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Original Article

Homogeneous Assays for LDL-C and HDL-C are Reliable in Both the Postprandial and Fasting State

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***Aim*: Most epidemiological and clinical studies calculated low-density lipoprotein-cholesterol (LDL-C) by Friedewald's formula which cannot be used in the postprandial samples. Although the homogeneous assays with poor analytical performance were withdrawn from the market, it remained unclear whether the currently available reagents for LDL-C and high-density lipoprotein-cholesterol (HDL-C) are as accurate for postprandial samples as for fasting samples.**

***Methods*: Fresh blood samples were collected from 59 non-diseased and 109 diseased subjects. Postprandial samples constituted 72.9% and 39.4% of these samples. LDL-C and HDL-C concentrations were measured using the homogeneous assays of four manufacturers (Denka Seiken, Wako, Kyowa Medex, and Sekisui Medical). Simultaneously, LDL-C and HDL-C concentrations were determined using the reference measurement procedures (RMPs) of the Centers for Disease Control and Prevention (CDC). Total errors were calculated using a routine method (TE_{com}) and via error component analysis (TE_{ECA}).**

***Results*: All homogeneous assays for LDL-C and HDL-C met the National Cholesterol Education Program (NCEP) requirements in terms of coefficient of variation, and TE_{com} in both non-diseased and diseased subjects. LDL-C and HDL-C values measured by the homogeneous assays were in good agreement with those measured by the RMPs in both fasting and postprandial samples. The TE_{com} and TE_{ECA} values of the postprandial samples were similar to those of fasting samples, although the TE_{ECA} values were up to 4.4-fold greater than the TE_{com} values.**

***Conclusions*: In both non-diseased and diseased subjects, the homogeneous assays for LDL-C and HDL-C of four manufacturers are as accurate for postprandial samples as for fasting samples.**

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Key words: Direct assay, Friedewald's formula, Standardization, Cholesterol Reference Method Laboratory Network (CRMLN), Dyslipidemia

Introduction

The homogeneous assays for low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) are innovative because they require neither extensive pretreatment nor a long

assay time[1-4]. Other strengths of such assays are excellent intra- and inter-assay reproducibility. These assays are also termed direct methods. To date, several original reagents have been developed in Japan by different manufacturers, based on various principles. Latecomer companies (distributors) purchase bulk reagents from the original manufacturers, and sell their products with different brand names[5, 6]. To ensure global standardization and harmonization of lipid laboratory tests, the Centers for Disease Control and Prevention (CDC) runs a manufacturer certification program with the aid of the Cholesterol Reference Method Laboratory Network (CRMLN)[7]. All commercial homogeneous assays produced by the original manufactures and their distributors have taken part in this program, and have passed the precision and accuracy requirements defined by the National Cholesterol Education Program (NCEP)[8, 9].

Despite these efforts, earlier studies revealed that some homogenous assays exhibited poor analytical performance in patients with common diseases, and even in disease-free subjects[5, 6, 10, 11]. At the end of 2016, these reagents were withdrawn from the Japanese market. A common problem was that when these reagents were used, hypertriglyceridemia often triggered a positive assay bias, especially in LDL-C measurements[5, 10]. In contrast to the LDL-C estimations afforded by various equations, homogeneous assays are applicable to postprandial samples with triglyceride (TG)-rich lipoprotein levels greater than those of the fasting state. However, no study has yet directly compared the accuracy of homogeneous assays of fasting samples with those of postprandial samples using the reference measurement procedures (RMPs) of the CDC, employing fresh sera collected from non-diseased and diseased subjects.

Aim

Our aim was to clarify whether homogeneous assays for LDL-C and HDL-C are as accurate for postprandial samples as for fasting samples.

Methods

Study Subjects

Study participants were volunteers and outpatients at Osaka University Hospital (Osaka, Japan) and the National Cerebral and Cardiovascular Center (NCVC, Osaka, Japan). Both normolipidemic and dyslipidemic subjects were eligible provided that their lipoprotein concentrations were in a certain range [20 mg/dL ≤ LDL-C; 20 mg/dL ≤ HDL-C < 100 mg/dL; TG < 1,000 mg/dL]. In line with the exclusion criteria of our previous studies[5, 6], we did not enroll patients with serious infectious diseases, decompensated liver cirrhosis, or cholestatic liver diseases. A diagnosis of hyperlipidemia was made if the LDL-C level was >160 mg/dL, and/or the TG level >200 mg/dL, under conditions of ad libitum food intake. Normolipidemic subjects with no medical history were classified as non-diseased, whereas others were considered diseased. This study complied with the dictates of the latest version of the Declaration of Helsinki (thus, that amended at the 64th WMA General Assembly, Fortaleza, Brazil, October 2013[12]). At recruitment, written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committees of the individual institutions.

Study Protocol

Fresh venous blood was drawn and serum was collected after centrifugation, dispensed into screw-capped tubes, and immediately stored at 4℃. The next morning, an aliquot of each sample was conveyed to the Kyoto Prefectural University where LDL-C and HDL-C were measured using homogeneous assays. The remaining samples were subjected (at NCVC) to LDL-C and HDL-C measurements using the RMPs described below. During sample transport, we used a special cooling container that held the samples at 2−4℃ (without freezing)[5]. All measurements were performed on the day of sample arrival.

Assays for LDL-C and HDL-C

At Kyoto Prefectural University, we measured LDL-C and HDL-C concentrations using four homogeneous assays [LDL-C$_{HA}$ and HDL-C$_{HA}$]. The reagents were manufactured by Denka Seiken, Wako, Kyowa Medex, and Sekisui Medical (**Supplemental Table 1**). All reagents, calibrators, and controls were provided by these companies. All reagents except the reagent of Kyowa Medex (used in the LDL-C assay) were the same as those described in our previous studies[5, 6]. The Kyowa Medex reagent (MetaboLead LDL-C) was a modified version of the previous formulation (Determiner L; LDL-C). The newer version exhibits an improved specificity for LDL particles; interference by TG-rich lipoproteins is reduced. TG concentrations were determined using an enzymatic method.

We employed the same automated analyzer used

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Table 1. Study subjects.

	Non-diseased group (n=59)	Diseased group (n=109)
Age (years)	41.7±9.4	53.4±15.9
Gender (male, %)	64.4	68.8
Body height (cm)	167.1±7.5	166.4±8.9
Body weight (kg)	65.3±16.1	69.5±17.1
Body mass index (kg/m^2)	23.3±5.3	24.8±4.2
TC (mg/dL)	187.0±24.3 [124.1−237.6]	200.5±38.9 [110.0−325.6]
LDL-C$_{RMP}$ (mg/dL)	109.7±20.9 [60.0−159.4]	121.2±35.9 [51.7−230.0]
HDL-C$_{RMP}$ (mg/dL)	60.7±12.7 [38.2−98.9]	51.8±13.3 [21.8−88.7]
TG (mg/dL)	115.2±45.3 [30.8−194.2]	195.6±144.6 [32.9−880.4]
Non-fasting subjects (%)	72.9	39.4
Time since last meal (h)[1)]	2.8±1.9	2.6±1.8
Hypolipidemic agents taken (statin) (%)	0.0	56.9 (39.4)
Dyslipidemia (%)[2)]	0.0	88.1
Type Ⅰ/Ⅴ	0.0	0.9/1.8
Type Ⅱa/Ⅱb (FH)	0.0 (0.0)	64.2 (12.8)
Type Ⅲ	0.0	0.9
Type Ⅳ	0.0	20.2
Cardiovascular disease (%)	0.0	21.1
Diabetes mellitus (%)	0.0	8.3
Fatty liver/alcoholic liver injury (%)	0.0	1.8
Renal disease (%)	0.0	1.8
Hyperthyroidism (%)	0.0	0.9

Data are presented as means±SD [minima to maxima], or as percentages.
TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; RMP, reference measurement procedures; TG, triglyceride; FH, familial hypercholesterolemia
[1)] Values were calculated using the postprandial samples.
[2)] Phenotypes were determined by analysis of the pre-treatment lipid profiles in the fasting state, using the classification of Frederickson.

in previous studies (Hitachi-7170; overseas brand name Hitachi-917)[5, 6)]. We prepared all reagents for the homogeneous assays in a defined order, and measured LDL-C, HDL-C, and TG concentrations once in each cycle. We repeated this process three times, yielding triplicate measurements. These procedures were the same as employed in earlier studies[5, 6, 10, 11)].

At NCVC, LDL-C and HDL-C concentrations were measured using the RMPs [LDL-C$_{RMP}$ and HDL-C$_{RMP}$] employed by the CRMLN[5, 6)]. In brief, the top fraction of ultracentrifuged serum (d>1.006, 18℃, 105,000×g, 18.5 h), which contained VLDL and chylomicrons, was separated from the bottom fraction, which contained LDL and HDL. The volume of the bottom fraction was adjusted to the original volume (5 mL) with 0.15 mol/L NaCl (Fraction 1). Then, a precipitate was obtained by addition of 40 μL of a heparin solution (5,000 units/mL) and 50 μL $MnCl_2$ (1.0 mol/L). The supernatant (Fraction 2, containing only HDL) was recovered after centrifugation of this mixture. After adjusting both volumes to the baseline levels, and measurement of cholesterol concentrations in both Fraction 1 [F1]$_{chol}$ and Fraction 2 [F2]$_{chol}$ using the Abell-Kendall method[13)], the LDL-C$_{RMP}$ concentration was defined as [F1]$_{chol}$ minus [F2]$_{chol}$, and the HDL-C$_{RMP}$ concentration as [F2]$_{chol}$.

Determination of Precision Using Pooled Sera

We prepared the pooled sera, dispensed the pool into screw-capped tubes, and stored the tubes at −80℃. After thawing, we measured LDL-C and HDL-C concentrations using all of the homogeneous assays (**Supplemental Table 1**) 30 times within a single day to determine among-run coefficients of variance (CVs), and on 21 different days to determine between-run CVs. Bias was assessed by subtracting the LDL-C$_{RMP}$ (or HDL-C$_{RMP}$) from the LDL-C$_{HA}$ (or HDL-C$_{HA}$). Bias percentages were calculated as (bias/LDL-C$_{RMP}$ or HDL-C$_{RMP}$)×100.

Statistical Analysis

We calculated two different total errors (TE$_{com}$

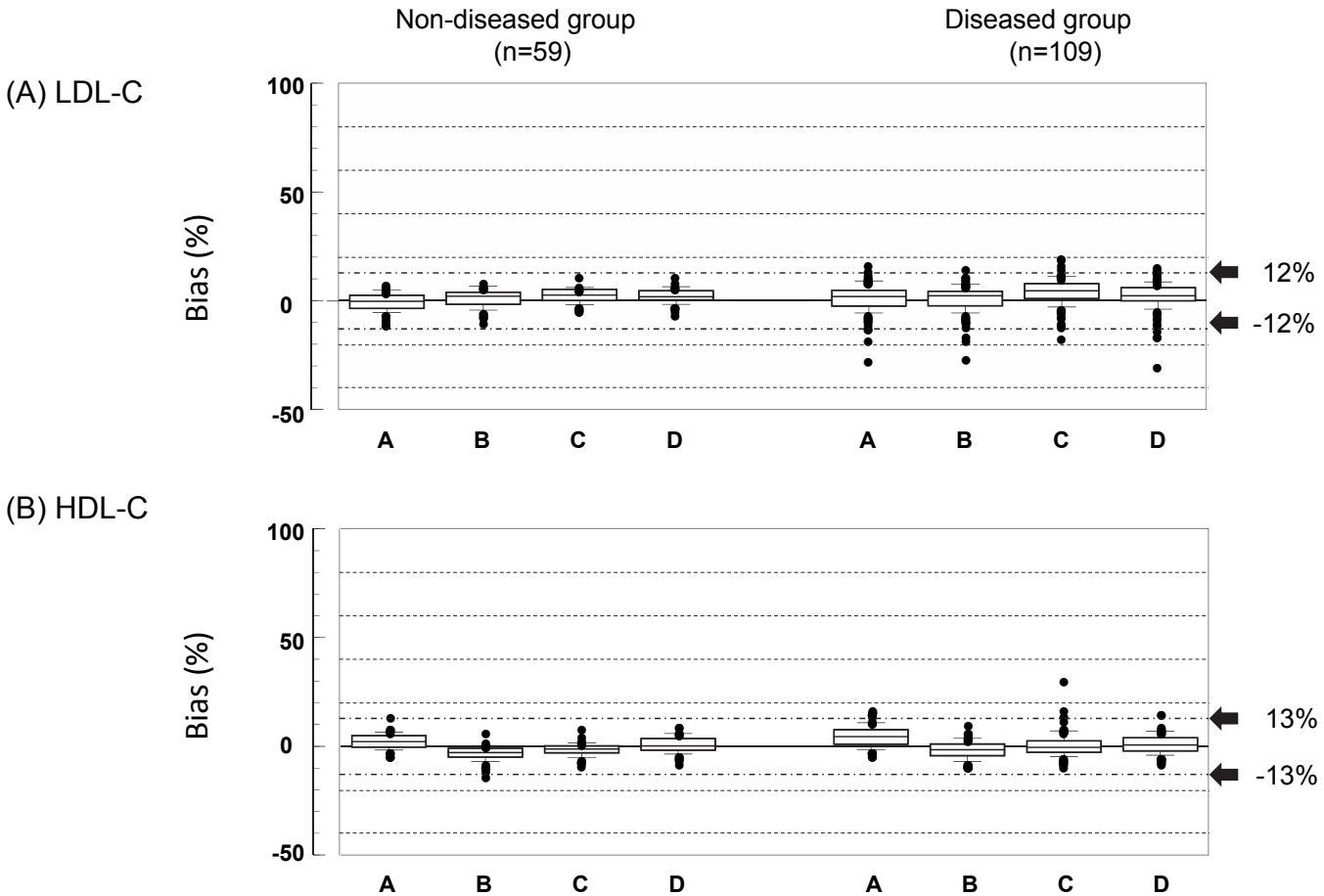

Fig. 1. Box-and-whisker plots of the %bias of the low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) homogeneous assays (HAs) in the non-diseased and diseased groups

The LDL-C and HDL-C concentrations of fresh blood samples were simultaneously measured using HAs and the reference measurement procedures (RMPs) of the Centers for Disease Control and Prevention (CDC), as described in the Methods. %bias is the percentage deviation of the HA value from the RMP value.
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

and TE_{ECA}). TE_{com} was defined as |bias (%)| + 1.96 × between-run CV (%)[14].

For error component analysis, we followed the modified methods of Nilsson[15]. The details have been described elsewhere[5]. Briefly, error component analysis is based on the hypothesis that analytical errors are derived from three components: the inter-assay CV (CV_b), the intra-assay CV (CV_e), and a CV derived from sample-specific effects (CV_d). CV_{ECA} was calculated as the square root of $[(CV_b)^2 + (CV_e)^2 + (CV_d)^2]$ where CV_b equals the between-run CV of the log-transformed data from the pooled sera. We also calculated the mean bias [ECA], the among-run CV (CV_e), and the subject-specific CV (CV_d), using the log-transformed LDL-C_{RMP} and LDL-C_{HA}, or HDL-C_{RMP} and HDL-C_{HA} of fresh samples. From these log-transformed variations, TE_{ECA} was defined as |bias [ECA] (%)| + 1.96 × CV_{ECA} (%). It should be noted that the %bias [ECA] and CV_{ECA} differ from the %bias and between-run CV used to calculate the conventional TE_{com}. We evaluated the CVs and TEs as dictated by the NCEP (**Supplemental Table 2**)[8, 9].

Results

Background of Study Participants

We collected fresh blood samples from 183 subjects. A total of 15 samples were excluded on the basis of the exclusion criteria, and/or because some essential data were missing. Finally, 59 non-diseased and 109 diseased participants were selected for further analysis (**Supplemental Fig. 1**). Those in the diseased group had common diseases treated in the outpatient clinic, although one patient had type Ⅲ hyperlipidemia and one a lipoprotein lipase deficiency (**Table 1**). More than half the samples were obtained in the postpran-

Table 2. Percentage of samples fulfilling the NCEP total error requirements for single LDL-CHA and HDL-CHA determinations.

Group	Non-diseased group (fasting, $n=16$/postprandial, $n=43$)				Diseased group (fasting, $n=66$/postprandial, $n=43$)			
Manufacturer	A	B	C	D	A	B	C	D
LDL-CHA								
Fasting samples	100.0	97.7	100.0	100.0	93.0	88.4	88.4	95.4
Postprandial samples	93.8	100.0	93.8	100.0	89.4	89.4	92.4	93.9
All samples	98.3	98.3	98.3	100.0	90.8	89.0	90.8	94.5
HDL-CHA								
Fasting samples	97.7	100.0	100.0	100.0	83.7	95.4	95.4	100.0
Postprandial samples	100.0	100.0	100.0	87.5	92.4	97.0	100.0	100.0
All samples	98.3	100.0	100.0	96.6	89.0	96.3	98.2	100.0

Percentages are based on the TE_{com} values of the first of triplicate LDL-CHA or HDL-CHA measurements on individual samples.
LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; HA, homogeneous assay
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

dial state. The samples exhibited wide ranges of TG, LDL-C, and HDL-C concentrations (**Supplemental Table 3**). In the diseased group, 7.3% of patients had TG levels >400 mg/dL.

Precision of the Homogeneous Assays

Upon analyses of pooled sera, the homogeneous assays for LDL-CHA and HDL-CHA exhibited excellent reproducibilities. For all reagents, the among-run CVs for LDL-CHA and HDL-CHA were less than 1%. The between-run CVs for LDL-CHA and HDL-CHA were about 1.0% (**Supplemental Table 4**), lower than the acceptable imprecisions of the NCEP (LDL-C, ≤4%; HDL-C, ≤4%) (**Supplemental Table 2**).

Accuracies of the Homogeneous Assays

1) Percentage Bias in Triplicate Measurements

Each bias was obtained by subtracting the LDL-CRMP from the mean of the triplicate LDL-CHA, or the HDL-CRMP from the mean of the triplicate HDL-CHA. For all reagents tested, both the non-diseased and diseased groups exhibited very low median %biases for LDL-CHA and HDL-CHA (**Fig. 1**). In the non-diseased group, the %biases for LDL-CHA and HDL-CHA lay close to the zero line for all reagents. Most individual %biases were ≤12% for LDL-C and ≤13% for HDL-C. Even in diseased patients, only a small number of samples (slightly) exceeded these values.

2) TE of Single Measurements

In this analysis, we used the first LDL-CHA or HDL-CHA obtained using triplicate measurements, and determined whether or not the calculated TEs of individual samples fulfilled the NCEP TE requirements. In the non-diseased group, all reagents for LDL-C and HDL-C surpassed the 95% acceptance criterion when fasting and postprandial samples were assayed together (**Table 2**). Little difference in analytical performance was evident between fasting and postprandial samples.

In the diseased group, the LDL-C reagents fulfilled the NCEP TE requirements in 90−95% of samples. The HDL-C reagents made by manufacturers B, C, and D met the acceptance criteria when used to assay all samples, independent of fasting status.

3) Comparison of Fasting and Postprandial Samples

For all reagents, the LDL-CHA concentrations agreed well with the LDL-CRMP concentrations in both fasting and postprandial samples, whether or not the subjects were diseased. In scatter plots, the postprandial data (**Fig. 2, red circles**) were well-superimposed on the fasting data (**Fig. 2, yellow circles**). Bland-Altman plots indicated an absence of systematic errors in the LDL-CHA concentrations. Similar findings were observed when fasting and postprandial HDL-CHA levels were assayed. In both the non-diseased and diseased groups, the postprandial data were compatible with the fasting data (**Fig. 3**).

As in previous studies, we combined the data from fasting and postprandial subjects and evaluated the analytical performance of the LDL-C and HDL-C assay reagents using the TE_{ECA} approach. In addition, we calculated TE_{com} values to allow additional comparisons. For both LDL-CHA and HDL-CHA, the TE_{com} values were much lower than the NCEP TE requirements (LDL-C, ≤12%; HDL-C, ≤13%) for all reagents (**Table 3**). The maximum TE_{com} values were about 7% for both LDL-CHA and HDL-CHA. On the other hand, the TE_{ECA} was 1.1−3.6-fold greater than the TE_{com} in the non-diseased group, and 1.4−4.4-fold greater in

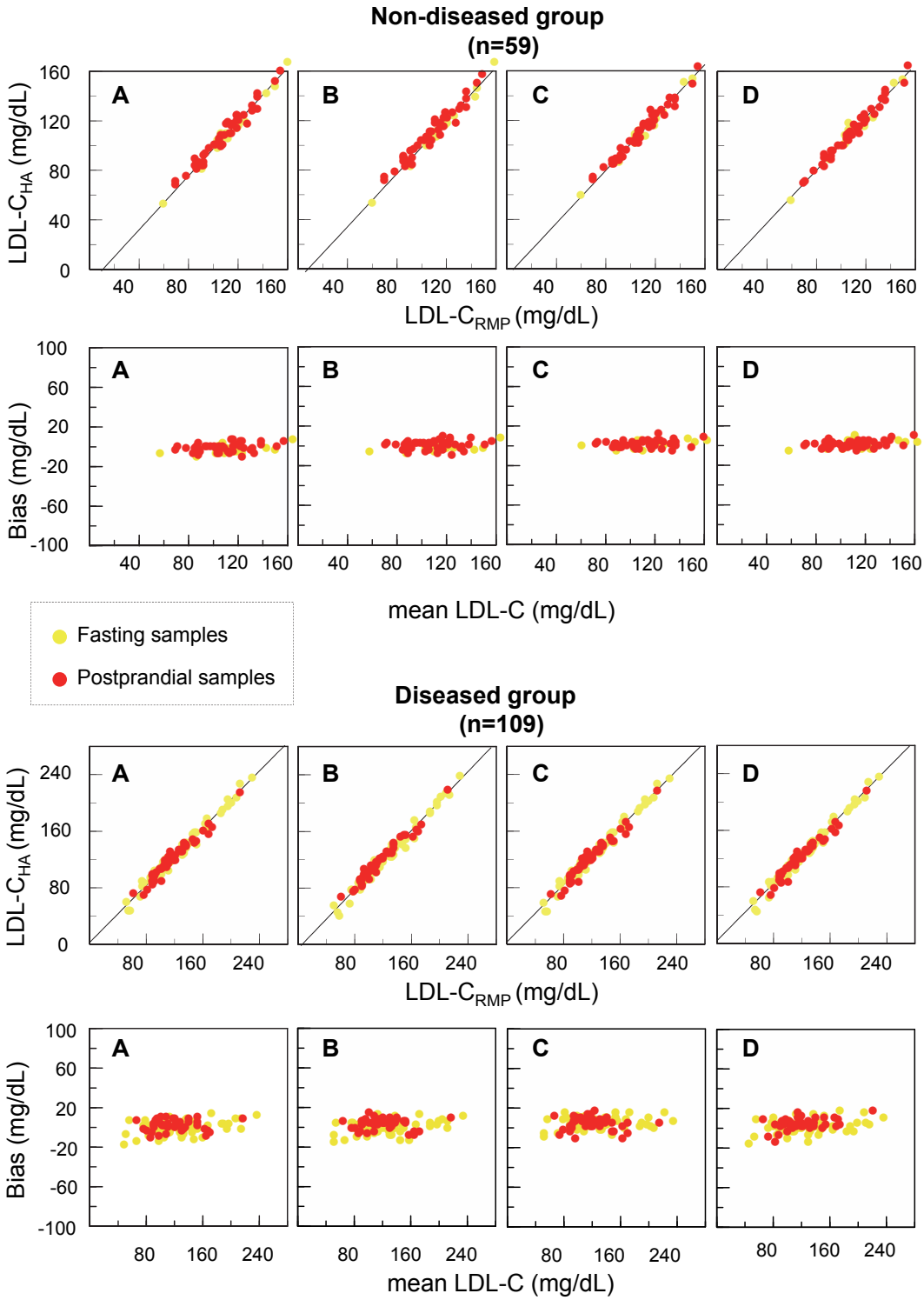

Fig. 2. Scatter plots and Bland-Altman plots of the LDL-C values measured by four HAs, and the RMPs of the CDC, in the non-diseased and diseased groups

Fresh blood samples were obtained from non-diseased and diseased subjects of either fasting (yellow circles) or postprandial (red circles) status. LDL-C levels were determined using the HAs and the RMPs of the CDC. For each group, scatter plots are shown in the upper panels, and Bland-Altman plots in the lower panels. In Bland-Altman plots, the X-axis indicates the mean LDL-C values determined by the HAs and the RMPs of the CDC, and the Y-axis indicates the difference in LDL-C levels between the two methods.
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

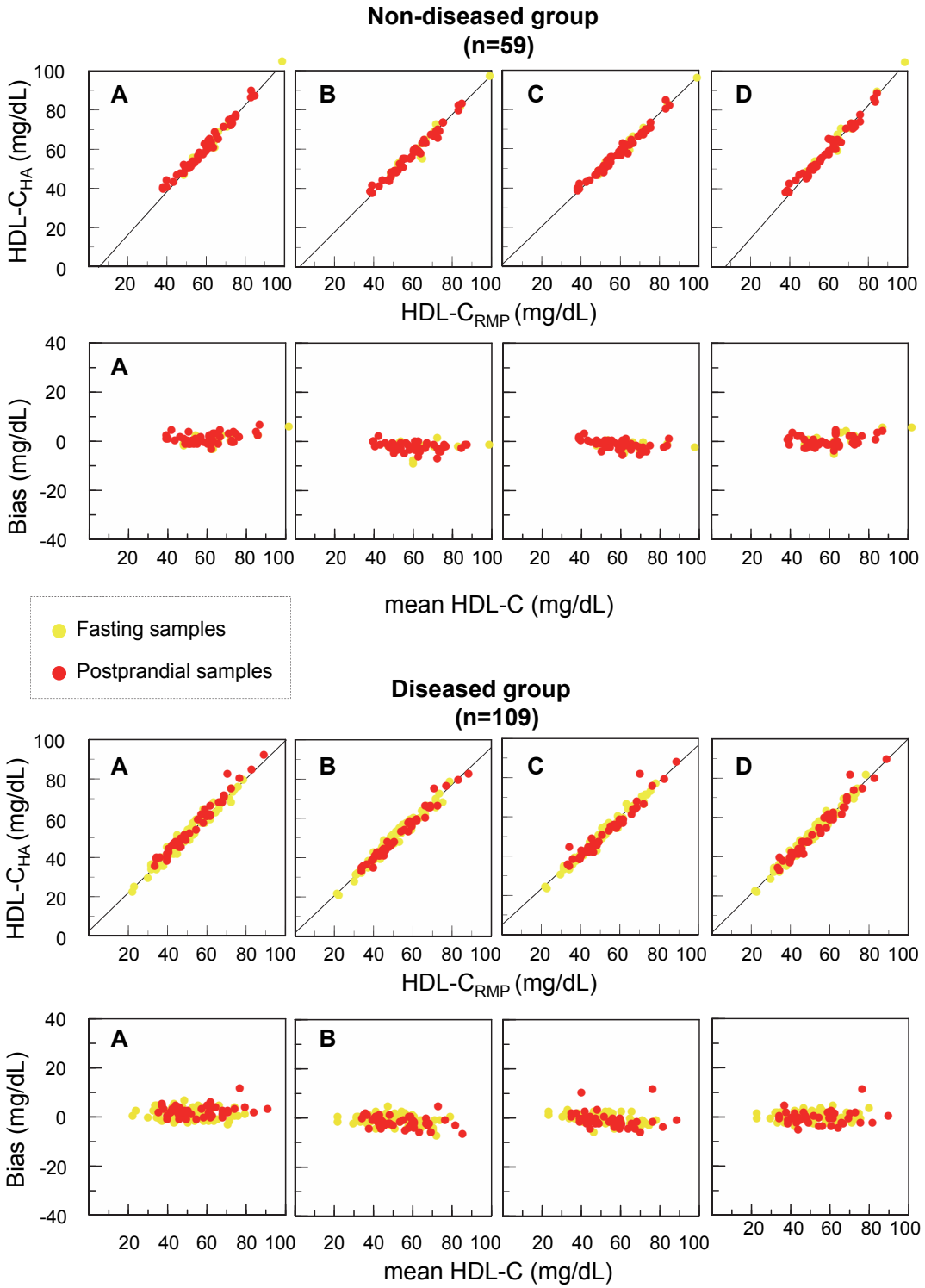

Fig. 3. Scatter plots and Bland-Altman plots of HDL-C values measured by four Has, and the RMPs of the CDC in the non-diseased and diseased groups

Fasting (yellow circles) and postprandial (red circles) samples from non-diseased and diseased subjects were subjected to HDL-C measurement using four HAs and the RMPs of the CDC. Scatter plots (upper panels) and Bland-Altman plots (lower panels) were drawn, as described in the legend to Fig. 2.
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

Table 3. Comparisons of the analytical performances of homogeneous assays for LDL-C and HDL-C between the non-diseased and diseased groups.

Group	Non-diseased group ($n=59$)				Diseased group ($n=109$)			
Manufacturer	A	B	C	D	A	B	C	D
LDL-C$_{HA}$								
Between-run CV (%)[#1]	1.01	0.99	1.44	0.96	–	–	–	–
Mean bias (%)	−0.34	1.26	2.31	2.38	1.16	1.06	4.41	2.22
TE$_{com}$ (%)	2.31	3.20	5.13	4.26	3.13	3.00	7.23	4.11
CV$_b$ (%)[#1]	0.22	0.22	0.31	0.21	–	–	–	–
CV$_e$ (%)[#2]	0.59	0.58	0.53	0.51	0.70	0.59	0.61	0.55
CV$_d$ (%)[#2]	4.00	3.25	3.00	3.97	6.92	5.81	6.66	6.40
CV$_{ECA}$ (%) ($=\sqrt{CV_b^2+CV_e^2+CV_d^2}$)	4.05	3.31	3.06	4.01	6.96	5.84	6.69	6.42
Mean bias [ECA][#3] (%)	−0.11	0.46	0.49	0.26	0.20	0.90	0.40	0.18
TE$_{ECA}$[#4] (%)	8.31	6.95	6.49	8.12	13.84	12.35	13.51	12.76
HDL-C$_{HA}$								
Between-run CV (%)	1.27	0.77	1.27	1.29	–	–	–	–
Mean bias (%)	2.44	−3.01	−1.44	0.80	4.74	−1.56	0.46	1.15
TE$_{com}$ (%)	4.92	4.51	3.93	3.33	7.23	3.07	2.95	3.68
CV$_b$ (%)	0.27	0.17	0.27	0.28	–	–	–	–
CV$_e$ (%)	0.61	0.54	0.55	0.58	0.70	0.60	0.69	0.67
CV$_d$ (%)	3.09	2.38	3.21	2.97	4.61	4.37	4.37	3.99
CV$_{ECA}$ (%) ($=\sqrt{CV_b^2+CV_e^2+CV_d^2}$)	3.16	2.45	3.27	3.04	4.67	4.41	4.43	4.06
Mean bias [ECA] (%)	0.61	−0.33	0.20	−0.74	1.19	0.13	0.28	−0.40
TE$_{ECA}$ (%)	6.80	5.13	6.61	6.70	10.34	8.77	8.96	8.36

LDL-C, low-density lipoprotein-cholesterol; CV, coefficient of variance; HDL-C, high-density lipoprotein-cholesterol; HA, homogeneous assay
[#1] Between-run CVs were calculated using the LDL-C$_{HA}$ and HDL-C$_{HA}$ values of pooled sera measured on each of 21 days. These values differ from the CV$_b$ values used in error component analysis.
[#2] CV$_e$ and CV$_d$ were calculated by error component analysis, as described in ref. #5.
[#3] Mean bias [ECA] was calculated by error component analysis, and differs from the mean bias which was used for TE$_{com}$ assessment.
[#4] TE$_{ECA}$ was calculated using CV$_{ECA}$, whereas TE$_{com}$ was calculated using between-run CV employing the following equation (TE = |mean bias (%)| + 1.96 × CV).
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

the diseased group. With the exception of the reagents of manufacturer C in the non-diseased group, the TE$_{ECA}$ values of the LDL-C reagents were greater than those of the HDL-C reagents. Although the TE$_{ECA}$ values of all HDL-C reagents met the NCEP TE requirements in both the non-diseased and diseased groups, the TE$_{ECA}$ values for the LDL-C reagents fulfilled the NCEP TE requirements only in the non-diseased group. Although the TE$_{ECA}$ values of the LDL-C reagents exceeded the cut-offs in the diseased group, the differences between the TE$_{ECA}$ values and these cut-offs ranged from 0.76% to 1.84%.

Finally, we separately compared the analytical performances of the homogenous assays used to assess fasting and postprandial samples. Both the TE$_{com}$ and TE$_{ECA}$ values of fasting and postprandial data were very similar for all reagents in both groups (**Table 4**). For all reagents, except the LDL-C reagent of manufacturer C, the TE$_{com}$ and TE$_{ECA}$ values were less than the NCEP TE requirements.

Discussion

This study indicates that the homogeneous assays for LDL-C and HDL-C from all four manufacturers are as accurate for postprandial samples as for fasting samples. We found that the TE$_{com}$ values for all reagents were less than 8% for the LDL-C reagents, and less than 9% for the HDL-C reagents, used to assay both fasting and postprandial samples in both the non-diseased and diseased groups (**Table 4**). Scatter plots and Bland-Altman plots showed that none of the homogeneous assays for LDL-C and HDL-C differed in terms of reactivity when used to assay fasting and postprandial samples (**Figs. 2** and **3**).

The LDL-C concentration is one of the most important risk markers of cardiovascular diseases, and is recognized as such in many countries[16-22]. Although

Table 4. Comparison of the analytical performances of homogeneous assays for LDL-C and HDL-C between fasting and postprandial samples of non-diseased and diseased groups.

Group	Non-diseased group (fasting, $n=16$/postprandial, $n=43$)				Diseased group (fasting, $n=66$/postprandial, $n=43$)			
Manufacturer	A	B	C	D	A	B	C	D
LDL-C$_{HA}$								
Mean bias (%)	−1.82/0.21	−0.95/2.09	1.63/2.57	1.90/2.55	0.03/2.88	−0.29/3.13	4.57/4.16	1.25/3.71
TE$_{com}$ (%)	3.80/2.18	2.89/4.03	4.45/5.40	3.79/4.44	2.00/4.85	2.24/5.07	7.39/6.99	3.14/5.60
CV$_c$ (%)	0.60/0.56	0.58/0.57	0.54/0.50	0.52/0.47	0.67/0.72	0.48/0.64	0.60/0.62	0.46/0.61
CV$_d$ (%)	3.92/3.30	3.11/3.03	2.80/3.42	3.97/2.36	4.90/6.01	5.58/5.16	5.92/6.73	4.37/5.55
CV$_{ECA}$ (%) ($=\sqrt{CV_b^2+CV_c^2+CV_d^2}$)[#2]	3.97/3.36	3.17/3.09	2.86/3.47	4.01/2.42	4.95/6.06	5.61/5.20	5.96/6.76	4.40/5.59
Mean bias [ECA] (%)	0.02/−0.44	0.51/0.32	0.55/0.36	0.46/−0.24	0.62/−0.07	0.88/0.91	0.72/0.20	0.67/−0.14
TE$_{ECA}$ (%)	7.80/7.03	6.72/6.38	6.16/7.16	8.32/4.98	10.32/11.94	11.88/11.10	12.40/13.45	9.29/11.10
HDL-C$_{HA}$								
Mean bias (%)	1.78/2.79	−3.07/−2.99	−1.59/−1.38	1.23/0.65	4.10/5.74	−1.15/−2.21	0.94/−0.27	0.83/0.12
TE$_{com}$ (%)	3.96/5.27	4.57/4.49	4.07/3.87	3.76/3.17	6.58/8.22	2.65/3.71	3.43/2.76	4.35/2.65
CV$_c$ (%)	0.61/0.62	0.49/0.65	0.50/0.65	0.58/0.60	0.64/0.74	0.57/0.61	0.75/0.64	0.57/0.72
CV$_d$ (%)	3.40/2.47	2.57/2.21	3.29/3.58	2.98/3.23	3.69/4.64	6.04/3.21	4.79/3.74	3.93/4.14
CV$_{ECA}$ (%) ($=\sqrt{CV_b^2+CV_c^2+CV_d^2}$)	3.46/2.56	2.62/2.31	3.34/3.65	3.04/3.30	3.75/4.70	6.07/3.27	4.85/3.81	3.98/4.21
Mean bias [ECA] (%)	0.72/0.33	−0.31/−0.39	0.17/0.26	−0.73/−0.78	1.43/1.04	−0.09/0.27	0.00/0.47	−0.55/−0.31
TE$_{ECA}$ (%)	7.50/5.35	5.45/4.92	6.72/7.41	6.69/7.25	8.78/10.25	11.99/6.68	9.51/7.94	8.35/8.56

LDL-C, low-density lipoprotein-cholesterol; CV, coefficient of variance; HDL-C, high-density lipoprotein-cholesterol; HA, homogeneous assay
Data in each cell are the calculated values for fasting (left) and postprandial (right) samples.
All parameters were calculated as described in the legend to Table 3.
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical

LDL-C concentrations are often calculated using Friedewald's formula[23], the accuracy depends on the TG concentration[24]. This is attributable to an increase in chylomicron levels[25], and compositional changes of TG-rich lipoproteins, in patients with hypertriglyceridemia[24, 26]. To overcome these problems, several modified equations have been proposed by different groups[24, 27-32]. However, most methods, including that of Friedewald, require subjects to fast overnight to exclude a negative bias in LDL-C measurements[27-33]. In clinical practice, it is sometimes difficult to obtain fasting samples. For example, almost 90% of patients with acute coronary syndrome (ACS) have not fasted for a sufficiently long period to allow LDL-C levels to be calculated[34]. In outpatients with diabetes mellitus, postprandial samples are preferred, because postprandial hyperglycemia and hyperlipidemia are established risk factors for atherosclerotic disorders[35-39]. Homogeneous assays are useful to determine the lipid profiles of postprandial patients.

In our previous study, we examined circadian changes in LDL-C and HDL-C concentrations determined by the homogeneous assays[40]. Although the mean TG concentration increased by 20.9% in the control group, and by 30.9% in the patients with coronary artery disease, LDL-C and HDL-C concentrations did not increase during the day. Instead, LDL-C and HDL-C decreased by 1.6 to 6.0%, and from 0.0% to 6.0% from the fasting state. These reductions were comparable to that in total cholesterol. Therefore, it is strongly suggested that homogeneous assays for LDL-C and HDL-C are not affected by postprandial increase in TG concentration. These results agree well with those of the present study.

To date, only two groups (including our group) have explored the accuracy of homogeneous assays for LDL-C and HDL-C using fresh sera from non-diseased and diseased subjects of various backgrounds[5, 6, 10, 11]. Both groups employed homogeneous assays to measure LDL-C$_{HA}$ and HDL-C$_{HA}$ levels, and also measured LDL-C$_{RMP}$ and HDL-C$_{RMP}$ using the RMPs of the CDC in a single central laboratory of a CRMLN institution. Both groups found that some homogeneous assays were of poor analytical performance[5, 6, 10, 11]. These studies prompted the manufacturers and distributors to withdraw defective reagents from the Japanese market. Therefore, all currently available homogeneous assays for LDL-C use the four original reagents (examined in the present study) or products derived therefrom. The LDL-C homogeneous assay of Kyowa

Medex employs a modified version of the prior formulation[5]. Here, this new LDL-C assay was shown for the first time to exhibit satisfactory accuracy. In terms of HDL-C assays, six original reagents (including four examined in the present study) and their derivatives remain on the Japanese market. However, homogenous assays exhibit significant diversity in terms of LDL or HDL reactivities when sera from specific patients with extremely low or high lipoprotein concentrations are assayed, and when sera from those with highly abnormal lipoprotein compositions (such as patients with cholestatic liver diseases) are evaluated[5, 6, 10, 11, 26, 41, 42]. Therefore, homogeneous assays should only be used to screen for dyslipidemia in, and evaluate therapies for, subjects without disease and those with common disorders. The results of our two studies indicate that the reagents tested here in patients with TG concentrations < 1,000mg/dL are reliable.

In the present study, we calculated two distinct TE values: TE_{com} and TE_{ECA}. In general, TE_{com} is used for quality assurance surveillance[14]. Furthermore, TE_{com} was adopted by the CDC when establishing certification protocols for manufacturers of LDL-C and HDL-C assay reagents[7-9]. Total error is a concept reflecting precision and accuracy simultaneously. Precision refers to reproducibility upon multiple measurements, whereas accuracy refers to how close the measured values are to the true values[14]. In the present study, the TE_{com} values of all reagents were much lower than the NCEP requirements (12% for LDL-C and 13% for HDL-C), whether or not the study subjects were fasting or diseased. The TE_{com} data showed that all tested reagents exhibited satisfactory analytical performance.

It is rather puzzling that the TE_{ECA} values were much worse in the study of Miller than in our current work, even though both groups ran error component analysis using similar protocols[5, 6, 10, 11]. We speculate that the measurement errors recorded by Miller *et al.*[10, 11] might have been accidentally exaggerated for the following reasons. The TE_{ECA} is based on the hypotheses that errors are exhibited by the log-transformed variations of three components; CV_b, CV_e, and CV_d. CV_d originates from patient-sample-specific effect, and is calculated as the mean square successive difference (MSSD) between the sorted measurement values, as follows:

$$s^2_{MSSD} = \frac{1}{2(n-1)} \cdot \sum_{i=1}^{n-1} (v_{i+1} - v_i)^2$$

The major assumption of this equation is that $V_{i+1} - V_i$ is both very small and continuous[5, 15]. However, not every TE_{ECA} based on the MSSD is valid in diseased samples when LDL measurements exhibit discrete distributions. All previous publications, including ours[5, 6, 10, 11], that employed the MSSD violated this continuity assumption (**Supplemental Table 2**). From the equation above, as the TE is a quadratic function of the MSSD, the TE_{ECA} is usually higher than the TE_{com} if the distributions are discrete. As shown in **Table 3**, this is in line with the fact that the TE_{com} and TE_{ECA} exhibited large discrepancies, although conventional assessments of LDL and HDL measurement accuracies were in the permissive ranges.

We should be aware that most epidemiological and clinical studies have used Friedewald's formula for LDL-C estimation although there are several methods to measure LDL-C concentrations. In the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), the first study to demonstrate the significant reduction in coronary artery disease by LDL-C lowering with cholestylamine, LDL-C concentrations were determined by ultracentrifugation and subsequent precipitation of apoB-containing lipoproteins[43]. The CDC's RMP of LDL-C measurement is based on this method with some modifications. In the early clinical trials with statins such as the West of Scotland Coronary Prevention Study (WOS-COPS)[44] and the Scandinavian Simvastatin Survival Study (4S)[45], LDL-C concentration was measured by the method of the LRC-CPPT[43]. Subsequently, many clinical trials and cohort studies used Friedewald's formula for LDL-C estimation. After the development of homogeneous assays in the late 1990s, some clinical trials including the Management of Elevated cholesterol in the primary prevention Group of Adult Japanese (MEGA) Study[46], the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction 22 (PROVE IT-TIMI 22) trial[47], and the Study of Coronary Atheroma by Intravascular Ultrasound: Effect of Rosuvastatin versus Atorvastatin (SATURN)[48], LDL-C concentrations were determined basically by the Friedewald's formula, but by the homogeneous assays when TG concentrations exceeded 300 or 400 mg/dL. More recently, some clinical and epidemiological studies used a homogeneous assay as the only method to measure LDL-C concentration[49-53]. Therefore, we need to accumulate more evidence that LDL-C concentration determined by homogeneous assays is a significant predictor of cardiovascular diseases.

We conclude that the homogeneous assays for LDL-C and HDL-C available from four manufacturers are as accurate for postprandial samples as for fasting samples. Although our series of studies on homogeneous assays has encouraged the withdrawal and improvement of reagents exhibiting poor analytical performance, future reagents require extensive preclinical evaluation to avoid unnecessary confusion.

Acknowledgments

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Conflict of Interest

We identify all potential conflicts of interests associated with this study even when the total amount of research funding and/or honoraria received did not exceed that which the Japan Atherosclerosis Society requires members to disclose. Takashi Miida received research funding from Denka Seiken and Wako, and honoraria from Denka Seiken, Kyowa Medex, and Sekisui Medical. Satoshi Hirayama received research funding from Denka Seiken and Wako. Daisaku Masuda and Shizuya Yamashita received research funding from Kyowa Medex. However, the data presented in this article were not given to the manufacturers prior to submission, and the manufacturers were not involved in data analysis.

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Supplemental Table 1. The reagents and calibrators used to measure LDL-C and HDL-C concentrations.

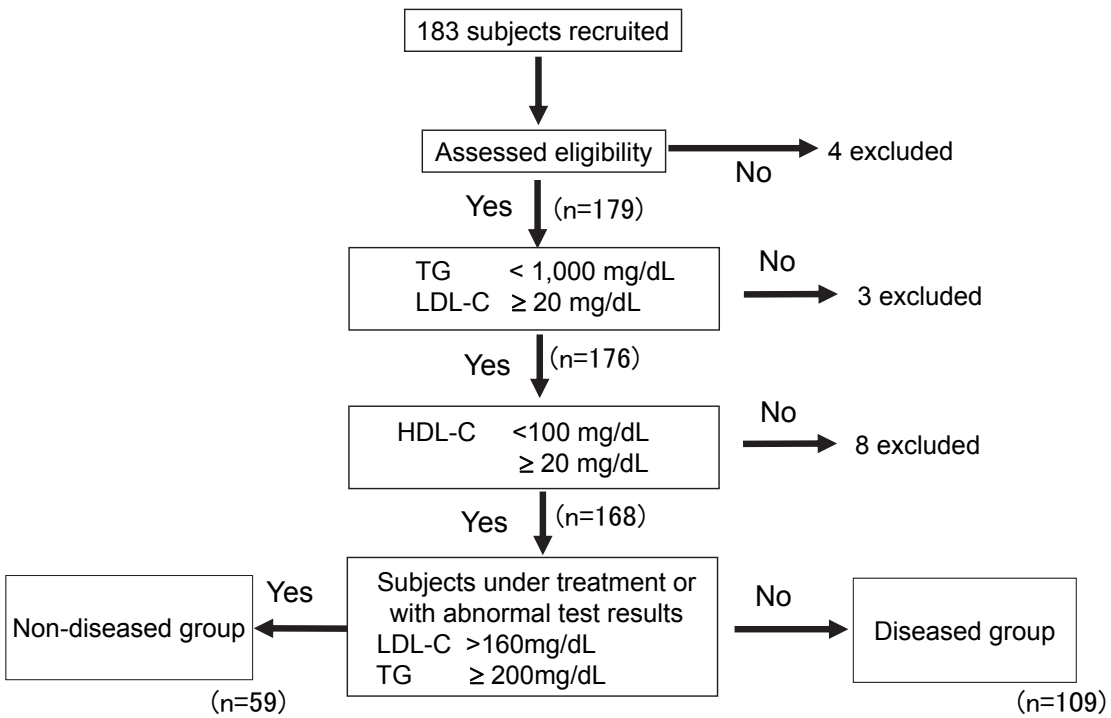
	Reagent			
Manufacturer	A	B	C	D
LDL-C				
Brand Name	LDL-EX (N) "SEIKEN"	L-Type LDL-C M	MetaboLead LDL-C	Cholestest LDL
Same reagent as in refs. #5, 6	Yes	Yes	No (modified formula)	Yes
Principle	Selective elimination method	Selective elimination method	Selective solubilization method	Selective elimination method
Calibrator	Lipid Calibrator	Multi Calibrator Lipids	MetaboLead Standard Serum	Cholestest N Calibrator
Control	Lipid Control	Lipids Control Set	MetaboLead Control Serum	Cholestest Control
Sample vol. (μL)	2.4	2.4	3.0	2.4
Reagent vol. (μL) R1/R2	180/60	210/70	180/60	240/80
Abs WL, 2nd/primary (nm)	700/600	700/600	700/600	660/546
Assay mode	2 Point End	2 Point End	2 Point End	2 Point End
Calibration	Linear	Linear	Linear	Linear
HDL-C				
Brand Name	HDL-EX "SEIKEN"	L-Type HDL-C M(2)	MetaboLead HDL-C	Cholestest N HDL
Principle	Selective elimination method	Selective elimination method	Selective inhibition method	Selective solubilization method
Calibrator	Lipid Calibrator	Multi Calibrator Lipids	MetaboLead Standard Serum	Cholestest N Calibrator
Control	Lipid Control	Lipids Control Set	MetaboLead Control Serum	Cholestest Control
Sample vol. (μL)	2.4	2.4	3.6	2.4
Reagent vol. (μL) R1/R2	180/60	210/70	180/60	240/80
Abs WL, 2nd/primary (nm)	700/600	700/600	700/600	700/600
Assay mode	2 Point End	2 Point End	2 Point End	2 Point End
Calibration	Linear	Linear	Linear	Linear
TG				
Brand Name		L-Type Triglyceride H		
Calibrator		Multi Calibrator Lipids		
Control		QAP Trol		
Sample Vol. (μL)		2.0		
Reagent Vol. (μL) R1/R2		180/90		
Abs WL, 2nd/primary (nm)		700/600		
Assay mode		2 Point End		
Calibration		Linear		

LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; R1, reagent 1; R2, reagent 2
Vol, volume; Abs WL, Absorption wavelength.
Each reagent was evaluated on a Hitachi 917 platform, sold in Japan under the brand name "Hitachi 7170".
A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.

Supplemental Table 2. The NCEP requirements for LDL-C and HDL-C measurements.

LDL-C	
Between-run CV (%)	≤4.0%
|Mean bias| (%)	≤4.0%
R^2	>0.975
TE_{com}	≤12.0%
HDL-C	
Between-run CV (%)	≤4.0%
|Mean bias| (%)	≤5.0%
R^2	>0.975
TE_{com}	≤13.0%

LDL-C, low-density lipoprotein-cholesterol; CV, coefficient of variation; HDL-C, high-density lipoprotein-cholesterol
TE_{com} is defined as=|mean bias (%)|+1.96×CV (=between-run CV).



Supplemental Fig. 1. The flow chart summarizing enrollment of the study subjects.

Supplemental Table 3. Comparisons of TG, LDL-C, and HDL-C distributions among various studies.

Principal investigator [Reference]	Miller *et al*.[#1] [2010, ref. #10; 2011, ref. #11]	Miida *et al*.[#2] [2012, ref. #5; 2014, ref. #6]	Present study
Number of samples (postprandial samples, %)			
Non-diseased group	37 (5, 13.5%)	49 (15, 30.6%)	59 (43, 72.9%)
Diseased group	138 (43, 31.2%)	124 (55, 44.4%)	109 (43, 39.4%)
Total	175 (48, 27.4%)	173 (70, 40.5%)	168 (86, 51.2%)
TG (mg/dL) (Non-diseased/diseased)			
≥ 1,000	3 (0/3)	(5, excluded)	(3, excluded)
600 ~ 999	1 (0/1)	9 (0/9)	3 (0/3)
400 ~ 599	3 (0/3)	8 (0/8)	5 (0/5)
200 ~ 399	23	29 (0/29)	34 (0/34)
< 199	145 } 168 (37/131)	127 (49/78)	126 (59/67)
LDL-C$_{RMP}$ (mg/dL)			
≥ 300	1 (0/1)	1 (0/1)	0 (0/0)
200 ~ 299	4 (0/4)	4 (0/4)	5 (0/5)
100 ~ 199	155 (36/119)	119 (37/82)	116 (43/73)
50 ~ 99		46 (12/34)	47 (16/31)
20 ~ 49	12 (1/11)	3 (0/3)	0 (0/0)
< 20	1 (0/1)	–	(1, excluded)
LDL-C$_{RMP}$, not available	2 (0/2)	–	–
HDL-C$_{RMP}$ (mg/dL)			
≥ 100	1 (0/1)	(1/5)[#3]	(6, excluded)
80 ~ 99	7 (5/2)	18 (10/8)	7 (5/2)
60 ~ 79		41 (22/19)	52 (27/25)
40 ~ 59	162 (32/130)[#1]	87 (16/71)	85 (23/62)
20 ~ 39		21 (0/21)	24 (4/20)
< 20	4 (0/4)	–	(3, excluded)
HDL-C$_{RMP}$, not available	1 (0/1)		

LDL-C$_{RMP}$, low-density lipoprotein-cholesterol concentration determined by the reference measurement procedure (RMP); HDL-C$_{RMP}$, high-density lipoprotein-cholesterol concentration determined by the RMP.

[#1] The sample numbers given with the ranges of individual lipoproteins were determined using the data of the two publications, including supplemental data.

[#2] The sample numbers given with the ranges of individual lipoproteins were counted using original data.

[#3] Those with HDL-C > 100mg/dL were included in the study subjects in ref. #5, but excluded in ref. #6.

Supplemental Table 4. Among-run and between-run CVs, determined using frozen pooled sera.

	Reagent			
Manufacturer	A	B	C	D
LDL-C				
Among-run CV (%)[#1]	0.51	0.71	0.38	0.54
Between-run CV (%)[#2]	1.01	0.99	1.44	0.96
HDL-C				
Among-run CV (%)	0.86	0.69	0.44	0.77
Between-run CV (%)	1.27	0.77	1.27	1.29
TC				
Among-run CV (%)	–	0.44	–	–
Between-run CV (%)		1.02		
TG				
Among-run CV (%)	–	0.75	–	–
Between-run CV (%)		1.62		

LDL-C, low-density lipoprotein-cholesterol; CV, coefficient of variation; HDL-C, high-density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride

We measured LDL-C and HDL-C concentrations of pooled sera, in triplicate, on 21 different days, to derive between-run CVs; or 30 times on the same day to derive among-run CVs.

A, Denka Seiken; B, Wako; C, Kyowa Medex; D, Sekisui Medical.