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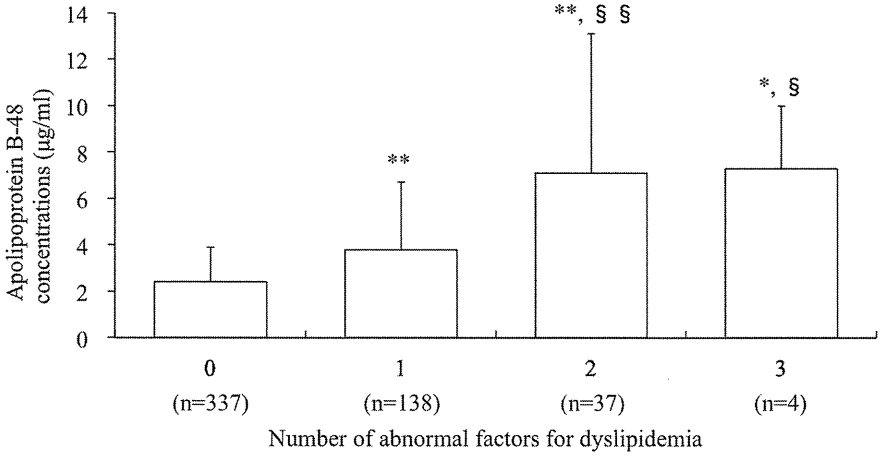

Fig. 2. Comparison of the apolipoprotein B-48 concentrations according to the cumulative number of abnormal factors for dyslipidemia.

The number of abnormal factors for dyslipidemia (a high LDL-C concentration [LDL-C ≥140 mg/dL], high TG concentration [TG ≥150 mg/dL] or low HDL-C concentration [HDL-C <40 mg/dL]) was counted in all patients. The apo B-48 concentrations were compared between four groups: patients with no abnormal factors (n=337) and those with one (n=138), two (n=37) and three abnormal factors (n=4). The values indicate the mean ± standard deviation, as follows: no abnormal factors=2.4±1.5 μg/mL, one abnormal factor=3.8±2.9 μg/mL, two abnormal factors=7.1±6.0 μg/mL, three abnormal factors=7.3±2.7 μg/mL. Statistical significance was assessed using the Mann-Whitney U test. $^{*}p<0.01$, $^{**}p<0.001$ against patients with no abnormal factors, $^{\S}p<0.05$, $^{\S\S}p<0.001$ against patients with one abnormal factor.

women: the apo B-48 concentrations of the 48 postmenopausal patients were higher than those of the 183 premenopausal patients, while the mean value of the postmenopausal patients was increased, drawing near the average observed in men (3.2±2.0 μg/mL vs 2.2±1.8 μg/mL, $p<0.001$). When all subjects were classified according to BMI, 111 patients with a BMI of ≥25 kg/m^2 were found to exhibit a statistically significantly high apo B-48 concentration in comparison with that observed in the 405 patients with a BMI of <25 kg/m^2 (4.4±3.7 μg/mL vs 2.8±2.4 μg/mL, $p<0.001$, Mann-Whitney U test) (**Fig. 1B**). The number of abnormal factors for dyslipidemia (a high LDL-C concentration [LDL-C ≥140 mg/dL], high TG concentration [TG ≥150 mg/dL] or low HDL-C concentration [HDL-C <40 mg/dL]) was counted in all patients. The apo B-48 concentrations in the patients with one (n=138), two (n=37) or three (n=4) abnormal factors for dyslipidemia were significantly higher than those observed in the patients with no abnormal factors for dyslipidemia (n=337) (**Fig. 2**). The 24 patients with MetS displayed significantly higher apo B-48 concentrations than the 492 patients without MetS (6.5±4.3 μg/mL vs 3.0±2.6 μg/mL, $p<0.001$, Mann-Whitney U test) (**Fig. 3A**)[16]. In addition, a positive correlation was observed between the apo B-48

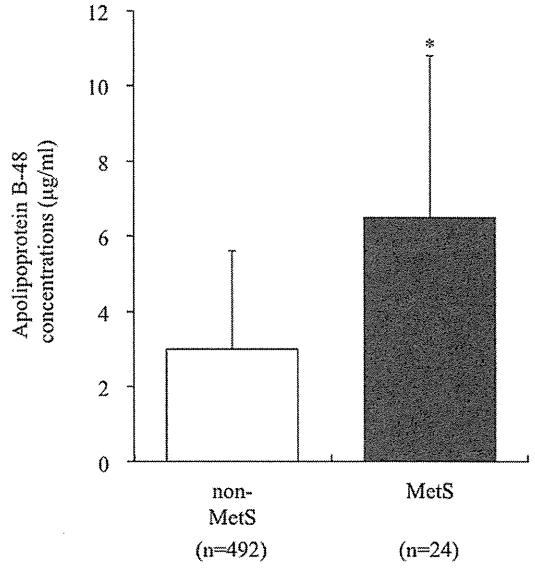

Fig. 3A. Comparison of the apolipoprotein B-48 concentrations in the subjects with or without metabolic syndrome (MetS).

The subjects were divided into two groups, MetS (n=24) and non-MetS (n=492), according to the criteria of the Japanese Society of Internal Medicine. The values indicate the mean ± standard deviation, as follows: non-MetS=3.0±2.6 μg/mL and MetS=6.5±4.3 μg/mL. Statistical significance was assessed using the Mann-Whitney U test. $^{*}p<0.001$

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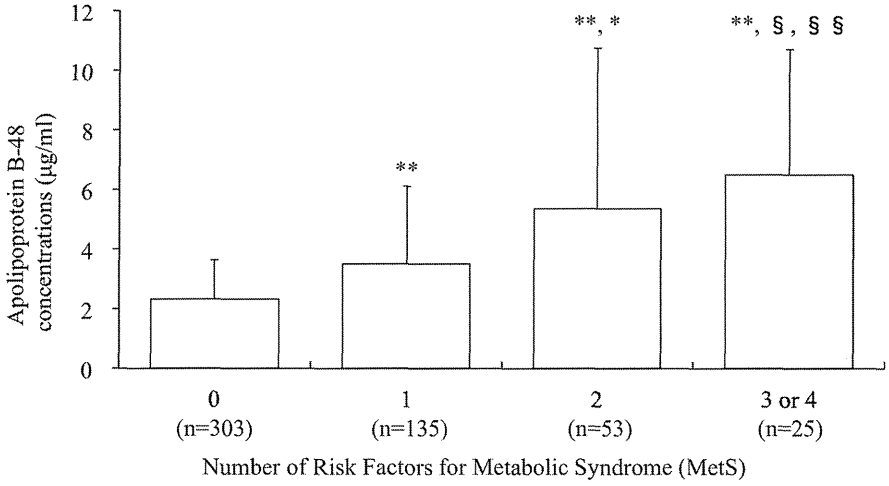

Fig. 3B. Comparison of the apolipoprotein B-48 concentrations according to the cumulative number of risk factors for metabolic syndrome (MetS).

The subjects were divided into four groups: patients with no risk factors ($n=303$) and those with one ($n=135$), two ($n=53$) and three or four risk factors ($n=25$), according to the number of abnormal factors for MetS (waist circumference, a high BP status, high TG/low HDL-C concentrations, a high FPG concentration). The values indicate the mean ± standard deviation, as follows: no risk factors = 2.3 ± 1.3 μg/mL, one risk factor = 3.5 ± 2.6 μg/mL, two risk factors = 5.4 ± 5.4 μg/mL, three or four risk factors = 6.5 ± 4.2 μg/mL. Statistical significance was assessed using the Mann-Whitney U test. $^{**}p<0.001$ against patients with no risk factors, $^{*}p<0.01$, $^{\S}p<0.001$ against patients with one risk factor, $^{\S\S}p<0.05$ against patients with two risk factors.

concentration and the number of risk factors for the components of MetS (hypertension, including a high BP status, hypertriglyceridemia, low HDL-cholesterolemia and a high fasting glucose level) (**Fig. 3B**).

Calculation of the Upper Reference Limit for the Apo B-48 Concentration in the Patients with Normolipidemia

The upper reference limit and reference interval for the apo B-48 concentration were calculated in 337 patients without parameters of abnormal lipid metabolism, as no differences in data were observed between the 152 pre- and 24 postmenopausal normolipidemic patients, as shown in **Fig. 4**; namely, the mean value among the postmenopausal patients increased (2.1 ± 1.2 μg/mL vs 2.6 ± 1.8 μg/mL, not statistically significant) approaching the average observed in the 161 men (2.7 ± 1.7 μg/mL vs 2.6 ± 1.8 μg/mL, not statistically significant). We estimated the upper reference limit for the apo B-48 concentration in 332 normolipidemic patients, excluding those with a mean value of ± 2.58 SD. The calculated mean value and range of mean ± 1.96 SD were 2.04 μg/mL (reference value) and 0.74 to 5.65 μg/mL (reference interval), respectively. Based on these results, we consider 5.7 μg/mL to be the optimum apo B-48 upper reference limit (**Fig. 5**). The reference interval and upper reference limit for the apo B-48 concentration were determined according to the results obtained with the CLEIA system (Fujirebio, Inc., Tokyo, Japan).

Discussion

The occurrence of a high TG concentration after a meal, or postprandial hypertriglyceridemia, is a risk factor for atherosclerosis. Meal-derived TG elevation results from the assembly of CMs, which contain a large quantity of TG in each particle in comparison with VLDL. CMs are immediately hydrolyzed to CM-remnants in patients with normolipidemia, whereas an abnormally high concentration of CM-remnants is observed six hours after meal intake in those with postprandial hypertriglyceridemia. Therefore, the accumulation of CM-remnants due to postprandial hypertriglyceridemia is one of the most serious risk factors for the development of arteriosclerosis-related diseases[17]. Several CM-remnant assay methods have been reported, including the retinyl palmitate method, the combination method employing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting and the remnant-like particle-cholesterol assay method[18]. However, these

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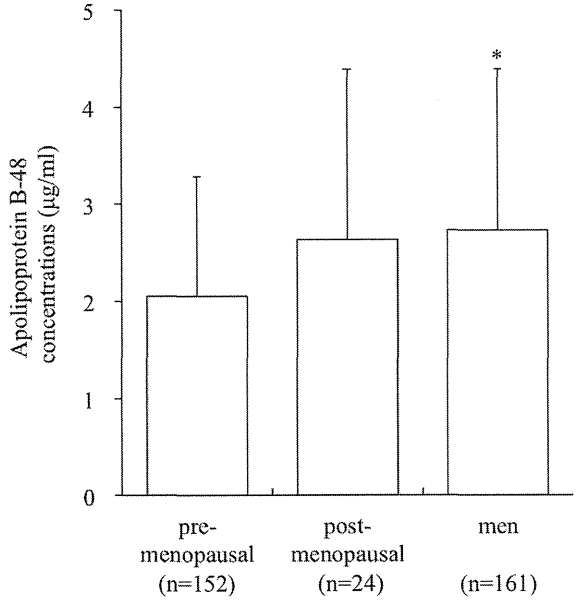

Fig. 4. Comparison of the apolipoprotein B-48 concentrations in the patients with normolipidemia.

The apolipoprotein B-48 concentrations in 161 men and 176 women (152 premenopausal patients and 24 postmenopausal patients) were compared. The values indicate the mean ± standard deviation, as follows: premenopausal = 2.1 ± 1.2 μg/mL, postmenopausal = 2.6 ± 1.8 μg/mL, men = 2.7 ± 1.7 μg/mL. Statistical significance was assessed using the Mann-Whitney *U* test. $^{*}p < 0.001$ against premenopausal women.

methods are associated with problems related to instability, complexity, reproducibility and inaccuracy regarding the assay target[19, 20]. In contrast, apo B-48 is a component of CMs and CM-remnants; therefore, the apo B-48 concentration is a direct marker of alteration of the meal-derived TG concentration, although the apo B-48 concentration in the peripheral blood is approximately one-fiftieth or one-hundredth of the apo B-100 concentration. Several assay methods for measuring the apo B-48 concentration using polyclonal antibodies and/or monoclonal antibodies have been reported[21, 22]. However, as the amino acid sequence of apoB-48 is completely identical to the N-terminal side of apoB-100, it is very difficult to prepare monoclonal and polyclonal antibodies. As a result, the accuracy of these ELISA methods is insufficient for the measurement of apo B-48. On the other hand, an accurate ELISA method was recently developed with the cooperation of Sakai *et al.*[9] using a highly specific monoclonal antibody to the C-terminal of apo B-48 established by Uchida *et al.*[23]. This ELISA system was subsequently improved to create a fully-automated assay system based on CLEIA[10].

In this study, we determined the reference level for the apoB-48 concentration using serum samples obtained from healthy individuals with normolipidemia. Namely, normolipidemic patients were selected by applying the diagnostic criteria for dyslipidemia of the Japan Atherosclerosis Society: (a) an LDL-C level of ≥ 140 mg/dL, (b) a TG level of ≥ 150 mg/dL and (c) an HDL-C level of < 40 mg/dL (Guidelines for the diagnosis and prevention of atherosclerotic cardiovascular disease for the Japanese)[15]. We then used the CLSI recommended method to calculate the reference level. Briefly, we estimated the upper reference limit and reference interval for the apo B-48 concentration in 332 normolipidemic patients, excluding those with a mean value above ± 2.58 SD. We thus determined the reference level for the apo B-48 concentration to be 2.04 μg/mL, the reference interval to range from 0.74 to 5.64 μg/mL and the upper reference limit to be 5.7 μg/mL. Incidentally, a different apo B-48 measuring kit (Human apo B-48 ELISA, Shibayagi, Gunma, Japan) is currently available in Japan. Therefore, the upper reference limit and reference interval for the apo B-48 concentration determined in this study should be restricted to the results obtained using the CLEIA system (Fujirebio, Inc., Tokyo, Japan). We then attempted to determine whether abnormal CM-remnant metabolism was present in the normolipidemia group. When the apo B-48 concentrations of all health checkup patients were measured, a high apo B-48 concentration was observed in the following order: men, postmenopausal women and premenopausal women. The apo B-48 concentrations also differed according to the presence or absence of obesity or MetS. The TG and LDL-C concentrations, which are affected by the apo B-48 concentrations, also differed between men and women and between pre- and postmenopausal women. The upper reference limit and reference interval for the apo B-48 concentration were estimated in patients with normolipidemia; this group also contained patients with hypertension, obesity and hyperglycemia, all of which may affect lipoprotein metabolism. In this study, we examined patients who received their annual health checkup; it was not assumed that these patients had severe metabolic disorders. Therefore, it is necessary to conduct separate studies of different patient groups, including those with relatively severe metabolic disorders.

Recent reports have highlighted the clinical usefulness of the apo B-48 concentration as a screening marker of type Ⅲ hyperlipidemia in patients with accumulated CM-remnants[9, 24] and parameter of the CM-remnants status in those with diabetes mellitus (DM) exhibiting carotid artery plaque[25]. Additionally, correlations have been reported between the apo B-48

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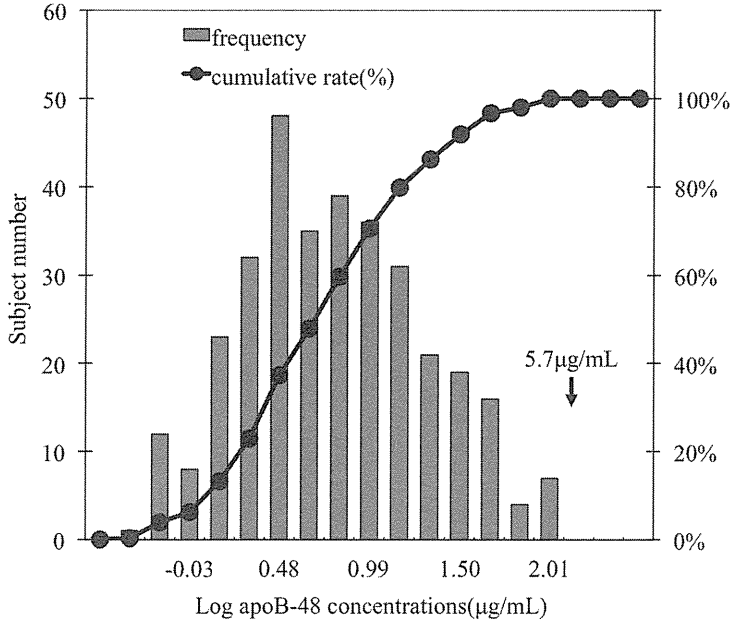

Fig. 5. Distribution of the apolipoprotein B-48 concentrations in the patients with normolipidemia.

The apolipoprotein B-48 concentration is expressed as the log concentration. The upper limit among the 332 patients with normolipidemia was found to be 5.7 μg/mL.

concentration and the carotid intima-media thickness in normotriglyceridemic (100<TG<150 mg/dL) subjects[26] as well as the status of kidney dysfunction in DM patients[27] and the incidence of CAD in ischemic heart disease patients in comparison with other risk factors, such as hypertriglyceridemia, low HDL-cholesterolemia, hypertension and/or hypoadiponectinemia[28]. Furthermore, an elevated incidence of CAD is observed in patients with a high apo B-48 concentration and the risk factors described above. Ultimately, this apo B-48 assay may have numerous applications in future studies.

Conclusion

Based on the results of this multicenter study of Japanese normolipidemic patients not taking any medications, the upper reference limit for the apo B-48 concentration in a fasting state is 5.7 μg/mL, as the mean value was found to be 2.04 μg/mL (reference value) and the mean ± 1.96 SD ranged from 0.74 to 5.65 μg/mL (reference interval).

Study Limitations

The limited number of subjects treated at two clinical facilities likely affected the results of this study.

Acknowledgements

We gratefully acknowledge the superior office work and technical assistance of Ms. Kyoko Ozawa and Ms. Risa Wada. We also appreciatively acknowledge Fujirebio, Inc. for measuring the samples using high quality standards.

Conflicts of Interest

Fujirebio, Inc. shared the costs of apo B-48 measurement. All authors have no other conflicts of interest to disclose.

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Author Contributions

M. Nishida, T. Arai, H. Yoshida, K. Yamauchi-Takihara, T. Moriyama, N. Tada and S. Yamashita supervised the progress of the clinical trial and D. Masuda, H. Hanada, N. Tada and S. Yamashita undertook the examination of the data and the preparation of this article.

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Original Article

Revised System to Evaluate Measurement of Blood Chemistry Data From the Japanese National Health and Nutrition Survey and Prefectural Health and Nutrition Surveys

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ABSTRACT

Background: We developed a monitoring system that uses total errors (TEs) to evaluate measurement of blood chemistry data from the National Health and Nutrition Survey (NHNS) and Prefectural Health and Nutrition Surveys (PHNS).

Methods: Blood chemistry data from the NHNS and PHNS were analyzed by SRL, Inc., a commercial laboratory in Tokyo, Japan. Using accuracy and precision from external and internal quality controls, TEs were calculated for 14 blood chemistry items during the period 1999–2010. The acceptable range was defined as less than the upper 80% confidence limit for the median, the unacceptable range as more than twice the cut-off value of the acceptable range, and the borderline range as the interval between the acceptable and unacceptable ranges.

Results: The TE upper limit for the acceptable and borderline ranges was 5.7% for total cholesterol (mg/dL), 9.9% for high-density lipoprotein cholesterol (mg/dL), 10.0% for low-density lipoprotein cholesterol (mg/dL), 10.4% for triglycerides (mg/dL), 6.6% for total protein (g/dL), 7.6% for albumin (g/dL), 10.8% for creatinine (mg/dL), 6.5% for glucose (mg/dL), 9.7% for γ-glutamyl transpeptidase (U/L), 7.7% for uric acid (mg/dL), 8.7% for urea nitrogen (mg/dL), 9.2% for aspartate aminotransferase (U/L), 9.5% for alanine aminotransferase (U/L), and 6.5% for hemoglobin A1c (%).

Conclusions: This monitoring system was established to assist health professionals in evaluating the continuity and comparability of NHNS and PHNS blood chemistry data among survey years and areas and to prevent biased or incorrect conclusions.

Key words: monitoring system; accuracy; precision; total error

INTRODUCTION

In November every year, the Japanese Ministry of Health, Labour, and Welfare conducts the National Health and Nutrition Survey (NHNS) in 300 unit areas. In addition, some local governments conduct an independent Prefectural Health and Nutrition Survey (PHNS) of extended samples, according to the procedures used for the NHNS. All blood samples collected in the NHNS, and some blood samples obtained in the PHNS, are analyzed by SRL Inc., a commercial laboratory in Tokyo, Japan, and measurements are performed using the same analytic system.

All measurement is subject to error. Errors are not always constant and can differ by survey year depending on variations in many factors, including the principles underlying the method, analytic instruments, reagents, calibrator, medical technologist, and other laboratory conditions.[1,2] Even if the external and internal quality controls used at SRL are sound, measurement errors are inevitable.

The monitoring system described in this study outlines principles that can be used by physicians and other health professionals who are interested in the continuity and comparability among survey years, or in the statistical results for components of physical examinations, in the

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annual NHNS and PHNS reports. Using these principles, they can determine by themselves if the results after 2011 can be used, should be used with care, or cannot be recommended for use according to the newly established TE criteria, which are based on external and internal quality controls at SRL during the 12-year period 1999–2010. The criteria for TEs were developed for use in monitoring during 2011–2015 but not for evaluating past data. Because the results of the analysis of collected data are open to the public but information on analytic errors is not, we hoped to prevent researchers from reaching biased or incorrect conclusions in their evaluations.

In 2008, we reported tentative monitoring principles that could be used to compare blood chemistry data obtained by the NHNS.[3] However, after 2008, more PHNS data became available, to allow for evaluation of local plans in Health Japan 21. In addition, the number of blood chemistry items in the NHNS varies and has tended to increase. Finally, the Metabolic Syndrome-Focused Health Checkups Program[4] in Japan began throughout the country in 2008. Due to these developments, we decided to revise the 2008 monitoring system.

METHODS

Blood chemistry items

In this study, 14 blood chemistry items (method, unit of measure at SRL) were evaluated: total cholesterol (TC) (enzymatic, mg/dL), high-density lipoprotein cholesterol (HDL-C) (homogeneous, mg/dL), low-density lipoprotein cholesterol (LDL-C) (homogeneous, mg/dL), triglycerides (enzymatic, mg/dL), total protein (Biuret, g/dL), albumin (bromcresol green, g/dL), creatinine (enzymatic, mg/dL), glucose (enzymatic, mg/dL), γ-glutamyl transpeptidase (γ-GT, γ-GTP) (Japanese Committee for Clinical Laboratory Standards [JSCC] recommended method, U/L), uric acid (enzymatic, mg/dL), urea nitrogen (enzymatic, mg/dL), aspartate aminotransferase (AST, GOT) (JSCC recommended, U/L), alanine aminotransferase (ALT, GPT) (JSCC recommended, U/L), and hemoglobin A1c (HbA1c) (latex agglutination-turbidimetric immunoassay [LA], %).

External and internal quality control

SRL participates in the External Quality Assessment of Clinical Laboratories (EQACL) program of the Japan Medical Association (JMA)[5] and the Lipid Standardization Program of the US Centers for Disease Control and Prevention/ Cholesterol Reference Method Laboratory Network (CDC/ CRMLN). SRL also has an internal quality control system that uses 2 concentrations of quality-control materials.

Accuracy

Regarding accuracy (%bias) in Table 2, the evaluation method described in the 2010 annual report on EQACL by the JMA[5] was as follows: (1) values that deviate by 3 SDs or more from the center are removed, the mean and SD are obtained according to the measurement method used by the laboratories that participated in the survey, and the coefficient of variation (CV) is calculated according to the measurement method; (2) measurement methods are arranged in order of increasing CV; (3) measurement methods with a high rank in at least 80% of laboratories are selected; (4) the mean of data from laboratories using the measurement methods selected in the previous step is calculated, 1-way analysis of variance is used to calculate intra-method variation (expressed as SD), and a common CV is obtained; and (5) the common CV is corrected for the report unit width and a corrected common CV is obtained. Using both the adjusted mean obtained from this iterative truncation method and measurement values obtained by SRL, %bias according to samples was calculated and the mean of multiple %bias (accuracy) was calculated as an index of systematic error.[6]

Precision

Regarding precision (CV%) in Table 2, SD described in the EQACL represents dispersion in all participants, not the precision of measurement by SRL. Therefore, we were given data on the assayed values for 2 concentrations of internal quality control sera that were collected during a 1-month period, including values in November every year, randomly sampled 1 measurement value/day ($n = 1$) for 20 days, after which we calculated CV from the mean value and SD as an index of random error.[7]

Total error and relevant criteria

Subsequently, TE was calculated from accuracy and precision. Regarding total error (%) in Table 2, the equation used was "accuracy (absolute value of %bias) + precision ($1.96 \times$ CV)", which is used by the US National Cholesterol Education Program (NCEP) and the Lipid Standardization Program by CDC/CRMLN.[6] The acceptable range of TE for each blood chemistry item was defined as less than the upper 80% confidence limit for the median of the 12-year period, as calculated by the nonparametric Bootstrap method (BC_a method).[8–10] Bootstrap method analyses were conducted using SAS, version 13 (SAS Institute, Inc., Cary, NC, USA). The unacceptable range was defined as more than twice the cut-off value of the acceptable range, based on evaluation criteria adopted by the US College of American Pathologists (CAP).[11] The interval between the acceptable and unacceptable ranges was classified as the borderline range. Thus, using these TE criteria, we have created a 3-level assessment of test performance.

Use in evaluating performance in 2011

We collected the results of EQACL evaluations and SRL internal quality control data in 2011 and attempted to evaluate SRL test performance in 2011 using the proposed TE criteria.

Table 1. Annual changes in numbers of assayed samples and blood chemistry items in the National Health and Nutrition Survey in Japan

Analyte	Year												*Application in 2011*
	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	
No. of assayed samples	5492	5743	5592	5413	5327	3921	3877	4319	4020	4517	4300	3930	*3515*
Total cholesterol	○	○	○	○	○	○	○	○	○	○	○	○	○
HDL cholesterol	○	○	○	○	○	○	○	○	○	○	○	○	○
LDL cholesterol	—	—	—	—	—	—	—	—	○	○	○	○	○
Triglycerides	○	○	○	○	○	○	○	○	○	○	○	○	○
Total protein	○	○	○	○	○	○	○	○	○	○	○	○	○
Albumin	—	—	—	—	○	○	○	○	○	○	○	○	○
Creatinine	—	○	—	—	—	—	—	—	—	○	○	○	○
Glucose	○	○	○	○	○	○	○	○	○	○	○	○	○
γ-GT (γ-GTP)	—	○	—	—	—	—	—	—	—	—	—	○	○
Uric acid	—	○	—	—	—	—	—	—	—	—	—	○	○
Urea nitrogen	—	○	—	—	—	—	—	—	—	—	—	—	—
AST (GOT)	—	—	—	—	—	—	—	—	—	—	—	○	○
ALT (GPT)	—	—	—	—	—	—	—	—	—	—	—	○	○
HbA1c	—	—	—	○	○	○	○	○	○	○	○	○	○

White circles show blood chemistry items assayed in the corresponding year.
Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; γ-GT (γ-GTP), γ-glutamyl transpeptidase; AST (GOT), aspartate aminotransferase; ALT (GPT), alanine aminotransferase; HbA1c, hemoglobin A1c.

Criteria for CDC/CRMLN lipid standardization

To evaluate lipid measurement, the following NCEP criteria were used: TC—accuracy within 3% of target value for CDC/CRMLN reference measurement procedure, precision as CV of 3% or less, and TE of 9% or less; HDL-C—accuracy within 5% of target value, precision as CV 4% or less, and TE of 13% or less; LDL-C—accuracy within 4% of target value, precision as CV of 4% or less, and TE of 12% or less.[12]

Implementation survey for PHNS

In 2007, our study group surveyed prefectural governments regarding implementation of their PHNS, including dietary intake surveys and blood examination, and collected additional data on the number of blood samples they entrusted to SRL for analysis in 2011.[13]

RESULTS

Table 1 shows annual changes in blood chemistry items measured and number of analyzed NHNS samples assayed at SRL during 1999–2010. Items measured every year since 1999 were TC, HDL-C, triglycerides, total protein, and glucose. LDL-C, albumin, creatinine, and HbA1c were recently added to these 5 items. Other items, such as γ-GT (γ-GTP), uric acid, urea nitrogen, AST (GOT), and ALT (GPT), have been measured infrequently. The average number of assayed samples in the NHNS was 4704 during 1999–2010.

Table 2 shows measurement performance at SRL, based on the EQACL of the JMA. On the basis of these calculations, criteria for acceptable, borderline, and unacceptable ranges were established, as shown in the column labeled Proposed TE Criteria.[10] The upper limit of TE in the new acceptable and borderline ranges for each item was 5.7% for TC, 9.9% for HDL-C, 10.0% for LDL-C, 10.4% for triglycerides, 6.6% for total protein, 7.6% for albumin, 10.8% for creatinine, 6.5% for glucose, 9.7% for γ-GT (γ-GTP), 7.7% for uric acid, 8.7% for urea nitrogen, 9.2% for AST (GOT), 9.5% for ALT (GPT), and 6.5% for HbA_{1C}. Concerning the acceptable TE range, 50% of the evaluation limits (1 side) of the CAP evaluation criteria, which are widely used worldwide, was adopted and is shown as a reference in the column labeled CAP TE in Table 2.[11] TE criteria for HbA_1c were not established in the CAP survey. Although the acceptable range for γ-GT (γ-GTP) is expressed as SD in the CAP evaluation criteria, 7.5% was used as the corresponding value.

A 2007 implementation survey showed that 25 (53.2%) of the 47 prefectures in Japan independently performed blood examinations. Blood examinations were entrusted to SRL by 21 of the 25 prefectures and to a local laboratory by the other 4. A total of 15 096 samples from the 21 prefectures were analyzed by SRL. This number was 3.2 times the mean sample number (4704) of the NHNS (Table 1). Additionally, according to the 2011 survey, 20 (42.6%) of the 47 prefectures performed blood examinations.

Blood examinations were entrusted to SRL by 15 of the 20 prefectures and to a local laboratory by the other 5. A total of 7063 samples from the 15 prefectures were analyzed by SRL. This number was 1.5 times the average sample number of the NHNS (Table 1). The survey of the current situation in each prefecture was not conducted systematically, and measurement items are different for each prefecture.

In 2011, urea nitrogen was not assayed in the NHNS or PHNS; thus, there was a total of 13 items. When TE was calculated for each SRL item in 2011 to establish proposed TE

Table 2. SRL performance based on JMA external quality assessment and SRL internal quality control system (unit, %)

Analyte	Performance	Measurement performance by SRL during observation period: Year 1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	Median (LL, UL of 80% CL)	Proposed TE Criteria: Acceptable	Borderline	Unacceptable	*Application to new data: Performance in 2011*	*Evaluation by proposed TE criteria in 2011*	(For reference) CAP TE Criteria
Total cholesterol	Accuracy (%bias)	0.19	−0.48	0.27	0.34	−0.15	−0.06	0.13	−0.82	−1.31	−1.45	−0.82	−0.66	−0.32 (−0.74, 0.04)				*0.19*		
	Precision (CV%)	1.7	1.6	1.3	1.1	1.6	1.0	1.2	1.0	0.7	0.8	0.7	0.7	1.1 (0.9, 1.3)				*0.8*		
	Total Error (%)	3.6	3.6	2.7	2.5	3.3	2.1	2.4	2.7	2.7	3.0	2.2	2.0	2.7 (2.5, 2.9)	<2.9	2.9–5.7	≥5.8	*1.8*	*acceptable*	5.0
HDL cholesterol	Accuracy (%bias)	−0.19	−1.57	−1.09	1.60	0.02	−0.33	0.70	1.29	−2.89	−0.90	−0.17	−0.68	−0.26 (−0.79, −0.08)				*−2.00*		
	Precision (CV%)	2.4	1.8	1.6	2.1	2.0	1.5	1.6	2.3	1.5	1.8	1.3	1.7	1.8 (1.6, 1.9)				*1.7*		
	Total Error (%)	4.9	5.1	4.2	5.7	4.0	3.2	3.8	5.7	5.8	4.4	2.7	4.0	4.3 (4.0, 5.0)	<5.0	5.0–9.9	≥10.0	*5.3*	*Borderline*	15.0
LDL cholesterol	Accuracy (%bias)	—	—	—	—	—	—	—	—	−0.39	1.95	−2.45	0.50	0.06 (−1.42, 1.23)				*0.63*		
	Precision (CV%)	—	—	—	—	—	—	—	—	1.2	2.0	0.9	1.4	1.3 (1.1, 1.7)				*1.1*		
	Total Error (%)	—	—	—	—	—	—	—	—	2.7	5.9	4.2	3.2	3.7 (3.0, 5.0)	<5.0	5.0–10.0	≥10.1	*2.8*	*acceptable*	15.0
Triglycerides	Accuracy (%bias)	1.91	−0.58	−1.34	0.37	1.56	−0.12	−0.36	0.00	−0.97	−1.10	−1.86	−1.67	−0.47 (−1.04, −0.06)				*−0.18*		
	Precision (CV%)	1.8	2.3	2.4	2.6	2.3	1.5	1.4	2.3	1.0	1.0	1.1	1.2	1.7 (1.3, 2.3)				*1.6*		
	Total Error (%)	5.5	5.2	6.1	5.5	6.2	3.0	3.1	4.6	2.9	3.1	4.0	4.0	4.3 (3.6, 5.3)	<5.3	5.3–10.4	≥10.5	*4.4*	*acceptable*	12.5
Total protein	Accuracy (%bias)	−0.27	−0.12	0.46	−0.24	−0.14	−0.28	0.19	−0.07	−0.39	1.59	−0.58	1.78	−0.13 (−0.26, 0.06)				*3.21*		
	Precision (CV%)	1.4	1.0	0.9	1.5	2.0	1.6	1.4	1.5	1.5	1.6	1.0	1.3	1.5 (1.4, 1.5)				*1.3*		
	Total Error (%)	3.0	2.1	2.2	3.2	4.1	3.4	2.9	3.0	3.3	4.7	2.5	4.3	3.1 (3.0, 3.4)	<3.4	3.4–6.6	≥6.7	*5.8*	*Borderline*	5.0
Albumin	Accuracy (%bias)	−2.43	−0.75	0.45	−1.12	0.64	0.12	−0.06	0.11	1.05	−0.28	−1.14	0.46	0.03 (−0.52, 0.29)				*5.19*		
	Precision (CV%)	1.7	1.3	2.0	1.8	1.9	1.2	1.6	1.1	0.9	1.2	1.0	1.2	1.3 (1.2, 1.6)				*1.0*		
	Total Error (%)	5.8	3.3	4.4	4.6	4.4	2.5	3.2	2.3	2.8	2.6	3.1	2.8	3.1 (2.8, 3.8)	<3.8	3.8–7.6	≥7.7	*7.1*	*Borderline*	5.0
Creatinine	Accuracy (%bias)	−2.24	1.93	−0.08	−0.34	0.15	0.19	−0.76	−0.55	−0.76	−1.25	−0.54	−4.18	−0.55 (−0.76, −0.21)				*−2.77*		
	Precision (CV%)	1.5	2.6	3.7	2.0	1.9	2.3	1.8	2.3	1.7	2.3	1.3	1.8	2.0 (1.8, 2.3)				*1.7*		
	Total Error (%)	5.1	7.1	7.2	4.3	3.9	4.8	4.3	5.0	4.1	5.8	3.1	7.7	4.9 (4.3, 5.5)	<5.5	5.5–10.8	≥10.9	*6.1*	*Borderline*	7.5
Glucose	Accuracy (%bias)	0.42	−0.58	−0.39	−0.31	0.17	−0.06	0.76	0.53	−0.83	−0.04	0.01	−0.74	−0.05 (−0.35, 0.09)				*−0.47*		
	Precision (CV%)	1.4	1.0	1.7	1.2	1.4	1.4	1.4	1.5	1.5	0.8	0.8	1.0	1.4 (0.8, 0.8)				*1.1*		
	Total Error (%)	3.1	2.5	3.7	2.7	3.0	2.7	3.5	3.5	3.8	1.6	1.6	2.7	2.9 (2.7, 3.3)	<3.3	3.3–6.5	≥6.6	*2.6*	*acceptable*	5.0
γ-GT (γ-GTP)	Accuracy (%bias)	0.74	−0.01	−0.24	0.82	0.37	−0.13	−0.48	−0.83	−1.50	0.45	−0.75	−1.04	−0.19 (−0.62, 0.18)				*−1.39*		
	Precision (CV%)	1.8	1.8	1.6	1.7	2.3	1.3	2.0	2.1	1.9	2.0	2.5	2.1	2.0 (1.8, 2.1)				*1.8*		
	Total Error (%)	4.2	3.5	3.4	4.2	4.8	2.7	4.4	5.0	5.2	4.4	5.7	5.2	4.4 (4.2, 4.9)	<4.9	4.9–9.7	≥9.8	*4.9*	*acceptable*	7.5
Uric acid	Accuracy (%bias)	0.21	−0.59	−0.43	0.25	−0.26	0.81	−0.44	0.88	−0.44	−0.56	0.31	1.26	−0.03 (−0.44, 0.28)				*1.11*		
	Precision (CV%)	2.1	2.1	1.4	1.5	1.4	1.4	1.8	1.5	1.6	1.1	1.3	1.6	1.5 (1.1, 1.1)				*1.1*		
	Total Error (%)	4.4	4.8	3.2	3.2	3.1	3.6	4.0	3.8	3.6	2.7	2.9	4.4	3.6 (3.2, 3.9)	<3.9	3.9–7.7	≥7.8	*3.3*	*acceptable*	8.5
Urea nitrogen	Accuracy (%bias)	−1.69	0.16	0.25	1.74	−0.17	0.75	−0.33	0.69	−2.86	—	—	1.58	0.21 (−0.25, 0.69)				*not assayed*		
	Precision (CV%)	1.3	1.2	1.2	1.7	1.8	1.1	1.9	1.4	1.5	—	—	1.5	1.5 (1.3, 1.6)				*not assayed*		
	Total Error (%)	4.3	2.6	2.7	5.1	3.7	3.0	4.1	3.4	5.8	—	—	4.5	3.9 (3.3, 4.4)	<4.4	4.4–8.7	≥8.8	*not assayed*		4.5
AST (GOT)	Accuracy (%bias)	3.03	−0.43	0.21	−0.07	1.37	0.59	−0.60	0.25	−1.25	0.51	0.71	0.64	0.38 (0.07, 0.62)				*−0.37*		
	Precision (CV%)	1.7	1.8	1.3	1.1	2.1	1.4	1.9	1.5	2.2	1.5	1.6	2.2	1.7 (1.5, 1.9)				*1.8*		
	Total Error (%)	6.3	4.0	2.7	2.3	5.5	3.4	4.4	3.3	5.6	3.5	3.8	5.0	3.9 (3.4, 4.6)	<4.6	4.6–9.2	≥9.3	*3.9*	*acceptable*	10.0
ALT (GPT)	Accuracy (%bias)	2.81	−0.22	0.38	−1.43	−0.08	1.48	1.06	−0.64	−1.47	0.95	0.88	0.37	0.38 (−0.15, 0.92)				*−1.12*		
	Precision (CV%)	1.4	1.7	1.4	1.4	2.3	1.5	2.3	2.2	2.2	1.6	1.8	2.2	1.8 (1.6, 2.2)				*2.3*		
	Total Error (%)	5.5	3.6	3.2	4.2	4.5	4.4	5.5	4.9	5.8	4.1	4.4	4.7	4.5 (4.3, 4.8)	<4.8	4.8–9.5	≥9.6	*5.6*	*Borderline*	10.0
HbA_1c	Accuracy (%bias)	—	—	−0.39	0.52	0.01	2.25	1.01	1.28	−0.34	−1.08	−0.14	−0.26	−0.07 (−0.30, 0.52)				*0.12*		
	Precision (CV%)	—	—	1.1	1.1	1.0	1.2	1.1	1.0	1.4	1.2	1.4	1.6	1.2 (1.1, 1.3)				*2.0*		
	Total Error (%)	—	—	2.5	2.7	2.0	4.6	3.2	3.2	3.1	3.4	2.9	3.4	3.1 (2.8, 3.3)	<3.3	3.3–6.5	≥6.6	*4.0*	*Borderline*	

Accuracy as an index of systematic error is expressed as %bias calculated based on JMA criteria.
Precision as an index of random error is expressed as CV calculated from SRL internal quality control data.
Total error is calculated as the sum of accuracy and precision, ie, absolute value of %bias + 1.96 × CV.
Abbreviations: JMA, Japan Medical Association; CAP, College of American Pathologists; TE, total error; LL, lower limit; UL, upper limit; CL, confidence limit; HDL, high-density lipoprotein; LDL, low-density lipoprotein; γ-GT (γ-GTP), γ-glutamyl transpeptidase; AST (GOT), aspartate aminotransferase; ALT (GPT), alanine aminotransferase; HbA1c, hemoglobin A1c.

Table 3. SRL performance based on CDC/CRMLN Lipid Standardization Program (unit, %)

Analyte	Performance	CDC Criteria	Year											Average	SD
			1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010		
Total cholesterol	Accuracy (%bias)	±3.0	0.00	−1.30	0.00	−0.90	0.30	−0.10	−0.90	−0.90	−0.90	−0.30	−0.50	0.10	−0.45	0.52
	Precision (CV%)	3.0	0.5	0.6	0.6	0.5	0.5	0.6	0.4	0.4	0.4	0.5	0.4	0.3	0.48	0.10
	Total Error (%)	9.0	1.0	2.5	1.2	1.9	1.3	1.4	1.7	1.7	1.7	1.3	1.3	0.8	1.48	0.45
HDL cholesterol	Accuracy (%bias)	±5.0	0.70	0.70	2.00	2.00	1.00	1.00	1.20	1.20	1.20	−1.00	0.00	0.00	0.83	0.85
	Precision (CV%)	4.0	1.0	1.0	1.3	1.3	1.7	1.7	1.1	1.1	1.1	1.0	0.7	0.7	1.14	0.32
	Total Error (%)	13.0	2.7	2.7	4.6	4.6	4.4	4.4	3.4	3.4	3.4	3.0	1.4	1.4	3.28	1.12
LDL cholesterol	Accuracy (%bias)	±4.0				−0.60	−0.60	−0.70	−0.70	0.30	0.30	1.70	−1.40	−1.40	−0.34	0.98
	Precision (CV%)	4.0				1.2	1.2	0.7	0.7	0.4	0.4	0.6	0.6	0.6	0.71	0.30
	Total Error (%)	12.0				3.0	3.0	2.1	2.1	1.1	1.1	2.9	2.6	2.6	2.28	0.75

Accuracy as an index of systematic error is expressed as %bias calculated based on CDC criteria.
Precision as an index of random error is expressed as CV calculated based on lipid standardization criteria of CDC.
Total error is calculated as the sum of accuracy and precision, ie, absolute value of %bias + 1.96 × CV.
Abbreviations: CDC, Centers for Disease Control and Prevention; CRMLN, Cholesterol Reference Method Laboratory Network; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

criteria, the evaluation was acceptable for 7 items (53.8%) —TC, LDL-C, triglycerides, glucose, γ-GT (γ-GTP), uric acid, and AST (GOT)—and borderline for 6 items (46.2%), namely, HDL-C, total protein, albumin, creatinine, ALT (GPT), and HbA_1c. No item was evaluated as unacceptable (Table 2).

Table 3 shows the measurement performance of SRL for TC, HDL-C, and LDL-C, based on the criteria of the Lipid Standardization Program by CDC/CRMLN. In each standardization year, performance satisfied the CDC/CRMLN criteria for clinical laboratories.

DISCUSSION

In standardization—the most advanced system of quality control assessment—target values are obtained by using globally accepted definitive or reference measurement procedures. However, in the EQACL, measurement values are collected from all participants and, after statistical analysis, adjusted mean values are obtained and used as an index of accuracy. A similar data processing method is used in external quality control assurance programs in Western countries.[14,15] This method statistically excludes extreme outliers and misreports, which improves the reliability of adjusted mean values as indices of accuracy. Such adjusted means do not represent physicochemical accuracy, as such, but are often used for practical purposes as consensus values in clinical surveys. Consensus values are often used as a substitute for accuracy when there is no established reference method, or when a reference method exists but is not used due to its complexity or technical difficulty. In this respect, we have no objection to the use of consensus values at many laboratories, such as those derived from approximately 3000 participants in the EQACL of the JMA.[5]

The sources of error in measured values include changes in: the underlying principles of the measurement method, analytic devices, sample status (fresh, frozen), reagents or reagent reactivity, calibrators and their value assignments, the skill of analytical technologists, and other laboratory conditions.[1,2,5,6] Measurement error can result in clinical examination-derived discontinuities with previously obtained results in surveys (such as retrospective case-control studies), which could markedly affect annual follow-up. In this study, we conducted detailed follow-up surveys of these factors to avoid discontinuities derived from clinical examinations. A disadvantage of using the mean value of an external quality assessment as an index of accuracy is that the method routinely used during each period has a direct influence on measurement values. For example, when an analytic method based on new measurement principles is developed and adopted at clinical laboratories, due to convenience and/or cost and time savings, changes in mean value are sometimes observed along with analytic errors.

Case 1: The routine analytic method for HDL-C changed from a precipitation method using polyanions and cations to a homogeneous method using detergent or surfactant. The new method has been adopted by many laboratories, and age-related changes in mean HDL-C values have been reported since the switch. In this former case, changes in mean HDL-C values were observed and, as a consequence, analytic errors change.[16–19]

Case 2: There has been increasing demand for more-precise creatinine analysis for people with diabetes mellitus and renal disorders, and the calibrator is changing from the old, water-soluble standard to a new serum-based reference material with high accuracy, as confirmed by gas chromatography/isotope dilution/mass spectrometry. Additionally, in many laboratories the creatinine method has changed from the classic Jaffe method to newly developed enzymatic methods. Changes in mean creatinine values have been observed with these new methods and, inevitably, analytic errors also change.[20,21]

The survey protocol agreed by the Ministry of Health, Labour, and Welfare in Japan and SRL stipulates that the same analytic system for the NHNS (BioMajesty 8060 device No. 1, JEOL Ltd.; installed in the SRL Medical Ultimate Quality Service [MUQS] Laboratory) should also be used for

blood examinations that are independently entrusted by prefectures to SRL. This protocol allows PHNS and NHNS results to be monitored in the same manner and permits PHNS data to be added to NHNS. The sample numbers of the PHNS are generally larger than those of the NHNS. However, there are 2 limitations in the use of PHNS data: the measured items differ according to prefecture, and it is possible that the analytic laboratory was changed from SRL to a local laboratory or from a local laboratory to SRL. Therefore, before using PHNS results as additional data, the laboratory responsible for the results should be confirmed. In this study, only samples measured by SRL were included.

In this study, on the basis of quality control results, target TE values for the subsequent 5 years were determined. Specifically, the acceptable limit was defined as the upper 80% confidence limit of TE. TE values above this limit were considered to be in the borderline or unacceptable range, and a caution was issued. The probability of including borderline or unacceptable ranges using these target values remains at 10% even if performance remains equal to that during the previous 12-year period. Assuming annual improvements in performance, approximately 50% of TE values in the subsequent 5-year period are expected to be within the acceptable range. In quality control, there are no absolute criteria for quality, and quality is improved by daily efforts to repeatedly establish and meet criteria. Our monitoring system uses past data to establish target values for a subsequent 5-year period, and adjustments are made by revising target values at 5-year intervals. The system is thus compatible with the idea of quality control. The TE limit for the acceptable and borderline ranges was established for monitoring during 2011–2015, not for its application to past data. Application to the year 2011 (Table 2) confirms the suitability of the proposed TE criteria. When TE falls within the acceptable or borderline ranges, annual continuity and comparability of survey results can be regarded as satisfactory. However, when TE falls within the unacceptable range, measurement values should be used with caution.

Precision is an index of the reproducibility of measurement values obtained by a laboratory. In this study, since TE was calculated using an equation, CV was limited to a singlicate value ($n = 1$) in internal quality control sera for 20 days. CV was calculated from 2 types of commercially available internal quality control serum in SRL. However, if there was a difference of 10% or more in CV between the concentrations of internal quality control materials, the higher CV was used.[7]

In lipid standardization by CDC/CRMLN,[12] the accuracy, precision, and TE for SRL measurements of TC, HDL-C, and LDL-C met CDC criteria (Table 3) for clinical laboratory use. Therefore, concerning these 3 lipid items, all results in the NHNS and the results in some PHNS can be compared with results in Western countries. However, only results obtained during the previous 9-year period are available for LDL-C, and it is desirable to use these results as a reference.

In conclusion, we used TE criteria to develop a revised 3-level assessment of test performance and evaluated the continuity and comparability of 14 blood chemistry items assayed at SRL for the NHNS and PHNS in Japan. To further improve reliability, TE performance criteria should be updated every 5 years.

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Evaluation of four different equations for calculating LDL-C with eight different direct HDL-C assays

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ABSTRACT

Background: Low-density lipoprotein cholesterol (LDL-C) is often calculated (cLDL-C) by the Friedewald equation, which requires high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG). Because there have been considerable changes in the measurement of HDL-C with the introduction of direct assays, several alternative equations have recently been proposed.

Methods: We compared 4 equations (Friedewald, Vujovic, Chen, and Anandaraja) for cLDL-C, using 8 different direct HDL-C (dHDL-C) methods. LDL-C values were calculated by the 4 equations and determined by the β quantification reference method procedure in 164 subjects.

Results: For normotriglyceridemic samples (TG < 200 mg/dl), between 6.2% and 24.8% of all results exceeded the total error goal of 12% for LDL-C, depending on the dHDL-C assay and cLDL-C equation used. Friedewald equation was found to be the optimum equation for most but not all dHDL-C assays, typically leading to less than 10% misclassification of cardiovascular risk based on LDL-C. Hypertriglyceridemic samples (≥200 mg/dl) showed a large cardiovascular risk misclassification rate (30%–50%) for all combinations of dHDL-C assays and cLDL-C equations.

Conclusion: The Friedewald equation showed the best performance for estimating LDL-C, but its accuracy varied considerably depending on the specific dHDL-C assay used. None of the cLDL-C equations performed adequately for hypertriglyceridemic samples.

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1. Introduction

In fasting human plasma, cholesterol is primarily associated with three major lipoprotein classes, namely, VLDL, LDL, and HDL [1,2]. Because LDL is a pro-atherogenic lipoprotein, whereas HDL is anti-atherogenic, the measurement of cholesterol on these 2 different types of lipoproteins is routinely performed for cardiovascular disease (CVD) risk assessment and for monitoring patients on lipid-lowering therapy [3–6]. The "gold standard" or reference method for LDL-C is called the "beta quantification" procedure, which requires the use of ultra-centrifugation to first remove chylomicrons and VLDL, followed by measurement of cholesterol in the LDL and HDL containing "bottom" fraction, selective precipitation of LDL, and measurement of the HDL cholesterol (HDL-C) in the supernatant [7]. The beta quantification procedure is labor intensive and time consuming, thus making it impractical for routine clinical laboratories. With the advent of dlDL-C assays, many laboratories now use a procedure in which cholesterol in non-LDL fractions is either masked or consumed, thus allowing the direct measurement of LDL-C without the physical separation and removal of LDL from the sample. dlDL-C assays offer many advantages, such as good precision and complete automation, and they do not require a fasting sample [6].

The other common procedure for LDL-C determination involves its estimation from fasting samples, using other lipid and lipoprotein

Abbreviations: dlDL-C, direct LDL cholesterol; dHDL-C, direct HDL cholesterol; rHDL-C, HDL cholesterol measured by the reference method; rLDL-C, LDL cholesterol measured by the reference method; cLDL-C, calculated LDL cholesterol; NCEP, National Cholesterol Education Program.

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parameters. The most widely used equation for estimating LDL-C is the Friedewald equation (LDL-C = TC − (HDL-C) − (TG/5)), which requires the measurement of serum TC, HDL-C, and TG [8]. The basis for this equation is that in a fasting sample, most cholesterol is either in LDL, HDL, or VLDL; therefore, one can calculate LDL-C by subtracting HDL-C and VLDL-C from TC. When concentration units are in mg/dl, the term TG/5 provides an estimate of VLDL-C [8,9]. Although it is only an approximation, the Friedewald equation is still commonly used for estimating LDL-C because of the extra cost involved in performing dlDL-C assays and because of the lack of specificity of some dlDL-C assays, particularly in patients with dyslipidemias [10,11].

The Friedewald equation for estimating LDL-C is also known to have many limitations. It is inaccurate in patients with hypertriglyceridemia, particularly when TG > 400 mg/dl [6]. It does not perform well for patients with type III hyperlipidemia because the *TG*/5 term for estimating VLDL-C is inaccurate when there is enrichment of lipoproteins with triglycerides [8]. Furthermore, the Friedewald equation can only be used on fasting samples because it does not account for cholesterol in chylomicrons that form in the post-prandial state [6]. The Friedewald formula also does not take into account cholesterol in intermediate-density lipoproteins or on Lipoprotein (a); therefore, cholesterol in these lipoprotein fractions are incorporated into the LDL-C value, although these same lipoprotein fractions are also frequently measured as LDL-C by the beta quantification procedure [2].

Another important limitation of the Friedewald equation is that it depends on accurate measurement of HDL-C. In the past 10 years, most clinical laboratories have switched from precipitation-based methods for measuring HDL-C to fully automated dHDL-C assays [10]. Like the direct assays for LDL-C, dHDL-C assays can be affected by various disease conditions and in some cases can have substantial biases compared to rHDL-C [10,11]. In an effort to overcome Friedewald formula limitations, several alternative equations for estimating LDL-C, which utilize the newer dHDL-C assays, have been proposed [12–14]. There has been no systematic study, however, on the performance of these alternative cLDL-C equations with all the current dHDL-C assays. In addition, many of the original studies first describing these alternative equations for cLDL-C did not compare the results to the β quantification reference procedure for LDL-C [12–14].

2. Materials and methods

2.1. Study design and patient samples

Participants for the study were recruited from the National Institutes of Health (Bethesda, MD) or the Virginia Commonwealth University Medical Center (Richmond, VA), with approval of their respective institutional review boards. Data from a previous study [10] on 145 participants with TG levels < 200 mg/dl and 19 subjects with TG levels ≥ 200 and < 400 mg/dl were used in the analysis. The study population contains 37 healthy control subjects, with the majority of the remaining subjects recruited from specialty clinics for dyslipidemia and cardiovascular disease and thus are representative of the population of patients for which accurate lipid testing is important for making clinical decisions on the use of lipid-lowering drug. Approximately one quarter of the subjects were not fasting for at least at 10 h. Details of the lipid profile, diagnosis, and use of lipid-lowering medication of each participant in the study has been previously described [10].

2.2. Lipid and lipoprotein analysis

Ultracentrifugation reference method procedures for LDL-C and HDL-C were performed at the CDC (Atlanta, GA), as previously described [10]. Direct HDL-C methods [Denka Seiken, Niigata, Japan; Kyowa Medex, Tokyo, Japan; Sekisui Medical (formerly Daiichi), Tokyo, Japan; Serotec, Hokkaido, Japan; Sysmex International Reagents, Hyogo, Japan; UMA, Shizuoka, Japan; Wako Pure Chemical Industries, Osaka, Japan; and Roche Diagnostics, Indianapolis, IN (distributor of Kyowa Medex reagents with Roche calibrator and controls)] were done on a Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN), using manufacturer-specific parameters. TC was measured with Roche reagent on a Siemens Advia 1650 analyzer (Roche Diagnostics, Indianapolis, IN). Triglycerides were measured without glycerol blanking, using Siemens Advia reagents on an Advia 1650 analyzer (Siemens Diagnostics, Tarrytown, NY). All analyses were done on fresh (<48 h) serum samples stored at 4 °C. The calibration traceability of the TC and TG methods to their respective reference methods was certified by the Centers for Disease Control and Prevention (CDC) Lipid Standardization Program.

2.3. Data analysis

Total error for cLDL-C was calculated, according to the following equation: Total error (%) = [(cLDL-C − rLDL-C) / rLDL-C] × 100. Misclassifications for CVD risk were determined by the difference in risk classification based on rLDL-C versus cLDL-C for the following NCEP risk categories based on LDL-C values: optimal for secondary prevention (<70 mg/dl), optimal (71 to 100 mg/dl), near optimal (101 to 130 mg/dl), borderline high (131 to 160 mg/dl), high (161 to 190 mg/dl), and very high (>190 mg/dl) [4,15]. The comparison of the results for cLDL-C versus rLDL-C was done by the Pearson correlation method. All values are reported in mg/dl units, and multiplication of the following conversion factors can be used to convert to SI units (mmol/l): total cholesterol, HDL-C, LDL-C: 0.0259; and triglycerides: 0.0113. Chi-square analysis was used to determine the optimum equation for cLDL-C for each of the dHDL-C methods based on the total number of subjects misclassified into an incorrect CVD risk category based on LDL-C. JMP Software (SAS Institute, Cary, NC) was used for statistical analysis. A $P < .05$ were considered statistically significant. Because the Anandaraja equation unlike the other 4 equations does not use HDL-C as a variable [8,12–14], only one cLDL-C value was calculated for each patient, whereas 8 different values of cLDL-C were calculated with the other three equations; one for each of the 8 different dHDL-C assays evaluated in this study.

3. Results

3.1. Equations for calculating LDL-C

Inspection of the 4 original equations for cLDL-C [8,12–14], after some minor algebraic manipulations, showed that the different formulas have significant similarities (Table 2). With the exception of the Anandaraja equation, they all use TC, TG, and HDL-C as variables for predicting LDL-C. The Friedewald and Vujovic equation are the most similar and only differ in the coefficient for the TG term. The Chen equation contains a coefficient less than one in front of the TC and HDL-C terms and has a smaller coefficient for the TG variable compared to the other equations. The Anandaraja equation does not contain HDL-C as a variable and therefore would not be affected by errors related to the measurement of HDL-C. The Anandaraja equation, however, does contain a relatively large fixed negative term of −28 and a different set of coefficients for the TC and TG variables than the Friedewald equation. The large fixed term that is subtracted from total cholesterol in the Anandaraja equation will act like HDL-C, so we also included it in our analysis to determine the accuracy of the equation in estimating LDL-C.

3.2. Comparison of cLDL-C equations for normotriglyceridemic subjects

The mean and range of lipid and lipoprotein values, as determined by reference method procedures, for the study participants are shown in Table 1. The performance of the 4 different cLDL-C formulas on this population was evaluated, using 8 different methods for dHDL-C for samples with TG < 200 mg/dl (Tables 3 and 4). Samples with TG > 200 mg/dl have previously been shown to lead to inaccuracies in HDL-C

measurement [10,11], and hence a separate analysis was done for samples below and above this cut point for triglycerides.

When compared to rLDL-C, the Friedewald equation for cLDL-C had correlation coefficients close to unity ($R^2 = 0.98$), with the UMA ($R^2 = 0.96$) and Wako ($R^2 = 0.97$) assays having slightly lower R^2 values. All dHDL-C assays showed a small negative proportional bias (slope = 0.94 to 0.98), but 7 out of 8 assays had a relatively large positive fixed bias (intercept = 5.52 to 11.59 mg/dl). The percent of results that exceeded the NCEP 12% total error goal for LDL-C [2] was relatively small (7.6 to 14.5%) when the Friedewald equation was used to calculate LDL-C, except for the Wako dHDL-C assay in which 24.8% of the results exceeded the total error goal.

When cLDL-C was calculated by the Chen equation, values of R^2 similar to the Friedewald equation were observed, but a slight positive proportional bias instead of a negative bias was present, and overall a smaller fixed bias was observed for most dHDL-C assays (intercept = −2.76 to 5.01 mg/dl). Most of the direct assays used for estimating cLDL-C by the Chen equation showed similar percentage of results (6.2–16.6%), exceeding the total error goal compared to the Friedewald equation, except for the UMA and Wako assays in which 22.8% and 24.8% results, respectively, exceeded the total error goal.

In the case of the Vujovic equation, values of R^2 and slopes were also very similar to the Friedewald equation, although in general smaller fixed biases were observed (intercept = −0.29 to 7.43 mg/dl) for the various dHDL-C assays. For 3 of the dHDL-C assays, namely, Denka, Kyowa, and Wako, the % of results exceeding the total error goal for LDL-C was lower than that for the Friedewald equation but was still in the range of 6.9–10.3%.

The Anandaraja equation appeared to be inferior to the Friedewald equation and all the other equations in terms of its relatively poor R^2 (0.88), large negative proportional error (0.87), and large positive fixed bias (11.1 mg/dl) (Table 4). Using the Anandaraja equation for cLDL-C, 44% of the results exceeded the total error goal.

3.3. Comparison of cLDL-C equations for hypertriglyceridemic subjects

When samples with TG between 200 and 400 mg/dl were analyzed, none of the equations for cLDL-C, including the Friedewald equation, showed good agreement with rLDL-C (Tables 3 and 4). In general, 30%–45% of the results exceeded the recommended total error for LDL-C, and none of the cLDL-C equations appeared to show a clear advantage over the others. The Chen and Vujovic equations were best for calculating LDL-C when the Wako dHDL-C test was used in hypertriglyceridemic samples, but in both cases, over 20% of the results still exceeded the total error goal recommendation.

3.4. Cardiac risk factor misclassification with cLDL-C equations

3.4.1. Normotriglyceridemic samples

Percent misclassification for each cLDL-C equation was determined for normotriglyceridemic samples (TG <200 mg/dl) (Fig. 1A–D). Each subject was classified into a CVD risk category based on rLDL-C and compared to the risk classification obtained when LDL-C was calculated by the 4 equations. Depending on the dHDL-C assay used in the calculation, there was a wide variation (5.6%–16.6%) in the degree of CVD risk misclassification when the Friedewald equation was used (Fig. 1A). The Vujovic and Chen equations also had a similar wide variation in the % of CVD risk misclassifications, depending on the dHDL-C assay used, although the Vujovic (Fig. 1B) equation tended to misclassify more subjects into a higher risk category, whereas the Chen equation (Fig. 1C) misclassified more subjects into a lower risk category. The Anandaraja equation showed the most misclassifications (27.6%) (Fig. 1D) and also displayed an overall bias for underestimating LDL-C, leading to misclassifying more patients into a lower CVD risk category.

In order to identify the best equation for each dHDL-C assay, rates of total misclassifications were compared for the combination of each one of the eight dHDL-C assays with each one of three equations (Friedewald, Vujovic and Chen). Roche, Serotec, Daiichi, Denka, and UMA dHDL-C assays all had the lowest rates of total misclassifications (5.6%, 6.9%, 7.6%, 9.7%, and 15.8%, respectively) when cLDL-C was estimated with Friedewald equation. In only 3 cases did one of the alternative equations yield a lower rate of misclassification with a particular dHDL-C assay than the Friedewald equation. The Sysmex dHDL-C assay yielded the lowest number of misclassifications when Chen equation was used (8.9%), but this difference was not statistically different compared to the misclassification rate obtained with Friedewald equation (10.4%). The Wako and Kyowa dHLD-C assays showed a trend toward less misclassifications (10.3% and 11.1%, respectively) when used in the Vujovic equation compared to the Friedewald equation, but again the difference was not statistically significant. The Anandaraja equation misclassified a total of 27.6% of normotriglyceridemic subjects, which was statistically the highest among the 4 equations investigated.

In hypertriglyceridemic (200 mg/dl > TG < 400 mg/dl) samples (Fig. 2A–D), a much greater % of total misclassifications, typically more than 40%, were observed for all cLDL-C equations. There was no statistical advantage for 1 cLDL-C equation over another, in terms of total %misclassification, in this population.

Table 1
Mean lipid and lipoprotein values in study participants.

Test	Triglycerides < 200 mg/dl ($n = 145$)	Triglycerides ≥ 200 and < 400 mg/dl ($n = 19$)
TG (mg/dl)	101 (41.2; 10–190)	263 (57.4; 208–386)
TC (mg/dl)	169 (55.2; 19–409)	198 (49.4; 120–275)
HDL-C (mg/dl)	49 (17.5; 20–126)	38 (8.7; 24–57)
LDL-C (mg/dl)	103 (45.1; 3–301)	112 (41.6; 49–196)

Numbers in parentheses refer to standard deviation and range of measured values. LDL-C and HDL-C values were determined by their respective reference methods.

4. Discussion

The following are the 3 main findings from this study: (1) the use of different dHDL-C assays has a profound effect on the accuracy of calculated LDL-C, (2) the different equations for cLDL-C can produce variable results and the optimum equation for calculating cLDL-C depends on which dHDL-C assay is used, and (3) the Friedewald equation has the best overall performance for calculating LDL-C.

Because 3 measurements, namely, TC, HDL-C, and TG, are typically used in the calculation of LDL-C, errors from any of these measurements can affect the accuracy of cLDL-C [2,6]. Based on College of American Pathologists participant summary reports for the Accuracy Based Lipid Survey (ABL-A 2011), which uses commutable fresh frozen serum samples as survey material, most TC and TG assays showed relatively good agreement with their reference methods, but dHDL-C assays do not as closely agree with rHDL-C. Even greater discrepancies between dHDL-C assays and rHDL-C were found in a recent study [10] when samples from patients with dyslipidemias were analyzed. Most dHDL-C assays were found to yield results that exceed their recommended total error goal of 13% [16]. It is not surprising, therefore, that a wide range of misclassification was found for the different cLDL-C equations, depending on the dHDL-C used (Figs. 1A–C and 2A–C). As would be expected, the dHDL-C assays that best matched their reference method appeared, in general, to yield more accurate cLDL-C results. Interestingly, however, there were some exceptions when a poorer-performing dHDL-C assay performed better with a particular cLDL-C equation. For example, the Wako dHDL-C test, which had the greatest percentage of results that exceeded the total error goal (Table 3), showed the most percentage of misclassifications for cLDL-C by the Friedewald and Chen equations but the least misclassifications for the Vujovic equation (Fig. 1A–C). Because the Vujovic equation tended to have a positive bias and overestimated cLDL-C (Fig. 1B), this bias was partially compensated by the negative bias in the Wako dHDL-C test.

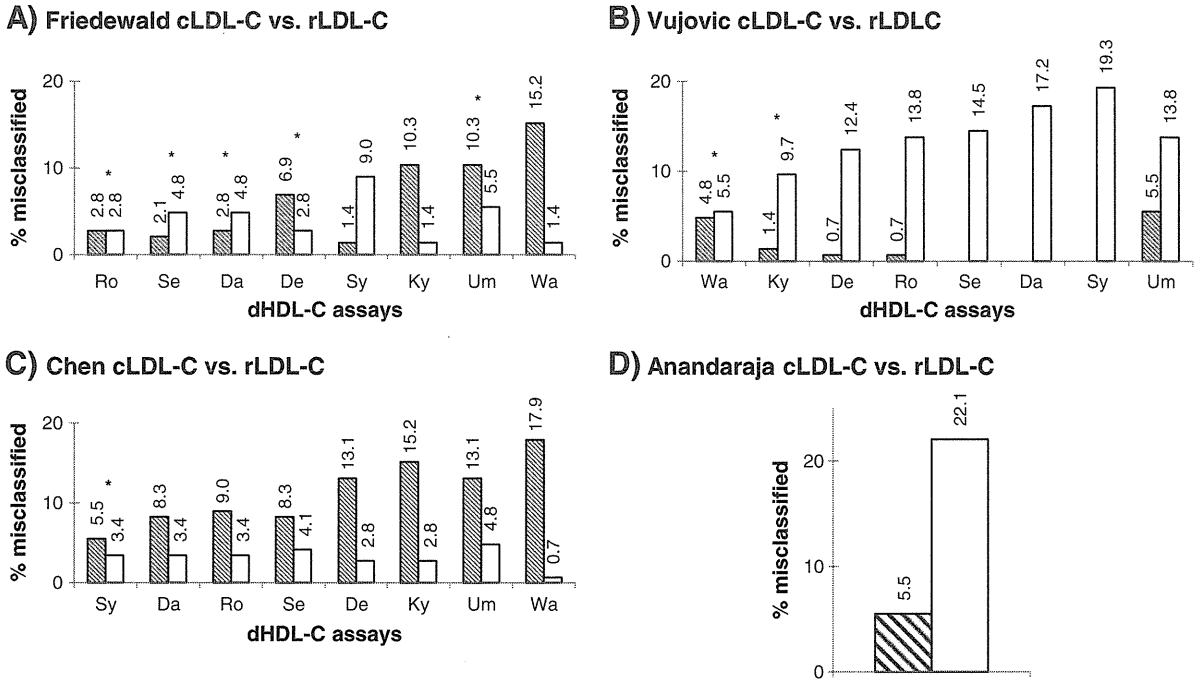

Fig. 1. Cardiovascular disease risk misclassification by various equations for cLDL-C in samples with TG < 200 mg/dl. Subjects (n = 145) with normal serum triglycerides (<200 mg/dl) were classified into cardiovascular risk categories based on LDL-C performed by the reference method and by the indicated equation and dHDL-C assay. Results are shown as % of participants who were misclassified by cLDL-C into either a lower risk category (hatched bars) or higher risk category (open bars). dHDL-C assays are displayed in ascending order according to increasing total misclassifications. Asterisks (*) indicate the equation that produced the lowest total % misclassification for the indicated dHDL-C assay when compared to other equations, using the same dHDL-C assay. dHDL-C assays: Da, Daiichi; De, Denka; Ky; Kyowa; Ro; Roche; Se; Serotec; Sy, Sysmex; Um, UMA; Wa, Wako.

Overall, the Friedewald equation for calculating LDL-C was either the best or equivalent to the other equations, in terms of CVD risk classification (Fig. 1A-D). Only in the cases of the Wako and Kyowa dHLD-C assays with the Vujovic equation and the Sysmex dHDL-C assay with the Chen equation, were fewer misclassifications observed with these equations compared to the Friedewald equation, although the differences were

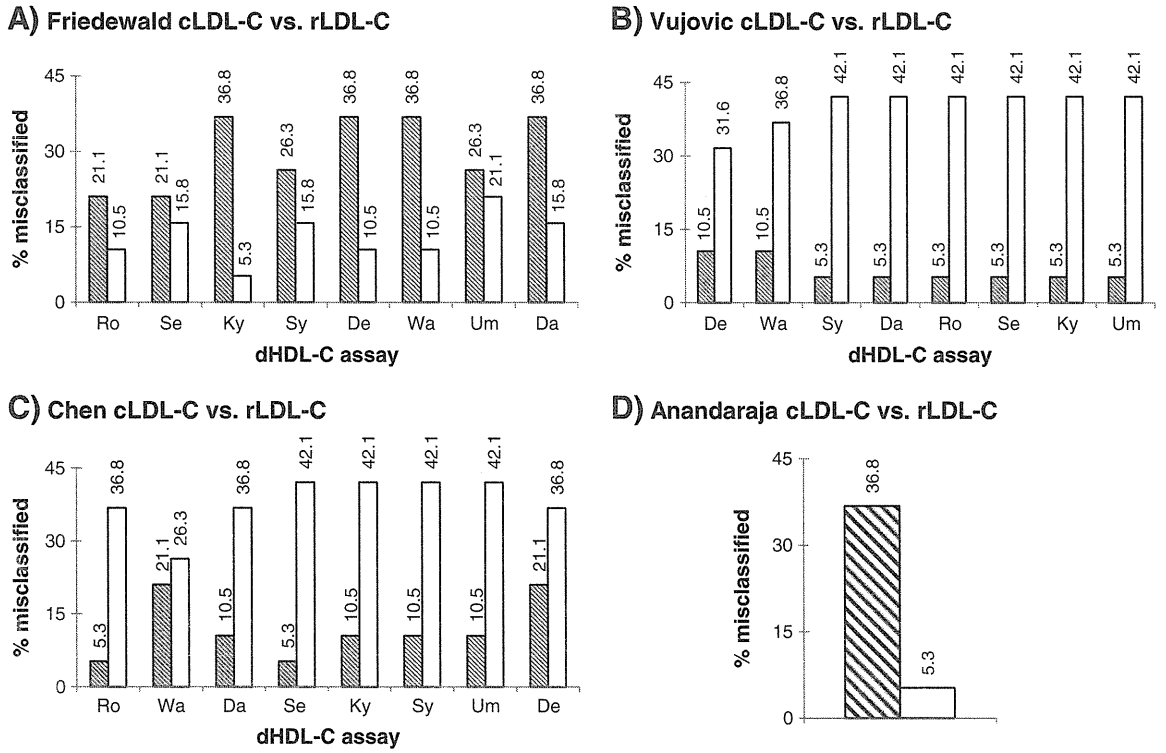

Fig. 2. Cardiovascular disease risk misclassification by various equations for cLDL-C in samples with TG between 200 and 400 mg/dl. Subjects (n = 19) with serum triglycerides between 200 and 400 mg/dl were classified into cardiovascular risk categories based on LDL-C performed by the reference method and by the indicated equation and dHDL-C assay. Results are shown as % of participants who were misclassified by cLDL-C into either a lower risk category (hatched bars) or a higher risk category (open bars). dHDL-C assays are displayed in ascending order, according to increasing total misclassification. dHDL-C assays: Da, Daiichi; De, Denka; Ky; Kyowa; Ro; Roche; Se; Serotec; Sy, Sysmex; Um, UMA; Wa, Wako.

Table 2
Formulas for calculation of LDL cholesterol.

Formula name	Original equation	Alternative form of equation
Friedewald	cLDL-C = TC − HDL-C − (TG/5)	cLDL-C = TC − HDL-C − (0.2 × TG)
Vujovic	cLDL-C = TC − HDL-C − (TG/6.58)	cLDL-C = TC − HDL-C − (0.152 × TG)
Chen	cLDL-C = (0.9 × Non-HDL-C) − (0.1 × TG)	cLDL-C = (0.9 × TC) − (0.9 × HDL-C) − (0.1 × TG)
Anandaraja	cLDL-C = (0.9 × TC) − (0.9 × TG/5) − 28	cLDL-C = (0.9 × TC) − 28 − (0.18 × TG)

Formulas are in form for units mg/dl.

not statistically significant (Fig. 1A-C). The Anandaraja equation was the least accurate for calculating cLDL-C, most likely because it does not contain a variable for HDL-C and only uses TC and TG. The Anandaraja equation does have a large constant negative term (Table 2), but this fixed term does not fully compensate for the variable amounts of HDL-C present in patient samples. Based on our analysis, there does not appear to be any advantage of the Anandaraja equation over the other cLDL-C equations except for perhaps its simplicity and reduced cost (no need for HDL-C testing). This potential advantage, however, is limited by the fact that HDL-C is frequently measured in CVD risk assessment and in the monitoring of lipid-lowering therapy.

It is well known that the Friedewald equation performs poorly in hypertriglyceridemic samples and is not recommended when TG >400 mg/dl [6]. Others have shown, however, that its performance steadily decreases with increasing TG [17,18]. For subjects with TG between 200 and 400 mg/dl, the Friedewald equation and all other equations in this study yielded relatively inaccurate cLDL-C results (Tables 3 and 4, Fig. 2A–D), with 21%–47% of the results exceeded the total error goal. dlDL-C tests are not as adversely affected by high TG and have been shown to be superior for CVD risk classification compared to cLDL-C by the Friedewald equation in hypertriglyceridemic samples [11]. The results from this study and previous findings therefore suggest that it may be best not to use any of the equations for calculating LDL-C in patients when TG > 200 mg/dl and instead use a dlDL-C assay. Alternatively, one could use non-HDL-C (TC − HDL-C) or apolipoprotein B, which, as other studies have shown, may be better cardiovascular biomarkers than LDL-C in hypertriglyceridemic subjects [4,15,19,20].

There are several limitations to this study that are important to note. First, only one specific assay was used for TC and TG (Roche and Siemens Advia reagents, respectively) in the calculations for LDL-C. As discussed above, the use of different TC and TG methods are not as likely, however,

Table 3
Comparison of cLDL-C by various equations to rLDL-C.

	TG < 200 mg/dl (n = 145)							
Direct HDL-C assay	Daiichi	Denka	Kyowa	Roche	Serotec	Sysmex	UMA	Wako
Friedewald Equation								
R^2	0.98	0.98	0.98	0.98	0.98	0.98	0.96	0.97
Slope	0.95*	0.98*	0.96*	0.95*	0.95*	0.95*	0.94*	0.94*
Intercept (mg/dl)	5.58*	5.52*	7.99*	6.10*	6.10*	3.95*	9.24*	11.59*
Mean total error, %(SD)	−1.4 (8.3)	−4.3 (11.7)	−5.9 (12.7)	−2.9 (10.7)	−2.6 (15.7)	0.6 (8.3)	−4.0 (12.2)	−8.7 (18.1)
% > total error goal[a]	8.3	12.4	13.8	10.3	7.6	9	14.5	24.8
Chen equation								
R^2	0.98	0.97	0.98	0.98	0.98	0.98	0.95	0.97
Slope	1.04*	1.06*	1.04*	1.04*	1.03*	1.04*	1.02*	1.03*
Intercept (mg/dl)	−1.05	−1.11	1.59	−0.36	−0.32	−2.76	3.18	5.01*
Mean total error, % (SD)	−2.0 (7.4)	−4.7 (9.7)	−6.1 (10.3)	−3.4 (8.8)	−3.2 (13.1)	−0.3 (8.4)	−4.4 (13.0)	−8.6 (14.3)
% > total error goal[a]	6.2	9	16.6	9	9.7	7.6	22.8	24.8
Vujovic equation								
R^2	0.98	0.98	0.98	0.98	0.98	0.98	0.95	0.97
Slope	0.94*	0.96*	0.95*	0.94*	0.94*	0.95*	0.93*	0.93*
Intercept (mg/dl)	1.38	1.32	3.94*	2.01	2.04	−0.29	5.39*	7.43*
Mean total error, % (SD)	4.8 (7.7)	1.9 (10.7)	0.3 (11.6)	3.3 (9.7)	3.6 (14.7)	6.8 (8.4)	2.2 (13.3)	−2.5 (16.5)
% > total error goal[a]	9.7	6.9	10.3	12.4	13.8	17.9	19.3	10.3
	200 mg/dl < TG < 400 mg/dl (n = 19)							
Friedewald equation								
R^2	0.94	0.94	0.95	0.95	0.95	0.94	0.94	0.94
Slope	0.91*	0.92*	0.91*	0.90*	0.89*	0.91*	0.91*	0.91*
Intercept (mg/dl)	14.39*	15.96*	15.53*	13.29*	13.83*	12.50	12.61	17.98*
Mean total error, % (SD)	−5.1 (11.3)	−8.0 (11.6)	−5.6 (11.0)	−3.8 (10.7)	−3.7 (11.0)	−3.0 (10.6)	−3.1 (11.4)	−9.8 (12.8)
% > total error goal[a]	42.1	36.8	36.8	36.8	36.8	31.6	36.8	47.4
Chen equation								
R^2	0.93	0.93	0.93	0.94	0.93	0.93	0.92	0.93
Slope	1.00*	1.02*	1.01*	1.00*	0.99*	1.00*	1.00*	1.01*
Intercept (mg/dl)	−6.43	−5.61	−6.13	−7.57	−6.42	−7.91	−8.03	−3.12
Mean total error, % (SD)	7.5 (12.2)	4.8 (10.8)	7.0 (11.7)	8.6 (11.5)	8.7 (11.6)	9.3 (12.4)	9.3 (13.0)	3.2 (10.4)
% > total error goal[a]	42.1	31.6	36.8	31.5	36.8	42.1	42.1	21.1
Vujovic equation								
R^2	0.93	0.94	0.94	0.95	0.94	0.94	0.93	0.94
Slope	0.91*	0.92*	0.91*	0.91*	0.89*	0.91*	0.91*	0.91*
Intercept (mg/dl)	1.37	2.51	1.59	0.25	1.16	−0.29	−0.31	4.81
Mean total error, % (SD)	9.8 (10.9)	6.9 (10.0)	9.2 (10.4)	11.1 (10.2)	11.2 (10.4)	11.9 (10.9)	11.8 (11.6)	5.1 (10.2)
% > total error goal[a]	42.1	31.6	42.1	42.1	47.4	42.1	42.1	26.3

* P value < 0.05.
[a] Percentage of results that exceeded the error goal of 12% for LDL-C determination as recommended by the National Cholesterol Education Program.

Table 4
Comparison of cLDL-C by Anandaraja equation to rLDL-C.

	TG < 200 mg/dl (n = 145)	200 mg/dl < TG < 400 mg/dl (n = 19)
R^2	0.88	0.95
Slope	0.87*	0.92*
Intercept (mg/dl)	11.13*	17.71*
Mean total error, % (SD)	−1.4 (53.1)	−10.8 (12.6)
% > total error goal[a]	44.1	42.1

* P value < 0.05.
[a] Percentage of results that exceeded the error goal of 12% for LDL-C determination as recommended by the National Cholesterol Education Program.

to significantly affect the calculation of LDL-C as much as dHDL-C assays because of their better standardization. The TC and TG methods used in this study also did not differ from their respective reference methods by more than 2% [10,11]. Another limitation is that only a small number of subjects with hypertriglyceridemia were examined, although based on the relatively poor accuracy observed in this small subset (Fig. 2A–D), it is unlikely that any of the equations for calculating LDL-C even in patients with a moderate increase in TG (200 mg/dl > TG < 400 mg/dl) will be fully satisfactory. Most of the direct HDL-C assays begin to show errors in samples with TG > 200 mg/dl [10,11], and hence this cut point was used in this study but comparing the cLDL-C equations at a lower TG thresholds may reveal an advantage for some equations for over the others. Another limitation of this study is that other equations besides the ones tested have been described for estimating LDL-C [17,18,21], but we have tried to focus here on the more recent and more popularly used cLDL-C equations. Finally, the samples collected for this study were largely obtained from patients with dyslipidemia and cardiovascular disease and may not be representative of the general population. The accurate determination of LDL-C in subjects with dyslipidemia, however, is obviously critical for the effective use of LDL-C in identifying and managing patients at risk for CVD.

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LDL cholesterol performance of beta quantification reference measurement procedure☆

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ABSTRACT

Background: Accurate measurement of blood lipids is crucial in cardiovascular disease risk management. The Centers for Disease Control and Prevention (CDC) Cholesterol Reference Method Laboratory Network (CRMLN) has assured the accuracy of these measurements for >20 years using beta quantification (BQ) method as reference measurement procedure (RMP) for high- and low-density lipoprotein cholesterol (HDL-C, LDL-C). Only limited data exist about the performance of the BQ RMP.

Methods: Bottom fraction cholesterol (BFC), HDL-C, and LDL-C results after ultracentrifugation from the CDC lipid reference laboratory and the Japanese CRMLN laboratory were compared using 280 serum samples measured over the past 15 years. Data were compared statistically using method comparison and bias estimation analysis.

Results: Regression analysis between CDC (x) and Osaka (y) for BFC, HDL-C, and LDL-C were $y = 0.988x + 1.794$ ($R^2 = 0.997$), $y = 0.980x + 1.118$ ($R^2 = 0.994$), and $y = 0.987x + 1.200$ ($R^2 = 0.997$), respectively. The Osaka laboratory met performance goals for 90% to 95% of the CDC reference values.

Conclusions: The BQ method by the Osaka CRMLN laboratory is highly accurate and has been stable for over 15 years. Accurate measurement of BFC is critical for the determination of LDL-C.

1. Introduction

Increased concentrations of low-density lipoprotein cholesterol (LDL-C) are associated with an increased risk for the development of cardiovascular diseases (CVDs), especially coronary heart disease (CHD) [1,2]. Other major risk factors include hypertension, diabetes mellitus, smoking, and chronic kidney diseases [3,4]. Interventions to decrease LDL-C levels can improve the risk of CVD and result in reductions in atherosclerotic lesions [5–8]. Because of the strong and positive association between LDL-C and CVD, 2013 ACC/AHA Guideline on the Treatment of Blood Cholesterol to Reduce Atherosclerotic Cardiovascular Risk in Adults [9], the Third Report of the U.S. National Cholesterol Education Program (NCEP) [10,11], the European Atherosclerosis Society [12], and Japan Atherosclerosis Society Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases 2012 [13] focused primarily on LDL-C for the categorization and treatment of dyslipidemia. Thus, measuring LDL-C has been the cornerstone of cardiovascular risk assessment and prevention for the past decades.

The precise and accurate measurement of LDL-C is of particular importance for correctly and consistently classifying individuals at risk for CVD as outlined in clinical guidelines for subsequent treatment of patients. The precision and accuracy of LDL-C measurements needed to assure that appropriate patient care was established by the NCEP [14]. The beta quantification (BQ) procedure, which relies on ultracentrifugation (UC) to separate apo B lipoprotein (apo B) particles

☆ Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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