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Apolipoprotein C-III Proteoforms, Plasma Lipids and Cardiovascular Risk in the Multiethnic Study of Atherosclerosis (MESA)

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Abstract

Background: Apolipoprotein C-III (apoC-III) is an important regulator of triglyceride metabolism and was associated with cardiovascular risk in several cohorts. ApoC-III is present in four major proteoforms, a native peptide ($C\text{-}III_{0a}$), and glycosylated proteoforms with zero ($C\text{-}III_{0b}$), one ($C\text{-}III_1$, most abundant) or two ($C\text{-}III_2$) sialic acids, which may differentially modify lipoprotein metabolism. We studied the relationships of these proteoforms with plasma lipids and cardiovascular risk.

Methods: ApoC-III proteoforms were measured by mass spectrometry immunoassay in baseline plasma samples of 5,791 participants of Multiethnic Study of Atherosclerosis, an observational community-based cohort. Standard plasma lipids were collected for up to 16 years and cardiovascular events (myocardial infarction, resuscitated cardiac arrest or stroke) were adjudicated for up to 17 years.

Results: ApoC-III proteoform composition differed by age, gender, race/ethnicity, BMI and fasting glucose. Notably, $C\text{-}III_1$ was lower in older participants, men and Blacks and Chinese (versus Whites), and higher in obesity and diabetes. In contrast, $C\text{-}III_2$ was higher in older participants, men, Blacks and Chinese, and lower in Hispanics and obesity. Higher $C\text{-}III_2$ to

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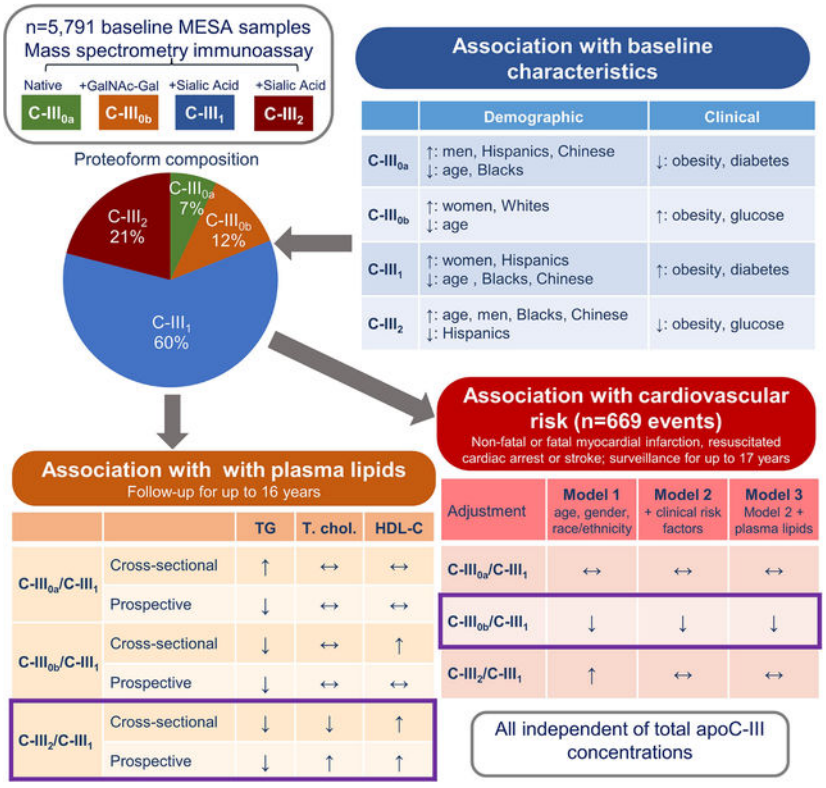
[A]Equal contribution

Disclosures: Jeremy Furtado is currently an employee of Biogen.

C-III$_1$ ratio (C-III$_2$/III$_1$) was associated with lower triglycerides and higher HDL in cross-sectional and longitudinal models, independently of clinical and demographic risk factors and total apoC-III. The associations of C-III$_{0a}$/III$_1$ and C-III$_{0b}$/III$_1$ with plasma lipids were weaker and varied through cross-sectional and longitudinal analyses. Total apoC-III and C-III$_2$/III$_1$ were positively associated with CVD risk (n=669 events, Hazard Ratios: 1.14 [95%CI: 1.04–1.25] and 1.21 [1.11–1.31], respectively), however the associations were attenuated after adjustment for clinical and demographic characteristics (1.07 [0.98–1.16]; 1.07 [0.97–1.17]). In contrast, C-III$_{0b}$/III$_1$ was inversely associated with CVD risk even after full adjustment including plasma lipids (0.86 [0.79–0.93]).

Conclusions: Our data indicate differences in clinical and demographic relationships of apoC-III proteoforms, and highlight the importance of apoC-III proteoform composition in predicting future lipid patterns and CVD risk.

Graphical Abstract:



Introduction

Apolipoprotein C-III (ApoC-III) is a major apolipoprotein attached to triglyceride-rich lipoproteins (TRLs), remnant cholesterol particles and is also present in LDL and HDL.[1] ApoC-III is a key regulator of lipid metabolism, which increases TRLs levels by inhibiting triglyceride clearance and stimulating VLDL production.[2–6] ApoC-III may also directly modulate endothelial function and was shown to promote monocyte adhesion to endothelial cells.[7,8] Consistent with these metabolic and vascular actions, several studies have demonstrated a strong association of higher total apoC-III levels

in plasma or various lipoprotein particles with increased triglycerides, dyslipidemia, inflammation, atherosclerosis, and cardiovascular disease (CVD).[9–16] Conversely, loss of function mutations in *APOC3* are associated with decreases in plasma triglyceride levels, coronary atherosclerosis and CVD risk.[17–19] Inhibition of apoC-III production by antisense oligonucleotides reduced plasma triglycerides and improved dyslipidemia.[20,21]

In circulation, apoC-III appears in four major proteoforms, a native peptide ($C\text{-}III_{0a}$), and O-glycosylated (on Threonine 74) proteoforms containing zero ($C\text{-}III_{0b}$), one ($C\text{-}III_1$) or two ($C\text{-}III_2$) sialic acids.[22,23] In preclinical studies, apoC-III_2 showed greater affinity to VLDL, but less potent inhibition of triglyceride clearance than apoC-III_1.[24,25] ApoC-III complexes with higher relative $C\text{-}III_2$ content were less potent inhibitors of LPL-mediated lipolysis and hepatocyte VLDL uptake.[26] In endothelial cells, sialylation was shown necessary for the proinflammatory effect of apoC-III.[27] In humans, plasma concentrations and production rates of sialylated apoC-IIIs showed stronger association with plasma triglycerides than non-sialylated proteoforms.[27–29] Higher plasma triglycerides were associated with a greater proportion of $C\text{-}III_1$ and lower percentages of $C\text{-}III_2$ in adolescents with insulin resistance and in adults with prediabetes and type 2 diabetes.[26,30]

To extend these initial cross-sectional findings of apoC-III proteoform associations with dyslipidemia into a more general population and to assess the role of apoC-III proteoform composition in longitudinal changes in lipids and prediction of CVD risk, we utilized samples and data from the Multi-Ethnic Study of Atherosclerosis (MESA). Specifically, we (a) identified key demographic and clinical characteristics affecting apoC-III proteoforms composition; (b) determined the cross-sectional and longitudinal associations of apoC-III proteoform composition with plasma lipids; and (c) tested the association between apoC-III proteoform composition and incident CVD.

Methods

Study design and population

Data used in this study were obtained from the Multi-Ethnic Study of Atherosclerosis (MESA) (https://www.mesa-nhlbi.org) in accordance with their published data access policies, including an approved written proposal. The data that support the findings of this study are available from the corresponding author upon reasonable request.

The MESA study is a multicenter longitudinal study to examine factors associated with subclinical CVD and the progression from subclinical to clinical CVD in individuals aged 45 to 84 years, of non-Hispanic White, African American, Hispanic and Chinese American race/ethnicity, and without known CVD at the enrollment.[31] Institutional review boards at each of the six MESA study sites (Columbia University, New York, NY; Johns Hopkins University, Baltimore, MD; Northwestern University Chicago, IL; University of California at Los Angeles, Los Angeles, CA; University of Minnesota, St. Paul, MN; and Wake Forest University, Winston-Salem, NC) approved the study protocol and informed consent was obtained from all study participants. The present study which used existing MESA data and plasma samples was approved by the Phoenix VA Health Care System Institutional Review Board. MESA clinical exams occurred in 2000 to 2002 (Exam 1), 2002 to 2004

(Exam 2), 2004 to 2006 (Exam 3), 2005 to 2007 (Exam 4), 2010 to 2012 (Exam 5) and 2016 to 2018 (Exam 6). For MESA, and the current study, Exam 1 data was considered baseline. Demographic information, medical history and physical measures were obtained through standardized protocols as described previously.[31] For the present analysis, obesity was defined as BMI ≥ 30 kg/m^2 in White, Hispanic and African Americans, and as BMI ≥ 27.5 kg/m^2 in Chinese Americans. Classification of diabetes was based on a fasting glucose > 6.99 mmol/l or use of hypoglycemic medications. Hypertension was defined as having systolic blood pressure ≥ 140 mmHg or using antihypertensive medications. Blood samples were obtained after a 12-hour fast. Blood biomarkers were measured at the MESA central laboratory at the University of Minnesota.

Outcomes

Outcomes for the present study included plasma triglycerides, and total, LDL and HDL cholesterol collected at each exam, and hard CVD, including definite myocardial infarction, resuscitated cardiac arrest and fatal or non-fatal stroke. Details on cardiovascular events surveillance has been previously reported.[32] Additional details on the MESA study's follow-up methods and event adjudication are available on the MESA web site at http://www.mesa-nhlbi.org. The present analysis includes CVD events reported through end of 2017, i.e., for up to 17 years of follow-up.

ApoC-III proteoform composition

ApoC-III proteoform composition was measured in 5,791 available samples from Exam 1 by mass-spectrometry immunoassay (MSIA).[26] Prior to running the assays, 3 μL of thawed plasma was diluted with 117 μL of PBS, 0.1% Tween (PBST). Then, 40 μL of this diluted plasma was mixed with 120 μL of PBST, yielding 160 μL of analytical sample that was plated onto a 96-well plate. Samples were run in batches of 96; each batch contained 90 analytical samples and 6 quality control samples (two distinct plasma samples aliquoted in triplicate). ApoC-III protein was captured by 250 aspiration and dispensing cycles (100 μL each) of analytical sample using immunoaffinity columns derivatized with anti-apoC-III antibody (Academy Biomedical Co, Houston, TX). Captured apoC-III was then eluted directly onto a 96-well formatted matrix-assisted laser desorption/ionization (MALDI) target using a 5 μL of MALDI matrix solution (33% aqueous acetonitrile and 0.4% trifluoroacetic acid saturated with sinapinic acid). Bruker Autoflex III MALDI-TOF instrument (Bruker, Billerica, MA) was utilized to acquire linear mass spectra from each sample spot. The mass spectra were first externally calibrated with protein calibration standards and then internally calibrated using the highest intensity apoC-III signals. The spectra were baseline subtracted and smoothed using Flex Analysis software (Bruker Daltonics). Areas under the peaks signals were integrated using Zebra 1.0 software (Intrinsic Bioprobes Inc., Tempe, AZ). Percent abundance of each apoC-III proteoform was obtained by dividing individual proteoform peak areas by the integrated peak area of all proteoforms. Mean coefficients of variation for intraassay and between-assay replicates, respectively, were 6.2% and 6.1% for C-III$_{0a}$, 8.2% and 8.2% for C-III$_{0b}$, 1.5% and 1.7% for C-III$_1$, and 3.5% and 3.8% for C-III$_2$. Plasma concentrations of total apoC-III were measured in baseline samples by sandwich ELISA as part of a previous study.[33] Further details on MSIA materials and instruments are in Supplemental methods.

Measurement of plasma lipids

In fasting blood samples from all visits, triglycerides were measured using a glycerol-blanked enzymatic method (Trig/GB; Roche Diagnostics, Indianapolis, IN). Plasma HDL cholesterol was measured by the cholesterol oxidase method (Roche Diagnostics) after precipitation of non–HDL-C magnesium/dextran. In those with triglycerides <4.5 mmol/l (400 mg/dl), LDL cholesterol levels were calculated by the Friedewald equation.

Statistical analyses

Statistical analyses were conducted using SAS (v9.4, SAS Institute, Cary, NC) and R (v3.4.1). P-values <0.05 were considered statistically significant. For statistical analyses, apoC-III proteoforms were expressed as the percentage of each individual proteoform when assessed as univariate variables. When examined in the multivariate models, apoC-III proteoform composition was expressed as additive natural log-ratios (ALR) of less abundant proteoforms to the most abundant C-III$_1$ to allow accurate estimation of relationships among variables that sum to a constant value as occurs with compositional data.[34,35] Covariates were chosen for their known prior association with apoC-III proteoforms or study outcomes.

Pearson correlations were used to describe the associations between total plasma apoC-III concentration and percentages of individual proteoforms. Multiple linear regression was used to test the relationships of percentages of individual apoC-III proteoforms with clinical and demographic characteristics. Models were run unadjusted and then adjusted in a two-stage approach: (1) the associations with non-modifiable demographic characteristics, including age (defined in 10-year increments), gender and race/ethnicity were examined in multivariable models; (2) the associations with modifiable clinical characteristics, including BMI, obesity, fasting plasma glucose, diabetes, use of lipid-lowering medications and kidney function were tested individually in models adjusted for the above-listed demographic characteristics and their significant interactions. The association of overall apoC-III proteoform composition with clinical and demographic characteristics was tested by multivariate linear regression (MANOVA), using ALRs of apoC-III proteoforms to C-III$_1$ as a multivariate outcome variable. Pillai's trace value was calculated to estimate the percent of variance in apoC-III proteoform composition explained by differences in the levels of covariates.

The cross-sectional associations between ALRs of apoC-III proteoforms and plasma lipids were tested by multiple linear regression models before and after adjusting for total apoC-III concentrations, age, gender, race/ethnicity, BMI, diabetes status, fasting glucose, lipid-lowering therapy and eGFR. The associations between ALRs of apoC-III proteoforms and longitudinal changes in plasma lipids were tested by mixed linear regression for repeated measures with random intercept and fixed effect of time, before and after adjusting for age, gender, race/ethnicity, and baseline and follow-up BMI, diabetes status, fasting glucose, lipid-lowering therapy, tobacco use and eGFR. In secondary analyses, we also tested the cross-sectional and longitudinal associations of plasma lipids with percentages of individual proteoforms.

To evaluate the association between apoC-III measures and CVD risk, we first estimated Kaplan-Meier curves for the survival time to the first event stratified by lowest and highest quartiles of baseline total apoC-III concentrations and ALRs of apoC-III proteoforms to C-III$_1$. Cox proportional hazard regression was used to assess the association between ALRs of apoC-III proteoforms to C-III$_1$ and incident CVD events. Proportional hazard assumptions were confirmed by inspecting Kaplan-Meier curves and calculating Schoenfeld residuals. These analyses were run adjusted for total apoC-III only (Model 1), and then adjusted for age, gender, race/ethnicity, smoking status, BMI, diabetes, systolic blood pressure, eGFR, and use of antihypertensive and lipid lowering medications (Model 2), and further adjusted for plasma triglycerides and HDL cholesterol levels (Model 3). To determine if the relationship of apoC-III proteoform composition to CVD risk was similar in different age, gender, and race or ethnic groups, we modelled the interaction of these variables with ALRs of apoC-III proteoforms to C-III$_1$. We also tested the associations of CVD risk with percentages of individual proteoforms. All continuous variables in the regression models were natural log transformed and scaled to a mean of zero and standard deviation of one.

Concordance statistics (C-index), category-free net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were calculated to quantify improvement in 10-year CVD risk prediction after adding apoC-III proteoforms measures (based on 10-year follow-up survival estimates) to the Pooled Cohort Equation (PCE) estimator of CVD risk in asymptomatic adults.[36] The confidence intervals were computed using 100 bootstrap samples.

Results

The final sample for this analysis included 5,790 participants after exclusion of one sample due to excess oxidation. Participants included nearly equal numbers of men and women and were racially and ethnically diverse, with relatively low rates of diabetes, and, on average, normal kidney function and near optimal plasma lipids (Table 1). Although nearly 50% had a history of hypertension, mean blood pressure levels were on average well within normal ranges. At baseline, 17% of participants were on lipid-lowering medications which was primarily statins (91% of the time).

C-III$_1$ was the most abundant proteoform (median, 60% of total peak area), followed by C-III$_2$ (21 %), C-III$_{0b}$ (12 %) and C-III$_{0a}$ (7 %)(Figure S1). Percentages of all proteoforms correlated only modestly to moderately with total apoC-III concentration; inversely for C-III$_2$ ($r= -0.30$) and positively for other proteoforms ($r= 0.07$, C-III$_{0a}$; $r= 0.14$, C-III$_{0b}$ and $r= 0.25$, C-III$_1$) (all $p<0.0001$, Table S1).

The percentages of apoC-III proteoforms were associated with all tested clinical and demographic characteristics in the unadjusted models (Table S2). In adjusted models, we first examined the relationship of apoC-III proteoform composition with non-modifiable demographic characteristics (Table 2). C-III$_2$ was higher in older participants; C-III$_{0b}$ and C-III$_1$ were higher in women; and compared with Whites, C-III$_2$ was higher in Blacks, C-III$_{0a}$ and C-III$_1$ were higher in Hispanics and C-III$_{0a}$ and C-III$_2$ were higher in Chinese. As indicated by Pillai's trace values, 9% of the variance in the apoC-III proteoform composition

in the model was explained by age, 6% by gender and 18% by race/ethnicity (Table 2). The association of apoC-III proteoform composition with both age and race/ethnicity differed by gender ($p<0.0001$ for interaction, Figure S2). Additionally, among women in the age group of 45 to 54 years with known menopausal status, those who had gone through menopause had significantly lower C-III$_2$ and higher C-III$_1$ (Figure S3).

Next, we tested the relationship of apoC-III proteoform composition with clinical variables. After adjustment for age, gender, race/ethnicity and *total* apoC-III concentrations, apoC-III proteoforms were associated with BMI, obesity, fasting plasma glucose and/or diabetes status, tobacco use, use of lipid lowering therapy and eGFR (Table 3). C-III$_{0a}$ and C-III$_2$ were lower while C-III$_{0b}$ and C-III$_1$ were higher in those with higher BMI and obesity, and in those with higher fasting glucose and/or diabetes. C-III$_2$ was higher and all other proteoforms were lower in those receiving lipid lowering therapy. C-III$_2$ was also higher in current tobacco users and those with worse kidney function.

The number of participants with available lipid measurements at each exam is shown in Table S3. In baseline cross-sectional models including total apoC-III and all ALRs of apoC-III proteoforms to C-III$_1$, plasma triglycerides were positively associated with total apoC-III and negatively associated with C-III$_2$/C-III$_1$ (Figure 1A). In longitudinal models (also including all apoC-III measures), follow-up triglyceride levels (adjusted for baseline levels) were positively associated with total apoC-III and negatively associated with all proteoform ratios, with the largest negative estimate for C-III$_2$/C-III$_1$ (Figure 1B). In cross-sectional models, total cholesterol was positively associated with total apoC-III and negatively associated with C-III$_2$/C-III$_1$ (Figure 1C). In longitudinal models, total cholesterol was positively associated with C-III$_2$/C-III$_1$ (Figure 1D). LDL cholesterol was positively associated with total apoC-III and negatively associated with C-III$_2$/C-III$_1$ in cross-sectional models. In longitudinal models, LDL cholesterol was positively associated with total apoC-III after adjustment, and was negatively associated with C-III$_{0a}$/C-III$_1$ (Figures 1E and F). In cross-sectional analyses, HDL cholesterol was positively associated with total apoC-III, C-III$_{0b}$/C-III$_1$ and C-III$_2$/C-III$_1$ (Figure 1G). In longitudinal analyses, HDL cholesterol was negatively associated with total apoC-III, and positively associated with C-III$_{0a}$/C-III$_1$ and C-III$_2$/C-III$_1$ (Figure 1H). In analyses of individual apoC-III proteoform percentages, higher relative amounts of C-III$_1$ and lower C-III$_2$ were associated with higher triglycerides and lower HDL in both cross-sectional and longitudinal models (Figure S4).

We also tested whether the associations of clinical and demographic characteristics with plasma triglycerides and HDL-cholesterol were influenced by adjustment for total apoC-III and apoC-III proteoform ALRs (Table 4). The negative association of age with plasma triglycerides was nullified after adjustment for total apoC-III and became positive after further adjustment for proteoform ALRs. The negative association of Black race with plasma triglycerides was weaker after adjustment for total apoC-III and further attenuated upon adjustment for proteoform ALRs. Higher plasma HDL levels persisted in Black participants and were unchanged after adjustment for total apoC-III, but this HDL elevation was reduced after further adjustment for proteoform ALRs. Adjustment for proteoform ALRs also attenuated the positive association of BMI with plasma triglycerides.

A total of 669 participants developed a CVD event out of 5,766 with available surveillance data through the end of 2017 (median time to an event 8.5 years). Kaplan-Meier curves showed significant differences in CVD risk between those with high and low C-III_{0b}/C-III_1 and C-III_2/C-III_1, while there were no significant differences between those with high and low total apoC-III and C-III_{0a}/C-III_1 (Figure 2A). In Cox regression models, C-III_{0b}/C-III_1 was inversely associated with CVD risk in the minimally adjusted model (containing all proteoform ALRs and total apoC-III) and after adjustments for baseline clinical and demographic characteristics, plasma triglycerides and HDL cholesterol (Figure 2B). Higher C-III_2/C-III_1 was also associated with higher CVD risk in the minimally adjusted model, however the association was attenuated after further adjustment for clinical characteristics (Figure 2B). These associations of apoC-III proteoforms with CVD were similar between different demographic subgroups, except for a weaker association of C-III_{0b}/C-III_1 with CVD in Whites (HR 0.88 [95% CI 0.77–1.001], p=0.002 for interaction) (Table S4). In analyses of individual apoC-III proteoform percentages, higher CVD risk was associated with lower C-III_{0b} and higher C-III_2 in all models (Figure S5).

The significant inverse association between C-III_{0b}/C-III_1 and CVD was also observed for the 10-year CVD risk (Figure S6). Addition of total apoC-III and proteoform ALRs to the 10-year PCE estimator did not change the C-index but improved NRI, i.e., the proportion of individuals correctly upgraded or downgraded in their eventual risk by 24%, and relative IDI, i.e., the difference between slopes of prediction curves by 16% for CVD risk ($p<0.0001$ for both) (Table S5).

Discussion

The present analyses revealed relationships of apoC-III proteoform composition with multiple demographic and clinical characteristics, including age, gender, race/ethnicity, obesity and fasting glucose status, cross-sectional and longitudinal changes in plasma lipids as well as incident CVD events. Most notably, C-III_2 levels were higher in older individuals, in males and in Black and Chinese participants. Higher C-III_2 levels were also associated with leaner phenotype and lower fasting glucose levels, more favorable plasma lipid profiles but not with future CVD events. CVD risk was, however, inversely related to baseline C-III_{0b}, a proteoform that showed a relative weak association with typical clinical and demographic determinants of CVD risk, including plasma lipids. Importantly, all of these associations were independent of total plasma apoC-III levels, suggesting posttranslational apoC-III proteoforms may be influenced by clinical characteristics and may have distinct roles in regulation of plasma lipids levels and future CVD risk.

The cross-sectional associations between apoC-III proteoforms and plasma triglycerides shown in this community-based setting are consistent with previous observations in smaller cohorts comprised of individuals with various degrees of impaired glucose regulation.[26,30,37] The novel finding of an inverse association between baseline C-III_2/C-III_1 and longitudinal changes in plasma lipids supports a direct role for the type of apoC-III sialylation in long-term regulation of triglyceride metabolism. The inverse associations of C-III_2/C-III_1 with plasma triglycerides was paralleled by a positive association between C-III_2/C-III_1 and HDL cholesterol. Although this could be explained by increased cholesterol transfer from VLDL

to HDL due to improved triglyceride clearance with this apoC-III proteoform pattern,[38] further studies are needed to test the possibility of direct effects of apoC-III sialylation on HDL metabolism.

ApoC-III proteoform composition was also associated with several other metabolic characteristics. C-III_2 and C-III_{0a} were lower while C-III_1 and C-III_{0b} were higher in obese participants. Recently, Mendoza et al. demonstrated that three years of dietary weight-loss intervention was associated with significant reductions in plasma concentrations of total apoC-III and all apoC-III proteoforms except for C-III_2.[39] In that study, C-III_2 concentrations trended higher after the intervention, indicating even greater, and presumably significant increases in the *relative* C-III_2 amounts. In addition to their relationships with obesity, C-III_1 was higher while C-III_2 was lower in participants with higher fasting glucose in our study. In the liver, lipoproteins with higher amounts of C-III_2 are cleared primarily by heparan sulfate proteoglycans (HSPGs), whereas lipoproteins containing more C-III_1 are preferentially cleared by LDL-receptor (LDL-R) and LDL-R related protein 1 (LRP1).[40] The translocation of LRP1 from intracellular vesicles to the plasma membrane depends on intact insulin action.[41] Thus, the membrane LRP1 content may be reduced in insulin resistance, potentially leading to retention of C-III_1 enriched lipoproteins in circulation. Further supporting the role of insulin resistance in altering apoC-III proteoform composition, treatment with insulin-sensitizing drug pioglitazone increased C-III_2 and reduced C-III_1 in persons with prediabetes.[26] Altogether these data indicate that altered apoC-III proteoform composition may help explain the link between insulin resistance and obesity with high triglycerides and low HDL levels.

The finding of higher C-III_2 levels in Black participants is consistent with previous observation from a smaller clinical trial cohort of individuals with prediabetes.[26] Significant differences in composition of apoC-III proteoforms between Whites and Hispanics or Chinese provide additional evidence for a broader role of racial and ethnic background in posttranslational apoC-III processing. Moreover, our analyses indicate that higher C-III_2 may be a key factor explaining lower triglyceride and higher HDL cholesterol levels in Blacks. Here we show for the first time that apoC-III proteoforms also differ by age and gender and that higher apoC-III_2 may in part underly the previously shown decline in triglycerides in elderly.[42] We have also observed that premenopausal women had even higher C-III_2 and lower C-III_1 compared with postmenopausal women, suggesting involvement of sex hormones in controlling apoC-III proteoforms composition.

Our analyses demonstrated a strong inverse association of CVD risk with C-III_{0b}, which did not show a particularly strong relationship with typical CVD risk factors, including plasma lipid levels. In a recent report, however, higher C-III_{0b} when transported in LDL particles was associated with a less proatherogenic phenotype characterized by larger LDL size and a lower lipoprotein-insulin resistance score.[43] C-III_{0b} did not show an association with incident CVD in our previous analyses of a subcohort of Veterans Affairs Diabetes Trial (VADT) [26]; however, that was a far smaller, predominantly White and much higher CVD risk cohort. Of note, as suggested by the present data, the association between C-III_{0b} and CVD appears weaker in Whites.

Lower C-III_{0b} may indicate reduced glycosylation of its precursor C-III_{0a} and/or increased downstream formation of sialylated apoC-III proteoforms. Greater methylation, i.e., epigenetic modification typically suppressing gene expression, of the N-Acetylgalactosaminyltransferase 2 (*GALNT2*) gene catalyzing O-glycosylation of C-III_{0a} (which in turn may decrease formation of C-III_{0b}) is associated with increased risk of coronary heart disease.[44] Loss of function mutations in *GALNT2* are also associated with lower HDL cholesterol levels.[45] Nevertheless, the association between C-III_{0b} and CVD risk remained significant even after adjustment for both plasma triglycerides and HDL cholesterol, indicating involvement of more complex facets of lipid metabolism, beyond standard plasma lipid levels, and potential lipid-unrelated mechanisms.

Higher C-III_2/C-III_1, indicating increased sialylation, was associated with increased CVD risk in the minimally adjusted model. However, the association was attenuated after adjustment for clinical and demographic risk factors. Given the strong positive association of C-III_2 with age, male gender and Black race, the apparent CVD risk associated with increased C-III_2/C-III_1 may be secondary to these demographic factors. However, the positive association between the individual percentage of C-III_2 and CVD in the fully adjusted model suggests a direct adverse effect of increased apoC-III sialylation in the cardiovascular system. In preclinical studies, sialic acid triggered myocardial injury and promoted coagulation while sialylation was required for proinflammatory action of apoC-III in endothelial cells.[27,46,47] In humans, both CVD or thrombotic events were positively associated with sialic acid levels.[48,49]

There is increasing awareness of the complex relationships between apolipoproteins, lipid levels and CVD risk. Recent mass spectrometry analysis using samples from several clinical trials demonstrated that levels of several apolipoproteins including apoC-III, apoC-I, apoE and apoB were associated with a 2–3 fold higher risk of coronary heart disease after adjustment for traditional clinical risk factors, including plasma lipids.[50] Our data show that apoC-III proteoform composition may account for some of the additional variation in both lipid levels and CVD risk. Moreover, apoC-III proteoform levels differed substantially between gender and racial/ethnic groups and accounted for some of their differences in plasma lipid levels. Thus, measurement of apolipoprotein modifications may further improve personalized CVD risk evaluation.

Besides the prognostic value for future plasma lipids and CVD risk, assessment of apoC-III proteoform composition may have potential therapeutic implications. Inhibition of apoC-III expression with the first-generation antisense oligonucleotide volanesorsen in individuals with hypertriglyceridemia was associated with profound increases in C-III_2/C-III_1.[40] It is possible that this may in part explain the triglyceride-lowering action of volanesorsen. On the other hand, the increase in relative amount of C-III_2 may warrant some caution regarding the long-term CVD effects of this type of lipid-lowering therapy.

A major strength of the present study was the measurement of apoC-III proteoform composition in a large demographically diverse and systematically observed longitudinal cohort. The cohort size allowed robust statistical modeling with adjustment for many relevant covariates as well as analyses in several subgroups. For almost all participants

we were able to match apoC-III mass spectrometry results with total plasma concentrations measured previously by enzymatic assay.[14] Similar to the previous report spanning 11 years of follow-up,[13] the association between total apoC-III levels in plasma and incident CVD over 17-year follow period was abolished once adjusted for clinical and demographic covariates. By including apoC-III proteoforms measures with total apoC-III in additive models we were able to demonstrate their independence and added prognostic value to plasma lipids and CVD risk.

Although the cohort size and comprehensive phenotyping permitted robust statistical modeling, the study conclusions are based on association analyses. Many of the novel relationships need to be examined by more direct mechanistic models to confirm causality and to identify underlying pathways. Previous studies have showed that the associations of apoC-III with atherosclerosis depend on the type of lipoproteins carrying apoC-III.[14,51] In fact, apoC-III carried on HDL was shown to be a better predictor of both subclinical and clinical coronary atherosclerosis than total apoC-III in plasma.[13,14] We were unable to ascertain whether the associations between apoC-III proteoform composition and study outcomes differ by lipoprotein species. However, according to a recent report, the distribution of apoC-III proteoforms is relatively uniform across different lipoprotein species.[43] Thus, the measurement of apoC-III proteoforms in whole plasma appears to be a valid overall indicator of their distribution in distinct lipoproteins.

In summary, our results suggest that apoC-III proteoforms may provide additional information beyond that indicated by total apo-CIII measurements. Measuring apoC-III proteoforms may be a useful prognostic tool for future lipid patterns and cardiovascular risk. Greater understanding of the factors regulating their concentrations and composition may not only provide new insights into lipid metabolism and differences in CVD risk, but may in turn lead to development of new therapeutic strategies for dyslipidemia and prevention of CVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms:

ALR	additive log-ratio
apoC-III	apolipoprotein C-III
CVD	cardiovascular disease
HR	hazard ratio
LDL-R	LDL receptor
LRP1	LDL receptor related protein 1
MESA	Multi-Ethnic Study of Atherosclerosis
MSIA	mass spectrometry immunoassay
TRL	triglyceride-rich lipoproteins

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Highlights

- ApoC-III proteoform composition was associated with age, sex and race/ethnicity
- Higher relative amount of disialylated apoC-III (C-III_2) was associated with more favorable cardiometabolic profile, including lower BMI, lower fasting plasma glucose and triglycerides levels, and higher plasma HDL cholesterol levels
- Increased relative amount of glycosylated and non-sialylated apoC-III (C-III_{0b}) was associated with reduced cardiovascular risk
- The relationship of apoC-III proteoform composition with clinical and demographic characteristics, and cardiovascular risk was independent of total apoC-III concentrations

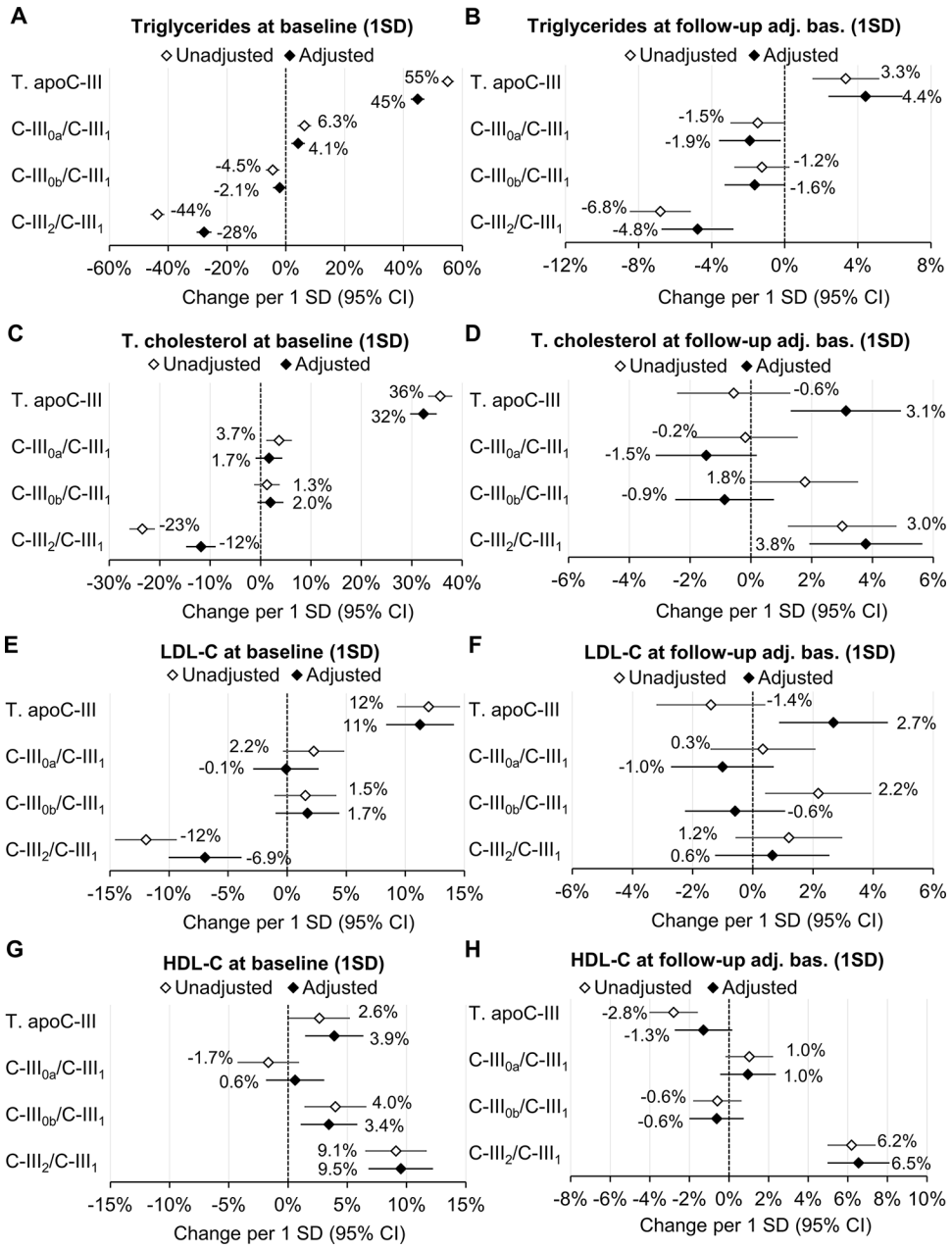

Figure 1.

Cross-sectional (baseline; left panels) and longitudinal (follow-up adjusted for baseline and time of follow-up; right panels) relationships of baseline total apoC-III concentrations, and log-ratios of apoC-III proteoforms to C-III$_1$ (all included in the same additive model) with plasma lipids. Multiple linear regression models were run unadjusted (total apoC-III and proteoforms ratios in separate models) and adjusted (total apoC-III and proteoforms ratios in the same model) for baseline age, gender, race/ethnicity, BMI, diabetes status, fasting glucose, tobacco use, lipid-lowering therapy and eGFR. Longitudinal mixed regression models for repeated measures were further adjusted for BMI, diabetes status, fasting glucose, tobacco use, lipid-lowering therapy and eGFR at each follow-up exam. Symbols

and labels are β-estimates. All 95% CI not crossing the zero-x-axis value are consistent with $p<0.05$. All apoC-III and lipid measures were natural log-transformed and scaled to 1 SD, e.g., an increase of 1 SD in $C\text{-}III_2/C\text{-}III_1$ (adjusted model) was associated with reductions of 28% and 4.5% of 1 SD in baseline and follow-up plasma triglycerides, respectively.

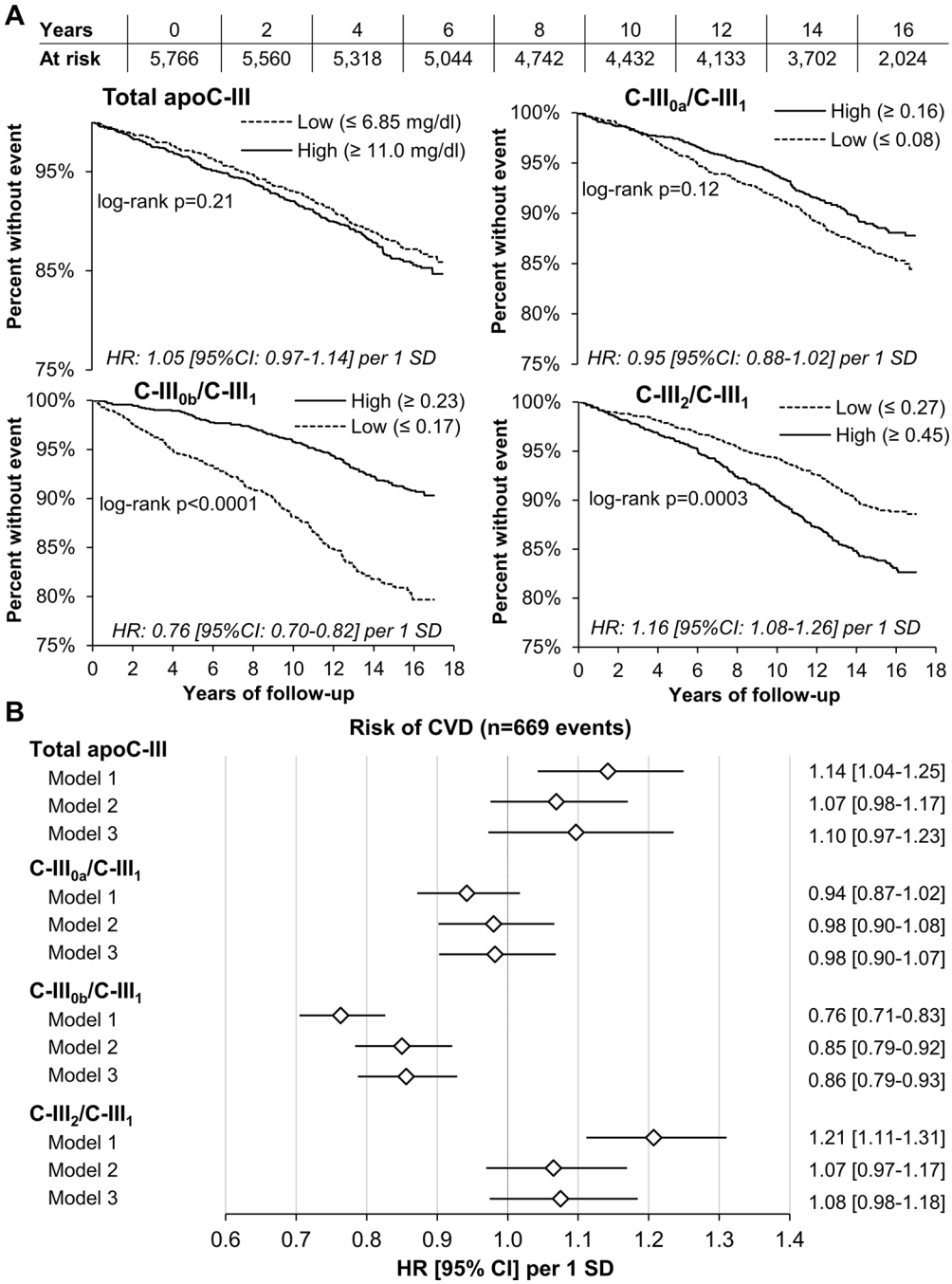

Figure 2. Association between baseline total apoC-III concentrations and log-ratios of apoC-III proteoforms to apoC-III$_1$ (most abundant), and CVD events.

Panel A: Kaplan-Meier curves of top (High) and bottom (Low) quartile of apoC-III measures for entire follow-up period. Unadjusted Cox proportional hazard ratios (HR) and 95% confidence intervals (95% CI) per 1 SD of apoC-III measures are listed in each figure. **Panel B:** Cox proportional risk models of CVD risk. Model 1 included total apoC-III and apoC-III proteoforms ratios; Model 2 also included baseline age, gender, race/ethnicity, BMI, diabetes, systolic blood pressure, use of antihypertensive and lipid lowering

medications, smoking status and eGFR, and Model 3 was further adjusted for plasma triglycerides and HDL cholesterol. All apoC-III measures were natural log-transformed and expressed per 1 SD unit.

Table 1:

Clinical and demographic characteristics of the cohort at baseline.

Variable	n	Mean ± SD or %
Age (Years)	5,790	63 ± 10
Non-Hispanic Whites	2,152	37%
Blacks	1,670	29%
Hispanics	1,264	22%
Chinese	704	12%
Women	3,017	52%
Former tobacco use	2136	37%
Current tobacco use	723	13%
BMI (kg/m^2)	5.790	28.3 ± 5.5
Obesity	1,916	33%
Hypertension	2,634	46%
Antihypertensive medication use	2,190	38%
Systolic Blood Pressure (mmHg)	5,788	127 ± 22
Diastolic Blood Pressure (mmHg)	5,788	72 ± 10
Fasting glucose (mmol/l)	5,780	5.44 ± 1.72
Diabetes	744	13%
Diabetes medication use	574	10%
Triglycerides (mmol/l)	5,783	1.48 ± 0.90
Total cholesterol (mmol/l)	5,783	5.02 ± 0.93
HDL-cholesterol (mmol/l)	5,780	1.32 ± 0.39
LDL-cholesterol (mmol/l)	5,710	3.03 ± 0.83
Lipid-lowering therapy use	973	17%
Statins	891	15%
Fibrates	64	1.1%
eGFR (ml/min/1.73 m^2)	5,780	89 ± 21
Total apoC-III (mg/dl)	5,784	9.4 ± 4.1

Table 2.

ApoC-III proteoforms by age, gender and race/ethnicity.

Variable	N	$C\text{-}III_{0a}$		$C\text{-}III_{0b}$		$C\text{-}III_{1}$		$C\text{-}III_{2}$	
		% peak area Mean ± SD	% SD change β [95% CI]	% peak area Mean ± SD	% SD change β [95% CI]	% peak area Mean ± SD	% SD change β [95% CI]	% peak area Mean ± SD	% SD change β [95% CI]
Age group	*Pillai's Trace = 0.09, p<0.001*								
45–54 years	1,560	7.6 ± 3.6	0	13 ± 2.6	0	60 ± 5.2	0	20 ± 6.1	0
55–64 years	1,562	7.7 ± 3.7	0.3 [−6.5, 7.1]	12 ± 2.6	−6.6 [−13, −0.1]	60 ± 5.2	2.3 [−4.3, 8.9]	20 ± 6.1	1.0 [−5.3, 7.5]
65–74 years	1,785	7.4 ± 3.5	−6.8 [−13, −0.3]	11 ± 2.6	−42 [−48, −35]	59 ± 5.4	−10 [−17, −4.1]	22 ± 6.5	30 [24, 37]
75–84 years	883	7.2 ± 3.5	−12 [−19, −3.8]	10 ± 2.4	−78 [−86, −71]	58 ± 5.8	−25 [−33, −17]	24 ± 7.1	57 [50, 65]
Gender	*Pillai's Trace = 0.06, p<0.001*								
Men	2,773	7.8 ± 3.7	0	11 ± 2.6	0	58 ± 5.2	0	23 ± 6.6	0
Women	3,017	7.2 ± 3.4	−17 [−22, −12]	12 ± 2.7	25 [20, 29]	60 ± 5.3	40 [36, 45]	20 ± 6.3	−37 [−41, −32]
Race/ethnicity	*Pillai's Trace = 0.18, p<0.001*								
White	2,152	7.1 ± 3.5	0	12 ± 2.7	0	60 ± 5.2	0	20 ± 6.2	0
Black	1,670	6.6 ± 3.0	−14 [−20, −7.7]	11 ± 2.6	−48 [−54, −42]	58 ± 5.6	−44 [−50, −38]	24 ± 6.7	65 [59, 71]
Hispanic	1,264	8.2 ± 3.6	31 [24, 38]	12 ± 2.6	−15 [−21, −8.1]	61 ± 4.7	7.9 [1.4, 14]	19 ± 5.5	−16 [−22, −9.9]
Chinese	704	9.6 ± 4.0	64 [56, 73]	11 ± 2.6	−42 [−51, −35]	57 ± 5.0	−58 [−66, −50]	22 ± 6.1	32 [24, 39]

Data are means ± SD, and percent difference in apoC-III proteoforms (scaled to 1 SD of natural log-transformed values) by each demographic variable compared with the reference group (indicated as "0"). Multiple linear regression was used to test the association of each individual apoC-III proteoform with age, gender and race/ethnicity (all included in the same multivariable model). For example, $C\text{-}III_2$ was higher in Blacks compared to Whites by 65% of 1 SD. Pillai's Trace values indicate the variance in overall apoC-III proteoform composition (defined as additive log-ratios of apoC-III proteoforms to $C\text{-}III_1$) explained by differences in the levels of the demographic variables tested by multivariate analysis of variance (MANOVA). For example, race/ethnicity accounted for 0.18, i.e., 18% of the variance in proteoform composition. All 95% CI not crossing zero value are consistent with $p<0.05$.

Table 3:

Relationship of key clinical characteristics with apoC-III proteoform composition.

Variable	$C\text{-}III_{0a}$ (% SD)	*P-value*	$C\text{-}III_{0b}$ (% SD)	*P-value*	$C\text{-}III_{1}$ (% SD)	*P-value*	$C\text{-}III_{2}$ (% SD)	*P-value*	*Pillai's trace*
BMI (1 SD)	−10 [−13, −7.7]	*<0.001*	12 [9.4, 15]	*<0.001*	27 [25, 30]	*<0.001*	−22 [−24, −19]	*<0.001*	*0.10*
Obesity	−17 [−22, −11]	*<0.001*	14 [8.7, 20]	*<0.001*	40 [35, 45]	*<0.001*	−31 [−36, −26]	*<0.001*	*0.05*
Fasting glucose (1 SD)	−13 [−16, −11]	*<0.001*	2.7 [0.2, 5.2]	*0.034*	17 [15, 20]	*<0.001*	−8.2 [−11, −5.8]	*<0.001*	*0.03*
Diabetes	−45 [−52, −37]	*<0.001*	6.1 [−1.2, 13]	*0.10*	21 [14, 28]	*<0.001*	−0.1 [−7.0, 6.9]	*0.96*	*0.02*
Lipid lowering therapy	−33 [−40, −26]	*<0.001*	−9.4 [−16, −2.9]	*0.004*	−7.0 [−13, −0.5]	*0.034*	24 [18, 30]	*<0.001*	*0.02*
Tobacco use									*0.003*
Former (vs. never)	2.0 [−3.6, 7.7]	*0.47*	−1.6 [−7.0, 3.8]	*0.56*	2.8 [−2.6, 8.2]	*0.31*	−1.2 [−6.3, 3.9]	*0.64*	
Current (vs. never)	−0.7 [−8.7, 7.4]	*0.87*	−9.2 [−17, −1.5]	*0.020*	−15 [−23, −7.2]	*<0.001*	17 [9.6, 24]	*<0.001*	
eGFR (1 SD)	3.2 [0.5, 5.9]	*0.018*	3.5 [0.9, 6.1]	*0.008*	9.3 [6.8, 12]	*<0.001*	−10 [−13, −7.8]	*<0.001*	*0.007*

Data are β-estimates [95% CI] of percent difference in 1 SD of natural log-transformed of each apoC-III proteoform for each category, or 1 SD of natural log-transformed continuous variables. Multiple linear regression models were adjusted for age, gender and race/ethnicity, interaction terms of age and race/ethnicity with gender, and total apoC-III concentrations. For example, after these adjustments, an increase of 1 SD in BMI was associated with an increase of 27% of 1 SD in relative amounts of $C\text{-}III_1$. Pillai's trace values indicate the variance in overall apoC-III proteoform composition (tested as additive log-ratios to $C\text{-}III_1$) explained by differences in the levels of the clinical characteristics, assessed by multivariate analysis of variance (MANOVA).

Table 4.

Relationship of clinical and demographic characteristics with plasma triglycerides and HDL cholesterol before (Model 1) and after adjustment for total apoC-III (Model 2) and log-ratios of apoC-III proteoforms to C-III1 (additive model, Model 3).

Characteristic	Triglycerides (% SD)			HDL-cholesterol (% SD)		
	Model 1	*Model 2*	*Model 3*	*Model 1*	*Model 2*	*Model 3*
Age (1 SD)	−3.3 [−5.9, −0.8]	−0.7 [−2.9, 1.5]	2.4 [0.3, 4.6]	7.4 [4.9, 9.8]	7.5 [5.0, 9.9]	7.0 [4.5, 9.5]
Women	−4.0 [−8.9, 0.9]	−19 [−23, −14]	−27 [−31, −23]	80 [75, 84]	79 [74, 84]	82 [78, 87]
Blacks (vs. Whites)	−53 [−59, −46]	−29 [−35, −24]	−11 [−16, −5.8]	16 [9.9, 21]	16 [11, 22]	11 [4.6, 17]
Hispanics (vs. Whites)	21 [15, 28]	23 [18, 29]	21 [16, 27]	−17 [−23, −11]	−17 [−23, −11]	−16 [−23, −10]
Chinese (vs. Whites)	29 [21, 37]	30 [23, 37]	35 [28, 42]	−29 [−36, −21]	−29 [−36, −21]	−31 [−39, −23]
BMI (1 SD)	19 [17, 22]	18 [16, 21]	12 [10, 15]	−26 [−28, −23]	−26 [−28, −23]	−24 [−26, −21]
Fasting glucose (1 SD)	17 [14, 21]	12 [8.9, 15]	8.8 [6.0, 12]	−8.5 [−12, −5.4]	−8.8 [−12, −5.6]	−7.4 [−11, −4.2]
Diabetes	−1.5 [−11, 8.3]	−4.1 [−12, 4.2]	4.3 [−3.6, 12]	−11 [−20, −1.6]	−11 [−20, −1.7]	−14 [−23, −4.6]
Lipid lowering therapy	12 [5.7, 19]	2.4 [−3.1, 7.9]	9.7 [4.4, 15]	−1.7 [−7.7, 4.4]	−2.0 [−8.1, 4.1]	−3.9 [−10, 2.2]
Former tobacco use	−2.6 [−8.0, 2.7]	−4.2 [−8.7, 0.3]	−4.7 [−8.9, −0.4]	8.8 [3.8, 14]	8.8 [3.8, 14]	8.9 [4.0, 14]
Current tobacco use	28 [20, 36]	25 [19, 32]	29 [23, 35]	−21 [−28, −14]	−21 [−28, −14]	−22 [−29, −15]
eGFR (1 SD)	−6.4 [−8.9, −3.8]	−0.3 [−2.5, 1.9]	−3.3 [−5.4, −1.2]	4.0 [1.6, 6.4]	1.8 [−0.5, 4.1]	5.3 [2.8, 7.7]

Data are β-estimates [95% CI] of percent changes of 1 SD of natural log-transformed plasma lipids by clinical category or 1 SD of continuous variables. For example, a 53% reduction (of 1 SD) in plasma triglycerides in Blacks (vs. Whites) was attenuated to a 29% reduction (of 1 SD) in Model 2 and to 11% in Model 3. All 95% CI not crossing zero value are consistent with $p<0.05$.