Apolipoprotein C-III Protein Concentrations and Gene Polymorphisms in Type 1 Diabetes: Associations With Lipoprotein Subclasses

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Serum apolipoprotein C-III (apoCIII) concentration and apoCIII gene polymorphisms have been shown to be a risk factor for cardiovascular disease; however, the underlying mechanisms remain unclear. In addition, no studies have been performed that address these issues in type 1 diabetes. The current study investigated apoCIII protein and apoCIII gene variation in a normotriglyceridemic (82 ± 57 mg/dL) population of patients with type 1 diabetes, the Diabetes Control and Complications Trial/Epidemiology of Diabetes Intervention and Complications (DCCT/EDIC) cohort. Blood samples were obtained in 409 patients after an overnight fast. Serum apoCIII concentration was highly correlated with multiple changes in lipids and lipoproteins that resulted in an adverse cardiovascular disease risk profile. Higher apoCIII concentrations were associated ($P < .0001$) with increased triglycerides ($r = 0.78$), total ($r = 0.61$) and low-density lipoprotein (LDL) ($r = 0.40$) cholesterol, apoA-I ($r = 0.26$), and apoB ($r = 0.50$), and these relationships persisted after controlling for age, gender, body mass index (BMI), and hemoglobin A_{1c} (HbA_{1c}). Nuclear magnetic resonance (NMR) lipoprotein subclass analyses demonstrated that apoCIII was correlated with an increase in very-low-density lipoprotein (VLDL) subclasses ($P = .0001$). There also was a highly significant positive relationship between serum apoCIII concentration and the LDL particle concentration in both men ($r = 0.49$, $P = .001$) and women ($r = 0.40$, $P = .001$), and a highly significant negative relationship between serum apoCIII levels and average LDL particle size in both men ($r = -0.37$, $P = .001$) and women ($r = -0.22$, $P = .001$) due primarily to an augmentation in the small L1 subclass ($r = 0.42$, $P = .0001$). Neither the $T^{-455}\rightarrow C$ polymorphism affecting an insulin response element in the apoCIII gene promoter nor a *SacI* polymorphism in the 3′UTR were associated with any alterations in circulating apoCIII concentrations, serum lipids, apolipoprotein concentrations, lipoprotein composition, or parameters measured by NMR lipoprotein subclass analyses. In summary, elevated apoCIII concentration was associated with risk factors for cardiovascular disease in normolipidemic type 1 diabetic patients through associated changes in lipoprotein subfraction distributions, which were independent of apoCIII genotype.

STUDIES INVOLVING both gene variation and circulating protein levels have implicated apoCIII as a risk factor for cardiovascular disease.[1,2] However, the mechanism(s) whereby apoCIII might contribute to increased cardiovascular disease risk has not been identified. The apoCIII gene has been mapped to chromosome 11, where it exists in a gene cluster with the apolipoprotein AIV (apoAIV) and apolipoprotein AI (apoAI) genes. Studies of sequence variation at the apoAI/CIII/AIV locus have demonstrated association with hypertriglyceridemia,[3,4] low high-density lipoprotein (HDL) cholesterol,[5,6] and ischemic heart disease.[3,5-8] The human apoCIII gene itself has been found to be polymorphic.[9] One common polymorphism in the 3′UTR of the apoCIII gene is a *SacI* restriction fragment polymorphism. In addition, at nucleotide −455, a 5′ promoter polymorphism substitutes a T for C in an insulin response element and is associated with the loss of insulin-mediated downregulation of apoCIII gene expression.[10] This observation in HepG2 cells transfected with an apoCIII gene promoter-reporter gene construct would predict that impaired suppression of gene transcription by insulin would lead to increased apoCIII synthesis in humans carrying this polymorphism. Higher apoCIII would then mediate an increase in triglyceride-rich lipoproteins via an effect to reduce lipolytic catabolism. Consistent with this prediction, transgenic mice hyperexpressing apoCIII were found to display hypertriglyceridemia.[11-13] However, while apoCIII promoter polymorphisms were found to be associated with increased plasma triglycerides in aboriginal Canadians,[14] this association was not observed in a population of Italian schoolchildren,[15] in subjects from the Atherosclerosis Risk in Communities (ARIC) study,[16] nor in populations living in the Netherlands[17] or in northern France.[18] Similarly, reports that apoCIII polymorphisms are observed in patients with severe hypertriglyceridemia or familial combined hyperlipidemia[9,19] have not been confirmed consistently.[17,20]

There is a wealth of information regarding the effects of

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apoCIII on lipoprotein metabolism.[2,21,22] However, comparatively less is known regarding the effects of apoCIII genetic variation on lipids and lipoproteins in diabetic patients,[23-27] and no studies have investigated type 1 diabetic patients. In addition, little information is available in diabetics or nondiabetics regarding the relationship between apoCIII gene polymorphisms and circulating concentrations of apoCIII.[18,28] To better understand these issues, we investigated the effects of serum apoCIII protein levels and apoCIII gene polymorphisms on lipids and lipoproteins in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) cohort of type 1 diabetic patients.[29] Our study included a comprehensive characterization of lipoproteins, including lipoprotein chemical composition, as well as subclass distribution and particle size assessed by nuclear magnetic resonance (NMR).

MATERIALS AND METHODS

Patients and Blood Samples

A blood sample was collected after an overnight fast of at least 8 hours and prior to insulin administration from 409 type 1 diabetic patients consecutively appearing for their biennial evaluation of lipid levels and exam according to the DCCT/EDIC research protocol.[29] Serum samples for measurement of total cholesterol, HDL cholesterol, and triglycerides concentrations were shipped on dry ice to the trial's Central Biochemistry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis. Plasma for other lipids and lipoprotein analyses, and blood cells for DNA analyses, were sent to the Medical University of South Carolina. For lipoprotein studies, blood was placed on ice in polypropylene tubes containing a solution of lipoprotein preservatives comprised of 2.8 mmol/L\L EDTA, 62 μmol/L chloramphenicol, 50 μg/mL gentamycin sulfate, 10 mmol/L ϵ-amino-caproic acid, and 100 mmol/L 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (final concentrations). Samples were immediately centrifuged at 3,000 rpm for 25 minutes to sediment blood cells and plasma samples were then shipped with ice packs to Charleston via overnight courier. The isolation of lipoproteins was initiated within 24 hours to 4 days after the blood collection. To minimize in vitro modification, isolated lipoprotein samples were kept cool, handled under yellow lighting, and stored under a nitrogen atmosphere. Isolated lipoprotein preparations were subsequently stored at −80°C until analysis.

Clinical characteristics of the 409 patients are listed in Table 1. These characteristics do not differ significantly from those in the 1,301 patients who comprise the overall DCCT/EDIC cohort (data not shown). We determined that the serum apoCIII concentration in those patients taking medications known to lower lipid concentrations (n = 27) did not significantly differ from that in those patients not taking this type of medication. We further determnined that apoCIII concentration in those patients taking antihypertension medications, whether an angitensin enzyme-converting (ACE)-inhibitor (n = 72) or other classes (n = 12), did not differ from those patients not taking these medications regardless of apoCIII genotype or gender. The studies were approved by the Institutional Review Boards at all DCCT/EDIC Clinical Study Centers and Medical University of South Carolina, and all patients signed an informed consent.

Genetic Analyses

Blood samples were collected from each patient using 2.8 mmol/L EDTA as an anticoagulant. Genomic DNA was isolated from peripheral blood using a DNA isolation kit (Gentra Systems, Minneapolis, MN). Genotyping of the $T^{-455}\rightarrow C$ polymorphism (numbered from the transcriptional start site) in the apoCIII promoter was conducted using manual DNA sequencing. A 411-bp polymerase chain reaction (PCR) amplification product was generated from genomic DNA using the following primers: apoCIII-pro-5′ (5′-GGATTGAAACCCAGAGAT-3′) and apoCIII-pro-3′ (5′-GGGAAATCCCTAGGAGACT-3′). *Taq* DNA polymerase was purchased from GIBCO-BRL (Gaithersburg, MD). Reaction samples (20 μL each) were denatured for 3 minutes at 95°C and PCR was performed for 40 cycles, each cycle consisting of 3 segments: denaturation at 95°C for 30 seconds, primer annealing at 60°C for 60 seconds, and primer extension at 72°C for 60 seconds (at 1.5 mmol/L $MgCl_2$). An additional extension period of 5 minutes at 72°C for 60 seconds was applied upon completion of the 40 cycles. All PCR reactions were performed in a Perkin-Elmer 9600 thermal cycler (Foster City, CA). Sequencing was performed manually using the primer apoCIII 5′UTR*f* with the Amplicycle DNA sequencing kit according to the manufacturer's instructions (Perkin-Elmer).

The polymorphism in the 3′UTR of the apoCIII gene was genotyped by restriction digestion of a 513-bp PCR product generated using the following primers: apoCIII 3′UTR*f*, 5′-GTTTGACTTGTGCTGGGGGTT-3′; and apoCIII 3′UTR*r*, 5′-TGTCCAGCTTTATTGGGAGG-3′. PCR was performed for 35 cycles as described above with the exception of a primer annealing temperature of 60°C for 30 seconds and primer extension at 72°C for 30 seconds. This PCR product was directly digested using the *SacI* restriction endonuclease (New England Biolabs, Beverly, MA) in 25-μL volumes for 2 hours at 37°C. The *SacI* restriction endonuclease is an isoschizomer of *SstI*. Thus, it recognizes the same restriction site as *SstI*, which has been used previously by others to identify the 3′UTR polymorphism resulting from a C→G base substitution 40 bp downstream from the end of the coding sequence. Digested products were resolved by electrophoresis on 2% agarose gels and visualised with ethidium bromide (0.5 μL/mL) under UV light. Restriction digestion of the 513-bp PCR product with *SacI* results in 2 DNA fragments of 383 and 130 bp in GG homozygotes and a single fragment of 513 bp in CC homozygotes, reflecting lack of the restriction site.

Table 1. Clinical and Biochemical Characteristics of 409 Patients Studied in the DCCT/EDIC

Clinical Parameter	Mean	SD
Gender (M/F)	219/190	
Age (yr)	39	7
Duration of type 1 diabetes (yr)	18	5
BMI (kg/m^2)	26.5	4.0
Waist/hip circumference ratio	0.84	0.08
Current HbA_{1c} (%)	8.3	1.4
Mean HbA_{1c} during DCCT* (%)	8.1	1.4
Treatment status during DCCT (% intensive treatment group)	49	
Serum triglycerides (mg/dL)	82	57
% with serum triglycerides >200 mg/dL	4	
Total cholesterol (mg/dL)	187	35
% with total cholesterol >200 mg/dL	35	
LDL cholesterol (mg/dL)	114	30
% with LDL cholesterol >130 mg/dL	28	
HDL cholesterol (mg/dL)	56	14
% with HDL cholesterol <35 mg/dL	3	
ApoAI (mg/dL)	136	24
ApoB (mg/dL)	84	23
ApoCIII (mg/dL)	10.1	5.1

*Intervention phase of DCCT study. Patients currently are followed in the nonintervention DCCT/EDIC study.

Preparation and Chemical Characterization of Lipoprotein Fractions

Lipoprotein fractions were isolated by sequential ultracentrifugation of each plasma sample to isolate the triglyceride-rich lipoprotein classes comprised of very-low-density and intermediate-density lipoproteins (VLDL and IDL, $d < 1.019$ g/mL), low-density lipoproteins (LDL, $1.019 < d < 1.063$ g/mL), and HDL ($1.063 < d < 1.21$ g/mL). The solvent density of plasma and ultracentrifugation infranatants was increased using solid potassium bromide (KBr). Saline containing the lipoprotein preservative solution described above and KBr to raise solution density, was used to adjust sample volume. Samples were centrifuged in heat sealed tubes in a Ti70 rotor (Beckman Instruments, Palo Alto, CA) spun at 65,000 rpm for 22 hours at 10°C, and the floated lipoprotein fractions were harvested by tube slicing. The isolated lipoprotein fractions were washed and concentrated by centrifugation at their isolation density in a SW41 rotor (Beckman Instruments) spun at 41,000 rpm for 24 hours at 10°C. Isolated lipoprotein fractions were dialyzed against nitrogen-purged 0.15 mol/L NaCl, 0.3 mmol/L EDTA, pH 7.4 at 4°C, in the dark, with 3 changes of buffer over 24 hours. The lipoprotein fraction was then sterile filtered through a 0.22-μm filter, and stored in the dark under nitrogen at 4°C until further analysis. Aliquots of isolated lipoproteins were extracted with chloroform/methanol (2:1, vol/vol) and the free and total cholesterol, triglyceride, phospholipid phosphorous, and protein concentrations in each isolated lipoprotein fraction were determined as described previously.[30]

NMR Analyses

The lipoprotein subclass profile was determined using NMR analysis of the fasting serum sample collected from each patient. The basis for NMR analysis of lipoprotein subclasses is that each lipoprotein particle in plasma within a given diameter range "broadcasts" a distinctive lipid NMR signal, the intensity of which is proportional to its bulk lipid mass concentration.[31] The methodology used in this study to acquire and process the NMR data has been described in detail,[31-35] and consisted of 3 steps: (1) acquisition of 250-MHz proton NMR spectra of the serum specimens at 45°C, using a Bruker WM-250 spectrometer; (2) deconvolution of the lipid methyl group signal envelope appearing in these spectra at approximately 0.8 ppm, yielding the derived signal amplitudes broadcast by 18 modeled lipoprotein subclasses; and (3) conversion of these signal amplitudes to lipoprotein subclass concentrations using experimentally determined standards that relate the signal amplitudes of isolated subfraction standards to their chemically measured cholesterol and triglyceride concentrations. LDL and HDL subclass distributions determined by gradient gel electrophoresis and NMR have been shown to be closely related.[32] A "particle size index," describing the mass-weighted average size of particles within each lipoprotein class, was calculated by weighting each subclass concentration by a numerical size designation (1 to 6 for VLDL and IDL, 1 to 3 for LDL, and 1 to 5 for HDL), with larger values representing larger particle subclasses.

Serum Apolipoproteins and Other Assays

Levels of apoB and apoAI in serum were determined using nephlometry (Array 360; Beckman Instruments, La Jolla, CA) with commercially available reagent systems (APB Apolipoprotein B Reagent and APA Apolipoprotein AI Reagent; Beckman Instruments). ApoCIII concentration in serum was determined using a competition enzyme-linked immunosorbent assay (ELISA) method described previously.[36] Briefly, aliquots of the density fraction $d < 1.02$ g/mL isolated from pooled plasma were used as coating material. Aliquots of the coating material were stored at −80°C and were never refrozen. Purified apoCIII was used to calibrate the concentration of apoCIII in a pooled plasma sample that was subsequently used as a standard for all assays. The standard curve was fitted to a 4-parameter sigmoidal curve. The inter- and intra-assay variability for the assay was less than 7%. Blood glucose, hemoglobin A_{1c} (HbA_{1c}), and serum cholesterol, triglycerides, and HDL cholesterol concentrations were determined by previously described methods.[37]

Statistical Methods

Deviation from Hardy-Weinberg equilibrium was assessed by chi-square test (2 *df*). The standardized pairwise linkage disequilibrium statistic (D′) was calculated to summarize the degree of association between the 5′ and 3′ apoCIII gene polymorphisms.[38] Haplotypes were constructed by maximum likelihood methods.[39] The hypothesis that clinical variables, lipid levels, and lipoprotein composition differed among apoCIII genotypes was tested using analysis of variance (ANOVA) and analysis of covariance (ANCOVA). ANCOVA *F* test was used to determine whether 2 or more adjusted least square means were significantly different between genotypes or across genotypes. The Tukey-Kramer method of approximation was applied for a multiple comparison adjustment for *P* values. Simple Pearson correlation was performed between apoCIII and other continuous variables. Single and multiple linear regression models were used to assess the association between apoCIII and other continuous clinically relevant variables, where the clinical variables were considered as dependent and apoCIII as independent. All significant tests were 2-sided and were considered statistically significant at $P < .05$. SAS software (Version 8; SAS Institute, Cary, NC) was used in all statistical analyses.

RESULTS

Relationships Between ApoCIII Protein Levels and Clinical Variables, Lipids, and Lipoproteins

Table 2 details the simple regressions between serum apoCIII levels with lipids and lipoproteins in the DCCT/EDIC cohort of patients with type 1 diabetes. With respect to the traditional lipid panel, apoCIII was highly correlated ($P = .0001$) with serum triglycerides ($r = .78$), total cholesterol ($r = .61$), and calculated LDL cholesterol ($r = .40$), but not with HDL cholesterol ($r = 0.07$; $P = .265$).

To more precisely study independent effects of apoCIII on these important parameters, we employed multiple regression analyses according to 3 models (Table 2). The first model adjusted for effects of age, duration of diabetes, gender, and DCCT treatment group. All of the relationships noted in the simple regression analyses remained significant and were largely undiminished. The second model adjusted for effects of body mass index (BMI) and HbA_{1c} as 2 additional factors, and, again, the statistical relationships between the multiple parameters and apoCIII remained strong. Notably, the regressions with triglycerides ($r = 0.74$), total cholesterol ($r = 0.558$), and apoB ($r = 0.431$) remained highly significant ($P = .0001$). In addition, a weak positive correlation with HDL cholesterol was now statistically significant ($r = 0.091$; $P = .047$) in this model. Because apoCIII concentration was very highly correlated with triglycerides, we analyzed a third regression model that also adjusted for triglycerides. ApoCIII levels were still independently related ($P = .001$) to total cholesterol ($r = 0.309$), LDL cholesterol ($r = 0.158$), and apoB ($r = 0.467$), and the relationship with HDL cholesterol and apoAI became stronger such that apoCIII could now explain 15.8% of the variability in HDL ($r = 0.397$) and 17.5% of the variability in apoAI ($r = 0.419$).

Table 2. Simple and Multiple Regression Analyses for Serum ApoCIII Protein Level

	Simple Regression			Multiple Regression*			Multiple Regression†			Multiple Regression‡		
Dependent Variable	Slope	*P*	Semipartial R^2 (%)	Slope	*P*	Semipartial R^2 (%)	Slope	*P*	Semipartial R^2 (%)	Slope	*P*	Semipartial R^2 (%)
BMI (kg/m^2)	0.09	.0171	1.4	0.09	.0180	1.4						
HbA_{1c} (% hemoglobin)	0.07	.0001	6.4	0.07	.0001	6.5						
Total cholesterol (mg/dL)	415	.0001	37.4	4.07	.0001	35.7	3.95	.0001	31.2	3.48	.0001	9.6
HDL cholesterol (mg/dL)	0.15	.26	0.3	0.12	.34	0.2	0.26	.0467	0.8	1.79	.0001	15.8
LDL cholesterol (mg/dL)	2.39	.0001	16.3	2.33	.0001	15.5	2.10	.0001	11.6	1.53	.0006	2.5
Triglycerides (mg/dL)	8.60	.0001	61.4	8.63	.0001	61.3	8.51	.0001	54.7			
ApoA-I (mg/dL)	1.22	.0001	6.8	1.17	.0001	6.1	1.29	.0001	7.0	3.23	.0001	17.5
ApoB (mg/dL)	2.26	.0001	24.6	2.24	.0001	24.2	2.03	.0001	18.5	1.23	.0001	2.8

*Multiple regression adjusts for age, duration of diabetes, gender, and DCCT treatment group.

†Multiple regression adjusts for age, duration of diabetes, gender, DCCT treatment group, HbA_{1c}, and BMI.

‡Multiple regression adjusts for age, duration of diabetes, gender, DCCT treatment group, HbA_{1c}, BMI, and triglycerides.

ApoCIII Protein and the NMR Lipoprotein Subclass Profile

To study more intensively the relationships of apoCIII with lipoproteins, serum apoCIII levels and the NMR lipoprotein subclass profile were measured in the same fasting blood sample. ApoCIII concentrations were found to have important associations with lipoprotein subfraction distributions that are pertinent to cardiovascular disease risk. Because we have determined that lipoprotein subfraction concentrations in type 1 diabetic patients differ significantly between men and women,[40] we segregated the data based on gender. As detailed in Table 3, there were significant, positive associations between the serum apoCIII levels and the plasma concentrations of large, medium, and small VLDL subclasses (all $P = .0001$) regardless of gender, which was consistent with the strong relationship with serum triglycerides in Table 2. A positive correlation with apoCIII was also observed with the IDL fraction ($P = .0001$), another triglyceride-rich lipoprotein, in men, but not in women. Importantly, apoCIII levels also appeared to be associated with specific LDL subfractions. Increasing serum apoCIII concentrations were significantly associated with increments in small, dense LDL (L1; $P = .0001$), but not larger sized LDL (L3 and L2) in both men and women. Serum apoCIII concentration was positively correlated ($P = .05$) with the concentration of larger, relatively cardioprotective HDL (H3 + H4 + H5) in women, but not men. It was not correlated with concentrations of the smaller, less cardioprotective (H1 + H2) HDL fractions in either gender. The very strong association between apoCIII and triglyceride concentration in the entire cohort (Table 2) was not influenced by gender.

Serum apoCIII levels also influenced LDL particle concen-

Table 3. Relationship Between Serum ApoCIII Levels and Lipoprotein Subclass Analyses Determined by NMR

	Males		Females	
Lipoprotein Subclass*	Correlation Coefficient (*r*)	*P* Value	Correlation Coefficient (*r*)	*P* Value
Chylomicrons	0.06	NS	−0.02	NS
Large VLDL	0.58	<.001	0.60	<.001
Medium VLDL	0.66	<.001	0.66	<.001
Small VLDL	0.41	<.001	0.60	<.001
IDL	0.40	<.001	0.13	NS
L3	−0.07	NS	−0.03	NS
L2	−0.05	NS	0.04	NS
L1	0.46	<.001	0.31	<.001
Large HDL	0.01	NS	0.21	.002
Small HDL	−0.05	NS	0.01	NS
Plasma triglycerides	0.74	<.001	0.78	<.001

Abbreviation: NS, not significant.

*Concentrations of chylomicron and VLDL subclasses are expressed in units of triglyceride (mg/dL). Large VLDL represents the combination of NMR subclasses V5 + V6; medium VLDL represents the combination of NMR subclasses V3 + V4; small VLDL represents the combination of NMR subclasses V1 + V2. Concentrations of IDL, LDL, and HDL subclasses are expressed in units of cholesterol (mg/dL). L3 are large LDL, L2 medium LDL and L3 small LDL. Large HDL represents the combination of NMR-determined subclasses H5 + H4 + H3; small HDL represents the combination of NMR-determined subclasses H2 + H1. For each lipoprotein class, the subclass number increases with particle size.

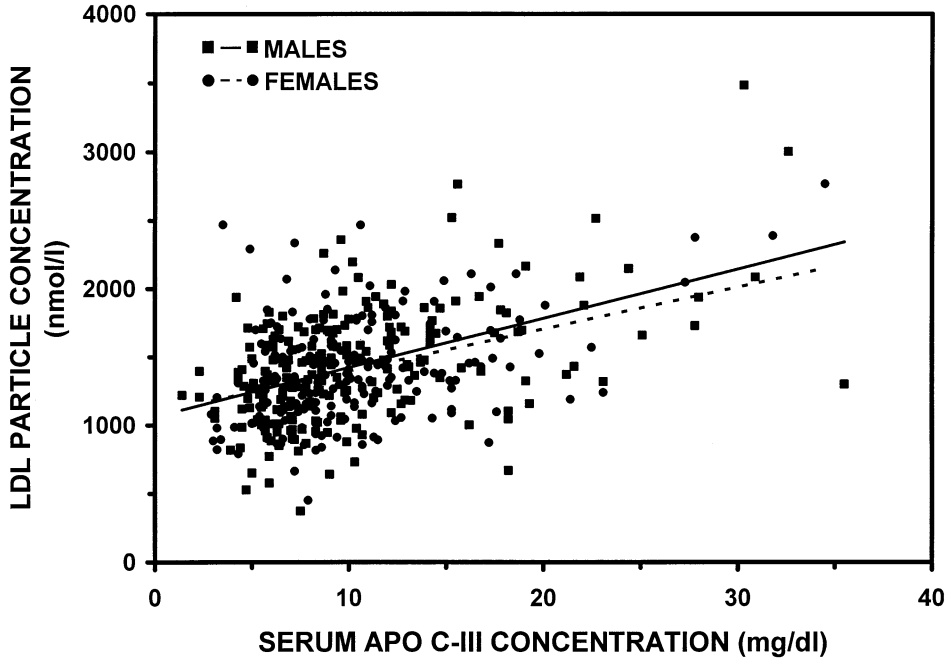

Fig 1. Relationship between serum apoCIII levels and LDL particle concentration. Fasting blood samples were obtained from 409 sequentially studied patients with type 1 diabetes in the DCCT/EDIC cohort. The figure shows the simple correlation between the serum level of apoCIII and LDL particle concentration assessed by NMR in individual male ($r = 0.49$) and female ($r = 0.40$) patients ($P = .001$).

tration and size determined using NMR spectroscopy. There was a highly significant positive relationship between serum apoCIII concentration and the LDL particle concentration in both men ($r = 0.49$, $P = .001$) and women ($r = 0.40$, $P = .001$) (Fig 1). In addition, there was a highly significant negative relationship between serum apoCIII levels and average LDL particle size in both men ($r = -0.37$, $P = .001$) and women ($r = -0.22$, $P = .001$) (Fig 2). Both relationships are

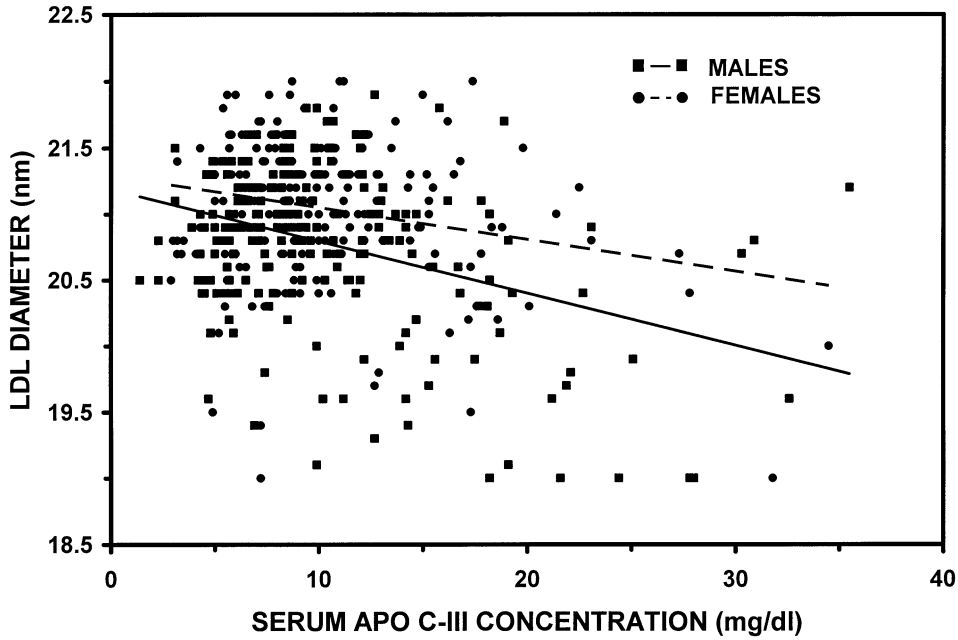

Fig 2. Relationship between serum apoCIII levels and LDL particle size. Fasting blood samples were obtained from 409 sequentially studied patients with type 1 diabetes in the DCCT/EDIC cohort. The figure shows the simple relationship between the serum level of apoCIII and mean LDL particle size assessed by NMR in individual male ($r = -0.37$) and female ($r = -0.22$) patients ($P = .005$).

Table 4. Effects of ApoCIII Genotype at Position −455 on Lipoprotein Subclass Concentration and Particle Size, and Serum Triglyceride and ApoCIII Concentrations

	ApoCIII −455 Genotype								
	TT			TC			CC		
	Male	Female	*P* Value	Male	Female	*P* Value	Male	Female	*P* Value
Chylomicron*	0.4 ± 0.9	0.6 ± 1.2	NS	0.2 ± 0.4	1.0 ± 2.1	NS	0.6 ± 2.1	1.2 ± 1.7	NS
Large VLDL	12.4 ± 36.4	5.4 ± 18.1	NS	10.7 ± 26.2	3.8 ± 8.9	NS	22.9 ± 50.8	2.0 ± 2.6	NS
Medium VLDL	25.5 ± 31.2	15.9 ± 29.9	.001	24.5 ± 25.7	18.7 ± 17.6	.03	29.1 ± 33.3	22.0 ± 34.1	.002
Small VLDL	23.8 ± 14.9	18.1 ± 16.6	.01	24.1 ± 15.3	20.5 ± 12.8	.001	25.3 ± 10.9	19.3 ± 12.1	.03
IDL	2.2 ± 4.3	2.3 ± 3.5	NS	2.4 ± 4.1	3.0 ± 4.6	.02	2.5 ± 3.8	1.6 ± 2.8	NS
L3	55.4 ± 41.5	70.0 ± 30.2	.004	55.1 ± 33.8	67.9 ± 27.6	.0002	53.7 ± 39.6	71.3 ± 38.5	.002
L2	33.9 ± 33.2	18.7 ± 25.9	.04	28.3 ± 32.6	18.8 ± 26.2	.05	38.5 ± 45.1	20.1 ± 25.3	NS
L1	31.6 ± 33.4	33.7 ± 34.0	NS	36.0 ± 34.1	36.5 ± 31.4	NS	31.9 ± 32.7	23.8 ± 19.1	.01
Large HDL	27.4 ± 12.6	40.4 ± 12.4	<.0001	27.8 ± 14.5	38.4 ± 14.3	<.0001	24.0 ± 14.6	36.9 ± 15.9	<.0001
Small HDL	19.3 ± 5.2	15.4 ± 5.3	<.0001	20.1 ± 5.3	15.1 ± 6.3	<.0001	19.6 ± 8.5	15.8 ± 6.0	<.0001
Mean VLDL size (nm)	48.1 ± 15.4	50.4 ± 19.8	NS	48.3 ± 15.6	49.1 ± 16.7	NS	50.5 ± 17.2	52.1 ± 18.2	NS
Mean LDL size (nm)	20.8 ± 0.6	21.1 ± 0.6	<.0001	20.8 ± 0.6	21.0 ± 0.5	.0002	20.8 ± 0.6	21.1 ± 0.5	<.0001
Mean HDL size (nm)	8.9 ± 0.4	9.2 ± 0.4	<.0001	8.8 ± 0.4	9.2 ± 0.5	<.0001	8.8 ± 0.4	9.2 ± 0.4	<.0001
Serum triglycerides (mg/dL)	91 ± 71	82 ± 87	NS	88 ± 58	78 ± 38	.04	109 ± 83	82 ± 55	.04
Serum apoCIII (mg/dL)	11.3 ± 6.9	11.3 ± 5.9	NS	10.6 ± 5.5	10.9 ± 4.2	NS	9.5 ± 4.6	10.6 ± 5.3	NS

*Concentrations of chylomicron and VLDL subclasses are expressed in units of triglyceride (mg/dL). Large VLDL represents the summed concentrations of NMR determined subclasses V5 + V6; medium VLDL represents the summed concentrations V3 + V4; small VLDL represents the summed concentrations of V1 + V2. Concentrations of IDL, LDL, and HDL subclasses are expressed in units of cholesterol (mg/dL). Large HDL represents the summed concentrations of NMR-determined subclasses H5 + H4 + H3; small HDL represents the combined concentrations of subclasses H2 + H1. Values expressed as mean ± SD. There were no statistically significant differences in the values between genotypes regardless of gender.

consistent with the observation that apoCIII was coupled with increments in the small, dense L1 subclass shown in Table 3. These changes in LDL as reflected in the NMR lipoprotein subclass profile are strongly associated with increased cardiovascular risk, and provide a mechanism for the epidemiological association between apoCIII and cardiovascular disease events noted by previous authors in nondiabetic and type 2 diabetic subjects.[41-46,48] While it was clear that apoCIII was correlated with apoB, total cholesterol, and calculated LDL cholesterol from the traditional lipid panel, the effect on LDL particle size and particle concentration could not be appreciated from these data, and could only be assessed using the NMR profile.

ApoCIII Genotype

Given the important relationships between apoCIII protein level and the lipid and lipoprotein subfraction profile, we examined whether apoCIII gene polymorphisms influenced these same parameters. ApoCIII genotype did not influence any of the clinical parameters reported in Table 1 for either the apoCIII -455 polymorphism or the *SacI* polymorphism (data not shown). The concentrations of lipoprotein subclasses differed significantly between men and women as we observed previously in this type 1 diabetic cohort.[40] However, the apoCIII −455 genotype did not influence the concentration of lipoprotein subclasses or mean VLDL, LDL or HDL particle size, as shown in Table 4. There were also no significant differences in lipoprotein subclasses and mean lipoprotein particle size among *SacI* genotype subgroups (data not shown). The significant changes in NMR-determined lipoprotein subfraction concentrations and sizes as a function of gender we previously identified in type 1 diabetic patients[40] were consistently exhibited for both the apoCIII −455 polymorphism (Table 4) and the *SacI* polymorphism (data not shown). In addition, neither the apoCIII −455 (Table 4) nor the *SacI* polymorphisms (data not shown) influenced serum triglyceride concentration in this group of predominantly normolipidemic type 1 diabetic subjects. Finally, there were no significant differences in the apoCIII concentration in serum among the apoCIII genotype subgroups at position −455 regardless of gender (Table 4).

There was no significant relationship between apoCIII genotype at −455 and protein, free or esterified cholesterol, triglyceride, or phospholipid contents of the triglyceride-rich lipoprotein fraction, which included both VLDL and IDL (data not shown). There also were no significant differences in the chemical composition of LDL or HDL by genotype at position −455 (data not shown). The *SacI* genotype had no effect on composition of any of the lipoprotein fractions (data not shown). The lack of associations between genotypes and apoCIII concentrations, as well as all other lipid/lipoprotein parameters, persisted after controlling for HbA_{1c}.

DISCUSSION

The results of this study show no association between apoCIII gene polymorphisms, including both the 5′ promoter (−455) and the 3′ *SacI* polymorphisms, and circulating levels of apoCIII protein in type 1 diabetic patients. Thus, the 5′ promoter polymorphism (−455) that disrupts an insulin response element[9,10] did not appear to affect apoCIII gene ex-

pression in type 1 diabetes. Furthermore, apoCIII gene polymorphisms were not associated with any alterations in serum triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, apoAI, apoB, or any of the parameters in the NMR lipoprotein subclass profile regardless of gender (Table 4). Some[9,14,19] but not all[15-18,20] previous studies have shown a significant association between apoCIII genotype and plasma triglyceride concentrations in non-diabetic humans. Those positive results are consistent with observations that the T→C base change at position −455 within an insulin response element abolishes the effect of insulin to suppress promoter activity in transfected HepG2 cells.[10] However, the current results show no relationship between the apoCIII −455 polymorphism and either serum apoCIII or triglyceride levels, and indicate that mechanisms other than the insulin regulatory element may determine apoCIII expression in type 1 diabetic patients. The current data are consistent with previous studies in type 2 diabetic patients showing that promoter[16,23] and 3′[23] apoCIII gene variation does not influence triglyceride levels, but did have an effect in nondiabetic subjects.[16] The combined data suggest that the apoCIII polymorphism could influence serum triglycerides in nondiabetic but not diabetic individuals. Diabetic patients in poor glycemic control frequently have elevated triglyceride levels; this might obscure a relationship with apoCIII polymorphism. However, this does not appear to be the case in our study. In the DCCT/EDIC cohort of patients, triglyceride levels were normal, and the subgroups divided according to apoCIII genotype had similar HbA_{1c} values, and apoCIII polymorphisms were not associated with alterations in serum triglycerides even after controlling for HbA_{1c}. Thus, there may exist heterogeneity in the effects of apoCIII gene variants in diabetic and nondiabetic individuals.

We further demonstrated for the first time that apoCIII level is associated with changes in lipoprotein subfraction concentration regardless of apoCIII genotype. One previous study of nondiabetic subjects[47] demonstrated that apoCIII $T^{-455}\rightarrow C$ polymorphism in men, but not women, was associated with differences in triglyceride levels which were reflected by significant differences in VLDL particle size and the concentrations of VLDL subfractions V6, V5, V4, and V3. No significant impact of apoCIII $T^{-455}\rightarrow C$ polymorphism on the average diameter of LDL or on LDL subfraction concentration were observed. ApoCIII concentration was not measured, however. In contrast, we demonstrated (Table 4) in type 1 diabetic patients that apoCIII level did not differ as a function of apoCIII $T^{-455}\rightarrow C$ polymorphism (Table 4). Nonetheless, there was a significant, positive association of apoCIII with the concentrations of large, medium and small VLDL regardless of gender (Table 3). Male, but not female, carriers of the less common S2 allele of the *SstI* polymorphism (*SacI* is an isoschizomer of *SstI*) had significantly lower concentrations of large LDL (L3) and a significant reduction in LDL particle size.[48] In the present study, we did not identify a significant difference in LDL particle size as a function of apoCIII $T^{-455}\rightarrow C$ polymorphism (Table 4) or *SacI* polymorphism (data not shown). However, there was a significant negative association of apoCIII concentration with LDL particle diameter in both men and women (Fig 2). Furthermore, there was a significant association of apoCIII concentration with LDL particle number in both males and females (Fig 1).

Previous studies have demonstrated a significant association between serum apoCIII concentration and cardiovascular risk. Both the Cholesterol-Lowering Atherosclerosis Study (CLAS) and the Monitored Atherosclerosis Regression Study (MARS) demonstrated that apoCIII predicted progression of angiographic coronary artery disease.[41,42] In the Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) study, post–myocardial infarction patients had increased apoCIII in apoB-containing lipoproteins and reduced apoCIII in non–apoB-containing lipoproteins compared with healthy controls.[43] In the Cholesterol and Recurrent Events (CARE) trial, VLDL+LDL-associated apoCIII was a stronger predictor of coronary heart disease events than plasma triglycerides per se.[44] In cross-sectional analyses, higher serum apoCIII concentrations were associated with increased severity of cardiovascular disease in sample populations of normotensive, non-obese, nondiabetic subjects who had undergone coronary angiography[45] and in patients with type 2 diabetes.[46]

Despite these strong epidemiological data, the mechanism by which alterations in the concentrations of apoCIII-containing lipoprotein subfractions might promote atherogenesis has not been elucidated. In the present study, we have observed a significant relationship between circulating apoCIII protein concentration independent of lipoprotein carrier form and atherogenic changes in lipids and lipoproteins in type 1 diabetic patients. Specifically, apoCIII concentrations were highly correlated with serum triglycerides and total cholesterol, and moderately correlated with LDL cholesterol, apoAI, and apoB levels, using both simple regression analyses and after adjusting for multiple covariables including age, gender, BMI, waist/hip ratio, and HbA_{1c}. Since the relationship between apoCIII and triglycerides was so pronounced, we examined whether the apoCIII correlations with other lipids and lipoproteins may arise secondarily due to a primary effect on triglycerides. The analyses clearly indicated that apoCIII was associated with a more atherogenic lipid/lipoprotein profile, independent of triglycerides (Table 2).

Other potential effects of apoCIII on lipoproteins only became manifest in our study when studying NMR lipoprotein subclass profiles that assess lipoprotein size, subclasses, and particle concentrations. There was a significant negative association of serum apoCIII concentration with LDL particle diameter determined using NMR spectroscopy. The association of apoCIII concentration with decreased LDL size resulted from the significant positive association of apoCIII concentration with that of small dense LDL (L1), without changes in the large and medium size LDL fractions (L3 and L2) (Table 3). In addition, increased apoCIII concentrations were associated with an augmentation in LDL particle concentration (Fig 1). Previous studies have shown a significant relationship between atherosclerosis risk and both decreased size and increased particle concentration for LDL.[35,49-53] The current study demonstrates for the first time that increased apoCIII levels are associated with increases in the circulating concentrations of small dense LDL particles in a cohort of type 1 diabetic patients. Our observations, considered together with the previous reports, indicate that the mechanism by which the apoCIII concentration confers increased cardiovascular disease risk may arise in large

part via effects on LDL particle size and concentration. Furthermore, the significant associations we observed between apoCIII and specific LDL subfractions might also be related to the important impact of LDL B:C on cardiovascular risk in diabetes.[54]

Theoretical mechanisms by which apoCIII might increase the number of small dense LDL particles with less cholesterol content have been developed through data detailing the metabolism of triglyceride-rich lipoproteins. Hypertriglyceridemia is associated with increased plasma VLDL and apoCIII production.[55] If VLDL enters the plasma from liver with increased content of apoCIII on the surface of the particle, this could inhibit lipoprotein lipase activity and, thus, increase the residence time of the VLDL particles.[56,57] Experimental data also indicate that the higher content of apoCIII could interfere with the cellular uptake of these abnormal VLDL to form VLDL remnants.[56] Prolonged circulation of VLDL by both these mechanisms would allow for greater exchange of VLDL triglyceride for the cholesterol ester of LDL and HDL. This exchange process between triglyceride-rich and cholesterol-rich particles is quite active in human plasma and is the function of the cholesterol ester exchange protein (CETP). The plasma triglyceride in LDL and HDL can then be metabolised by lipases lining the vascular space, particularly the extracellular hepatic triglyceride lipase. When the triglycerides are removed from LDL, the particle is left with a smaller core of hydrophobic lipids, a smaller diameter and a higher relative content of protein for greater density.

In conclusion, in type 1 diabetes, circulating apoCIII levels were highly correlated with multiple changes in lipids and lipoproteins that resulted in an adverse cardiovascular disease risk profile. Higher serum apoCIII concentrations were associated with increased triglycerides, total and LDL cholesterol, apoA1, and apoB. NMR lipoprotein subclass analyses demonstrated that VLDL subclasses were increased by apoCIII, and that there were important effects on LDL including decreased LDL size and an increase in LDL particle concentration due primarily to an augmentation in the small L1 subclass. Further follow-up of the DCCT/EDIC cohort should reveal whether apoCIII influences the rate of cardiovascular disease events in type 1 diabetes.

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