Table 2
Association of lipid measures with the presence of CAC (CAC score >0), adjusted for non-lipid risk factors in apparently healthy Japanese men aged 4079 years: SESSA, Shiga, 2006–2008.

	First quartile	Second quartile	Third quartile	Fourth quartile	*P* For trend
Standard lipids					
TC					
Range, mg/dl	<187	187–206	207–230	≥231	
Adjusted OR (95% CI)	1.00	0.98 (0.62–1.53)	1.19 (0.76–1.89)	1.82 (1.14–2.90)	0.006
LDL-C					
Range, mg/dl	<106.0	106.0–123.3	123.4–146.5	≥146.6	
Adjusted OR (95% CI)	1.00	1.47 (0.94–2.31)	1.35 (0.86–2.13)	2.06 (1.28–3.30)	0.005
HDL-C					
Range, mg/dl	<47	47–56	57–68	≥69	
Adjusted OR (95% CI)	1.00	0.83 (0.52–1.32)	0.50 (0.31–0.81)	0.56 (0.34–0.91)	0.011
TG					
Range, mg/dl	<74	74–99	100–144	≥145	
Adjusted OR (95% CI)	1.00	1.17 (0.75–1.83)	1.03 (0.66–1.61)	2.02 (1.24–3.29)	0.004
Non-HDL-C					
Range, mg/dl	<126	126–147	148–171	≥172	
Adjusted OR (95% CI)	1.00	1.43 (0.91–2.23)	1.53 (0.96–2.42)	2.08 (1.29–3.36)	0.003
Lipoprotein particle concentrations					
LDL-P					
Total					
Range, nmol/l	<1010	1010–1269	1270–1540	≥1541	
Adjusted OR (95% CI)	1.00	1.50 (0.95–2.35)	1.59 (1.00–2.5 s)	2.01 (1.24–3.23)	0.004
Large					
Range, nmol/l	<453	453–633	634–830	≥831	
Adjusted OR (95% CI)	1.00	0.79 (0.50–1.24)	0.92 (0.58–1.46)	0.91 (0.57–1.44)	0.847
Small					
Range, nmol/l	<103	103–516	517–841	≥842	
Adjusted OR (95% CI)	1.00	1.17 (0.75–1.84)	1.41 (0.88–2.25)	1.87 (1.14–3.05)	0.008
IDL					
Range, nmol/l	<41	41–90	91–162	≥163	
Adjusted OR (95% CI)	1.00	0.85 (0.54–1.32)	0.81 (0.52–1.27)	0.89 (0.56–1.41)	0.788
HDL-P					
Total					
Range, μmol/l	<29.8	29.8–33.3	33.4–37.4	≥37.5	
Adjusted OR (95% CI)	1.00	1.13 (0.71–1.81)	0.66 (0.42–1.06)	1.04 (0.62–1.75)	0.766
Large					
Range, μmol/l	<4.3	4.3–6.5	6.6–9.7	≥9.8	
Adjusted OR (95% CI)	1.00	0.74 (0.47–1.18)	0.64 (0.39–1.05)	0.68 (0.41–1.12)	0.185
Medium					
Range, μmol/l	<4.9	4.9-7.2	7.3-10.2	≥10.3	
Adjusted OR (95% CI)	1.00	1.23 (0.78–1.94)	1.27 (0.81–1.99)	1.36 (0.85–2.17)	0.238
Small					
Range, μmol/l	<15.4	15.4-18.7	18.8-22.1	≥22.2	
Adjusted OR (95% CI)	1.00	1.14 (0.71–1.82)	0.97 (0.61–1.54)	0.66 (0.42–1.05)	0.060
VLDL-P					
Total					
Range, nmol/l	<27.5	27.5–49.7	49.8–83.9	≥84.0	
Adjusted OR (95% CI)	1.00	1.06 (0.68–1.66)	1.17 (0.74–1.84)	1.82 (1.13–2.95)	0.009
Large					
Range, nmol/l	<0.3	0.3-0.5	0.6-1.5	≥1.6	
Adjusted OR (95% CI)	1.00	0.99 (0.63–1.55)	0.72 (0.46–1.13)	0.98 (0.61–1.57)	0.743
Medium					
Range, nmol/l	<7.7	7.7-17.9	18.0-35.4	≥35.5	
Adjusted OR (95% CI)	1.00	1.28 (0.82–2.00)	1.21 (0.78–1.89)	1.70 (1.06–2.73)	0.042
Small					
Range, nmol/l	<12.6	12.6-25.4	25.5-46.5	≥46.6	
Adjusted OR (95% CI)	1.00	0.74 (0.47–1.15)	1.52 (0.96–2.41)	1.46 (0.92–2.34)	0.017
Lipoprotein particle size					
LDL-P					
Range, nm	<20.6	20.6–20.9	21.0–21.2	≥21.3	
Adjusted OR (95% CI)	1.00	0.66 (0.41–1.06)	0.69 (0.44–1.09)	0.59 (0.36–0.97)	0.041
HDL-P					
Range, nm	<8.8	8.8–9.2	9.3–9.6	≥9.7	
Adjusted OR (95% CI)	1.00	0.70 (0.44–1.12)	0.64 (0.40–1.01)	0.66 (0.40–1.10)	0.115
VLDL-P					
Range, nm	<40.4	40.4–43.3	43.4–47.3	≥47.4	
Adjusted OR (95% CI)	1.00	0.95 (0.58–1.56)	1.21 (0.72–2.03)	0.67 (0.40–1.12)	0.120
Ratios					
TC/HDL-C					
Range	<2.93	2.93–3.63	3.64–4.45	≥4.46	
Adjusted OR (95% CI)	1.00	1.26 (0.81–1.96)	1.67 (1.05–2.64)	2.27 (1.37–3.78)	0.001
LDL-C/HDL-C					
Range	<1.65	1.65–2.19	2.20–2.88	≥2.89	
Adjusted OR (95% CI)	1.00	1.21 (0.78–1.89)	1.62 (1.02–2.56)	1.73 (1.06–2.85)	0.016

Table 2 (*continued*)

	First quartile	Second quartile	Third quartile	Fourth quartile	*P* For trend
LDL-P/HDL-P					
Range	<0.029	0.029–0.036	0.037–0.048	≥0.049	
Adjusted OR (95% CI)	1.00	1.28 (0.82–2.01)	1.86 (1.17–2.96)	1.92 (1.18–3.17)	0.004

Odds ratios were adjusted for age, smoking status (former, current), ethanol consumption (g/day), body mass index, blood glucose, systolic blood pressure, medication status (hypertension, diabetes), and type of CT. Abbreviations: CI, confidence interval; OR, odds ratio; other abbreviations are as in Table 1.

associated with CAC. Among single standard lipids, non-HDL-C had a large OR for the presence of CAC. All of the standard lipids were also significantly associated with CAC. As shown in Table 2, ORs for NMR measures were of approximately similar magnitude to those for standard lipids or ratios. However, the TC/HDL-C ratio had the largest OR for the presence of CAC among all of the indices of lipid or lipoprotein that we studied.

The ORs of CAC per 1-SD increase in lipid indices adjusted for non-lipid risk factors and type of CT are shown in the Fig. 1. Among the NMR-measured lipoproteins, the strength of association with CAC was largest for total LDL-P concentration, although it was not predominant over that for standard lipids. Among the single standard lipids, non-HDL-C had a large OR per 1-SD increase for the presence of CAC. TC/HDL-C ratio had the largest OR per 1-SD increase for the presence of CAC among all of the indices of lipid or lipoprotein that we studied.

In the model that included non-lipid risk factors plus other lipids (as continuous variables), the association of total LDL-P concentration with CAC was attenuated (Supplementary Table II). In particular, no significant association of total LDL-P concentration was found after adjustment for TC/HDL-C ratio or non-HDL-C when LDL-P was examined either as a quartile (highest quartile OR, 1.37; 95% CI, 0.74–2.54 and highest quartile OR, 1.36; 95% CI, 0.66–2.80, respectively) or a continuous variable (OR per 1-SD increase, 1.07; 95% CI, 0.84–1.35 and OR per 1-SD increase, 1.03; 95% CI, 0.76–1.39, respectively). Whereas, TC/HDL-C ratio was still significantly associated with CAC after adjustment for total LDL-P concentration when TC/HDL-C ratio was examined both as a quartile (highest quartile OR, 2.01; 95% CI, 1.04–3.89) and a continuous variable (OR per 1-SD increase, 1.30; 95% CI, 1.01–1.68).

4. Discussion

In this cross-sectional study among an apparently healthy Japanese population, we found that lipoprotein particle profiles assessed by NMR were significantly associated with the presence of CAC after adjustment for non-lipid risk factors. This magnitude of association was comparable, but not superior, to that of standard lipids. Among the NMR-measured lipoproteins, the strength of association with CAC was largest for total LDL-P concentration. However, this association was weaker than that of either non-HDL-C or TC/HDL-C. To the best of our knowledge, this is the first study to directly compare NMR-measured lipoproteins with standard lipids and ratios in relation to CAC within a Japanese or an Asian population.

Our results support 2 prospective population-based studies, which reported that routine measurement of NMR lipoproteins is similar in incident CVD risk prediction to non-HDL-C and TC/HDL-C ratio, and is not recommended when a standard lipid panel is available [5,6]. However, in these previous studies, one was conducted among only women [5], and the other was a nested case–control study design [6]. Surprisingly, 2 cross-sectional studies that compared these lipid indices in association with subclinical atherosclerosis reported a stronger relation of NMR-based indices than standard lipids [7,8]. Therefore, only a limited number of Western studies directly compared the risk estimates of clinical CVD or subclinical atherosclerosis between NMR-based lipoprotein profiles and standard lipids, and showed conflicting results. Some prospective studies of the Japanese and US general populations also suggested NMR-based lipoprotein profiles as alternative lipid measures for improved risk assessment of CVD, but this was not assessed in comparison with non-HDL-C or lipid ratios [31–33]. Consistent with the prospective Western studies with hard

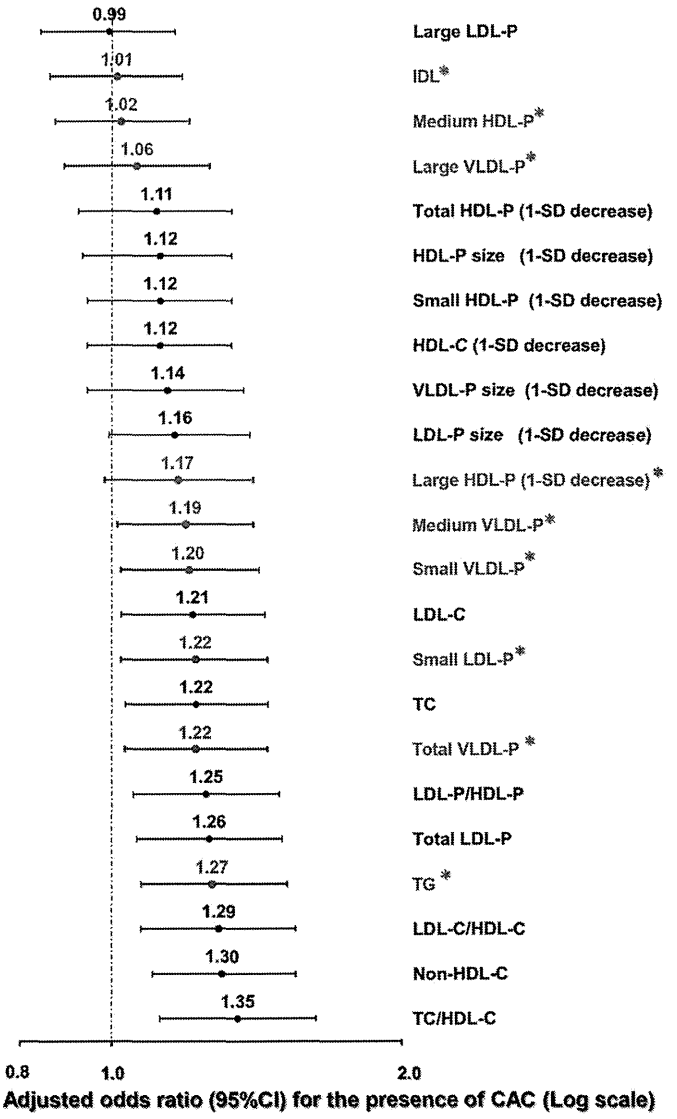

Fig. 1. Adjusted odds ratios and 95% CIs for the presence of coronary artery calcification (CAC score >0) per 1-standard deviation (SD) increase in lipid indices (per 1-SD decrease in the case of large HDL-P, total HDL-P, HDL-C, small HDL-P, HDL-P size, VLDL-P size, and LDL-P size) in apparently healthy Japanese men aged 40–79 years: SESSA. Adjustment for age, smoking status (former, current), ethanol consumption (g/day), body mass index, blood glucose, systolic blood pressure, medication status (hypertension, diabetes), and type of CT was performed. *Lipid indices with skewed distributions were log-transformed. Abbreviations: all abbreviations are as in Table 2.

endpoints [5,6], the association of CAC with NMR measures was comparable, but not predominant, over that with standard lipids in our study. This was the case in the Japanese general population where the burden of CAD, subclinical atherosclerosis, and lipid profiles are quite different compared with Western populations.

Non-HDL-C, defined as TC minus HDL-C, includes all of the potentially atherogenic lipid fraction (LDL-C, lipoprotein [a], IDL cholesterol, and VLDL remnants). TC/HDL ratio gives identical results to non-HDL-C/HDL-C ratio (note that TC/HDL ratio = 1 plus non-HDL-C/HDL-C ratio), and thus exclusively consists of non-HDL-C divided by an anti-atherogenic denominator (HDL-C). Inclusion of all of the atherogenic lipid fractions in the calculation of non-HDL-C and TC/HDL-C ratio may then provide an improved assessment of CVD risk. In fact, a prospective cohort study in the Japanese population showed that the predictive value of non-HDL-C for incident CAD is almost similar to that of LDL-C and recommended non-HDL-C as an alternative screening marker for primary prevention of CAD in the Japanese community [34].

Our data also support current guidelines that recommend the use of a standard lipid panel, especially non-HDL-C or TC/HDL-C ratio, for CVD risk assessment in clinical practice. [23,24,35,36] European, North American, and Japanese guidelines recommend the use of standard lipids for CVD risk assessment in asymptomatic individuals. Non-HDL-C is introduced as another means to refine risk estimation beyond LDL-C from Friedwald's formula, especially in combined hyperlipidemia, diabetes, metabolic syndrome, or chronic kidney disease [23,24,36]. TC/HDL ratio is useful for CVD risk estimation [23,24]. By contrast, a statement involving an international panel of lipid experts proposed that CVD risk may be more closely related to atherogenic lipoprotein particle concentrations than to LDL-C [37].

Our study has several limitations. First, the study design was cross-sectional. Therefore, causal and longitudinal relationships were not addressed. Second, we did not examine CVD outcome, although our data are consistent with large prospective studies with assessment of clinical CVD. Finally, our analyses were limited to apparently healthy Japanese men, and our results should not be generalized to women, other races, and patient populations.

In conclusion, the overall association of CAC with NMR-measured lipoprotein indices is comparable, but not superior to, that with standard lipids in a community-based sample of asymptomatic Japanese men who do not use lipid-lowering medication. Non-HDL-C and TC/HDL ratio are strongly associated with the presence of CAC. Therefore, our data support the clinical use of standard lipids, particularly non-HDL-C and TC/HDL ratio, which are highly effective and readily available for routine CVD risk assessment.

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Conflict of interest

There is no potential conflict of interest that relates to the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can http://dx.doi.org/10.1016/j.atherosclerosis.2014.07.019.

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High-density lipoprotein particle concentration and subclinical atherosclerosis of the carotid arteries in Japanese men☆

Maryam Zaid [a], Akira Fujiyoshi [a,*], Katsuyuki Miura [a,b], Robert D. Abbott [b], Tomonori Okamura [c], Naoyuki Takashima [a], Sayuki Torii [a], Yoshino Saito [a], Takashi Hisamatsu [a,b], Naoko Miyagawa [a], Takayoshi Ohkubo [a,d], Aya Kadota [b], Akira Sekikawa [e], Hiroshi Maegawa [f], Yasuyuki Nakamura [a,g], Kenichi Mitsunami [h], Hirotsugu Ueshima [a,b], For the SESSA Research group

[a] Department of Public Health, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga, 520-2192, Japan
[b] Center for Epidemiologic Research in Asia, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga, 520-2192, Japan
[c] Department of Preventive Medicine and Public Health, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku, Tokyo, 160-8582, Japan
[d] Department of Hygiene and Public Health, Teikyo University School of Medicine, 2-11-1 Kaga Itabashi-ku, Tokyo, 173-8605, Japan
[e] Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, 130 De Soto Street, Pittsburgh, PA, 15261, USA
[f] Division of Endocrinology and Metabolism, Department of Internal Medicine, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga, Japan
[g] Department of Cardiovascular Epidemiology, Kyoto Women's University, 35 Kitahiyoshi-cho, Imakumano, Higashiyama-ku, Kyoto, 605-8501, Japan
[h] Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga, 520-2192, Japan

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ABSTRACT

Objective: The association of high-density lipoprotein particle (HDL-P) with atherosclerosis may be stronger than that of HDL-cholesterol (HDL-C) and independent of conventional cardiovascular risk factors. Whether associations persist in populations at low risk of coronary heart disease (CHD) remains unclear. This study examines the associations of HDL-P and HDL-C with carotid intima-media thickness (cIMT) and plaque counts among Japanese men, who characteristically have higher HDL-C levels and a lower CHD burden than those in men of Western populations.

Methods: We cross-sectionally examined a community-based sample of 870 Japanese men aged 40–79 years, free of known clinical cardiovascular disease (CVD) and not on lipid-lowering medication. Participants were randomly selected among Japanese living in Kusatsu City in Shiga, Japan.

Results: Both HDL-P and HDL-C were inversely and independently associated with cIMT in models adjusted for conventional CHD risk factors, including low-density lipoprotein cholesterol (LDL-C) and diabetes. HDL-P maintained an association with cIMT after further adjustment for HDL-C ($P < 0.01$), whereas the association of HDL-C with cIMT was noticeably absent after inclusion of HDL-P in the model. In plaque counts of the carotid arteries, HDL-P was significantly associated with a reduction in plaque count, whereas HDL-C was not.

Conclusion: HDL-P, in comparison to HDL-C, is more strongly associated with measures of carotid atherosclerosis in a cross-sectional study of Japanese men. Findings demonstrate that, HDL-P is a strong correlate of subclinical atherosclerosis even in a population at low risk for CHD.

1. Introduction

Many studies have reported an inverse association between high-density lipoprotein cholesterol (HDL-C) and coronary heart disease (CHD) [1–3]. This has led to the notion that cardiovascular risk may drop significantly once HDL-C levels are increased [4]. However, recent trials involving pharmacological increases in HDL-C levels have reported no significant effects on the reduction of

☆ Institution where work was performed: Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga, 520-2192, Japan.

* Corresponding author.
E-mail address: afujiy@belle.shiga-med.ac.jp (A. Fujiyoshi).

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carotid intima-media thickness (cIMT) [5], the progression of coronary atherosclerosis [6], or any other cardiovascular measurement [7,8]. Also, a large mendelian randomization study has shown that some polymorphisms associated with genetically higher HDL-C levels do not lower risk of myocardial infarction [9]. Lack of improved cardiovascular outcomes with increased HDL-C has stressed the view that increasing HDL-C levels may not directly translate to decreases in cardiovascular risk [10] and, thus has led to a surge of interest in identifying other features of HDL that can be targeted for assessing cardiovascular risk.

Recently, total HDL particle (HDL-P) concentration has been shown to be a marker of reduced cardiovascular risk [11–13] and some evidence suggests that this is independent of HDL-C [12]. However, studies on HDL-P were largely limited to Western populations, which are known to have a higher risk of CHD and lower levels of HDL-C than less vulnerable regions of Asia, particularly Japan [14–16]. Whether associations persist in these regions at lower risk for CHD and with higher HDL-C levels remains unclear. Our objective is to evaluate the association of HDL-C and HDL-P with subclinical atherosclerosis in a population-based sample of Japanese men.

2. Methods

2.1. Study participants

The Shiga Epidemiological Study of Subclinical Atherosclerosis (SESSA) aims to examine various factors associated with subclinical atherosclerosis. The design of this study is described elsewhere [17]. In brief, from 2006 to 2008, 1094 Japanese men aged 40–79 years were randomly selected from the general population in Kusatsu City, Shiga, Japan. After excluding those on lipid-lowering medications (n = 168) and missing information on HDL-P, HDL-C or lipid-lowering medications (n = 56), 870 remained for analysis in the current report. All participants provided written informed consent. The study complies with *the Declaration of Helsinki* and was approved by the Institutional Review Board of Shiga University of Medical Science, Otsu, Japan.

Factors collected through physical examinations include height, weight, blood pressure, and a variety of other measures. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Blood pressure was measured twice in a seated position after a 5 min rest, using an automated sphygmomanometer (BP-8800; Omron Colin, Tokyo, Japan). The average of two measurements was used.

Hypertension was defined as systolic blood pressure (SBP) ≥140 mm Hg, diastolic blood pressure (DBP) ≥90 mm Hg, or as the use of antihypertensive medications. Diabetes mellitus (DM) was defined as a hemoglobin A1c (HbA1c) ≥6.1% (Japan Diabetes Society criteria; equivalent to HbA1c ≥ 6.5% in National Glycohemoglobin Standardization Program) [18] a fasting glucose ≥6.99 mmol/l (126 mg/dL), or the use of antidiabetic medications.

A self-administered questionnaire was used to collect data on medical history, medication use, smoking, alcohol intake, and other lifestyle behaviors with confirmation by trained technicians.

2.2. Laboratory measurements

Blood samples were drawn from participants after a 12-h fast and centrifuged soon after coagulation. Standard lipids, including total cholesterol and triglycerides (TG), were measured using enzymatic techniques. HDL-C was measured after heparin-calcium precipitation. Measurements were standardized according to guidelines from the Center for Disease Control and Prevention/Cholesterol Reference Method Laboratory Network (CDC/CRMLN) [19]. Friedewald's formula was used to estimate low-density lipoprotein cholesterol (LDL-C) levels in men with TG < 4.52 mmol/l (400 mg/dl). For higher TG levels, LDL-C was treated as missing.

HDL-P concentration was determined by nuclear magnetic resonance (NMR) spectroscopy using serum samples stored at −80 °C [20], and shipped on dry ice to LipoScience Inc (Raleigh, North Carolina, US). Concentrations were obtained from amplitudes of distinct spectroscopic NMR signals of the lipid methyl group, characteristic of each subclass. Reproducibility of NMR-measured HDL-P has been examined and measurements have a coefficient of variation <2% [21].

2.3. Intima-media thickness and plaque counts of carotid arteries

Ultrasound measurements of the carotid arteries were performed by sonographers following an established protocol of the Ultrasound Research Laboratory at the University of Pittsburgh [17,22]. A Toshiba XarioSSA-660A scanner (Toshiba Medical Systems, Japan), equipped with a 7.5 MHz linear-array imaging probe, was used for high-resolution B-mode ultrasound of the carotid arteries. Sonographers scanned both right and left carotid arteries.

In both arteries, the IMT of the common carotid artery (CCA), carotid bulb, and internal carotid artery were measured. For the CCA segment, both near and far walls were examined 1 cm proximal to the bulb. For the bulb and internal carotid artery segments, only far walls were examined. cIMT was defined as the mean of the eight IMT values measured in both arteries.

Plaque was defined as focal thickening lesion (>10% protrusion compared to adjacent areas) with an IMT of ≥1 mm. The total number of plaques in CCA, bulb, and internal carotid artery of both left and right carotid arteries were counted.

2.4. Statistical analyses

Participant demographics were described according to quartile of HDL-P and HDL-C. P-values for trend across the quartiles were determined either using linear regression when a response variable is continuous (such as age), or using logistic regression when it is categorical (such as current smoker or not).

A dose–response relationship between HDL measures and subclinical atherosclerosis was investigated by obtaining adjusted means of cIMT and plaque counts across quartiles of HDL-P and HDL-C using linear regression. We then calculated a difference in cIMT per 1 standard deviation (SD) increase in HDL-P or HDL-C, treating them as continuous variables.

For carotid plaque, we modeled plaque count as an overdispersed integer response following a negative binomial distribution. Regression coefficients have been transformed to percentages, indicating the percent reduction (or excess) in plaque counts per 1 SD increase in HDL-P or HDL-C.

In regression models, we chose the following adjusting covariates as they are established cardiovascular risk factors: age (years), SBP (mmHg), hypertension medication (yes/no), current smoker (yes/no), current alcohol intake (g/day), DM (yes/no), LDL-C (mmol/l) [this set was defined as "base covariates"] and HDL-P or HDL-C (mmol/l).

Analyses were performed using SAS version 9.3 (SAS Institute, Cary, North Carolina) and two-tailed *P*-values of <0.05 were considered significant.

3. Results

3.1. Study participants and characteristic trends with HDL-P and HDL-C

Characteristics of study participants according to quartiles of HDL-P and HDL-C are displayed in Table 1A and 1B. Mean (SD) characteristics of all participants included 63.3 (10) years for age, 834 (184)μm for cIMT and 2.4 (2.4) for plaque count (75.4% of all participants had presence of plaque ≥1). Men with higher HDL-P tended to be younger, leaner, have less prevalence of DM, and consumed more alcohol. The same was also true for HDL-C with the exception of age. Additionally, men with higher HDL-C tended to have less prevalence of hypertension and were less likely to be current smokers. Among lipids, HDL-P and HDL-C were positively related to each other. LDL-C was negatively associated with both HDL-P and HDL-C.

3.2. HDL-P and HDL-C associations with cIMT and carotid plaque

Results of quartile analyses are depicted in Fig. 1. With adjustment for base covariates, higher quartiles of HDL-P and HDL-C were both associated with smaller cIMT (panels A & B, dashed blue lines). The overall inverse relationship of HDL-P was maintained with further adjustments for HDL-C (panel A, solid red line). In contrast, the observed inverse association of HDL-C was noticeably absent after adjustments for HDL-P (panel B, solid red line). Higher quartiles of HDL-P were associated with lower mean plaque count in both models (panel C), with and without adjustments for HDL-C. Across quartiles of HDL-C, an association with plaque counts was absent (panel D).

In Table 2, a 1 SD increase in HDL-P and HDL-C was associated with 47.2 μm and 22.1 μm lower cIMT, respectively, (unadjusted models). In models adjusted for base covariates, 22.1 μm and 11.1 μm lower cIMT was estimated per 1 SD increase in HDL-P and HDL-C, respectively. After adjustment for HDL-C, the estimated cIMT differences in relation to HDL-P remained significant. In contrast, differences in cIMT with HDL-C were absent when adjusted for HDL-P.

Table 3 depicts the estimated reduction or excess in total number of carotid artery plaque counts per 1 SD increase in HDL-P and HDL-C. In unadjusted models, a 1 SD increase in HDL-P and HDL-C was associated with 20.4% and 8.8% reduction in total plaques, respectively. HDL-P was associated with significant reductions in plaque counts even after adjustment for base covariates and HDL-C. Here, a 1 SD increase in HDL-P was associated with 10.4% reduction in number of plaques in the final model adjusted for HDL-C. In contrast, HDL-C had no significant associations with carotid artery plaque in any of the adjusted models.

Table 1A
Characteristics of participants (n = 870), aged 40–79 years, across quartiles of HDL particle concentration, 2006–2008, Kusatsu, Shiga, Japan.

Characteristic	Quartile of HDL-P				*P* trend
	1	2	3	4	
Age, years	68.2 ± 8.3	64.4 ± 9.7	60.7 ± 10.2	60.0 ± 9.6	<0.001
Body mass index, kg/m^2	23.9 ± 3.2	23.1 ± 2.8	23.3 ± 3.0	23.1 ± 2.8	0.016
SBP, mmHg	138 ± 16	133 ± 19	133 ± 19	140 ± 22	0.089
Hypertension, %[a]	57.7	47.0	46.1	53.2	0.803
Diabetes, % (Type 2)	21.8	17.7	16.6	17.0	0.033
Current smoker, %	34.6	36.3	27.2	35.8	0.512
Alcohol intake (g/day)	12.3 ± 18.4	17.3 ± 20.8	23.4 ± 25.2	43.4 ± 34.1	<0.001
Triglycerides, mmol/l	1.36 ± 0.70	1.31 ± 0.74	1.38 ± 1.00	1.53 ± 1.11	0.010
LDL-C, mmol/l[b]	3.31 ± 0.87	3.33 ± 0.80	3.33 ± 0.71	3.05 ± 0.83	<0.001
HDL-C, mmol/l	1.20 ± 0.31	1.44 ± 0.34	1.62 ± 0.36	1.88 ± 0.47	<0.001

Values are mean ± SD, or % (as indicated).
SBP, systolic blood pressure; HDL, high-density lipoprotein; HDL-C, HDL cholesterol; HDL-P, HDL particle; LDL, low-density lipoprotein cholesterol.
Quartiles of HDL-P are as follows (1): 13.9–29.8 μmol/l; n = 220, (2): 29.9–33.4 μmol/l; n = 215, (3): 33.5–37.8 μmol/l; n = 217, and (4): 37.9–68.9 μmol/l; n = 218. P-values for trend were obtained using linear regression (for continuous variables) or logisitc regression (for categorical variables) as per 1 unit increase in HDL-P.
[a] Hypertension is defined as SBP ≥140 mmHg or DBP ≥90 mmHg or use of anti-hypertensive medication. Diabetes is defined as glycated hemoglobin ≥6.5% (NGSP) or fasting glucose ≥ 6.99 mmol/l or use of anti-diabetic medication.
[b] LDL-C was calculated by Friedewald equation. [LDL-C (mg/dl) = total cholesterol (mg/dl) - HDL cholesterol (mg/dl) -triglyceride (mg/dl)/5].

Table 1B
Characteristics of participants (n = 870), aged 40–79 years, across quartiles of HDL cholesterol concentration, 2006–2008, Kusatsu, Shiga, Japan.

Characteristic	Quartile of HDL-C				*P* trend
	1	2	3	4	
Age, years	64.1 ± 9.5	63.7 ± 9.5	62.2 ± 10.6	63.4 ± 10.3	0.129
Body mass index, kg/m^2	24.6 ± 3.0	23.9 ± 2.8	23.0 ± 2.9	21.9 ± 2.6	<0.001
SBP, mmHg	137 ± 17	136 ± 19	136 ± 18	135 ± 23	0.168
Hypertension, %[a]	55.2	50.5	56.4	41.4	0.008
Diabetes, % (Type 2)	20.8	22.0	20.5	9.5	0.001
Current smoker, %	42.5	32.7	35.0	23.3	<0.001
Alcohol intake (g/day)	18.4 ± 23.8	19.0 ± 24.2	27.9 ± 30.0	30.9 ± 30.9	<0.001
Triglycerides, mmol/l	1.91 ± 1.01	1.49 ± 1.11	1.23 ± 0.63	0.96 ± 0.43	<0.001
LDL-C, mmol/l[b]	3.36 ± 0.85	3.47 ± 0.82	3.18 ± 0.72	3.02 ± 0.79	<0.001
HDL-P, μmol/l	28.9 ± 4.6	32.5 ± 4.2	36.4 ± 5.7	39.0 ± 7.1	<0.001

Values are mean ± SD, or % (as indicated).
Quartiles of HDL-C are as follows (1): 0.67–1.19 mmol/l; n = 212, (2): 1.22–1.45 mmol/l; n = 214, (3): 1.46–1.78 mmol/l; n = 234, and (4): 1.81–3.88 mmol/l; n = 210. P-values for trend were obtained using linear regression (for continuous variables) or logisitc regression (for categorical variables) as per 1 unit increase in HDL-C.
[a] Hypertension is defined as SBP ≥140 mmHg or DBP ≥90 mmHg or use of anti-hypertensive medication. Diabetes is defined as glycated hemoglobin ≥6.5% (NGSP) or fasting glucose ≥ 6.99 mmol/l or use of anti-diabetic medication.
[b] LDL-C was calculated by Friedewald equation. [LDL-C (mg/dl) = total cholesterol (mg/dl) - HDL cholesterol (mg/dl) -triglyceride (mg/dl)/5].

4. Discussion

4.1. HDL and carotid atherosclerosis

In this cross-sectional study of Japanese men, free of clinical CVD and not on lipid-lowering medication, the inverse association of HDL-P with cIMT was independent of conventional cardiovascular risk factors, including HDL-C. In contrast, the association of HDL-C with cIMT was attenuated with adjustments for these factors and was absent after adjustment for HDL-P. Furthermore, higher HDL-P, but not higher HDL-C, was inversely and independently associated with lower number of carotid artery plaque after adjustment for cardiovascular risk factors. We demonstrated stronger associations of HDL-P, compared to HDL-C, with two different measures of carotid atherosclerosis (i.e. cIMT and carotid plaque) among a community-based sample of Japanese men. Whether effects of HDL-P are more noticeable in the higher ranges of HDL-C, normally thought to be atheroprotective, warrants consideration.

Our findings are consistent with those of other studies [11–13]. The Multi-Ethnic Study of Atherosclerosis (MESA) [12], in the United States, for example, reported a significant inverse association of cIMT with HDL-P, but not with HDL-C after adjustments for each other and known risk factors. The Woman's Health Study (WHS), however, did not find a significant inverse association of HDL-P with CHD [23] and instead, only confirmed the inverse association between HDL-C and cardiovascular risk. Possible explanations for the difference in findings may not only be due to the

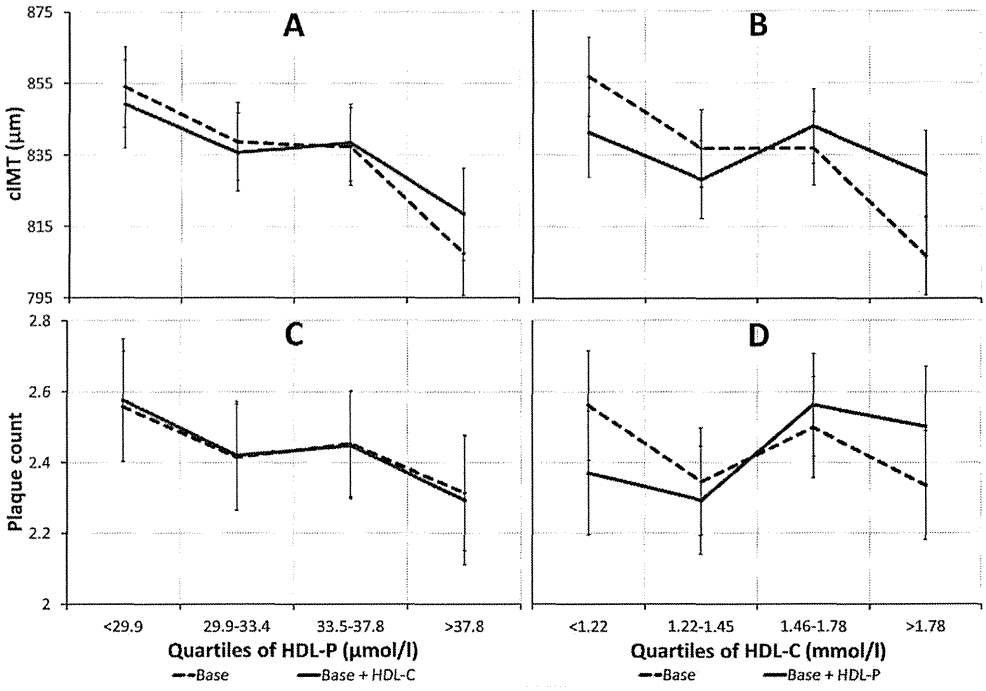

Fig. 1. Adjusted mean cIMT (n = 870) and plaque count across quartiles of HDL-P and HDL-C. Means were adjusted for base covariates [Base]: age (years), systolic blood pressure (mmHg), hypertension medication (yes/no), smoking status (yes/no), alcohol intake (g/day), diabetes (yes/no) and LDL-C (mg/dl), or adjusted for base covariates and HDL-C (mmol/l) or HDL-P (μmol/L) [Base + HDL-C or HDL-P]. P-values are for linear trend. All linear trends are significant $p < 0.05$, except for HDL-C models: Base + HDL-P ($p = 0.886$) for cIMT and Base ($p = 0.763$) and Base + HDL-P ($p = 0.187$) for plaque counts. Error bars represent standard error of mean.

Table 2
Estimated reduction (−) or excess (+) in cIMT per 1 standard deviation increase in HDL-P or HDL-C (n = 870), 2006–2008.

Parameter	Model	cIMT (μm)	95% CI	*P* value
HDL-P	Unadjusted	−47.2	−59.0, −35.3	<0.001
	Base covariates	−22.1	−34.4, −9.9	<0.001
	Base covariates + HDL-C	−22.8	−37.9, −7.7	0.003
HDL-C	Unadjusted	−22.1	−34.3, −10.0	<0.001
	Base covariates	−11.1	−22.1, 0.0	0.050
	Base covariates + HDL-P	+1.0	−12.6, +14.6	0.886

Base covariates include: age, SBP, hypertension medication (yes/no), and smoking status (yes/no), alcohol intake, LDL-C, diabetes (yes/no) and HDL-P or HDL-C. 1 standard deviation of HDL-P = 6.7 μmol/l and of HDL-C = 0.45 mmol/l.
cIMT, carotid intima-media thickness; CI, confidence interval.

sample population, but also to the randomized clinical trial study design of WHS, involving low-dose aspirin and vitamin E in primary prevention of CVD and cancer.

It is noteworthy that Japanese populations have higher HDL-C levels [16] and lower risk of CHD compared to populations of Western countries [24]. In addition, we previously reported significantly lower measurements of cIMT and higher levels of HDL-P among Japanese men compared to Caucasian men in the US [15]. Despite having a different cardiovascular risk profile, we found that in Japanese men, HDL-P, but not HDL-C, was significantly inversely associated with two measures of carotid atherosclerosis. Hence, our finding, together with results of other studies, suggests that HDL-P may be a novel marker for, and may possibly play a biological role against, the pathogenesis of atherosclerosis.

We have also analyzed HDL size subclass: small (7.3–8.2 nm), medium (8.2–8.8 nm), and large (8.8–13 nm) and their associations with cIMT and carotid plaque counts. However, we found no significant associations of any size with either measure of subclinical atherosclerosis in models adjusted for HDL-C. An atheroprotective effect of subclass size is also controversial [25] and is in need of focused attention.

Table 3
Estimated percent reduction (−) or excess (+) in total number of carotid artery plaque count per 1 standard deviation increase in HDL-P or HDL-C (n = 870), 2006–2008.

Parameter	Model	Estimate (%)	95% CI	*P* value
HDL-P	Unadjusted	−20.4	−27.4, −13.5	<0.001
	Base covariates	−7.8	−15.1, −0.5	0.037
	Base covariates + HDL-C	−10.4	−19.7, −1.1	0.029
HDL-C	Unadjusted	−8.8	−15.7, −1.9	0.012
	Base covariates	−2.0	−8.5, +4.5	0.552
	Base covariates + HDL-P	+3.7	−4.5, +11.9	0.380

Base covariates include: age, SBP, hypertension medication (yes/no), and smoking status (yes/no), alcohol intake, LDL-C, diabetes (yes/no) and HDL-P or HDL-C. 1 standard deviation of HDL-P = 6.7 μmol/l and of HDL-C = 0.45 mmol/l.

4.2. Potential mechanisms

The failure of recent randomized controlled trials on HDL-C-increasing drugs for CVD prevention resulted in questioning a causal protective role of HDL-C, which may only be an indicator of cardioprotective mechanisms at work. Nevertheless, the cardioprotective association of HDL is far from being ruled out. It has been suggested that increased particle concentrations of HDL may be indicative of higher reverse cholesterol transport activity [12]. The reverse cholesterol transport pathway mediates the efflux of cholesterol from peripheral cells to the liver [26] which is believed to be a key process in preventing plaque formation and progression [27] and, thus, many CVDs. Indeed, macrophage-specific cholesterol efflux was found to have a strong inverse association with cIMT and CHD [28]. Furthermore, recent studies have found that HDL-P, and not HDL-C, concentrations are positively associated with cholesterol efflux in patients with type 2 diabetes [29] and

patients undergoing coronary angiography [30]. These findings parallel our results, with total HDL-P having inverse associations with cIMT. It may be that serum HDL-P concentration is more closely related to the performance rate of cholesterol efflux in the reverse cholesterol transport pathway than HDL-C, with more particles being analogous to increased pathway activity. How HDL affects the cholesterol transport and protects against CVD may depend on its structure and composition, leading to a variety of biological activities, such as anti-inflammation, antioxidation, and vasodilation [25], all of which cannot be assessed by HDL-C alone [26, 31].

4.3. Limitations and strengths

As our study is cross-sectional and observational, causality cannot be proven in the associations of HDL-P with cIMT and carotid plaque counts. Other limits of our study include the study population being restricted to men of a single ethnic group. However, this is not without its advantage, as homogeneity in a population minimizes confounding from genetic variation. The size of carotid plaques was also not taken into account. Total plaque count may not define the grade or vulnerability of plaques, nevertheless it has been reported that the presence of plaque, alone, in the carotid arteries is positively associated with increased risk of cardiovascular events [36]. Thus, carotid plaque count can be used as an alternate indicator of subclinical atherosclerosis [37]. The main protein component of HDL particle, apolipoprotein A-1, is a strong predictor of CHD and a key player in reverse cholesterol transport [32,33]. Unfortunately, apolipoprotein A-1 levels were not measured in our serum samples and thus we were unable to look at possible confirmatory associations of HDL-P with cIMT and plaque.

This is the first study, of which we are aware of, to report a significant and inverse association of HDL-P, and not HDL-C, with plaque count in the carotid arteries, even after adjustments for conventional risk factors. This finding, as well as the confirmatory finding of cIMT associations, is in agreement with most literature published on HDL-P and both clinical CVD and subclinical atherosclerosis [11,12,38]. The fact that we could identify a relationship of HDL-P with cIMT and plaque in a population at low risk of CHD, indicates that HDL-P may be an important predictor of subclinical atherosclerosis and perhaps even more so in populations at high risk. Presently, there are few population-based studies on HDL-P, let alone any on a Japanese population, that characteristically has higher serum HDL-C levels compared to those of Western populations. Thus, our findings provide additional information to the current modest body of knowledge in this area.

5. Conclusion

In a community-based sample of Japanese men, free of clinical CVD, HDL-P was associated with measures of carotid atherosclerosis (cIMT and plaque count) independent of lipids or lipoproteins and other traditional CVD risk factors. In contrast, associations with HDL-C were absent after accounting for HDL-P. There is need for more scrutiny towards the properties of HDL in general, in order to better understand its involvement in CVD risk processes.

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The SESSA research group

Chairperson: Hirotsugu Ueshima (Center for Epidemiologic Research in Asia, Department of Public Health, Shiga University of Medical Science, Otsu, Shiga).

Co-chairperson: Katsuyuki Miura (Department of Public Health, Shiga University of Medical Science, Otsu, Shiga).

Research members: Minoru Horie, Yasutaka Nakano, Takashi Yamamoto (Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu, Shiga), Emiko Ogawa (Health Administration Center, Shiga University of Medical Science, Otsu, Shiga), Hiroshi Maegawa, Itsuko Miyazawa (Division of Endocrinology and Metabolism, Department of Medicine, Shiga University of Medical Science, Otsu, Shiga), Kiyoshi Murata (Department of Radiology, Shiga University of Medical Science, Otsu, Shiga), Kenichi Mitsunami (Shiga University of Medical Science, Otsu, Shiga), Kazuhiko Nozaki (Department of Neurosurgery, Shiga University of Medical Science, Otsu, Shiga), Akihiko Shiino (Biomedical MR Science Center, Shiga University of Medical Science, Otsu, Shiga), Isao Araki (Kusatsu Public Health Center, Kusatsu, Shiga) Teruhiko Tsuru (Department of Urology, Shiga University of Medical Science, Otsu, Shiga), Ikuo Toyama (Unit for Neuropathology and Diagnostics, Molecular Neuroscience Research Center, Shiga University of Medical Science, Otsu, Shiga), Hisakazu Ogita, Souichi Kurita (Division of Medical Biochemistry, Department of Biochemistry and Molecular Biology, Shiga University of Medical Science, Otsu, Shiga), Toshinaga Maeda (Central Research Laboratory, Shiga University of Medical Science, Otsu, Shiga), Naomi Miyamatsu (Department of Clinical Nursing Science Lecture, Shiga University of Medical Science, Otsu, Shiga), Toru Kita (Kobe City Medical Center General Hospital, Kobe, Hyogo), Takeshi Kimura (Department of Cardiovascular Medicine, Kyoto University, Kyoto), Yoshihiko Nishio (Department of Diabetes, Metabolism, and Endocrinology, Kagoshima University, Kagoshima), Yasuyuki Nakamura (Department of Living and Welfare, and Cardiovascular Epidemiology, Kyoto Women's University, Kyoto), Tomonori Okamura (Department of Preventive Medicine and Public Health, School of Medicine, Keio University, Tokyo), Akira Sekikawa, Emma JM Barinas-Mitchell (Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA), Daniel Edmundowicz (Department of Medicine, Section of Cardiology, School of Medicine, Temple University, Philadelphia, PA, USA), Takayoshi Ohkubo (Department of Hygiene and Public Health, Teikyo University School of Medicine, Tokyo), Atsushi Hozawa (Preventive Medicine, Epidemiology Section, Tohoku University, Tohoku Medical Megabank Organization, Sendai, Miyagi), Nagako Okuda (Department of Health and Nutrition, University of Human Arts and Sciences, Saitama, Japan), Aya Kadota (Department of Public Health, Shiga University of Medical Science, Otsu, Shiga), Aya Higashiyama (Research and Development Initiative Center, National Cerebral and Cardiovascular Center, Suita, Japan), Shinya Nagasawa (Department of Epidemiology and Public Health, Kanazawa Medical University, Kanazawa, Ishikawa), Yoshikuni Kita(Tsuruga Nursing University), Akira Fujiyoshi, Naoyuki Takashima, Takashi Kadowaki, Sayaka Kadowaki (Department of Public Health, Shiga University of Medical Science, Otsu, Shiga), Yoshitaka Murakami (Department of Medical Statistics, Faculty of Medicine, Toho University, Tokyo, Japan), Robert D. Abbott, Seiko Ohno (Center for Epidemiologic Research in Asia, Shiga University of Medical Science, Otsu, Shiga), Takashi Hisamatsu (Center for Epidemiologic Research in Asia, Department of Public Health, Shiga University of Medical Science, Otsu, Shiga), Naoko Miyagawa, Sayuki Torii, and

Yoshino Saito (Department of Public Health, Shiga University of Medical Science, Otsu, Shiga).

Conflict of interest

None declared.

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Validation of homogeneous assays for HDL-cholesterol using fresh samples from healthy and diseased subjects☆

Takashi Miida[a,*], Kunihiro Nishimura[b], Tomonori Okamura[c], Satoshi Hirayama[a], Hirotoshi Ohmura[d], Hiroshi Yoshida[e], Yoh Miyashita[f], Masumi Ai[g], Akira Tanaka[h], Hiroyuki Sumino[i], Masami Murakami[i], Ikuo Inoue[j], Yuzo Kayamori[k], Masakazu Nakamura[l], Tsutomu Nobori[m], Yukihisa Miyazawa[n], Tamio Teramoto[o], Shinji Yokoyama[p]

[a] Department of Clinical Laboratory Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[b] National Cerebral and Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan
[c] Department of Preventive Medicine and Public Health, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[d] Department of Cardiology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[e] Department of Laboratory Medicine, Jikei University Kashiwa Hospital, 163-1 Kashiwashita, Kashiwa, Chiba 277-8567, Japan
[f] Center of Diabetes, Endocrine and Metabolism, Toho University Sakura Hospital Medical Center, 564-1 Shimoshizu, Sakura, Chiba 285-0841, Japan
[g] Department of Insured Medical Care Management, Tokyo Medical and Dental University Hospital, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[h] Laboratory of Clinical Nutrition and Medicine, Kagawa Nutrition University, 3-9-21 Chiyoda, Sakado, Saitama 350-0288, Japan
[i] Department of Clinical Laboratory Medicine, Gunma University Graduate School of Medicine, 3-39-22 Showa, Maebashi, Gunma 371-8511, Japan
[j] Department of Endcrinology and Diabetics, Saitama Medical University, 38 Morohongo Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
[k] Department of Health Sciences, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[l] National Cerebral and Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan
[m] Department of Molecular and Laboratory Medicine, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507 Japan
[n] Department of Laboratory Science, Faculty of Medical Technology, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
[o] Department of Internal Medicine, Teikyo University, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
[p] Nutritional Health Science Research Center and Department of Food and Nutritional Sciences, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan

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ABSTRACT

Background: High-density lipoprotein-cholesterol (HDL-C) is a negative risk factor for cardiovascular events. Although several homogeneous HDL-C assays are available, their accuracy has not been validated, particularly in subjects with disease. We aimed to clarify whether HDL-C concentrations measured by homogeneous assays [HDL-C (H)] agree with those determined by the reference measurement procedures [HDL-C (RMP)] using ultracentrifugation and precipitation with heparin-manganese reagent in fresh clinical samples.

Methods: HDL-C concentrations in samples from 48 healthy subjects and 119 subjects with disease were determined using 12 homogeneous assays and RMPs.

Results: All reagents showed excellent intra- and inter-assay CVs (<2.23%) for two pooled sera. Furthermore, the mean bias was within ±1.0% in nine reagents using samples from healthy subjects and in eight reagents using samples from subjects with disease. In a single HDL-C (H) determination, the total error requirement of the National Cholesterol Education Program (95% of results < 13%) was fulfilled in nine reagents using samples from healthy subjects and six reagents in those from subjects with disease. Error component analysis revealed that only one reagent exceeded ±10% total error in samples from healthy subjects, whereas four reagents exceeded this error in samples from subjects with disease. Correlations between HDL-C (H) and HDL-C (RMP) revealed that the slopes were within 1.00 ± 0.06 in six reagents in healthy subjects, and eight reagents in subjects with disease.

* Corresponding author. Tel.: +81 3 5802 1104; fax: +81 3 5684 1609.
E-mail address: tmiida@juntendo.ac.jp (T. Miida).

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Conclusions: Except for three reagents, HDL-C (H) agrees well with HDL-C (RMP) in subjects with common disease, but not in those with extremely low HDL-C or abnormal HDL composition.

1. Introduction

Lipoprotein profiles are closely associated with atherosclerotic disorders such as coronary artery disease and cerebrovascular disease, the major causes of death in industrialized countries [1,2]. Functionally, serum lipoproteins are divided into two groups: atherogenic and anti-atherogenic lipoproteins. Atherogenic lipoproteins consist of intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and remnant lipoproteins. Over a prolonged period of time, these lipoproteins accumulate in macrophages in the vascular walls; macrophages turn into foam cells, the main component of lipid-rich plaques. These are called "vulnerable plaques" because pro-inflammatory cells—including macrophages—degrade the fibrous cap of plaques using proteolytic enzymes, causing sudden rupture [3].

High-density lipoprotein (HDL) is the only circulating anti-atherogenic lipoprotein. HDL removes free cholesterol from somatic cells by accepting cell cholesterol via an exchange with the help of ATP-binding cassette transporter A1 (ABCA1) [4] and G1 (ABCG1) [5]. This reverse cholesterol transport pathway regresses vascular atheromatous plaques [6]. In clinical practice, the amount of HDL is expressed as the cholesterol concentration (HDL-C). Numerous cross-sectional and longitudinal epidemiological studies have shown that HDL-C is a strong negative risk factor for cardiovascular events [7,8]. In both primary and secondary prevention studies using statins, each 0.0259 mmol/L (1 mg/dL) increase in HDL-C level decreased the risk of coronary artery disease by 2–3% [9,10]. Since precipitation methods for HDL-C measurement are cumbersome and time consuming, HDL-C is measured by homogenous assays (so-called "direct assays") using a variety of principles in almost all clinical laboratories [11]. In general, homogeneous assays eliminate or inhibit non-HDL-C with the first reagents, and then solubilize HDL particles for measuring cholesterol with the second reagents. Although the performance of seven HDL-C homogeneous assays was investigated recently, the study subjects included a considerable number of patients with rare dyslipidemia and extremely low HDL-C [12]. Furthermore, there are additional novel assays based on different principles.

We examined the precision and accuracy of the HDL-C homogeneous assays that are commercially available at present. Using fresh blood samples from healthy subjects and subjects with disease, we compared the HDL-C concentrations determined using 12 homogeneous assays [HDL-C (H)] with those determined by the reference measurement procedures [HDL-C (RMP)].

2. Methods

2.1. Study subjects

This study was planned and carried out concurrently with the multi-center study of the precision and accuracy of homogeneous assays for LDL-cholesterol (LDL-C) published previously [13]. Initially, 173 subjects consisting of volunteers and patients with disease were recruited at the participating institutions. We excluded dyslipidemic patients with extremely low or high lipoprotein concentrations [triglyceride (TG) > 11.29 mmol/L (1000 mg/dL), LDL-C < 0.52 mmol/L (20 mg/dL), HLD-C < 0.52 mmol/L (20 mg/dL), and HDL-C > 2.59 mmol/L (100 mg/dL). In addition, we excluded patients with severe systemic infections, decompensated liver cirrhosis or cholestatic liver disease [13]. Healthy subjects ($n = 48$) were defined as normolipidemic healthy volunteers who had no abnormal laboratory tests or documented diseases. The remaining 119 persons were classified as subjects with disease, according to their medical history and lipoprotein profiles. At recruitment, written informed consent was obtained from all subjects.

The study protocol was reviewed and approved by the ethics committees of all participating institutions. This study was conducted according to the latest version of the Declaration of Helsinki.

2.2. Blood sampling and delivery

Fresh venous blood was drawn into vacuum tubes (Venoject II, VP-AS109K50, Terumo, Tokyo, Japan) from each subject regardless of time lapsed since the last meal. After the blood was allowed to clot, the serum was separated within an hour and poured into 50-mL plastic tubes (430290, Corning Japan, Tokyo) to equalize the blood components. Aliquots of samples were dispensed into screw-capped tubes and delivered to either SRL (Hachioji, Japan) for homogenous assays or Osaka Medical Center for Health Science and Promotion (OMC-HSP) (Osaka, Japan) for RMP [12]. Samples were placed in a cooling box containing refrigerant, and carried in a van equipped with a refrigerator. Temperatures were monitored continuously at two sites inside the box; temperatures were maintained between 2 and 4 °C within 24 h (Supplemental Fig. 1S).

2.3. HDL-C measurement

Within 24 h after blood collection, we measured HDL-C concentrations using 12 homogeneous assays, as well as RMP—described below. At SRL, the precision and accuracy of 12 homogeneous assays were evaluated (Reagent-A, Denka Seiken; Reagent-B, Wako; Reagent-C, Sysmex; Reagent-D, Serotec; Reagent-E, Fureiya; Reagent-F, Kyowa Medex; Reagent-G, Toyobo; Reagent-H, Shino-Test; Reagent-I, Sekisui Medical; Reagent-J, Ortho Clinical Diagnostics; Reagent-K, Siemens Healthcare, and Reagent-L, Beckman Coulter). Reagents-A–G, and Reagent-I were original homogenous assays, whereas Regent-H, Reagent-J, Reagent-K and Reagent-L were introduced products from other manufacturers (Supplemental Table S1). Reagents-A to -I were run on the same automated analyzer (Hitachi-917, also called Hitachi-7170 in Japan) that was used in the preceding studies [12,13]. The other three reagents were run on three different instruments manufactured by the distributors that produced the individual reagents. All of the reagents, calibrators and controls were supplied by the respective manufacturers and distributors. Under conditions of anonymity, the operators of SRL measured HDL-C (H) in triplicate as described previously [13]. In a preliminary study, we confirmed no cross contamination between cells, and no condensation during measurements using the Hitachi-910 instrument.

HDL-C was measured by RMP at OMC-HSP. First, two tubes prepared from each sample were ultracentrifuged at 18 °C, 105,000× g for 18.5 h. The bottom fraction was recovered by discarding the floating fraction using a tube slicer. After adding heparin-manganese solution to the bottom fraction, we removed the precipitate by centrifugation (1500× g for 30 min), and obtained the HDL fraction [14]. Finally, we measured cholesterol levels

of the supernatant in duplicate as HDL-C (RMP) by the Abell–Kendall method [15].

2.4. Statistical analysis

We determined three CVs (%): CV_b, derived from among-run variation using pooled serum; CV_e, derived from within-run variation using triplicate measurements; and CV_d, derived from patient-specific errors, as described previously. CV_t was calculated from these three CVs [12,13]. Bias was calculated by subtracting HDL-C (RMP) from HDL-C (H). Percentage total error (%TE) was calculated as the sum of %bias and CV_t multiplied by 1.96. We used the criteria of the National Cholesterol Educational Program (NCEP) for TE requirement for HDL-C measurement, where %bias, CV and % TE were less than 5, 4 and 13%, respectively [16].

3. Results

3.1. Subjects' characteristics

A total of 173 fresh samples were collected during the study period. Six subjects were diagnosed with hyper-α-lipoproteinemia with HDL-C > 2.59 mmol/L, and excluded from the analysis. Of six patients, two were diagnosed as heterozygotes for cholesteryl ester transfer protein (CETP) deficiency. Neither CETP activities nor CETP gene mutations were determined in the other patients. In all subjects, HDL-C ranged from 0.74 to 2.54 mmol/L, and TG ranged from 0.35 to 10.57 mmol/L (Table S2).

3.2. Homogeneous assay precision

In most reagents, the inter- and intra-assay CV values were less than 1.0% for pooled sera with HDL-C concentrations of 1.29 and 2.13 mmol/L (Table S3). The total CV values were 0.84–2.23%; this maximum value was about half of the target (4%) proposed by NCEP.

3.3. Relationship between HDL-C (H) and HDL-C (RMP)

In the healthy group, the %bias values of most samples were within 13% for all reagents, except Reagent-D. In contrast, a significant number of samples in subjects with disease exceeded 20% for Reagents-G and -L (Fig. 1). The medians deviated markedly from zero for Reagents-D and -G.

Scatter plots, % bias plots and Bland–Altman plots clearly showed that Reagents-G and -L had poorer analytical performance than the other reagents, particularly in the samples from subjects with disease. In most reagents, HDL-C (H) exhibited good correlation with HDL-C (RMP) in samples from healthy and diseased subjects (Fig. 2, upper panels). However, the intercepts and slopes of the linear regression lines ranged from −0.059 to 0.210 mmol/L and from 0.90 to 1.12 in the samples from healthy subjects, and from −0.016 to 0.316 mmol/L and from 0.89 to 1.01 in samples from subjects with disease (Table 1). In Bland–Altman plots, the absolute bias positively correlated with the mean value of HDL-C (H) and HDL-C (RMP) concentrations for Reagent-D (Fig. 3). There was marked diversity in the absolute bias independent of the mean HDL-C values for Reagents-G and -L.

3.4. Total error for single measurements

In samples from healthy subjects, five reagents fulfilled the requirement of NCEP in all samples, while Reagent-D failed in almost 40% of samples (Table 2-A). In samples from subjects with disease, nine reagents reached 90% agreement between HDL-C (H) and HDL-C (RMP). These reagents showed good agreement in even samples from patients with severe hypertriglyceridemia or type III hyperlipidemia (Table S4). In contrast, the percentages of agreement in Reagents-D, -G, and -L were markedly lower.

3.5. Error component analysis

This analysis was less informative than those using scatter plots and Bland–Altman plots. In samples from healthy subjects, all reagents met the NCEP requirement (Table 2-B). In samples from

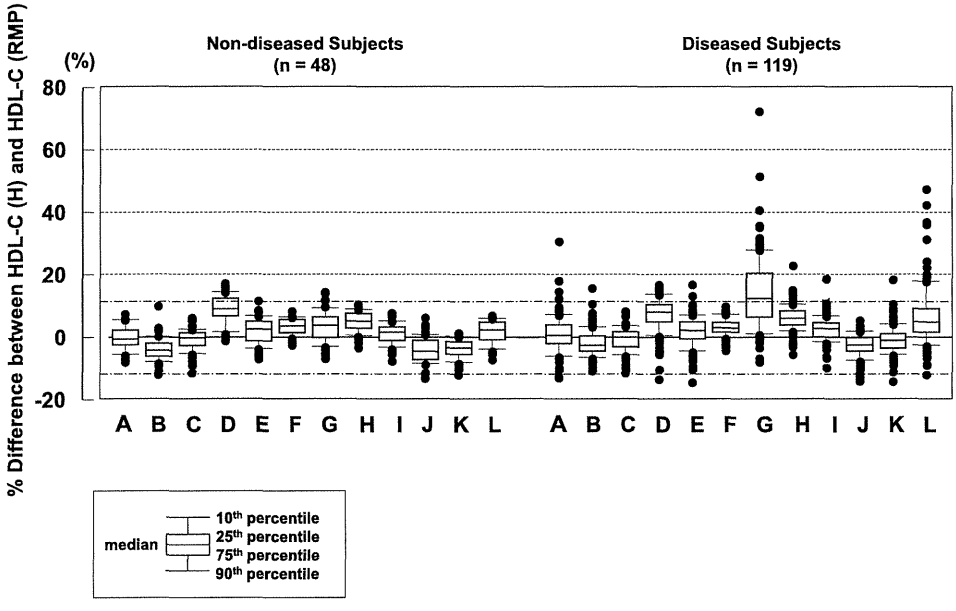

Fig. 1. Box-and-whisker plots of the percentage difference between HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. HDL-C concentrations in fresh serum samples were measured using 12 homogeneous assays and RMPs. Percentage differences were determined using the first measurements of individual reagents.

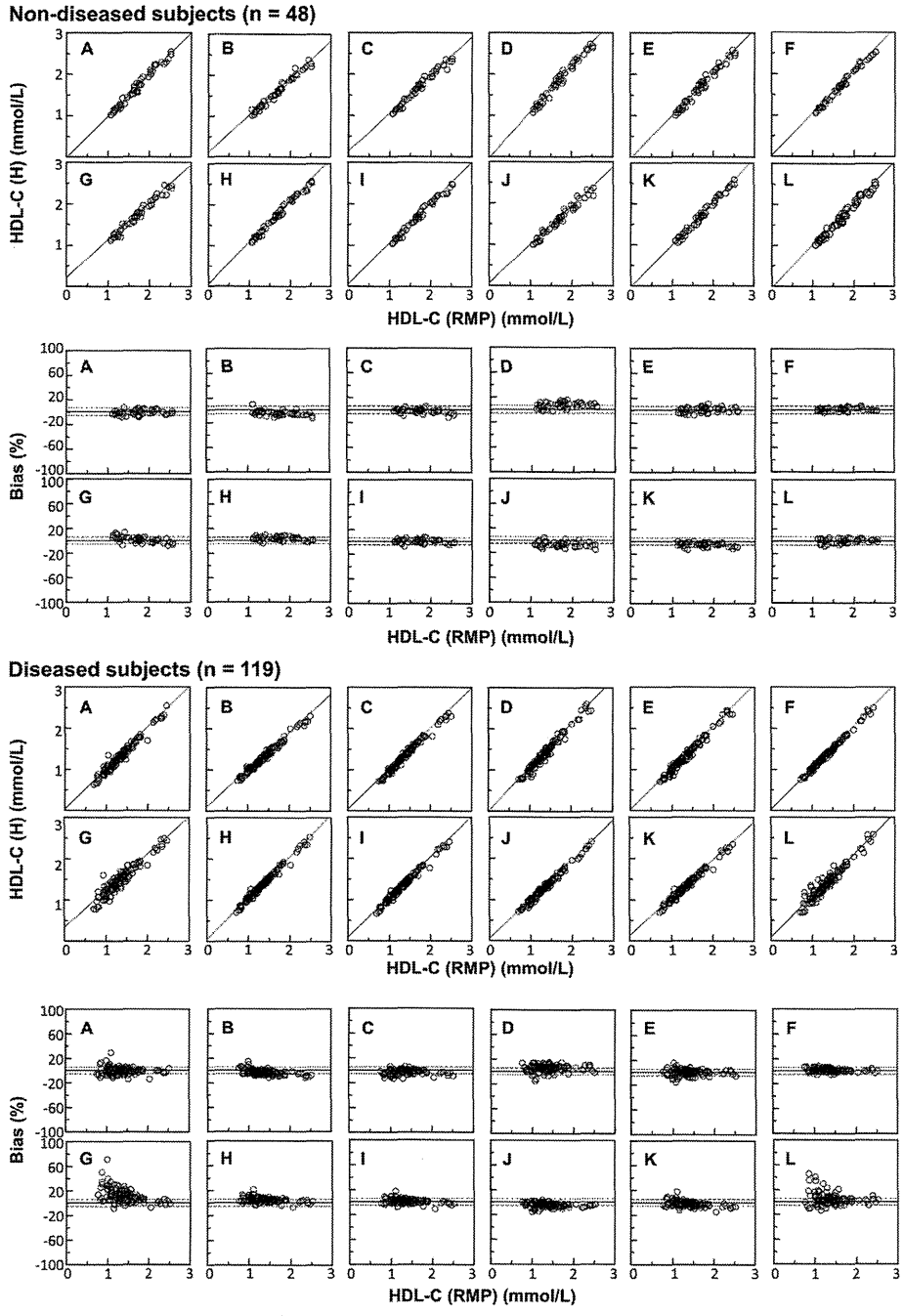

Fig. 2. Relationship between HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. Data from healthy and diseased subjects were plotted as scatter graphs (upper panels) and %bias graphs (lower panels).

subjects with disease, Reagents-G and -L did not meet the NCEP requirement. The CV_d value was the critical determinant of these unfavorable results.

4. Discussion

Our data indicates that the HDL-C (H) concentrations determined by most of the homogeneous assay reagents agree well with HDL-C (RMP) determined by CDC reference method procedures in samples from both healthy and diseased subjects. Nine of the twelve reagents achieved better than 90% agreement with the NCEP total error requirement for a single HDL-C determination (Table 1). In contrast, the HDL-C (H) measured using Reagents-G and -L did not match the HDL-C (RMP) data over a wide range of HDL-C concentrations (Fig. 2).

Standardization of homogenous HDL-C assays is problematic because no pure and stable HDL particles are available for use as a reference. Apolipoprotein A-I is a better predictor for atherosclerotic disorders than HDL-C, and is already standardized with the reference material [17]. However, apolipoprotein A-I is not measured as widely as HDL-C, partly due to the relatively high cost. In 1994, the Cholesterol Reference Method Laboratory Network (CRMLN) launched a HDL-C certification program for manufacturers [18] using the designated comparison method (DCM) and

Table 1
Relationships between HDL-C (H) and HDL-C (RMP) values.

Subjects	Reagent											
	A	B	C	D	E	F	G	H	I	J	K	L
Non-diseased subjects ($n = 48$)												
Sy/x	2.60	2.20	2.42	2.83	2.70	1.75	2.56	2.16	2.19	2.74	2.04	2.24
Intercept (mmol/L)	−0.041	0.108	0.107	−0.052	−0.059	0.005	0.210	0.060	0.050	0.058	0.073	−0.038
95% C.I. (mmol/L)	−0.107 to 0.030	0.025 to 0.191	0.020 to 0.194	−0.137 to 0.034	−0.140 to 0.022	−0.048 to 0.059	0.121 to 0.299	−0.006 to 0.127	−0.016 to 0.116	−0.041 to 0.156	0.004 to 0.141	−0.107 to 0.032
p-value	N.S.	0.0122	0.0167	N.S.	N.S.	N.S.	<0.0001	N.S.	N.S.	N.S.	0.0375	N.S.
Slope	1.02	0.90	0.93	1.12	1.06	1.03	0.91	1.01	0.98	0.92	0.92	1.04
95% C.I.	0.98–1.07	0.85–0.95	0.87–0.98	1.07–1.17	1.01–1.11	1.00–1.07	0.85–0.96	0.97–1.05	0.94–1.02	0.86–0.99	0.88–0.96	1.00–1.08
p-value	N.S.	0.0002	0.0109	<0.0001	0.0205	N.S.	0.0007	N.S.	N.S.	0.0158	0.0003	0.0367
Diseased subjects ($n = 119$)												
Sy/x	2.60	1.79	1.96	2.72	2.31	1.33	4.44	1.85	1.85	1.70	1.93	3.96
Intercept (mmol/L)	0.005	0.114	0.025	−0.016	0.009	0.038	0.316	0.081	0.076	0.033	0.113	0.082
95% C.I. (mmol/L)	−0.024 to 0.034	0.077 to 0.152	−0.019 to 0.069	−0.078 to 0.046	−0.042 to 0.059	0.008 to 0.067	0.231 to 0.401	0.041 to 0.121	0.038 to 0.109	0.003 to 0.063	0.073 to 0.154	−0.003 to 0.167
p-value	N.S.	<0.0001	N.S.	N.S.	N.S.	0.0140	<0.0001	0.0001	0.0002	<0.0318	<0.0001	N.S.
Slope	0.99	0.89	0.98	1.09	1.01	1.00	0.89	1.00	0.97	0.95	0.91	1.00
95% C.I.	0.97–1.01	0.87–0.92	0.94–1.01	1.04–1.13	0.98–1.05	0.98–1.03	0.84–0.95	0.97–1.03	0.94–1.00	0.93–0.97	0.88–0.93	0.94–1.06
p-value	N.S.	<0.0001	N.S.	0.0003.	N.S.	N.S.	0.0002	N.S.	0.0305	<0.0001	<0.0001	N.S.

A, Denka Seiken; B, Wako; C, Sysmex; D, Serotec; E, Fureiya; F, Kyowa Medex; G, Toyobo; H, Shino-Test; I, Sekisui Medical; J, Ortho Clinical Diagnostics; K, Siemens Healthcare Diagnostics; L, Beckman Coulter.
Sy/x, standard error of the estimate; C. I., confidence interval.

Centers for Disease Control and Prevention (CDC) reference method (CDCRM). Precipitation methods, such as DCM, cannot completely precipitate apolipoprotein-B-containing lipoproteins in hypertriglyceridemic samples [19]; therefore, we measured HDL-C (RMP) at OMC-HSP by CDCRM, which included removal of chylomicron and very-low density lipoprotein (VLDL) by slicing a tube after ultracentrifugation, and precipitation of LDL from the bottom fraction with heparin-manganese solution. Unlike some reagents for LDL-C homogeneous assays, high TG concentrations were not associated with discrepancies between HDL-C (H) and HDL-C (RMP) (Table S4, S5). In addition, the prevalence of postprandial samples did not increase in the discordant results from diseased subjects (Table S5). These results suggest that it is acceptable to use postprandial HDL-C (H) data for calculation of the non-HDL-C concentration.

Miller et al. conducted a similar study of homogenous assays for HDL-C where they also examined the accuracy of homogeneous assays for LDL-C [12]. In subjects with disease, our results of error component analysis were better than those of Miller's study, although data were comparable in healthy subjects. We excluded patients who might have abnormal HDL particles due to genetic dyslipidemia, severe systemic infection, and decompensated liver cirrhosis. Furthermore, we also excluded samples with severe hypertriglyceridemia (TG > 11.29 mmol/L) since chylomicrons float spontaneously on the top of samples, which may cause mechanical problems with the sampling probes. Error component analysis is not applicable to samples in which the mean successive difference between HDL-C (H) and HDL-C (RMP) is large or discontinuous [13]. Miller et al. evaluated samples from 138 subjects with disease, including those with lecithin-cholesterol acyltransferase (LCAT) deficiency ($n = 1$), Niemann–Pick disease type B ($n = 1$), and primary biliary cirrhosis ($n = 6$) [12]. In LCAT deficiency, apoA-I containing HDL decreased markedly, while apoE-containing HDL was comparatively preserved [20]. In Niemann-Pick type B, the sphingomyelin content of HDL particles was greater than that in healthy subjects [21]. In cholestatic liver diseases such as primary biliary cirrhosis, apoE-rich HDL increases markedly [22,23]. In the present study, we excluded samples from subjects with hyper-α-cholesterolemia (HDL-C > 100 mg/dL) and the abovementioned diseases. We reported previously that the mean apoE-rich HDL-cholesterol concentration was more than fourfold greater in hyper-α-cholesterolemia than in healthy controls [24]. Due to the difference in the measuring principles, reactivity to apoE-rich HDL may vary markedly among the HDL-C homogeneous assay reagents. Sugiuchi et al. measured HDL-C in PBC and cholesteryl ester transfer protein (CETP) deficiency using six homogeneous assays. HDL-C (H) ranged from 51 to 147 mg/dL in PBC, and from 157 to 192 mg/dL in CETP deficiency [23]. Miller's study is likely to have overemphasized the inaccuracy of homogenous assays since they included a relatively high percentage of samples with extremely low HDL-C or extremely abnormal HDL composition. However, it should be noted that our data certify the analytical performance of homogenous assays in samples only from subjects with common diseases.

No significant problems were identified in the error component analysis of most of the HDL-C homogeneous assay reagents (Table 2-B). Thus, HDL-C homogeneous assays showed generally acceptable accuracy in the healthy and diseased groups, except for those from a few commercial sources. However, we suggest the need for further improvement with respect to standardization when examining the data in a serial manner. The scatter plots showing the relationships between HDL-C (H) and HDL-C (RMP) revealed that the slopes and Y-intercepts of the regression lines did not fall within the satisfactory range (1.00 ± 0.03 for slope, and 0.00 ± 0.06 for intercept; Fig. 2, Table 1). The slopes ranged from 0.90 to 1.12 in the healthy group, and from 0.89 to 1.09 in the diseased group. Even after excluding Reagents-D, -G and -L, three other reagents had significantly

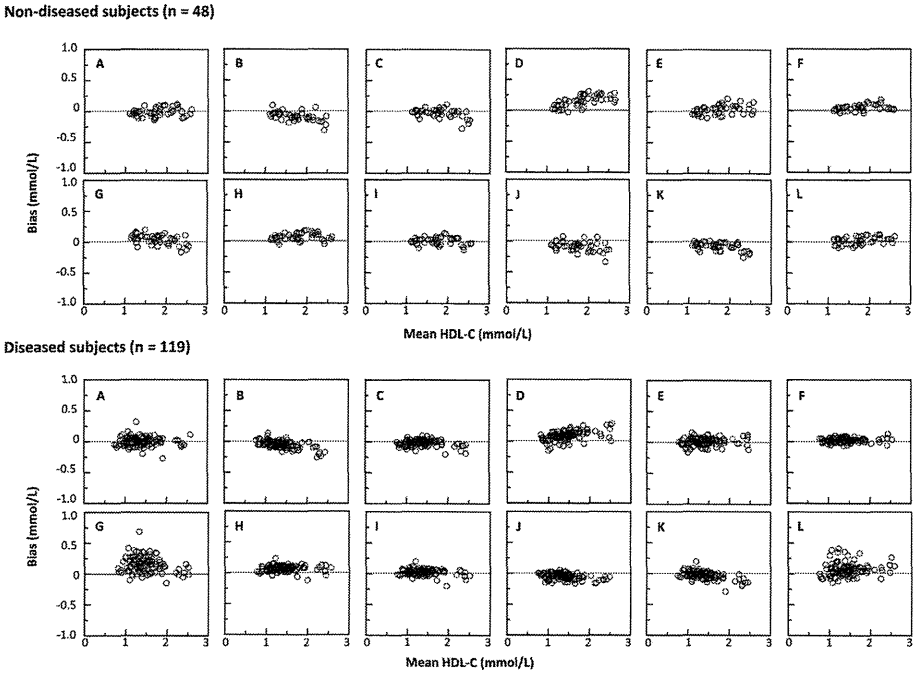

Fig. 3. Bland–Altman plots of HDL-C (H) and HDL-C (RMP) in samples from healthy and diseased subjects. The *X*-axis represents the mean HDL-C{=[HDL-C (H) + HDL-C (RMP)]/2}.

deviated slopes and Y-intercepts in samples from the healthy group, and four reagents deviated in the diseased group. Bland–Altman plots indicated the systemic proportional biases in Reagents-B, -D, -K, and -L in the diseased group (Fig. 3). Because we used HDL-C values to calculate LDL-C [25] and non-HDL-C, HDL-C values should be further standardized. We strongly suggest that the manufacturers re-evaluate the HDL-C values of their calibrators using the same fresh serum.

We conclude that HDL-C (H) concentrations measured using most of the homogeneous assay reagents (except for Reagents-D, -G, and -L) agree well with the HDL-C (RMP) concentration as determined by CDC reference method procedures in healthy and diseased subjects without extremely low HDL-C or abnormal HDL particles. The HDL-C homogeneous assays used in the described criteria enable accurate and rapid determination of HDL-C concentrations in the appropriate population.

Table 2
Accuracy of HDL-C (H) reagents evaluated with a single measurement (A) and error component analysis (B).

Subjects	Reagent											
	A	B	C	D	E	F	G	H	I	J	K	L
A: Percentage of samples that met the TE requirement of the NCEP for a single HDL-C determination[a]												
Non-diseased subjects ($n = 48$)	100.0	97.9	97.9	62.5	97.9	100.0	100.0	100.0	100.0	89.6	95.8	91.6
Diseased subjects ($n = 119$)	93.2	99.1	97.4	75.6	96.6	100.0	43.7	92.4	97.5	94.1	97.4	68.1
B: Error component analysis												
Non-diseased subjects ($n = 48$)												
CV_b (%)[b]	[1.2]	[0.7]	[1.2]	[1.4]	[0.9]	[0.8]	[1.2]	[1.0]	[0.9]	[1.1]	[1.0]	[2.9]
CV_e (%)	0.8	0.5	0.6	0.5	0.6	0.4	0.7	0.5	0.6	1.9	0.7	1.7
CV_d (%)	3.8	3.3	3.3	4.1	4.2	2.4	4.1	3.0	3.2	3.2	3.2	3.6
CV_t (%)	4.0	3.4	3.4	4.2	4.3	2.5	4.2	3.2	3.3	4.3	3.4	4.2
Mean bias (%) (SD)	−0.1 (3.9)	−0.9 (3.7)	−0.2 (3.5)	2.1 (4.6)	0.5 (4.2)	0.8 (2.6)	0.8 (5.0)	1.2 (3.2)	0.3 (3.2)	−1.0 (4.0)	−0.9 (3.2)	0.4 (3.7)
TE (%), for greater of positive or negative limit	8.1	−7.5	6.9	11.1	9.4	6.0	9.6	7.8	7.2	−9.3	−7.4	9.3
Diseased subjects ($n = 119$)												
CV_b (%)[b]	[1.2]	[0.7]	[1.2]	[1.4]	[0.9]	[0.8]	[1.2]	[1.0]	[0.9]	[1.1]	[1.0]	[2.9]
CV_e (%)	0.7	0.5	0.7	0.6	0.6	0.4	0.7	0.6	0.6	1.5	1.0	1.8
CV_d (%)	5.6	3.3	3.4	5.1	4.5	2.5	8.0	3.3	3.3	2.8	3.6	7.7
CV_t (%)	5.7	3.5	3.6	5.2	4.6	2.6	8.1	3.4	3.5	3.9	3.8	8.0
Mean bias (%) (SD)	0.3 (5.9)	−0.5 (4.2)	−0.2 (3.8)	1.8 (5.3)	0.4 (4.7)	0.8 (2.8)	3.3 (11.6)	1.6 (3.9)	0.7 (3.9)	−0.6 (3.6)	−0.2 (4.4)	1.5 (9.5)
TE (%), for greater of positive or negative limit	12.4	−7.1	7.3	13.0	10.0	6.2	21.4	8.7	7.9	−8.1	7.7	19.2

TE, total error.
Based on the method of our previous study [13], we calculated three different CV values, CV_b, CV_e and CV_d for (1) inter-assay variations, (2) intra-assay variations, and (3) variations due to subject sample-specific effects, respectively. CV_t was calculated as the square root of CV_b^2, CV_e^2 and CV_d^2.
[a] The TE requirement of the NCEP is 95% of results <13%.
[b] We determined CV_b using the pooled serum (Supplemental Table S3, Ref. [13]), and used the same CV_b values in both non-diseased and diseased groups.

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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.2013.12.033.

Conflict of interest

Investigators of the LDL-C Study Group received no remuneration for conducting this study.

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Selective evaluation of high density lipoprotein from mouse small intestine by an in situ perfusion technique[S]

Satoshi Yamaguchi,[1,*] Bo Zhang,[1,†] Takeshi Tomonaga,[§] Utako Seino,[**] Akiko Kanagawa,[§] Masaru Segawa,[††] Hironori Nagasaka,[§§] Akira Suzuki,[*] Takashi Miida,[***] Sohsuke Yamada,[†††] Yasuyuki Sasaguri,[†††] Takefumi Doi,[§§§] Keijiro Saku,[****] Mitsuyo Okazaki,[††††] Yoshihiro Tochino,[§§§§] and Ken-ichi Hirano[2,*]

Laboratory of Cardiovascular Disease,* Novel, Non-invasive and Nutritional Therapeutics (CNT), Graduate School of Medicine, Osaka University, Osaka, Japan; Department of Biochemistry,[†] Fukuoka University School of Medicine, Fukuoka, Japan; Laboratory of Proteome Research,[§] National Institute of Biomedical Innovation, Osaka, Japan; Niigata Prefectural Institute of Environmental Radiation Monitoring,** Niigata Branch, Niigata, Japan; Central Laboratory for Pathology and Morphology,[††] Fukuoka University School of Medicine, Fukuoka, Japan; Department of Pediatrics,[§§] Takarazuka City Hospital, Takarazuka, Japan; Department of Clinical Laboratory Medicine,*** Juntendo University School of Medicine, Tokyo, Japan; Department of Pathology and Cell Biology,[†††] School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan; Graduate School of Pharmaceutical Sciences,[§§§] Osaka University, Osaka, Japan; Department of Cardiology,**** Fukuoka University School of Medicine, Fukuoka, Japan; Professor Emeritus of Tokyo Medical and Dental University,[††††] Skylight Biotech Inc., Akita, Japan; Department of Metabolic Medicine,[§§§§] Graduate School of Medicine, Osaka University, Osaka, Japan

Abstract The small intestine (SI) is the second-greatest source of HDL in mice. However, the selective evaluation of SI-derived HDL (SI-HDL) has been difficult because even the origin of HDL obtained in vivo from the intestinal lymph duct of anesthetized rodents is doubtful. To shed light on this question, we have developed a novel in situ perfusion technique using surgically isolated mouse SI, with which the possible filtration of plasma HDL into the SI lymph duct can be prevented. With the developed method, we studied the characteristics of and mechanism for the production and regulation of SI-HDL. Nascent HDL particles were detected in SI lymph perfusates in WT mice, but not in ABCA1 KO mice. SI-HDL had a high protein content and was smaller than plasma HDL. SI-HDL was rich in TG and apo AIV compared with HDL in liver perfusates. SI-HDL was increased by high-fat diets and reduced in apo E KO mice. In conclusion, with our in situ perfusion model that enables the selective evaluation of SI-HDL, we demonstrated that ABCA1 plays an important role in intestinal HDL production, and SI-HDL is small, dense, rich in apo AIV, and regulated by nutritional and genetic factors.—Yamaguchi, S., B. Zhang, T. Tomonaga, U. Seino, A. Kanagawa, M. Segawa, H. Nagasaka, A. Suzuki, T. Miida, S. Yamada, Y. Sasaguri, T. Doi, K. Saku, M. Okazaki, Y. Tochino, and K. Hirano. **Selective evaluation of high density lipoprotein from mouse small intestine by an in situ perfusion technique. *J. Lipid Res.* 2014. 55: 905–918.**

Supplementary key words atherosclerosis • regulation • lipoprotein • in situ perfusion • intestine

HDL is unquestionably a major antiatherogenic factor worldwide, regardless of sex, race, and age (1, 2). Therefore, an increase in HDL levels has been an important goal that guides the development of novel therapies for atherosclerotic cardiovascular diseases (3) because it was reported that plasma HDL cholesterol (HDL-C) levels were reduced in patients with ischemic heart disease and stroke (4, 5). Strategies for raising HDL are now available. Because remarkable progress has been made in our understanding of the molecular mechanism of HDL production, enhanced production is one of the major strategies for raising HDL.

ABCA1, mutations of which are the genetic cause of Tangier disease and genetic HDL deficiency, is one of the

Abbreviations: ACN, acetonitrile; CE, cholesterol ester; CETP, cholesteryl ester transfer protein; C-HDL, HDL in mesenteric lymph obtained by conventional intestinal lymph cannulation experiments; CM, chylomicron; EM, electron microscopy; FC, free cholesterol; HDL-C, HDL cholesterol; L-HDL, HDL in liver perfusates; P-HDL, plasma HDL; PL, phospholipid; SI, small intestine; SI-HDL, small-intestine-derived HDL; TC, total cholesterol; TEM, transmission electron microscopy.

[1] S. Yamaguchi and B. Zhang contributed equally to this work.

[2] To whom correspondence should be addressed.
e-mail: khirano@cnt-osaka.com; khirano@cardiology.med.osaka-u.ac.jp

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prerequisite molecules for the production of HDL (6–8). We and others found that a human intestinal cell line, CaCo-2, expresses ABCA1 (9, 10) and secretes HDL though the involvement of ABCA1 (11). Experimental studies with rodents have demonstrated that the liver and small intestine (SI) are the major organs for ABCA1 expression and HDL production (12–16). Recent findings have shown that, although the overexpression of hepatic ABCA1 in experimental mouse models is not beneficial (17), the overexpression of intestinal ABCA1 is beneficial for atherosclerosis (18), indicating that HDL from SI may perform unique and distinct functions.

However, the characterization of SI-derived HDL (SI-HDL) and clarification of the mechanism that underlies its production and regulation remain elusive because selective evaluation of SI-HDL is difficult. Since the 1970s, various studies have demonstrated that HDL can be obtained from the intestinal lymph duct of anesthetized rodents in vivo, but its origin has been doubtful because it has been suggested that the source is either the secretion of HDL by the SI or the filtration of HDL from plasma through the blood capillary-lymph loop into the intestinal lymph duct (19–22).

In this regard, the group of Hayden et al. (16) recently reported the interesting and unexpected finding that HDL from the intestinal lymph duct was not reduced in SI-specific ABCA1 KO mice but was markedly reduced in liver-specific ABCA1 KO mice. The authors concluded that SI-HDL may be secreted directly into the circulation and that HDL in the intestinal lymph duct is predominantly derived from plasma. However, this conclusion is tenable only if a substantial quantity of plasma HDL, the majority of which is derived from the liver, passes through the loop and enters the intestinal lymph duct, so that the liver-specific disruption of ABCA1 affects the quantity of HDL obtained from the intestinal lymph duct more than does the SI-specific disruption of ABCA1.

To prevent the possible filtration of plasma HDL into the intestinal lymph duct and to realize the selective evaluation of SI-HDL, we developed an in situ perfusion technique using surgically isolated mouse SI. With this technique, we could demonstrate that the SI produces HDL, which reaches at least the SI lymph duct, and that the production of SI-HDL may be dynamically regulated.

METHODS

Animals

WT C57BL6/J mice, ABCA1 KO, and apo E KO mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed in a room under controlled temperature and humidity conditions and with free access to water and chow. Experiments were conducted when the male mice were aged from 12 to 16 weeks. Blood was drawn for the measurement of plasma lipid levels by enzymatic methods. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

In situ perfusion system for the evaluation of HDL production in the SI

We developed an in situ perfusion model in mice with isolated SI in which the arterial blood supply for the SI has been blocked for the assessment of HDL production in mouse SI (**Fig. 1A** and supplementary Fig. I). This novel in situ perfusion model can be used for the evaluation of HDL originated from the SI without intrusion by the filtration of HDL from plasma. The detailed experimental procedures are available in the form of video upon request. After the mice were deeply anesthetized, the abdominal cavity was opened, and the appropriate arteries except for the abdominal aorta were ligated to block the blood supply for the SI (Fig. 1A). Argatroban was injected intravenously as an anticoagulant (23), and 5 min after injection, the mice were euthanized for the removal of tissues including the heart and lungs from the thorax, except for the thoracic descending aorta. At this point, the SI would become ischemic.

To protect the SI from organ damage, the following procedures needed to be performed quickly. Two tubes were inserted into the abdominal aorta and portal vein by the following procedure. First, a needle (26G) connected to a tube was inserted antegrade through the thoracic descending aorta into the abdominal aorta, which was then ligated with the needle inserted at distal to the inserted portion to secure the needle as the inlet for perfusion (supplementary Fig. I). Next, the portal vein was cannulated as the outlet with an Intramedic PE50 tube (Becton Dickinson, Franklin Lakes, NJ) connected to a silicone tube (outside diameter 1.00 mm; inside diameter 0.50 mm; 30 cm length) (supplementary Fig. I). The intestinal lymph duct was then immediately cannulated to serve as the outlet with a needle (26G) connected to a silicon tube in the same manner as for the portal vein (supplementary Fig. I), while a buffer solution (DMEM without phenol red containing 0.03% BSA) aerated with 95% O_2 and 5% CO_2 was perfused slowly from the inlet. The interval when the mice were euthanized to circulation of the infusion had to be less than 5 min.

After all three tubes were secured, perfusion was started, and the flow rate of the perfusion buffer was increased to 0.5 ml/min. The body was kept at 35°C in Krebs Ringer buffer in a water bath, and sample collection by means of gravity was started from the two outlets. We confirmed that the motility of the intestines, glucose uptake, and an appropriate ratio of lymph to portal perfusates (1:50) could be maintained for up to 90 min in our system. However, due to the fragility and sensitivity of the SI (24, 25), prolonged perfusion seems to be problematic, and the SI showed organ damage such as edema and lower motility 90 min after perfusion. Therefore, we collected samples from 10 min through 60 min after the start of perfusion. The collected samples were pooled for the analyses.

The production of HDL from the SI was examined by analyzing HDL-apo AI and apo AI using non-SDS-PAGE and SDS-PAGE, respectively, followed by Western blot analysis of apo AI in SI lymph perfusates from WT mice and ABCA1 KO mice. Peptide mapping of HDL using LC/MS and the analyses of lipid and apo composition and size distribution of HDL were performed in plasma, SI lymph perfusates, and liver perfusates from WT mice. The effects of an ABCA1 inhibitor, glyburide (26) (Cayman), and an LCAT inhibitor, DTNB (27) (Cayman), on the formation of SI-HDL were examined in WT mice by adding them to the perfusion buffer. The effects of a high-fat diet on the production of SI-HDL were examined by measuring HDL-apo AI in SI lymph perfusates using non-SDS-PAGE followed by Western blot analysis of apo AI after WT mice were fed a high-fat diet for 4 weeks.

Western blot analyses

For the analysis of apos in SI lymph perfusates, plasma, and lipoprotein density fractions, samples were run on SDS-PAGE or