（別添 3）

Ⅱ．研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Kondo N, Shirai K.	"Microfinance and social capital	Kawachi I, Takao S, S. V. Subramanian	Social Capital and Health from global perspectives	Springer	New York	2013	
近藤尚己・白井こころ	マイクロ・ファイナンスと健康	高尾総司・近藤克則・白井こころ・近藤尚己	ソーシャルキャピタルと健康政策：地域における活用	日本評論社	東京	2013	
白井こころ	沖縄共同体社会における高齢者とソーシャルキャピタル	等々力英美・イチローカワチ	ソーシャルキャピタルと地域の力――沖縄から考える健康と長寿	日本評論社	東京	2013	
白井こころ	沖縄県民の社会参加活動と地域帰属意識 -沖縄県におけるソーシャルキャピタルとSocial Determinants of healthへの考察	安藤由美・鈴木規之	沖縄の社会構造と意識：沖縄総合社会調査2006による分析	九州大学出版会	福岡	2012	

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nakamura M, Iso H, Kitamura A, Imano H, Kiyama M, Yokoyama S, Kayamori Y, Koyama I, Nishimura K, Nakai M, Dasti M, Vesper HW, Teramoto T, Miyamoto Y	Total cholesterol performance of Abell-Levy-Brodie-Kendall reference measurement procedure: Certification of Japanese in-vitro diagnostic assay manufacturers through CDC's Cholesterol Reference Method Laboratory Network.	Clin Chim Acta	455	127-132	2015
Nakamura M, Yokoyama S, Kayamori Y, Iso H, Kitamura A, Okamura T, Kiyama M, Noda H, Nishimura K, Nakai M, Koyama I, Dasti M, Vesper HW, Teramoto T, Miyamoto Y	HDL cholesterol performance using an ultracentrifugation reference measurement procedure and the designated comparison method	Clin Chim Acta	439	185-190	2015

工藤弥春、野上恵子、小宮紘弥、辻京子、福島英彦、西田一明	都市部における住民主体の循環器疾患予防対策50周年の取り組み	日循予防誌	50 (1)	48-51	2015
Yamagishi K, Iso H, Tsugane S	Saturated Fat Intake and Cardiovascular Disease in Japanese Population	J Atheroscler Thromb	22 (5)	435-439	2015
Iso H, Imano H, Yamagishi K, Ohira T, Cui R, Noda H, Sato S, Kiyama M, Okada T, Hitsumoto S, Tanigawa T, Kitamura A	Fasting and non-fasting triglycerides and risk of ischemic cardiovascular disease in Japanese men and women: the Circulatory Risk in Communities Study (CIRCS)	Atherosclerosis	237 (1)	361-368	2014
Hirokawa K, Nagayoshi M, Ohira T, Kajiura M, Kitamura A, Kiyama M, Okada T, Iso H	Menopausal status in relation to cardiovascular stress reactivity in healthy Japanese participants	Psychosom Med	76 (9)	701-708	2014
Hiromi Mori, Isao Saito, Eri Eguchi, Koutatsu Maruyama, Tadahiro Kato, Takeshi Tanigawa	Heart rate variability and blood pressure among Japanese men and women: a community-based cross-sectional study.	Hypertension Research	37	779-784	2014
Nakamura M, Kayamori Y, Iso H, Kitamura A, Kiyama M, Koyama I, Nishimura K, Nakai M, Noda H, Dasti M, Vesper HW, Miyamoto Y	LDL cholesterol performance of beta quantification reference measurement procedure	Clin Chim Acta	431	288-293	2014
Sano F, Ohira T, Kitamura A, Imano H, Cui R, Kiyama M, Okada T, Yamagishi K, Sankai T, Tanigawa T, Kario K, Iso H	Heavy alcohol consumption and risk of atrial fibrillation. The Circulatory Risk in Communities Study (CIRCS)	Circ J	78 (4)	955-961	2014
Shimizu Y, Imano H, Ohira T, Kitamura A, Kiyama M, Okada T, Ishikawa Y, Shimamoto T, Yamagishi K, Tanigawa T, Iso H	Adult height and body mass index in relation to risk of total stroke and its subtypes: the Circulatory Risk in Communities Study	J Stroke Cerebrovasc Dis	23 (4)	667-674	2014
Yamagishi K, Hori M, Iso H	Fish and omega-3 polyunsaturated fatty acids in relation to risk of cardiovascular disease	Nihon Rinsho	71 (9)	1552-1557	2013

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Imano H, Iso H, Kiyama M, Yamagishi K, Ohira T, Sato S, Noda H, Maeda K, Okada T, Tanigawa T, Kitamura A	Non-fasting blood glucose and risk of incident coronary heart disease in middle-aged general population: the Circulatory Risk in Communities Study (CIRCS)	Prev Med	55 (6)	603-607	2012
Yamagishi K, Sato S, Kitamura A, Kiyama M, Okada T, Tanigawa T, Ohira T, Imano H, Kondo M, Okubo I, Ishikawa Y, Shimamoto T, Iso H	Cost-effectiveness and budget impact analyses of a long-term hypertension detection and control program for stroke prevention	J Hypertens	30 (9)	1874-1879	2012
Ohira T, Maruyama M, Imano H, Kitamura A, Kiyama M, Okada T, Maeda K, Yamagishi K, Noda H, Cui R, Masuda S, Kimura H, Tachikawa K, Ishikawa Y, Iso H	Risk factors for sudden cardiac death among Japanese: the Circulatory Risk in Communities Study	J Hypertens	30 (6)	1137-1143	2012
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Nakamura M, Kiyama M, Kitamura A, Ishikawa Y, Sato S, Noda H, Yoshiike N	Revised system to evaluate measurement of blood chemistry data from the Japanese National Health and Nutrition Survey and Prefectural Health and Nutrition Surveys	J Epidemiol	23 (1)	28-34	2013

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Total cholesterol performance of Abell–Levy–Brodie–Kendall reference measurement procedure: Certification of Japanese in-vitro diagnostic assay manufacturers through CDC's Cholesterol Reference Method Laboratory Network☆

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ABSTRACT

Background: Accurate measurement of total cholesterol (TC) is important for cardiovascular disease risk management. The US Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) perform Abell–Levy–Brodie–Kendall (AK) reference measurement procedure (RMP) for TC as a secondary reference method, and implement Certification Protocol for Manufacturers. Japanese CRMLN laboratory at Osaka performed the AK RMP for 22 years, and conducted TC certification for reagent/calibrator/instrument systems of six Japanese manufacturers every 2 years for 16 years. Osaka TC performance was examined and compared to CDC's reference values.

Methods: AK RMP involved sample hydrolysis, cholesterol extraction, and determination of cholesterol levels by spectrophotometry. The Certification Protocol for Manufacturers includes comparison with AK RMP using at least 40 fresh specimens. Demonstration of average bias ≤3% and total coefficient of variation ≤3% qualified an analytical system for certification.

Results: In the AK RMP used in the Osaka CRMLN laboratory, the regression equation for measuring TC was y (Osaka) = 1.000x (CDC) + 0.032 (n = 619, R^2 = 1.000). Six Japanese manufacturers had allowable performance for certification.

Conclusions: The AK RMP for TC measurement was accurate, precise, and stable for 22 years. Six Japanese manufacturers were certified for 16 years.

1. Introduction

The association between elevated total cholesterol (TC), due to increased low-density lipoprotein cholesterol concentrations, and the risk of premature coronary heart disease (CHD) has been well documented [1–3]. CHD is the major cause of death in developed countries; accurate and reproducible TC measurements are of particular importance for correctly and consistently classifying individuals who are at increased risk for this disease, as is outlined in the clinical guidelines for the diagnosis, treatment, monitoring, and prevention of dyslipidema [4–7].

In 1988, the US Laboratory Standardization Panel [8,9] recommended that cholesterol measurements be standardized so that values are traceable to the US Centers for Disease Control and Prevention (CDC) reference measurement procedure (RMP) for cholesterol, which is a modification of Abell–Levy–Brodie–Kendall (AK) method [10,11]. As a result cholesterol tests performed in patient care as well as in clinical studies used to define clinical decision levels are standardized to the same AK RMP. This enabled the correct interpretation of cholesterol values and efficient implementation of clinical guidelines and public health efforts.

The AK RMP is linked to the National Institute of Standards and Technology (NIST) method for total serum cholesterol, which involves gas chromatography-isotope dilution mass spectrometry (GC-IDMS) [12–15]. The GC-IDMS RMP has higher specificity and selectivity than the AK RMP. Thus, results obtained with this method are not interchangeable with results from the AK RMP [10,14]. Because AK RMP-based clinical decision levels are currently being used in patient care, CDC continues to operate the AK RMP and standardizes clinical tests to this method. At the same time, it established a GC-IDMS RMP [14] to meet the increasing need for more specific and selective clinical measurements.

Epidemiological and large-scale clinical studies have been performed in Japan to investigate the risk of cardiovascular disease (CVD) using lipid measurements similar to studies conducted in Europe and the United States. The limitations of lipid measurement in Japan have historically been the comparability and accuracy of the assayed results. To overcome this limitation and to achieve traceable, accurate, and stable lipid measurements over time, an epidemiological study group at Osaka Medical Center has participated in the World Health Organization (WHO)-CDC Cooperative Cholesterol-Triglyceride Standardization Program since April 1975 [15–17]. The standardization of TC measurement at Osaka was achieved through the CDC-NHLBI Lipid Standardization Program in the 1970s and 1980s using Zak assays [18,19] and enzymatic methods [20,21], which are routinely used to analyze cholesterol levels in clinical laboratories in Japan. In 1991, the AK RMP for cholesterol was introduced to the epidemiological laboratory at Osaka, and it was standardized through the Cholesterol Reference Method Laboratory Network (CRMLN) from July 1992 to July 2014. TC certification has been performed by the CDC and CRMLN for reagent manufacturers using the Total Cholesterol Certification Protocol for Manufacturers [22]. For clinical laboratories, TC certification has been performed using the Certification Protocol for Clinical Laboratories [23]. As a result, most Japanese manufacturers and many clinical laboratories standardized TC measurements to provide traceability to the CDC's AK RMP. In 2002, the Osaka laboratory established a GC-IDMS method similar to CDC's RMP [24]. The AK RMP and GC-IDMS method have both been used continuously and simultaneously through regular CRMLN surveillance under the same measurement conditions since July 2012 to the present.

In this study, we present the accuracy and imprecision of the AK RMP obtained at Osaka during the course of 22 years. Moreover, we outline an evaluation of the accuracy, precision, and total error of reagent/calibrator/instrument systems of 6 Japanese reagent manufacturers that participated in the TC certification program for manufacturers [22] every 2 years for 16 years.

2. Materials and methods

2.1. Materials

In the CRMLN survey, all standardization pools for TC were created using sera that were prepared according to the Clinical and Laboratory Standards Institute Document C37-A [25], which defines blood collection, clotting and processing conditions. This suggests that no preservatives or additives were added nor was the material lyophilized. CRMLN survey pools include round robin samples that were provided from a participating CRMLN laboratory, and which included native specimens from patients and 12.1% (75 of 619 runs) of all samples. All survey pools were blinded to the CRMLN laboratories. Samples were shipped frozen from the CDC, and stored at −70 °C for subsequent analysis.

In the TC Certification Protocol for Manufacturers [22], participating manufacturers collected 40 or more fresh specimens from individual fasting donors. The cholesterol concentration of these specimens were distributed over a clinically meaningful range, as close as possible to the following target distribution: 20% of samples from 120 mg/dL to 180 mg/dL, 30% of samples from 181 mg/dL to 220 mg/dL, 30% of samples from 221 mg/dL to 260 mg/dL, and 20% of samples from 261 mg/dL to 400 mg/dL. The minimum amount of serum needed per sample for the AK RMP analysis is 1.5 mL. Manufacturers analyzed the specimens using their reagent/calibrator/instrument system over 20 runs, with 2 samples per run. To estimate imprecision, manufacturers should provide quality control (QC) single data obtained from 20 separate runs. The recommended concentration range for the QC material is 200 mg/dL to 240 mg/dL.

In the Certification Protocol for Clinical Laboratories [23], clinical laboratories analyzed two fresh samples in each of the three concentration regions; namely region 1: 100 mg/dL and 200 mg/dL, region 2: 200 mg/dL and 240 mg/dL and region 3: >240 mg/dL. The samples were assayed using the AK RMP at Osaka.

TC measurements were conducted at the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, at the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and at the National Cerebral and Cardiovascular Center at Osaka continuously since April 2012 (all laboratories are referred to as the Osaka laboratory).

2.2. Methods

2.2.1. AK RMP for TC at the Osaka CRMLN laboratory

The AK RMP for TC measurement is a modification of the extraction procedure by Abell et al. [10] and the original method was improved at the CDC laboratory [11]. We used a Digiflex (ICN, Biomedicals, Inc.) automatic pipettor for aspirating and dispensing standard solution, sample, and reagent. Current RMP consists of saponification of a 0.250 mL serum sample with alcoholic potassium hydroxide at 50 °C for 1 h, extraction for 20 min with hexane using a mechanical shaker in a horizontal position, evaporation of an aliquot of extract connected with a vacuum oven, and color development with Liebermann–Burchard reagent (mixed reagent of acetic anhydride, glacial acetic acid, and concentrated sulfuric acid) at 620 nm using a spectrophotometer (Beckman DU600 and DU800). The AK RMP is calibrated using the NIST Standard Reference Material (SRM) of pure unlabeled cholesterol (SRM 911c). The working standard solutions of cholesterol in alcohol consist of 25.0, 50.0, 100.0, 200.0, 300.0, and 400.0 mg/dL concentrations.

2.2.2. TC performance criteria applied to the CRMLN laboratory using AK RMP

TC performance criteria applied to the CRMLN lipid reference laboratory are summarized in Table 1. Precision was evaluated in terms of coefficient of variation (CV, %), and accuracy (%bias versus CDC reference value) was evaluated in terms of deviation (%) from the CDC reference value.

2.2.3. Statistical criteria of TC certification for manufacturers

Statistical criteria of TC certification for manufacturers are summarized in Table 2A. As a reference, statistical criteria of TC certification for clinical laboratories are summarized in Table 2B.

Table 1
TC performance criteria applied to CRMLN lipid reference laboratory using AK RMP.

Lipid	Precision criterion	Accuracy criterion
TC	Coefficient of variation ≤ 1%	Bias (deviation from CDC reference value) ± 1%

CRMLN: Cholesterol Reference Method Laboratory Network. AK RMP: Abell–Levy–Brodie–Kendall Reference Measurement Procedure. TC: total cholesterol.
CDC: Centers for Disease Control and Prevention.

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

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Table 2A
Statistical criteria of TC certification for manufacturer.

Parameter	Criterion
r^2	>0.975
Bias at 200 mg/dl	≤3%
Bias at 240 mg/dl	≤3%
Average % bias	≤3%
Average absolute % bias	≤3%
Among-run CV	≤3%
Z-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

Table 2B
Statistical criteria of TC certification for clinical laboratory.

Parameter	Criterion
r^2	>0.975
Bias at 200 mg/dl	≤3%
Bias at 240 mg/dl	≤3%
Average % bias	≤3%
Average absolute % bias	≤3%
Among-run CV	≤3%
t-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

2.3. Statistical analysis

We used the protocol of NCCLS guideline EP9-A from the Clinical and Laboratory Standards Institute for bias estimation [26] and the STATA12 analysis program for all other calculations [27,28].

3. Results

3.1. Regression, accuracy and precision of TC by AK RMP at Osaka laboratory over time

In the AK RMP for TC measurement at Osaka, the CDC pooled sera with 219 different concentrations (lots) were analyzed among 619 survey samples with 165 survey runs, in which each survey run consisted of 3 to 4 different pools. There was one native sample from patients with low TC concentration ranged 27.7 to 83.4 mg/dL in the three to four provided pools: these samples were for the purpose of measuring the accuracy and precision of high-density lipoprotein cholesterol. The TC concentration ranged from 27.7 to 383.7 mg/dL: concentrations were analyzed during the course of 22 years between July 1992 and July 2014.

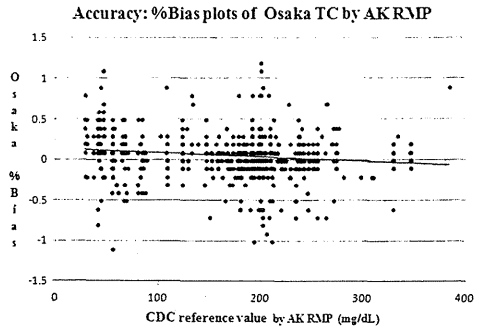

Fig. 1. Accuracy: %Bias plots of Osaka TC by AK RMP. Y-axis indicates the %bias vs. CDC reference value of Osaka TC by AK RMP and x-axis indicates CDC reference value by AK RMP (mg/dL). CDC: the US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

In the scatter plots of %bias between Osaka (y) and CDC (x), $y = 1.000x + 0.032$ ($n = 619$, $R^2 = 1.000$). This means that 200 mg/dL at the CDC corresponds to 200.03 mg/dL at Osaka. The p-value and 95% confidence interval (CI) for the slope were $p < 0.001$ and (1.000, 1.001), respectively. The p-value and 95% CI for the intercept were $p = 0.502$ and (−0.061, 0.124), respectively (Table 3).

In the scatter plots of accuracy, %bias vs. CDC at Osaka, y (Osaka) = −0.001 × (CDC reference value) + 0.141 (n: 619, $R^2 = 0.022$). The p-value and 95% CI for the slope were $p < 0.001$ and (−0.001, −0.000), respectively. The p-value and 95% CI for the intercept were $p < 0.001$ and (0.086, 0.195), respectively (Table 3). The Osaka laboratory met the acceptable accuracy goals within ± 1% compared to the CDC reference values for 99.0% of the samples (613 of 619) (Fig. 1., Table 3). The maximum %bias at Osaka AK RMP was +1.2% and the minimum was −1.1% among all 619 samples. The %bias between the reference values of the CDC and the measurements of the Osaka laboratory at a medical decision point of 200 mg/dL was only −0.06% at Osaka.

In the scatter plots of precision, CV(%) at Osaka, y (Osaka CV%) = −0.001 × (CDC reference value) + 0.321 (n: 619, $R^2 = 0.093$). The p-value and 95% CI for the slope were $p < 0.001$ and (−0.001, −0.001), respectively. The p-value and 95% CI for the intercept were $p < 0.001$ and (0.286, 0.357), respectively (Table 3). The Osaka laboratory met acceptable precision goals < 1% at CV for 99.2% of the samples (614 of 619). The maximum CV at the Osaka AK RMP was 1.6% (Fig. 2.).

Table 3
Regression, accuracy and precision of TC by Osaka AK RMP over time.

Parameter	TC method	Number of samples	Pass rate	Slope (95%CI)	Intercept (95%CI)	R^2	Time period
Regression	AK RMP	619		1.000 (1.000, 1.001) p < 0.001	0.032 (-0.061, 0.124) p = NS	1.000	July 1992 to July 2014 (22 y)
Accuracy (as %Bias vs. CDC)	AK RMP	619	99.0%	-0.001 (-0.001, -0.000) p < 0.001	0.141 (0.086, 0.195) p < 0.001	0.022	July 1992 to July 2014 (22 y)
Precision (as CV)	AK RMP	619	99.2%	-0.001 (-0.001, -0.001) p < 0.001	0.321 (0.286, 0.357) p < 0.001	0.093	July 1992 to July 2014 (22 y)

AK RMP: Abell–Brodie–Levy–Kendall Reference Measurement Procedure. TC: total cholesterol.
For pass rate, TC accuracy criterion as %bias is ± 1% vs. CDC target value and TC precision criterion as CV is ≤1%.

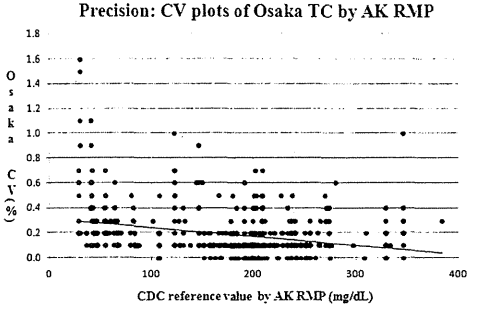

Fig. 2. Precision: coefficient of variation plots of Osaka TC by AK RMP. Y-axis indicates coefficient of variation (CV, %) of Osaka TC by AK RMP and x-axis indicates CDC reference value by AK RMP (mg/dL). CDC: the US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

3.2. Accuracy, precision and total error of Japanese manufacturers conducted by the TC Certification Protocol for Manufacturers

Six reagent manufacturers in Japan were evaluated between 1996 and 2012 eight times according to the TC Certification Protocol for Manufacturers [22], with regard to their analytical systems consisting of a reagent/calibrator/instrument. Their accuracy (mean %bias versus reference value by AK RMP), precision (among-run CV), and total error (absolute mean %bias + 1.96 among-run CV) are presented in Table 4A, Table 4B, and Table 4C, respectively.

3.3. %Bias plots of TC by Osaka AK RMP at each run over time

Fig. 3 shows the %bias compared to the CDC reference value plots of the Osaka AK RMP at each run from a total of 165 runs for 22 years. The minimum value of the %bias was −1.1%, whereas the maximum value was 1.2%. The x-axis indicates the survey run number every 20 runs, and the y-axis indicates the %bias versus CDC reference value of the Osaka AK RMP. The acceptable criterion for the accuracy of TC was within ± 1.0% compared to the reference value of CDC. Each survey run consisted of three to four CDC pools, including round robin samples provided from the CRMLN laboratory for TC analysis.

4. Discussion

The CRMLN maintains robust reference measurement systems with high quality reference materials, measurement procedures, and continuous monitoring. Established protocols and guidelines within CDC's CRMLN certification program allows manufacturers to perform measurement comparisons between the test method and the reference method to assess performance accuracy and imprecision. As shown in earlier reports, CRMLN successfully applies this principle to complex analytes such as HDL- and LDL-cholesterol as well as to analytes such as total cholesterol. Reliable data allows for consistent patient monitoring, treatment management, and improved worldwide public health efforts for CVD.

The AK RMP as operated at the CDC Laboratory and the Osaka laboratory is highly accurate and reliable for over 20 years. Measurement results obtained by both laboratories show excellent agreement, precision and stability. This ensures assay manufacturers calibrated by the Osaka CRMLN laboratory can produce measurement results that are highly accurate and comparable over time.

The main purpose of the CRMLN laboratories is to work with manufacturers to certify the accuracy and precision of cholesterol measurements of reagent/calibrator/instrument systems used in clinical laboratories [29]. This is in agreement with the US Laboratory Standardization Panel, which suggests that standardization is most effectively achieved through the manufacturers of analytical instruments and reagents [8,9]. Between 1996 and 2012, six reagent manufacturers in Japan were certified eight times with regard to their analytical systems consisting reagent/calibrator/instrument [22]. Standardization of 2,122 Japanese clinical laboratories has been performed by the Certification Protocol for Clinical Laboratories [23] between 1993 and 2014, and 98.2% of these participants met the certification criteria, which were derived from clinical needs. This high pass rate with clinical laboratories suggests that calibration of assay manufacturers by CRMLN laboratories is highly successful and effective. Lipid standardization activities have improved the accuracy of TC measurements [30]. All manufacturers and clinical laboratories with current certification are listed on CDC's-CRMLN web site (http://www.cdc.gov/labstandards/crmln.html).

Table 4A
Accuracy (mean %bias) of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

	Certification year for TC								
Manufacturer	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	0.6		-0.8	-0.4	-0.1	1.0	-0.3	-0.3	1.3
B	-1.2		-0.6	1.6	-0.4	1.4	1.0	-0.3	-0.1
C	1.0		-1.3	-1.0	0.6	-0.4	1.1	-0.1	-0.9
D	2.1	1.2		-1.1	-0.1	0.7	0.1	-0.5	0.5
E		−2.2		0.2	0.2	−0.2	1.8	0.5	−0.1
F		1.2		0.4	−0.4	0.0	0.5	0.3	0.0

Accuracy criterion: mean % bias ≦3% unit: %.

Table 4B
Precision (among-run CV) of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

	Certification year for TC								
Manufacturer	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	0.5		0.6	0.6	0.6	0.8	0.4	0.5	0.5
B	0.7		0.7	0.7	0.7	1.2	0.6	0.6	0.7
C	0.6		0.7	0.5	0.6	0.5	0.6	0.5	0.5
D	0.6	0.7		0.5	0.4	0.3	0.4	0.4	0.4
E		0.4		0.5	0.7	0.5	0.4	0.8	1.0
F		1.5		0.8	0.7	0.5	0.5	0.4	1.0

Precision criterion: among-run CV ≦ 3% unit: %.

Table 4C
Total error of 6 Japanese manufacturers conducted by TC Certification Protocol for Manufacturers.

Manufacturer	Certification year for TC								
	1996	1997	1998	2002	2004	2006	2008	2010	2012
A	1.5		2.0	1.5	1.3	2.6	1.1	1.2	2.3
B	2.6		2.1	3.0	1.7	3.9	2.3	1.5	1.4
C	2.2		2.7	2.0	1.8	1.4	2.3	1.0	1.8
D	3.3	2.7		2.2	0.8	1.4	0.9	1.3	1.3
E		3.0		1.3	1.6	1.3	2.6	2.1	2.1
F		4.2		1.9	1.8	1.0	1.5	1.1	2.0

Total error (absolute mean %bias + 1.96 among-run CV) criterion: ≦8.9% unit: %.

The AK RMP was proposed more than 60 years ago and is considered the secondary reference method for analyzing cholesterol. It has been used internationally as a reference procedure for measuring cholesterol at CDC, CRMLN laboratories, and in many epidemiological research institutes worldwide. Despite its widespread use, the AK method has limitations such as complex operations, the requirement of skilled technicians, use of hazardous reagents, and interferences related to the measurement of reactive substances other than cholesterol. These limitations can be minimized with current GC-IDMS RMPs.

Current clinical and public health decision points are based on measurements standardized to the AK RMP. Because results obtained with this RMP are not interchangeable with results obtained with GC-IDMS RMPs, and a reliable relationship between both types of RMPs has not been established yet. The AK RMP continues to be used to standardize tests in patient care and public health. At the same time, CDC together with CRMLN laboratories including Osaka laboratory is working to further establish mass spectrometry-based RMPs for TC and other lipid and lipoproteins [14,31–33]. Mass spectrometry-based RMPs for TC and triglycerides are already part of the CRMLN. This will help the clinical and public health communities to generate new data and clinical decision points that are linked to new more specific mass spectrometry-based RMPs.

5. Conclusions

We presented the performance of TC measurements using the AK RMP over the past 22 years and demonstrated the accuracy, precision, and long-term stability of this method. As examples of AK RMP application, we presented six reagent manufacturers in Japan that successfully certified their reagent/calibrator/instrument systems and participated in the TC Certification Protocol for Manufacturers for 16 years. The clinical laboratories in Japan demonstrated high achievement rates for TC certification. This accomplishment is one example on how CRMLN is improving clinical testing of cholesterol and other blood lipids not only in Japan but worldwide. These data as well as data shown in previous reports demonstrate that the same standardization principles can be applied to analytes such as TC as well as highly complex analytes such as HDL- and LDL-cholesterol.

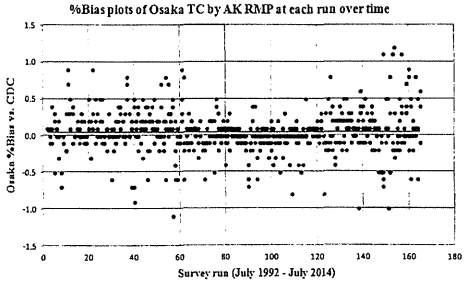

Fig. 3. %Bias plots of Osaka TC by AK RMP at each run over time. Y-axis indicates Osaka %bias vs. CDC reference value and x-axis indicates each survey run from total 165 runs (July 1992 - July 2014). CDC: The US Centers for Disease Control and Prevention. AK RMP: Abell–Kendall reference measurement procedure. TC: total cholesterol.

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No declared.

Conflict of interest

No authors have any financial, personal or professional relationships associated with other people or organizations.

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HDL cholesterol performance using an ultracentrifugation reference measurement procedure and the designated comparison method☆

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ABSTRACT

Background: Accurate high-density lipoprotein cholesterol (HDL-C) measurements are important for management of cardiovascular diseases. The US Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) perform ultracentrifugation (UC) reference measurement procedure (RMP) to value assign HDL-C. Japanese CRMLN laboratory (Osaka) concurrently runs UC procedure and the designated comparison method (DCM). Osaka performance of UC and DCM was examined and compared with CDC RMP.

Methods: CDC RMP involved UC, heparin-$MnCl_2$ precipitation, and cholesterol analysis. CRMLN DCM for samples containing <200 mg/dl triglycerides involved 50-kDa dextran sulfate-$MgCl_2$ precipitation and cholesterol determination.

Results: HDL-C regression equations obtained with CDC (x) and Osaka (y) were $y = 0.992x + 0.542$ ($R^2 = 0.996$) for Osaka UC and $y = 1.004x - 0.181$ ($R^2 = 0.998$) for DCM. Pass rates within ±1 mg/dl of the CDC target value were 91.9 and 92.1% for Osaka UC and DCM, respectively. Biases at 40 mg/dl HDL-C were +0.22 and −0.02 mg/dl for Osaka UC and DCM, respectively.

Conclusions: Osaka UC and DCM were highly accurate, precise, and stable for many years, assisting manufacturers to calibrate products for clinical laboratories to accurately measure HDL-C for patients, calculate non-HDL-C, and estimate low-density lipoprotein cholesterol with the Friedewald equation.

1. Introduction

A low high-density lipoprotein cholesterol (HDL-C) level is a strong biomarker for predicting the risk of cardiovascular diseases (CVD), as demonstrated by several epidemiological studies and clinical trials [1–3]. The US National Cholesterol Education Program (NCEP) estimated that each 1% increase in HDL-C may be associated with a 2–4% decrease in the risk of coronary heart disease (CHD), and clinical trials on low-density lipoprotein-lowering therapies have shown that concomitant increases in HDL-C confer an additional independent reduction in the risk of CHD [4]. HDL-C together with low-density lipoprotein cholesterol (LDL-C), total cholesterol, and triglycerides form a lipid panel that is measured in routine patient care to determine and monitor the risk of a patient developing CVD.

Accurate and reproducible HDL-C measurements are of particular importance for correctly and consistently classifying individuals at risk of CVD, as outlined in the clinical guidelines for the subsequent diagnosis, treatment, and prevention of patients [5–7]. Furthermore, the US NCEP reported [4] that the accuracy of HDL-C was particularly important because (a) the inverse association of HDL-C with the risk of CHD is expressed over a relatively narrow concentration range, (b) the medical decision cut-off point (40 mg/dl) for an increased risk of CHD is at the lower end of the HDL-C concentration range, at which small errors can have a strong impact on patient classification, and (c) the calculation of non-HDL-C [8,9] or LDL-C using the Friedewald equation [10]. Inaccurate HDL-C measurements also lead to errors in the estimation of LDL-C.

Previous studies recommended that the US Centers for Disease Control and Prevention (CDC) reference measurement procedure (RMP) should be used to achieve accurate HDL-C measurements. CDC RMP is a three-step procedure [11,12]: (1) ultracentrifugation (UC) at d = 1.006 kg/l to remove triglyceride-rich lipoproteins; (2) precipitation of apo B-containing lipoproteins from the ultracentrifugal infranatant with heparin-$MnCl_2$; (3) measurement of cholesterol in the heparin-$MnCl_2$ supernatant using the CDC reference method for cholesterol [13]. However, ultracentrifugal measurements of HDL-C have low sample throughput and require equipment that is not commonly available in routine clinical laboratories. Therefore, the Cholesterol Reference Method Laboratory Network (CRMLN) sought to implement a designated comparison method (DCM) [14–16] with the objective of better assisting reagent manufacturers in the calibration of their products so that clinical laboratories could more accurately measure HDL-C for patients, calculate non-HDL-C, and estimate LDL-C with the Friedewald equation.

The CDC UC method has been accepted as the most reliable RMP for HDL-C and the CRMLN DCM is an accurate, robust, transferable and practical method for clinical laboratories and manufacturers. As part of the CRMLN activities, the National Cerebral and Cardiovascular Center at Osaka, Japan has implemented and maintained 1) the UC method, which is same as CDC RMP, for 17 years since May 1997 and 2) DCM for 20 years since April 1994. We measured the performance of both HDL-C reference methods in terms of accuracy and reproducibility after many years using comparisons with CDC RMP.

2. Materials and methods

2.1. Materials

All standardization pools for HDL-C were prepared according to the Clinical Laboratory Standards Institute document C37-A (Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures; Approved Guideline), which implied that no preservatives or no additives were added. All survey pools were blinded to the CRMLN laboratories. They were shipped frozen from CDC and stored at −70 °C before analysis.

HDL-C assays were conducted in the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, in the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and in the National Cerebral and Cardiovascular Center at Osaka continuously from April 2012 (all laboratories were referred to as the 'Osaka' laboratory).

2.2. Methods

2.2.1. CDC reference measurement procedure for ultracentrifugation

The first step of CDC RMP employed preparative ultracentrifugation (Beckman Coulter, Optima L-70 K and/or Optima XE-90) to remove apo B-containing lipoproteins [11,12]. The methods at CDC and Osaka used 5.00 ml of serum at a density of d = 1.006 kg/l (0.195 mol/l NaCL solution) and a 50.4Ti rotor (Beckman Coulter). UC at CDC was carried out for 16.2 h at 120,000 ×g and 18 °C, and at Osaka for 18.5 h at 105,000 ×g and 18 °C. After UC, the top fraction (d < 1.006 kg/l) was removed using tube slicer and the bottom fraction (d > 1.006 kg/l) was quantitatively transferred to a 5.00 ml volumetric flask adjusting with 0.15 mol/l NaCl solution [14–16]. In the second step, 1.00 ml aliquots of the bottom fraction were precipitated with 40 μL heparin (sodium injection, 5000 USP units/ml, Baxter Healthcare Corporation) and 50 μL manganese reagents ($MnCl_2$ solution, 1.00 M ± 0.01 M, SIGMA) [17]. The precipitate was removed for 30 min at 1500 ×g and 4 °C [18–20]. In the third step, HDL-C was determined in the supernatant in duplicate measurements by the Abell–Kendall reference method for cholesterol [13]. The recovered cholesterol value was multiplied by 1.09 to account for the dilution introduced by the addition of the precipitation reagent. Four replicates from each sample were used in comparisons of assay performance.

2.2.2. CRMLN designated comparison method

DCM is a precipitation-based designated comparison method using 50-kDa dextran sulfate (DS)-$MgCl_2$ as the reagent. DS (stored at 2 to 8 °C. Kept tightly capped in a desiccator in a refrigerator after opening) was obtained from Warnick & Co. and was a special lot (lot#: 162176) for CRMLN use only. All CRMLN laboratories used the same DS lot to minimize potential lot-to-lot variations. $MgCl_2 \cdot 6H_2O$ (this reagent was highly hygroscopic and had to be dried. A larger amount than was needed was placed in a beaker and dried in an oven at 37 °C for at least one hour) and sodium azide (NaN_3) were obtained from Wako Pure Chemistries Inc. in Japan. The stock solution of DS contained 2.0 g/dl DS including 50 mg/dl NaN_3, while that of $MgCl_2$ contained 14.22 g/dl including 50 mg/dl NaN_3. The working reagent was prepared by mixing equal volumes. The working solution was stored at 2 and 8 °C [15,16]. Osaka laboratory previously confirmed that it was stable for 3 years.

In the first step, the samples and working reagent were equilibrated to room temperature and mixed at a ratio of 1.00 ml specimen and 0.10 ml working reagent. The samples for DCM required normotriglyceridemic sera including <200 mg/dl in triglycerides because of its limited sedimentation efficiency [16,17]. The samples were then incubated at room temperature for 10–30 min and centrifuged for 30 min at 4 °C at 1500 ×g. In the second step, clear supernatants were analyzed using the reference method for cholesterol [13]. The recovered HDL-cholesterol value in the DCM was multiplied by 1.1. HDL-C was assayed in the supernatant in duplicate measurements. Four replicates from one aliquot were used in comparisons of assay performance.

2.3. Performance criteria for HDL-C applied to CRMLN laboratories

The performance criteria for HDL-C applied to the CRMLN lipid reference laboratories are summarized in Table 1. Imprecision is evaluated not in coefficient variation (CV), but in standard deviation (SD, unit: mg/dl), and accuracy is evaluated in bias (mg/dl) from CDC reference value.

2.4. Statistical analysis

We used protocol EP9-A from the Clinical and Laboratory Standards Institute for bias estimation [21] and the STATA12 analysis program for all other calculations.

Table 1
Performance criteria applied to CRMLN lipid reference laboratory using UC method and DCM for HDL-C.

Lipid	Imprecision criterion	Accuracy criterion
HDL-C	Standard deviation ≤1 mg/dl	Bias ≤1 mg/dl

CRMLN: cholesterol reference method laboratory network. UC: ultracentrifugation. DCM: designated comparison method. HDL-C: high-density lipoprotein cholesterol.

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

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0009-8981/

3. Results

3.1. Accuracy

In the UC procedure at Osaka, the pooled serum with 160 different concentrations (lots) for HDL-C were analyzed among 626 survey samples with 154 survey runs, in which each survey run consisted of 3 to 5 different pools. They were analyzed for 17 years between May 1997 and January 2014. The concentration ranges were 26.9–78.9 mg/dl. In the scatter plots of bias (unit: mg/dl) between Osaka (y) and CDC (x), $y = -0.008x + 0.540$ ($R^2 = 0.017$). The p-values and 95% confidence interval (CI) of the slopes and intercepts were 0.001 and (−0.013, −0.003), and <0.001 and (0.296, 0.784), respectively (Table 2). The Osaka laboratory met acceptable accuracy goals for 91.9% (575 of 626 samples) within ±1 mg/dl of the CDC reference values (Fig. 1A). Biases between the target values of CDC and the measurements of Osaka at two medical decision points of 40 and 60 mg/dl were 0.22 and 0.06 mg/dl, respectively, both of which were slightly on the positive side. Although the bias and SD scattering of DCM appeared to be slightly better than that of CDC RMP, no significant differences (p-value: 0.05) were observed in the accuracy or precision of the 2 procedures.

In the DCM at Osaka, the pooled serum with 163 different concentrations (lots) for HDL-C were analyzed among 570 survey samples with 147 survey runs, in which each survey run consisted of 3 to 4 different pools. They were analyzed for 20 years between April 1994 and January 2014. The concentration ranges were 20.8–86.0 mg/dl. In the scatter plots of bias (unit: mg/dl) between Osaka (y) and CDC (x), $y = 0.004x - 0.181$ ($R^2 = 0.006$). The p-values and 95% CI of the slopes and intercepts were 0.065 and (−0.0002, 0.007), and 0.062 and (−0.370, 0.009), respectively (Table 2). The Osaka laboratory met acceptable accuracy goals for 92.1% (525 of 570 samples) within ±1 mg/dl of the CDC reference values (Fig. 1B). Biases between the target values of CDC and measurements of Osaka at two medical decision points of 40 and 60 mg/dl were −0.02 and +0.06 mg/dl, respectively, both of which were slightly biased.

3.2. Precision

In the scatter plots of SD between Osaka (y) and CDC (x), y (SD, mg/dl) = 0.002x (CDC reference value) + 0.270 [n: 626, $R^2 = 0.006$]. The p-value and 95% CI for the slope were 0.056 and (−0.00005, 0.0036), respectively. The p-value and 95% CI for the intercept were <0.001 and (0.179, 0.360), respectively (Table 2). The Osaka laboratory met acceptable precision goals for 97.9% (613 of 626 samples) within ±1 mg/dl. The maximum SD at Osaka UC was 2.3 mg/dl (Fig. 2C).

In the scatter plots of SD between Osaka (y) and CDC (x), y (SD, mg/dl) = 0.001x (CDC reference value) + 0.218 [n: 570, $R^2 = 0.005$].

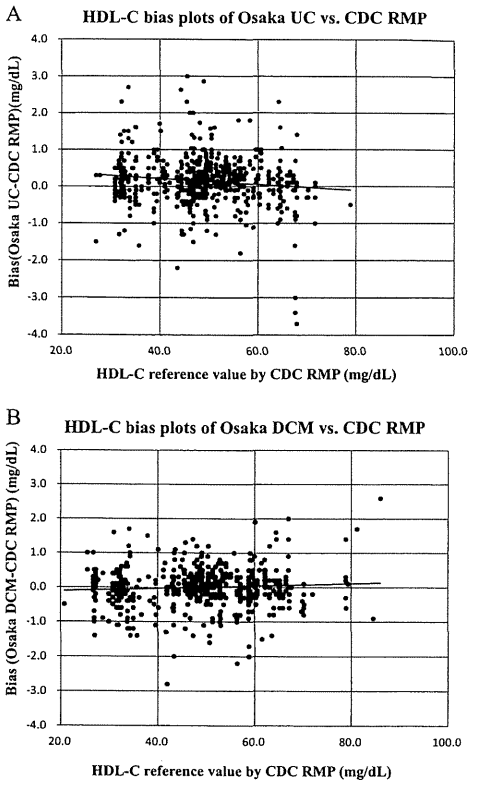

Fig. 1. A: HDL-C bias plots of Osaka UC vs. CDC RMP. The y-axis indicates the bias (mg/dl) of Osaka UC compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. UC: Ultracentrifugation. B: HDL-C bias plots of Osaka DCM vs. CDC RMP. The y-axis indicates the bias (mg/dl) of the Osaka DCM compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. DCM: Designated comparison method.

Table 2
Regression analysis of the bias between Osaka (y) and CDC (x) and imprecision for HDL-C over time (unit: mg/dl).

Parameter	HDL-C method	Number of samples	Slope (95%CI)	Intercept (95%CI)	R^2	Time period
Accuracy	UC	626	−0.008 (−0.013, −0.003) p = 0.001	0.540 (0.296, 0.784) p < 0.001	0.017	May 1997 to January 2014 (17 years)
	DCM	570	0.004 (−0.0002, 0.07) p = NS	−0.181 (−0.370, 0.009) p = NS	0.006	April 1994 to January 2014 (20 years)
Precision	UC	626	0.002 (−0.00005, 0.0036) p = NS	0.270 (0.179, 0.360) p < 0.001	0.006	May 1997 to January 2014 (17 years)
	DCM	570	0.001 (−0.0001, 0.002) p = NS	0.218 (0.162, 0.275) p < 0.001	0.005	April 1994 to January 2014 (20 years)

UC: ultracentrifugation. DCM: designated comparison method.

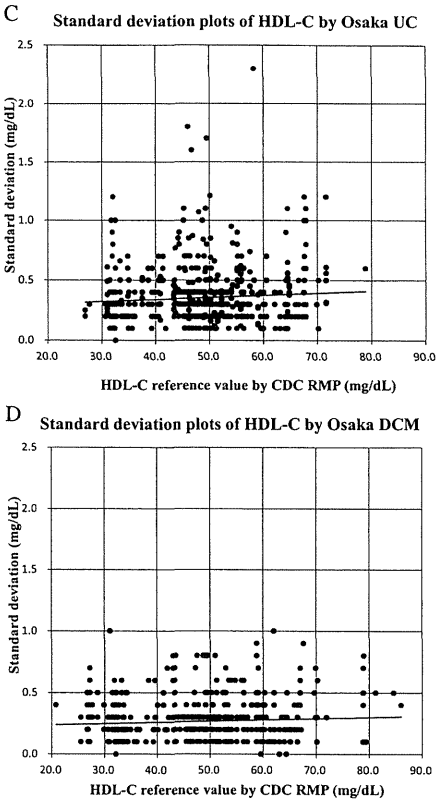

Fig. 2. C: Standard deviation plots of HDL-C by Osaka UC. The y-axis indicates the SD (mg/dl) of the Osaka UC method compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. D: Standard deviation plots of HDL-C by Osaka DCM. The y-axis indicates the SD (mg/dl) of the Osaka DCM compared to the CDC reference value and the x-axis indicates the CDC RMP HDL-C reference value. CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. DCM: Designated comparison method.

The p-value and 95% CI for the slope were 0.083 and (−0.0001, 0.002), respectively. The p-value and 95% CI for the intercept were <0.001 and (0.162, 0.275), respectively (Table 2). The Osaka laboratory met acceptable precision goals for 100.0% (all 570 samples) within ±1 mg/dl. The maximum SD at Osaka DCM was 1.0 mg/dl (Fig. 2D).

3.3. Long-term bias (mg/dl) plots by the UC method and DCM at Osaka

Fig. 3E shows the bias (mg/dl) plots of Osaka UC HDL-C vs. CDC RMP at each run for 17 years. The minimum value of the bias was −3.7 mg/dl while the maximum value was 3.0 mg/dl. The x-axis indicated the survey run number between May 1997 and January 2014 with 154 runs and the y-axis indicated the bias (mg/dl) of Osaka UC HDL-C vs. CDC RMP. The acceptable criteria for the accuracy of HDL-C were within ±1.0 mg/dl of the target value of CDC. Each survey run consisted of 3 to 5 CDC pools for the HDL-C analysis.

Fig. 3F shows the bias (mg/dl) plots of Osaka DCM HDL-C vs. CDC RMP at each run for 20 years. The minimum value of the bias was −2.8 mg/dl while the maximum value was 2.6 mg/dl. The x-axis indicated the survey run number between April 1994 and January 2014 with 147 runs and the y-axis indicated the bias (mg/dl) of Osaka DCM HDL-C vs. CDC RMP. The acceptable criteria for the accuracy of HDL-C were within ±1.0 mg/dl of the target value of CDC. Each survey run consisted of 3 to 4 CDC pools for the HDL-C analysis.

4. Discussion

Previous epidemiological studies and clinical trials were based on the results of large scale population studies using the UC method for HDL-C, which were, in turn, based on the heparin-$MnCl_2$ precipitation method. However, an inherent problem with this precipitation method is the inability to sediment all the centrifuged lipoproteins [18,19], which mainly affects triglyceride-rich lipoproteins included in turbid or milky diseased specimens. Therefore, the UC procedure merits the elimination of interference [20,22].

High-density lipoprotein (HDL) represents a mixture of heterogeneous macromolecules and physicochemical particles. No primary certified standards or measurement procedures are currently available for HDL-C in order to establish the metrological traceability of HDL-C measurements to SI. However, UC-based CDC RMP has been the reference method of HDL-C measurements for practical use. DCM was established to better meet needs related to faster sample turnaround and higher throughput [16]. Both methods are now used to assure the accuracy of testing performed in patient care and research. However, it is important to understand the limitations of the DCM, especially with samples containing high levels of triglycerides. Therefore, it will be necessary and important to maintain the UC-based reference method and its standardization when encountering diseased and complicated samples.

Iso et al. in the Circulatory Risk in Communities Study (CIRCS) have conducted epidemiological studies on the prevention of and reductions in cerebral strokes and heart diseases among Japanese individuals for over 50 years [2,9]. During this time, we have experienced various changes for HDL-C in assay principles from the old precipitation methods to new homogeneous methods, in instruments from manual operation to automatic analyzers, in reagents from strong acids to mild enzymes, and in calibrators from cholesterol standards in alcohol to serum-based materials. All these changes have influenced the precision and accuracy of HDL-C measurements. Therefore, it is of utmost importance to ensure reference methods providing an accuracy basis for clinical measurements remain consistent and stable over time. This is achieved by maintaining a network of reference laboratories. In the present study, we assessed the measurement performance and limits of the UC and DCM methods for HDL-C at Osaka.

The homogeneous HDL-C reagents now widely adopted have several advantages: they are fully automated on various analytical instruments, have good precision, triglycerides do not need to be measured, and non-fasting samples may potentially be used. However, Miller et al. [23] and Miida et al. [24] reported some limitations when comparing these assays against the UC-RMP. Deventer et al. found that non-HDL cholesterol showed improved accuracy for cardiovascular risk score classification over that of direct or calculated LDL cholesterol in a dyslipidemic population [25]. Non-HDL-C is calculated as [total cholesterol − HDL-C]. Therefore, accurate HDL-C will be a key factor in obtaining accurate non-HDL-C values. Since non-HDL-C was previously reported to be superior to LDL-C in predicting the risk of CVD risk [8,9], it will be recommended as a primary screening test in the future by Japanese authorities.

Recent discovery that serum/plasma HDL-C markedly and selectively increased by up to 15% over the past 20 years among Japanese individuals [26] raised concerns regarding consistencies in HDL-C measurements in Japan. According to the Japanese National Health and Nutritional Survey, the average HDL-C levels of males and females

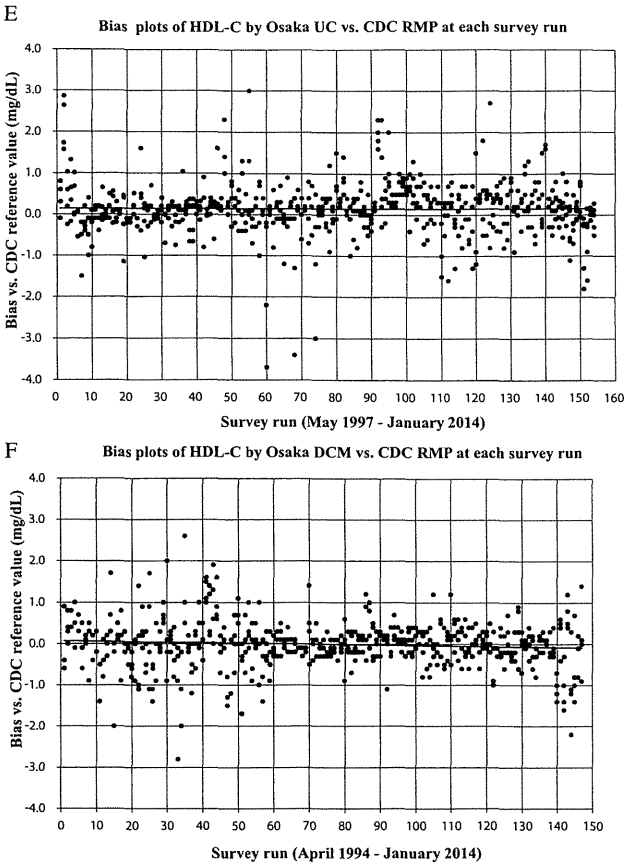

Fig. 3. E: Bias plots of HDL-C by Osaka UC vs. CDC RMP at each survey run. The y-axis indicates the bias (mg/dl) of Osaka UC compared to the CDC reference value and the x-axis indicates each survey run (May 1997–January 2014). CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol. F: Bias plots of HDL-C by Osaka DCM vs. CDC RMP at each survey run. The y-axis indicates the bias (mg/dl) of Osaka DCM compared to the CDC reference value and the x-axis indicates each survey run (April 1994–January 2014). CDC: The US Centers for Disease Control and Prevention. HDL-C: High-density lipoprotein cholesterol.

reached 55 and 65 mg/dl in 2012, which were markedly higher than those in Western countries [27]. We tentatively concluded that this could not be attributed to a drift in the standardization of HDL-C measurements in Japan because the increase was continuous over several time points when new assay reagents and systems were introduced. Furthermore, similar findings were reported for plasma apoA-I concentrations that were independently measured [26]. However, the underlying reasons for this phenomenon and its outcome on public health in Japan remain unknown. This is a unique and perhaps important finding for world public health; therefore, it should be extensively investigated in association with recent trends and changes in various aspects of Japanese lifestyles and medical/public health environments. It is also extremely important to monitor Japanese HDL-C levels carefully for years hereafter. Therefore, methods to measure HDL-C parameters must be established based on reliable standardization and stabilization for international consistency through CRMLN activities [16].

Since 1996, 7 Japanese reagent manufacturers have developed new homogeneous methods for HDL-C to replace the old precipitation-based methods [20]. These methods present new calibration challenges

Table 3
Performance criteria applied to clinical laboratory and manufacturer for HDL-C.

Parameter	Criterion
R^2	>0.975
Bias at 40 mg/dl	≤5%
Bias at 60 mg/dl	≤5%
Average % bias	≤5%
Average absolute % bias	≤5%
Among-run CV	≤4%
t-test of bias	Not significant at $\alpha = 5\%$
Within-method outliers	1 allowed
Between-method outliers	None allowed, but may eliminate one sample

because they use different principles that include detergents or surfactants to quantify HDL-C level. Homogeneous methods that do not require a sample pretreatment step are being introduced all over Japan and are used in many clinical laboratories. Based on the HDL Cholesterol Certification Protocol for Manufacturers (November 2002) by CRMLN, we conducted protocols 10 times every 2 years since 1996 using standardized DCM as a reference to Japanese manufacturers, and all manufacturers successfully met the performance criteria (Table 3). However, further accuracy improvements in homogeneous HDL-C methods will be required in several diseased samples derived from patients with dyslipidemia [28], which may require an increased use of the UC-RMP instead of the DCM. Unsolved issues associated with homogeneous methods remain and have yet to be examined in detail.

In conclusion, the UC method and the DCM for HDL-C at Osaka were found to be highly accurate, precise, and stable for many years, and assist reagent manufacturers in calibrating their products so that clinical and epidemiological laboratories can accurately measure HDL-C for patients and in research, calculate non-HDL-C, and estimate LDL-C with the Friedewald equation. DCM is a simpler equivalent reference method that has been consistent with CDC RMP for 20 years. However, the traceability of HDL-C should be accomplished by performing a method comparison with a fresh split sample because matrix interactions can severely affect HDL-C measurements.

Acknowledgments

This work was supported by a Health and Labour Sciences Research Grant, Japan (Comprehensive Research on Lyfestyle-Related Diseases Including Cardiovascular Diseases and Daibetes Mellitus) from the Minustry of Health, Labour, and Welfare of Japan. The authors would like to thank Dr. Katsuyuki Nakajima and Dr. Ikunosuke Sakurabayashi for their valuable comments and discussion, and Ms. Yukari Ichikawa for her excellent help in providing the references and manuscript.

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第10回日本心臓財団小林太刀夫賞受賞業績概要報告

都市部における住民主体の循環器疾患予防対策50周年の取り組み

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八尾市保健師一同・保健推進課を代表して

Ⅰ．はじめに

本報告では、大阪府八尾市が平成26年7月に開催された第50回日本循環器病予防学会・日本循環器管理研究協議会総会において、第10回日本心臓財団小林太刀夫賞を受賞する理由となった当市の循環器疾患予防対策をここに報告し、研究機関の先生方と地域住民の活動についても紹介したい。

Ⅱ．大阪府八尾市の概況

八尾市は大阪府中央部の東側に位置し、総面積41.71平方キロメートルである。昭和39年当時の人口は約15万7千人、その後人口の急激な流入があり昭和52年には約26万8千人に急増した。平成26年度現在では、人口269,759人、高齢化率25.7%である。東は信貴生駒山系を境に奈良県に、西は大阪市に接している。中小企業を中心にした「ものづくりのまち」であり、農業も盛んであり、花卉花木の他、枝豆や若ごぼうの特産品がある。

Ⅲ．予防対策のはじまり

八尾市の循環器疾患予防対策は、昭和37年に始まった。当時、脳卒中が死因の第一位を占め、さらに増加傾向を示していた。循環器疾患予防対策を推進するきっかけは、国民健康保険の赤字に悩んでいた同市が、増え続ける脳卒中を予防する手立てについて、大阪府立成人病センター集団検診第一部（現大阪がん循環器病予防センター、以下成人病センター）の小町喜男先生に相談したことであった。八尾市と成人病センター等の協議の結果、市内で脳卒中死亡率の高い地区からモデル地区を選定し、循環器健診を中心とする重点的な予防対策を地域ぐるみで実施することを決定した。また、地域の循環器疾患の実態を把握するとともに、対策の効果を評価するためにモデル地区住民全体を対象として脳卒中・虚血性心疾患（心筋梗塞・狭心症）の発症状況の調査を経年的に実施することにした。

昭和38年には、曙川地区をモデル地区として設定し、対策の進め方や各機関の連携・役割分担を協議するため成人病対策協議会を結成しその協議をふまえ、昭和39年に曙川地区にて第1回循環器健診を実施した。昭和40年には南高安地区を新たにモデル地区に加え、40～69歳の住民を対象に循環器健診を実施し、1,235人（受診率92.5%）が受診した。当時の検査項目は、問診・検尿・血圧測定・聴診・心電図検査・眼底検査・血液検査・糖負荷試験（尿糖陽性者のみ）と、当時の最先端の臨床的検査を採り入れた内容であった。健診後は受診者を対象に健診結果説明会を開き、健診で把握された要指導者に対しては、毎年健診を実施すると共に保健師が家庭訪問で生活指導、受診勧奨などを行った。

昭和41年から毎年新たにモデル地区以外の3地区を推進地区に選び、健診を実施し、要指導者に対しグループ指導を行った。しかし、推進地区の受診率はモデル地区と比較して20～40%と低く、全市を一巡するのに9年を要するなどの課題が残った。

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（〒581-0833　八尾市旭ヶ丘5-85-16）
受付日　2015年2月6日・受理日　2015年2月17日

Ⅳ．成人病予防会の発足

都市化が進み、住民の生活様式や考え方が多様化したことに伴い、行政が住民に対する一方的なサービスとして健診・管理を提供するという対策の進め方では、住民の理解と協力が得難くなりつつあった。そのため従来の地域住民を対象とする循環器健診のあり方について再検討が必要になった。昭和50年、曙川地区については、勤務者の増加と共に人口の移動が激しくなり、次第に継続的な健診・管理の実施が困難となったためモデル地区を終了した。

昭和50年、成人病対策協議会にて「八尾市循環器集団健診実施要綱」が制定され、モデル地区以外の市民に対しても、自らの意思で自らの健康を管理しようとする人々を成人病予防会の会員として募り、その会員に対する健診・管理を行うこととした。会員を対象とした成人病センターの多項目健診が開始され、平成20年3月まで継続した。

昭和51年、成人病対策協議会で、他の地区に先駆け、モデル地区である南高安地区において、市と成人病センターは、南高安地区での成人病予防会の結成をめざし、健診を受ける住民側の体制を整えるため、住民の中から自らの努力で自らの健康を守る意思を持つ人を積極的に募った。

昭和52年、モデル地区において、自らの健康管理に熱意のある住民が主体となり、自分たちで運営する南高安地区成人病予防会が結成された。南高安地区成人病予防会活動の母体となる組織は、地区の自治振興委員会と婦人会であった。成人病予防会は、会員一人ひとりの健康増進のみではなく、地区住民全体の健康増進を目標とした。

昭和55年実施の健診結果により把握した要指導者に対し、健康教室への受講を勧め、健康教室を開催した。第1回の教室では、食事と栄養のバランスや減塩について、また運動やその他の日常生活などについて4ヶ月に渡り全8日間の教室を実施した。昭和57年には卒業生105名をもって健康教育OB会が結成され、その後正式に南高安地区成人病予防会の下部組織として位置づけられた。健康教室OB会は、昭和52年に解散した婦人会の役割を担っている。

このようにして循環器疾患予防対策は住民を主体とする成人病予防会方式に引き継がれた。

Ⅴ．法の改正と健診

昭和57年の老人保健法の制定、平成6年の地域保健法の制定をうけ、全市的な健診・管理体制に見直しが必要となった。そこで、八尾市衛生問題対策協議会成人病対策部会（昭和54年に成人病対策協議会が組織替え）において協議を重ね、一般地区の循環器健診と、モデル地区において実施している循環器健診は共に、老人保健法における基本健康診査として継続することとなった。一般地区において実施していた健診については、市保健センターと成人病セ

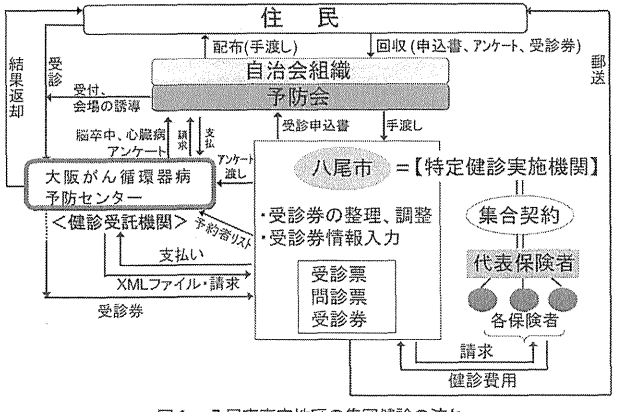

図1　八尾南高安地区の集団健診の流れ

ンターが集団健診方式で実施するとともに、八尾市医師会での個別健診を導入することになった。

さらに平成20年には老人保健法が高齢者の医療の確保に関する法律に改正され、基本健康診査は特定健診として実施することとなった。モデル地区においては、市保健センターが特定健診実施機関となり継続している。

モデル地区における集団健診では保険者の種類によらず満30歳以上の地域住民全員に受診機会を提供していること、自治会組織を通じて健診の申込書類の配布や回収、受診勧奨を行っていることが特徴的である。図1に健診の流れの略図を示す。

南高安地区成人病予防会が主体的に集団健診を中心とした予防活動を実施しており、その活動をがん循環器病予防センターや八尾市医師会、市等の関係機関が支援している。主な取り組みは図2の通りである。

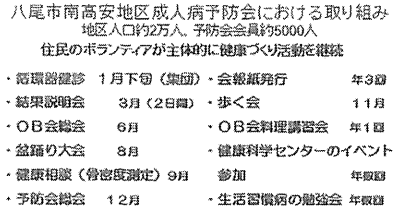

図2　南高安地区での健康づくり活動について

Ⅵ. モデル地区における循環器疾患予防対策の効果

こうした活動の成果として南高安地区ではより多くの住民が健診を受診し、脳卒中発生率が減少した（図3）。

また、最近の疾患予防活動の評価として、平成23年度に行われた大阪府の行動変容推進事業にて、国民健康保険の特定健診データと医療費データの分析を行い、南高安地区と他地区との間で各指標を比較することで効果検証を行った。

特定健診受診率は、図4に示すように全体でみると南高安地区では他地区に比べ8.5%高かった。年齢区分別にみると、南高安地区と他地区の差は40歳代で10%、50歳代で9.6%と特に壮年層で差が大きい傾向を示した。

高血圧や糖尿病の有所見率は、図5、6に示すように全体でみると南高安地区では他地区に比べ低く、いずれの年齢区分でも同様の傾向であった。

医療費については、被保険者1人あたりの月平均の医療費を算出したところ、図7に示すように、40－74歳計でみると、医療費全体で南高安地区では他地区よりも、被保険者1人あたり2,286円／月低額であった。南高安地区と他地区との差は、40歳代、50歳代の年齢区分でより大きく、南高安地区全体で年間約1.4億円の抑制効果があり、長期にわたり実施している南高安地区の循環器疾患予防対策の有効性が明らかになった。

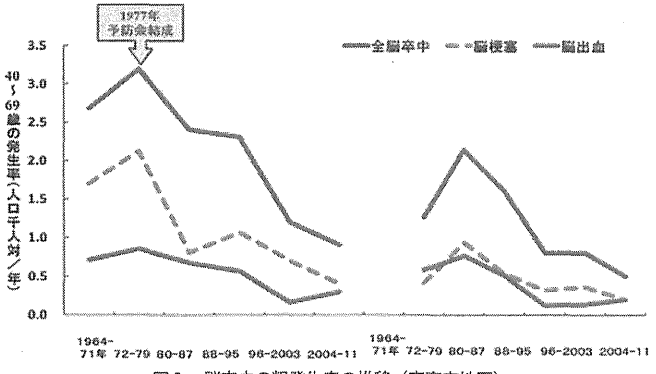

図3　脳卒中の粗発生率の推移（南高安地区）

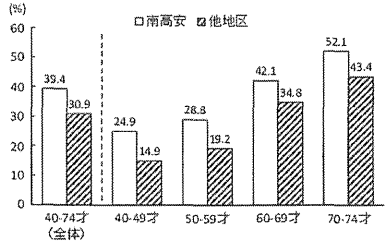

図4　特定健診受診率の比較／男女計（H22年度）

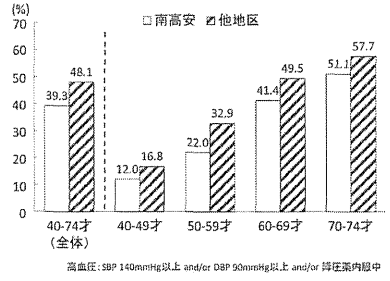

図5　高血圧者の割合の比較／男女計（H22～24年度）

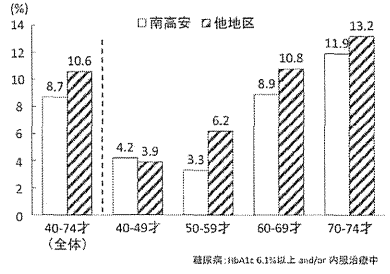

図6　糖尿病者の割合の比較／男女計（H22～24年度）

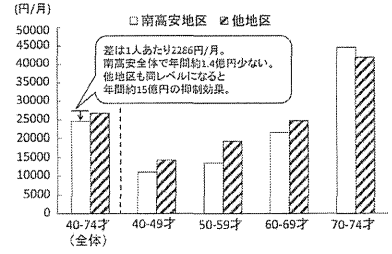

図7　被保険者1人あたり医療費の比較／男女計（H23年1月、2月、6月審査分の平均値）

Ⅷ. 今後の取り組み

南高安地区における循環器病予防対策の成果をもとに、平成24年にモデル地区以外の3地区で集団健診や健康づくり推進員育成の取り組みを始めた。地区組織の協力を得ながら、より身近なところで健診や結果説明会・講座を行い、受診率の向上・予防の推進・地域住民の健康意識の向上をめざしている。また、平成25年度には市内15箇所での健康相談を開催し、翌年度からは、市内の出張所11か所に、週に2日間保健師を配置し、地域住民の健康づくり活動の全市的な展開に取り組んでいる。地域での身近な健康相談の窓口を設置し、地域組織や関係機関と連携を密にすることで、各地域の健康課題を把握し、予防対策をさらに進めたい。

以上示した予防対策の成果は、対策をはじめて今日までの52年間、多くの方々のご協力・ご尽力の積み重ねによって得られたもので、私たちを支えてくださった筑波大学名誉教授の小町喜男先生、大阪がん循環器病予防センターの木山昌彦先生、大阪大学大学院医学系研究科社会医学講座公衆衛生学の磯博康先生、北村明彦先生、今野弘規先生をはじめ、多くの医師、保健師、栄養士、医療関係者そして住民の皆様に厚くお礼を申し上げます。

文　献

1）嶋本喬，飯田稔編著．地域における循環器疾患の疫学研究と予防対策の発展　秋田・大阪における40年の歩み．東京：財団法人日本公衆衛生協会，2007; 55-74.

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Fasting and non-fasting triglycerides and risk of ischemic cardiovascular disease in Japanese men and women: The Circulatory Risk in Communities Study (CIRCS)

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ABSTRACT

Background: Non-fasting triglycerides were reported to have a greater impact on risk of ischemic cardiovascular events than fasting triglycerides. However, evidence from Asia, where the prevalence of dyslipidemia is generally lower, has been limited.
Methods: We used 1975–1986 baseline surveys to investigate cohort data of 10,659 (4264 men and 6395 women) residents aged 40–69 years, initially free from ischemic heart disease and stroke, in four Japanese communities. Serum triglyceride concentrations at baseline were obtained for 2424 fasting (≥8 h after meal) and 8235 non-fasting (<8 h after meal) participants.
Results: During the 22-year follow-up, 284 (165 men and 119 women) developed ischemic heart disease and 666 (349 men and 317 women) ischemic stroke. After adjustment for age, sex and known cardiovascular risk factors, multivariable hazard ratios (95%CI) of ischemic cardiovascular disease (ischemic heart disease and ischemic stroke) for the highest versus lowest quartiles of triglycerides were 1.71 (1.14–2.59), *P* for trend = 0.013, for fasting participants and 1.60 (1.25–2.05), *P* for trend <0.001, for non-fasting participants. The positive associations did not differ between fasting and non-fasting men, while they were strong for non-fasting women. They were stronger for ischemic heart disease than for ischemic stroke. After further adjustment for HDL-cholesterol, these associations were slightly attenuated, but remained statistically significant.
Conclusion: Non-fasting as well as fasting triglycerides are predictive of risk of ischemic cardiovascular disease for Japanese men, as are non-fasting triglycerides for women.

1. Introduction

Although the impact of total and LDL-cholesterols on ischemic cardiovascular disease has been well established [1], the impact of triglycerides has remained controversial. Large meta-analyses, primarily performed in western countries [2–4], but not all [5], have identified moderate and statistically significant associations between triglycerides and risk of ischemic heart disease, stroke, or cardiovascular events, even after adjustment for cardiovascular risk factors including body mass index, diabetes mellitus and HDL-cholesterol. The evidence for Asian populations is limited, but a previous study of ours [6,7] and a meta-analysis by Asia Pacific Cohort Studies Collaboration [8] detected an independent relationship between triglycerides and risk of coronary heart disease. Furthermore, emerging evidence from western countries supports the notion that non-fasting triglycerides, a postprandial state of lipid profile, is an even better predictor of ischemic cardiovascular disease [9–12].

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High levels of non-fasting triglycerides reflect increased residues from chylomicrons and very low density lipoproteins. These cholesterol-containing and triglyceride-rich lipoprotein residues penetrate the arterial intima and are trapped within the arterial wall, leading to the development of atherosclerosis [13–15]. It remains to be determined, however, whether populations such as Japanese, with lower levels of total- or LDL-cholesterol and triglycerides, run a similar potential risk of high postprandial triglyceride levels.

Associations between non-fasting and fasting triglycerides and risk of incident cardiovascular disease in Asian countries have not been investigated systematically in any cohort studies. We hypothesized that non-fasting triglycerides constitute a better predictor for ischemic cardiovascular disease than fasting triglycerides in Asian populations whose prevalence of dyslipidemia is lower than that in western populations. To test our hypothesis, we examined the data of the Circulatory Risk in Communities Study (CIRCS), community-based prospective study of approximately 10,000 middle-aged Japanese men and women.

2. Methods

2.1. Study population

The surveyed population comprised 11,370 residents aged 40–69 years in four communities: Ikawa town (a rural community in Akita Prefecture in northwestern Japan), the Minami-Takayasu district in Yao City (a southwestern suburb in Osaka Prefecture), Noichi town, (a rural community in Kochi Prefecture in southwestern Japan) and Kyowa town (a rural community in Ibaraki Prefecture in central Japan) [16,17]. The baseline surveys were conducted in 1975–1980, 1975–1984, 1975–1980, and 1981–1986, respectively. The total census populations aged 40–69 years old in the four communities were, respectively, 2291 in 1975, 5538 in 1980, 3599 in 1975, and 5408 in 1980. The study participation rate was 65%. After the exclusion of participants with a history of coronary heart disease and/or stroke at baseline, the data for the remaining 10,659 subjects were analyzed. This study was approved by the ethics committees of the Osaka Medical Center for Health Science and Promotion and of Osaka University.

2.2. Follow-up and ascertainment of cases

Follow-up lasted until the end of 2005 for Kyowa and Noichi, 2009 for Ikawa and 2008 for Minami-Takayasu, and was terminated at the first incident of ischemic heart disease and stroke, exit from the community or death. Persons who moved out of the communities during the follow-up numbered 822 (8%), and 3597 (34%) persons died. These were censored at the date of moving out or the date of death. The median follow-up was 22.3 years for coronary heart disease and 22.2 years for ischemic stroke.

The details of endpoint determination have been described in previous CIRCS reports [16,17]. For all the residents, cardiovascular disease end points were ascertained from death certificates, national insurance claims, reports by local physicians, reports by public health nurses and health volunteers, and annual cardiovascular risk surveys. To confirm the diagnosis, all living patients were telephoned, visited or invited to take part in risk factor surveys, or a medical history was obtained from their families. In addition, medical records in the local clinics and hospitals were reviewed. In case of death with certain underlying causes of death (ICD 9 classification codes: 410–414, 428 and 429), histories were obtained from families and/or attending physicians and medical records were reviewed.

The criteria for ischemic heart disease, i.e. definite and probable myocardial infarctions, angina pectoris and sudden cardiac death within 1 h of onset were modified from those of the World Health Organization Expert Committee [1], as previously reported by us [16].

The criterion for incident stroke was a focal neurological disorder with rapid onset and persisting for at least 24 h or until death [17]. The determination of stroke subtypes was performed primarily by using CT/MRI findings, which were available for 81% of the stroke cases. Strokes that were diagnosed clinically but showed no lesion on CT/MRI films were classified based on the clinical criteria. Ischemic stroke was used as an outcome in this study. The final diagnosis for ischemic heart disease and ischemic stroke were made by a panel of 3–4 physicians participating in this study who were blinded to the data from the risk factor survey.

2.3. Baseline examination

Blood was drawn into a plain, siliconized glass tube and the serum was separated immediately after centrifugation. Fasting was not required. The time intervals since the last meal were: 0 – <1 h (3.0%), 1 – <2 h (20.7%), 2 – <3 h (43.7%), 3 – <8 h (9.9%), and ≥8 h (22.7%). Fasting was defined as ≥8 h after the last meal.

Serum triglycerides were measured with the fluorometric method using Autoanalyzer II (Technicon, Tarrytown, NY, U.S.A.) and serum total cholesterol was measured with the direct Lieberman–Burchard method using Autoanalyzer II for the period 1975–1979 and Autoanalyzer SMA-12/60 from 1979 to 1986 at the Osaka Medical Center for Cancer and Cardiovascular Diseases [6,16]. For 55% of the total sample (5880 subjects), HDL-cholesterol after heparin-manganese precipitation was measured at the same laboratory with the direct Liebermann–Burchard method. The Osaka Medical Center laboratory has been standardized by the Lipid Standardization Program, conducted by the Centers for Disease Control (Atlanta, GA), and successfully met the criteria for both reproducibility and accuracy of triglycerides and cholesterol measurements [18].

Serum total cholesterol was measured with the enzymatic method using Olympus AU 2700 at the lipid reference laboratory of the Osaka Medical Center for Health Science and Promotion, which is an international member of the US National Cholesterol Reference Method Laboratory Network. This laboratory has been certified since 1975 by the CDC-NHLBI Lipid Standardization Program conducted by the Centers for Disease Control and Prevention [18] and successfully met the performance criteria for both reproducibility and accuracy of serum triglycerides, total cholesterol and HDL-cholesterol measurements [19].

Blood pressures were measured by trained physicians using standard mercury sphygmomanometers and unified epidemiological methods [20]. Hypertension was defined as systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥100 mmHg and/or use of antihypertensive medication, while normotension was defined as systolic blood pressure <140 mmHg, diastolic blood pressure <90 mmHg and no antihypertensive medication use. All others were classified as borderline hypertension. Height was measured with the subjects in stocking feet and their weight while wearing light clothing. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m^2).

An interview was conducted to ascertain the number of cigarettes smoked per day, usual weekly intake of ethanol measured in units of *go* (a Japanese traditional unit of volume corresponding to 23 g ethanol), and menopausal status for women.

Serum glucose values were classified into three categories (diabetic, prediabetic and normal types). Diabetic type was defined

Table 1
Baseline characteristics of subjects according to quartiles of serum triglycerides.

	Fasting (≥8 h)					Non-fasting (<8 h)				
Triglycerides quartiles	1 (low)	2	3	4 (high)	*P* for difference	1 (low)	2	3	4 (high)	*P* for difference
Range of triglycerides, mmol/L	0.33–0.94	0.95–1.29	1.30–1.88	1.89–14.29		0.26–0.95	0.96–1.30	1.31–1.86	1.87–23.71	
Range of triglycerides, mg/dL	29–83	84–114	115–166	167–1266		23–84	85–115	116–165	166–2100	
Median triglycerides, mmol/L	0.77	1.12	1.52	2.47		0.77	1.12	1.54	2.47	
Median triglycerides, mg/dL	68	99	135	219		68	99	136	219	
Men										
No. at risk	247	228	225	259		807	773	797	928	
Age, year	55.8 (0.6)	55.9 (0.6)	54.4 (0.6)	52.5 (0.6)	<0.001	54.1 (0.3)	53.6 (0.3)	52.6 (0.3)	50.8 (0.3)	<0.001
Body mass index, kg/m^2	21.5 (0.2)	22.7 (0.2)	23.4 (0.2)	24.8 (0.2)	<0.001	21.7 (0.1)	22.4 (0.1)	22.9 (0.1)	24.0 (0.1)	<0.001
Systolic blood pressure, mmHg	142 (1)	144 (1)	142 (1)	147 (1)	0.005	134 (1)	136 (1)	137 (1)	141 (1)	<0.001
Diastolic blood pressure, mmHg	82 (1)	85 (1)	85 (1)	88 (1)	<0.001	80 (0.4)	81 (0.5)	83 (0.4)	85 (0.4)	<0.001
Use of antihypertensive medication, %	15	21	22	22	0.147	8	11	12	16	<0.001
Hypertensives, %	55	60	60	74	<0.001	37	43	50	56	<0.001
Serum total cholesterol, mmol/L	4.26 (0.06)	4.50 (0.06)	4.88 (0.06)	5.17 (0.05)	<0.001	4.43 (0.03)	4.56 (0.03)	4.78 (0.03)	5.12 (0.03)	<0.001
Serum HDL-cholesterol, mmol/L	1.62 (0.04)	1.49 (0.04)	1.45 (0.04)	1.33 (0.04)	<0.001	1.59 (0.02)	1.52 (0.02)	1.43 (0.02)	1.30 (0.02)	<0.001
Diabetes mellitus, %	26	22	30	31	0.141	4	4	6	7	0.010
Current smoking, %	69	69	63	68	0.579	64	65	65	68	0.312
Ethanol intake, g/day	27.9 (1.7)	25.6 (1.7)	27.5 (1.8)	29.4 (1.6)	0.463	29.4 (1.0)	27.9 (1.0)	28.9 (0.9)	29.7 (0.9)	0.541
Women										
No. at risk	358	384	377	346		1255	1291	1280	1104	
Age, year	51.1 (0.4)	53.9 (0.4)	56.1 (0.4)	56.9 (0.5)	<0.001	50.0 (0.2)	52.1 (0.2)	53.7 (0.2)	54.8 (0.2)	<0.001
Body mass index, kg/m^2	22.8 (0.2)	23.5 (0.2)	24.4 (0.2)	25.4 (0.2)	<0.001	22.3 (0.1)	23.1 (0.1)	23.7 (0.1)	24.6 (0.1)	<0.001
Systolic blood pressure, mmHg	138 (1)	141 (1)	142 (1)	145 (1)	<0.001	130 (1)	132 (1)	135 (1)	138 (1)	<0.001
Diastolic blood pressure, mmHg	80 (1)	83 (1)	84 (1)	85 (1)	<0.001	77 (0.3)	78 (0.3)	80 (0.3)	82 (0.3)	<0.001
Use of antihypertensive medication, %	19	26	26	30	0.005	8	10	13	17	<0.001
Hypertensives, %	50	61	60	67	<0.001	32	36	40	49	<0.001
Serum total cholesterol, mmol/L	4.58 (0.05)	4.87 (0.04)	5.20 (0.04)	5.53 (0.05)	<0.001	4.71 (0.02)	4.93 (0.02)	5.12 (0.02)	5.41 (0.02)	<0.001
Serum HDL-cholesterol, mmol/L	1.64 (0.02)	1.51 (0.02)	1.42 (0.02)	1.32 (0.03)	<0.001	1.61 (0.01)	1.52 (0.01)	1.45 (0.01)	1.34 (0.01)	<0.001
Diabetes mellitus, %	17	16	17	20	0.149	2	2	4	6	<0.001
Current smoking, %	7	9	6	11	0.118	5	7	8	8	0.005
Ethanol intake, g/day	1.3 (0.4)	1.8 (0.4)	1.1 (0.4)	1.3 (0.4)	0.526	1.4 (0.2)	1.1 (0.2)	1.2 (0.2)	1.3 (0.2)	0.865
Postmenopausal, %	63	69	71	72	0.001	56	57	60	62	<0.001

Values were presented as means ± standard errors or proportions, adjusted for age and community.
Non-fasting serum glucose values were also adjusted for time since last meal.

as a fasting glucose of 7.0 mmol/L or more, and/or a non-fasting glucose of 11.1 mmol/L or more, and/or use of medication for diabetes. Prediabetic type was defined as a fasting glucose of 6.1–6.9 mmol/L, and/or a non-fasting glucose of 7.8 mmol/L or more, without medication use for diabetes. All others were classified as normal type.

2.4. Statistical analyses

Analysis of covariance was used to test for differences in age-adjusted means and prevalence of baseline characteristics in terms of serum triglyceride categories, as well as stratification by fasting status. Person-years were calculated as the sum of individual follow-up time until the occurrence of incident ischemic heart disease, ischemic stroke, death, emigration, or the end of follow-up. Cox proportional hazards regression models were used to calculate the sex-specific and sex-adjusted hazard ratios and 95% confidence intervals (CIs) for incident ischemic cardiovascular disease using the risk for persons with the lowest category of serum triglycerides as reference. A test for trend of association between serum triglycerides and ischemic cardiovascular disease was also conducted using the median values of triglyceride levels for each category. When the assumption of proportional hazards was tested, no violation of the proportionality principle was found.

The hazard ratios were also calculated in relation to fasting status. The initial model was adjusted only for age and sex, while the variables for multivariable adjustment comprised age, sex (for total participants), community, systolic blood pressure, antihypertensive medication use, serum total cholesterol level (mg/dL), sex-specific quartiles of BMI (kg/m^2), smoking status (never, former and current 1–24 or ≥25 cigarettes per day), and alcohol intake (never, former, and current < 46, 46–68 or ≥ 69 g ethanol per day), serum glucose category (normal, impaired glucose tolerance and diabetes), time since last meal (<2, 2, 3–7 and 8 h or more), and for women menopausal status (pre- and post-menopause). Further adjustment for HDL-cholesterol (mmol/L) was conducted with a subsample for whom data on HDL-cholesterol were available. Sex interaction with the association between serum triglycerides and risk of ischemic cardiovascular disease was tested using a cross-product term of sex (0 or 1) and triglyceride levels (continuous) for the model.

All statistical analyses were performed with the SAS System for Windows (version 9.2; SAS Inc, Cary, NC). All *P*-values for statistical tests were two-tailed, and values of <0.05 were regarded as statistically significant.

3. Results

Table 1 lists sex-specific, age-adjusted mean values and prevalence of selected cardiovascular risk factors at baseline in relation to serum triglyceride quintiles and stratified by fasting status. For both fasting and non-fasting status, triglyceride levels were positively associated with body mass index, systolic and diastolic blood pressure levels, antihypertensive medication, hypertension, serum total cholesterol levels and serum glucose levels, and inversely associated with HDL-cholesterol levels for both sexes. Triglycerides levels were inversely associated with age for men, and positively with age, current smoking and postmenopausal status for women. Fasting subjects were 1–2 years younger and had lower means of body mass index and blood pressure levels, and higher prevalence of diabetes mellitus than non-fasting participants.

During the median 22-year follow-up totaling 232,947 person-years, there were 284 documented cases of incident ischemic heart diseases (165 men and 119 women) and 666 of incident ischemic strokes (349 men and 317 women).

Table 2 shows age- and community-adjusted and multivariable hazard ratios of ischemic cardiovascular disease (ischemic heart disease and ischemic stroke) by serum triglyceride quartile, per 1 mmol/L (88.6 mg/dL) increment of triglycerides, and for ≥2.26 mmol/L (200 mg/dL) and 1.69–2.25 mmol/L (150–199 mg/dL) of triglycerides compared to <1.69 mmol/L (150 mg/dL) of triglycerides. Serum triglyceride levels were positively associated with risk of ischemic cardiovascular disease for both men and women. After adjustment for conventional cardiovascular risk factors except HDL-cholesterol levels, the association with risk of ischemic cardiovascular disease weakened but remained statistically significant for both men and women. The multivariable hazard ratios (95%CIs) of ischemic cardiovascular disease for the highest versus lowest quartile of triglycerides were 1.48 (1.11–1.97); *P* for trend = 0.001 for men, 1.73 (1.24–2.40), *P* for trend < 0.001 for women and 1.65 (1.33–2.04), *P* for trend < 0.001 for total subjects. The positive association tended to be more evident for women than for men, although the sex interaction was not statistically significant (*P* for interaction = 0.535). As shown in Supplemental Table 1 the positive association with triglycerides was stronger for ischemic heart disease than for ischemic stroke for both men and women. For example, the multivariable hazard ratios (95%CIs) of ischemic heart disease for the highest versus lowest triglyceride quartile were 1.68 (1.02–2.76), *P* for trend = 0.012 for men, 2.11 (1.12–4.00), *P* for trend = 0.001 for women, and 1.93 (1.31–2.83), *P* for trend < 0.001 for total subjects, while those of ischemic stroke were 1.37 (0.98–1.92), *P* for trend = 0.025, and 1.53 (1.05–2.23), *P* for trend = 0.037, and 1.48 (1.15–1.90), *P* for trend < 0.001, respectively.

The multivariable hazard ratios associated with 1 mmol/L increment of triglycerides and for two or three other triglyceride categories showed similar results. For example, the risk after multivariable adjustment of ischemic cardiovascular disease was 11% for 1 mmol/L increment of triglycerides, 33% higher for ≥1.69 mmol/L, 45% higher for ≥2.26 mmol/L and 21% higher for 1.69–2.25 mmol/L of triglycerides, compared to <1.69 mmol/L of

Table 2
Hazard ratios (HR) of ischemic cardiovascular disease (ischemic heart disease and ischemic stroke) according to quartiles of serum triglycerides.

	Person years	No of events	Age and community-adjusted HR (95%CI)	Multivariable HR (95%CI)
Men				
Triglyceride quartiles				
Q1 (low)	21,939	108	1.00	1.00
Q2	20,185	95	0.99 (0.75–1.31)	0.94 (0.71–1.24)
Q3	20,047	129	1.48 (1.14–1.93)†	1.35 (1.03–1.77)*
Q4 (high)	23,085	157	1.77 (1.37–2.28)‡	1.48 (1.11–1.97)†
P for trend			<0.001	0.001
HR per 1 mmol/L increment	85,256	489	1.12 (1.06–1.18)‡	1.06 (0.99–1.13)
Triglycerides				
<1.69 mmol/L	57,040	301	1.00	1.00
≥1.69 mmol/L	28,216	188	1.52 (1.26–1.83)‡	1.32 (1.07–1.62)†
Triglycerides				
<1.69 mmol/L	57,040	301	1.00	1.00
1.69–2.25 mmol/L	12,181	79	1.40 (1.09–1.80)†	1.30 (1.00–1.69)*
≥2.26 mmol/L	16,035	109	1.61 (1.29–2.02)‡	1.33 (1.04–1.71)*
Women				
Triglyceride quartiles				
Q1 (low)	38,015	65	1.00	1.00
Q2	38,280	92	1.21 (0.88–1.67)	1.17 (0.85–1.62)
Q3	34,915	124	1.62 (1.19–2.20)†	1.46 (1.06–2.00)*
Q4 (high)	30,245	144	2.04 (1.51–2.77)‡	1.73 (1.24–2.40)†
P for trend			<0.001	<0.001
HR per 1 mmol/L increment	141,454	425	1.28 (1.19–1.38)‡	1.24 (1.13–1.36)‡
Triglycerides				
<1.69 mmol/L	102,716	253	1.00	1.00
≥1.69 mmol/L	38,738	172	1.47 (1.20–1.79)‡	1.27 (1.03–1.57)*
Triglycerides				
<1.69 mmol/L	102,716	253	1.00	1.00
1.69–2.25 mmol/L	20,629	72	1.19 (0.91–1.55)	1.09 (0.83–1.42)
≥2.26 mmol/L	18,109	100	1.78 (1.41–2.27)‡	1.49 (1.15–1.93)†
Total subjects				
Triglyceride quartiles				
Q1 (low)	59,954	173	1.00	1.00
Q2	58,465	187	1.09 (0.88–1.34)	1.05 (0.85–1.29)
Q3	54,962	253	1.56 (1.28–1.91)‡	1.43 (1.17–1.76)‡
Q4 (high)	53,330	301	1.94 (1.60–2.36)‡	1.65 (1.33–2.04)‡
P for trend			<0.001	<0.001
HR per 1 mmol/L increment	226,711	914	1.17 (1.12–1.21)‡	1.11 (1.06–1.17)‡
Triglycerides				
<1.69 mmol/L	159,756	554	1.00	1.00
≥1.69 mmol/L	66,955	360	1.54 (1.34–1.76)‡	1.33 (1.15–1.54)‡
Triglycerides				
<1.69 mmol/L	159,756	554	1.00	1.00
1.69–2.25 mmol/L	32,811	151	1.32 (1.10–1.59)†	1.21 (1.01–1.46)*
≥2.26 mmol/L	34,144	209	1.75 (1.49–2.06)‡	1.45 (1.21–1.73)‡

Test for significance: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.
Q1; 0.26–0.95 mmol/L (23–84 mg/dL), Q2; 0.96–1.30 mmol/L (85–115 mg/dL), Q3; 1.31–1.86 mmol/L (116–165 mg/dL), Q4; 1.87–23.71 mmol/L (166–2100 mg/dL).
Multivariable hazard ratio adjusted for age, sex, community, quartiles of body mass index, systolic blood pressure, use of antihypertensive medication, serum total cholesterol, cigarette smoking status, alcohol intake category, serum glucose category, time since last meal and for women, menopausal status.

Table 3
Multivariable hazard ratios (HR) of ischemic cardiovascular disease (ischemic heart disease and ischemic stroke) according to quartiles of serum triglycerides, stratified by fasting status.

	Person years	No of events	Age and community-adjusted HR (95%CI)	Multivariable HR (95%CI)
Men				
Fasting triglycerides				
Quartiles				
Q1 (low)	5017	30	1.00	1.00
Q2	4445	35	1.36 (0.83–2.23)	1.32 (0.80–2.19)
Q3	4390	34	1.47 (0.89–2.43)	1.47 (0.87–2.49)
Q4 (high)	4929	46	1.84 (1.15–2.96)*	1.75 (1.03–3.07)*
P for trend			0.015	0.056
HR per 1 mmol/L increment	18,781	145	1.08 (0.96–1.23)	1.07 (0.92–1.24)
Triglycerides				
< 1.69 mmol/L	12,881	92	1.00	1.00
≥ 1.69 mmol/L	5900	53	1.39 (0.98–1.96)	1.31 (0.89–1.95)
Triglycerides				
< 1.69 mmol/L	12,881	92	1.00	1.00
1.69–2.25 mmol/L	2451	23	1.41 (0.89–2.24)	1.35 (0.83–2.19)
≥ 2.26 mmol/L	3449	30	1.37 (0.90–2.08)	1.28 (0.79–2.08)
Non-fasting triglycerides				
Quartiles				
Q1 (low)	16,793	78	1.00	1.00
Q2	15,764	59	0.83 (0.59–1.16)	0.77 (0.55–1.09)
Q3	15,815	97	1.50 (1.10–2.04)*	1.28 (0.93–1.77)
Q4 (high)	18,104	110	1.73 (1.28–2.35)‡	1.34 (0.95–1.88)
P for trend			<0.001	0.016
HR per 1 mmol/L increment	66,476	344	1.13 (1.06–1.19)‡	1.05 (0.98–1.14)
Triglycerides < 1.69 mmol/L	44,159	209	1.00	1.00
≥1.69 mmol/L	22,317	135	1.59 (1.27–1.99)‡	1.29 (1.01–1.66)*
Triglycerides < 1.69 mmol/L	44,159	209	1.00	1.00
1.69–2.25 mmol/L	9731	56	1.42 (1.05–1.91)*	1.24 (0.91–1.70)
≥2.26 mmol/L	12,586	79	1.75 (1.34–2.28)‡	1.34 (1.00–1.80)
Women				
Fasting triglycerides				
Quartiles				
Q1 (low)	8461	15	1.00	1.00
Q2	8581	24	1.14 (0.59–2.17)	1.01 (0.52–1.97)
Q3	7678	45	1.98 (1.09–3.58)*	1.81 (0.97–3.37)
Q4 (high)	6930	36	1.59 (0.86–2.96)	1.36 (0.70–2.64)
P for trend			0.132	0.342
HR per 1 mmol/L increment	31,650	120	1.10 (0.93–1.31)	1.05 (0.86–1.27)
Triglycerides				
< 1.69 mmol/L	22,924	75	1.00	1.00
≥ 1.69 mmol/L	8726	45	1.12 (0.77–1.63)	1.04 (0.67–1.50)
Triglycerides				
< 1.69 mmol/L	22,924	75	1.00	1.00
1.69–2.25 mmol/L	4277	23	1.19 (0.74–1.91)	1.11 (0.69–1.80)
≥ 2.26 mmol/L	4449	22	1.05 (0.64–1.71)	0.89 (0.53–1.50)
Non-fasting triglycerides				
Quartiles				
Q1 (low)	29,349	50	1.00	1.00
Q2	29,652	68	1.23 (0.85–1.78)	1.19 (0.82–1.73)
Q3	27,576	80	1.46 (1.02–2.09)*	1.28 (0.88–1.87)
Q4 (high)	23228	107	2.23 (1.57–3.17)‡	1.87 (1.28–2.73)†
P for trend			<0.001	<0.001
HR per 1 mmol/L increment	109,805	305	1.35 (1.25–1.47)‡	1.32 (1.20–1.47)‡
Triglycerides				
< 1.69 mmol/L	79,792	178	1.00	1.00
≥ 1.69 mmol/L	30,013	127	1.66 (1.31–2.10)‡	1.43 (1.11–1.84)†
Triglycerides				
< 1.69 mmol/L	79,792	178	1.00	1.00
1.69–2.25 mmol/L	16,353	49	1.20 (0.87–1.65)	1.09 (0.79–1.52)
≥ 2.26 mmol/L	13,660	78	2.22 (1.69–2.93)‡	1.85 (1.37–2.50)‡
Total subjects				
Fasting triglycerides				
Quartiles				
Q1 (low)	13,477	45	1.00	1.00
Q2	13,026	59	1.29 (0.87–1.90)	1.22 (0.82–1.82)
Q3	12,069	79	1.81 (1.25–2.63)†	1.73 (1.17–2.56)†
Q4 (high)	11,858	82	1.87 (1.29–2.72)†	1.71 (1.14–2.59)*
P for trend			<0.001	0.013
HR per 1 mmol/L increment	50,430	265	1.12 (1.02–1.24)*	1.08 (0.96–1.21)
Triglycerides				
< 1.69 mmol/L	35,805	167	1.00	1.00
≥ 1.69 mmol/L	14,625	98	1.34 (1.04–1.72)*	1.22 (0.93–1.62)

(continued on next page)

Table 3 (*continued*)

	Person years	No of events	Age and community-adjusted HR (95%CI)	Multivariable HR (95%CI)
Triglycerides				
< 1.69 mmol/L	35,805	167	1.00	1.00
1.69–2.25 mmol/L	6727	46	1.39 (1.00–1.93)	1.31 (0.93–1.84)
≥ 2.26 mmol/L	7898	52	1.30 (0.94–1.78)	1.14 (0.80–1.62)
Non-fasting triglycerides				
Quartiles				
Q1 (low)	46,143	128	1.00	1.00
Q2	45,415	127	1.00 (0.78–1.28)	0.96 (0.75–1.23)
Q3	43,391	177	1.49 (1.18–1.88)‡	1.30 (1.02–1.66)*
Q4 (high)	41,332	217	1.98 (1.58–2.49)‡	1.60 (1.25–2.05)‡
P for trend			<0.001	<0.001
HR per 1 mmol/L increment	176,281	649	1.18 (1.13–1.23)‡	1.12 (1.06–1.18)‡
Triglycerides				
< 1.69 mmol/L	123,951	387	1.00	1.00
≥ 1.69 mmol/L	52,330	262	1.65 (1.41–1.94)‡	1.38 (1.16–1.65)‡
Triglycerides				
< 1.69 mmol/L	123,951	387	1.00	1.00
1.69–2.25 mmol/L	26,084	105	1.32 (1.06–1.64)*	1.18 (0.94–1.48)
≥ 2.26 mmol/L	26,246	157	2.00 (1.65–2.42)‡	1.59 (1.29–1.96)‡

Test for significance: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.
Q1; 0.26–0.95 mmol/L (23–84 mg/dL), Q2; 0.96–1.30 mmol/L (85–115 mg/dL), Q3; 1.31–1.86 mmol/L (116–165 mg/dL), Q4; 1.87–23.71 mmol/L (166–2100 mg/dL).
Multivariable hazard ratio adjusted for the same variables shown in Table 2.

triglycerides in all subjects. The associations with triglycerides were stronger for ischemic heart disease than for ischemic stroke (Supplemental Table 1).

As shown in Supplemental Table 2, we conducted a sub-analysis with further adjustment for HDL-cholesterol ($n = 5880$). The results were similar to those for total subjects shown in Table 2 and Supplemental Table 1, except for associations with ischemic heart disease for total subjects, which were consistent and significantly positive.

Table 3 shows the associations between triglyceride levels and risk of ischemic cardiovascular disease by fasting status. The associations were positive and did not differ substantially for fasting and non-fasting men, while they were more marked for non-fasting women and total subjects than for fasting subjects. The multivariable hazard ratios (95%CIs) of ischemic cardiovascular disease for the highest versus lowest triglyceride quartiles were 1.75 (1.03–3.07), *P* for trend = 0.056 for fasting men, and 1.34 (0.95–1.88), *P* for trend = 0.016 for non-fasting men, while that for ≥1.69 mmol/L of triglycerides versus the lower levels was 1.31 (0.89–1.95) for fasting men and 1.29 (1.01–1.66) for non-fasting men; that for ≥2.26 mmol/L of triglycerides versus <1.69 mmol/L of triglycerides was 1.28 (0.79–2.08) and 1.34 (1.00–1.80). The corresponding hazard ratios for women were 1.36 (0.70–2.64), *P* for trend = 0.342 and 1.87 (1.28–2.73), *P* for trend < 0.0001; 1.04 (0.67–1.50) and 1.43 (1.11–1.84); and 0.89 (0.53–1.50) and 1.85 (1.37–2.50), respectively, indicating that the significant positive associations were limited to non-fasting women. The corresponding hazard ratios for total subjects were 1.71 (1.14–2.59), *P* for trend = 0.013 and 1.60 (1.25–2.05), *P* for trend < 0.0001; 1.22 (0.80–1.62) and 1.38 (1.16–1.65); and 1.14 (0.80–1.62) and 1.59 (1.29–1.96), respectively. When we looked into ischemic heart disease and ischemic stroke, separately, these positive associations were more evident for non-fasitng than for fasting men, women and total subjects (Supplemental Table 3).

Further adjustment for HDL-cholesterol levels did not result in substantial changes for total subjects (Supplemental Table 4). For example, the multivariable hazard ratios (95%CIs) of ischemic cardiovascular disease for the highest versus lowest quartiles of triglycerides were 1.40 (0.70–2.81), *P* for trend = 0.517 for fasting persons, and 1.46 (1.02–2.08), *P* for trend = 0.005 for non-fasting persons; those for triglycerides ≥1.69 mmol/L versus the lower levels were 0.91 (0.55–1.49) for fasting persons and 1.49 (1.15–1.92) for non-fasting persons; and those for triglycerides ≥2.26 mmol/L and 1.69–2.25 mmol/L versus the lower levels were 0.92 (0.50–1.72) and 0.89 (0.47–1.67), respectively for fasting persons, and 1.62 (1.19–2.21) and 1.36 (0.99–1.87) for non-fasting persons.

4. Discussion

For our large, long-term prospective cohort of Japanese middle-aged residents, we found that, independent of other major cardiovascular risk factors, serum triglycerides levels were positively associated with risk of ischemic cardiovascular disease, and of either ischemic heart disease or ischemic stroke. These associations were more marked for ischemic heart disease than for ischemic stroke. When stratified by fasting status, these associations did not differ substantially between fasting and non-fasting men, but were more marked for non-fasting than for fasting women. For the subsample analysis (55% of total participants) with further adjustment for HDL-cholesterol levels, the results were similar. To the best of our knowledge, this is the first study conducted in an Asian country to examine the associations of non-fasting and fasting triglycerides with risk of cardiovascular disease for individuals who do not live in a western environment and are characterized by a lower prevalence of dyslipidemia than seen in western countries.

The stronger association between triglycerides and risk of ischemic heart disease than risk of ischemic stroke found in our study was consistent with the finding from a previous meta-analysis [5]. Further, the stronger association between triglycerides and risk of ischemic cardiovascular disease for women than for men in the present study was also consistent with the finding of a previous meta-analysis [2], but not with that of another which reported no sex difference [3]. That stronger association for women, in particular non-fasting ones, could be in part due to the smaller variability of non-fasting triglycerides in women than in men [21] because of the estrogen effect [22]. This smaller variability allowed for the representation of long-term non-fasting triglyceride values, so that the association was less attenuated.

As for the mechanisms involved in the positive association between non-fasting triglycerides and risk of ischemic cardiovascular disease, a high postprandial triglyceride level is closely linked with delayed clearance of chylomicron remnants [13,23]. These remnants with enhanced triglycerides and cholesterol esters are taken up into the arterial wall by means other than the LDL-cholesterol receptors, and they have been found to be as atherogenic as LDL-

cholesterol in animal experiments [12]. It is uncertain, however, whether postprandial increments in triglycerides levels differ for men and women. One study showed that the postprandial increment was larger for men than for women [24], while another study indicated that there was no difference in the increments did not differ when the quantity of visceral adipose tissue was equal [25].

High triglyceride levels in either the postprandial or fasting state are associated with increased small LDL particles [26], which may be more atherogenic than larger LDL particles because of increased susceptibility to oxidation [27]. High triglycerides levels are also associated with increased concentrations of factor VII and plasminogen activator inhibitor [13], as well as with enhanced insulin resistance [28] and blood leukocyte counts [29], all of which may accelerate atherosclerotic and thrombotic processes.

Our study's finding that the positive association between triglycerides and risk of ischemic heart disease was similar for fasting and non-fasting men was in agreement with the previous finding by a cohort study of American men enrolled in the Multiple Risk Factor Intervention Trial [9]. Two cohort studies of Japanese men showed a positive association between fasting triglycerides and risk of ischemic heart disease [30,31]. The positive association between triglycerides and risk of ischemic heart disease among women in our study was stronger for non-fasting than fasting status, and this was also consistent with the finding by a cohort study of American women enrolled in the trial of Women's Health Study [10]. However, our study succeeded in providing an integrated picture of potentially differential impacts of fasting and non-fasting triglycerides on risk of ischemic cardiovascular outcomes for men and women in a single cohort.

Our study has several strengths other than its large sample size, prospective design and long-term follow-up. First, we used incident cases of ischemic heart disease and ischemic stroke as the target endpoint because they may be more directly related with triglycerides than are fatal outcomes only. Second, ours was a community-based study of residents, so that our findings are likely to be able to be extrapolated to Japanese populations in general. Third, we examined sex-specific associations of triglycerides with incident ischemic cardiovascular disease stratified by fasting status, which allowed us to examine the impact of postprandial triglyceride concentrations on risk of ischemic cardiovascular disease.

There are, however, several limitations to this study. First, the study participants were not randomly assigned to fasting or non-fasting status because of the observational design. Non-fasting participants were slightly younger and had lower body mass index and blood pressure levels than their fasting counterparts, but other cardiovascular risk factors were similar, and these risk factors were adjusted for the multivariable analyses. Second, over 80 percent of the participants were non-fasting. Thus, our findings pertain primarily to postprandial triglycerides and ischemic cardiovascular disease, as in several previous studies [32–36]. This means that the findings of the investigation to detect the actual associations between fasting triglycerides and risk of ischemic cardiovascular disease were less robust, in particular for women. Third, the single measurement of triglycerides at baseline may have made the associations biased towards the null value because of their random measurement variations, so that the actual associations would be stronger.

Nevertheless, our findings support the usefulness of non-fasting triglyceride measurements in clinical practice and during population screening examinations because patients and participants do not need to attain fasting status, since non-fasting and fasting triglycerides have been shown to be equally predictive for ischemic cardiovascular disease for men, and even more predictive for women. Because over two-thirds of a person's life time is characterized by a non-fasting status, non-fasting triglycerides may reflect more individualized lipid profiles and metabolic status.

In conclusion, non-fasting as well as fasting serum triglycerides were found to be predictive for the risk of ischemic cardiovascular disease for middle-aged Japanese men, as were non-fasting triglycerides for women. The positive associations were stronger for ischemic heart disease than for ischemic stroke.

Author contributions

The authors made the following contributions: H.I.(first author) researched data, conducted the analyses and drafted the manuscript; H.I. (second author) researched data, contributed to the discussion and edited the manuscript; K.Y. researched data, coordinated the implementation of research at Kyowa and contributed to the discussion; T.Ohira, R.C., H.N., T.Okada, S.H., T.T. researched data and contributed to the discussion; S.S. researched data, coordinated the implementation of research at Ikawa and contributed to the discussion; M.K. researched data, coordinated the implementation of research at Minami-Takayasu and contributed to the discussion; A.K. researched data, coordinated the implementation of research at Noichi and Ikawa, and contributed to the discussion. None of the authors has any personal or financial conflict of interest to declare.

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Competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2014.08.028.

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Menopausal Status in Relation to Cardiovascular Stress Reactivity in Healthy Japanese Participants

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Objective: To investigate the hypothesis that postmenopausal women demonstrate greater cardiovascular stress reactivity during mental stress tasks than do both premenopausal women and men. **Methods:** The study included 979 Japanese participants (338 men and 641 women [238 postmenopausal]) aged 16 to 82 years. Blood pressure, heart rate, heart rate variability, and peripheral blood flow were measured at rest and during a mirror drawing stress task and a maze task. Differences between measured variables during tasks and at rest were calculated and considered to represent reactivity to stress. Analyses were adjusted for age and other potential confounding factors. **Results:** After adjusting for multiple factors, significant group effects were found for systolic blood pressure (SBP), diastolic blood pressure, heart rate, low-frequency (LF), LF/high-frequency, and peripheral blood flow (effect size: partial $\eta^2 = 0.015$, 0.011, 0.013, 0.013, 0.008, and 0.009, respectively). Postmenopausal women were more reactive than men to stress for SBP (15.4 ± 0.8 versus 11.7 ± 0.6 mm Hg), diastolic blood pressure (10.4 ± 0.6 versus 8.0 ± 0.5 mm Hg), heart rate (2.7 ± 0.5 versus 0.7 ± 0.4 beats/min), LF (23.0 ± 5.2 versus 3.2 ± 3.8 ms^2/Hz), and peripheral blood flow (−39.0 ± 3.8 versus −25.9 ± 2.8 Laser Doppler Perfusion Units) and more reactive than premenopausal women ($p < .050$) for SBP (15.4 ± 0.8 versus. 12.4 ± 0.5 mm Hg) and LF/high-frequency (1.7 ± 0.1 versus 1.3 ± 0.1). **Conclusions:** Postmenopausal Japanese women evidenced greater cardiovascular stress reactivity during mental stress tasks than did Japanese men or premenopausal women. Cardiovascular hyperreactivity could play a role in the higher risks of cardiovascular diseases in postmenopausal women. **Key Words:** menopausal status, cardiovascular stress reactivity, heart rate variability, blood pressure, heart rate, peripheral blood flow.

SBP = systolic blood pressure; **DBP** = diastolic blood pressure; **LF** = low-frequency; **HF** = high-frequency; **MDS** = mirror drawing stress; **ECG** = electrocardiogram; **pre** = pretask interval; **post** = posttask interval.

INTRODUCTION

There is evidence that more than 90% of coronary artery disease morbidity among women occurs in postmenopausal women (1). Estrogen deficiency is associated with altered vasomotor reactivity, and estrogen replacement normalizes this condition (2). Thus, there is some indirect evidence that estrogen has an impact on cardiovascular diseases.

Another potential contributor to the development of coronary heart disease is increased cardiovascular and neuroendocrine responses to mental stress (3). Such responses may trigger clinical events in individuals with disease through hemodynamic, vasoconstrictive, or coagulation factors (4). Cardiovascular reactivity to mental stress has been demonstrated to be a predictor of atherosclerosis and cardiac events (5,6). A systematic review based on the results of several prospective studies concluded that higher cardiovascular reactivity could lead to increased risk of cardiovascular outcomes such as high blood pressure, carotid atherosclerosis, carotid intima thickness, and increased left ventricular mass (7). The most commonly used indices of cardiovascular reactivity are blood pressure and heart rate. An increase in resting heart rate is associated with an increased risk of cardiovascular mortality in men aged 70 to 90 years (8). Heart rate variability is a widely used method for studying cardiac autonomic modulation of heart rate (9). Low resting heart rate variability is thought to reflect excessive sympathetic and inadequate parasympathetic modulation of heart rate and is a strong predictor of mortality among patients with coronary heart disease (10–12).

Cardiovascular and neuroendocrine responses to mental stress may be influenced by reproductive hormone status (13). Several studies have examined associations between menopausal status and cardiovascular reactivity to mental stress and reported that postmenopausal women have higher blood pressure and decreased parasympathetic modulation of heart rate during mental stress than do premenopausal women and/or men (5,13,14). However, these studies involved small samples (10–16 postmenopausal women) and failed to adjust statistically for possible confounding variables. Furthermore, there have been no general population studies of Asian participants.

The purpose of the present study was to investigate whether menopausal status is associated with cardiovascular reactivity to mental stress in healthy Japanese population. We postulated that postmenopausal women have increased cardiovascular reactivity to mental stress tasks compared with premenopausal women and men.

METHODS

Study Participants

The study included 979 Japanese people (338 men and 641 women) aged 16 to 82 years who underwent mental health checkups at the Osaka Medical Center for Health Science and Promotion between 2001 and 2009. The mental health checkups were conducted to examine associations of mental stress levels with somatic and psychological symptoms. The participants were drawn from companies around the Osaka area, Japan, and via the Web site of the Osaka Medical Center for Health Science and Promotion. The study was explained to potential participants, and only those who gave written consent were enrolled.

Most of the participants were employed persons (98% of men and 93% of women). Among the employed participants, the major occupation was teachers (54% of men and 62% of women). Unemployed persons made up 1.5% of the male and 6% of the female participants. Among the unemployed women, 65% stated that they were housewives.

Of the 641 women, 238 (37.1%) of those who answered "Yes" to the question "Have you already reached menopause?" were postmenopausal. Menopausal status was defined as the last menstrual period having been at least 1 year earlier. Of those who said they had already reached menopause, 20 women with a less than 1-year interval between current age and age at menopause were excluded from the analyses.

No participant had a history of stroke or myocardial infarction. There were 25 participants (16 men and 9 women) who had diabetic mellitus and 63 (27 men and 36 women) taking medications for hypertension, including 9 participants (4 men and 5 women) who had both diseases. They were excluded from the analyses. Finally, the data of 880 participants (299 men, 388 premenopausal women, and 193 postmenopausal women) were analyzed. Relevant participant variables are detailed in Table 1.

This study was approved by the Ethics Committee of the Osaka Medical Center for Health Science and Promotion. All participants signed informed consent forms before participating.

Experimental Tasks

The experimental tasks consisted of a modified mirror drawing stress (MDS) task and a maze task, both of which are frequently used to examine cardiovascular reactivity in a laboratory setting. In the MDS task, a complex pathway is presented to participants on a computer screen for 2 minutes (13,15). Participants are asked to trace the pathway with a mouse as accurately and as rapidly as possible. The horizontal and/or vertical axis controls of the mouse are reversed. The maze task (Amthat; Brain Medical, Japan) is designed to assess perceptual functioning, especially thinking ability, in elderly people. A maze is presented on a computer screen for 2 minutes. Participants are required to study the maze and plan how to reach the goal by passing through invisible walls in a grid composed of five lines and five columns. There were 2-minute intervals between tasks, during which cardiovascular reactivity was not assessed. The sequence of these tasks was fixed as follows: pretask interval, MDS task, posttask interval, maze task, and posttask interval.

Measurements

To assess cardiovascular reactivity, the systolic blood pressure (SBP; in mm Hg) and diastolic blood pressure (DBP; in mm Hg) were measured by tonometry and the heart rate (in beats/min) by electrocardiogram (ECG; BP-508SD; Omron Colin, Kyoto, Japan) during the pretask interval (pre), the MSD task, the maze task, and the posttask interval (post), each item having a duration of 2 minutes. Participants' ECGs were monitored from electrodes on the left subclavicular area and right lower chest. The R-R intervals were measured by a Memcal, which analyzes data while eliminating abnormal cardiac rhythms. When atrial fibrillation was present and/or more than 10% of recorded cardiac rhythm was abnormal, those data were omitted from the analyses. Power spectral analysis for the R-R intervals on the ECGs was performed every 128 beats to yield low-frequency (LF; 0.04–0.15 Hz) and high-frequency (HF) components (0.15–0.40 Hz) and their ratio (LF/HF).

Peripheral blood flow (Laser Doppler Perfusion Units) was measured by a laser Doppler blood-flowmeter (PriFlux PF-4000; Perimed, Stockholm, Sweden) on the third finger during the above-described experimental periods. Blood flow was obtained from the product of red blood cell counts and blood flow velocity. The laser Doppler blood-flowmeter allows measurement of intracapillary blood flow at a depth of about 0.5 mm from the skin surface (16,17).

Blood was drawn into a plain, siliconized glass tube and the serum separated immediately by centrifugation. Time since the last meal ranged from 0.2 to 21.2 hours; fasting was defined as at least 8 hours since eating. Serum glucose concentration was measured by the hexokinase method. Serum total cholesterol concentration was measured by the Liebermann-Burchard direct method. All blood measurements were performed by an autoanalyzer (Hitachi 7250; Hitachi, Tokyo, Japan) in the laboratory of the Osaka Medical Center for Health Science and Promotion.

Hypertension was defined as SBP at least 140 mm Hg, and/or DBP at least 90 mm Hg, and/or use of antihypertensive medication. Diabetes mellitus was defined as a fasting blood glucose concentration of at least 126 mg/dl or a nonfasting concentration of at least 200 mg/dl, and/or the use of medication for diabetes mellitus. Hyperlipidemia was defined as total cholesterol ≥ 220 mg/dl and/or use of medication for hyperlipidemia.

Height in stockinged feet and weight in light clothing were measured. Body mass index (BMI) was calculated as weight (kg) divided by the square of the height in meters (m^2). Participants were interviewed to ascertain smoking status (never, ex-smokers, and current smokers) and consumption of alcohol status (never, ex-drinkers, and current drinkers). Physical activity was evaluated according to the criteria of the Japan Arteriosclerosis Longitudinal Study (18). Participants were asked whether they had regularly performed physical exercise for more than 15 minutes within the previous 3 months and were categorized as physically active if they answered "Yes."

Statistical Analyses

Analysis of variance (ANOVA) was performed to compare age and BMI between the three groups (premenopausal women, postmenopausal women, and men). A χ^2 test was performed to compare categorical variables between the three groups. A 3 × 4 repeated ANOVA was performed to compare measured variables between the three groups and between the four task periods (pre, MDS task, maze task, and post). Because values for measured variables were higher during pretask than during posttask periods, the latter were considered as rest periods. Differences between variables during MDS tasks and posttask periods (MDS task-post) and during maze tasks and posttask periods (maze task-post) were calculated to provide measures of reactivity to stress. A 3 × 2 repeated ANOVA was performed to compare reactivity to stress between the three groups and two task periods. A 3 × 2 repeated analysis of covariance was performed adjusting for age, BMI, smoking status, and hypertension status. Bonferroni post hoc test was performed. All statistical analyses were performed with SPSS version 16. All probability values for statistical tests were two tailed, and p values less than .05 were regarded as statistically significant.

Age-matched analyses were performed to compare premenopausal and postmenopausal women, adjusting for BMI, smoking status, and hypertension status. In all, 76 premenopausal women were selected case by case to match 76 postmenopausal women based on age (age range, 42–58 years).

Multiple regression analyses were also performed on reactivity to MDS and maze tasks (outcome variables), whereas menopausal status (premenopausal as

TABLE 1. Demographic Variables of Premenopausal and Postmenopausal Women and Men

	Premenopausal Women (n = 388)	Postmenopausal Women (n = 193)	Men (n = 299)	p
Age, y, M (SD)	41.7[c] (7.7)	54.2[a] (4.5)	45.5[b] (9.4)	<.001
Body mass index, kg/m^2, M (SD)	22.0[b] (3.5)	22.3[b] (3.2)	23.6[a] (3.2)	<.001
Hypertension, %	5.4	11.4	20.4	<.001
Hyperlipidemia, %	26.0	57.5	33.1	<.001
Current smoker, %	10.8	7.8	25.8	<.001
Current drinker, %	38.9	41.5	63.5	<.001
Physically active, %	42.8	52.3	54.5	.005

Superscript letters a, b, and c indicate a significant difference between the values according to Bonferroni post hoc test ($p < .05$).

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a reference), age, BMI, drinking consumption, smoking status, hypertension status, hyperlipidemia status, diabetes mellitus status, and physical activity were treated as predictor variables.

RESULTS

Crude means of measured variables are detailed in Table 2. The main findings are that mean SBP and DBP were higher in men than in both premenopausal and postmenopausal women. Mean LF/HF was higher in men and postmenopausal women than in premenopausal women, whereas mean LF and HF was higher in premenopausal women than in postmenopausal women, and mean HF was also higher in premenopausal women than in men. The main effects of tasks on measured variables were as follows: mean SBP, DBP, and LF/HF were higher during both MDS and maze task periods than during pretask and posttask periods, whereas mean LF, HF, and peripheral blood flow during both MDS and maze task periods were lower than those during pretask and posttask periods. Mean heart rate during posttask period was lower than those during other periods. Mean SBP was higher in postmenopausal than in premenopausal women during both tasks, whereas there were no significant differences between postmenopausal and premenopausal women during pretask and posttask periods. Mean DBP during the maze task was higher in postmenopausal than in premenopausal women, whereas there were no significant differences between postmenopausal and premenopausal women during pretask and posttask periods. In men, mean heart rate during the MDS task did not differ from those during pretask and posttask periods. Mean LF/HF during the MDS task was higher in postmenopausal than in premenopausal women, whereas there were no significant differences between postmenopausal and premenopausal women during other periods. Mean peripheral blood flow during the maze task and posttask periods was significantly higher in postmenopausal than in premenopausal women.

TABLE 2. Cardiovascular Responses to Stress Challenge Tasks in Premenopausal Women, Postmenopausal Women, and Men

	Premenopausal Women		Postmenopausal Women		Men		Group Mean Value		Results of ANOVA		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Effect	F	*p*
Systolic blood pressure, mm Hg											
Pretask	111.4[bf]	0.8	114.4[bf]	1.1	122.3[af]	0.9	116.0[f]	0.5	Group	37.84	<.001
MDS task	120.8[cd]	1.0	126.4[bd]	1.5	133.0[ad]	1.2	126.8[d]	0.7	Task	473.47	<.001
Maze task	119.3[ce]	1.1	124.3[be]	1.5	130.1[ae]	1.2	124.6[e]	0.7	Group × Task	4.35	<.001
Posttask	108.1[bg]	0.8	109.7[bg]	1.2	119.3[ag]	1.0	112.4[g]	0.6			
Mean value	114.9[c]	0.9	118.7[b]	1.2	126.2[a]	1.0					
Diastolic blood pressure, mm Hg											
Pretask	68.1[bf]	0.6	70.4[bf]	0.9	78.1[af]	0.7	72.2[f]	0.4	Group	54.63	<.001
MDS task	75.2[bd]	0.8	78.2[bd]	1.1	86.2[ad]	0.9	79.9[d]	0.5	Task	373.37	<.001
Maze task	71.7[ce]	0.8	75.3[be]	1.1	82.0[ae]	0.9	76.3[e]	0.6	Group × Task	3.84	<.001
Posttask	64.8[bg]	0.7	66.5[bg]	1.0	75.7[ag]	0.8	69.0[g]	0.5			
Mean value	70.0[b]	0.7	72.6[b]	1.0	80.5[a]	0.8					
Heart rate, beats/min											
Pretask	73.3[d]	0.5	73.2[d]	0.7	73.3[d]	0.6	73.3[d]	0.4	Group	0.04	.96
MDS task	73.6[d]	0.6	74.2[d]	0.8	73.4	0.7	73.7[d]	0.4	Task	43.35	<.001
Maze task	73.1[d]	0.6	74.0[d]	0.8	73.4[d]	0.6	73.5[d]	0.4	Group × Task	4.63	<.001
Posttask	71.8[e]	0.5	70.9[e]	0.7	72.6[e]	0.6	71.7[e]	0.4			
Mean value	72.9	0.5	73.1	0.7	73.2	0.6					
LF, ms^2/Hz											
Pretask	59.1	3.1	44.6	4.3	63.7	3.4	55.8[e]	2.1	Group	6.01	.003
MDS task	76.5	4.3	62.3	6.1	70.3	4.9	69.7[d]	3.0	Task	14.59	<.001
Maze task	78.2	4.1	62.2	5.8	75.5	4.7	71.9[d]	2.8	Group × Task	1.23	.29
Posttask	59.9	3.1	46.1	4.4	67.6	3.5	57.9[e]	2.1			
Mean value	68.4[a]	2.7	53.8[b]	3.8	69.2[a]	3.1					
HF, ms^2/Hz											
Pretask	65.1	2.6	51.9	3.7	52.6	3.0	56.5[d]	1.8	Group	9.90	<.001
MDS task	41.0	1.8	29.2	2.5	31.4	2.0	33.9[e]	1.2	Task	112.18	<.001
Maze task	41.6	1.8	32.8	2.6	33.3	2.1	35.9[e]	1.3	Group × Task	0.80	.57
Posttask	66.4	2.7	51.0	3.8	53.5	3.0	57.0[d]	1.9			
Mean value	53.5[a]	1.9	41.2[b]	2.7	42.7[b]	2.2					
LF/HF											
Pretask	1.2[be]	0.1	1.3[be]	0.1	1.5[ae]	0.1	1.3[e]	0.0	Group	15.17	<.001
MDS task	2.5[bd]	0.1	3.0[ad]	0.1	3.1[ad]	0.1	2.9[d]	0.1	Task	279.55	<.001
Maze task	2.4[bd]	0.1	2.7[d]	0.1	3.0[ad]	0.1	2.7[d]	0.1	Group × Task	2.44	.024
Posttask	1.2[be]	0.1	1.2[be]	0.1	1.5[ae]	0.1	1.3[e]	0.0			
Mean value	1.8[b]	0.1	2.0[a]	0.1	2.3[a]	0.1					
Peripheral blood flow, PU											
Pretask	162.3[e]	4.2	174.2[e]	6.0	168.1[d]	4.8	168.2[e]	2.9	Group	2.79	.062
MDS task	135.8[f]	4.1	147.1[g]	5.8	147.5[e]	4.7	143.4[g]	2.8	Task	119.33	<.001
Maze task	140.7[bf]	4.3	160.3[af]	6.1	149.1[e]	4.9	150.0[f]	3.0	Group × Task	3.01	.006
Posttask	169.1[bd]	4.2	190.0[ad]	5.9	173.7[d]	4.8	177.6[d]	2.9			
Mean value	152.0	4.0	167.9	5.6	159.6	4.5					

MDS = mirror drawing stress; LF = low-frequency; HF = high-frequency; PU = Laser Doppler Perfusion Units; SE = standard error; ANOVA analysis of variance. Data shown are unadjusted (crude) means and SEs of the mean.

Superscript letters a, b, and c indicate a significant difference between the values according to Bonferroni post hoc test, when compared between menopausal statuses ($p < .05$).

Superscript letters d, e, f, and g indicate a significant difference between the values according to Bonferroni post hoc test, when compared between task and rest periods ($p < .05$).

Table 3 shows the mean differences between task and posttask periods (task-post). The main finding was that reactivity to stress for SBP and heart rate was greater in postmenopausal than in premenopausal women and men, whereas for DBP and peripheral blood flow, these parameters were greater in postmenopausal women than in men. Reactivity to stress for LF was greater in premenopausal and postmenopausal women than in men, and for the LF/HF ratio, it was greater in postmenopausal than in premenopausal women. On the other hand, for peripheral blood flow, it was greater in postmenopausal women than in men during MDS task periods. There were no significant differences between tasks in peripheral blood flow in men. Adjustment for the multiple covariates did not alter these findings.

According to age-matched analyses, the main finding concerned reactivity to stress as reflected by the LF/HF ratio ($F(1,128) = 7.55$, $p = .007$; premenopausal: mean = 1.19, standard error (SE) = 0.18; postmenopausal: mean = 1.91, SE = 0.18). An interaction of group and task was found in reactivity to stress for heart rate ($F(1,130) = 3.96$, $p = .049$; premenopausal: mean = 4.34, SE = 0.74, and mean = 3.49, SE = 0.68, for both MDS and maze tasks, respectively; postmenopausal: mean = 2.52, SE = 0.76, and mean = 3.06, SE = 0.69, respectively).

Table 4 shows the results of regression analysis of the association between menopausal status, sex, and multiple covariates on the reactivity to stress of the cardiovascular risk factors. Postmenopausal women had greater reactivity to stress for SBP and LF/HF than did premenopausal women, whereas the opposite trend was observed for peripheral blood flow. Men were less reactive to stress for heart rate and LF than were premenopausal women.

DISCUSSION

Our findings show that postmenopausal women are more reactive to stress for SBP, DBP, heart rate, and peripheral blood flow than are men and more reactive to stress for SBP and LF/HF than are premenopausal women. Adjustment for multiple confounding factors did not alter these findings. A meta-analysis of prospective studies revealed that increased cardiovascular reactivity to acute mental stress has an adverse effect on future cardiovascular risk status (19). Our findings suggest that menopause is significantly associated with greater changes in cardiovascular reactivity, which could be linked to greater risks of cardiovascular diseases in postmenopausal women.

Men had greater blood pressure during mental stress tasks and at baseline than did women; they also had greater LF/HF than premenopausal women. These findings are consistent with those of previous studies (5,13,15). Mean values of HF in premenopausal women were greater than those in postmenopausal women and men. It is widely accepted that HF is a reflection of cardiac parasympathetic activity (20). Because it is fully acknowledged that LF is influenced by both sympathetic and parasympathetic activities, the LF/HF ratio has been proposed as a measure of sympathetic-parasympathetic activity balance (21). The present findings are consistent with a previous report of stronger sympathetic nervous activity and weaker parasympathetic nervous activity in men than in women (15). However, so far it is not clear why such sex differences emerge in responses of cardiovascular reactivity to mental stress. One possible explanation for these sex differences is that they are mediated by hormonal mechanisms involving estrogen and progesterone. For example, greater reactivity to mental stress according to heart rate and/or blood pressure (22,23) and LF and LF/HF (15,24) has been observed during the luteal phase, during which concentrations of these hormones are greater than during the follicular phase. However, a recent review stated that interpretations of LF and LF/HF ratio components are controversial because the LF component is mainly determined by the parasympathetic system (25) and the LF/HF ratio does not accurately reflect sympathetic-parasympathetic balance (20). Therefore, interpretations of heart rate variability should be made with care.

According to our findings, postmenopausal women are more reactive to stress than premenopausal women and men. Heart rate reactivity did not differ between premenopausal and postmenopausal women, but both had higher heart rate reactivity than did men. It is noteworthy that mean values of cardiovascular measures of reactivity were greater in men than in women, whereas changes in cardiovascular measures in response to mental stress were greater in women. Reactivity to stress may be augmented by menopausal status beyond these sex differences. For example, in the present study, postmenopausal women were more reactive to stress for SBP and LF/HF than were premenopausal women. These results are also consistent with the findings of previous studies (5,13,26). Greater changes in blood pressure reactivity during mental stress in postmenopausal women than in premenopausal women could be attributable to hormonal differences between those two groups. Owen et al. (13) reported that lower concentrations of estrogen and greater follicle-stimulating hormone are associated with greater blood pressure during mental stress tasks.

In relation to postmenopausal women, the effects of aging on cardiovascular responses cannot be ignored because

TABLE 3. Comparison of Reactivity to Stress Between Premenopausal Women, Postmenopausal Women, and Men

	Crude									Multi-Adjusted								
	Premenopausal Women		Postmenopausal Women		Men		Results of ANOVA			Premenopausal Women		Postmenopausal Women		Men		Results of ANCOVA		
	Mean	SE	Mean	SE	Mean	SE	Effect	F	*p*	Mean	SE	Mean	SE	Mean	SE	Effect	F	*p*
Systolic blood pressure, mm Hg																		
[MDS task]–[posttask]	12.7	0.7	16.7	0.9	13.7	0.7	Group	9.57	<0.001	13.2	0.7	16.3	1.0	13.3	0.8	Group	6.75	0.001
[Maze task]–[posttask]	11.1	0.5	14.6	0.7	10.7	0.6	Task	33.58	<0.001	11.7	0.5	14.5	0.8	10.1	0.6	Task	5.11	0.024
Mean value	11.9[b]	0.5	15.7[a]	0.7	12.2[b]	0.6	Group × Task	1.34	0.26	12.4[b]	0.5	15.4[a]	0.8	11.7[b]	0.6	Group × Task	1.88	0.15
Diastolic blood pressure, mm Hg																		
[MDS task]–[posttask]	10.4	0.5	11.7	0.8	10.5	0.6	Group	3.58	0.028	10.6	0.6	11.9	0.8	10.2	0.6	Group	4.72	0.009
[Maze task]–[posttask]	6.9	0.4	8.8	0.5	6.3	0.4	Task	133.77	<0.001	7.2	0.4	9.0	0.6	5.9	0.4	Task	7.50	0.006
Mean value	8.7	0.4	10.3[a]	0.6	8.4[b]	0.5	Group × Task	1.40	0.25	8.9	0.4	10.4[a]	0.6	8.0[b]	0.5	Group × Task	1.44	0.24
Heart rate, beats/min																		
[MDS task]–[posttask]	1.8	0.4	3.4	0.5	0.8	0.4	Group	8.82	<0.001	2.2	0.4	2.7	0.6	0.7	0.4	Group	5.51	0.004
[Maze task]–[posttask]	1.3	0.3	3.1	0.4	0.9	0.3	Task	1.73	0.19	1.6	0.3	2.7	0.5	0.8	0.3	Task	3.93	0.048
Mean value	1.5[b]	0.3	3.2[a]	0.4	0.9[b]	0.4	Group × Task	0.95	0.39	1.9	0.3	2.7[a]	0.5	0.7[b]	0.4	Group × Task	1.53	0.22
LF, ms^2/Hz																		
[MDS task]–[posttask]	16.6	3.9	16.2	5.6	2.7	4.4	Group	3.39	0.034	17.3	4.2	18.4	6.3	0.4	4.6	Group	5.33	0.005
[Maze task]–[posttask]	18.5	4.1	16.1	5.8	7.9	4.6	Task	0.56	0.45	14.1	4.3	27.6	6.5	6.1	4.8	Task	10.37	0.001
Mean value	17.5[a]	3.3	16.2	4.6	5.3[b]	3.7	Group × Task	0.20	0.82	15.7[a]	3.5	23.0[a]	5.2	3.2[b]	3.8	Group × Task	1.14	0.28
HF, ms^2/Hz																		
[MDS task]–[posttask]	−25.4	2.2	−21.8	3.1	−22.2	2.5	Group	1.38	0.25	−23.2	2.4	−24.5	3.5	−23.3	2.6	Group	0.05	0.95
[Maze task]–[posttask]	−24.8	2.1	−18.2	2.9	−20.2	2.3	Task	3.68	0.055	−23.1	2.2	−20.2	3.3	−21.0	2.4	Task	4.83	0.028
Mean value	−25.1	2.0	−20.0	2.8	−21.2	2.3	Group × Task	0.59	0.56	−23.2	2.1	−22.3	3.2	−22.2	2.3	Group × Task	0.96	0.38
LF/HF																		
[MDS task]–[posttask]	1.3	0.1	1.9	0.1	1.5	0.1	Group	6.07	0.002	1.4	0.1	1.7	0.2	1.5	0.1	Group	3.55	0.029
[Maze task]–[posttask]	1.2	0.1	1.6	0.1	1.5	0.1	Task	3.87	0.049	1.2	0.1	1.7	0.1	1.4	0.1	Task	0.74	0.39
Mean value	1.3[b]	0.1	1.7[a]	0.1	1.5	0.1	Group × Task	0.96	0.38	1.3[b]	0.1	1.7[a]	0.1	1.5	0.1	Group × Task	0.14	0.87
Peripheral blood flow (PU)																		
[MDS task]–[posttask]	−33.3[e]	2.8	−42.9[be]	3.9	−26.3[a]	3.1	Group	3.25	0.039	−31.5[ae]	2.9	−45.7[be]	4.4	−26.8[a]	3.2	Group	3.81	0.023
[Maze task]–[posttask]	−28.4[d]	2.3	−29.6[d]	3.3	−24.6	2.6	Task	25.74	< 0.001	−26.8[d]	2.5	−32.3[d]	3.7	−25.0	2.7	Task	0.62	0.43
Mean value	−30.8	2.4	−36.3[b]	3.3	−25.4[a]	2.7	Group × Task	5.91	0.003	−29.1	2.5	−39.0[b]	3.8	−25.9[a]	2.8	Group × Task	4.73	0.009

MDS = mirror drawing stress; LF = low frequency; HF = high frequency; PU = Laser Doppler Perfusion Units; SE = standard error; ANOVA = analysis of variance; ANCOVA = analysis of covariance.
Crude data present unadjusted means and SEs, and multiadjusted data present means and SEs adjusted for age, body mass index, smoking status, and hypertension status.
Superscript letters a and b indicate a significant difference between the values according to Bonferroni post hoc test, when compared between menopausal statuses ($p < .05$).
Superscript letters d and e indicate a significant difference between the values according to Bonferroni post hoc test, when compared between task and rest periods ($p < .05$).

TABLE 4. Multivariate Regression Analyses of the Association Between Menopausal Status, Sex, Age, and Biobehavioral Measures as Related to Cardiovascular Reactivity to Stress

	Reactivity in SBP Pressure		Reactivity in DBP Pressure		Reactivity in Heart Rate		Reactivity in LF		Reactivity in HF		Reactivity in LF/HF		Reactivity in Peripheral Blood Flow	
	Standardized β	*p*	Standardized β	*p*	Standardized β	*p*	Standardized β	*p*	Standardized β	*p*	Standardized β	*p*	Standardized β	*p*
Postmenopausal[a], yes	0.12	.004	0.08	.053	0.05	.24	0.05	.30	0.00	>.99	0.11	.010	−0.08	.071
Men[a]	−0.03	.47	−0.05	.19	−0.10	.014	−0.08	.043	0.02	.63	0.06	.11	0.02	.59
Age	0.04	.36	−0.01	.74	0.09	.028	−0.11	.010	0.08	.047	0.00	.97	0.08	.057
Body mass index	0.04	.24	0.03	.45	−0.03	.43	0.01	.85	0.01	.68	−0.01	.72	−0.06	.097
Hypertension, yes	0.20	<.001	0.21	<.001	0.16	<.001	0.01	.79	0.02	.65	0.10	.007	0.02	.60
Hyperlipidemia, yes	−0.04	.31	−0.05	.19	−0.01	.70	0.05	.17	0.03	.46	0.02	.57	−0.05	.18
Current drinker, yes	−0.02	.59	0.00	.94	0.01	.86	−0.01	.71	−0.06	.091	−0.01	.84	0.05	.13
Current smoker, yes	−0.04	.28	−0.04	.27	−0.03	.37	0.08	.029	0.08	.034	0.03	.40	0.08	.018
Physically active, yes	−0.02	.61	−0.02	.56	0.04	.26	−0.02	.59	0.03	.39	−0.03	.37	0.01	.75
F	6.71	<.001	5.50	<.001	5.62	<.001	2.25	.017	1.70	.086	2.52	.008	2.76	.003
R	0.65		0.05		0.06		0.02		0.02		0.03		0.03	
Adjusted R^2	0.06		0.04		0.05		0.01		0.01		0.02		0.02	
Durbin-Watson test	1.91		1.92		1.83		2.02		2.03		1.81		1.82	

SBP = systolic blood pressure; DBP = diastolic blood pressure; LF = low frequency; HF = high frequency.
[a] Premenopausal women as a reference.

progressive reduction in estrogen concentrations may be an inevitable consequence of the aging process. It has been pointed out that age-related changes in regional blood flow response are regulated by endothelium-derived nitric oxide (27). The capacity of vascular endothelium to generate nitric oxide declines with aging. Consistent with this point, when measured as reactivity to 30 seconds of standing, the velocity of blood flow in the middle cerebral artery reportedly falls less in older than in younger participants (28). In heat stress conditions, older men have an attenuated skin vasoconstrictor response to the unloading of baroreceptors (29). It has been proposed that the inability of baroreceptors to modulate skin blood flow may play a role in the decreased ability of the elderly to properly regulate central blood volume and maintain blood pressure during blood pressure challenges. However, these basic findings are drawn from related studies that included only men or in which sex differences were not considered. In the present study, older men had smaller reactivity to stress in peripheral blood flow than did younger men, whereas the reactivity was greater in postmenopausal women (data not shown). In women, menopause may have a larger impact on cardiovascular responses to stress tasks than aging per se.

Furthermore, life-style habits may have effects on cardiovascular reactivity to mental stress. Although heart rate variability declines with advancing age in women, physically active women have significantly greater heart rate variability in all age groups (30). In the present study, however, adjustment for physical activity did not affect the findings. Chronic diseases related to cardiovascular diseases should also be considered. In the Framingham Heart Study, it was found that all heart rate variability measures, except for LF/HF, were significantly reduced in participants with hypertension than in those without hypertension (31). There was no significant change in the associations between postmenopausal status and cardiovascular reactivity to mental stress after adjustment for hypertension status. Possible impacts of oral contraceptives and hormone replacement therapy on cardiovascular measures have been postulated (32,33). In the present study, only two women (0.3%) were taking oral contraceptives and 16 (2.5%) were taking hormone replacement therapy. When those women were excluded from the analyses, the results were essentially the same (data not shown). Furthermore, 87 women (15.0%) had undergone surgery for uterine or ovarian problems (no information concerning whether these organs were removed). When those women were excluded from the analyses, the results were essentially the same (data not shown).

Several limitations of this study must be addressed. First, the effects of aging per se on cardiovascular reactivity to mental stress could not be discriminated clearly from those of postmenopausal status. Although age-matched analyses attenuated the significance of the associations between menopause status and cardiovascular reactivities, greater reactivity to stress for the LF/HF ratio persisted among postmenopausal women compared with their counterparts. Hormonal data, such as concentrations of estrogen and follicle-stimulating hormone, were not assessed in the present study. Second, sympathetic and

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parasympathetic modulation of reactivity to stress may depend on task characteristics. Two different tasks were used in the present study to assess reactivity to psychological stress. The procedure of conducting these two tasks was not counterbalanced. The latter task, namely, the maze task, may confer less psychological stress than the MDS task. Because self-reported measures of stress do not evaluate whether tasks are stressful for participants, their subjective stress levels for each task should have been assessed. Third, because the participants in this study had attended the center to check their stress levels, they may have had some interest in their mental health or actually had mental health problems. Thus, because we cannot assume that they were representative of the overall Japanese population, selection bias may have affected the results. We found no association between severity of depressive symptoms and cardiovascular reactivity (data not shown).

The present study has several strengths. Analyses were performed on a large sample of healthy Japanese participants. No participants had major histories of stroke or myocardial infarction, which might have influenced cardiovascular reactivity to mental stress. Furthermore, various potential confounders were taken into account in the analyses. Cardiovascular reactivity to mental stress was evaluated by various measures including blood pressure, heart rate, heart rate variability, and peripheral blood flow. It was also compared using two different tasks. Given that it has been shown that task involvement is directly associated with the magnitude of acute cardiovascular reactivity (34), use of multiple tasks would have diminished task-dependent differences in reactivity.

In conclusion, the present study showed that postmenopausal women have greater changes in cardiovascular reactivity to mental stress than do premenopausal women and men. Their autonomic reactivity to mental stress as measured by heart rate variability was also associated with menopausal status. Postmenopausal women may be at additional risk for cardiovascular diseases, beyond the risk of aging per se. The potential protective effects of high estrogen concentrations on cardiovascular reactivity should be further investigated.

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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 over 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 according to the hydrated density at d = 1.006, has been the established reference measurement procedure (RMP) for HDL-C and LDL-C [15,16]. BQ RMP performed at the U.S. Centers for Disease Control and Prevention (CDC) and Cholesterol Reference Method Laboratory Network (CRMLN) is considered the highest order RMP for this analyte. For over 15 years, the National Cerebral and Cardiovascular Center at Osaka, Japan has standardized their LDL-C BQ RMP through participation in the CRMLN. Members of the CRMLN are required to meet stringent performance criteria for precision and accuracy to allow both calibration and calibration verification of routine assays. Few reports are available on the performance of BQ RMP.

Using data obtained between May 1997 and October 2012, the precision and accuracy for HDL-C and LDL-C as measured at the Osaka laboratory were determined. We determined the fixed and/or proportional bias and correlations between the CDC and Osaka laboratories, and assessed factors that may affect results obtained with the BQ method by verifying relationships among bottom fraction cholesterol (BFC) – one major component of the BQ procedure, HDL-C, and LDL-C.

☆ 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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Table 1
Performance criteria applied to CRMLN lipid reference laboratory using BQ RMP.

Lipid	Precision	Accuracy
BFC	CV ≤ 1.5%	±(CDC LDL-C reference value × 0.02 + HDL-C bias vs. CDC) [max = ±2 mg/dL or 0.04 (HDL-C reference value) if smaller]
HDL-C	SD ≤ 1 mg/dL	±CDC HDL-C reference value × 0.04
LDL-C	CV ≤ 1.5%	±CDC LDL-C reference value × 0.02

CRMLN: Cholesterol Reference Method Laboratory Network. BQ RMP: Beta quantification reference measurement procedure.
CDC: US Centers for Disease Control and Prevention.
BFC: Bottom fraction cholesterol.

2. Material and methods

2.1. Materials

All materials were prepared according to Clinical Laboratory Standards Institute (CLSI) document C37-A. This implies that no preservatives or no additives were added. In this study, 67 different pool concentrations (lots) were used among the 280 survey samples provided by the CDC as part of the CRMLN monitoring surveys. One lot (bq47) was used 8 times over 2.5 years, which represented the longest period any lot was used. All CDC survey pools were blinded to the CRMLN participants. The pools were shipped frozen and stored at −70 °C before BQ analysis, and they were analyzed between May 1997 and October 2012 in 70 survey runs, with each survey run consisting of 3 to 5 different pools.

Measurements were conducted in the Osaka Medical Center for Cancer and Cardiovascular Diseases between July 1997 and June 2001, in the Osaka Medical Center for Health Science and Promotion between July 2001 and March 2012, and in the National Cerebral and Cardiovascular Center at Osaka continuously since April 2012 (all laboratories are referred to as 'Osaka laboratory').

2.2. Ultracentrifugation

BQ employs preparative ultracentrifuge (Beckman Coulter, Optima L-70K) to remove the chylomicrons and very-low-density lipoproteins (VLDL) of apo B-containing lipoproteins [17]. The methods at CDC and Osaka used 5 ml of serum per sample at a density of d = 1.006 kg/L (0.195 mol/L NaCL solution) and a 50.4 Ti rotor (Beckman Coulter) for UC. UC was carried out at CDC for 16.2 hours at 120,000 ×g, and 18 °C, and at Osaka for 18.5 hours, 105,000 ×g, and 18 °C. After UC, chylomicrons and VLDL in the top fraction (d < 1.006 kg/L) were removed and the remaining bottom fraction (d > 1.006 kg/L) including high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), and lipoprotein(a) (Lp(a)) was quantitatively transferred to a 5.00 mL volumetric flask and adjusted for volume with 0.15 mol/L NaCL solution [14,15]. The total cholesterol in this bottom fraction (BFC) was determined from one aliquot.

2.3. HDL-C precipitation

One mL aliquots of the apo B-containing lipoproteins in the bottom fraction were precipitated with 40 μL heparin (sodium injection, 5000 USP units/mL, Baxter Healthcare Corp.) and 50 μL manganese reagents (manganese(II) chloride solution, 1.00 mol/L ± 0.01 mol/L, SIGMA). The precipitate was removed by centrifugation for 30 min at 1500 ×g, 4 °C [18]. HDL-C was determined in the supernatant in duplicate measurements by the Abell–Kendall RMP [19]. LDL-C was calculated as the difference between BFC and HDL-C. A total of 8 replicate values per sample were obtained, and the mean of these replicates is used for comparison of assay performance.

2.4. Performance criteria

Performance criteria applied to the CRMLN lipid reference laboratories are summarized in Table 1. Because the LDL-C is the difference between BFC and HDL-C, the bias criterion for BFC was determined by the allowable bias for LDL-C and HDL-C and was considered to be ± the sum of the allowable HDL-C and LDL-C bias.

Table 2
Measurement performance of the CRMLN laboratory at Osaka determined with 280 pooled sera measured between May 1997 and October 2012 in 70 survey runs.

Statistical item	BFC	HDL-C	LDL-C
Mean precision as %CV (SD)	0.60 (0.342)	1.01 (0.605)	0.85 (0.461)
Mean bias as % (SD)	−0.12 (0.853)	0.45 (1.708)	−0.34 (1.148)
Pass rate for imprecision (N)	95.4% (267)	95.4% (267)	91.8% (257)
Pass rate for bias (N)	91.4% (256)	94.6% (256)	89.6% (251)
Absolute bias (%)	0.63 ± 0.589	1.23 ± 1.270	0.86 ± 0.830
Bias in mg/dL (95% CI)	0.34 (0.14, 0.53)	−0.16 (−0.26, −0.07)	0.49 (0.32, 0.66)
Limits of agreement in mg/dL	−2.87 to 3.54	−1.76 to 1.43	0.31 to 0.66
Slope (95% CI)	0.988 (0.981, 0.995)	0.980 (0.971, 0.989)	0.987 (0.980, 0.993)
Intercept (95% CI)	1.794 (0.581, 3.006)	1.118 (0.676, 1.560)	1.200 (0.388, 2.011)
Correlation coefficient as R^2	0.997	0.994	0.997

CRMLN: Cholesterol Reference Method Laboratory Network.
BFC: Bottom fraction cholesterol.