Original Article

Validity of a Novel Method for Estimation of Low-Density Lipoprotein Cholesterol Levels in Diabetic Patients

Hideto Chaen[1], Shigesumi Kinchiku[2], Masaaki Miyata[1], Shoko Kajiya[2], Hitoshi Uenomachi[2], Toshinori Yuasa[1], Kunitsugu Takasaki[1] and Mitsuru Ohishi[1]

[1]Department of Cardiovascular Medicine and Hypertension, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan
[2]Uenomachi-Kajiya Clinic, Kagoshima, Japan

Aim: Low-density lipoprotein cholesterol (LDL-C) is routinely estimated using the Friedewald equation [LDL-C(F)]. A novel method for LDL-C [LDL-C(M)] estimation recently proposed by Martin *et al.* was reported to be more accurate than the Friedewald formula in subjects in the United States. The validity of LDL-C(M) in different races and patients with diabetes mellitus (DM) has not been elucidated. The purpose of this study was to validate the LDL-C(M) estimates in Japanese population with type 2 DM by comparing with LDL-C(F) and directly measured LDL-C [LDL-C(D)].
Methods: Both LDL-C(M) and LDL-C(F) levels were compared against LDL-C(D) measured by selective solubilization method in 1,828 Japanese patients with type 2 DM.
Results: On linear regression analysis, LDL-C(M) showed a stronger correlation than that shown by LDL-C(F) ($R = 0.979$ vs. $R = 0.953$, respectively) with LDL-C(D). We further analyzed the effect of serum triglyceride (TG) concentrations on the accuracy of LDL-C(F) and LDL-C(M). Although LDL-C levels showed a positive correlation with TG levels, the LDL-C(F) levels tended to show a greater divergence from LDL-C(D) levels than that shown by LDL-C(M) with changes in TG levels.
Conclusion: We for the first time demonstrated a more useful measurement of LDL-C levels estimated by Martin's method than that estimated by the Friedewald equation in Japanese patients with DM.

Key words: Low-density lipoprotein cholesterol, Friedewald formula, Diabetes mellitus

Introduction

Coronary artery disease (CAD) is a major public health issue in industrialized countries[1]. Cohort studies and randomized clinical trials have demonstrated causal association between the serum low-density lipoprotein cholesterol (LDL-C) levels and the development and progression of CAD[2-5]. Increased LDL-C concentration is a major risk factor for CAD[6]. Clinical practice guidelines recommend evaluation of LDL-C levels as a key element of routine patient care[7].

Friedewald equation is a recommended method for estimation of LDL-C levels [LDL-C(F)][8], which estimates LDL-C as total cholesterol (TC) − high-density lipoprotein cholesterol (HDL-C) − [triglyceride (TG)/5] in mg/dl in the fasting state and with TG < 400 mg/dl. Although TG/5 represents the very low-density lipoprotein cholesterol (VLDL-C), simple division of TG concentrations by 5 does not provide an accurate estimation of VLDL-C and thereby that of LDL-C.

In the Lipid Research Clinics Prevalence Study[9], the mean TG:VLDL-C ratios ranged from 5.2 to 8.9 across clinics. Therefore, Martin *et al.*[10] proposed a novel method for LDL-C estimates [LDL-C(M)], which is derived as TC − HDL-C − (TG/adjustable factor) in mg/dl. The adjustable factor was determined as the strata-specific median TG:VLDL-C ratios from a dataset of 900,605 subjects in the United States

Address for correspondence: Masaaki Miyata, Department of Cardiovascular Medicine and Hypertension, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima City, Kagoshima 890-8520, Japan
E-mail: miyatam@m3.kufm.kagoshima-u.ac.jp

TG Levels (mg/dL)	Non-HDL-C (mg/dL)					
	<100	100-129	130-159	160-189	190-219	≧220
7-49	3.5	3.4	3.3	3.3	3.2	3.1
50-56	4.0	3.9	3.7	3.6	3.6	3.4
57-61	4.3	4.1	4.0	3.9	3.8	3.6
62-66	4.5	4.3	4.1	4.0	3.9	3.9
67-71	4.7	4.4	4.3	4.2	4.1	3.9
72-75	4.8	4.6	4.4	4.2	4.2	4.1
76-79	4.9	4.6	4.5	4.3	4.3	4.2
80-83	5.0	4.8	4.6	4.4	4.3	4.2
84-87	5.1	4.8	4.6	4.5	4.4	4.3
88-92	5.2	4.9	4.7	4.6	4.4	4.3
93-96	5.3	5.0	4.8	4.7	4.5	4.4
97-100	5.4	5.1	4.8	4.7	4.5	4.3
101-105	5.5	5.2	5.0	4.7	4.6	4.5
106-110	5.6	5.3	5.0	4.8	4.6	4.5
111-115	5.7	5.4	5.1	4.9	4.7	4.5
116-120	5.8	5.5	5.2	5.0	4.8	4.6
121-126	6.0	5.5	5.3	5.0	4.8	4.6
127-132	6.1	5.7	5.3	5.1	4.9	4.7
133-138	6.2	5.8	5.4	5.2	5.0	4.7
139-146	6.3	5.9	5.6	5.3	5.0	4.8
147-154	6.5	6.0	5.7	5.4	5.1	4.8
155-163	6.7	6.2	5.8	5.4	5.2	4.9
164-173	6.8	6.3	5.9	5.5	5.3	5.0
174-185	7.0	6.5	6.0	5.7	5.4	5.1
186-201	7.3	6.7	6.2	5.8	5.5	5.2
202-220	7.6	6.9	6.4	6.0	5.6	5.3
221-247	8.0	7.2	6.6	6.2	5.9	5.4
248-292	8.5	7.6	7.0	6.5	6.1	5.6
293-399	9.5	8.3	7.5	7.0	6.5	5.9
400-13975	11.9	10.0	8.8	8.1	7.5	6.7

Fig. 1. Median TG:VLDL-C ratios disaggregated by non-HDL-C and TG levels (180-cell).

TG, triglyceride; VLDL-C, very low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; cited from reference #10.

(**Fig. 1**), which appeared to provide more accurate measure of LDL-C than that calculated with Friedewald formula. However, the effect of factors, such as race, obesity, diabetes, and insulin resistance, which may contribute to variance in the TG:VLDL-C ratio, has not been assessed.

Homogenous assays for LDL-C have been developed, and the direct measurement of LDL-C [LDL-C(D)] by a selective solubilization method demonstrated a good agreement with LDL-C(F) in the Framingham Offspring Study[11]. Zhang *et al.* also reported a high correlation between LDL-C(D) and LDL-C(F) in patients with hypercholesterolemia[12].

The validity of LDL-C(M) in different races and patients with type 2 diabetes mellitus (DM) has not been elucidated. The purpose of this study was to validate LDL-C(M) by comparing with LDL-C(F) and LDL-C(D) in Japanese patients with type 2 DM.

Methods

Patients

A total of 1,828 consecutive patients with type 2 DM and fasting serum TG levels <400 mg/dl at the Uenomachi-Kajiya Clinic were enrolled in the study. Regarding the use of antilipidemic agents, 369 patients (20.2%) were treated with statins, 47 (2.6%) with fibrates, 21 (1.1%) with ezetimib, and 12 (0.7%)

with eicosapentaenoic acids. With respect to antidiabetic medications, 1,112 patients (60.8%) were being treated with oral antidiabetic agents and 272 patients (14.9%) with insulin therapy.

The study protocol was approved by the institutional ethics committee at the Kagoshima University Hospital. Informed consent was obtained from all patients prior to their enrolment in the study.

Laboratory Measurements

Blood samples were collected after 12 h of fasting. Serum levels of TC (mg/dl), TG (mg/dl), and HDL-C (mg/dl) were measured by enzymatic methods; reagents used were T-CHO (Kainos Laboratories, Tokyo, Japan), TG-II (Kainos Laboratories, Tokyo, Japan), and MetaboLead HDL-C (Kyowa Medex, Tokyo, Japan). LDL-C(D) (mg/dl) was directly measured by the selective solubilization methods with the use of reagent MetaboLead LDL-C (Kyowa Medex, Tokyo, Japan). LDL-C(F) was calculated by the Friedewald equation: i.e., $TC - HDL-C - TG/5$. LDL-C(M) was calculated by the equation: $TC - HDL-C - TG/adjustable\ factor$, where adjustable factor was the strata-specific TG:VLDL-C ratio based on TG and non-HDL-C levels (**Fig. 1**)[10]. Martin et al. performed multiple linear analysis examining the extent to which TG:VLDL-C was explained by information in the standard lipid profile such as TG, non-HDL, TC/HDL, TG/HDL, TG/TC, and age and sex in 900,605 Americans. Results from this analysis guided the choice of TG and non-HDL for stratification to determine strata-specific median TG:VLDL-C ratios. Glycosylated hemoglobin (HbA1c) levels were determined by cation-exchange HPLC ADAMS A1c HA-8180 analyzer (Arkray, Inc., Kyoto, Japan).

Statistical Analysis

Data are expressed as mean ± standard deviation. Between-group differences with respect to continuous variables were assessed by paired t-test. Logistic regression analysis was performed to assess the relationship between two continuous variables. Statistical analyses were performed with JMP ver.11 (SAS Institute, Cary, USA) at Kagoshima University. A value of $p < 0.05$ was considered indicative of a statistically significant between-group difference.

Results

Patient Characteristics

Baseline characteristics of the study population are summarized in **Table 1**. The mean age of patients ($n = 1828$) was 60.6 ± 11.9 years; of these, 1,067 (58.4%) patients were male. Mean levels of LDL-

Table 1. Baseline patient characteristics ($n = 1,828$)

Age (years)	60.6 ± 11.9
Sex (male/female)	1,067/761
BMI (kg/m^2)	22.6 ± 3.5
HbA1c (%)	7.6 ± 1.7
Total cholesterol (mg/dl)	207.7 ± 36.6
LDL-C(D) (mg/dl)	128.6 ± 32.4
LDL-C(F) (mg/dl)	118.4 ± 33.5
LDL-C(M) (mg/dl)	122.0 ± 32.0
HDL-cholesterol (mg/dl)	59.5 ± 16.3
Non-HDL cholesterol (mg/dl)	148.2 ± 36.5
TG (mg/dl)	148.8 ± 80.9
TG:VLDL-C	5.38 ± 0.96

BMI, body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C(M), LDL-C calculated by Martin's method; TG, triglyceride; TG:VLDL-C, ratio of triglyceride to very low-density lipoprotein cholesterol.

C(D), LDL-C(F), and LDL-C(M) were 128.6 ± 32.4, 118.4 ± 33.5, and 122.0 ± 32.0 mg/dl, respectively.

Distribution of TG:VLDL-C Ratio

Based on strata of TG and non-HDL-C values, a 180-cell table of median TG:VLDL-C value was derived (**Fig. 1**), and LDL-C(M) levels were estimated. **Fig. 2** demonstrates the distribution of TG:VLDL-C ratio in the present study. The median TG:VLDL-C ratio was 5.4 (interquartile range, 4.7 − 5.9). The 5th − 95th percentile was 4.0 − 7.0.

Correlation between LDL-C(D) and LDL-C(F) or LDL-C(M)

Linear regression analysis revealed a good correlation of both LDL-C(F) and LDL-C(M) with LDL-C(D); however, LDL-C(M) levels showed a better fit with LDL-C(D) compared with that shown by LDL-C(F) (**Fig. 3**). In addition, we checked this correlation in patients with well controlled (HbA1c < 7.0%, HbA1c = 6.3 ± 0.5%, $n = 811$) and those with poorly controlled DM (HbA1c ≥ 7.0%, HbA1c = 8.6 ± 1.6%, $n = 1017$). As shown in **Fig. 4**, LDL-C(M) demonstrated a better correlation with LDL-C(D) than LDL-C(F) in patients regardless of how well the diabetes is controlled.

Effect of TG on Measurements of LDL-C(F) and LDL-C(M)

The effect of serum TG concentrations on the divergence of LDL-C(M) and LDL-C(F) levels from that of LDL-C(D) was assessed individually (**Fig. 5**). Although LDL difference showed a positive correla-
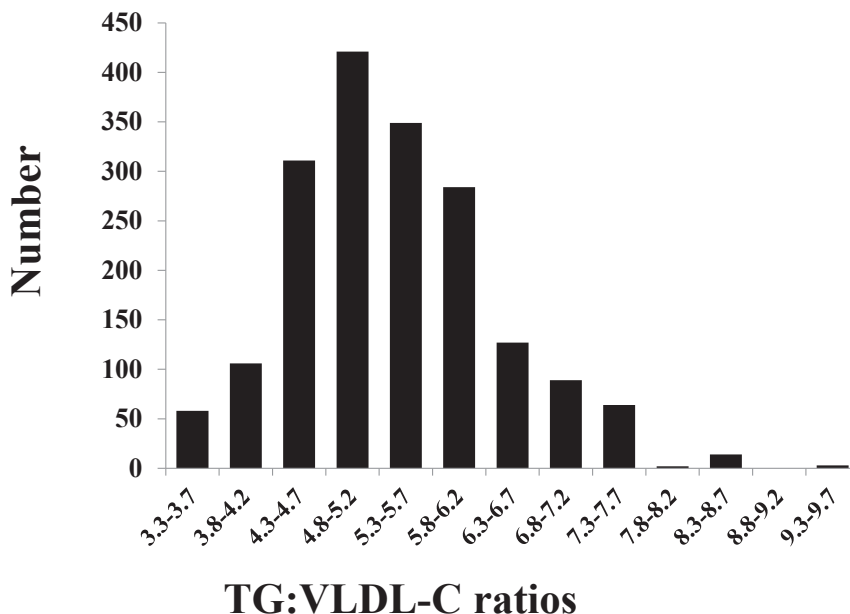
Fig. 2. Distribution of TG:VLDL-C ratio.

TG, triglyceride; VLDL-C, very low-density lipoprotein cholesterol.
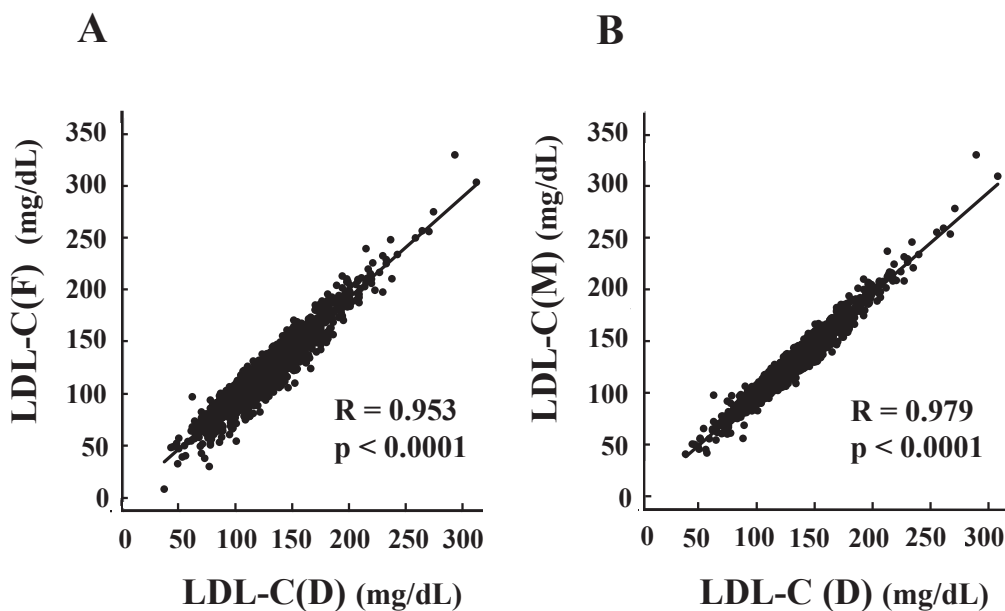
A

B

Fig. 3. Comparison of correlation between LDL-C(D) and LDL-C(F) or LDL-C(M) levels.

(A) LDL-C(F) versus LDL-C(D). (B) LDL-C(M) versus LDL-C(D). LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C (M), LDL-C calculated by Martin's method.

tion with TG levels, the effect of TG level on the divergence in LDL-C(F) levels from the LDL-C(D) level was greater than that shown by LDL-C(M). Furthermore, we compared the three LDL measurements after stratification of dataset by TG levels: TG < 150

mg/dl and TG ≥ 150 mg/dl groups (**Fig. 6**). Although significant differences among three LDL measurements were observed in both TG < 150 mg/dl and TG ≥ 150 mg/dl groups, the differences between the three LDL measurements were larger in TG ≥ 150 mg/
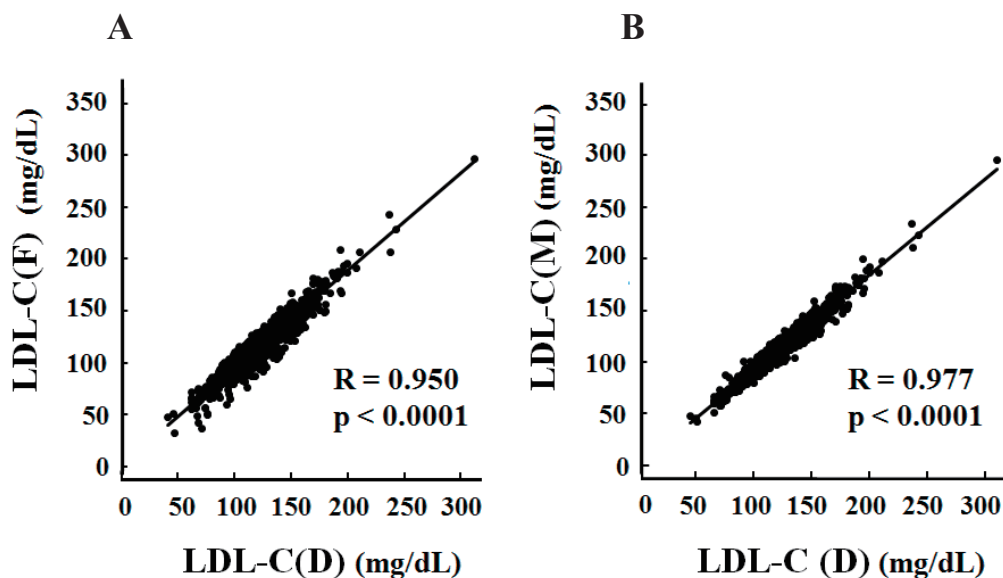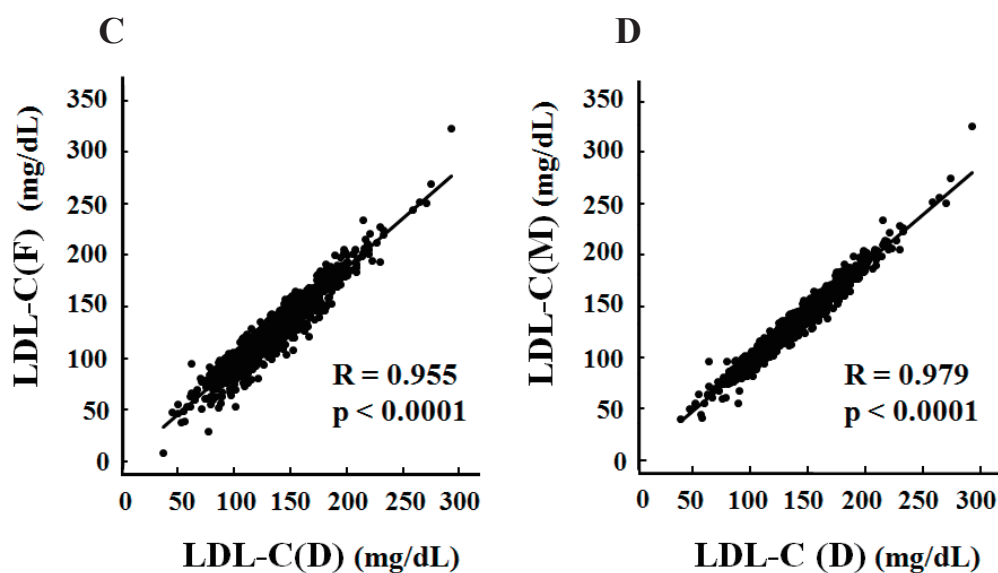
HbA1c < 7.0%

A

B

HbA1c ≥ 7.0%

C

D

Fig. 4. Comparison of correlation between LDL-C(D) and LDL-C(F) or LDL-C(M) levels in patients with a good (HbA1c <7.0%) and poor (HbA1c ≥7.0%) control of DM.

(A) LDL-C(F) versus LDL-C(D) in patients with HbA1c <7.0%. (B) LDL-C(M) versus LDL-C(D) in patients with HbA1c <7.0%. (C) LDL-C(F) versus LDL-C(D) in patients with HbA1c ≥7.0%. (D) LDL-C(M) versus LDL-C(D) in patients with HbA1c ≥7.0%. LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C (M), LDL-C calculated by Martin's method.

dl groups compared with those in TG <150 mg/dl groups.

Concordance in Guideline Classification

We analyzed the concordance between calculated LDL-C with respect to LDL-C(D) using guidelines of the Japanese Society of Atherosclerosis[13] in TG <150
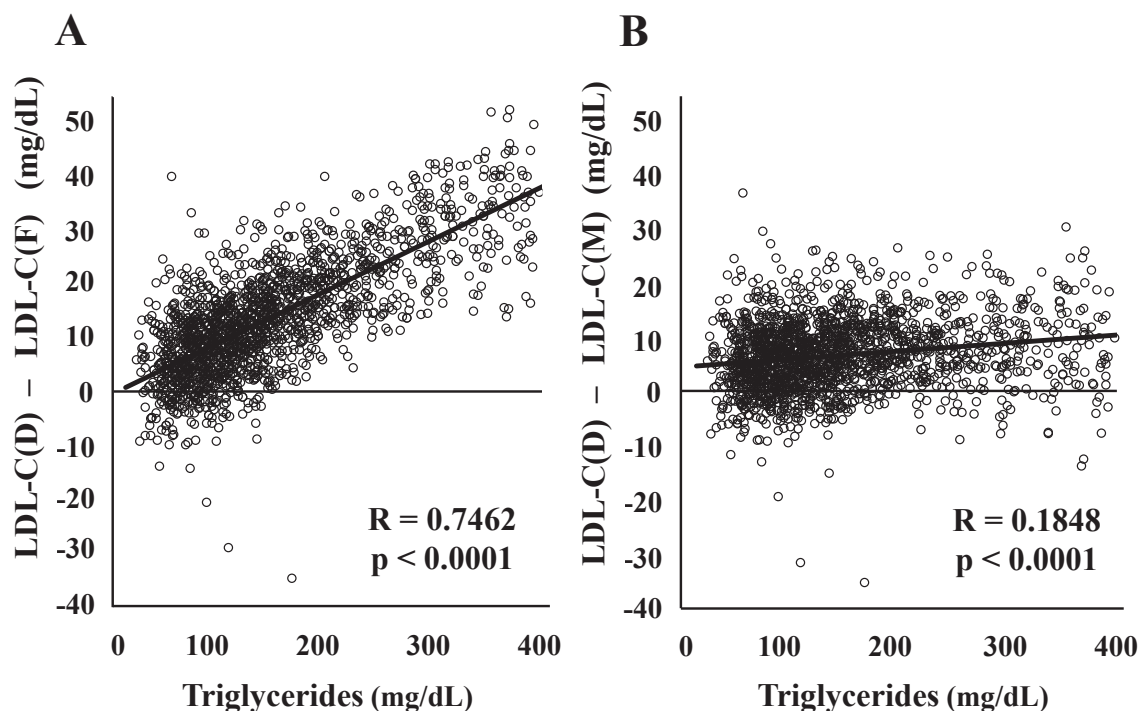
Fig. 5. Effect of serum triglycerides concentrations on the LDL difference.

(A) Triglycerides versus LDL-C(D) − LDL-C(F). (B) Triglycerides versus LDL-C(D) − LDL-C(M). LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C(M), LDL-C calculated by Martin's method.

mg/dl and TG ≥150 mg/dl groups (**Table 2** and **Fig. 7**). In the TG <150 mg/dl group, the concordance was similar for both LDL-C(F) and LDL-C(M) (72.1% and 72.7%, respectively). In contrast, LDL-C(M) demonstrated a better concordance than LDL-C(F) levels in TG ≥150 mg/dl group (63.6% and 37.2%, respectively).

Discussion

We measured serum levels of TC, HDL-C, TG, and LDL-C(D) and calculated the LDL-C(F) and LDL-C(M) in 1,828 Japanese patients with DM. LDL-C(D) demonstrated a good correlation with both LDL-C(F) and LDL-C(M); LDL-C(M) demonstrated a better fit to LDL-C(D) compared with LDL-C(F). In addition, the effect of TG on the LDL difference was greater in LDL-C(F) compared with that on LDL-C(M); the difference between three LDL measurements was greater in TG ≥150 mg/dl group compared with that in TG <150 mg/dl group. In addition, LDL-C(M) demonstrated a better concordance compared with LDL-C(F) in the TG ≥150 mg/dl group, whereas the concordance was similar for both LDL-C(F) and LDL-C(M) in the TG <150 mg/dl group.

The current guidelines on the use of LDL-C for cardiovascular risk assessment are largely based on epidemiological studies that have established the link between cholesterol and cardiovascular disease[14-16]. Most of these studies used beta-quantification methods or the Friedewald equation for measurements of LDL-C. The gold standard method for LDL-C measurement is ultracentrifugation followed by beta-quantification [LDL-C(BQ)][17]. However, beta-quantification is too expensive and inconvenient for routine clinical application[18]. Therefore, the Friedewald formula is widely used to derive LDL-C from measurements of TC, TG, and HDL-C levels in the fasting state.

In 1972, the Friedewald formula was first developed based on a study involving with 448 patients with familial hyperlipoproteinemia or their relatives[8]. The Friedewald formula is easy to calculate and is commonly used in clinical and research settings as it precludes the use of ultracentrifugation. However, it has several limitations. First, lipid levels should be measured in the fasting state. Second, it is not recommended for use in patients with TG >400 mg/dl. Third, an underestimation of LDL-C(F) level has been shown at low LDL-C[19, 20] and high TG levels[21, 22].

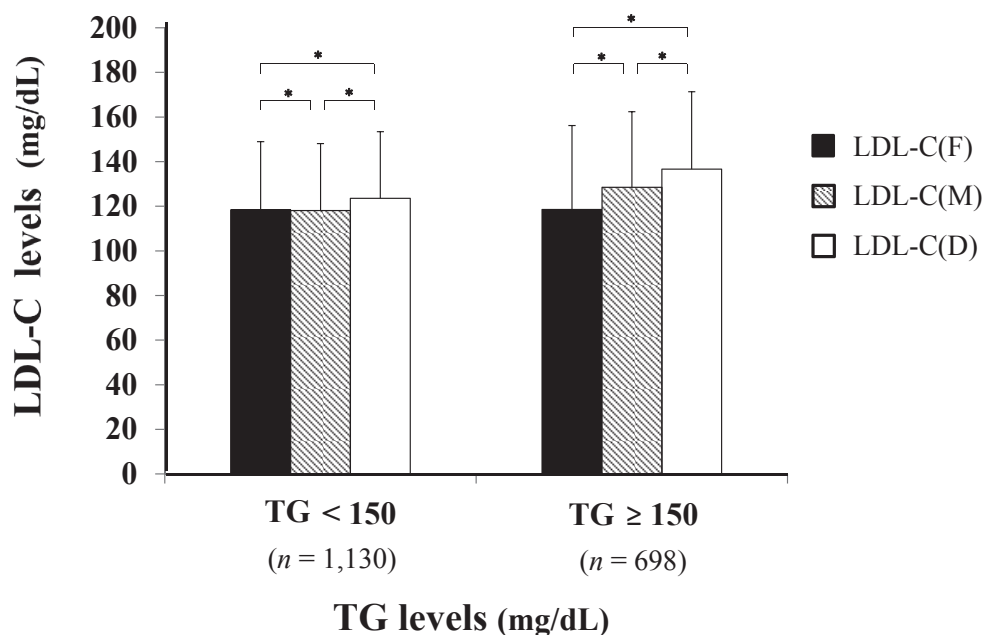Delong *et al.* proposed a fixed factor of 6 rather

Fig. 6. Comparison of the three LDL-C measurements disaggregated by TG levels (TG < 150 mg/dl and TG ≥ 150 mg/dl).

*indicates $p < 0.0001$. LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C(M), LDL-C calculated by Martin's method.

than 5 for the TG:VLDL-C ratio[9]. However, considering individual variance in the TG:VLDL-C ratios, any fixed factor may not account for the variance in TG:VLDL-C ratios. Recently, Martin et al.[10] proposed a novel method for calculation of an adjustable factor for the TG:VLDL-C ratio based on the individual's TG and non-HDL-C levels; the method that was developed based on a dataset of 900,605 subjects in the United States and that classifies patients with superior concordance to LDL-C(D) compared with that with the Friedewald method, especially in patients with elevated TG concentrations. The 180-cell strata approach could be coded into an online calculator (http://ldlcalculator.com/), smartphone application, or automated laboratory reporting system. In the present study, LDL-C(M) demonstrated a stronger correlation with LDL-C(D) compared with LDL-C(F) and a better concordance compared with LDL-C(F) in patients with TG ≥ 150 mg/dl. Recently Rim JH et al.[23] compared the accuracy of 10 equations to calculate LDL-C, including the Friedewald formula and Martin's method. They directly measured LDL-C by a homogeneous direct assay using reagents from Sekisui Medical Corporation in 168,212 Korean hospital patients and subjects in the general population. They reported that Martin's methods and the DeLong equation showed the highest intraclass correlation, indicating

the best agreement with direct LDL-C measurement. In contrast, our study demonstrated the usefulness of LDL-C(M) in Japanese patients with diabetes who had higher TG levels than the general population. In addition, Dansethakul et al.[24] compared seven formulas for LDL-C calculation, including the Friedewald formula but not Martin's method, with direct LDL-C homogenous enzymatic measurement in 1,768 Thai. They reported that their new formula; $0.9955TC - 0.9853HDL\text{-}C - 0.1998TG + 7.1449$, exhibited a good r of 0.971 with the direct LDL-C measurement.

Several reports have suggested that LDL-C(F) may be inaccurate in patients with type 2 DM because of the tendency for having high TG levels. Rubies-Prat et al.[25] compared LDL-C(F) and LDL-C(BQ) levels in 61 patients with type 1 DM, 50 patients with type 2 DM, and 116 healthy control subjects. The mean difference between LDL-C(F) and LDL-C(BQ) was significantly greater in patients with type 2 DM and not in type 1 DM compared with that in controls. Hirany et al.[26] compared LDL-C(F), LDL-C(D), and LDL-C(BQ) in 148 diabetic patients and found the direct LDL-C assay as being more reliable and accurate method than the Friedewald formula for LDL-C determination in diabetic patients. Although the Martin's method is not known to be accurate in specific diseases such as DM, we demonstrated that LDL-

Table 2. Concordance of calculated and directly measured LDL-C in guideline classification

		TG < 150 (n = 1,130)					TG ≥ 150 (n = 698)				
		LDL-C(D)					LDL-C(D)				
		< 100 n = 234	100-199 n = 287	120-139 n = 305	140-159 n = 178	160- n = 126	< 100 n = 76	100-119 n = 158	120-139 n = 162	140-159 n = 151	160- n = 151
	< 100	**231**	85	2	0	0	**76**	126	21	0	0
	100-119	3	**195**	120	1	0	0	**32**	101	19	1
LDL-C(F)	120-139	0	7	**175**	51	0	0	0	**40**	103	11
	140-159	0	0	8	**120**	32	0	0	0	**29**	56
	160-	0	0	0	6	**94**	0	0	0	0	**83**
	< 100	**232**	81	0	0	0	**73**	48	0	0	0
	100-119	2	**203**	124	3	0	3	**108**	61	1	0
LDL-C(M)	120-139	0	3	**176**	55	0	0	2	**100**	83	3
	140-159	0	0	5	**117**	33	0	0	1	**67**	52
	160-	0	0	0	3	**93**	0	0	0	0	**96**

TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C(M), LDL-C calculated by Martin's method.

C(M) may be more useful than LDL-C(F) in patients with type 2 DM. LDL-C(F) includes intermediate-density lipoprotein cholesterol (IDL-C); theoretically, LDL-C(M) also includes IDL-C. In addition, LDL-C(BQ) is LDL-C (1.006 < d < 1.063) and also includes IDL-C. Furthermore, LDL-C(D) includes IDL-C. These four LDL-C measurements including IDL-C and IDL-C would be increased in type III dyslipidemia and diabetic nephropathy with advanced CKD. Therefore, in these diseases, the influence of IDL-C on measurement of LDL-C should be considered.

Direct measurement of LDL-C levels results in a plus bias compared with LDL-C(BQ) when samples with both high TG and lower cholesterol are used[27]. The old version of the direct assay of LDL-C (Determiner L LDL-C) was not accurate. The modified version of the reagent (MetaboLead LDL-C) used in the present study provides a better measure of LDL-C[28].

Limitations

There are several limitations in the present study. First, we used LDL-C(D) instead of LDL-C(BQ) as the standard reference measurement. Although LDL-C(BQ) had been used as the gold standard for LDL-C measurement, the modern generation of direct methods for determination of LDL-C has shown good reproducibility, has an advantage of being single step
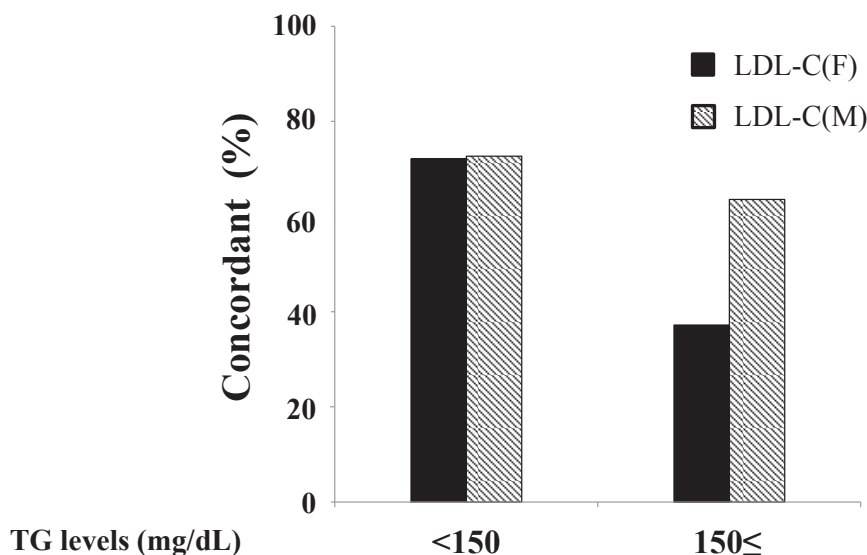
procedures, and is not sensitive to variations in TG levels to the same extent[29]. Watanabe *et al.* reported the MetaboLead LDL can be used to measure serum LDL-C levels, which are close to those measured by the modified BQ method[28]. Second, although the Martin's method can estimate LDL-C in patients with TG ≥ 400 mg/dl, we excluded these patients to compare with LDL-C(F). The validity of Martin's method in patients with TG ≥ 400 mg/dl was not assessed in the present study. Third, we have no data from Japanese without diabetes and could not determine whether Martin's method is applicable to such individuals. Therefore, further study is necessary to analyze the validity of Martin's method in the general Japanese population.

Conclusion

We for the first time demonstrated that LDL-C levels estimated by Martin's method were more useful than those estimated by the Friedewald equation in Japanese patients with type 2 DM. This novel method can be easily incorporated in the existing laboratory reporting systems at virtually no extra cost and may be useful in clinical and research settings.

Conflicts of Interests

The authors declare that they have no conflicts

Fig. 7. Comparison of concordance between LDL-C(F) and LDL-C(D) and that between LDL-C(M) and LDL-C(D) disaggregated by TG level (TG < 150 mg/dl and TG ≥ 150 mg/dl).

LDL-C, low-density lipoprotein cholesterol; LDL-C(D), LDL-C measured by direct assay; LDL-C(F), LDL-C calculated by Friedewald formula; LDL-C(M), LDL-C calculated by Martin's method; TG, triglyceride.

of interest.

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