Serum apolipoproteins and apolipoprotein-defined lipoprotein subclasses: a hypothesis-generating prospective study of cardiovascular events in T1D

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Abstract APOB, APOC3, and APOE and apolipoprotein-defined lipoprotein subclasses (ADLSs; based on qualitative apolipoprotein complement) have been associated with dyslipidemia and CVD. Our main objective was to define associations of serum apolipoproteins and ADLSs with "any CVD" and "major atherosclerotic cardiovascular events" (MACEs) in a prospective study of T1D. Serum apolipoproteins and ADLSs (14 biomarkers in total) were measured in sera (obtained between 1997 and 2000) from a subset ($n = 465$) of the Epidemiology of Diabetes Interventions and Complications cohort. Prospective associations of "any CVD" (myocardial infarction, stroke, confirmed angina, silent myocardial infarction, revascularization, or congestive heart failure) and MACEs (fatal or nonfatal myocardial infarction or stroke), over 5,943 and 6,180 patient-years follow-up, respectively, were investigated using Cox proportional hazards models that were unadjusted and adjusted for risk factors. During 15 years of follow-up, 50 "any CVD" events and 24 MACEs occurred. Nominally significant positive univariate associations with "any CVD" were APOB, APOC3 and its subfractions [heparin precipitate, heparin-soluble (HS)], and ADLS-defined Lp-B. In adjusted analyses, APOC3-HS remained nominally significant. Nominally significant positive univariate associations with MACEs were APOC3 and its subfractions and Lp-B:C; those with total APOC3 and APOC3-HS persisted in adjusted analyses. However, these associations did not reach significance after adjusting for multiple testing. There were no significant associations of APOA1, APOA2, APOE, or other ADLSs with either "any CVD" or MACEs. These hypothesis-generating data suggest that total serum APOC3 and APOC3 in HDL are potentially important predictive biomarkers for any CVD and MACEs in adults with T1D.—Basu, A., I. Bebu, A. J. Jenkins, J. A. Stoner, Y. Zhang, R. L. Klein, M. F. Lopes-Virella, W. T. Garvey, M. J. Budoff, T. J. Lyons, and the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. **Serum apolipoproteins and apolipoprotein-defined lipoprotein subclasses: a hypothesis-generating prospective study of cardiovascular events in type 1 diabetes.** ***J. Lipid Res.*** **2019.** 60: **1432–1439.**

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Abbreviations: ADLS, apolipoprotein-defined lipoprotein subclass; AER, albumin excretion rate; DCCT, Diabetes Control and Complications Trial; EDIC, Epidemiology of Diabetes Interventions and Complications; HP, heparin precipitate; HS, heparin-soluble; IMT, intima-media thickness; LDL-C, LDL cholesterol; MACE, major adverse cardiac event; MUSC, Medical University of South Carolina; SBP, systolic blood pressure; TG, triacylglycerol.

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The incidence and prevalence of T1D is increasing globally, and despite modern management of associated risk factors, remains associated with greatly increased morbidity and mortality from CVD (1–3). Serum lipids and apolipoprotein concentrations are important biomarkers of CVD, and several studies have identified the clinical utility of serum apolipoprotein levels and measurements of apolipoprotein-defined lipoprotein subclasses (ADLSs) in predicting vascular complications of T2D (4–8), complementing known associations of CVD with conventional lipids; however, such data are limited in the T1D population.

In studies of apolipoprotein concentrations in plasma in nondiabetic cohorts, APOB has been positively associated with CVD events (9, 10). Similar associations have been observed with APOC3, which is involved in the transport and catabolism of triacylglycerols (TGs) (11). The distribution of APOC3 between APOB- and non-APOB-containing particles can be elucidated by heparin precipitation. Thus, APOC3 in APOB-containing lipoproteins can be precipitated using heparin (APOC3-HP), whereas that in APOA1-containing lipoproteins remains in the supernatant, i.e., is heparin-soluble (APOC3-HS). A higher APOC3-HS:APOC3-HP ratio (APOC3 ratio) is considered a useful index of the peripheral catabolism of TG-rich lipoproteins (6). Serum APOE is less clearly associated with atherogenic risk (12): it enhances the uptake of TG-rich particles by remnant receptors primarily in the liver (13). APOB-containing lipoproteins include LDL, IDL, and VLDL, whereas APOA1-containing lipoproteins are predominantly associated with HDL.

ADLS analysis relates particle function and metabolism: it defines particles according to their qualitative apolipoprotein complement, and the subclass names reflect the apolipoproteins present on the particle (14). ADLS thus provides information about the distribution of apolipoproteins among particle classes, in contrast to the "simple" quantification of apolipoproteins in whole plasma that lacks this information. Based on the ADLS nomenclature, lipoproteins can be categorized into two families: the APOA1 family overlaps with HDL, includes two subclasses (Lp-A1, Lp-A1:A2), and usually has antiatherogenic potential; the APOB family includes VLDL, IDL, and LDL and five subclasses (Lp-B, Lp-B:E, Lp-B:C, Lp-B:C:E, and Lp-A2:B:C:D:E) and generally has proatherogenic effects (15). The Monitored Atherosclerosis Regression Study and the Cholesterol Lowering Atherosclerosis Study demonstrated that high levels of Lp-B and Lp-B:C particles predict coronary events even when adjusted for conventional lipid profiles (16, 17). Prospective data on the associations of serum apolipoproteins and ADLS with CVD events in T1D are lacking and are the subject of our current analysis.

In 1993, the Diabetes Control and Complications Trial (DCCT; 1983–1993) demonstrated that intensive diabetes management for an average of 6.5 years, with an emphasis on glycemic control, dramatically reduced the onset and progression of the microvascular complications of diabetes (18). Few CVD events had occurred by the time DCCT ended in 1993 because the study cohort was still young (mean ± SD: 34 ± 7 years) and with a relatively short duration of T1D (mean ± SD: 12 ± 4 years) (19). Furthermore, the risk of early events was reduced by the original DCCT entry criteria, which mandated an absence of CVD events or CVD risk factors such as hypertension and hyperlipidemia. In the continuing follow-up phase of DCCT, the Epidemiology of Diabetes Interventions and Complications (EDIC) study, implemented in 1994, HbA1c levels converged and became almost identical between the former DCCT randomization groups, but despite this, over the ensuing years carotid intima-media thickness (IMT) progressed more slowly in the former intensive versus "conventional" management group, and there were fewer clinical CVD events (20, 21), a phenomenon called "metabolic memory" (19). We previously reported cross-sectional and some longitudinal associations between novel lipoprotein-related risk factors and carotid IMT in the DCCT/EDIC cohort (5, 22, 23), but the longitudinal associations of these detailed metrics with macrovascular disease events in T1D have not been explored. Here we report longitudinal associations of serum ADLS and individual apolipoproteins with "any CVD" and major adverse cardiac events (MACEs) in this T1D cohort.

METHODS

The DCCT/EDIC study cohort and related methods have been previously described (18). Briefly, the DCCT was a randomized study started in 1983 that was designed to compare the rates of microvascular complications between participants assigned to receive intensive therapy ($n = 711$) aimed at lowering glycemic values to near the nondiabetic range and participants assigned to conventional therapy ($n = 730$) aimed at maintaining clinical well-being with no specific glucose targets. At baseline, the DCCT study cohort consisted of a primary prevention cohort [1–5 years of diabetes duration, no retinopathy based on fundus photography, and albumin excretion rate (AER) <40 mg/24 h] and a secondary intervention cohort (1–15 years of diabetes duration, minimal to moderate nonproliferative retinopathy, and AER <200 mg/24 h). In 1993, at the end of the DCCT, all participants were instructed in intensive therapy and referred back to their healthcare providers; 97% of the surviving DCCT cohort then enrolled in the EDIC study, a follow-up observational study, and 94% of the surviving DCCT participants were still actively participating after 20 years of follow-up in EDIC.

In 1996, a collaboration between the Medical University of South Carolina (MUSC) and DCCT/EDIC was initiated to identify vascular risk factors. In total, 25 of 28 DCCT/EDIC clinical centers participated, and from 1997 to 2000 (EDIC years 4–6), serum samples were shipped overnight on dry ice to MUSC; on arrival, aliquots were promptly prepared and stored at −70°C until analysis. The study, which meets Declaration of Helsinki guidelines, was approved by the MUSC Institutional Review Board and all participating DCCT/EDIC centers. Each participant gave written informed consent. Of the 1,441 DCCT participants, 968 agreed to participate in the MUSC study, but cost and resource considerations precluded the determination of ADLS and apolipoprotein concentrations in all of these participants. The present study therefore utilized a previously described subset ($n = 465$)

(24–26). Briefly, those with abnormal albuminuria (AER >40 mg/24 h), an increased Early Treatment Diabetic Retinopathy Study score (53/<53 or higher), or elevated carotid atherosclerosis (≥25% stenosis at a carotid lesion) were included (i.e., all available cases meeting one or more of these definitions were sampled), together with a larger group of subjects free of all of these complications. The three disease categories (albuminuria, retinopathy, carotid stenosis ≥25%) were combined and reweighted to reflect the demographic and vascular disease status of the entire EDIC cohort at EDIC year 6 (see Statistical Analysis below).

CVD risk factors

DCCT/EDIC study visits included a detailed medical history that included demographic and behavioral risk factors, medical outcomes, and a physical examination that included measurements of height, weight, sitting blood pressure, and pulse rate. Blood samples were collected at each visit and assayed centrally at the DCCT/EDIC Central Biochemistry Laboratory (University of Minnesota) for HbA1c using high-performance ion-exchange liquid chromatography. Fasting lipids [TGs and LDL cholesterol (LDL-C) and HDL cholesterol] were measured annually during DCCT and in alternate years during EDIC and were evaluated centrally (27). Total cholesterol, TG, and HDL cholesterol levels were determined using previously reported enzymatic methods (23). LDL-C was estimated using to the Friedewald equation (27).

Variables were fixed (e.g., sex) or time-dependent (e.g., HbA1c), and the latter were captured either as the current (most recent) value or as the updated mean from baseline. The updated mean is the weighted average of prior values using weights proportional to the time interval between the measurements.

Apolipoprotein and ADLS measurements

We evaluated a subset ($n = 465$) of the DCCT/EDIC cohort using fasting samples collected from 1997 to 2000 (EDIC years 4–6), with participants selected on the basis of retinopathy, nephropathy, and carotid stenosis status. For all measures, sampling weights were calculated on the basis of the relative distribution of retinopathy, nephropathy, and carotid stenosis in this subcohort and in the full EDIC cohort and were further adjusted for sex and original DCCT cohort (primary prevention vs. secondary intervention).

Apolipoproteins were quantified by electroimmunoassays for APOA1, APOA2, APOB, APOC3, and APOE (14). APOC3 was measured in whole serum before and after heparin precipitation. The precipitation step enables the determination of APOC3 bound to APOA1-containing lipoproteins (APOC3-HS) and APOC3 bound to APOB-containing lipoproteins (APOC3-HP) (28). We also report APOC3-HS/HP (APOC3 ratio) as a useful index of the catabolism of TG-rich lipoproteins (29).

For the determination of APOB-containing ADLS, 100 µl whole plasma was mixed with buffer solution and then sequentially treated with polyclonal antisera to APOA2, followed by antisera to APOE, and finally with antisera to APOC3, with overnight incubations at each step followed by centrifugation to separate precipitates and supernatants (30). The determination of APOB in the first precipitate and all supernatants enabled the calculation of Lp-B, Lp-B:C, [Lp-B:E + Lp-B:C:E], and Lp-A2:B:C:D:E subclasses, each expressed according to its APOB content (30). Lp-A1 and Lp-A1:A2 were measured by differential turbidimetry and defined according to APOA1 content (31). ADLS assays were conducted in the Lipid and Lipoprotein Laboratory at the Oklahoma Medical Research Foundation using previously described procedures (30, 32).

Cardiovascular outcomes

CVD events were ascertained on the basis of medical history, electrocardiogram, and available medical records and adjudicated by a committee masked to DCCT treatment group and HbA1c levels. The primary CVD outcome ("any CVD") was defined as the time to the first occurrence of CVD death, nonfatal myocardial infarction, nonfatal stroke, subclinical myocardial infarction on electrocardiogram, angina confirmed by ischemic changes with exercise tolerance testing or by clinically significant obstruction on coronary angiography, revascularization (with angioplasty or coronary artery bypass), or congestive heart failure (paroxysmal nocturnal dyspnea, orthopnea, or marked limitation of physical activity caused by heart disease) (33–36). The secondary CVD outcome, MACE, included only the time to fatal or nonfatal myocardial infarction or stroke, whichever occurred first. Participants free of a CVD event were administratively censored as of December 31, 2013 (the date of the last CVD data lock). Participants with "any CVD" events ($n = 11$) and MACEs ($n = 6$) prior to the study sample collection time point were excluded from these analyses, while all subsequent incident CVD events until censoring were included in the analyses.

Statistical analysis

Summary statistics (counts and percentages for binary variables and medians and quartiles for continuous variables) of the baseline (i.e., EDIC years 4–6) characteristics were used to describe the participants and to assess whether, after adjusting for the sampling weights, the 465 participants with available apolipoprotein and ADLS measurements included in these analyses were representative of the entire DCCT/EDIC cohort. All statistical analyses used sampling weights computed on the basis of retinopathy, nephropathy, and carotid stenosis status and data were further adjusted for sex and the original DCCT cohort.

Summary statistics were used to describe the lipoprotein measures both overall and separately by the initial DCCT treatment group, while the Wilcoxon test allowed for the sampling weights to test for differences between the two DCCT treatment groups. Kaplan-Meier survival curves were used to describe the time-to-event outcomes (i.e., "any CVD" and MACE). Cox proportional hazards models (separately for "any CVD" and MACEs) were used to assess the association between each lipoprotein measure and the risk of CVD. The power to detect associations in time-to-event analyses is dictated by the number of events. Given the relatively small number of incident "any CVD" events among the 465 participants in our study, and informed by our previous work on risk factors for CVD in the DCCT/EDIC cohort (35), a set of prespecified models was considered: model 1 (unadjusted); model 2 (age and mean HbA1c); model 3 (age, mean HbA1c, and log TGs); model 4 (age, mean HbA1c, and LDL); and model 5 [age, mean HbA1c, log TGs, systolic blood pressure (SBP), and pulse rate]. HbA1c, LDL, pulse rate, and SBP were defined using the updated mean values, with weights proportional to the time between visits. For TGs, we used the most recent value, as this has previously been shown to be most strongly associated with CVD events (35), and data were log-transformed. Given the lower number of MACEs, only models 1–4 were considered for the MACE outcome. All analyses were performed using R, and $P \leq 0.05$ was considered nominally significant. Given the exploratory nature of our analyses, the reported P values were computed without adjustment for multiple testing.

RESULTS

Table 1 presents the characteristics of the 465 participants from 1997 to 2000 (EDIC years 4–6), with values presented after adjustment for sampling weights, and compares these characteristics to the characteristics of the full DCCT/EDIC cohort at the same time point. For the 465 participants,

TABLE 1. Participant characteristics of the full cohort subgroup (EDIC 1997–2000)

Variable	Study Subgroup (n = 465)	Entire EDIC Cohort (n = 1,389)[a]
Group [% intensive (n)]	47 (219)	50 (695)
Cohort [% primary (n)]	50 (233)	50 (695)
Sex [% men (n)]	53 (246)	53 (736)
Smoking [% (n)]	17 (79)	17 (236)
Age (years)	40.8 (34.5, 46.4)	40.5 (35.2, 45.5)
BMI (kg/m^2)	26.3 (23.9, 28.8)	26.4 (24.0, 29.1)
Mean HbA1c (%)	8.2 (7.4, 9.3)	8.0 (7.3, 8.8)
Mean total cholesterol (mg/dl)	185 (165, 202)	183 (164, 203)
Mean HDL (mg/dl)	50 (44, 59)	51 (45, 60)
Mean LDL (mg/dl)	115 (98, 133)	113 (97, 131)
TGs (mg/dl)	74 (53, 104)	72 (53, 106)
Mean SBP (mm/Hg)	117 (112, 123)	116 (111, 122)
Mean DBP (mm/Hg)	75 (72, 78)	74 (71, 78)
Mean pulse rate (bpm)	74 (70, 78)	74 (69, 78)
Any CVD[b] [% (n)]	11 (50)	12 (174)
MACEs[b] [% (n)]	5 (24)	6 (83)

Data presented in parentheses for continuous variables are medians (first and third quartiles). Weighted averages were used to account for complications, sex, and the DCCT cohort (primary prevention vs. secondary intervention). DBP, diastolic blood pressure.

[a]The total number of individuals in the cohort varied depending on availability.

[b]Incident events (events after EDIC year 6).

sampling weights were computed on the basis of retinopathy, nephropathy, and carotid stenosis status and further adjusted for sex and original DCCT cohort (primary vs. secondary prevention). After weight adjustment, 47% of the participants were from the original DCCT intensive treatment group, 50% were from the original primary prevention cohort, 53% were males, and 17% were current smokers, and the study subset became representative of the full cohort. Among the 465 participants in this study, by the end of 2013, there were 50 incident "any CVD" events over a total follow-up of 5,942 patient-years [rate of 8.4 events (any) per 1,000 individuals at risk for 1 year] and 24 MACEs over a total follow-up of 6,180 patient-years (rate of 3.9 events per 1,000 individuals at risk for 1 year). **Figure 1** shows the survival probability curves for the time to "any CVD" event and MACE in our cohort.

Summaries of the apolipoprotein and ADLS biomarkers stratified by initial DCCT randomization groups (intensive vs. conventional) are presented in **Table 2**. Only Lp-A1 was borderline nominally different, being higher in the former conventional versus intensive group (P = 0.049), and APOA1 also tended to be higher in the former conventional versus intensive group (P = 0.06).

Table 3 shows associations of 14 apolipoprotein and ADLS biomarkers with the risk of "any CVD" in T1D. In model 1 (unadjusted), higher levels of APOB (P = 0.004), total APOC3 (P < 0.0001), APOC3-HP (P = 0.001), APOC3-HS (P < 0.0001), and Lp-B (P = 0.001) were nominally associated with a higher risk of "any CVD". After adjusting for age and mean HbA1c (model 2), all of these variables remained nominally associated with "any CVD" except APOC3-HP, which revealed a positive trend (P = 0.07). When further adjusted for TG levels (model 3), only APOC3-HS remained nominally associated with "any CVD" (P = 0.019), and when adjusted for LDL-C (model 4), both total APOC3 and APOC3-HS emerged as nominally significant (P = 0.022 and P = 0.001, respectively). In the final adjusted model for age, mean HbA1c, mean SBP, mean pulse, and current (log) TGs (model 5), only APOC3-HS was nominally associated with the risk of "any CVD" (P = 0.012).

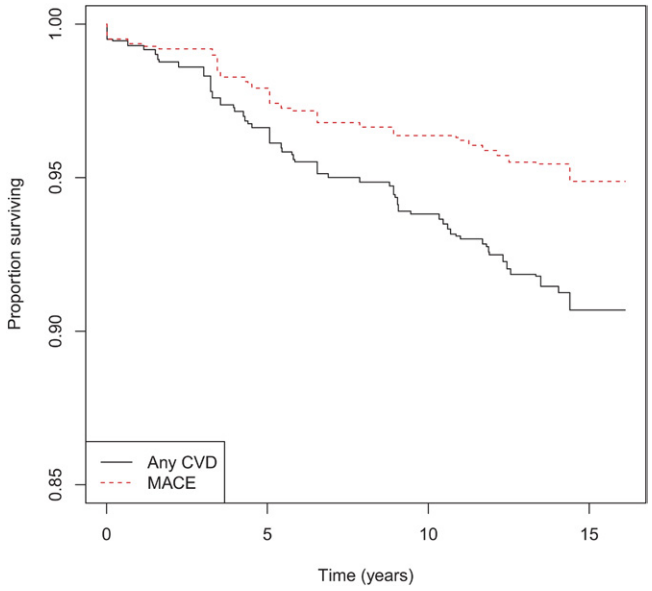

Fig. 1. Kaplan-Meier survival curves for the time to "any CVD" and time to MACEs.

The results were somewhat similar for the association of apolipoprotein and ADLS biomarkers with MACEs, as shown in **Table 4**. In model 1, higher levels of total APOC3 (P = 0.001), APOC3-HP (P = 0.007), APOC3-HS (P = 0.004), and Lp-B:C (P = 0.046) were nominally associated with MACEs. When adjusted for age and mean HbA1c (model 2), total APOC3 and APOC3-HS remained nominally significant (P = 0.013 and P = 0.026, respectively), and Lp-B:C showed a positive trend (P = 0.06). When further adjusted for serum TGs (model 3), none of the biomarkers were nominally significant, and when adjusted for LDL-C (model 4), both total APOC3 and APOC3-HS emerged as nominally significant (P = 0.026 and P = 0.027, respectively).

Additional analyses (not shown) addressed the association between the biomarkers and the risk of CVD after further adjustment for the initial DCCT treatment group, AER, duration of diabetes, hypertension, smoking status, and use of statins, all of which were assessed in 2000 (EDIC year 6). Due to the small number of events, these potential risk factors were included separately as additional covariates in the models described in Table 3 (model 5 for "any CVD" event) and Table 4 (model 4 for MACEs). The results were not much different from those above.

DISCUSSION

Overall, our analyses revealed that APOC3 and its subfractions showed the most consistent and nominally significant prospective associations with risks of "any CVD" event and MACEs in models adjusted for traditional cardiovascular risks and conventional lipids. Among the other

TABLE 2. Serum levels of serum apolipoproteins and ADLSs for EDIC years 4–7 stratified by initial DCCT randomization group

Biomarker	Overall (n = 465)	Intensive Group (n = 219)	Conventional Group (n = 246)	P
APOB (mg/dl)	72.5 (62.4, 84.8)	73.3 (64.4, 84.4)	71.8 (61.4, 85.3)	0.53
APOA1 (mg/dl)	146.1 (126.8, 163.6)	141.3 (121.9, 163.7)	149.9 (133.9, 162.9)	0.06
APOA2 (mg/dl)	35.1 (27.0, 43.2)	33.2 (25.5, 42.7)	36.5 (28.4, 43.9)	0.13
APOE (mg/dl)	4.3 (3.6, 4.8)	4.2 (3.5, 4.8)	4.4 (3.7, 4.9)	0.55
Total APOC3 (mg/dl)	9.3 (7.6, 11.1)	9.3 (7.6, 11.1)	9.3 (7.7, 11.2)	0.79
APOC3-HP (mg/dl)	2.3 (1.8, 3.1)	2.4 (1.8, 3.1)	2.3 (1.8, 3.1)	0.87
APOC3-HS (mg/dl)	6.8 (5.6, 8.1)	6.8 (5.6, 8.1)	6.8 (5.7, 8.1)	0.57
APOC3 ratio	2.8 (2.2, 3.5)	2.8 (2.1, 3.4)	2.9 (2.3, 3.5)	0.25
Lp-B (mg APOB/dl)	35.6 (29.2, 41.4)	36.2 (30.0, 42.6)	34.8 (28.7, 41.0)	0.31
Lp-B:C (mg APOB/dl)	11.6 (9.0, 14.4)	11.9 (9.2, 14)	11.1 (8.9, 14.6)	0.40
Lp-A2:B:C:D:E (mg APOB/dl)	12.9 (10.3, 15.6)	12.3 (10.2, 15.6)	13.0 (10.3, 15.6)	0.92
Lp-B:E + Lp-B:C:E (mg APOB/dl)	11.8 (8.9, 15.2)	11.7 (8.9, 15.1)	11.9 (8.9, 15.2)	0.95
Lp-A1 (mg APOA1/dl)	39.9 (34.8, 44.4)	38.9 (33.4, 43.7)	40.8 (36.0, 44.8)	0.05
Lp-A1:A2 (mg APOA1/dl)	107.4 (92.4, 120.4)	103.5 (87.5, 120.4)	109.6 (96.5, 120.8)	0.09

Data presented in parentheses are medians (first and third quartiles). Weighted averages were used to account for complications, sex, and the DCCT cohort (primary prevention vs. secondary intervention).

apolipoproteins measured in our cohort, APOB showed a positive association with "any CVD" event but not with MACEs after adjusting for age and mean HbA1c. Among the ADLS biomarkers, only Lp-B showed a positive association with "any CVD" event, but no biomarkers were associated with MACEs after adjusting for age and mean HbA1c. These findings add new data on the role of APOC3 in predicting CVD risks in individuals with T1D.

APOC3 is synthesized in the liver. It is found on the surface of VLDL, LDL, and HDL particles and has been independently associated with hypertriglyceridemia and cardiovascular events. Various mechanisms for APOC3 atherogenicity have been proposed: it leads to the decreased clearance of APOB from the circulation (37), formation of small, dense LDL (38), and stimulation of hepatic formation of TG-rich VLDL (39). In a meta-analysis of 11 studies that included 2,832 cases with cardiovascular events, each 5 mg/dl increase in total APOC3 was associated with a 33% increase in the risk of cardiovascular events (40). In our study, we observed the risk to increase by approximately 10% for every mg/dl increase in total APOC3 for "any CVD" event and for MACEs. In a recent report of 4,659 participants with CVD risk factors in the Multi-Ethnic Study of Atherosclerosis, HDL particles containing APOC3 were positively associated with coronary artery calcification in women (41). Our findings are consistent with these previous observations, as we observed nominally significant positive associations of the HDL-containing subfraction of APOC3 (APOC3-HS) with "any CVD" event and with MACEs: this persisted in our most rigorously adjusted model, which adjusted for SBP and pulse, both prespecified variables in our analytic plan. Recently, again in DCCT/EDIC, we reported prospective associations of APOC3-HP with carotid IMT in men (17); similar positive associations of APOC3 with carotid IMT have been reported in a healthy population (42) and with coronary heart disease in high-risk adults, including those with T2D (43).

TABLE 3. Cox models for the associations of apolipoproteins and ADLSs with the risk of "any CVD"

	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]		Model 5[e]	
Variable	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
APOB (mg/dl)	1.02 (1.01, 1.03)	**0.004**	1.02 (1.00, 1.04)	**0.027**	1.01 (0.99, 1.03)	0.30	1.01 (0.98, 1.04)	0.25	1.01 (0.98, 1.03)	0.39
APOA1 (mg/dl)	1.01 (0.99, 1.02)	0.19	1.00 (0.98, 1.01)	0.92	1.00 (0.98, 1.02)	0.77	1.00 (0.98, 1.02)	0.86	1.00 (0.98, 1.02)	0.79
APOA2 (mg/dl)	1.02 (0.99, 1.04)	0.15	1.01 (0.98, 1.03)	0.44	1.01 (0.98, 1.04)	0.37	1.01 (0.98, 1.04)	0.36	1.01 (0.98, 1.04)	0.28
APOE (mg/dl)	1.13 (0.87, 1.46)	0.35	0.99 (0.73, 1.35)	0.96	0.92 (0.67, 1.24)	0.57	1.01 (0.72, 1.43)	0.94	0.88 (0.64, 1.23)	0.47
Total APOC3 (mg/dl)	1.15 (1.08, 1.22)	**<0.0001**	1.10 (1.03, 1.18)	**0.003**	1.07 (0.98, 1.18)	0.12	1.10 (1.01, 1.19)	**0.022**	1.07 (0.98, 1.17)	0.11
APOC3-HP (mg/dl)	1.18 (1.07, 1.32)	**0.001**	1.11 (0.98, 1.23)	0.07	1.00 (0.87, 1.15)	0.97	1.08 (0.95, 1.21)	0.21	0.99 (0.86, 1.15)	0.97
APOC3-HS (mg/dl)	1.28 (1.14, 1.45)	**<0.0001**	1.24 (1.11, 1.39)	**0.0002**	1.19 (1.03, 1.37)	**0.019**	1.26 (1.11, 1.45)	**0.001**	1.18 (1.04, 1.35)	**0.012**
APOC3 ratio	0.73 (0.48, 1.12)	0.15	0.76 (0.51, 1.14)	0.18	0.98 (0.57, 1.69)	0.94	0.83 (0.55, 1.26)	0.38	1.05 (0.62, 1.77)	0.84
Lp-B (mg APOB/dl)	1.04 (1.01, 1.06)	**0.001**	1.03 (1.01, 1.06)	**0.014**	1.02 (0.98, 1.05)	0.22	1.02 (0.98, 1.06)	0.16	1.02 (0.98, 1.05)	0.32
Lp-B:C (mg APOB/dl)	1.06 (0.99, 1.13)	0.08	1.05 (0.97, 1.14)	0.18	1.02 (0.94, 1.09)	0.64	1.02 (0.92, 1.14)	0.64	1.02 (0.94, 1.11)	0.67
Lp-A2:B:C:D:E (mg APOB/dl)	1.06 (0.99, 1.14)	0.12	1.05 (0.97, 1.14)	0.22	1.03 (0.94, 1.11)	0.51	1.04 (0.95, 1.13)	0.39	1.03 (0.95, 1.11)	0.44
Lp-B:E + Lp-B:C:E (mg APOB/dl)	1.03 (0.97, 1.08)	0.26	1.00 (0.95, 1.05)	0.91	0.99 (0.93, 1.05)	0.74	0.98 (0.92, 1.05)	0.57	0.98 (0.92, 1.05)	0.69
Lp-A1 (mg APOA1/dl)	1.01 (0.97, 1.05)	0.64	0.98 (0.94, 1.02)	0.35	0.98 (0.94, 1.02)	0.42	0.98 (0.94, 1.03)	0.46	0.98 (0.94, 1.02)	0.38
Lp-A1:A2 (mg APOA1/dl)	1.01 (0.99, 1.03)	0.12	1.00 (0.98, 1.02)	0.64	1.00 (0.98, 1.02)	0.50	1.00 (0.98, 1.02)	0.61	1.00 (0.98, 1.03)	0.49

P values in bold are nominally significant ($P < 0.05$). HR, hazard ratio.
[a] Unadjusted.
[b] Adjusted for age and mean HbA1c.
[c] Adjusted for age, mean HbA1c, and TGs (log-transformed).
[d] Adjusted for age, mean HbA1c, and LDL-C.
[e] Adjusted for age, mean HbA1c, mean SBP, mean pulse rate, and current TGs (log-transformed).

TABLE 4. Cox models for the associations of serum apolipoprotein and ADLS biomarkers and the risk of MACEs

	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]	
Variable	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
APOB (mg/dl)	1.01 (0.99, 1.03)	0.12	1.01 (0.99, 1.04)	0.27	1.00 (0.98, 1.03)	0.80	1.01 (0.97, 1.05)	0.45
APOA1 (mg/dl)	1.00 (0.99, 1.02)	0.30	0.99 (0.98, 1.01)	0.41	0.99 (0.98, 1.01)	0.55	0.99 (0.98, 1.00)	0.37
APOA2 (mg/dl)	1.00 (0.97, 1.04)	0.66	0.98 (0.95, 1.02)	0.44	0.98 (0.91, 1.07)	0.48	0.98 (0.95, 1.02)	0.44
APOE (mg/dl)	1.20 (0.85, 1.69)	0.28	1.07 (0.64, 1.80)	0.78	0.99 (0.61, 1.62)	0.98	1.09 (0.63, 1.88)	0.75
Total APOC3 (mg/dl)	1.15 (1.05, 1.25)	**0.001**	1.10 (1.02, 1.19)	**0.013**	1.07 (0.95, 1.19)	0.22	1.11 (1.01, 1.22)	**0.026**
APOC3-HP (mg/dl)	1.16 (1.04, 1.29)	**0.007**	1.08 (0.96, 1.22)	0.17	0.93 (0.77, 1.13)	0.50	1.07 (0.94, 1.22)	0.26
APOC3-HS (mg/dl)	1.28 (1.08, 1.53)	**0.004**	1.19 (1.02, 1.39)	**0.026**	1.13 (0.93, 1.36)	0.19	1.22 (1.02, 1.45)	**0.027**
APOC3 ratio	0.82 (0.46, 1.43)	0.48	0.81 (0.47, 1.37)	0.43	1.05 (0.51, 2.15)	0.90	0.83 (0.49, 1.39)	0.48
Lp-B (mg APOB/dl)	1.02 (0.99, 1.05)	0.11	1.02 (0.98, 1.06)	0.30	1.00 (0.96, 1.03)	0.94	1.01 (0.97, 1.06)	0.51
Lp-B:C (mg APOB/dl)	1.08 (1.00, 1.17)	**0.046**	1.09 (0.99, 1.20)	0.06	1.05 (0.97, 1.14)	0.21	1.09 (0.96, 1.25)	0.16
Lp-A2:B:C:D:E (mg APOB/dl)	1.02 (0.92, 1.13)	0.72	1.01 (0.89, 1.15)	0.81	0.98 (0.87, 1.12)	0.83	1.00 (0.88, 1.15)	0.92
Lp-B:E + Lp-B:C:E (mg APOB/dl)	1.02 (0.95, 1.09)	0.46	0.99 (0.92, 1.07)	0.91	0.98 (0.91, 1.07)	0.74	0.98 (0.89, 1.07)	0.69
Lp-A1 (mg APOA1/dl)	1.01 (0.96, 1.07)	0.58	0.97 (0.92, 1.03)	0.35	0.97 (0.92, 1.03)	0.42	0.97 (0.92, 1.03)	0.37
Lp-A1:A2 (mg APOA1/dl)	1.01 (0.99, 1.03)	0.15	0.99 (0.98, 1.01)	0.66	0.99 (0.98, 1.02)	0.81	0.99 (0.97, 1.01)	0.57

P values in bold are nominally significant ($P < 0.05$). HR, hazard ratio.
[a]Unadjusted.
[b]Adjusted for age and mean HbA1c.
[c]Adjusted for age, mean HbA1c, and TGs (log-transformed).
[d]Adjusted for age, mean HbA1c, and LDL-C.

We did not observe any association of APOC3 ratio with CVD events or MACEs, possibly due to the absence of associations of APOC3 in LDL and VLDL (i.e., APOC3-HP) and APOB with cardiovascular outcomes in adjusted models. Our findings in this study add to the existing literature and suggest that APOC3, as well as the APOC3 content of HDL, may represent a therapeutic target in individuals with T1D.

Among the other apolipoproteins measured, we observed a nominally significant positive association of APOB with "any CVD" event but not with MACEs in models adjusted for age and HbA1c. We previously found a positive association of APOB with IMT (17), and APOB has been associated with a risk for stroke and increased IMT in other populations with and without diabetes (44, 45). No significant associations of HDL-containing APOA1, APOA2, or APOE with either "any CVD" or MACEs were found in our cohort; this may be explained by the overall low prevalence of dyslipidemia at baseline and the low number of events in the follow-up period.

Among the ADLS biomarkers, Lp-B was nominally associated with "any CVD" (models 1 and 2), and Lp-B:C was positively associated with MACEs (model 1, $P = 0.046$; model 2, $P = 0.06$). APOB-containing ADLSs, especially Lp-B and Lp-B:C, have shown positive correlations with risk for coronary artery disease in subjects with hypercholesterolemia (46) and macrovascular disease in subjects with T2D (47). Elevated Lp-B:C was shown to be highly correlated with the progression of atherosclerosis, reflected by coronary artery calcium scores, in patients with rheumatoid arthritis (48). We previously showed that Lp-B was prospectively associated with increased IMT in men, but no previous studies have reported associations with CVD events and MACEs in a T1D population. In our study, in models adjusted for blood pressure and/or conventional lipids, ADLSs were not significantly associated with future CVD, perhaps because of the strong collinearity among atherogenic lipids that contribute to CVD.

The strengths of our study include the detailed clinical characterization and long-term follow-up of DCCT/EDIC participants, the rigorous design of the parent study, the detailed measures of apolipoproteins and ADLSs, and the definition and rigorous validation of "any CVD" and MACE end points. Blood and urine samples were collected and maintained under stringent conditions after collection, and ADLS and apolipoprotein assays were conducted in a single laboratory with robust quality control.

Study limitations include the necessity, due to assay cost, of including only a subset of the EDIC cohort and the fact that the DCCT/EDIC study comprises predominantly Caucasian (North American) participants and thus has limited generalizability to other populations. ADLS assays require large investments of time, labor, and funds, and standardization across laboratories is challenging: therefore, ADLS assays are currently applicable only for research and not for clinical use. The power to detect associations between risk factors and subsequent outcomes in Cox proportional hazards models is a function of the effect size (i.e., hazard ratio) and the number of observed events. The relatively small number of CVD events in our study (50 "any CVD" events and 24 MACEs) may have limited our power to detect associations between the biomarkers and the risk of CVD. Adjustment for confounding factors in the prospective analyses may have been imperfect. No adjustment for multiple testing was performed, and therefore the results should be interpreted with caution: applying the Holm correction for 14 tests would require that the smallest *P* value be $\leq 0.05/14 = 0.0035$ to reach significance, with larger cutoff values for the other *P* values. For example, APOB, total APOC3, APOC3-HP, APOC3-HS, and Lp-B would remain significant after adjustment for the 14 tests (i.e., biomarkers) in the unadjusted models for "any CVD".

In conclusion, we provide new, biologically plausible evidence of prospective associations of serum total APOC3 and APOC3 in HDL (APOC3-HS) with cardiovascular

events in individuals with T1D. These associations provide information beyond that yielded by conventional lipid/lipoprotein measures and may help to elucidate new biomarkers, pathogenic mechanisms, and therapeutic targets for the prevention of cardiovascular events in T1D.

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