



Apolipoprotein-defined lipoprotein subclasses, serum apolipoproteins, and carotid intima-media thickness in T1D^S

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Abstract Circulating apolipoprotein-defined lipoprotein subclasses (ADLS) and apolipoproteins predict vascular events in the general and type 2 diabetes populations, but data in T1D are limited. We examined associations of ADLS, serum apolipoproteins, and conventional lipids with carotid intima-media thickness (IMT) measured contemporaneously and 6 years later in 417 T1D participants [men: n = 269, age 42 ± 6 y (mean ± SD); women: n = 148, age 39 ± 8 y] in the Epidemiology of Diabetes Interventions and Complications study, the follow-up of the Diabetes Control and Complications Trial (DCCT). Data were analyzed by multiple linear regression stratified by sex, and adjusted for time-averaged hemoglobin A1C, diabetes duration, hypertension, BMI, albuminuria, DCCT randomization, smoking, statin treatment, and ultrasound devices. In cross-sectional analyses, lipoprotein B (Lp-B), Lp-B:C, Lp-B:E+Lp-B:C:E, Apo-A-II, Apo-B, Apo-C-III-HP (heparin precipitate; i.e., Apo-C-III in Apo-B-containing lipoproteins), and Apo-E were positively associated with common and/or internal carotid IMT in men, but only Apo-C-III (total) was (positively) associated with internal carotid IMT in women. In prospective analyses, Lp-B,

Apo-B, and Apo-C-III-HP were positively associated with common and/or internal carotid IMT in men, while Lp-A-I:AI and Apo-A-I were inversely associated with internal carotid IMT in women. The only significant prospective association between conventional lipids and IMT was between triacylglycerols and internal carotid IMT in men. **CONCLUSIONS** ADLS and apolipoprotein concentrations may provide sex-specific biomarkers and suggest mechanisms for IMT in people with T1D.—Basu, A., A. J. Jenkins, J. A. Stoner, Y. Zhang, R. L. Klein, M. F. Lopes-Virella, W. T. Garvey, D. S. Schade, J. Wood, P. Alaupovic, T. J. Lyons, and The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group. **Apolipoprotein-defined lipoprotein subclasses, serum apolipoproteins, and carotid intima-media thickness in T1D.** *J. Lipid Res.* 2018. 59: 872–883.

This project was funded by National Institutes of Health Grant R01DK080043 and American Diabetes Association Grant 7-12-CT-46. DCCT/EDIC has been supported by Division of Diabetes Endocrinology and Metabolic Diseases of the National Institute of Diabetes and Digestive and Kidney Diseases U01 cooperative agreement grants (1982–1993 and 2011–2016) and contracts (1982–2011); and through support by the National Eye Institute, the National Institute of Neurological Disorders and Stroke, the Genetic Clinical Research Centers Program (1993–2007), and the Clinical Translational Science Center Program (2006–present; Bethesda, MD). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors declare no competing interests.

Manuscript received 23 August 2017 and in revised form 15 February 2018.

Published, *JLR Papers in Press*, March 25, 2018

DOI <https://doi.org/10.1194/jlr.P080143>

Abbreviations: ADLS, apolipoprotein-defined lipoprotein subclass; AER, albumin excretion rate; Apo-C-III ratio, ratio of Apo-C-III HS to Apo-C-III HP; HbA_{1C}, hemoglobin A1C; HP, heparin precipitate; HS, heparin-soluble; IMT, intima-media thickness; Lp-A-I, lipoprotein whose only apolipoprotein is Apo-A-I; Lp-B, lipoprotein whose only apolipoprotein is Apo-B; MUSC, Medical University of South Carolina; TG, triacylglycerol.

This trial was registered at clinicaltrials.gov as NCT00360815 and NCT00360893.

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^SThe online version of this article (available at <http://www.jlr.org>) contains a supplement.

T1D has been associated with shorter life expectancy and increased risk of atherosclerotic events than in the general population (1, 2). Dyslipidemia is an independent vascular risk factor, and both quantitative and qualitative changes in lipoproteins are implicated (3, 4). Lipid-lowering therapies, especially “statins,” substantially reduce the risk of cardiovascular events in people with either T1D or T2D (5). Thus, serum lipids and lipoproteins are important biomarkers for atherosclerosis progression, and detailed lipid and lipoprotein subclasses, such as those based on size and density, are known to extend and complement associations of conventional lipid profiles with the vascular complications of diabetes (4, 6, 7). Apolipoprotein-defined lipoprotein subclass (ADLS) analysis represents an approach relating to particle function and metabolism: it defines particles according to their qualitative apolipoprotein complement, and the subclass names reflect the apolipoproteins present on the particle (8). Lipoproteins can be categorized into two “families,” containing either Apo-A-I or Apo-B. The Apo-A-I family overlaps with HDL, includes two subclasses [lipoprotein A-I (Lp-A-I) and Lp-A-I:A-II], and usually has antiatherogenic potential (9, 10). The Apo-B family includes VLDL, IDL, and LDL; includes five subclasses [lipoprotein B (Lp-B), Lp-B:E, Lp-B:C, Lp-B:C:E, and Lp-A-II:B:C:D:E]; and generally has proatherogenic effects (8, 9). Findings from lipid-lowering trials, such as the Monitored Atherosclerosis Regression Study and the Cholesterol Lowering Atherosclerosis Study, supported the significance of ADLS, especially high levels of Lp-B and Lp-B:C particles, in predicting coronary events, and these associations remained significant when adjusted for conventional lipid profiles (11, 12). However, associations between ADLS and carotid intima-media thickness (IMT), a clinically useful surrogate marker of atherosclerosis, have not been explored in T1D.

ADLS analyses may be compared with, and complemented by, simpler clinically available plasma or serum concentrations of apolipoproteins, measures that contain no information about the distribution of apolipoproteins among particle subclasses [for example, circulating ApoB represents the total amount in VLDL, IDL, LDL, and lipoprotein (a)]. In addition to ApoB, high plasma concentrations of Apo-C-III have been associated with CVD risk (13). Apo-C-III is involved in the transport and catabolism of triacylglycerols (TGs) and can be bound to Apo-A-I-containing lipoproteins which are heparin-soluble (Apo-C-III HS) or to Apo-B-containing lipoproteins, which are precipitated by heparin (Apo-C-III HP). ApoC-III reduces clearance of lipoproteins by inhibiting lipoprotein lipase and by blocking the binding of Apo-E to hepatic receptors (14–16). The ratio of Apo-C-III HS to Apo-C-III HP (“Apo-C-III ratio”) is considered a useful index of the peripheral catabolism of TG-rich lipoproteins, with higher values being considered favorable (17). Apo-E modulates TG metabolism, and enrichment of Apo-B-containing lipoproteins with Apo-E

accelerates uptake of the particles via remnant receptors, increasing atherogenicity (18).

The Diabetes Control and Complications Trial (DCCT) aimed to determine effects of intensive vs. “conventional” diabetes therapy on the development and progression of diabetic microvascular complications (19). The study cohort comprised 1,441 T1D patients who, at enrollment in 1983–1989, were aged 13–39 years and were free of overt CVD. A primary prevention cohort comprised patients with T1D for 1–5 years and no diabetes-related complications. A secondary intervention cohort comprised patients with T1D for 1–15 years, mild to moderate nonproliferative retinopathy, and urinary albumin excretion rate (AER) <200 mg/day. In 1994, the Epidemiology of Diabetes Interventions and Complications study (EDIC), the longitudinal observational follow-up of the DCCT cohort, was initiated to assess the long-term effects of the prior DCCT intervention on cardiovascular and other complications (20); EDIC is ongoing. Carotid IMT measures were obtained at EDIC years 1 (1994–1996), 6 (1998–2000), and 12 (2004–2006); the ADLS and apolipoprotein measures reported in this study were measured on one occasion at EDIC year 6 and related cross-sectionally and prospectively to IMT at years 6 and 12. We hypothesized that Apo-B-containing lipoproteins, and serum Apo-B and Apo-C-III, are positively associated with IMT, whereas Apo-A-containing lipoproteins and serum Apo-A exhibit protective associations with IMT. The present study is the first to examine the relationship of ADLS and apolipoprotein concentrations with common and internal carotid IMT in T1D patients and complements our recent study of associations with albuminuria (21).

METHODS

In 1996, a collaboration between the Medical University of South Carolina (MUSC) and DCCT/EDIC was initiated to identify vascular risk factors. Twenty-five of 28 DCCT/EDIC clinical centers participated, and in 1997–1999, serum samples were shipped overnight on ice to MUSC; on arrival, aliquots were promptly prepared and stored at -80°C until analysis. The study, which meets Declaration of Helsinki guidelines, was approved by the Institutional Review Boards at MUSC and all participating DCCT/EDIC centers. Each subject gave written informed consent. Of the 1,441 DCCT participants, 968 agreed to participate in the MUSC program, but cost and resource considerations precluded determination of ADLS and apolipoprotein concentrations in all of these. The present study therefore utilized a previously described subset ($n = 465$) (18–20), of whom 417 had IMT measures available. Briefly, all those with abnormal albuminuria (AER > 40 mg/24 h), increased Early Treatment Diabetic Retinopathy Study retinopathy score (≥ 10), or elevated carotid atherosclerosis ($\geq 25\%$ stenosis at a carotid lesion) were included (i.e., all available cases were sampled), together with a larger group of subjects free of all of these complications. The three disease categories (albuminuria, retinopathy, and carotid stenosis $\geq 25\%$) were combined and re-weighted to reflect the demographic and vascular disease status of the entire EDIC cohort (see Statistical Analyses below).

Carotid ultrasonography and image analysis

Common and internal carotid IMT measurements in EDIC have previously been described in detail (22–24). In this substudy,

associations of lipoprotein measures with common and internal carotid IMT were assessed cross-sectionally at EDIC year 6 and prospectively at EDIC year 12. The IMT measures defined the maximum IMT for each individual. Reliability measures for IMT readers at EDIC years 6 and 12 have been reported previously (24): briefly, for common carotid IMT, intrareader and inter-reader coefficients of reliability were >0.93 and >0.81 respectively, and for internal carotid IMT, >0.93 and >0.90.

ADLS measures and serum apolipoproteins

The concept of ADLS addresses the functional significance of apolipoprotein heterogeneity that is not evident from conventional density-based lipoprotein classification methods (8, 9). This heterogeneity is an important determinant of metabolism and, thus, atherogenicity. Assays were conducted in the Lipid and Lipoprotein Laboratory at the Oklahoma Medical Research Foundation using previously described procedures (25, 26). Briefly, for Apo-B-containing particles, 100 μ l of whole plasma was mixed with buffer solution, then sequentially treated with polyclonal antisera to apo A-II, followed by antisera to Apo-E, and finally with antisera to Apo-C-III, with overnight incubations at each step followed by centrifugation to separate the precipitates and supernatants (26). Lp-B, Lp-B:C, (Lp-B:E + Lp-B:C:E), and Lp-A-II:B:C:D:E particle levels were expressed according to Apo-B content (26). Lp-A-I and Lp-A-I:A-II were measured by differential turbidimetry and defined according to Apo-A-I content (27). Quantification of Apo-C-III bound to Apo-A- and to Apo-B-containing lipoproteins was performed on heparin-manganese supernatants (Apo-C-III-HS) and precipitates (Apo-C-III-HP), respectively (14). Apolipoproteins were quantified by electroimmunoassays for Apo-A-I, Apo-A-II, Apo-B, Apo-C-III, and Apo-E (9).

Conventional lipid profiles, hemoglobin A1C, and other clinical measurements

These assays were performed at the DCCT/EDIC Central Biochemistry Laboratory (University of Minnesota). Total cholesterol, triglyceride, and HDL-cholesterol levels were determined by using previously reported enzymatic methods (28). LDL-cholesterol was estimated according to the Friedewald equation. Hemoglobin A1C (HbA_{1C}) was measured by high-performance ion exchange liquid chromatography (29).

Statistical analyses

In order to generate statistical estimates representative of the entire EDIC cohort, the distributions of cross-tabulated retinopathy, nephropathy, and carotid stenosis disease categories were determined for both the sampled subset and for the entire EDIC cohort. Inverse probability of selection weights was then calculated for each combination of disease status measures, by dividing the relative frequency among the entire EDIC cohort by the relative frequency among the sampled subset. The resulting weights were used in all statistical analyses (30, 31).

Common and internal carotid IMT, ADLS, most apolipoprotein concentrations, conventional lipids, and clinical and demographic factors measured at EDIC year 6, if normally distributed, were analyzed as continuous variables, presented as means \pm SD, and compared according to sex using Student *t*-tests. Variables with skewed distributions [diabetes duration, urinary AER, triglycerides, and Apo-C-III (total and HP)], were summarized as medians and interquartile ranges; the sexes were compared by using the Wilcoxon rank sum test. Data expressed as proportions, e.g., smoking (current), prior statin use at EDIC year 6, and original DCCT randomization group and status (primary or secondary at DCCT baseline), were compared between the sexes using a χ^2 test.

Multiple linear regression analyses were performed to examine associations of the EDIC year 6 lipoprotein-related measures (ADLS, apolipoprotein concentrations, and conventional lipid profiles) with the two dependent variables: common and internal carotid IMT. These associations were assessed in cross-sectional analyses using IMT data from EDIC year 6 and prospective analyses using IMT data from EDIC year 12, again stratified by sex. Results of unadjusted (univariate) analyses and adjusted (multivariate) analyses are presented in the tables. For the adjusted analyses, each lipoprotein measure was included as an independent variable in the linear model simultaneously with a fixed group of covariates (also from EDIC year 6): diabetes duration, smoking (yes/no), DCCT treatment group, hypertension (systolic blood pressure \geq 140 mmHg, diastolic blood pressure \geq 90 mmHg, or treatment with antihypertensive medications), BMI, AER, HbA_{1C}, and ultrasound imaging device. Prior statin use, defined at EDIC year 6 and, for prospective analyses, also at EDIC year 12, was also included in the adjusted models. A similar modeling approach was used for the analysis of IMT changes over 6 years (EDIC year 12 minus year 6 IMT) and their associations with EDIC year 6 lipoprotein-related measures. Variables with skewed distributions were log-transformed (natural log) to satisfy linear modeling assumptions.

Two other analyses are presented as text in the Results. First, a “final combined model” was fit that included all ADLS, serum apolipoprotein concentrations, and conventional lipid measures that were significant at the 0.05 α level: this was to identify measures that were independently associated with IMT: two-tailed *P* values < 0.05 were considered statistically significant. Second, to take multiple comparisons into account, the *P* value for significance was taken as \leq 0.001 based on a Bonferroni adjustment. Data were analyzed by using SAS/STAT software (Version 9.4; SAS Institute Inc., Cary, NC).

RESULTS

Subject characteristics

Oversampling of participants with evidence of vascular complications resulted in more men than women in the study subset. As shown in **Table 1**, at EDIC year 6 “baseline” in men compared with women, age, BMI, systolic blood pressure, estimated glomerular filtration rate, triglycerides, LDL- and nonHDL-cholesterol were significantly higher, and HDL-cholesterol was significantly lower. Among the ADLS-related measures, men had significantly higher Lp-B and Lp-B:C and significantly lower Lp-A-I and Lp-A-I:A-II than women. Among apolipoprotein concentrations, men had higher Apo-C-III HP, lower Apo-C-III ratios, and lower Apo-A-I than women (all *P* < 0.05). In view of all these differences, for the presentation of data below, men and women were analyzed separately.

ADLS measures, serum apolipoproteins and carotid IMT: cross-sectional associations (EDIC year 6)

Cross-sectional associations of lipids/lipoproteins (ADLS, serum apolipoprotein concentrations, and conventional lipids) with common and internal carotid IMT at EDIC year 6 are shown in **Tables 2** and **3**, respectively. In men, by unadjusted analysis, Lp-B, Lp-B:C, Apo-B, and Apo-C-III HP were positively associated with common carotid IMT, while Apo-C-III ratio was inversely associated (Table 2). In

TABLE 1. EDIC year 6 characteristics (1997–2000) of the reported subset of EDIC participants (n = 417), according to sex

Characteristics	Men (n = 269) (mean ± SD) ^a	Women (n = 148) (mean ± SD) ^a	P ^b
Age (years)	41.5 ± 5.9	39.0 ± 8.3	0.0015
Intensive treatment (%)	48	44	0.42
Secondary Intervention (%)	53	50	0.65
Duration of diabetes (years) ^a	17 (15, 22)	18 (16, 23)	0.051
Current cigarette smoker (%)	16	14	0.59
Any prior statin use (%)	6	3	0.14
Hypertension (%)	41	26	0.0019
BMI (kg/m ²)	27.4 ± 3.5	26.1 ± 4.8	0.0026
Blood pressure (mmHg)			
Systolic	123 ± 11	119 ± 18	0.0049
Diastolic	77 ± 7	75 ± 11	0.054
AER (mg/24 h) ^a	11.5 (8.6, 31.7)	11.5 (5.7, 17.3)	0.14
Glomerular filtration rate (ml/min)	92.6 ± 18.5	87.8 ± 18.6	0.010
HbA _{1c} (%) [weighted mean]	8.2 ± 1.0	8.2 ± 1.5	0.71
ADLS measures			
Lp-B (mg ApoB/dl)	37.2 ± 9.5	35.2 ± 11.4	0.049
Lp-B:C (mg ApoB/dl)	12.1 ± 3.5	11.2 ± 4.5	0.033
Lp-B:E + Lp-B:C:E (mg ApoB/dl)	11.7 ± 4.0	12.4 ± 4.6	0.13
Lp-A-II:B:C:D:E (mg ApoB/dl)	13.1 ± 3.8	13.2 ± 5.8	0.90
Lp-A-I (mg ApoA-I/dl)	39.2 ± 6.7	40.8 ± 9.1	0.043
Lp-A-I:A-II (mg ApoA-I/dl)	101.9 ± 21.2	111.7 ± 26.1	<0.0001
Serum apolipoproteins			
Apo-B (mg/dl)	74.1 ± 15.5	71.9 ± 20.7	0.24
Apo-A-I (mg/dl)	141.1 ± 26.6	152.6 ± 33.7	0.0002
Apo-A-II (mg/dl)	35.4 ± 11.1	36.5 ± 12.4	0.40
Apo-C-III total (mg/dl) ^a	9.4 (7.4, 11.0)	9.2 (7.8, 11.1)	0.94
Apo-C-III HP (mg/dl) ^a	2.4 (1.9, 3.2)	2.1 (1.6, 2.9)	0.019
Apo-C-III HS (mg/dl)	6.8 ± 2.0	7.2 ± 2.2	0.12
Apo-C-III ratio ^c	2.7 ± 0.7	3.2 ± 1.1	<0.0001
Apo-E (mg/dl)	4.3 ± 1.0	4.1 ± 1.0	0.095
Conventional lipids			
Total cholesterol (mg/dl)	190.3 ± 30.8	187.9 ± 37.8	0.49
Triglyceride (mg/dl) ^a	79 (57, 118)	66 (48, 87)	<0.0001
LDL-cholesterol (mg/dl)	117.5 ± 24.7	111.4 ± 34.6	0.044
Non-HDL-cholesterol (mg/dl)	138.8 ± 31.3	126.3 ± 39.1	0.0005
HDL-cholesterol (mg/dl)	51.5 ± 11.1	61.5 ± 16.0	<0.0001
Carotid IMT			
Common carotid IMT (mm)	0.677 ± 0.118	0.607 ± 0.099	<0.0001
Internal carotid IMT (mm)	0.836 ± 0.356	0.693 ± 0.298	<0.0001

^aIf the distribution of a continuous variable is skewed, median and the interquartile range (Q₁, Q₃) are presented.

^bComparison between men and women: *t*-test for difference in means, Wilcoxon rank sum test for difference in medians, and χ^2 test for difference in proportions. *P* values are shown in bold if significant (<0.05).

^cApo-C-III ratio is the ratio of Apo-C-III-HS:Apo-C-III-HP (an index of peripheral catabolism of TG-rich lipoproteins)

adjusted analysis, the positive associations of Lp-B:C and Apo-B persisted, and, in addition, Lp-B:E + Lp-B:C:E, Apo-AII, and Apo-E became statistically significant. Also in men, by unadjusted analysis, all conventional lipid profile measures, except triglyceride and HDL-cholesterol, were associated with common carotid IMT, but only total cholesterol remained in adjusted analysis. In the final combined model, Apo-E (slope = 0.038, SE = 0.013, *P* = 0.005) and Apo-B (slope = 0.0027, SE = 0.0009, *P* = 0.0028) remained significant among the lipid/lipoprotein measures. For women, there was a significant inverse association between Apo-C-III ratio and common carotid IMT (unadjusted analysis), but no other significant associations were observed.

Broadly similar cross-sectional associations were seen for internal carotid IMT (Table 3). In men, by unadjusted analyses, Lp-B, Lp-B:C, (Lp-B:E + Lp-B:C:E), Apo-B, Apo-C-III total, and Apo-C-III HP were positively associated with internal carotid IMT, while Apo-C-III ratio was inversely associated. All of the significant associations, except those for

total Apo-C-III (*P* = 0.051) and Apo-C-III ratio (*P* = 0.14), persisted in adjusted analyses. Also in men, by unadjusted analysis, all conventional lipid measures except HDL-cholesterol were significantly associated with internal carotid IMT, and nonHDL cholesterol and (inversely) HDL-cholesterol were associated in adjusted analysis. In the final combined model, only Lp-B:C remained significant in men. In women, by unadjusted analysis, there was a significant association between Apo-C-III HP and internal carotid IMT, and in adjusted analyses, for total Apo-C-III; only total cholesterol was significant among conventional lipid profile measures. In the final combined model, Apo-C-III HP was no longer significantly associated with internal carotid IMT in women.

ADLS measures, serum apolipoproteins, and carotid IMT: prospective associations (EDIC year 12)

Prospective associations of ADLS, plasma apolipoprotein concentrations, and conventional lipid profiles with

TABLE 2. EDIC Year 6 ADLS and serum apolipoprotein measures vs. common carotid IMT^b at EDIC year 6 (linear regression coefficients)

Variables	Men (n = 269)						Women (n = 148)					
	Unadjusted			Adjusted ^a			Unadjusted			Adjusted ^a		
	Slope	SE	P	Slope	SE	P	Slope	SE	P	Slope	SE	P
ADLS measures												
Lp-B (mg ApoB/d)	0.0030	0.0010	0.004	0.0016	0.0012	0.17	-0.00001	0.0012	0.99	-0.0001	0.0013	0.95
Lp-B:C (mg ApoB/dl)	0.0086	0.0028	0.002	0.0080	0.0029	0.0056	-0.0017	0.0031	0.59	-0.0030	0.0032	0.36
Lp-B:E + Lp-B:C:E (mg ApoB/dl)	0.0044	0.0026	0.089	0.0064	0.0027	0.018	-0.0015	0.0030	0.62	-0.0004	0.0030	0.89
Lp-A-II:B:C:D:E (mg ApoB/dl)	0.0048	0.0025	0.063	0.0032	0.0027	0.24	-0.0014	0.0024	0.57	-0.0010	0.0029	0.74
Lp-A-I (mg ApoA-I/dl)	0.0013	0.0015	0.39	0.0013	0.0016	0.41	-0.0023	0.0015	0.12	-0.0011	0.0015	0.47
Lp-A-I:A-II (mg ApoA-I/dl)	-0.0004	0.0005	0.44	0.00001	0.0005	0.99	-0.0010	0.0005	0.061	-0.0004	0.0005	0.44
Serum apolipoproteins												
Apo-B (mg/dl)	0.0022	0.0006	<0.001	0.0017	0.0007	0.014	-0.0002	0.00007	0.73	-0.0003	0.0008	0.74
Apo-A-I (mg/dl)	-0.0002	0.0004	0.55	0.00004	0.0004	0.92	-0.0008	0.0004	0.062	-0.0003	0.0004	0.43
Apo-A-II (mg/dl)	0.0008	0.0012	0.53	0.0031	0.0013	0.019	-0.0013	0.0011	0.22	-0.00003	0.0011	0.98
Apo-C-III total (mg/dl) ^b	0.047	0.033	0.15	0.031	0.034	0.37	-0.015	0.042	0.72	0.0050	0.045	0.91
Apo-C-III HP (mg/dl) ^b	0.062	0.026	0.020	0.036	0.029	0.23	0.051	0.030	0.098	0.032	0.031	0.31
Apo-C-III HS (mg/dl)	0.0062	0.0049	0.21	0.0057	0.0052	0.28	-0.0041	0.0061	0.50	0.0002	0.0063	0.98
Apo-C-III ratio ^c	-0.026	0.013	0.045	-0.0065	0.014	0.63	-0.034	0.012	0.005	-0.017	0.012	0.17
Apo-E (mg/dl)	0.016	0.013	0.23	0.037	0.014	0.0075	0.0088	0.013	0.51	0.019	0.014	0.18
Conventional lipids												
Total cholesterol (mg/dl)	0.0009	0.0003	0.003	0.0007	0.0003	0.036	0.00005	0.0004	0.89	-0.0001	0.0004	0.87
Triglyceride (mg/dl) ^b	0.033	0.020	0.090	0.014	0.020	0.49	0.0088	0.027	0.75	0.0030	0.028	0.92
LDL cholesterol (mg/dl)	0.0009	0.0004	0.023	0.0005	0.0004	0.19	0.0003	0.0004	0.46	0.0001	0.0005	0.77
Non-HDL cholesterol (mg/dl)	0.0009	0.0003	0.004	0.0006	0.0003	0.058	0.0003	0.0004	0.49	0.0001	0.0004	0.87
HDL cholesterol (mg/dl)	0.00003	0.0009	0.97	0.0003	0.0009	0.71	-0.0011	0.0009	0.21	-0.0006	0.0009	0.48

P values are in bold if significant (<0.05).

^aModels were adjusted for DCCT randomization, AER, HbA_{1c}, diabetes duration, hypertension, BMI, and current smoking at EDIC year 6. The regression models were also adjusted for statin use (any time from DCCT baseline to EDIC year 6), ultrasound devices, and image readers at EDIC year 6.

^bTriglyceride, Apo-C-III total, and Apo-C-III HP and common carotid IMT measures were natural log-transformed.

^cApo-C-III ratio: the ratio of Apo-C-III-HS:Apo-C-III-HP (an index of peripheral catabolism of TG-rich lipoproteins).

common and internal carotid IMT are shown in **Tables 4 and 5**, respectively. In men, by unadjusted analysis, baseline Lp-B, Lp-B:C, Lp-A-II:B:C:D:E, Apo-B, and nonHDL cholesterol were significantly positively associated with year 12 common carotid IMT, while the Apo-C-III HP ($P = 0.065$) and Apo-C-III ratio (inverse, $P = 0.054$) exhibited trends (Table 4). By adjusted analyses, Apo-B and Lp-B remained significant, but neither remained in the final combined model. For women, there were no statistically significant prospective associations between any lipid/lipoprotein measure and subsequent common carotid IMT with or without adjustment (Table 4).

Prospective associations with internal carotid IMT are shown in Table 5. In men, in unadjusted analyses, (Lp-B:E + Lp-B:C:E), Lp-A-II:B:C:D:E, Apo-B, total Apo-C-III, Apo-C-III HP, and Apo-C-III HS were significantly and positively associated with internal carotid IMT 6 years later, with a trend toward a negative association for the Apo-C-III ratio. By adjusted analysis, Apo-C-III-HP and triglyceride levels were significantly associated, but did not persist in the final adjusted model. Also in men, by unadjusted analysis, total cholesterol and nonHDL cholesterol were prospectively associated with internal carotid IMT, but the significance did not persist in adjusted analysis or in the final adjusted model. In women, there was a positive association of Apo-C-III-HP with future internal carotid IMT by unadjusted analysis and negative associations for Lp-A-I:A-II and Apo-A-I in adjusted analyses, but significance did not persist in the final adjusted model. There were no statistically significant associations with conventional lipoprotein measures.

In the final, most rigorous analysis, accounting for multiple comparisons (and adjusting α -level to 0.001), associations of serum Apo-B with common carotid IMT in men retained significance, observed in unadjusted models and in both cross-sectional and prospective analyses (Tables 2 and 4).

We also sought associations of the lipoprotein measures with the change in IMT between EDIC years 6 and 12: only a few scattered associations of borderline significance were seen, regardless of statistical approach (data not shown).

DISCUSSION

The DCCT/EDIC is the world's largest and best-characterized longitudinal study to address the evolution of the vascular complications of T1D (19, 20, 32). Participants have undergone repeated measures of common and internal carotid IMT, including measurements at EDIC year 6 (1998–2000), approximately contemporaneous with the blood samples used for our lipoprotein analyses (1997–1999), and 6 years later at EDIC year 12, enabling us to relate lipoprotein characteristics to carotid IMT measurements both cross-sectionally and prospectively. Note that the IMT data show the “means of maximum IMT” for each participant and thus relate to the extent of plaque, if present.

As in our previous work, some of which included NMR-determined lipoprotein subclasses and certain individual serum apolipoprotein levels (4, 6, 28), associations were

TABLE 3. EDIC year 6 ADLS and serum apolipoprotein measures vs. internal carotid IMT^b at EDIC year 6 (linear regression coefficients)

Variables	Men (n = 269)						Women (n = 148)					
	Unadjusted			Adjusted ^a			Unadjusted			Adjusted ^a		
	Slope	SE	P	Slope	SE	P	Slope	SE	P	Slope	SE	P
ADLS measures												
Lp-B (mg ApoB/dl)	0.0057	0.0022	0.010	0.0050	0.0025	0.050	0.0014	0.0024	0.57	0.0006	0.0028	0.84
Lp-B:C (mg ApoB/dl)	0.015	0.0059	0.015	0.017	0.0063	0.0070	0.0002	0.0062	0.97	-0.0016	0.0071	0.82
Lp-B:E + Lp-B:C:E (mg ApoB/dl)	0.0139	0.0054	0.011	0.015	0.0058	0.0095	0.0001	0.0060	0.99	0.0043	0.0065	0.51
Lp-A-II:B:C:D:E (mg ApoB/dl)	0.0037	0.0055	0.50	-0.0038	0.0059	0.53	0.0083	0.0048	0.089	0.0061	0.0062	0.33
Lp-A-I (mg ApoA-I/dl)	-0.0002	0.0031	0.96	-0.0019	0.0034	0.59	-0.0011	0.0031	0.73	0.0006	0.0033	0.85
Lp-A-I:A-II (mg ApoA-I/dl)	-0.0004	0.0010	0.72	-0.0001	0.0011	0.94	-0.0011	0.0011	0.30	-0.0004	0.0011	0.74
Serum apolipoproteins												
Apo-B (mg/dl)	0.0041	0.0014	0.003	0.0035	0.0015	0.019	0.0012	0.0013	0.39	0.0010	0.0016	0.55
Apo-A-I (mg/dl)	-0.0004	0.0008	0.62	-0.0003	0.0009	0.76	-0.0007	0.0008	0.37	-0.0002	0.0009	0.83
Apo-A-II (mg/dl)	-0.0004	0.0027	0.89	0.0022	0.0029	0.44	-0.0040	0.0022	0.07	-0.0021	0.0023	0.36
Apo-C-III total (mg/dl) ^b	0.16	0.070	0.019	0.15	0.075	0.051	0.14	0.083	0.086	0.21	0.094	0.025
Apo-C-III HP (mg/dl) ^b	0.17	0.056	0.002	0.17	0.063	0.0094	0.12	0.061	0.047	0.12	0.066	0.077
Apo-C-III HS (mg/dl)	0.019	0.011	0.071	0.015	0.011	0.20	0.014	0.012	0.27	0.022	0.014	0.10
Apo-C-III ratio ^c	-0.056	0.027	0.039	-0.043	0.029	0.14	-0.027	0.025	0.27	-0.0094	0.027	0.73
Apo-E (mg/dl)	0.0012	0.029	0.68	0.024	0.030	0.42	0.024	0.028	0.39	0.034	0.031	0.27
Conventional lipids												
Total cholesterol (mg/dl)	0.0015	0.0007	0.024	0.0012	0.0007	0.092	0.0015	0.0008	0.062	0.0018	0.0009	0.048
Triglyceride (mg/dl) ^b	0.094	0.041	0.024	0.080	0.044	0.068	0.034	0.056	0.54	0.055	0.062	0.38
LDL cholesterol (mg/dl)	0.0018	0.0008	0.028	0.0017	0.0009	0.066	0.0017	0.0009	0.061	0.0016	0.0010	0.13
Non-HDL cholesterol (mg/dl)	0.0018	0.0007	0.005	0.0017	0.0007	0.017	0.0015	0.0008	0.057	0.0015	0.0009	0.11
HDL cholesterol (mg/dl)	-0.0033	0.0019	0.086	-0.0042	0.0020	0.036	-0.00004	0.0018	0.98	0.0015	0.0020	0.45

^aP-values are in bold if significant (<0.05).

^bModels were adjusted for DCCT randomization, AER, HbA_{1c}, diabetes duration, hypertension, BMI, and current smoking at EDIC year 6. The regression models were also adjusted for statin use (any time from DCCT baseline to EDIC year 6), ultrasound devices, and image readers at EDIC year 6.

^cTriglyceride, Apo-C-III total, and Apo-C-III HP and common carotid IMT measures were natural log-transformed.

^dApo-C-III ratio is the ratio of Apo-C-III-HS:Apo-C-III-HP (an index of peripheral catabolism of TG-rich lipoproteins).

TABLE 4. EDIC year 6 ADLS and serum apolipoprotein measures vs. common carotid IMT^b at EDIC year 12 (linear regression coefficients)

Variables	Men (n = 269)						Women (n = 148)					
	Unadjusted			Adjusted ^a			Unadjusted			Adjusted ^a		
	Slope	SE	P	Slope	SE	P	Slope	SE	P	Slope	SE	P
ADLS measures												
Lp-B (mg ApoB/dl)	0.0035	0.0012	0.0046	0.0029	0.0015	0.044	-0.0011	0.0011	0.32	-0.0025	0.0016	0.11
Lp-B:C (mg ApoB/dl)	0.0076	0.0034	0.025	0.0072	0.0038	0.061	-0.0031	0.0029	0.29	-0.0027	0.0036	0.46
Lp-B:E + Lp-B:C:E (mg ApoB/dl)	0.0049	0.0031	0.12	0.0050	0.0035	0.15	-0.0009	0.0028	0.75	-0.0003	0.0037	0.92
Lp-A-II:B:C:D:E (mg ApoB/dl)	0.0076	0.0031	0.014	0.0054	0.0034	0.11	-0.0021	0.0023	0.35	-0.0022	0.0031	0.49
Lp-A-I (mg ApoA-I/dl)	0.0006	0.0018	0.73	0.0011	0.0020	0.60	-0.0008	0.0014	0.96	0.0014	0.0015	0.38
Lp-A-I:A-II (mg ApoA-I/dl)	-0.0008	0.0006	0.17	0.0002	0.0006	0.79	-0.0006	0.0005	0.25	-0.00003	0.0006	0.95
Serum apolipoproteins												
Apo-B (mg/dl)	0.0026	0.0008	0.001	0.0022	0.0009	0.014	-0.0007	0.0006	0.28	-0.0013	0.0010	0.17
Apo-A-I (mg/dl)	-0.0005	0.0004	0.22	0.0001	0.0005	0.86	-0.0003	0.0004	0.37	0.0001	0.0004	0.85
Apo-A-II (mg/dl)	-0.0009	0.0015	0.54	0.0005	0.0016	0.77	-0.0007	0.0010	0.50	-0.0001	0.0011	0.96
Apo-C-III total (mg/dl) ^b	0.026	0.040	0.52	-0.0027	0.042	0.95	0.011	0.041	0.78	-0.046	0.047	0.34
Apo-C-III HP (mg/dl) ^b	0.057	0.031	0.065	0.018	0.035	0.60	0.018	0.029	0.54	-0.051	0.035	0.14
Apo-C-III HS (mg/dl)	0.0064	0.0057	0.26	0.0049	0.0061	0.43	0.0015	0.0058	0.79	-0.0028	0.0066	0.68
Apo-C-III ratio ^c	-0.028	0.015	0.054	-0.0070	0.017	0.68	-0.0043	0.012	0.71	0.016	0.013	0.22
Apo-E (mg/dl)	0.0022	0.016	0.89	0.016	0.023	0.48	0.010	0.013	0.41	-0.0020	0.016	0.90
Conventional lipids												
Total cholesterol (mg/dl)	0.0007	0.0004	0.075	0.0005	0.0004	0.30	0.0002	0.0004	0.51	0.0001	0.0004	0.81
Triglyceride (mg/dl) ^b	0.021	0.024	0.37	-0.0069	0.025	0.78	-0.0001	0.026	0.99	-0.0084	0.032	0.79
LDL cholesterol (mg/dl)	0.0008	0.0005	0.086	0.0007	0.0006	0.25	0.0005	0.0004	0.25	0.0005	0.0005	0.35
Non-HDL cholesterol (mg/dl)	0.0007	0.0004	0.049	0.0004	0.0004	0.43	0.0004	0.0004	0.33	0.0003	0.0005	0.58
HDL cholesterol (mg/dl)	-0.0006	0.0011	0.55	0.0007	0.0012	0.54	-0.0006	0.0008	0.50	-0.0006	0.0009	0.53

P-values are in bold if significant (<0.05).

^aModels were adjusted for DCCT randomization, AER, HbA_{1c}, diabetes duration, hypertension, BMI, and current smoking at EDIC year 6. The regression models were also adjusted for statin use (any time from DCCT baseline to EDIC year 12), ultrasound devices, and image readers at EDIC year 12.

^bTriglyceride, Apo-C-III total, and Apo-C-III HP and common carotid IMT measures were natural log-transformed.

^cApo-C-III ratio is the ratio of Apo-C-III-HS:Apo-C-III-HP (an index of peripheral catabolism of TG-rich lipoproteins).

TABLE 5. EDIC Year 6 ADLS and serum apolipoprotein measures vs. internal carotid IMT^a at EDIC year 12 (linear regression coefficients)

Variables	Men (n = 269)						Women (n = 148)					
	Unadjusted			Adjusted ^a			Unadjusted			Adjusted ^a		
	Slope	SE	P	Slope	SE	P	Slope	SE	P	Slope	SE	P
ADLS measures												
Lp-B (mg ApoB/dl)	0.0039	0.0026	0.13	-0.0018	0.0029	0.55	-0.0030	0.0036	0.40	-0.0018	0.0050	0.72
Lp-B:C (mg ApoB/dl)	0.013	0.0069	0.071	0.0043	0.0077	0.58	-0.0031	0.0092	0.74	-0.0049	0.012	0.67
Lp-B:E + Lp-B:C:E (mg ApoB/dl)	0.0146	0.0063	0.022	0.0067	0.0070	0.34	-0.0029	0.0090	0.75	-0.0051	0.012	0.66
Lp-A-II:B:C:D:E (mg ApoB/dl)	0.016	0.0063	0.014	0.0083	0.0068	0.22	0.0032	0.0073	0.66	-0.0038	0.0098	0.70
Lp-A-I (mg ApoA-I/dl)	0.0004	0.0036	0.90	-0.0026	0.0041	0.52	-0.0023	0.0046	0.61	-0.0065	0.0048	0.18
Lp-A-I:A-II (mg ApoA-I/dl)	-0.0003	0.0012	0.81	0.0001	0.0013	0.97	-0.0016	0.0016	0.32	-0.0037	0.0017	0.031
Serum apolipoproteins												
Apo-B (mg/dl)	0.0039	0.0016	0.014	0.0004	0.0018	0.83	-0.0008	0.0020	0.68	-0.0015	0.0030	0.63
Apo-A-I (mg/dl)	-0.0004	0.0009	0.70	-0.0003	0.0010	0.76	-0.0011	0.0012	0.36	-0.0026	0.0013	0.045
Apo-A-II (mg/dl)	-0.0003	0.0030	0.92	0.0021	0.0032	0.52	-0.0015	0.0033	0.65	-0.0060	0.0033	0.075
Apo-C-III total (mg/dl) ^b	0.20	0.081	0.015	0.16	0.085	0.061	0.19	0.12	0.13	0.025	0.14	0.86
Apo-C-III HP (mg/dl) ^b	0.20	0.066	0.002	0.18	0.072	0.015	0.19	0.091	0.038	0.039	0.11	0.72
Apo-C-III HS (mg/dl)	0.028	0.012	0.024	0.023	0.013	0.074	0.015	0.018	0.41	-0.0012	0.020	0.95
Apo-C-III ratio ^c	-0.060	0.032	0.063	-0.047	0.036	0.19	-0.044	0.037	0.23	-0.010	0.040	0.80
Apo-E (mg/dl)	0.041	0.032	0.20	0.070	0.043	0.11	0.041	0.041	0.31	-0.028	0.049	0.57
Conventional lipids												
Total cholesterol (mg/dl)	0.0016	0.0008	0.047	0.0004	0.0009	0.64	0.0018	0.0012	0.11	0.0008	0.0014	0.56
Triglyceride (mg/dl) ^b	0.12	0.048	0.013	0.10	0.049	0.039	0.034	0.081	0.68	0.0094	0.099	0.92
LDL cholesterol (mg/dl)	0.0017	0.0010	0.080	0.0000	0.0012	0.97	0.0020	0.0013	0.13	0.0011	0.0017	0.52
Non-HDL cholesterol (mg/dl)	0.0019	0.0008	0.016	0.0007	0.0009	0.42	0.0019	0.0012	0.10	0.0011	0.0015	0.47
HDL cholesterol (mg/dl)	-0.0027	0.0022	0.21	-0.0022	0.0024	0.36	-0.0002	0.0026	0.93	-0.0007	0.0029	0.81

^a P-values are in bold if significant (<0.05).

^b Models were adjusted for DCCT randomization, AER, HbA_{1c}, diabetes duration, hypertension, BMI, and current smoking at EDIC year 6. The regression models were also adjusted for statin use (any time from DCCT baseline to EDIC year 12), ultrasound devices, and image readers at EDIC year 12.

^c Triglyceride, Apo-C-III total, and Apo-C-III HP and common carotid IMT measures were natural log-transformed.

^d Apo-C-III ratios the ratio of Apo-C-III:HS:Apo-C-III:HP (an index of peripheral catabolism of TG-rich lipoproteins).

much more numerous in men than in women. One reason may be that men, compared with women, have more atherogenic lipoprotein profiles and a higher prevalence of vascular complications, including renal disease (which itself worsens the lipoprotein profile). Also, men have increased common and internal carotid IMT compared with women (33–35). Men therefore comprised a majority of our cohort. For these reasons, as in all our previous studies, the sexes were analyzed separately.

In our cross-sectional analyses in men, the ApoB-containing subclasses, Lp-B, Lp-B:C, and (Lp-B:E + Lp-B:C:E), showed significant positive associations with common and/or internal carotid IMT. We previously reported similar associations with albuminuria, predominantly in men, in the same cohort (21), and in the present study we included albuminuria in the adjusted analyses. Relating these subclasses to conventional, density-defined lipoprotein classes, Lp-B is found in the (proatherogenic) LDL class, whereas Lp-B:C and (Lp-B:E + Lp-B:C:E), which showed the strongest associations with IMT, are distributed among the LDL, IDL, and VLDL classes and are highly atherogenic: those containing Apo-E represent atherogenic VLDL remnants (36–38). Apo-B-containing ADLS subclasses, especially Lp-B and Lp-B:C, have shown positive correlations with risks of coronary artery disease in patients with hypercholesterolemia (36) and with macroangiopathy in patients with T2D (37). These particles are efficiently taken up by macrophages (9, 38). Lp-B:C correlates strongly with atherosclerosis progression as reflected by coronary artery calcium scores in patients with rheumatoid arthritis (39), but no previous studies have reported these associations with carotid IMT in a T1D population. Likewise, the positive associations of (Lp-B:E + Lp-B:C:E) with IMT in men with T1D in our present study is consistent with our prior observation in DCCT-EDIC that the density-defined equivalent of these ADLS, IDL (VLDL remnants), showed the same association when measured by NMR (4).

Among serum apolipoproteins in men, Apo-B and measures of Apo-C-III were strongly associated with common and internal carotid IMT in cross-sectional analyses. Elevated Apo-B has been a recognized risk factor for coronary heart disease (40, 41) and has been associated with carotid IMT in cohorts with the metabolic syndrome and T2D (42, 43). Total Apo-C-III, Apo-C-III in Apo-B-containing particles (Apo-C-III HP), and an index of the efficiency of triglyceride lipolysis, Apo-C-III ratio (inversely), were all associated with IMT. Apo-C-III can impair plasma lipoprotein metabolism by inhibiting lipoprotein lipase and by blocking the binding of Apo-E to hepatic receptors, thus reducing clearance of Apo-B and triglyceride-rich VLDL particles (15, 16); it also stimulates monocyte-endothelial adhesion and ensuing inflammation (44). In a meta-analysis of retrospective and prospective studies, 5 mg/dl increases in total or “non-HDL Apo-C-III” were associated with 33% and 148% increases, respectively, in relative risk for cardiovascular events in high-risk populations (45). The observed associations of serum Apo-C-III, including its distribution between VLDL and HDL, with IMT are consistent with the association with Lp-B:C discussed above. In

the present study, Apo-E and Apo-A-II were also associated with common but not internal carotid IMT in men. Among conventional profiles in men, cross-sectional significance with common carotid IMT was observed only for total cholesterol, and significance with internal carotid IMT was observed for LDL-cholesterol and nonHDL-cholesterol. We therefore conclude that ADLS and detailed apolipoprotein concentrations, particularly those involving Apo-C-III, represent potential biomarkers and mechanistic clues for early atherosclerosis in T1D men.

Fewer women than men were included in the study, and the extent of their IMT was less. Perhaps for these reasons, few cross-sectional associations between lipoprotein measures and IMT were observed in women, and it is not possible to draw firm conclusions. The paucity of associations is in concert with a smaller study of carotid IMT in 58 women with systemic lupus erythematosus (46). A possible association of Apo-C-III with IMT would require confirmation in a larger study, and associations may develop in the future as age and duration of diabetes advance.

For our prospective analyses, in men, univariate analyses were generally similar to, and consistent with, the cross-sectional findings. The associations with Apo-C-III, while significant for internal carotid IMT, fell just short of statistical significance for common carotid IMT. For women, univariate associations were again scattered and inconclusive. In multivariate analyses, very few significant associations remained in either sex, and those observed were again scattered and had, in general, *P* values of lesser significance than the cross-sectional analyses. Given these reservations, and even though all observed associations were biologically plausible, conclusions cannot be drawn with confidence; longer studies are needed and are in progress.

The decreased statistical significance in adjusted vs. univariate prospective analyses could be caused by several confounding factors. We measured lipids and lipoprotein variables at a single time point (EDIC year 6), and, although we adjusted for statin use during the ensuing 6 years, differing durations and intensities of statin therapy and changes in lifestyle and/or other medications could not be taken into account and could have confounded the prospective analyses. Likewise, although we adjusted for prior DCCT randomization group, “metabolic memory” resulting from differing prior glycemic control during DCCT (47, 48) or earlier in EDIC may have had variable long-lasting effects on IMT progression between EDIC years 6 and 12 (23). Note that the lack of statistically significant associations in the multivariate prospective analyses were not confined to ADLS and apolipoproteins, but extended to conventional lipid profiles as well.

The multiple statistical tests performed raise concern for an inflated type I error rate beyond the nominal 0.05 α level. Clearly, multiple comparisons were made in the course of the statistical analyses. When we applied the most conservative Bonferroni adjustment ($\alpha \leq 0.001$, accounting for the number of comparisons), only serum Apo-B remained significantly associated with common carotid IMT, and only in men and in unadjusted models, a finding consistent with evidence that ApoB is superior to any conventional

cholesterol measure (49). However, to explore markers and mechanisms, we consider this stringent level of significance to be excessively conservative: many of the lipoprotein measures are interdependent. For this reason, we presented data in the tables without formal α -level adjustment [as we have done in the past in similar analyses (50, 51)]. We also emphasize that all of the associations shown in the tables are biologically plausible.

We found only scattered and borderline associations between lipoprotein measures and the “change in IMT” between EDIC years 6 and 12. There was little progression of IMT over this time period, likely due, in part, to significant introduction of statin treatment. Clearly, longer follow-up will be needed for a more robust assessment of predictive biomarkers.

The strengths of our study include the outstanding clinical characterization of DCCT/EDIC participants, the rigorous design of the parent study, the detailed measures of ADLS, apolipoproteins and conventional lipid measurements, and the definition of associations with IMT in both cross-sectional and prospective analyses. The study used samples maintained under stringent conditions since collection, and the assays were conducted in a single laboratory with robust quality control. Important covariate risk factors were taken into account, including statin use (although with the caveat above). The study is the first to define these associations in people with T1D.

The weaknesses of the study include the necessity, due to assay cost, of including only a subset of the EDIC cohort, the smaller number of women than men, and limited generalizability to other populations. Adjustment for confounding factors in the prospective analyses may have been imperfect. The assays reported, particularly ADLS, require large investments of time, labor, and funds, and standardization across laboratories is challenging: consequently, ADLS analyses are applicable only for research, not for clinical use. Prolonged sample storage could affect ADLS measurements and might result in nondetection of associations. However, in this and other studies using stored sera (11, 12, 52, 53), plausible associations were identified, coherent with, and as frequent as those with conventional lipid profiles measured near the time of sample collection. Also, precipitation of apo-B-containing particles using antibodies against other apolipoproteins was similar in extent to that in prior studies, suggesting that particles remained intact. Finally, we recognize the large heterogeneity in lipid and lipoprotein composition: our classification is based on major apolipoproteins only, and other complex variations in composition and posttranslational modifications are known to confer multiple biological effects (54, 55).

CONCLUSIONS

We provide sex-specific and biologically plausible evidence for associations of both ADLS and detailed measures of serum apolipoproteins with common and/or internal carotid IMT, both cross-sectionally and, to a lesser extent, prospectively in people with T1D. Associations were much

more frequent in men, and most involved Apo-B-containing particles. Both particle and apolipoprotein data point to an important (adverse) role for Apo-C-III. These associations complement and extend the information yielded by conventional lipid/lipoprotein measures and may help to elucidate new biomarkers and pathogenic mechanisms. Future analyses will relate these detailed measures to “hard” cardiovascular events that are now accruing among DCCT/EDIC participants. ■

The authors wish to thank all DCCT/EDIC participating centers and Dr. Barbara Braffett at the EDIC Data Coordinating Center for assistance with the review process and final approval by the DCCT/EDIC Publications and Presentations Committee. Industry contributors that have had no role in the DCCT/EDIC study but have provided free or discounted supplies or equipment to support participants’ adherence to the study are as follows: Abbott Diabetes Care (Alameda, CA), Animas (Westchester, PA), Bayer Diabetes Care (North America Headquarters, Tarrytown, NY), Becton Dickinson (Franklin Lakes, NJ), CanAm (Atlanta, GA), Eli Lilly (Indianapolis, IN), Lifescan (Milpitas, CA), Medtronic Diabetes Minneapolis, MI), Omron (Shelton CT), OmniPod[®] Insulin Management System (Bedford, MA), Roche Diabetes Care (Indianapolis, IN), and Sanofi-Aventis (Bridgewater, NJ).

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