



Association of High-Density Lipoprotein Subclasses with Carotid Intima-Media Thickness: Shimane CoHRE Study

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Aims: Recent studies suggested that subclasses of high-density lipoprotein (HDL) may be a better biomarker to predict the risk of atherosclerotic disorders. We aimed to examine the association of HDL2- and HDL3-cholesterol (HDL2-C and HDL3-C) with carotid intima-media thickness (IMT) using a new method to quantify the HDL-C subclasses.

Methods: Participants were 657 Japanese subjects (434 women) who received a health examination (mean age: 73 years). Serum samples were analyzed by the homogenous assay for HDL-C and HDL3-C. HDL2-C was calculated indirectly by subtracting HDL3-C from HDL-C. HDL3-C measured by this assay was well correlated with that measured by ultracentrifugation ($r=0.898$, $p<0.001$). The maximum IMT (max-IMT) and plaque score (PS) were evaluated by ultrasonography following the standard protocol.

Results: HDL3-C was associated with age both in men ($r=-0.322$, $p<0.0001$) and women ($r=-0.315$, $p<0.0001$). In a simple regression analysis, max-IMT showed an inverse association with HDL3-C, whereas no significant association was observed with HDL2-C. A multiple linear regression analysis indicated, however, that the association between HDL3-C and max-IMT was not significant in both aged and younger populations when age was included in the analysis. Further, not only HDL2-C but also HDL3-C was not a significant predictor of 'atherosclerotic arteries' defined as the max-IMT ≥ 1.5 mm. Similar results were observed in the analysis on PS.

Conclusions: Neither HDL3-C nor HDL2-C was significantly associated with carotid atherosclerosis in the Japanese population in this study.

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Key words: High-density lipoprotein (HDL), Cholesterol, Atherosclerosis, Intima-media thickness (IMT)

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Introduction

A high level of plasma HDL-C has long been believed to associate with a reduced number of cardiovascular events in general populations as well as in populations with high risks for cardiovascular events¹⁻⁸. However, this concept is now controversial

because of recent evidence against the beneficial role of HDL⁹⁻¹². In this context, it was of interest that a recent cohort study suggested that two HDL-C subclasses, HDL2 and 3, had opposite effects on the plaque formation; an inverse association between HDL3-C and plaque area and a positive association between HDL2-C and plaque thickness were found in the US population¹³.

In spite of increasing attention to physiological roles of the HDL subclasses, a limited number of reports have so far been available on the role of the HDL-C subclasses in cardiovascular diseases probably due to methodological difficulty in quantification of

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the subclasses^{1, 14-17}); it is necessary to separate the two HDL subclasses by ultracentrifugation [HDL2 (1.063–1.125) and HDL3 (1.125–1.210)]¹⁸⁻²⁰. Measurement of plasma concentration of HDL subclasses was therefore a laborious and time-consuming process, which hampers quantification of HDL2 and HDL3 in large-scale clinical and epidemiological studies. In this context, it was groundbreaking that a novel reagent was introduced to separate HDL2 from HDL3, which made it possible to quantify them using a versatile automatic analyzer without ultracentrifugation²¹. Further, a new assay kit was recently developed and kindly provided (Kyowa Medex Co., Ltd. Tokyo, Japan), which established a simple method to determine precise concentrations of HDL2-C and HDL3-C²².

In this study, we therefore employed the new method and examined the association of HDL2-C and HDL3-C with the intima-media thickness (IMT) in the carotid artery, which is a good surrogate marker for the systemic atherosclerosis, in a community-dwelling Japanese population.

Methods

Subjects

Inclusion criteria of participants of the present study were all individuals aged 35 or older who received health examinations conducted on Oki Island in 2015. Although we invited all the subjects according to the criteria, the participants in this study were mostly over 50 years of age due to a highly aging society in this area. The exclusion criterion was having severe disorders, such as advanced cancer, heart failure, frailty and renal failure with serum creatinine (Cr) > 2 mg/dL. Participants were considered to have these diseases when they had already been diagnosed by medical doctors.

Based on these criteria, a total of 657 subjects (223 males and 434 females) were assigned to this study. Histories of smoking, hypertension, diabetes mellitus, and hypercholesterolemia were obtained through an interview. Blood pressure and fasting blood glucose measured on site were not included in the criteria for the diagnosis. This study was a part of the cohort study (Shimane CoHRE Study) conducted by the Center for the Community-based Health Research and Education, Shimane University. The Shimane CoHRE study started in 2006, and the population employed in this study was not the same as those in our previous reports performed for different aims^{23, 24}.

Ethics

Written informed consent was obtained from each participant. The study protocol was approved by

the local ethics committee of Shimane University.

Data Collection

Disease history, medication and information about lifestyle such as smoking, alcohol consumption and regular exercise were obtained by a questionnaire. Smokers were defined as subjects smoking daily (smokers: 1, and non-smokers: 2). Habitual drinkers were defined as subjects drinking alcohol regularly (habitual drinkers: 1, and non-habitual drinkers: 2). As for a habit of regular exercise, subjects self-reporting to have daily exercise were categorized as subjects having “regular exercise” (regular exercise: 1, and non-regular exercise: 2). It was recommended to include “ex-smoker” in the smoking status because smoking was shown to be a risk for atherosclerotic disorders even many years after quitting²⁵. However, as our database included many missing data on previous smoking history, only the current smoking status was employed in the analysis.

Blood pressure was measured twice after taking a rest for at least 15 min. The lower of the two measurements was taken as the blood pressure measured at site. Venous blood was collected after overnight fasting. A serum sample was separated and kept frozen at –80°C until the measurement of HDL-C and the subclasses. Biochemical measurements of triglyceride (TG), low density lipoprotein cholesterol (LDL-C), and Cr were performed by standard enzymatic methods. Hemoglobin A1c (HbA1c) was determined by high-performance liquid chromatography (HPLC). Estimated glomerular filtration rate (eGFR) was calculated by the following formula revised by the working group of Japanese Chronic Kidney Disease Initiative²⁶; $eGFR \text{ (mL/min/1.73 m}^2\text{)} = 194 \times (\text{serum Cr, mg/dL})^{-1.094} \times \text{age}^{-0.287} \times (0.739, \text{ if female})$.

Intima-media thickness (IMT) in the carotid artery was measured with a high-resolution, real-time ultrasonography with a 7.5-MHz transducer (Vivid I or LOGIQ e, GE, Tokyo, Japan). The measurement of the arterial wall thickness was performed at four segments in the bilateral carotid arteries; at 1.5-cm distal to the bifurcation in the internal carotid artery (S1), at the bifurcation (S2), at 0–1.5 cm (S3) and 1.5–3.0 cm (S4) proximal to the bifurcation in the common carotid artery. The maximal value among the eight measurements at S1–4 of the bilateral carotid arteries was analyzed as max-IMT in the present study. Eight measurements at S1–4 of the bilateral carotid arteries were summed up as plaque score (PS) if they were 1.1 cm or more.

HDL2-C and HDL3-C Measurements

The measurement of total HDL-C was per-

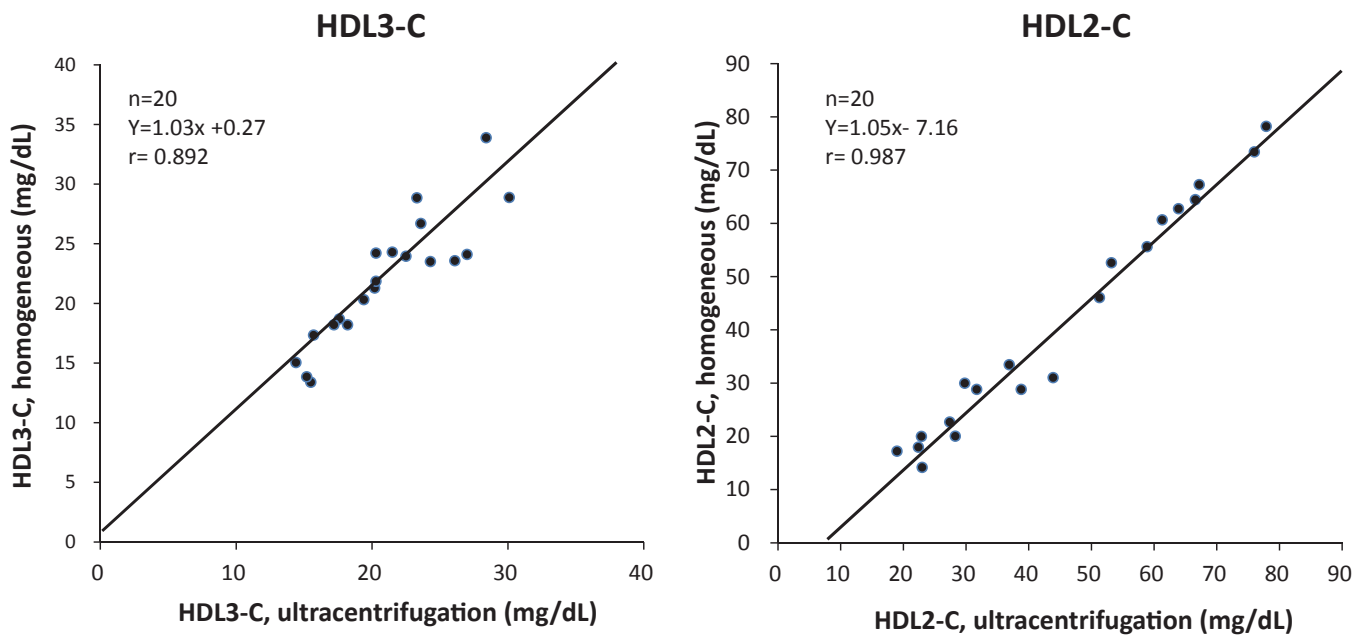


Fig. 1. Correlation between HDL-C subclasses determined by the homogeneous method and by ultracentrifugation.

formed using a commercial assay kit, MetaboLead® HDL-C (Kyowa Medex Co., Ltd. Tokyo, Japan) and a general auto-analyzer (JCA-BM6070, Nihon Denshi Co., Japan). The measurement of HDL3-C was performed using the homogeneous method developed by Kyowa Medex Co., Ltd.²²⁾. In brief, not only LDL and VLDL but also HDL2 were coagulated in a solution with an adequate concentration of polyanions and magnesium because HDL2 has a higher positive charge compared with HDL3. Accordingly, HDL3-C reacted with cholesterol-related enzymes, such as cholesterol esterase and cholesterol oxidase, preferentially, and the HDL3-C level was quantified. The HDL2-C level was then determined by subtraction of HDL3-C from the total HDL-C level. The detection limit for total HDL-C and HDL3-C were 0.03 and 0.05 $\mu\text{mol/L}$, and the relative standard deviation (SD) was <4.7 and <2.9%, respectively. The intra- and inter-assay coefficients of variations in the HDL3-C measurement were 1.9% and 2.1%, respectively.

In order to validate the homogeneous method described above, the concentrations of the HDL subclasses measured by this method were compared with those by the ultracentrifugation method in 20 samples. Measurement by the ultracentrifugation was carried out in an outsourcing laboratory (SRL, Inc., Japan) according to the modified protocol of the methods reported previously.^{27, 28)}; a plasma sample was cooled on ice immediately and separated into two tubes with potassium bromide (KBr). After adjustment for the density ($d=1.063$ kg/L with KBr for

HDL-C, and $d=1.125$ kg/L with KBr and sodium chloride (NaCl) for HDL3-C), the sample was centrifuged at $223,000 \times g$ (L-60, Beckman-Coulter, Fullerton, CA) with Type 50.4 Ti Rotor (Beckman-Coulter) at 10°C for four h. After aspiration of 40% of the supernatant (upper layer), the remaining sample was used for the measurement of cholesterol (Wako Pure Chemical Industries, Ltd.) to determine the concentration of the total HDL-C and the HDL3-C subfraction, respectively. The HDL2-C subfraction was determined by the following formula:

$$\text{HDL2-C} = [(\text{HDL-C}) - (\text{HDL3-C}) \times 1.54^*] \times 0.6^{**}$$

*; coefficients for dilution, and **; correction of the volume for aspiration

In addition, we determined the HDL3-C concentration using single precipitation methods to compare the homogeneous method developed by Kyowa Medex Co., Ltd. In the Warnick method, 50 μg of HDL3 reagent (19.1 g/L Dextran sulfate and 1.95 M magnesium chloride (MgCl_2)) was added to 0.5 mL serum. After vortex mixing, the mixture was placed at room temperature for 15 min and centrifuged at $12,000 \times g$ for five min. Cholesterol concentrations of the supernatant in the isolated HDL3 were measured by the cholesterol assay. In the Hirano method, 60 μg of HDL3 reagent (12 g/L Dextran sulfate, 8.25 g/L heparin and 98.7 g/L MgCl_2) was added to 0.3 mL serum²⁹⁾. After vortex mixing, the mixture was placed to centrifuge at 10,000 rpm for 10 min. The supernatant was used for the measurement of HDL3-C.

Table 1. Baseline characteristics of participants

	Men	Women	P
N	223	434	
Age, y	72.1 ± 7.9	72.8 ± 7.6	0.241
BMI, kg/m ²	23.5 ± 2.9	23.0 ± 3.3	0.074
SBP, mmHg	129.9 ± 15.9	134.1 ± 16.3	0.002
DBP, mmHg	75.8 ± 9.5	76.3 ± 9.5	0.520
Hypertension, %	42.0	46.8	0.508
Smoking, %	15.0	0.2	<0.001
Exercise, %	43.9	38.2	0.158
Alcohol habits, %	62.1	15.2	<0.001
Diabetes, %	12.4	7.1	0.021
Lipid lowering therapy, %	19.3	33.9	<0.001
LDL-C, mg/dL	114.4 ± 27.2	128.9 ± 28.4	<0.001
HDL-C, mg/dL	58.6 ± 15.9	61.2 ± 14.6	0.036
HDL2-C, mg/dL	38.2 ± 15.2	40.3 ± 14.7	0.087
HDL3-C, mg/dL	20.4 ± 3.8	20.9 ± 3.6	0.100
TG, mg/dL	93.3 [87.1, 97.7]	95.5 [91.2, 97.7]	0.640
HbA1c (NGSP), %	5.87 ± 0.56	5.84 ± 0.42	0.329
eGFR, mL/min/1.73 m ²	69.2 ± 16.4	69.4 ± 15.1	0.835
max-IMT, mm	1.99 [1.87, 2.12]	1.65 [1.59, 1.71]	<0.001
Plaque score	4.61 [4.41, 4.83]	3.95 [3.85, 4.06]	<0.001

Data was expressed as the mean ± SD or the mean [95% confident interval].
Statistical significance was examined either by Student's *t*-test or χ^2 -test.

Statistics

Data were expressed as mean ± SD. Because of a skewed distribution, the serum TG level and max-IMT were analyzed after logarithmic (log) transformation. Pearson's correlation coefficient and analysis of variance (ANOVA) were employed in univariate analyses between max-IMT and other variables. Then, the multiple linear regression and the multiple logistic regression analyses were performed to examine an association of max-IMT with the HDL subclasses. In the logistic regression analysis, subjects were divided into two groups according to their max-IMT following a clinical criterion³⁰⁻³²; subjects with max-IMT ≥ 1.5 mm were categorized as "atherosclerotic". As for PS, those over the median (>4.0) were categorized as "atherosclerotic". All statistical analyses were performed with the use of the IBM SPSS Statistic software (SPSS Statistics 21). Statistical significance was defined as $p < 0.05$.

Results

Validation of the Homogenous Method to Quantify the HDL-C Subclasses

In order to validate the new measurement method, levels of HDL-C subclasses determined by the homogenous method were compared with those

by ultracentrifugation. As shown in **Fig. 1**, a strong correlation was obtained between the two methods [$r=0.89$, $p<0.001$ for HDL3-C and $r=0.99$, $p<0.001$ for HDL2-C], indicating that the homogenous method was reliable as a quantification method of the HDL subclasses, at least in the ranges shown in **Fig. 1**. In addition, in terms of HDL3-C, the correlation of the homogenous method with the ultracentrifugation method was significantly better than the Warnick method and at least comparable to the Hirano method (**Supplementary Fig. 1**).

Demographic Data of the Studied Population

The baseline characteristics of the 657 participants (223 men and 434 women) were shown in **Table 1**. LDL-C, HDL-C and HDL2-C were significantly higher in women than in men, though subjects taking anti-hyperlipidemic drugs were found more in women. HDL3-C tended to be higher in women than in men, though it did not reach a significant level. Max-IMT and the ratio of smokers and diabetes mellitus were significantly higher in men than in women.

Impact of the Level of HDL-C Subclasses on Max-IMT and PS

A simple regression analysis showed that max-IMT and PS showed an inverse correlation with

Table 2A. Simple regression analysis for max-IMT

variables	All		Men		Women	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age	0.341	<0.0001	0.363	<0.0001	0.359	<0.0001
BMI	-0.084	0.032	-0.252	0.0002	-0.023	0.638
SBP	0.029	0.466	-0.079	0.241	0.137	0.004
DBP	-0.145	0.0002	-0.256	0.0001	-0.075	0.120
LDL-C	-0.099	0.011	-0.038	0.570	-0.060	0.210
HDL-C	-0.069	0.077	-0.016	0.815	-0.078	0.105
HDL2-C	-0.032	0.412	0.021	0.758	-0.043	0.376
HDL3-C	-0.153	<0.0001	-0.150	0.026	-0.140	0.004
LogTG	0.03	0.449	-0.035	0.603	0.085	0.078
HbA1c (NGSP)	0.111	0.004	0.038	0.574	0.162	0.0008
eGFR	-0.111	0.005	0.036	0.593	-0.163	0.0007

Pearson's correlation coefficient (*r*) is shown with *p* value.

Table 2B. Simple regression analysis for Plaque score

variables	All		Men		Women	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age	0.368	<0.0001	0.408	<0.0001	0.384	<0.0001
BMI	-0.073	0.064	-0.220	<0.001	-0.023	0.639
SBP	0.016	0.679	-0.090	0.181	0.135	0.005
DBP	-0.142	<0.001	-0.263	<0.0001	-0.064	0.186
LDL-C	-0.122	0.002	-0.096	0.156	-0.053	0.275
HDL-C	-0.124	0.002	-0.095	0.158	-0.117	0.015
HDL2-C	-0.078	0.046	-0.043	0.526	-0.078	0.107
HDL3-C	-0.191	<0.0001	-0.226	<0.001	-0.154	0.001
LogTG	0.009	0.814	-0.097	0.151	0.099	0.039
HbA1c (NGSP)	0.128	0.001	0.107	0.113	0.137	0.005
eGFR	-0.163	<0.0001	-0.138	0.041	-0.186	0.0001
Max-IMT	0.771	<0.0001	0.781	<0.0001	0.742	<0.0001

Pearson's correlation coefficient (*r*) is shown with *p* value.

HDL3-C but not with HDL2-C (**Table 2A** and **2B**). This correlation was still observed when the analysis was done in men and women separately. HDL-C showed an inverse correlation with max-IMT and PS as well, which was, however, weaker when compared with HDL3-C. HDL3-C showed a modest inverse association with age in men as well as in women whereas HDL2-C did not (**Table 3**). Interestingly, HDL3-C was not associated with HDL2-C, although HDL3-C was significantly associated with DBP, LDL-C, and HDL-C (**Table 3**).

In spite of the significant correlation found in the simple regression, multiple linear regression analysis under adjustment of confounding factors indicated that HDL3-C was not associated with max-IMT as

well as PS (**Table 4A** and **4B**). Age, male gender, and smoking in the younger population and male gender, HbA1c, and lower BMI in the elderly population were factors positively associated with max-IMT (**Table 4A**). In addition, multiple logistic regression analysis showed that in both the younger and elder population, HDL3-C was not selected as an independent factor to identify "atherosclerotic" subjects with max-IMT ≥ 1.5 mm under adjustment with covariates (**Table 5A**). These findings were consistent with those on PS (**Table 4B** and **5B**), suggesting that the apparent association between HDL3-C and max-IMT as well as PS in a simple regression analysis was mainly due to a strong confounder, i.e., age.

Table 3. Simple regression analysis for HDL2-C and HDL3-C

	HDL3-C				HDL2-C			
	Men		Women		Men		Women	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
Age	-0.322	<0.0001	-0.315	<0.0001	-0.006	0.935	-0.078	0.103
BMI	0.084	0.213	0.193	<0.0001	-0.275	<0.0001	-0.282	<0.0001
SBP	0.096	0.152	0.078	0.103	0.073	0.279	-0.064	0.186
DBP	0.315	<0.0001	0.200	<0.0001	0.131	0.051	-0.074	0.125
LDL-C	0.312	<0.0001	0.296	<0.0001	-0.133	0.047	-0.071	0.139
HDL-C	0.262	<0.0001	0.155	0.0012	0.965	<0.0001	0.968	<0.0001
HDL2-C	0.038	0.573	-0.062	0.194				
HDL3-C					0.038	0.573	-0.062	0.194
TG	-0.019	0.781	0.017	0.726	-0.547	<0.0001	-0.521	<0.0001
HbA1c (NGSP)	0.073	0.277	0.082	0.087	-0.048	0.476	-0.143	0.003

Pearson's correlation coefficient (*r*) is shown with *p* value.

Effects of Medication

Since medication, such as statins and fibrates, has not been considered in the analysis above, we further assessed the relationship between HDL3-C and max-IMT in subjects without lipid-lowering medication ($n=465$). Multiple linear regression analysis indicated that HDL3-C was not associated with max-IMT (Table 6A), although HDL3-C significantly and inversely correlated with max-IMT in a simple regression analysis (data not shown). These findings were consistent with the results of multiple logistic regression analysis (Table 6B).

Discussion

We found that serum concentration of HDL-C subclasses measured by the homogenous assay was well correlated with that measured by ultracentrifugation, and that not HDL2-C but HDL3-C was inversely associated with age. Although HDL3-C was inversely correlated with max-IMT as well as PS in a simple regression, the significant correlation was not observed under adjustment with confounding factors including age. These findings suggested that serum level of HDL-C subclasses was not independently associated with carotid IMT in this population.

Although HDL-C has been considered to be protective against atherosclerosis and cardiovascular events, recent evidence does not support this idea^{10, 33-35}. In this context, HDL2 and HDL3 have attracted much attention as they may possess key roles to explain the discrepancy of the role of HDL. In fact, previous studies showed that the HDL3-C level was inversely associated with coronary and peripheral arterial diseases, plaque formation or plaque area, and cardiovascular

events and mortality^{13, 16, 36-41}. However, as findings inconsistent with those above have been reported as well⁴²⁻⁴⁶, pathophysiological roles of the HDL subclasses have not been confirmed yet. Concerning the relationship between HDL subclasses and atherosclerosis, some investigators have reported that the HDL2-C level is inversely associated with carotid artery wall thickness^{42, 45}, while the HDL3-C level has been reported to be inversely associated with carotid plaque area¹³ and with plaques in the femoral sites³⁶. Taken together, the role of HDL-C subclasses in plaque formation and prevention against cardiovascular events are still controversial. On the other hand, according to recent studies regarding total HDL-C level, it was inversely associated with carotid IMT especially in middle-aged men⁴⁷ and was protective against coronary heart disease unless it was not very high⁴⁸.

In the present study, the serum HDL2-C level was higher than the HDL3-C level, which was consistent with previous reports in Japanese populations^{21, 49}. In contrast, in Caucasians and Hispanics, the HDL3-C level was higher than the HDL2-C level^{13, 14, 16, 45, 50}. Dietary factors may affect the HDL-C subclass level. In this context, we should keep in mind that fish is a common food in Japan especially in the Oki Islands. Previous studies suggested that the serum HDL2-C level was influenced by dietary intake of saturated fatty acids^{50, 51}. However, a twin study indicated that effects of long-term dietary habits on the serum lipid levels were determined largely by the genetic factor⁵². In addition, because polymorphisms of the hepatic lipase and the cholesteryl ester transfer protein genes can affect the serum level of HDL-C and of the subclasses^{53, 54}, the discrepancy between Japanese/Asians and Caucasians/Hispanics in terms of the serum level

Table 4A. Multiple linear regression analysis for max-IMT

variables	≤73 years-old (n=346)				>73 years-old (n=311)			
	beta	SE	std beta	p	beta	SE	std beta	p
Age	0.009	0.002	0.271	<0.0001	0.002	0.003	0.041	0.501
Gender *	-0.066	0.023	-0.185	0.004	-0.089	0.028	-0.230	0.001
HDL3-C	0.002	0.003	0.045	0.415	-0.004	0.003	-0.082	0.194
HDL2-C	0.005	0.001	0.047	0.465	-0.0002	0.001	-0.018	0.796
LDL-C	0.0001	0.0003	0.019	0.739	0.0001	0.001	0.0001	0.999
LogTG	0.092	0.049	0.111	0.067	0.024	0.071	0.023	0.739
BMI	-0.002	0.003	-0.039	0.487	-0.007	0.004	-0.122	0.046
DBP	-0.0001	0.001	-0.007	0.895	-0.002	0.001	-0.098	0.103
HbA1c (NGSP)	0.016	0.019	0.046	0.384	0.050	0.022	0.129	0.027
eGFR	0.001	0.001	0.092	0.086	0.0002	0.001	0.015	0.802
Alcohol habits	0.003	0.021	0.008	0.895	0.002	0.029	0.004	0.958
Smoking	-0.094	0.035	-0.156	0.007	0.030	0.075	0.023	0.689
Exercise	-0.019	0.018	-0.054	0.301	<0.0001	0.021	-0.0001	0.999

SE: standard error of beta, std beta: standardized beta, * 1: men, 2: women

Table 4B. Multiple linear regression analysis for Plaque score

variables	≤73 years-old (n=346)				>73 years-old (n=311)			
	beta	SE	std beta	p	beta	SE	std beta	p
Age	0.008	0.001	0.315	<0.0001	0.002	0.002	0.050	0.400
Gender *	-0.044	0.015	-0.182	0.004	-0.081	0.020	-0.281	0.0001
HDL3-C	-0.001	0.002	-0.017	0.757	-0.004	0.002	-0.113	0.066
HDL2-C	-0.0004	0.001	-0.057	0.361	-0.0004	0.001	-0.047	0.497
LDL-C	0.0001	0.0002	0.016	0.777	-0.0001	0.0001	-0.025	0.667
LogTG	-0.003	0.03	-0.005	0.933	0.015	0.051	0.020	0.767
BMI	-0.001	0.002	-0.017	0.769	-0.006	0.003	-0.138	0.019
DBP	0.0002	0.001	0.019	0.718	-0.001	0.001	-0.092	0.112
HbA1c (NGSP)	0.021	0.013	0.087	0.093	0.036	0.016	0.127	0.024
eGFR	0.0001	0.0004	0.009	0.852	-0.0001	0.001	-0.012	0.830
Alcohol habits	0.005	0.014	0.022	0.703	0.006	0.021	0.020	0.774
Smoking	-0.052	0.023	-0.126	0.027	0.009	0.054	0.009	0.870
Exercise	-0.003	0.012	-0.014	0.792	0.021	0.015	0.078	0.165

SE: standard error of beta, std beta: standardized beta, * 1: men, 2: women

of the HDL-C subclasses may be explained by the different genetic background among the population studied. Furthermore, a recent study indicated that efflux capacity of HDL-C was also different between Asians and Caucasians, which might be explained by the difference in the ratio between HDL3 and HDL2⁵⁵.

This is the first report showing a significant correlation between age and HDL3-C. It is of note that HDL2-C showed no correlation with age at all (**Table 3**). This observation seemed reliable because we obtained consistent results both in men and women. The correlation between HDL3-C and age may be

related to sedentary life style in an aged population. Although, to the best of our knowledge, disease prevalence on the Oki Islands was not largely deviated from the average of Japanese populations, future study is needed to evaluate the association between disease prevalence and the HDL3-C level. Further, this observation is not consistent with those of previous reports showing that not HDL3-C but HDL2-C positively correlated with age in Caucasian/Hispanic populations^{56, 57}. This discrepancy between Japanese and Caucasians/Hispanics is again enigmatic. Further studies on precise metabolisms of the HDL-C sub-

Table 5A. Multiple logistic regression analysis for max-IMT

variables	≤73 years-old (n=346)				>73 years-old (n=311)			
	OR	95%CI		p	OR	95%CI		p
Age	1.143	1.077	1.213	<0.0001	1.058	0.981	1.142	0.143
Gender *	0.436	0.238	0.796	0.007	0.408	0.196	0.849	0.016
HDL3-C	0.989	0.924	1.059	0.749	0.968	0.891	1.052	0.444
HDL2-C	0.996	0.977	1.016	0.702	1.012	0.990	1.035	0.277
LDL-C	1.005	0.997	1.014	0.233	1.002	0.993	1.012	0.628
LogTG	1.899	0.502	7.181	0.345	4.157	0.682	25.33	0.122
BMI	0.980	0.904	1.064	0.633	0.964	0.882	1.055	0.428
DBP	1.002	0.976	1.028	0.892	0.993	0.965	1.022	0.638
HbA1c (NGSP)	1.275	0.779	2.086	0.334	1.702	0.941	3.080	0.079
eGFR	1.020	1.003	1.038	0.020	1.014	0.994	1.034	0.185
Alcohol habits	0.672	0.379	1.191	0.173	0.713	0.337	1.051	0.377
Smoking	0.297	0.108	0.815	0.018	0.808	0.084	7.811	0.854
Exercise	0.875	0.541	1.415	0.585	1.155	0.678	1.968	0.596

A dichotomous variable according to max-IMT (i.e., max-IMT ≥ 1.5 mm vs. < 1.5 mm) was examined in the analysis.

OR: odds ratio, 95%CI: 95% confidence interval, * 1: men, 2: women

Table 5B. Multiple logistic regression analysis for Plaque score

variables	≤73 years-old (n=346)				>73 years-old (n=311)			
	OR	95%CI		p	OR	95%CI		p
Age	1.125	1.058	1.197	0.0002	1.049	0.980	1.124	0.170
Gender *	0.495	0.271	0.906	0.023	0.410	0.209	0.803	0.009
HDL3-C	0.986	0.918	1.059	0.706	0.949	0.876	1.027	0.194
HDL2-C	0.987	0.967	1.007	0.193	1.001	0.981	1.022	0.900
LDL-C	1.001	0.992	1.010	0.899	0.996	0.987	1.005	0.361
LogTG	1.809	0.464	7.050	0.393	0.994	0.191	5.182	0.995
BMI	0.983	0.904	1.070	0.697	0.960	0.883	1.044	0.340
DBP	1.000	0.973	1.026	0.972	0.998	0.972	1.025	0.903
HbA1c (NGSP)	1.723	1.028	2.887	0.039	1.260	0.735	2.160	0.400
eGFR	1.007	0.990	1.025	0.394	1.001	0.983	1.019	0.908
Alcohol habits	1.248	0.702	2.220	0.450	0.888	0.440	1.789	0.739
Smoking	0.694	0.278	1.735	0.435	0.499	0.052	4.739	0.545
Exercise	1.190	0.726	1.951	0.490	1.192	0.724	1.960	0.490

A dichotomous variable according to plaque score (i.e., plaque score >4.0 vs. ≤4.0) was examined in the analysis.

OR: odds ratio, 95%CI: 95% confidence interval, * 1: men, 2: women

classes are essential to solve these questions. In addition, as our population was rather aged (mean age was 73 years); it may be necessary to examine the levels of the HDL-C subclasses in a younger generation.

Although the present study could not show a significant association of HDL2 or HDL3 with max-IMT, this observation did not negate pathophysiological significance of HDL3 in atherosclerosis; as age is a strong confounder, it may be necessary to design studies to dissociate effects of HDL3 from those of age.

Furthermore, we did not consider the presence of ex-smokers in the analysis, which could be a limitation of our study. It might affect the association of HDL sub-fractions with carotid atherosclerosis, because it is well known that smoking decreases HDL-C whereas cessation of smoking increases HDL-C. In addition, roles of HDL subclasses in the cholesterol metabolism may need to be clarified in experimental studies. Recent studies demonstrated that the cholesterol efflux capacity of macrophages was inversely associated with

Table 6A. Multiple linear regression analysis for max-IMT in 453 subjects without lipid lowering therapy

variables	Without lipid lowering therapy (<i>n</i> =453)			
	beta	SE	std beta	<i>p</i>
Age	0.009	0.001	0.359	<0.00001
Gender *	-0.095	0.020	-0.249	<0.00001
HDL3-C	-0.002	0.003	-0.033	0.523
HDL2-C	0.0004	0.0006	0.031	0.551
LDL-C	0.0003	0.0003	0.044	0.376
LogTG	0.052	0.047	0.057	0.268
BMI	-0.004	0.003	-0.059	0.196
DBP	-0.001	0.001	-0.041	0.366
HbA1c (NGSP)	0.024	0.018	0.057	0.190
eGFR	0.001	0.0006	0.072	0.129
Alcohol habits	-0.007	0.019	-0.019	0.713
Smoking	-0.079	0.034	-0.109	0.020
Exercise	-0.014	0.016	-0.038	0.383

SE: standard error of beta, std beta: standardized beta, * 1: men, 2: women

Table 6B. Multiple logistic regression analysis for max-IMT in 453 subjects without lipid lowering therapy

variables	Without lipid lowering therapy (<i>n</i> =453)			
	OR	95%CI		<i>p</i>
Age	1.127	1.087	1.168	<0.0001
Gender *	0.341	0.200	0.583	0.0001
HDL3-C	0.950	0.890	1.015	0.129
HDL2-C	1.008	0.992	1.025	0.338
LDL-C	1.010	1.002	1.018	0.016
LogTG	1.790	0.535	5.994	0.345
BMI	1.010	0.940	1.085	0.780
DBP	1.002	0.980	1.024	0.890
HbA1c (NGSP)	1.364	0.855	2.177	0.193
eGFR	1.018	1.003	1.034	0.018
Alcohol habits	0.604	0.357	1.021	0.060
Smoking	0.244	0.090	0.664	0.006
Exercise	1.037	0.680	1.581	0.867

A dichotomous variable according to max-IMT (i.e., max-IMT \geq 1.5 mm vs. <1.5 mm) was examined in the analysis.

OR: odds ratio, 95%CI: 95% confidence interval, * 1: men, 2: women

carotid IMT, with the risk of angiographic coronary artery disease and with the incident of coronary heart disease, independently of HDL-C levels⁵⁸⁻⁶⁰). It is of interest to examine a correlation between cholesterol efflux capacity and the ratio HDL3-C/HDL2-C in a future study.

In conclusion, we found that the serum level of HDL3-C was inversely associated with age, and that the HDL-C subclass was not independently associated

with max-IMT and PS in a general Japanese population. Direct measurement of HDL-C subclasses by the homogenous assay is a simple and reliable method, which can be applied in epidemiological and clinical studies.

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Conflict of Interest Statement

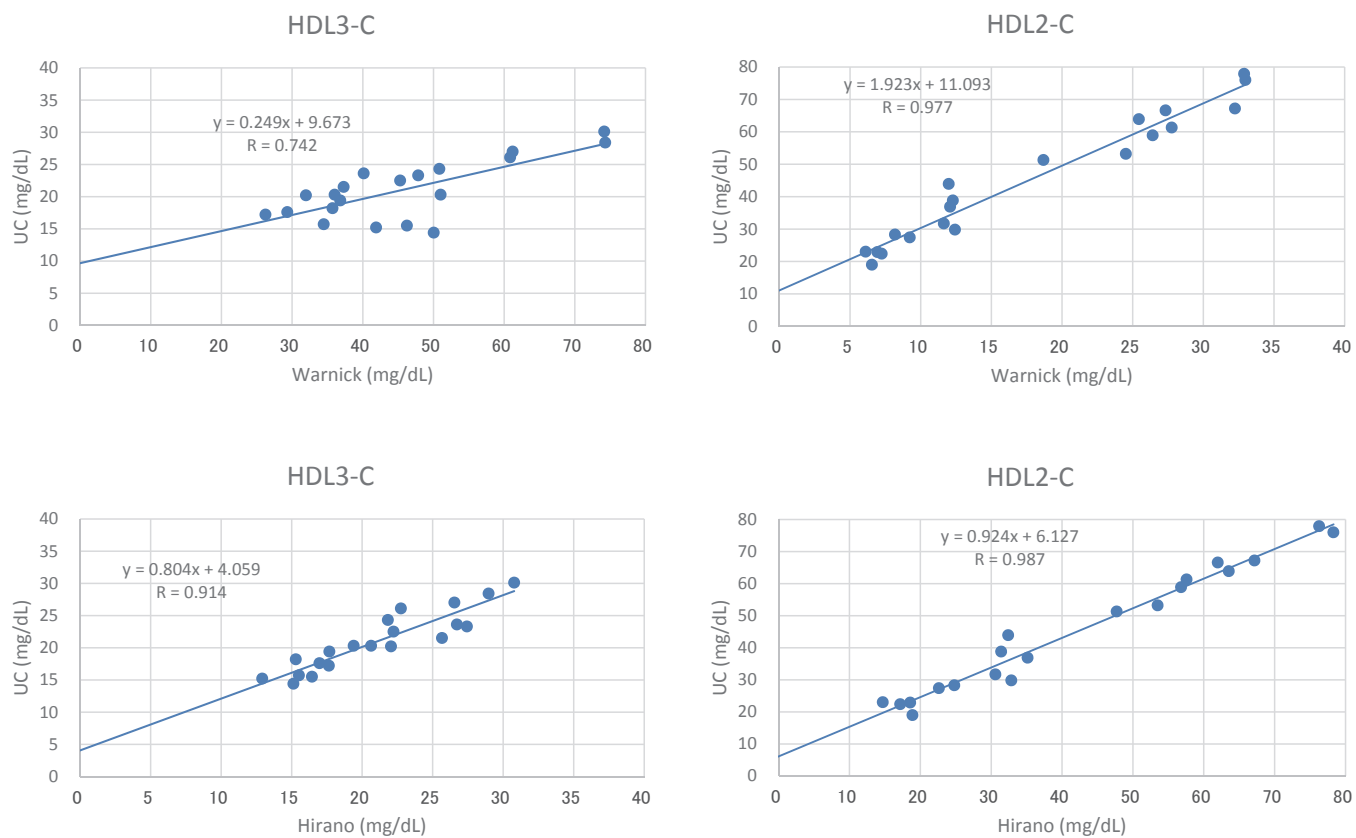
All authors have no conflicts of interest.

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Supplementary Fig. 1. Correlation between HDL-C subclasses determined by the Warnick and Hirano methods and by ultracentrifugation (UC).