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Subfractions of High-Density Lipoprotein Cholesterol and Carotid Intima-Media Thickness: The Northern Manhattan Study

Eduard Tiozzo, PhD[*,1,2], **Hannah Gardener, ScD**[*,2], **Barry I. Hudson, PhD**[3], **Chuanhui Dong, PhD**[2], **David Della-Morte, MD, PhD**[2,4,5], **Milita Crisby, MD, PhD**[6], **Ronald B. Goldberg, MD**[7], **Mitchell S.V. Elkind, MD, MS**[8], **Ying Kuen Cheung, PhD**[9], **Clinton B. Wright, MD, MS**[2], **Ralph L. Sacco, MD, MS, MS**[2,10], **Moise Desvarieux, MD, PhD**[11,12], and **Tatjana Rundek, MD, PhD**[2]

Eduard Tiozzo: etiozzo@med.miami.edu; Hannah Gardener: hgardener@med.miami.edu; Barry I. Hudson: bhudson@med.miami.edu; Chuanhui Dong: cdong@med.miami.edu; David Della-Morte: ddellamorte@med.miami.edu; Milita Crisby: milita.crisby@ki.se; Ronald B. Goldberg: rgoldber@med.miami.edu; Mitchell S.V. Elkind: mse13@cumc.columbia.edu; Ying Kuen Cheung: yc632@columbia.edu; Clinton B. Wright: cwright@med.miami.edu; Ralph L. Sacco: rsacco@med.miami.edu; Tatjana Rundek: trundek@med.miami.edu

[1]Department of Psychiatry and Behavioral Sciences, Miller School of Medicine, University of Miami, Miami, FL, USA

[2]Department of Neurology, Miller School of Medicine, University of Miami, Miami, FL, USA

[3]Division of Endocrinology, Diabetes and Metabolism, University of Miami, Miami, FL, USA

[4]Department of System Medicine, University of Rome Tor Vergata, Rome, Italy

[5]IRCCS San Raffaele Pisana, Rome, Italy

[6]Karolinska Institute, Department of Neurobiology, Care Sciences and Society, Stockholm, Sweden

[7]Diabetes Research Institute and Lipid Disorder Clinic, University of Miami, Miami, FL, USA

[8]Department of Neurology, Columbia University College of Physicians and Surgeons, New York, NY, USA

[9]Department of Biostatistics, Mailman School of Public Health, Columbia University, New York, NY, USA

[10]Department of Public Health Sciences, University of Miami, Miami, FL, USA

[11]Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA

[12]Centre de Recherche Épidémiologie et Statistique Sorbonne Paris Cité (CRESS-UMR1153), Paris, France

Abstract

Correspondence: Eduard Tiozzo, PhD, University of Miami, Department of Psychiatry and Behavioral Sciences & Department of Neurology, 1120 NW 14th street, Miami, FL 33136, Telephone: +305-243-6912, Fax: 305-243-1619, etiozzo@miami.edu.
*co-first authorships

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Background and Purpose—Recent drug trials have challenged the "HDL-cholesterol anti-atherosclerotic hypothesis", suggesting that total level of HDL-C may not be the best target for intervention. HDL-cholesterol (HDL-C) subfractions may be better markers of vascular risk than total levels of HDL-C. The objective of this cross-sectional study was to investigate the relation between HDL2-C and HDL3-C fractions and carotid intima-media thickness (cIMT) in the population-based Northern Manhattan Study.

Methods—We evaluated 988 stroke-free participants (mean age 66±8 years; 60% women; 66% Hispanic, and 34% Non-Hispanic) with available data on HDL-C subfractions using precipitation method and cIMT assessed by a high-resolution carotid ultrasound. The associations between HDL-C subfractions and cIMT were analyzed by multiple linear regression models.

Results—The mean HDL2-C was 14 ± 8 mg/dl, HDL3-C 32 ± 8 mg/dl, and the mean total HDL-C was 46 ±14 mg/dl. The mean cIMT was 0.90 ± 0.08 mm. After controlling for demographics and vascular risk factors, HDL2-C and total HDL-C were inversely associated with cIMT (per 2SDs, beta= -0.017, p=0.001 and beta= -0.012, p=0.03, respectively). The same inverse association was more pronounced among those with diabetes (per 2SDs, HDL2-C: beta= -0.043, p=0.003; HDL-C: beta= -0.029, p=0.02). HDL3-C was not associated with cIMT.

Conclusions—HDL2-C had greater effect on cIMT then HDL3-C in this large urban population. The effect of HDL2-C was especially pronounced among individuals with diabetes. More research is needed to determine anti-atherosclerotic effects of HDL-C subfractions and their clinical relevance.

Keywords

HDL-C; HDL subfractions; carotid intima-media thickness; atherosclerosis

Introduction

High density lipoprotein cholesterol (HDL-C) is one of the most commonly measured biomarkers integrated into public health prevention guidelines. Major epidemiological studies have demonstrated strong, inverse and independent relationships between HDL-C and cardiovascular disease (CVD) and stroke (1-3). However, several recent clinical trials have challenged the value of raising HDL-C pharmacologically and the validity of the "HDL-C anti-atherosclerotic hypothesis" (4-6). In addition to HDL-C "quantity", HDL-C "quality", such as HDL-C subfractions and their function may have differential effects on atherosclerosis and CVD risk. Variability of the levels of HDL-C subfractions and their function in total HDL-C may in part explain unexpected results of HDL-C-based interventions.

HDL-C consists of two principal subfractions, larger size (8.7-12.5 nm), more buoyant (density; 1.06-1.13 g/mL) HDL2-C and smaller (<8.7 nm), less buoyant (density; 1.13-1.21 g/mL) HDL3-C (7). High-density lipoprotein subfractions differ in their biological activities and responses to lifestyle changes and drug therapy (8). The decreased risk of CVD associated with HDL-C has been predominantly linked to HDL2-C (9-11). However, a more protective effect of HDL3-C over HDL2-C (12-14), or equal benefits of both subfractions for CVD, have also been reported (15).

Low HDL-C has been associated with increased carotid intima-media thickness (cIMT), a marker of subclinical atherosclerosis in multiple studies (16;17). Nonetheless, this relationship has not always been consistent (18;19), suggesting that total levels of HDL-C alone may not fully explain HDL-related atherosclerotic risk. The associations of HDL-C subfractions with cIMT have been less investigated and remain inconclusive (20).

The aim of this study was to investigate the relationship between HDL2-C and HDL3-C subfractions and cIMT in a large urban and multi-ethnic stroke-free cohort.

Methods

Study Participants

Stroke-free participants of the Northern Manhattan Study (NOMAS), an ongoing, prospective, population-based study of stroke incidence, vascular risk factors and cognitive decline underwent high-resolution B-mode ultrasound imaging as part of the Oral Infections and Vascular Disease Epidemiology Study (INVEST). Details on subject ascertainment, extensive assessments, and methods used in NOMAS and INVEST are described elsewhere (21;22). Of 3,298 participants in NOMAS, 1,552 (47%) had data available on HDL-C subfractions and 988 (63%) of those participants with HDL-C subfractions had cIMT measurements. The levels of HDL-subfractions were performed consecutively for 47% of the cohort and based on the available funds. NOMAS and INVEST were approved by the Institutional Review Boards of Columbia University Medical Center and the University of Miami. All participants gave written informed consent to participate in the study.

Baseline Evaluation

Data were collected through interviews with trained research assistants in English or Spanish. Physical and neurological examinations were conducted by study neurologists. Race-ethnicity was based upon self-identification through a series of questions modeled after the US census and conforming to standard definitions outlined by Directive 15 (23). Standardized questions were adapted from the Behavioral Risk Factor Surveillance System by the Centers for Disease Control regarding hypertension, type 2 diabetes mellitus (DM), smoking, and cardiac conditions (24). Blood pressure was measured with mercury sphygmomanometers and appropriately-sized cuffs. Hypertension was defined as a blood pressure ≥140/90 mmHg (based on the average of two measurements during one sitting), the patient's self-reported hypertension, or use of anti-hypertensive medications. Diabetes mellitus was defined by fasting glucose ≥126 mg/dl, the patient's self-reported diabetes, or use of insulin or oral anti-diabetic medication. Body mass index (BMI) was calculated in kg/m^2. Smoking was categorized as never smoking, former smoking, and current (within the past year) smoking. Mild-moderate alcohol use was defined as current drinking of >1 drink per month and ≤2 drinks per day. Physical activity was defined as the frequency and duration of 14 different recreational activities during the 2-week period before the interview, as described previously (21).

HDL2-C, HDL3-C, and Total HDL-C Measurements

Blood samples were drawn after an overnight fast. Plasma levels of cholesterol and triglycerides (TGs) were measured using standardized enzymatic procedures with a Hitachi 705 automated spectrophotometer (Boehringer Mannheim, Mannheim, Germany). HDL-C was measured after precipitation of plasma apo B-containing lipoproteins with phosphotungstic acid. The inter-assay coefficient of variation in our laboratory was 3% for HDL-C. HDL2-C and HDL3-C were determined in plasma by sequential precipitation using heparin-manganese and dextran sulphate (25). Apo B-containing lipoproteins were precipitated in the first reaction using heparin-manganese chloride at final concentrations of 1.26 mg/ml and 0.091 M, respectively. The supernatant (total HDL-C) was removed; an aliquot saved for analysis, and dextran sulphate (mol wt 15,000; Genzyme, Cambridge, MA) was added to precipitate HDL2-C, which was estimated by subtracting HDL3-C from HDL-C. Following centrifugation, the supernatant (HDL3-C) was removed and analyzed for cholesterol content.

Carotid Ultrasound

High-resolution carotid B-mode ultrasound (GE LogIQ 700, 9/13-MHz linear-array transducer) was performed by trained and certified sonographers using standardized and validated scanning and reading protocols as described previously (18). Carotid IMT in all carotid segments was measured off-line using an automated computerized edge detection image analysis system, *M'Ath* (*Intelligence in Medical Technologies, Inc., Paris, France*) in the areas free of plaque. Plaque was defined as a focal wall thickening or protrusion in the lumen more than 50% greater than the surrounding thickness. Carotid IMT was calculated as a composite measure of the near and the far wall of IMT in the common carotid artery (CCA), internal carotid artery (ICA), and bifurcation of both sides of the neck, and expressed as a mean of the maximum measurements of the 12 carotid sites in mm within an individual. The reliability of carotid measurements in our laboratory has been high and reported previously (26).

Statistical Analysis

The primary exposures of interest (HDL2-C, HDL3-C, and total HDL-C) were assessed as continuous variables. First, we examined these variables in relation to the demographics, anthropometrics, lifestyle and vascular risk factors, among all participants with data available on all three HDL variables and cIMT (n=988) using ANOVA. Next, we examined the associations of HDL2-C, HDL3-C, and total HDL-C with cIMT (n=988) using linear regression models with cIMT as the dependent variable. We constructed 2 models; Model 1 controlled for race/ethnicity, age, sex, low-density lipoprotein cholesterol (LDL-C), TGs and cholesterol lowering medications. Model 2 was additionally controlled for BMI, smoking, alcohol use, physical activity, hypertension, diabetes, and the time span from baseline to carotid ultrasound. Based on abnormalities in HDL subfraction distribution in diabetes, consisting of the increased prevalence of small HDL3-C and accelerated turnover and remodelling of large HDL2-C particles (27), we examined the interactions between all three HDL variables and diabetes in relation to cIMT. This exploratory analysis of potential effect modification by diabetes was conducted by including interaction terms in fully adjusted

models, and stratified analyses were conducted as appropriate. Finally, the interaction between HDL variables and cIMT by three carotid segments were analysed. All analysis of HDL2-C and HDL3-C were mutually adjusted. SAS version 9.1 (SAS institute, Cary, NC) was used for statistical analysis and $p<0.05$ was considered significant.

Results

The mean age of the study population (n=988) was 66±8 years; 60% were women: 66% Hispanic, and 34% non-Hispanic. The prevalence of smoking was 15%, diabetes 21%, and hypertension 72%. Mean LDL-C was 129.47 ± 34.10, mean TGs was 130.39 ± 65.85, and 15% of the population were taking cholesterol lowering medication. The mean values of total HDL-C, HDL2-C and HDL3-C by demographics and vascular risk factors are presented in Table 1. The mean HDL2-C was 14 ± 8 mg/dl, HDL3-C 32 ± 8 mg/dl, and the mean total HDL-C was 46 ±14 mg/dl. The mean total HDL-C was similar in the NOMAS participants with carotid ultrasound measurements but without available HDL subfractions (n= 806; 47 mg/dl), suggesting unbiased selection of our sub-cohort sample. The mean cIMT in the sample was 0.90 ± 0.08 mm (CCA: 0.92 ± 0.10 mm; ICA: 0.84 ± 0.09 mm; Bifurcation: 0.94 ± 0.10 mm).

A strong positive correlation was observed for total HDL-C with HDL2-C ($r=.81$, $p<0.01$) and for total HDL-C with HDL3-C ($r=.75$, $p<0.01$), while a weaker positive correlation was observed between HDL2 and HDL3 ($r=.34$, $p<0.01$).

Table 2 shows the association between the HDL variables and cIMT. After controlling for demographic and vascular risk factors including LDL-C and TG, HDL2-C and total HDL-C were inversely and significantly associated with cIMT (Model 2, $p=0.001$ and $p=0.03$, respectively). HDL3-C levels were not statistically significant associated with cIMT after full adjustment (Model 2, $p=0.81$).

We checked and found no significant interaction between traditional risk factors (sex, age, race/ethnicity, BMI, diabetes, hypertension, alcohol, smoking, and physical activity) and HDL-C variables in relation to cIMT. Suggested interactions were found only between diabetes and HDL2-C ($p=0.07$), and between diabetes and total HDL-C ($p=0.07$). Stratified analyses by diabetes status showed that the inverse associations for total HDL-C and HDL2-C subfraction with cIMT were stronger among participants with diabetes (Table 3).

Table 4 shows the association between HDL subfractions and HDL-C with cIMT by different carotid segments. Suggested interactions were observed in all carotid segments for HDL2-C, intima carotid artery and bifurcation for HDL-C, and no carotid segments for HDL-3.

Discussion

In the present study we have shown that HDL2-C and total HDL-C are inversely associated with cIMT independent of demographics and vascular risk factors among stroke-free individuals from an urban multi-ethnic stroke-free cohort. These associations were stronger

among participants with diabetes. No significant association was observed between HDL3-C and cIMT after adjusting for vascular risk factors.

Our findings are similar to a small clinical trial involving 21 healthy middle-aged individuals (28), where HDL2 was inversely related to carotid IMT. The same study, however, concluded that HDL subfractions may be more closely related to cIMT than total HDL-C. Our results suggest similar association between HDL2-C and total HDL-C with cIMT. In a sample of Japanese American people who were not using cholesterol and glucose lowering medications, but including individuals with dyslipidemia and type 2 diabetes, no significant association was found between total HDL-C and both HDL-C subfractions with cIMT (20). In a Finnish study (29) a significant association with cIMT was limited only to HDL-C, among asymptomatic members of low HDL-C families. According to the same study total HDL-C was a more critical predictor for cIMT than other lipid variables, including HDL2-C and HDL3-C. The results of Multi-Ethnic study of Atherosclerosis (MESA) suggested the absence of an inverse association of HDL-C with cIMT or CVD, after adjusting for LDL and HDL particles, thus identifying complex intersecting functions and atheroprotective mechanisms within HDL structure (19). The inconsistency of study findings could be explained by the differences in health status of study populations, such as the absence or presence of type 2 diabetes and whether the condition is treated or not. Type 2 diabetes reduces the levels of total HDL-C and HDL2-C subfraction and increases both relatively or absolutely HDL-3 subfraction (30). Our participants with type 2 diabetes had lower levels of total HDL-C and both HDL2-subfractions, compared to those without type 2 diabetes.

Our findings suggest the potential protective role of the HDL2-C subfraction for subclinical atherosclerosis among those with diabetes. In pro-atherosclerotic and pro-inflammatory conditions, HDL2-C has been inversely associated with cardiometabolic risk factors such as homeostatic model assessment, fasting glucose, and C-reactive protein (20;31). This can be explained by impaired efflux of cellular cholesterol in diabetes. A universal shift from HDL2-C toward HDL3-C and consequently a change in reverse cholesterol transport has been reported in diabetes (32). This conversion is facilitated by key factors in the HDL remodeling process whereby suppressed lecithin-cholesterol acyltransferase and enhanced hepatic lipase and CETP can impair maturation of HDL3-C into HDL2-C or enhance production of HDL3. Even elevated levels of HDL3-C from type 2 diabetics can display significantly reduced antioxidative capacity, linked to oxidative stress, glycaemia and hypertrygliceridemia (33). The relative levels of HDL2-C (30%) and HDL3-C (70%) of total HDL-C were similar among individuals with and without diabetes in our study. The HDL2-C is the more variable subfraction and more protective of atherosclerosis through its role in efflux of cellular cholesterol, stimulated by ATP binding cassette transporter. Similar ratio of HDL2-C/HDL3-C in our study suggests that the inverse association between HDL2-C and cIMT that was present among individuals with diabetes may be more related to altered HDL2-C quality rather than HDL-C quantity. Since our work has not assessed functionality of the HDL-C subfractions, no further explanation about the inverse association between HDL2-C and cIMT can emerge from this work.

Reverse cholesterol transport is considered one of the most important antiatherogenic functions of total HDL-C. Since the level of plasma HDL-C alone does not reliably predict

the degree of reverse cholesterol transport, the atherogenic quality may be better defined by HDL-C subfractions and their functionality (34). A causal relationship between circulating HDL-C levels and risk of atherosclerosis and CVD remains uncertain despite strong evidence for this association from observational studies. In MESA total HDL-C alone did not fully explain HDL-C-related risk. Similarly, raising HDL-C with medications in several recent pharmacotherapeutic interventional trials did not uniformly translate into lower risk of CVD events (4) or atherosclerosis (35). Dysfunctional and pro-inflammatory HDL-C, even after adjusting for quantitative HDL-C levels, was associated with cIMT in South Asian immigrants in the U.S (36). All these findings indicate the need for further evaluation of HDL quality, such as HDL-C subfractions, or even more specific measures of HDL-C function.

In our previous work (12), we observed an inverse association between HDL3-C and carotid plaque. Formation of carotid plaque is highly related to inflammation, endothelial dysfunction and smooth muscle cell proliferation. The same atherogenic mechanisms have been targeted by HDL3-C particles via inhibition of vascular cell adhesion molecule-1 expression (37) and LDL-C oxidation associated with higher paraxonase antioxidative activity (27). Carotid IMT however, may be distinct from plaque in the biologic and pathophysiologic effects on CVD (16) and it is not necessarily an intermediate lesion between normal wall structure and atherosclerotic plaque development as recently reported in the NOMAS (38). Intima media thickness, unlike carotid plaque, is more associated with hypertensive medial hypertrophy or thickening of smooth muscle cells although it is s not only a lesion represented by smooth muscle cell proliferation. It also represent a large part of the complex, depending on genetics, age and modifiable risk factors (39). Our data supports distinct atheroprotective properties of HDL-C subfractions in relation to carotid plaque and cIMT. Higher HDL2-C levels seem to be more atheroprotective for cIMT and HDL3-C for carotid plaque in our population.

Current cholesterol lowering therapeutic goals target total HDL-C without specific recommendations for HDL-C components. More research is needed to determine if the levels of HDL-C subfractions are clinically relevant beyond the levels of total HDL-C.

Strengths of the current study include the use of a large population, with available information on systematically collected and standardized measurements of vascular risk factors. Furthermore, our research contributes to knowledge of the role of HDL-C subfractions in subclinical atherosclerosis, a relatively understudied area in CVD. Our study also has several limitations, most notably its cross-sectional design, limiting inferences about temporality and causality. We also lacked information on changes over time in total HDL-C and its subfractions, especially in relation to changes in carotid IMT. Finally, we assessed the plasma levels of HDL-C subfractions without their biological activity and subclasses (e.g., HDL2a and HDL2b).

Conclusions

HDL-C subfractions may have distinct protective biological role against atherosclerosis, particularly in the presence of diabetes. Future studies are needed to determine more

conclusively the role of HDL2-C and HDL3-C subfractions and their functionality in atherosclerosis among individuals with different metabolic and CVD profiles.

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Table 1
Clinical characteristics of study participants

Demographics	N (%)	HDL-C (mg/dl) Mean (SD)	HDL2-C (mg/dl) Mean (SD)	HDL3-C (mg/dl) Mean (SD)
All	988	46 (14)	14 (8)	32 (8)
Race/ethnicity Hispanic	655	43 (12) *	13 (7) *	30 (7) *
Non-Hispanic	333	48 (15)	16 (9)	33 (9)
Age				
40-59	283	44 (13) *	13 (7) *	31 (8)
60-69	407	45 (14)	14 (8)	31 (7)
70-79	230	47 (15)	15 (9)	32 (9)
80+	68	48 (13)	16 (8)	32 (7)
Sex				
Male	397	41 (13) *	12 (7) *	29 (7) *
Female	591	48 (14)	15 (8)	33 (8)
BMI				
Normal	240	50 (16) *	16 (9) *	33 (8) *
Overweight	457	45 (14)	14 (8)	31 (8)
Obese	288	44 (12)	13 (7)	31 (7)
Smoking				
Current	152	44 (13)	13 (8)	30 (8)
Former	366	45 (14)	14 (9)	31 (8)
Never	470	46 (13)	14 (8)	32 (8)
Mild-moderate alcohol use				
Yes	381	47 (15) *	14 (8)	32 (8) *
No	607	45 (13)	14 (8)	31 (8)
Moderate-heavy physical activity				
Yes	95	49 (15) *	16 (9) *	33 (9) *
No	890	45 (14)	14 (8)	31 (8)
Cholesterol lowering medications				
Yes	150	47 (14)	14 (7)	33 (8)
No	838	45 (14)	14 (8)	31 (8)
Hypertension				
Yes	716	45 (14)	14 (8)	31 (8)
No	272	47 (14)	15 (9)	32 (8)
Diabetes				
Yes	203	43 (13) *	13 (8) *	30 (7) *
No	785	46 (14)	14 (8)	32 (8)
LDL-C (mg/dl)				
>160	818	46 (14) *	14 (8) *	32 (8)
≤160	166	44 (11)	12 (6)	32 (7)
TGs (mg/dl)				

Demographics	N (%)	HDL-C (mg/dl) Mean (SD)	HDL2-C (mg/dl) Mean (SD)	HDL3-C (mg/dl) Mean (SD)
>150	276	39(10) *	11(6) *	28(7) *
≤150	711	48(14)	15 (8)	33(8)

* ANOVA or t-test p<0.05 (categories of covariates were compared for the HDL variables as continuous measures). HDL-C, high-density lipoprotein; BMI, body mass index; LDL-C, low-density lipoprotein cholesterol; TGs, triglycerides

Table 2

The association of HDL2 and HDL3 subfractions and total HDL-C with carotid IMT

IMT change per 2 SDs	Beta, SE (n=988)	p-value
HDL2-C		
Model 1	-0.013, 0.005	0.02
Model 2	-0.017, 0.005	0.001
HDL3-C		
Model 1	-0.011, 0.006	0.05
Model 2	0.001, 0.006	0.81
HDL-C		
Model 1	-0.015, 0.006	0.01
Model 2	-0.012, 0.006	0.03

Linear regression model

Model 1; adjusted for race/ethnicity, age, sex, LDL-C, TGs, and cholesterol medication use,

Model 2; adjusted for Model 1 and BMI, smoking, alcohol, physical activity, hypertension, diabetes, time from baseline to carotid ultrasound

Table 3

The association between the HDL-C variables and carotid IMT stratified by diabetes mellitus status

IMT change per 2 SDs	Beta, SE (n=988)	p-value	Interaction p-value
HDL2-C			
Diabetics	-0.043, 0.014	0.003	0.07
Non-diabetics	-0.012, 0.006	0.04	
HDL3-C			
Diabetics	0.009, 0.014	0.52	1.00
Non-diabetics	0.002, 0.006	0.81	
HDL-C			
Diabetics	-0.029, 0.012	0.02	0.07
Non-diabetics	-0.008, 0.006	0.24	

Linear regression model

Fully adjusted for race/ethnicity, age, sex, LDL-C, TGs, and cholesterol medication use, BMI, smoking, alcohol, physical activity, hypertension, and time from baseline to carotid ultrasound

Table 4

The association of HDL2 and HDL3 subfractions and total HDL-C with carotid IMT stratified by carotid segment

IMT change per 2 SDs	CCA		ICA		Bifurcation	
	Beta, SE	p-value	Beta, SE	p-value	Beta, SE	p-value
HDL2-C						
Model 1	-0.012, 0.007	0.08	-0.011, 0.006	0.07	-0.014, 0.007	0.05
Model 2	-0.014, 0.007	0.03	-0.014, 0.006	0.03	-0.022, 0.007	0.001
HDL3-C						
Model	-0.015, 0.007	0.03	-0.010, 0.006	0.13	-0.008, 0.008	0.27
Model 2	-0.001, 0.007	0.85	-0.002, 0.007	0.74	0.006, 0.007	0.41
HDL-C						
Model 1	-0.014, 0.007	0.05	-0.015, 0.006	0.02	-0.016, 0.008	0.04
Model 2	-0.008, 0.007	0.27	-0.013, 0.007	0.04	-0.015, 0.007	0.04

CCA, common carotid artery; ICA, intima carotid artery

Linear regression model

Model 1; adjusted for race/ethnicity, age, sex, LDL-C, TGs, and cholesterol medication use,

Model 2; adjusted for Model 1 and BMI, smoking, alcohol, physical activity, hypertension, diabetes, and time from baseline to carotid ultrasound