in this study, it is reasonable that DHA, not EPA, has inhibitory effect on the LDL-R, one of target genes of SREBP-2, despite a comparable suppression of lipogenic genes. Suppressive effect of EPA on SREBP-1, not SREBP-2, is also consistent with a previous study [28].

Addition of EPA or DHA to HepG2 cells inhibited LDL-R mRNA expression, which is consistent with a previous report [29]. The discrepancy between EPA and DHA in the effects on the LDL-R therefore cannot be simply explained by the effects of the fatty acids on hepatocytes. Olano-Martin et al. [30] suggested

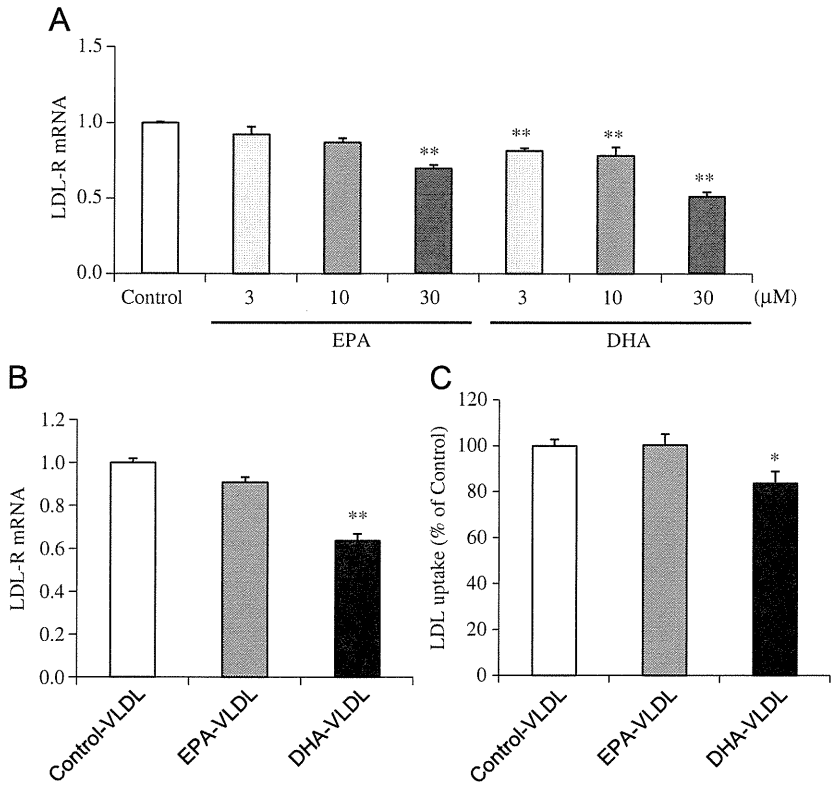

Fig. 3. Effects of EPA and DHA on LDL-R gene expression and LDL uptake in HepG2 cells. (A) HepG2 cells were incubated overnight with EPA-Na or DHA-Na (3–30 μM). (B, C) VLDLs were prepared from each group of fatty acid-treated hamsters. Cells were incubated overnight with control (OA)-VLDL, EPA-VLDL or DHA-VLDL (10 μg protein/mL) plus purified LPL (0.5 U/mL). (A, B) LDL-R mRNA and (C) LDL uptake in HepG2 cells, compared to average control values, are expressed as mean ± S.E.M. of individual samples of 3–4 wells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

that DHA-rich oil increased LDL-C level in apoE4 carriers because DHA-containing VLDL is a more potent competitor for the hepatic LDL-R compared to control-VLDL or EPA-containing VLDL. In this study, addition of VLDL, obtained from DHA-treated hamsters, to HepG2 cells significantly inhibited mRNA expression of the LDL-R and cellular LDL intake, while VLDL, obtained from EPA-treated hamsters, showed no such inhibition. These results suggest that DHA-induced inhibition of LDL-R expression in high fat diet-fed hamsters may not be due to the DHA fatty acid itself, but rather due to the activity of VLDL-containing DHA on the liver. n-3 PUFA oil has been reported to markedly increase LDL-C level in patients with hypertriglyceridemia [7]. Exposure of HepG2 cells to VLDL is thought to mimic the hypertriglyceridemia in high fat diet-fed hamsters in this study. The addition of VLDL to cultured hepatocytes has been reported to result in accumulation of TG in the cells [31], and the differential effects of EPA and DHA may therefore become obvious under these conditions.

DHA was found to increase plasma TG in this study, which is inconsistent with results obtained in several clinical studies. The reason for this is unknown. There appears to be no link between the increase in plasma TG and LDL-C levels since administration of n-3 PUFA oil was found to simultaneously reduce plasma TG level and increase LDL-C level. This is also supported by the fact that DHA increased LDL-C level even though it did not significantly increase plasma TG level (e.g. 1% (w/w) DHA).

Further study is needed to elucidate the precise mechanisms against differences in CETP activation and VLDL-mediated LDL-R suppression by DHA and EPA, which will require an intensive investigation of intracellular roles of each n-3 PUFA.

In conclusion, the n-3 PUFA oil-induced increase in LDL-C level in high fat diet-fed hamsters was demonstrated to be primarily mediated by DHA; EPA was found to have no LDL-C increasing-effect. In addition, the LDL-C increasing-effect of DHA appears to be associated, at least in part, with increased CETP activity and decreased expression of the hepatic LDL-R.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2013.01.001.

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Original Article

Carotid Artery Plaque and LDL-to-HDL Cholesterol Ratio Predict Atherosclerotic Status in Coronary Arteries in Asymptomatic Patients with Type 2 Diabetes Mellitus

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Aims: To investigate the clinical predictors of coronary atherosclerosis and to assess the utility of maximum-IMT for predicting coronary atherosclerosis in asymptomatic type 2 diabetic patients.
Methods: One hundred one Japanese patients with type 2 diabetes underwent computed tomography coronary angiography. Definitions of coronary artery stenosis and vulnerable coronary plaque were luminal narrowing of ≥ 50% and any coronary plaque with positive vessel remodeling and low attenuation, respectively. Carotid intima-media thickness (IMT) was assessed using B-mode ultrasound.
Results: Of the 101 patients, 40 had coronary artery stenosis without vulnerable coronary plaque, 7 had vulnerable coronary plaque without coronary artery stenosis, and 23 had coronary artery stenosis with vulnerable coronary plaque. Male sex ($p=0.031$), duration of diabetes ($p=0.024$), systolic blood pressure (SBP) ($p=0.039$), and the LDL/HDL ratio (LDL/HDL) ($p=0.013$) were independent predictors of coronary artery stenosis and the LDL/HDL ($p=0.042$) independently predicted vulnerable coronary plaque by logistic regression analyses. Areas under the curves in receiver operating characteristic curve analysis of the maximum-IMT, LDL/HDL, and these two parameters combined were 0.711 (95% CI 0.601-0.820), 0.618 (0.508-0.728), and 0.732 (0.632-0.831), respectively, for predicting coronary artery stenosis and 0.655 (0.537-0.773), 0.629 (0.504-0.754), and 0.710 (0.601-0.818), respectively, for predicting vulnerable coronary plaque.
Conclusions: Male sex, duration of diabetes, elevated SBP, and LDL/HDL were independent predictors of coronary artery stenosis. LDL/HDL was an independent predictor of vulnerable coronary plaque. Maximum-IMT predicted both coronary stenosis and vulnerable coronary plaque. Adding LDL/HDL improved the prediction of coronary artery stenosis and vulnerable coronary plaque.

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Key words; Coronary plaque, LDL-to-HDL cholesterol ratio, Computed tomography coronary angiography, Maximum carotid intima-media thickness

Introduction

Coronary artery disease (CAD) is a major cause of morbidity and mortality in patients with type 2 diabetes[1]. Although comprehensive and intensive management of multiple cardiovascular risk factors in type 2 diabetic patients is recommended to reduce the risk of cardiovascular events[2, 3], a considerable number of patients still develop CAD even under intensive management.

Acute coronary syndrome (ACS) is caused by disruption of an atherosclerotic plaque in two-thirds of those who experience it[4]. The characteristics of the

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culprit plaque responsible for ACS, designated as vulnerable coronary plaque, are large lipid cores, a thin fibrous cap, and positive vascular remodeling. Moreover, most lesions are not associated with significant coronary luminal stenosis before the event[5, 6]. Studies of asymptomatic type 2 diabetic patients revealed that silent myocardial ischemia existed irrespective of the number of traditional cardiovascular risk factors and was a cause of future cardiac events[7-10].

Coronary computed tomography angiography (CCTA) was proposed as an alternative imaging modality to evaluate patients with known or suspected CAD[6, 11]. CCTA accurately identifies the presence of obstructive CAD and can evaluate the composition of coronary plaque[6, 12-14]. Compared with intravascular ultrasound, 64-slice CCTA is equally able to detect and evaluate coronary atherosclerotic plaque[15]. The characteristics of culprit lesions in ACS, as shown by CCTA, are positive vessel remodeling and low-attenuation plaque[6]. Moreover, patients having plaque with these characteristics were shown to be at higher risk for development of ACS over time than patients without such plaque characteristics[6]. The application of CCTA has been limited to patients at high CAD risk because of exposure to contrast media and radiation. Screening for CAD using CCTA is controversial in asymptomatic type 2 diabetic patients[2]; however, identifying the clinical predictors of vulnerable coronary plaque and coronary artery stenosis is important for appropriate management of these patients.

Carotid ultrasonography is useful to assess the extent of systemic atherosclerosis[16-18]. Increased carotid intima-media thickness (IMT), maximum-IMT in particular, is a strong predictor of future myocardial infarction[16]. Complex carotid plaque characterized by low echoicity, an irregular surface, and/or ulceration is also associated with future cardiovascular events[19, 20]. Although carotid IMT is a good predictor of future myocardial infarction[16] and the presence of coronary artery stenosis[21], the usefulness of maximum-IMT for predicting CAD is unclear in high risk but asymptomatic type 2 diabetic patients, because the participants in a previous study were limited to the patients at high risk of coronary heart disease or had previously diagnosed coronary heart disease[21]. Moreover, maximum-IMT has high sensitivity but low specificity for the detection of coronary artery stenosis in asymptomatic patients with type 2 diabetes[22, 23]. This would cause an overestimation of coronary artery stenosis; therefore, it is important to examine the predictability of maximum-IMT for CAD and examine whether the addition of other markers, such as the LDL-cholesterol (LDL-C)/HDL-cholesterol (HDL-C) ratio, which is one of the most important variables related to coronary atherosclerosis[24], to carotid IMT could improve the prediction of CAD.

Aim

To investigate the clinical predictors of coronary atherosclerosis and to assess the utility of maximum-IMT for predicting coronary atherosclerosis in asymptomatic type 2 diabetic patients.

Methods

Subjects

We retrospectively analyzed data on patients with type 2 diabetes who underwent CCTA at University of Tsukuba Hospital from April 2009 to March 2011. Because of complications associated with CCTA, such as renal failure, allergy, and radiation-related issues, we reserve its use for those patients at high risk for CAD in whom the risk/benefit ratio indicates its use. Reasons for performing CCTA were maximum-IMT ≥ 1.1 mm, ischemic change on electrocardiogram (ECG), positive exercise ECG test results, or left ventricular wall motion abnormality on echocardiography. In most healthy individuals, the maximum-IMT was reported not to exceed 1.1 mm in Japan[25]. ECG abnormalities such as Q waves, ST-T changes, or negative T waves, positive exercise ECG test results, and left ventricular wall motion abnormality on echocardiography suggest the presence of CAD. Exclusion criteria were chest symptoms, known CAD, type 1 diabetes mellitus, comorbid endocrine disorders, serum triglyceride (TG) level ≥400 mg/dL, and ventricular and supraventricular arrhythmias. All patients had undergone a structured interview, physical examination, and laboratory analysis. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg or current use of antihypertensive agents. Diabetic retinopathy was diagnosed by ophthalmologists. Urinary microalbumin excretion ≥30 mg/day indicated diabetic nephropathy. Glomerular filtration rate was estimated (eGFR) by an equation modified for the Japanese as previously described[26]. Diabetic neuropathy was defined by reduced bilateral ankle vibration sensation (<10 s) and/or reduced or absent bilateral Achilles tendon reflex and/or impairment of light touch; other causes of neuropathy were excluded. This study was approved by the institutional ethics committee and conducted according to the Helsinki Declaration.

Laboratory Analysis

Blood samples were collected the morning after an overnight fast. Plasma levels of glucose and serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C), TG, and creatinine were determined by an automated analyzer (7700 clinical analyzer; Hitachi High-Technologies Corporation, Tokyo, Japan). Serum LDL-cholesterol (LDL-C) levels were calculated by the Friedewald equation when the plasma TG concentration did not exceed 400 mg/dL. HbA1c was measured by high-performance liquid chromatography (HLC-723G9; Tosoh Corporation, Tokyo, Japan). HbA1c values were converted from the Japanese Diabetes Society (JDS) values into National Glycohemoglobin Standardization Program (NGSP) equivalent values[27].

Assessment of CCTA

Coronary stenosis and vulnerable coronary plaque were assessed with a Philips Brilliance-64 scanner (Philips Medical Systems, Cleveland, OH, USA) with a 64×0.625-mm detector configuration. Scanning was performed at 120 kV and 600-1050 mA, 0.2 pitch, and with standard or sharp filters. 60 mL contrast agent (iopamidol 370 mg/mL; Schering AG, Berlin, Germany) was injected intravenously at a rate of 4 mL/s. When the signal density in the ascending aorta reached a predefined threshold of 100 Hounsfield units (HU), acquisition of CT data and an electrocardiogram trace were automatically started during a 7- to 9-s breath-hold. Patients whose heart rate was >70 beats/min were given oral metoprolol (20 mg) 1 h before the scan. Scans were analyzed using a Brilliance Workspace 3-D workstation (Philips Medical Systems). Each scan was analyzed independently by two experienced readers unaware of the patient's identity and clinical presentation. Luminal narrowing of 50% or more revealed by CCTA indicated coronary stenosis. Vulnerable coronary plaque was defined as positive vessel remodeling (remodeling index >1.10) and low-attenuation plaques (<50 HU) on CCTA[12, 28, 29]. Coronary artery lesions based on CCTA were categorized into four groups: 1) neither coronary artery stenosis nor vulnerable coronary plaque (stenosis [−]/vulnerability [−]); 2) coronary artery stenosis without vulnerable coronary plaque (stenosis [+]/vulnerability [−]); 3) vulnerable coronary plaque without coronary stenosis (stenosis [−]/vulnerability [+]); and 4) coronary artery stenosis with vulnerable coronary plaque (stenosis [+]/vulnerability [+]).

Assessment of Carotid Ultrasonography

B-mode examinations of the carotid arteries were performed by an ultrasound system with a 7.5/10-MHz linear-array transducer. Scanning of each common carotid artery (CCA) began just above the clavicle and then passed cephalically through the bifurcation as distally as possible. Carotid IMT was determined by the distance from the leading edge of the lumen-intima interface of the far wall to the leading edge of the media-adventitia interface of the near wall. Maximum-IMT was the greatest thickness measured on both sides of the CCA, bulbus and internal carotid artery, excluding the external carotid artery. Mean-IMT was measured for both CCAs, excluding the bulbus, and was defined as the average of 3 points that included the point of maximum-IMT and 2 points on either side (each point 1 cm distant from the point of maximum-IMT)[25]. The average mean-IMT for both sides was recorded. Carotid plaques were analyzed for their echogenic composition and surface characteristics and were considered to be complex carotid plaques if an irregular surface, a low echoic area, and/or ulceration were present[30].

Statistical Analysis

Categorical variables are expressed as numerals and percentages and were compared with the χ^2 test. Continuous variables are expressed as the mean ± SDs or median and interquartile range (IQR). Based on distribution, continuous variables were compared using unpaired Student's t test or the Mann-Whitney U test for two-group comparisons and ANOVA or Kruskal-Wallis tests for four-group comparisons. Logistic regression analyses identified variables related to coronary artery stenosis or vulnerable coronary plaque. Differences across tertiles of the maximum-IMT or the LDL/HDL ratio were analyzed with one-way ANOVA followed by the Bonferroni post hoc test. The ability of mean-IMT, maximum-IMT, LDL/HDL ratio, or a combination of those factors to predict coronary artery stenosis and vulnerable coronary plaque was examined by receiver operating characteristic (ROC) curve analyses. Area under the receiver operating characteristic curves (AUCs) and sensitivity, specificity, and positive and negative predictive values were calculated for detection of coronary artery stenosis and vulnerable coronary plaque. The effects of combinations of the maximum-IMT and LDL/HDL ratio for the presence of coronary plaque were assessed. All statistical analyses were performed by SPSS (version 15.0; Chicago, IL). Statistical significance was considered at $p<0.05$.

Table 1. Characteristics of study participants according to the combination of coronary artery stenosis and vulnerable coronary plaque

Coronary artery stenosis	(−)	(+)	(−)	(+)	*p* value
Vulnerable coronary plaque	(−)	(−)	(+)	(+)	
	n=31	*n*=40	*n*=7	*n*=23	
Age (years)	60±10	63±10	56±10	65±10	0.093
Male/female	12/19	30/10	5/2	16/7	0.012
BMI (kg/m^2)	25.9±6.0	26.1±5.4	27.7±3.6	26.0±4.4	0.870
Duration of diabetes (years)	7.0 (1.0-12.0)	8.5 (2.0-19.5)	9.0 (6.0-15.0)	12.0 (7.0-20.0)	0.081
Hypertension, n (%)	15 (48)	33 (83)	3 (43)	18 (78)	0.006
Systolic blood pressure (mmHg)	133±19	140±20	131±11	133±17	0.310
Diastolic blood pressure (mmHg)	77±11	80±10	76±8	73±13	0.090
Current smoking, n (%)	8 (26)	11 (28)	4 (57)	9 (39)	0.320
Family history of CAD, n (%)	5 (16)	8 (20)	2 (29)	6 (26)	0.784
Retinopathy, n (%)	5 (16)	20 (50)	2 (29)	10 (43)	0.025
Nephropathy, n (%)	10 (32)	20 (50)	2 (29)	8 (35)	0.378
Neuropathy, n (%)	15 (48)	23 (58)	2 (29)	15 (65)	0.311
HbA1c (%)	10.0±2.2	10.1±1.9	10.2±2.3	9.4±1.7	0.535
Fasting plasma glucose (mmol/L)	9.8±3.5	9.1±2.6	9.3±4.0	8.6±2.0	0.477
Total cholesterol (mmol/L)	5.04±1.13	4.89±1.13	4.73±0.97	5.21±1.54	0.724
LDL cholesterol (mmol/L)	2.98±0.77	3.00±0.96	2.84±0.93	3.39±1.33	0.367
HDL cholesterol (mmol/L)	1.37±0.57	1.19±0.40	1.11±0.13	1.05±0.23	0.054
LDL/HDL ratio	2.4±0.8	2.7±1.0	2.7±1.1	3.4±1.6	0.011
Triglycerides (mmol/L)	1.53±0.58	1.55±0.72	1.71±0.52	1.66±0.68	0.818
White cell count (×10^9/L)	6.0±1.8	7.1±2.2	7.3±1.7	7.0±1.8	0.143
eGFR (mL/min/1.73 m^2)	89±28	91±33	79±22	81±18	0.552
Medications, n (%)					
Insulin treatment	7 (23)	8 (20)	2 (29)	5 (22)	0.965
Sulfonylurea	18 (59)	19 (48)	3 (43)	12 (52)	0.800
Glinides	2 (6)	3 (8)	1 (14)	1 (4)	0.836
Metformin	11 (35)	14 (35)	4 (57)	11 (48)	0.547
Thiazolidinedione	2 (6)	6 (15)	1 (14)	4 (17)	0.630
α-Glucosidase inhibitor	7 (23)	5 (13)	3 (43)	8 (35)	0.117
Incretin-related therapies	4 (13)	7 (18)	0 (0)	1 (4)	0.330
Statin	8 (26)	12 (30)	4 (57)	12 (52)	0.111
Any antihypertensive agent	6 (19)	19 (48)	3 (43)	15 (65)	0.007
ACEI or ARB	6 (19)	17 (42)	3 (43)	13 (57)	0.041
Ca blocker	4 (13)	18 (45)	1 (14)	13 (57)	0.003
β blocker	1 (3)	4 (10)	0 (0)	2 (9)	0.598
Mean-IMT of common carotid artery (mm)	1.0 (0.8-1.3)	1.0 (0.9-1.2)	0.9 (0.8-0.9)	1.1 (0.9-1.4)	0.146
Maximum-IMT of carotid artery (mm)	1.6 (1.3-2.6)	2.4 (2.0-3.0)	2.3 (1.1-3.1)	2.6 (2.2-3.6)	0.001
Complex carotid plaque, n (%)	7 (22)	12 (30)	1 (14)	14 (61)	0.013

Data are the mean±SD or median (interquartile range). BMI, body mass index; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; hypertension, systolic blood pressure ≥140 and/or diastolic blood pressure ≥90 or treatment; LDL/HDL ratio, LDL-to-HDL cholesterol ratio; IMT, intima-media thickness; ACEI, angiotensin-converting-enzyme inhibitor; ARB: angiotensin II receptor blocker

Results

Characteristics of Subjects

Initially, 124 patients were enrolled; however, 18 patients were excluded because they had type 1 diabetes (*n*=3), known CAD (*n*=4), comorbid endocrine disorders (*n*=7), or hypertriglyceridemia (*n*=1), or were in a poor general condition (*n*=3). An additional 5 patients were excluded due to motion artifacts and/or clip artifacts, leaving 101 patients for analysis. Of the subject group, 97 had a maximum-IMT ≥1.1 mm, 31 had ischemic changes on the ECG, 24 had a

Table 2. Characteristics of study participants according to coronary artery stenosis or vulnerable coronary plaque

	all	Coronary artery stenosis		*p* value	Vulnerable coronary plaque		*p* value
		(−)	(+)		(−)	(+)	
	n=101	*n*=38	*n*=63		*n*=71	*n*=30	
Age (years)	62 ± 10	59 ± 10	64 ± 10	0.032	61 ± 10	63 ± 11	0.417
Male/female	63/38	17/21	46/17	0.004	42/29	21/9	0.304
BMI (kg/m^2)	26.1 ± 5.2	26.2 ± 5.6	26.0 ± 5.1	0.848	26.0 ± 5.6	26.4 ± 4.3	0.740
Duration of diabetes (years)	9.0 (2.5-15.0)	8.5 (1.8-12.3)	10.0 (4.0-20.0)	0.053	8.0 (2.0-13.0)	10.5 (6.8-20.0)	0.039
Hypertension, n (%)	69 (68)	18 (47)	51 (81)	<0.001	48 (68)	21 (70)	0.813
Systolic blood pressure (mmHg)	136 ± 19	133 ± 18	138 ± 19	0.228	137 ± 20	133 ± 15	0.257
Diastolic blood pressure (mmHg)	77 ± 11	77 ± 10	77 ± 12	0.772	78 ± 10	73 ± 12	0.029
Current smoking, n (%)	32 (32)	12 (32)	20 (32)	0.986	19 (27)	13 (43)	0.102
Family history of CAD, n (%)	21 (21)	7 (18)	14 (22)	0.648	13 (19)	8 (27)	0.344
Retinopathy, n (%)	37 (37)	7 (18)	30 (48)	0.003	25 (35)	12 (40)	0.648
Nephropathy, n (%)	40 (40)	12 (32)	28 (44)	0.200	30 (42)	10 (33)	0.402
Neuropathy, n (%)	55 (54)	17 (45)	38 (60)	0.128	38 (54)	17 (57)	0.772
HbA1c (%)	9.9 ± 2.0	10.1 ± 2.2	9.9 ± 1.9	0.678	10.1 ± 2.1	9.6 ± 1.9	0.271
Fasting plasma glucose (mmol/L)	9.2 ± 2.9	9.8 ± 3.5	8.9 ± 2.4	0.158	9.4 ± 3.0	8.8 ± 2.6	0.332
Total cholesterol (mmol/L)	5.00 ± 1.21	4.99 ± 1.10	5.00 ± 1.29	0.937	4.96 ± 1.12	5.10 ± 1.43	0.611
LDL cholesterol (mmol/L)	3.07 ± 1.01	2.42 ± 0.86	3.14 ± 1.12	0.368	2.99 ± 0.88	3.26 ± 1.25	0.214
HDL cholesterol (mmol/L)	1.21 ± 0.43	1.32 ± 0.53	1.14 ± 0.35	0.042	1.27 ± 0.49	1.07 ± 0.21	0.005
LDL/HDL ratio	2.8 ± 1.2	2.4 ± 0.9	3.0 ± 1.3	0.013	2.6 ± 0.9	3.3 ± 1.6	0.029
Triglycerides (mmol/L)	1.58 ± 0.65	1.56 ± 0.56	1.59 ± 0.70	0.835	1.54 ± 0.66	1.67 ± 0.64	0.347
White cell count (×10^9/L)	6.8 ± 2.0	6.3 ± 1.8	7.1 ± 2.1	0.066	6.7 ± 2.1	7.1 ± 1.8	0.348
eGFR (mL/min/1.73 m^2)	87 ± 28	87 ± 27	88 ± 29	0.984	90 ± 31	81 ± 19	0.105
Medications, n (%)							
Insulin treatment	22 (22)	9 (24)	13 (21)	0.719	15 (21)	7 (23)	0.806
Sulfonylurea	52 (51)	21 (55)	31 (49)	0.555	37 (52)	15 (50)	0.846
Glinides	7 (7)	3 (9)	4 (6)	0.767	5 (7)	2 (7)	0.946
Metformin	40 (40)	15 (40)	25 (40)	0.983	25 (35)	15 (50)	0.165
Thiazolidinedione	13 (13)	3 (8)	10 (16)	0.246	8 (11)	5 (17)	0.459
α-Glucosidase inhibitor	23 (23)	10 (26)	13 (21)	0.510	12 (17)	11 (37)	0.030
Incretin-related therapies	12 (12)	4 (11)	8 (13)	0.744	11 (16)	1 (3)	0.084
Statin use	36 (36)	12 (32)	24 (38)	0.508	20 (28)	16 (53)	0.016
Any antihypertensive agent	43 (43)	9 (24)	34 (54)	0.003	25 (35)	18 (60)	0.021
ACEI or ARB	39 (39)	9 (24)	30 (48)	0.017	23 (32)	16 (53)	0.048
Ca blocker	36 (36)	5 (13)	31 (49)	<0.001	22 (31)	14 (47)	0.133
β blocker	7 (7)	1 (3)	6 (10)	0.186	5 (7)	2 (7)	0.946
Mean-IMT of common carotid artery (mm)	1.0 (0.8-1.4)	0.9 (0.8-1.2)	1.1 (0.9-1.4)	0.173	1.1 (0.8-1.2)	1.0 (0.9-1.4)	0.489
Maximum-IMT of carotid artery (mm)	2.3 (1.6-3.1)	1.6 (1.2-2.7)	2.4 (2.1-3.2)	<0.001	2.1 (1.5-2.8)	2.6 (2.1-3.7)	0.014
Complex carotid plaque, n (%)	34 (34)	8 (21)	26 (41)	0.037	19 (27)	15 (50)	0.024

Data are the mean ± SD or median (interquartile range). BMI, body mass index; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; hypertension, systolic blood pressure ≥ 140 and/or diastolic blood pressure ≥ 90 or treatment; LDL/HDL ratio, LDL-to-HDL cholesterol ratio; IMT, intima-media thickness; ACEI, angiotensin-converting-enzyme inhibitor; ARB: angiotensin II receptor blocker

Table 3. Logistic regression models for variables associated with the presence of coronary artery disease and vulnerable coronary plaque

	B	SE	OR (95% CI)	*p* value
For coronary stenosis				
Age	0.056	0.033	1.06 (0.99-1.13)	0.091
Male sex	1.203	0.558	3.33 (1.12-9.95)	0.031
BMI	0.001	0.057	1.00 (0.90-1.12)	0.990
SBP	0.032	0.015	1.03 (1.00-1.06)	0.039
Duration of diabetes	0.074	0.033	1.08 (1.01-1.15)	0.024
Current smoking	−0.623	0.614	0.54 (0.16-1.79)	0.311
Family history of CAD	0.459	0.652	1.58 (0.44-5.68)	0.482
HbA1c	0.071	0.145	1.07 (0.81-1.43)	0.626
Maximum-IMT	0.358	0.373	1.43 (0.69-2.97)	0.338
LDL/HDL ratio	0.866	0.348	2.38 (1.20-4.71)	0.013
Complex carotid plaque	0.562	0.652	1.75 (0.49-6.30)	0.389
For vulnerable coronary plaque				
Age	0.003	0.031	1.00 (0.94-1.07)	0.920
Male sex	0.041	0.553	1.04 (0.35-3.08)	0.940
BMI	0.053	0.055	1.05 (0.95-1.18)	0.343
SBP	−0.012	0.016	0.99 (0.96-1.02)	0.446
Duration of diabetes	0.040	0.028	1.04 (0.98-1.10)	0.156
Current smoking	0.631	0.549	1.88 (0.64-5.52)	0.251
Family history of CAD	0.533	0.601	1.70 (0.52-5.54)	0.375
HbA1c	−0.164	0.148	0.85 (0.63-1.13)	0.267
Maximum-IMT	0.417	0.347	1.52 (0.77-2.99)	0.230
LDL/HDL ratio	0.464	0.229	1.59 (1.02-2.49)	0.042
Complex carotid plaque	0.579	0.583	1.79 (0.57-5.59)	0.320

BMI, body mass index; CAD, coronary artery disease; hypertension, SBP ≥140 and/or DBP ≥90 or treatment; LDL/HDL ratio, LDL-to-HDL cholesterol ratio; maximum-IMT, maximum carotid intima-media thickness

positive exercise ECG test, and 31 had a left ventricular wall motion abnormality on echocardiography. Some patients had two or more of these conditions. There were 15 patients with cerebrovascular disease and 5 patients with peripheral arterial disease. **Table 1** shows the baseline characteristics of study subjects according to the category of coronary artery lesion. Sex, hypertension, retinopathy, LDL/HDL ratio, use of any antihypertensive agent, maximum-IMT, and complex carotid plaque differed significantly among the following four groups: stenosis (−)/vulnerability (−), stenosis (+)/vulnerability (−), stenosis (−)/vulnerability (+), and stenosis (+)/vulnerability (+). As shown in **Table 2**, compared with the stenosis (−) group, the stenosis (+) group included more individuals who were older, male, had hypertension, had retinopathy, were taking any antihypertensive agent or who had complex carotid plaque, lower HDL-C levels, a higher LDL/HDL ratio, and higher maximum-IMT values; differences between the stenosis (+) and stenosis (−) groups were significant. In the vulnerable coronary artery (+) group compared with the vulnerability (−) group, the duration of diabetes was higher, values for the LDL/HDL ratio and maximum-IMT were higher, and the percentages of those with complex carotid plaque and who used statins were higher; these differences were significant; however, DBP and HDL-C were significantly lower in the vulnerable coronary artery (+) group than in the vulnerability (−) group (**Table 2**). Logistic regression analyses showed that male sex (odds ratio [OR] 3.33 [95% confidence interval 1.12-9.95], $p=0.031$); duration of diabetes (OR 1.08 [1.05-1.15], $p=0.024$); SBP (OR 1.03 [1.00-1.06], $p=0.039$); and LDL/HDL ratio (2.38 [1.20-4.71], $p=0.013$) independently predicted the presence of coronary artery stenosis after adjustments for age, body mass index (BMI), current smoking, family history of CAD, HbA1c, maximum-IMT, and complex carotid plaque (**Table 3**). The LDL/HDL ratio (OR 1.59 [1.02-2.49], $p=0.042$) independently predicted vulnerable coronary plaque adjusted by age, sex, BMI, SBP, duration of diabetes, current smoking, family

history of CAD, HbA1c, maximum-IMT, and complex carotid plaque (**Table 3**).

Combined Effects on Coronary Artery Lesions According to the Maximum-IMT and the LDL/HDL Ratio

Table 4 and **Fig. 1A** show combinations of the effects of the maximum-IMT and LDL/HDL ratio on coronary artery stenosis. According to the results of tertile analysis of maximum-IMT, 12 patients (34%) in the lowest tertile (T1), 26 (81%) in the middle tertile (T2), and 25 (74%) in the highest tertile (T3) had coronary artery stenosis ($p=0.001$). Analysis using the variance post-hoc test revealed a statistically significant difference between the T1 group and each of the other 2 groups in the maximum-IMT category regarding coronary artery stenosis ($p<0.001$ and $p=0.001$, respectively). In the LDL/HDL ratio category, 18 patients (55%) in T1, 20 (61%) in T2, and 25 (71%) in T3 had coronary artery stenosis ($p=0.352$). There was no significant difference in the frequency of coronary artery stenosis associated with an increase in the LDL/HDL ratio for each tertile of the maximum-IMT ($p=0.949$ for T1, $p=0.531$ for T2, and $p=0.512$ for T3). Increasing frequency of coronary stenosis was observed in the T2 and T3 categories of the maximum-IMT compared with the T1 category of the maximum-IMT for each category of the LDL/HDL ratio. Statistical significance was observed among the tertiles of the maximum-IMT in the T3 of the LDL/HDL ratio ($p=0.001$). There were, however, no significant differences in T1 and T2 of the LDL/HDL ratio ($p=0.166$ and $p=0.135$, respectively).

Table 4 and **Fig. 1B** show combinations of the effects on vulnerable coronary plaque according to the maximum-IMT and the LDL/HDL ratio. According to the results of tertile analysis of the maximum-IMT, 6 patients (17%) in T1, 11 (34%) in T2, and 13 (38%) in T3 had vulnerable coronary plaque. In the LDL/HDL ratio category, 7 patients (21%) in T1, 8 (24%) in T2, and 15 (43%) in T3 had vulnerable coronary plaque. Analysis of variance revealed no significant difference among the tertiles of maximum-IMT or LDL/HDL ($p=0.352$ and $p=0.107$, respectively). There was a difference in the frequency of vulnerable coronary plaque associated with increases in the LDL/HDL ratio for each category of the maximum-IMT. The frequency of vulnerable coronary plaque associated with increases in the LDL/HDL ratio was observed in the T1 and T3 categories of the maximum-IMT. According to tertile analysis of the LDL/HDL ratio in the T1 category of maximum-IMT, the LDL/HDL ratio was associated with improved prediction of vulnerable coronary plaque but without statistical significance ($p=0.078$). Increasing frequency of vulnerable coronary plaque was observed in the T2 and T3 categories of the maximum-IMT compared with the T1 category of the maximum-IMT for either T1 or T2 category of the LDL/HDL ratio; however, these relationships were not observed in the T3 category of the LDL/HDL ratio.

Predictive Value of Mean-IMT, Maximum-IMT, and LDL/HDL Ratio

Fig. 2A shows the AUCs for prediction of coronary artery stenosis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of variables for maximum-IMT are provided in **Table 5**. A maximum-IMT ≥1.7 mm predicted coronary artery stenosis with a sensitivity of 0.89, specificity of 0.55, PPV of 0.77, and NPV of 0.75 (AUC 0.711 [0.601-0.820]; $p<0.001$). An LDL/HDL ratio ≥3.0 predicted coronary artery stenosis with sensitivity of 0.40, specificity of 0.74, PPV of 0.71, and NPV of 0.42 (AUC 0.618 [0.508-0.728]; $p=0.048$). The combination of the maximum-IMT and LDL/HDL ratio improved these values (AUC 0.732 [0.632-0.831]; $p<0.001$). The combination of maximum-IMT ≥1.8 and LDL/HDL ratio ≥2.5 had sensitivity of 0.52, specificity of 0.87, PPV of 0.87, and NPV of 0.52; however, mean-IMT of CCA did not significantly discriminate the presence of coronary artery stenosis (AUC 0.581 [0.465-0.697]; $p=0.176$).

Fig. 2B shows the AUCs for the prediction of vulnerable coronary plaque. Sensitivities, specificities, PPVs, and NPVs of each parameter were as follows (**Table 5**): maximum-IMT ≥2.1 mm had sensitivity of 0.80, specificity of 0.45, PPV of 0.38, and NPV of 0.84 (AUC 0.655 [0.537-0.773], $p=0.014$) and an LDL/HDL ratio ≥3.0 had sensitivity of 0.50, specificity of 0.72, PPV of 0.43, and NPV of 0.77 (AUC 0.629 [0.504-0.754], $p=0.042$). These values were improved by the combination of the maximum-IMT and the LDL/HDL ratio (AUC 0.710 [0.601-0.818]; $p=0.001$). The combination of maximum-IMT ≥2.3 mm or LDL/HDL ratio ≥3.0 had sensitivity of 0.90, specificity of 0.44, PPV of 0.40, and NPV of 0.91; however, mean-IMT of CCA did not significantly discriminate the presence of vulnerable coronary plaque (AUC 0.543 [0.419-0.668]; $p=0.492$).

Discussion

This study had three major findings. First, male sex, duration of diabetes, elevated SBP, and the LDL/HDL ratio were independently associated with the

Table 4. Frequency of coronary plaque according to the tertile of Maximum-IMT and LDL/HDL-C ratio

	Maximum-IMT(mm)				
	Total	T1 (<2.0)	T2 (2.0-2.6)	T3 (≥2.7)	*p* for tertile of maximum-IMT
For coronary artery stenosis					
LDL/HDL ratio					
Total		12/35 (34)	26/32 (81)**	25/34 (74)*	0.001
T1 (<2.17)	18/33 (55)	5/14 (36)	6/8 (75)	7/11 (64)	0.166
T2 (2.17-3.06)	20/33 (61)	4/11 (36)	9/12 (75)	7/10 (70)	0.135
T3 (>3.06)	25/35 (71)	3/10 (30)	11/12 (92)*	11/13 (85)*	0.001
p for tertile of LDL/HDL ratio	0.352	0.949	0.531	0.512	
For vulnerable coronary plaque					
LDL/HDL ratio					
Total		6/35 (17)	11/32 (34)	13/34 (38)	0.352
T1 (<2.17)	7/33 (21)	1/14 (7)	3/8 (38)	3/11 (27)	0.220
T2 (2.17-3.06)	8/33 (24)	1/11 (9)	3/12 (25)	4/10 (40)	0.274
T3 (>3.06)	15/35 (43)	4/10 (40)	5/12 (42)	6/13 (46)	0.956
p for tertile of LDL/HDL ratio	0.107	0.078	0.698	0.654	

Data are expressed as n/N (%). *$p<0.01$ and **$p<0.001$ vs. T1 in maximum-IMT. IMT, intima-media thickness; LDL/HDL ratio, LDL-to-HDL cholesterol ratio

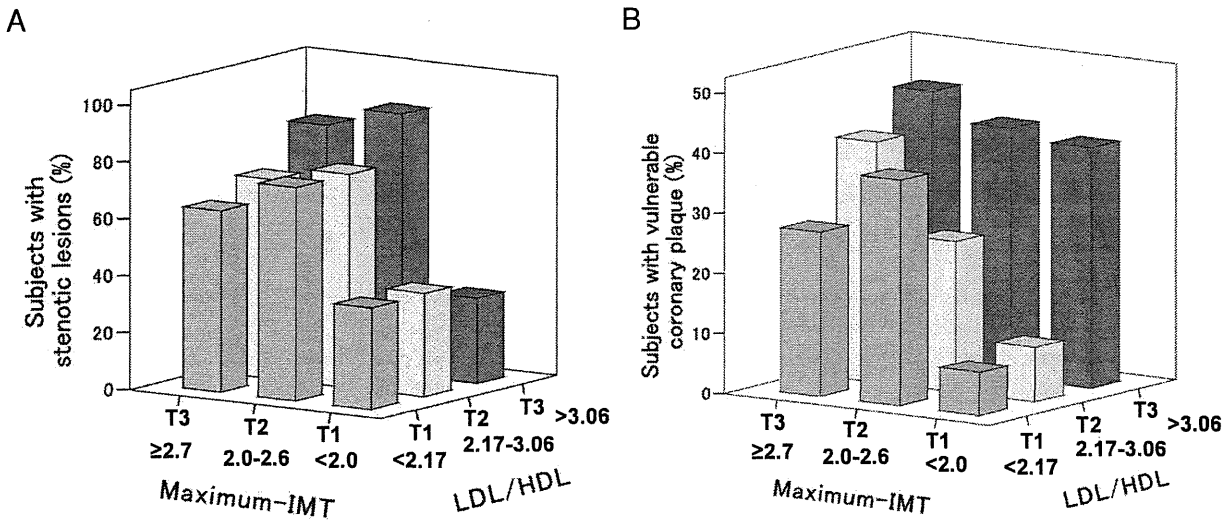

Fig. 1. A: Presence of coronary artery stenosis in different maximum-IMT categories according to 3 categories of the LDL-to-HDL cholesterol (LDL/HDL) ratio (tertile, T). In the T1 group in the maximum-IMT category, 34% of subjects had coronary artery stenosis whereas in the T2 and T3 groups, 81% and 74% of subjects, respectively, had coronary artery stenosis. In the T1 group in the LDL/HDL ratio category, 55% of subjects had coronary artery stenosis whereas in the T2 and T3 groups, 61% and 71% of subjects, respectively, had coronary artery stenosis.
B: Presence of vulnerable coronary plaque for different maximum-IMT categories according to 3 categories of LDL/HDL ratio (tertile, T). In the T1 group in the maximum-IMT category, 17% of subjects had vulnerable coronary plaque whereas in the T2 and T3 groups, 34% and 38% of subjects, respectively, had vulnerable coronary plaque. In the T1 group in LDL/HDL ratio category, 21% of subjects had vulnerable coronary plaque, whereas in the T2 and T3 groups, 24% and 43% of subjects, respectively, had vulnerable coronary plaque.

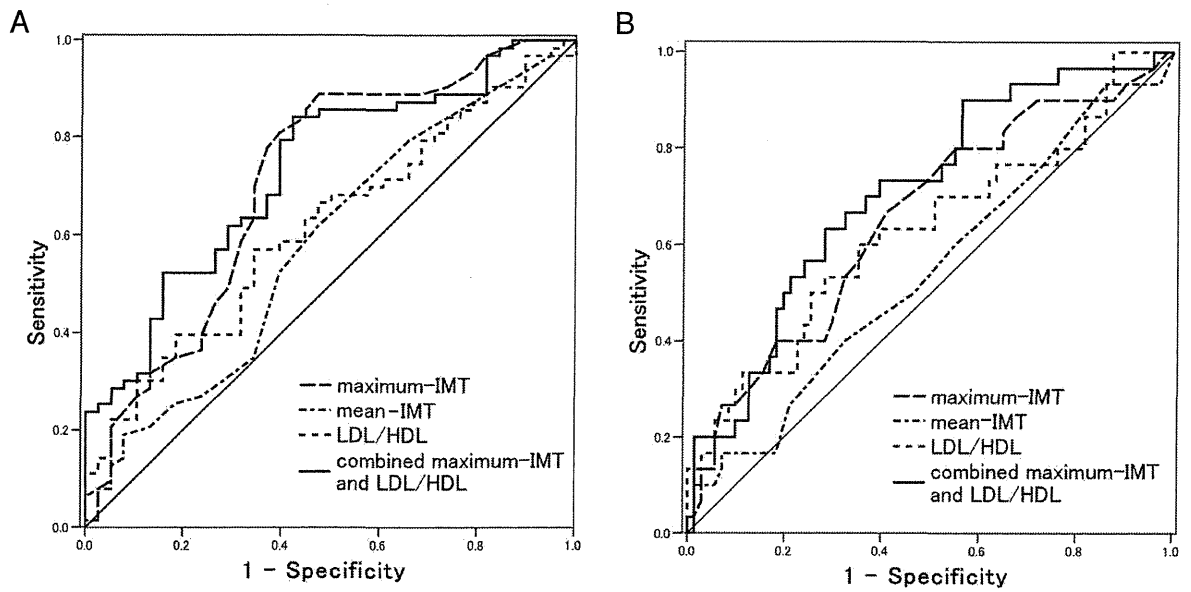

Fig. 2. A: Comparison among the AUCs of the mean-IMT, maximum-IMT, LDL-to-HDL cholesterol (LDL/HDL) ratio, and the combination of maximum-IMT and LDL/HDL ratio for the presence of coronary artery stenosis (A) and for the presence of vulnerable coronary plaque (B). A: The AUCs (95% confidence interval), cut-off values, sensitivities, and specificities were as follows: maximum-IMT 0.711 (0.601-0.820), 1.7mm, 0.89, and 0.55; LDL/HDL ratio 0.618 (0.508-0.728), 3.0, 0.40, and 0.74; and the combination of maximum-IMT and the LDL/HDL ratio 0.732 (0.632-0.831), 1.8 mm and 2.5, 0.52, and 0.87. B: The AUCs, cut-off values, sensitivities, and specificities were as follows: maximum-IMT 0.655 (0.537-0.773), 2.1 mm, 0.80, and 0.45; LDL/HDL ratio 0.629 (0.504-0.754), 3.0, 0.40, and 0.74; and the combination of maximum-IMT and LDL/HDL ratio 0.710 (0.601-0.818), 2.3 mm or 3.0, 0.90, and 0.44.

presence of coronary artery stenosis whereas the LDL/HDL ratio was independently associated with the presence of vulnerable coronary plaque. Second, maximum-IMT values predicted both coronary artery stenosis and vulnerable coronary plaque. Third, the combination of maximum-IMT and the LDL/HDL ratio had greater predictive value than each parameter alone, as shown by the AUC for predicting both coronary artery stenosis and vulnerable coronary plaque. To our knowledge, this is the first investigation of clinical predictors of vulnerable coronary plaque in high risk but asymptomatic patients with type 2 diabetes.

High LDL-C, low HDL-C, elevated blood pressure, hyperglycemia, smoking, and diabetic retinopathy are independent risk factors for CAD in type 2 diabetes[31, 32]. The TC/HDL ratio and LDL/HDL ratio were reported to be better predictors of future cardiovascular disease than single lipid parameters (TC, LDL-C, and HDL-C)[33]. Traditional risk factors were shown to have distinct effects on plaque stability: stable plaque was associated with age and hypertension in men and with elevated glycosylated hemoglobin and hypertension in women; plaque erosion with smoking in both sexes; and plaque rupture with elevated levels of serum TC in both sexes and with smoking and elevated TC/HDL ratio in men[4, 34]. Subjects with lower HDL-C were at high risk of cardiovascular disease regardless of whether statins were used[35]. These findings are consistent with our results; therefore, our results appear highly plausible, given the findings in those previous epidemiological and pathological studies.

Our finding of lower DBP in the vulnerability (+) group than in the vulnerability (−) group is probably due to a greater proportion of anti-hypertensive drug use in the former than the latter group. The higher rate of statin use in the vulnerability (+) than in the vulnerability (−) group reflects poorer control of LDL-C.

Carotid IMT is strongly predictive of future cardiovascular events[36] and is associated with the presence of coronary atherosclerotic plaque evident by coronary angiography[21]. Increased maximum-IMT was shown to be associated with the presence of coronary artery stenosis as assessed by CCTA in asymptomatic patients with type 2 diabetes[22, 23]. The maximum-IMT, which includes the CCA, bulbus, and internal carotid artery, was reported to have superior

Table 5. Sensitivity, specificity, PPV and NPV for identification of individuals with stenotic lesions at different maximum-IMT or LDL/HDL thresholds

	For coronary artery stenosis (n=63)				For vulnerable coronary plaque (n=30)			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Maximum-IMT (mm)								
1.2	0.97	0.18	0.66	0.78	0.93	0.10	0.30	0.78
1.3	0.94	0.24	0.67	0.69	0.90	0.13	0.30	0.75
1.4	0.90	0.29	0.68	0.65	0.90	0.18	0.32	0.81
1.5	0.89	0.34	0.69	0.65	0.90	0.23	0.33	0.84
1.6	0.89	0.45	0.73	0.71	0.90	0.28	0.35	0.87
1.7	0.89	0.55	0.77	0.75	0.87	0.32	0.35	0.85
1.8	0.86	0.58	0.77	0.71	0.83	0.35	0.35	0.83
1.9	0.84	0.58	0.77	0.69	0.80	0.35	0.34	0.81
2.0	0.81	0.63	0.78	0.67	0.80	0.41	0.36	0.83
2.1	0.78	0.66	0.79	0.64	0.80	0.45	0.38	0.84
LDL/HDL								
2.0	0.75	0.34	0.65	0.45	0.77	0.31	0.32	0.76
2.5	0.59	0.61	0.71	0.47	0.63	0.54	0.37	0.78
3.0	0.40	0.74	0.71	0.42	0.50	0.72	0.43	0.77
3.5	0.29	0.89	0.82	0.43	0.33	0.83	0.45	0.75
4.0	0.16	0.95	0.93	0.40	0.23	0.93	0.58	0.74

PPV, positive predictive value; NPV, negative predictive value; LDL/HDL ratio, LDL-to-HDL cholesterol ratio; Maximum-IMT, maximum carotid intima-media thickness

predictive value than the mean-IMT of the CCA for a cardiovascular event or coronary artery stenosis[16, 23]. Moreover, the relationship of CAD with characteristics of carotid plaque has been indicated. Complex carotid plaque was associated with complex coronary plaque[19]. Echolucent carotid plaque, a component of complex carotid plaque, was reported to be lipid-and macrophage-rich[37] and to predict future coronary events in stable CAD[19]. An irregular surface of carotid plaque was associated with vascular events[38]. Our data are consistent with these findings.

Interestingly, we found that the maximum-IMT had moderate predictive value not only for coronary artery stenosis but also for vulnerable coronary plaque in asymptomatic patients with type 2 diabetes. On the other hand, the LDL/HDL ratio could predict vulnerable coronary plaque but not coronary artery stenosis, especially in T1 of the maximum-IMT category. These data indicate that maximum-IMT is a useful predictor of both coronary artery stenosis and vulnerable coronary plaque and that the LDL/HDL ratio is a useful predictor of vulnerable coronary plaque in patients categorized as being in T1 of the maximum-IMT. Our data also suggest that the maximum-IMT has a threshold for the prediction of coronary artery stenosis because the prevalence of coronary artery stenosis clearly increased between T1 and T2 and was similar between T2 and T3 in the maximum-IMT category; however, we could not exclude the presence of coronary artery stenosis in the lowest maximum-IMT category because its prevalence was 34% in this group.

Kasami *et al.* showed that the cut-off level of maximum-IMT for predicting the presence of coronary artery stenosis was 1.90 mm with sensitivity of 0.93 and specificity of 0.55 in type 2 diabetic patients without a history of CAD[23]. A similar result was reported by Irie *et al.*: the maximum-IMT cut-off level was 1.55 mm with sensitivity of 0.90 and specificity of 0.46 in asymptomatic patients with type 2 diabetes[22]. Our result is comparable to these results, indicating that maximum-IMT is useful in screening for coronary artery stenosis.

We showed that the AUC of the combination of maximum-IMT and LDL/HDL ratio for predicting the presence of coronary artery stenosis was greater than the maximum-IMT alone; however, the maximum-IMT alone had higher sensitivity than the combined maximum-IMT and LDL/HDL ratio, which had higher specificity than the maximum-IMT. Therefore, the maximum-IMT alone is useful to rule out

coronary artery stenosis and the combination of maximum-IMT and LDL/HDL ratio is of use in ruling it in.

Our cut-off level of maximum-IMT for predicting vulnerable coronary plaque was 2.1 mm, which is plausible because maximum-IMT of the carotid artery ≥ 1.9 mm was shown to be associated with myocardial infarction[39]. Moreover, the maximum-IMT is superior to the LDL/HDL ratio for this purpose. Combining the two significantly improved the AUC for predicting the presence of vulnerable coronary plaque compared with the maximum-IMT alone. The combination of maximum-IMT ≥ 1.7 mm or LDL/HDL ratio ≥ 3.0 could predict vulnerable coronary plaque with sensitivity of 0.97 and specificity of 0.27. Prediction of the stenosis (−)/vulnerability (+) group is important, because angiographic encroachment of the lumen was reported as < 50% in ACS[5, 6], and patients who could not be included in that group cannot be identified using routine cardiac examinations. Five of the 7 patients in the stenosis (−)/vulnerability (+) group were detected using the combination of maximum-IMT ≥ 1.7 mm or LDL/HDL ratio ≥ 3.0 (data not shown).

Clinical Implications

Several cardiovascular risk scores have been proposed[40]. Although useful for predicting future cardiovascular events, their value is not high for predicting the presence of coronary artery stenosis[41]. Lee *et al.* showed that three cardiovascular risk scores (Framingham risk score, systematic coronary risk evaluation, and Chinese multi-provincial cohort study) predicted the presence of coronary artery stenosis detected by CCTA with a sensitivity of 0.61-0.70 and specificity of 0.55-0.66[41]. The current and previous studies showed that maximum-IMT can predict the presence of coronary artery stenosis with sensitivity of 0.84-0.93 and specificity of 0.46-0.57[22, 23]. Maximum-IMT appears superior to risk scores in screening for obstructive CAD. Moreover, we could predict the status of the coronary atherosclerotic burden with two simple measures: maximum-IMT and LDL/HDL ratio. This suggests that carotid ultrasonography is a worthwhile tool to predict the atherosclerotic status of coronary arteries.

Study Limitations

This study has limitations. First, it was cross-sectional with a relatively small number of subjects, all of which were Japanese. The number of patients was too small to assess clinically useful cut-off values. Our participants had poor glycemic and lipid control, and, although they were asymptomatic, they were at high risk for cardiovascular disease. We reserve the use of CCTA for those patients at high risk for CAD in view of the risk/benefit ratio for CCTA. Under most circumstances we could not ethically perform CCTA in patients with maximum-IMT < 1.1 mm for comparison with those with maximum-IMT ≥ 1.1 mm. In addition, because of our criteria for the performance of CCTA, we could not identify enough patients with good glycemic and lipid control for comparison with those with poor glycemic and lipid control. These issues could have introduced selection bias. However, our data regarding the cut-off values of maximum-IMT for the prediction of coronary artery stenosis are thought to be similar to those of previous studies[22, 23]; thus, we consider that our results could be applicable to patients with type 2 diabetes at low to moderate risk. Second, the clinical significance of vulnerable coronary plaque detected by CCTA has not been fully established. CCTA has failed to characterize lesions at risk of plaque erosion, which could be responsible for one-third of ACS[42]. Prospective studies are needed on the association between vulnerable plaque assessed by CCTA and development of future CAD. Third, methods for measuring IMT are not standardized[36]. Finally, IMT measurements were performed by two expert sonographers as routine clinical examinations, which allows for intra-sonographer differences. Our results should be confirmed in prospective studies with an appropriate sample size and subjects from various ethnic groups.

Conclusion

Male sex, duration of diabetes, elevated SBP, and the LDL/HDL were independent predictors of coronary artery stenosis and the LDL/HDL was an independent predictor of vulnerable coronary plaque. Maximum-IMT predicts both coronary stenosis and vulnerable coronary plaque and adding the LDL/HDL improved the prediction of coronary artery stenosis and vulnerable coronary plaques in asymptomatic patients with type 2 diabetes.

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K.F. developed the study design, researched the data, contributed to discussions, wrote the manuscript, and reviewed and edited the manuscript. H. Su. planned and supervised this research, researched the data, contributed to discussions, wrote the manuscript, and reviewed and edited the manuscript. A.S. researched the data, contributed to discussions, wrote the manuscript, and reviewed and edited the manuscript. S.K., Y.H., and K.S. developed the study

design, researched the data, contributed to discussions, and reviewed and edited the manuscript. H.I., K.K., S.Y., and A.T. researched the data, contributed to discussions, and reviewed and edited the manuscript. N.Y., H. So., and H. Sh. developed the study design, contributed to discussions, and reviewed and edited the manuscript.

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CARDIO VASCULAR DIABETOLOGY

ORIGINAL INVESTIGATION Open Access

Metabolic predictors of ischemic heart disease and cerebrovascular attack in elderly diabetic individuals: difference in risk by age

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Abstract

Background: High LDL-cholesterol (LDL-C) and glucose levels are risk factors for ischemic heart disease (IHD) in middle-aged diabetic individuals; however, the risk among the elderly, especially the very elderly, is not well known. The aim of this study was to identify factors that predict IHD and cerebrovascular attack (CVA) in the elderly and to investigate their differences by age.

Methods: We performed a prospective cohort study (Japan Cholesterol and Diabetes Mellitus Study) with 5.5 years of follow-up. A total of 4,014 patients with type 2 diabetes and without previous IHD or CVA (1,936 women; age 67.4 ± 9.5 years, median 70 years; <65 years old, n = 1,261; 65 to 74 years old, n = 1,731; and ≥ 75 years old, n = 1,016) were recruited on a consecutive outpatient basis from 40 hospitals throughout Japan. Lipids, glucose, and other factors related to IHD or CVA risk, such as blood pressure (BP), were investigated using the multivariate Cox hazard model.

Results: One hundred fifty-three cases of IHD and 104 CVAs (7.8 and 5.7/1,000 people per year, respectively) occurred over 5.5 years. Lower HDL-cholesterol (HDL-C) and female gender were correlated with IHD in patients ≥75 years old (hazard ratio (HR):0.629, $P < 0.01$ and 1.132, $P < 0.05$, respectively). In contrast, systolic BP (SBP), HbA1C, LDL-C and non-HDL-C were correlated with IHD in subjects <65 years old ($P < 0.05$), and the LDL-C/HDL-C ratio was correlated with IHD in all subjects. HDL-C was correlated with CVA in patients ≥75 years old (HR: 0.536, $P < 0.01$). Kaplan-Meier estimator curves showed that IHD occurred more frequently in patients <65 years old in the highest quartile of the LDL-C/HDL-C ratio. In patients ≥75 years old, IHD and CVA were both the most frequent among those with the lowest HDL-C levels.

Conclusions: IHD and CVA in late elderly diabetic patients were predicted by HDL-C. LDL-C, HbA1C, SBP and non-HDL-C are risk factors for IHD in the non-elderly. The LDL-C/HDL-C ratio may represent the effects of both LDL-C and HDL-C. These age-dependent differences in risk are important for developing individualized strategies to prevent atherosclerotic disease.

Trial registration: UMIN-CTR, UMIN00000516

Keywords: Elderly, Diabetes mellitus, Cardiovascular diseases, HDL-C, LDL-C/HDL-C ratio

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Introduction

Type 2 diabetes mellitus, dyslipidemia and aging are independent risk factors for cardiovascular diseases, such as ischemic heart disease (IHD). Within diabetic individuals, lipids, especially LDL-cholesterol (LDL-C), blood pressure (BP), and diabetic control are risk factors for IHD [1-4]. For example, the United Kingdom Prospective Diabetes Study (UKPDS) showed the importance of BP, lipids, and diabetic control in the prevention of IHD in newly diagnosed diabetic individuals(mean age 53 years, range 25–65 years), and subsequent studies have confirmed these findings [1,2]. However, the risk factors for IHD or cerebrovascular attack (CVA) in elderly diabetic individuals (older than 65 years), particularly in late elderly diabetic individuals (older than 75 years), have not been identified.

In Western countries, the evidence suggests that middle-aged diabetic individuals have an IHD risk similar to that of non-diabetic patients who have experienced a myocardial infarction, and the guidelines for diabetes treatment recommend that the LDL-C level should be less than 100 mg/dl, which is similar to the recommendation for the secondary prevention of myocardial infarction [5,6]. However, it is unknown whether the same risk exists for elderly diabetic individuals. Additionally, many guidelines recommend strict control of LDL-C levels to prevent atherothrombotic diseases, especially in diabetic patients, yet recommend the same HDL-cholesterol (HDL-C; 40 mg/dl) and triglyceride (TG; 150 mg/dl) levels as for non-diabetic individuals [5-7]. There are few reports on the absolute risk conferred by HDL-C and TG in elderly diabetic patients.

Additionally, diabetes can either develop in the elderly or continue through old age after an earlier onset. Even in elderly individuals without diabetes, postprandial hyperglycemia occurs because of a delay in insulin secretion in response to feeding and may contribute to an increase in the number of elderly diabetic patients [8]. The International Diabetes Federation (IDF) reports that the number of diabetic patients increased from 30 million in 1987 to 246 million in 2007 (7% of adults) and speculates that it will increase to 380 million by 2027 [9]. In Japan, 30% of diabetic individuals were elderly in 1997 (13% of the elderly suffered from diabetes mellitus), which increased to 40% in 2007 (17% of the elderly). Furthermore, individuals older than 75 (13 million) comprise over 10% of the total population. However, no large-scale investigations have focused on type 2 diabetes mellitus in the elderly, especially in the late elderly, or those older than 75 [10]. Thus, evaluating the metabolic predictors of atherosclerotic diseases, such as IHD and CVA, in elderly diabetic individuals is important. For these reasons, we organized the Japan Cholesterol and Diabetes Mellitus Study (JCDM) to evaluate which factors can predict IHD or CVA in diabetic patients, including the elderly. Our elderly sample population included 1,016 late elderly, who were older than 75 and performed independent daily life activities at outpatient clinics [11].

Materials and methods

Subjects

The JCDM is a prospective, cohort study that consists of 4,014 Japanese diabetic individuals from 40 hospitals throughout Japan who were recruited on a consecutive outpatient basis between September 2004 and March 2005 (1,936 women; mean age 67.4 ± 9.5 years, median age 70 years; Figure 1) [11]. The JCDM protocol, which is in accordance with the provisions of the Declaration of Helsinki, received ethical approval from the institutional review boards of all the participating institutes. Written informed consent was obtained from all patients. The criteria from the American Diabetes Association for type 2 diabetes mellitus diagnosis were used [6]. Patients with previous IHD (myocardial infarction, unstable angina pectoris, angioplasty, or bypass grafting) or CVA (recent stroke with admission within the past 24 months) were excluded, as were patients whose medical records concerning plasma lipids (TG, HDL-C and total cholesterol or LDL-C) were not provided. The other exclusion criteria were a history or complication of serious heart disease (e.g., severe arrhythmia, heart failure, cardiomyopathy, valvular disease, or congenital disease), serious hepatic or renal disease with admission within the past 24 months, malignant disease, intention to undergo surgery, any illness with a poor prognosis of less than one year, and judgment by the physician in charge that the patient was not suitable for the study.

At 24 months (2007), 92.3% of the enrolled patients were followed up, and 84.1% were followed up at 66 months (2010). Patients were divided into groups based on age at registration: younger than 65 (non-elderly, n = 1,267), 65 to 74 years old (early elderly, n = 1,731) and older than 75 (late elderly, n = 1,016). These age categories are used frequently in Japan for the study of elderly patients and for health care insurance purposes [12].

Outcome measurements

The primary endpoints were the incidence of IHD and CVA, specifically fatal and non-fatal myocardial infarction and other non-fatal events, including unstable angina pectoris, angioplasty, stenting, coronary artery bypass grafting and stroke. Detailed definitions of each event are shown below. Transient ischemic attacks were included only if definite focal lesions from the attack were confirmed by head CT or MRI.