Diabetic Retinopathy and Serum Lipoprotein Subclasses in the DCCT/EDIC Cohort

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Purpose. To determine associations between retinopathy status and detailed serum lipoprotein subclass profiles in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study (DCCT/EDIC) cohort.

Methods. Persons with type 1 diabetes (440 women, 548 men) from the DCCT/EDIC cohort were studied. Retinopathy was characterized by Early Treatment Diabetic Retinopathy Study (ETDRS) scores, hard exudate scores, and ETDRS scores minus the hard exudate component. Lipoproteins were characterized by conventional lipid profile, nuclear magnetic resonance lipoprotein subclass profile (NMR-LSP), apoA1, apoB, lipoprotein(a), and susceptibility of LDL to oxidation. Data were analyzed with and without the following covariates: age, gender, duration of diabetes, HbA_{1c}, albumin excretion rate (AER), creatinine clearance, hypertension, body mass index, waist-hip ratio, DCCT treatment group, smoking status.

Results. The severity of retinopathy was positively associated with triglycerides (combined cohort) and negatively associated with HDL cholesterol (men, combined cohort). NMR-LSP identified retinopathy as being positively associated with small and medium VLDL and negatively with VLDL size. In men only, retinopathy was positively associated with small LDL, LDL particle concentration, apoB concentration, and small HDL and was negatively associated with large LDL, LDL size, large HDL, and HDL size. No associations were found with apoA1, Lp(a), or susceptibility of LDL to oxidation. All three measures of retinopathy revealed the same associations.

Conclusions. NMR-LSP reveals new associations between serum lipoproteins and severity of retinopathy in type 1 diabetes. The data are consistent with a role for dyslipoproteinemia involving lipoprotein subclasses in the pathogenesis of diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2004;45:910–918) DOI:10.1167/iovs.02-0648

The pathogenesis of diabetic retinopathy is not completely understood, but established risk factors include poor glycemic control, hypertension, increasing age, and duration of diabetes.[1] Further identification of risk factors and determinants for retinopathy is important to improve understanding of disease mechanisms, and to facilitate new treatments and preventive strategies.

Cross-sectional studies have shown positive associations between the severity of retinopathy and conventional plasma lipid profiles, specifically total- and LDL-cholesterol levels and the LDL-HDL cholesterol ratio.[2] The Epidemiology of Diabetes Complications Study demonstrated that high triglycerides and high LDL at baseline are associated with subsequent progression of retinopathy over 2 years.[3] The Early Treatment Diabetic Retinopathy Study (ETDRS) showed that baseline risk factors for proliferative diabetic retinopathy include high triglycerides, supporting "the possibility that reducing elevated blood lipids. . . slow(s) the progression of retinopathy."[4] Consistent with this, lipid-lowering dietary[5] and drug[6] therapy may lead to regression of retinal hard exudates, and two studies concluded that a diet high in polyunsaturated fatty acids may confer protection against retinopathy, perhaps by modifying platelet function.[7]

Each of the three major lipoprotein classes that are measured in conventional lipid profiles, VLDL, LDL, and HDL, has a distinct diameter range and unique structural characteristics and functions. Very briefly, VLDL particles contain three apolipoproteins (B, C, and E), are triglyceride-rich, and transport triglycerides from the liver to other tissues. LDL particles contain only apolipoprotein B and deliver cholesterol to tissues. HDL particles contain apolipoproteins A, C, and E, and remove cholesterol from tissues. In conventional lipid profiles, plasma concentrations of each class are expressed in terms of its contribution to total cholesterol, providing only a crude description of a very complex system. Modifications of lipoproteins by glycation and oxidation[8] and/or variations in the size (i.e., diameter) distributions of lipoprotein particles within the major lipoprotein classes, are not reflected in conventional profiles. In the cases of LDL and HDL,[9] mean particle size is known to be inversely associated with vascular disease. For

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Supported by National Heart Lung Institute Research Grant P01 HL55782, by Juvenile Diabetes Research Foundation Grants 4-1998-272 and 996001; the American Diabetes Association; the Diabetes Research and Wellness Foundation, Inc. (Fairfax, VA); the Medical University of South Carolina General Clinical Research Center (Grant M01-RR-1070), through which John Bercik provided computer database support; GlaxoSmithKline, which provided logistic support; and Beckman Inc., which donated antisera for Lp(a) determination. DCCT/EDIC is sponsored by the Division of Diabetes, Endocrinology and Metabolic Diseases, National Institute of Diabetes and Digestive and Kidney Diseases, the National Institutes of Health, through cooperative arrangements and a research contract and receives additional support from the National Heart, Lung and Blood Institute; the National Eye Institute; and the General Clinical Research Program of the National Center for Research Resources.

Submitted for publication July 1, 2002; revised December 8, 2002, and June 22, September 16, and November 10, 2003; accepted November 21, 2003.

Disclosure: **T.J. Lyons**, None; **A.J. Jenkins**, None; **D. Zheng**, None; **D.T. Lackland**, None; **D. McGee**, None; **W.T. Garvey**, None; **R.L. Klein**, None

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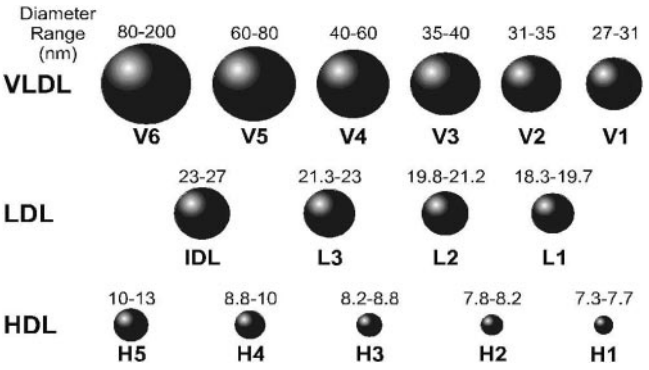

FIGURE 1. The NMR technique determines the concentrations (in milligrams per decaliter) of the 15 size-based lipoprotein subclasses shown. It also determines the mean particle diameters (in nanometers) of VLDL, LDL, and HDL, and the molar concentration of LDL particles in plasma. Lipoprotein classes and subclasses are structurally and metabolically distinct, differing by apolipoprotein content and lipid composition, as well as by size and density.

example, a preponderance of small LDL has been associated with diabetic nephropathy,[10] whereas both small LDL[11] and small HDL[12] have been associated with atherosclerosis. Studies addressing the associations of size-based lipoprotein subclasses with diabetic microvascular complications are few, and none concern diabetic retinopathy. Such studies have been hampered by the laborious techniques necessary to distinguish lipoprotein subclasses, which, until recently, required physical separation of subclasses. A new technique, nuclear magnetic resonance (NMR) analysis of whole serum, can rapidly determine concentrations of 15 different lipoprotein subclasses, designated according to particle size (Fig. 1), without physical separation of the subclasses.[13] In the present study, we performed the first cross-sectional assessment of the relationship between diabetic retinopathy and a comprehensive lipoprotein analysis, which included conventional lipid profiles, lipoprotein subclasses defined by NMR analysis, levels of apolipoproteins relevant to vascular disease (apo-A1 [present on HDL], apo-B [present on LDL, IDL, and VLDL]), and lipoprotein(a) [Lp(a)]), and the susceptibility of isolated LDL to in vitro oxidation.

METHODS

Study Subjects

The original cohort of the Diabetes Control and Complications Trial (DCCT) comprised 1441 volunteers (97% whites, 2% African American, 1% Hispanic or Native American) who, at study entry (1983–1989) were aged 13 to 39 years and had had type 1 diabetes for 1 to 15 years.[14] Subjects were observed until 1993 (average follow-up 6.5 years) on randomly assigned intensive (n = 711) or conventional (n = 730) treatments. The goal of the DCCT was to determine whether intensive therapy, aimed at normalizing blood glucose and HbA_{1c}, would prevent or delay complications, primarily retinopathy. In 1993, the trial was stopped because of a salutary effect of intensive therapy on retinal, renal, and neurologic outcomes.[14,15] Of the DCCT subjects, 1326 subsequently participated in the Epidemiology of Diabetes Intervention and Complications (EDIC) trial, a noninterventional, observational, longitudinal study.[16]

In 1996, a collaborative project between investigators at the Medical University of South Carolina (MUSC) and DCCT/EDIC investigators was implemented to identify new risk factors and mechanisms for vascular disease in type 1 diabetes. Twenty-five of the 28 DCCT/EDIC centers participated, and, from 1997 to 1999, this provided MUSC investigators with access to 1180 of the 1326 subjects still under follow-up. Of these, 968 consented and provided samples for the present study. Fasting serum was shipped overnight at 4°C to MUSC. Aliquots were frozen, stored at −70°C and later used to determine NMR lipoprotein subclass profiles (NMR-LSP), apoA1, apoB, and Lp(a). From fresh serum, LDL was isolated by ultracentrifugation, and the susceptibility of its protein and lipid components to copper-mediated in vitro oxidative stress was determined.

The study was approved by the Institutional Review Boards of MUSC and each DCCT/EDIC center, and adhered to the tenets of the Declaration of Helsinki. The clinical characteristics of the 968 subjects from whom NMR-LSP was obtained are summarized in Table 1. This cohort comprised 67% of the original DCCT cohort and 73% of subjects still under follow-up in DCCT/EDIC. Although sample bias is possible, the study cohort was representative of the entire DCCT cohort for all characteristics shown in Table 1, both at the time of DCCT close-out in 1993 and at the time of the present study.

EDIC Procedures

DCCT/EDIC subjects participated in annual evaluations, including resting electrocardiograms, arm blood pressure, and HbA_{1c} determined by high-performance liquid chromatography in the DCCT/EDIC central laboratory.[17] On alternate years, fasting lipid profiles and AER from 4-hour urine collections were determined in the central laboratory. Therefore, it took 2 years, in this case 1997 to 1999, for the entire DCCT/EDIC cohort to complete one cycle of the study at MUSC. The fasting blood samples sent to MUSC were obtained from the same samples as those sent to the DCCT/EDIC central laboratory for fasting lipid profiles.

Assessment of Retinopathy

In DCCT/EDIC, retinopathy was assessed every 4 years, with graders at the University of Wisconsin Reading Center unaware of DCCT randomization status.[14] For the present study, we used the scores obtained at the annual visit preceding the one at which the fasting blood sample was obtained. We used three different measures of retinopathy in our analyses, each derived from stereoscopic seven-field fundus photography according to the ETDRS protocol[18] used for DCCT/EDIC.[14] First, we used the "abbreviated final version of the ETDRS scale of diabetic retinopathy severity."[14,19] This provides a score on a scale of 1 to 23 for individual subjects, not for individual eyes (i.e., the score estimates severity using a composite of lesions in both eyes). Subjects were categorized into three groups by ETDRS severity, as defined in Table 2.

Second, we used scores for the presence and severity of hard exudates as defined by ETDRS.[20] This analysis was conducted because of known associations of hard exudate status (as an individual component of retinopathy) with conventional plasma lipid profiles (2–4), and because hard exudate status contributes only a relatively minor, "present/absent" component of the ETDRS score. Specifically, if present, hard exudates can raise ETDRS scores (which range from 1 to 23) only from ≤3 to 4 (if present in one eye) or to 5 (both eyes). The same score changes can also be caused by the presence of soft exudates and mild retinal hemorrhages. For ETDRS scores of 6 or above, hard exudate status does not contribute. In the separate system used specifically to assess hard exudates, scores were assigned to individual eyes (0, 1, 2, 3, 4, and 5 by increasing severity), and the score assigned to each subject was that of the more severely affected eye.

Third, we performed an analysis using ETDRS scores from which the (categorical) contribution of hard exudates had been removed, to determine whether the associations between total ETDRS scores and lipoprotein status would persist. This adjustment led to reductions in ETDRS scores (from 4 or 5 to 2 or 3) in only 23 subjects. As described in the following sections, all three analyses demonstrated essentially the same associations between retinal status and lipoprotein profiles. Therefore, only the associations of total ETDRS scores with lipoprotein profiles are presented in the tables.

TABLE 1. Clinical Characteristics of 968 DCCT/EDIC Study Subjects at Time of NMR-LSP Sample Acquisition (1997–1999), According to Gender and Former DCCT Randomization Group (Intensive or Conventional treatment)

	Women (n = 428)			Men (n = 540)		
	Intensive (n = 225) Mean (SE)	Conventional (n = 203) Mean (SE)	P	Intensive (n = 275) Mean (SE)	Conventional (n = 265) Mean (SE)	P
Age (y)	39.9 (0.5)	38.4 (0.5)	<0.05	39.9 (0.4)	40.2 (0.4)	NS
Duration of type 1 diabetes (y)	17.6 (0.3)	17.8 (0.4)	NS	17.5 (0.3)	16.8 (0.3)	NS
Body mass index (BMI, kg/m^2)	26.8 (0.3)	25.9 (0.3)	<0.05	27.3 (0.3)	27.0 (0.2)	NS
Waist-to-hip ratio	0.8 (0.0)	0.8 (0.0)	NS	0.9 (0.0)	0.9 (0.0)	NS
HbA_{1c} (percent hemoglobin, Hb)	8.2 (0.1)	8.2 (0.1)	NS	8.1 (0.1)	8.3 (0.1)	<0.05
Mean DCCT HbA_{1c} (% Hb)	7.3 (0.1)	9.1 (0.1)	<0.0001	7.2 (0.1)	9.0 (0.1)	<0.0001
Systolic BP (mm Hg)	116.9 (1.0)	115.0 (0.9)	NS	121.1 (0.7)	123.6 (0.9)	<0.05
Diastolic BP (mm Hg)	73.3 (0.6)	72.2 (0.6)	NS	76.4 (0.5)	77.3 (0.6)	NS
Total cholesterol (mg/dL)	189.0 (2.2)	187.1 (2.3)	NS	190.0 (2.2)	188.7 (2.2)	NS
HDL (conventional profile; chol, mg/dL)	63.0 (1.0)	62.7 (1.0)	NS	51.2 (0.8)	51.7 (0.7)	NS
LDL (conventional profile; chol, mg/dL)	110.9 (2.0)	108.8 (2.1)	NS	118.8 (1.9)	118.1 (1.9)	NS
Triglycerides (conventional profile; mg/dL)	75.7 (2.5)	78.0 (4.0)	NS	98.2 (4.1)	96.2 (4.5)	NS
Standard creatinine clearance (mL/min)	110.9 (1.6)	110.3 (1.7)	NS	120.6 (1.4)	119.2 (1.6)	NS
	% (SE)	% (SE)	P	% (SE)	% (SE)	P
BMI > 27.3 in women, > 27.8 in men (kg/m^2)	39.3 (0.2)	28.2 (0.6)	<0.02	38.5 (0.2)	36.3 (0.5)	NS
Hypertension*	26.1 (0.2)	26.2 (0.6)	NS	42.1 (0.2)	47.3 (0.5)	NS
AER > 40 mg/24 hours	6.8 (0.1)	16.8 (0.5)	0.001	11.5 (0.1)	20.5 (0.4)	<0.005
Smoker†	20.9 (0.2)	17.3 (0.5)	NS	20.3 (0.1)	18.3 (0.4)	NS
Taking lipid lowering medications†	4.0 (0.1)	3.9 (0.3)	NS	7.3 (0.1)	6.0 (0.2)	NS
Taking ACE inhibitor†	6.7 (0.1)	13.8 (0.5)	<0.02	12.4 (0.1)	17.7 (0.4)	NS

Probabilities refer to differences for each gender according to former randomization group. chol, cholesterol. ACE, angiotensin-converting enzyme.

* Hypertension is defined by previously documented or current SBP/DBP > 140/90 mm Hg.

† At time of blood sample collection.

Nuclear Magnetic Resonance Lipoprotein Subclass Profiles

Serum was separated by prompt centrifugation (3000 rpm, 20 minutes), shipped overnight on wet ice to MUSC, and stored at −70°C. Serum collected on Fridays was stored at 4°C for shipment the following Monday.

NMR-LSP was measured on first-thaw serum (250 μL) using a 400-MHz proton NMR analyzer at LipoScience Inc. (Raleigh, NC). The NMR technique entails measurement of the distinctive lipid methyl group signals broadcast by lipoprotein particles of differing sizes and has recently been described in detail.[13] Briefly, the measured amplitudes of the signals provide the concentrations of 15 lipoprotein subclasses, as defined in Figure 1. The diameter ranges for each subclass were established by calibration using purified subfractions isolated by ultracentrifugation and agarose gel filtration, in which particle size distribution was determined by "gold standard" techniques: electron microscopy for VLDL and LDL and polyacrylamide gradient gel electrophoresis for HDL. NMR-determined LDL subclass diameters are consistent with calculations based on lipid compositional data[21] and electron microscopy measurements,[22] but are approximately 5 nm smaller than those estimated by gradient gel electrophoresis.[23] Of the NMR-determined LDL subclasses, the smallest, L1, reflects levels of small, dense LDL. For the HDL subclasses, H5, H4, H3, H2, and H1 have been established as similar to gradient gel electrophoresis subclass designations 2b, 2a, 3a, 3b, and 3c,[30] respectively.[13] VLDL subclass levels are expressed as milligrams per decaliter triglyceride, and LDL and HDL subclass levels as milligrams per decaliter cholesterol.

In the present study, data for the six VLDL and the five HDL subclasses suggested that subclass grouping was appropriate to simplify data analysis, e.g., for HDL, the two smallest subclasses (H1, H2) exhibited similar associations which were opposite to those of the three larger subclasses (H3–H5). HDL subclasses were therefore grouped as small (H1+H2) and large (H3+H4+H5). Likewise, VLDL subclasses were grouped as small (V1+V2), medium (V3+V4), and large (V5+V6).

TABLE 2. Categories of Severity of Retinopathy According to ETDRS scores, as Used in Tables 3, 4, and 5

ETDRS Score	Retinopathy Description	Definition
Steps 1–3	None–Minimal	From no retinopathy (step 1) through to bilateral microaneurysms, i.e. "very mild" non-proliferative diabetic retinopathy (NPDR) (Step 3).
Steps 4–9	Mild–moderate nonproliferative	From unilateral "mild" NPDR (microaneurysms plus hard exudates, cotton wool spots, and/or mild hemorrhages) (step 4) through to bilateral "moderate" NPDR (moderately extensive intraretinal microvascular abnormalities (IRMA), severe retinal hemorrhages, or venous beading in one quadrant only) (step 9).
Steps 10–23	Preproliferative and proliferative	Unilateral "severe" NPDR (severe hemorrhages in four quadrants, venous beading in at least two quadrants, moderately severe (IRMA) in at least one quadrant, step 10), or worse.

TABLE 3. Associations of ETDRS Scores with Conventional Lipid Profiles

Conventional Lipid Profile (mg/dL)	ETDRS Score			Significance		
	1–3 (245 Females; 230 Males: Total 475)	4–9 (147 Females; 255 Males: Total 402)	10–23 (39 Females; 44 Males: Total 83)	P^1	P^2	P^3
Total Triglycerides						
Female	74.4 (2.9)	75.0 (3.2)	99.3 (12.0)	**<0.001**	NS	
Male	86.2 (4.0)	101.4 (4.3)	124.5 (14.7)	**<0.01**	NS	
Total	80.1 (2.5)	91.5 (3.0)	112.7 (9.7)	**<0.0001**	0.06	NS
LDL Cholesterol						
Female	107.8 (2.0)	113.3 (2.2)	115.4 (4.3)	**<0.05**	NS	
Male	115.4 (2.1)	119.2 (1.7)	125.6 (5.6)	NS	NS	
Total	111.5 (1.4)	117.0 (1.4)	120.7 (3.6)	**<0.01**	NS	NS
HDL Cholesterol						
Female	64.1 (1.0)	60.7 (0.9)	60.5 (2.5)	**<0.02**	NS	
Male	53.0 (0.9)	51.2 (0.8)	46.5 (1.2)	**<0.01**	**<0.01**	
Total	58.7 (0.7)	54.8 (0.6)	53.1 (1.5)	**<0.0001**	**<0.002**	NS

All probabilities <0.10 are shown; <0.05 in bold. Data are expressed as the mean ± SE. P^1, test for trend; quantitative dependent variable is ordinal ETDRS retinopathy score; independent variable is specified lipid parameter. P^2, test for trend; quantitative dependent variable is ordinal ETDRS retinopathy score, independent variable is specified lipid parameter adjusted for age, gender (if applicable), duration of diabetes, hypertension, AER, creatinine clearance, HbA_{1c}, BMI, WHR, and DCCT randomization group. P^3, test for interaction between specified lipid parameter and sex in predicting ETDRS retinopathy score; covariates as for P^2.

The average particle size (diameter in nanometers) of each lipoprotein class (VLDL, LDL, HDL) was calculated from the relative contributions of its constituent subclasses. LDL particle concentration (in nanomolar) was determined as the sum of the particle concentrations of the individual LDL subclasses (including IDL). The latter were calculated by relating the NMR signal amplitudes that corresponded to individual subclasses to those of isolated subclass standards.[20]

Apolipoproteins

Serum apoA1, apoB, and Lp(a) were measured at MUSC by nephelometry (Beckman, Brea, CA).

Susceptibility of LDL to Oxidation

Freshly isolated plasma (containing 2.8 mM EDTA, 62 μM chloramphenicol, 50 μg/mL gentamicin sulfate, 10 mM ϵ-amino-caproic acid, final concentrations) was shipped overnight at 4°C from DCCT/EDIC centers to MUSC. LDL (density 1.019–1.063 g/mL) was isolated by sequential ultracentrifugation as described.[24] Susceptibility to oxidation of the lipid component of LDL (n = 704) was determined by a modification of the method of Esterbauer et al.,[25] as described.[26] Results were expressed as the change in absorbance (Δ absorbance) at 234 nm from baseline to peak absorbance (measuring formation of conjugated dienes, reflecting change in content of lipid peroxides) after exposure to 5 μM Cu^{2+} ions. Susceptibility to oxidation of the protein component (n =710) was determined from fluorescence (excitation, 360 nm; emission 430 nm), expressed as the ratio of fluorescence after 24 hours of exposure to Cu^{2+} over that at baseline.

Statistical Analyses

For analyses examining differences between former DCCT/EDIC treatment groups (Table 1), we used a two-sample t-test assuming equal variances or a χ^2 test with 1 degree of freedom.

For analyses of associations between retinopathy scores (either ETDRS or exudate scores) and lipoprotein status, the ordinal score was treated as a dependent variable in the regression models, with each lipoprotein parameter as the independent variable. Three regression analyses were completely stratified by gender. In the first analysis (designated P^1 in Tables 3, 4, 5), univariate analyses between ordinal retinopathy scores and each lipoprotein parameter were completed. In the second (P^2 in the tables), a standard set of covariates was added to the model: age, duration of diabetes, HbA_{1c}, AER, creatinine clearance, hypertension, body mass index, waist-hip ratio, DCCT randomization group, smoking status, and for the combined cohort, gender. In the third analysis (P^3 in the tables), we determined whether the relationship between retinopathy scores and lipoprotein parameters was the same for men and women. The probabilities (P^1, P^2, P^3) reported in Tables 3, 4, and 5 represent tests of the linear relationship between retinopathy scores as dependent variables and the lipoprotein parameters.

At the time of DCCT close-out in 1993 (the most recent time point at which comparative data were available), our study cohort (n = 968) was representative of the entire DCCT/EDIC cohort, in that it did not differ from the total cohort according to any of the parameters shown in Table 1, or in its racial and ethnic composition.

RESULTS

Table 1 presents clinical characteristics of the 968 subjects with type 1 diabetes in whom NMR-LSP was performed, according to gender and former DCCT randomization group. As has been reported for the entire DCCT/EDIC cohort,[27] retinopathy remained more severe in subjects from the former conventional than the former intensive DCCT treatment group. This was the case, even though the difference in glycemic control between these groups, reflected by annual HbA_{1c} determination, narrowed dramatically between 1994 (year 1 of EDIC) and 1997 to 1999, the time of the present study.[16] By 1998, the difference in HbA_{1c} between former intensive and conventional treatment groups persisted for men only, and was small (0.2%) compared with the difference (1.7%) that was maintained (for both genders) during the DCCT.[14,15] For both former DCCT randomization groups, retinopathy was slightly but significantly more severe in men than in women (conventional group: $P < 0.02$; intensive group: $P < 0.05$; Kruskal-Wallis nonparametric test).

There were no differences in current lipid profiles (Table 1) between former DCCT intensive and conventional treatment groups; however, lipid profiles differed substantially between

Table 4. Associations of ETDRS Scores with NMR-Determined Lipoprotein Subclasses

NMR Lipoprotein Subclass (mg/dL)*	ETDRS Score			Significance		
	1–3 (237 Females; 231 Males: Total 468)	4–9 (147 Females; 255 Males: Total 402)	10–23 (35 Females; 42 Males: Total 77)	P^1	P^2	P^3
Large VLDL TG						
Female	5.6 (0.8)	5.8 (1.0)	10.5 (3.7)	**<0.02**	NS	
Male	9.6 (1.6)	14.6 (2.1)	17.4 (4.9)	NS	NS	
Total	7.6 (0.9)	11.4 (1.4)	14.3 (3.2)	**<0.05**	NS	NS
Medium VLDL TG						
Female	14.5 (1.2)	13.8 (1.6)	29.2 (6.4)	**<0.001**	**0.05**	
Male	26.3 (2.4)	31.0 (2.0)	43.3 (8.1)	**<0.01**	**<0.05**	
Total	20.4 (1.4)	24.7 (1.5)	36.9 (5.3)	**<0.0001**	**<0.01**	NS
Small VLDL TG						
Female	14.4 (0.9)	15.7 (1.2)	25.9 (3.4)	**<0.0001**	**<0.02**	
Male	19.5 (0.9)	22.4 (1.0)	34.7 (3.2)	**<0.0001**	**<0.0001**	
Total	16.9 (0.6)	19.9 (0.8)	30.7 (2.4)	**<0.0001**	**<0.0001**	NS
IDL Cholesterol						
Female	2.4 (0.3)	2.4 (0.4)	1.2 (0.4)	NS	NS	
Male	1.3 (0.2)	1.6 (0.3)	1.0 (0.4)	NS	NS	
Total	1.8 (0.2)	1.9 (0.2)	1.1 (0.3)	NS	NS	NS
L3 Cholesterol						
Female	64.9 (2.3)	75.3 (2.8)	72.4 (6.4)	NS	NS	
Male	59.5 (2.8)	52.4 (2.3)	43.4 (5.0)	**<0.01**	**<0.01**	
Total	62.2 (1.8)	60.8 (1.9)	56.6 (4.3)	0.10	NS	**<0.01**
L2 Cholesterol						
Female	31.3 (2.1)	28.3 (2.8)	28.2 (5.6)	NS	NS	
Male	37.8 (2.2)	45.9 (2.3)	46.4 (6.4)	NS	**0.05**	
Total	34.5 (1.5)	39.4 (1.8)	38.1 (4.4)	NS	NS	**<0.05**
L1 Cholesterol						
Female	25.9 (1.9)	26.2 (2.1)	29.2 (6.1)	NS	NS	
Male	28.4 (2.3)	31.0 (2.2)	49.0 (7.4)	**<0.001**	**<0.05**	
Total	27.1 (1.5)	29.3 (1.6)	40.0 (5.0)	**<0.001**	0.06	NS
Large HDL Cholesterol						
Female	44.2 (1.0)	40.9 (1.0)	39.6 (2.7)	**<0.05**	0.10	
Male	31.1 (1.0)	28.2 (0.9)	22.6 (1.8)	**<0.001**	**<0.001**	
Total	37.7 (0.8)	32.8 (0.7)	30.4 (1.8)	**<0.0001**	**<0.001**	NS
Small HDL Cholesterol						
Female	13.8 (0.4)	15.6 (0.5)	16.5 (1.1)	**<0.05**	NS	
Male	19.1 (0.4)	20.2 (0.4)	21.7 (1.0)	**<0.01**	**<0.05**	
Total	16.4 (0.3)	18.5 (0.3)	19.3 (0.8)	**<0.0001**	0.08	NS

Data are expressed as the mean ± SE. Definitions of probabilities are as in Table 3. TG, triglycerides.
* For subclass definitions, see Figure 1.

genders,[28] and, for this reason, data for men and women were analyzed separately. Tables 3 through 5 summarize the lipoprotein values for men, women, and the combined cohort, according to the categorical severity of retinopathy, as defined in Table 2. They also show probabilities representing the results of regression analyses that relate ordinal ETDRS score (used as a continuous variable) to each lipid parameter (as the independent variable). Three different regression analyses are reported as described earlier. For brevity, the multivariate analysis (P^2) will be emphasized in the text that follows. Table 3 presents the results for the conventional lipid profile. Although ETDRS scores were significantly related to triglyceride levels in univariate analyses, multivariate analyses (P^2) exhibited only a borderline association present in the combined cohort only. LDL cholesterol tended to increase in both genders with more severe retinopathy, but this was not significant in the multivariate analyses. HDL cholesterol was inversely associated with ETDRS score in both genders with univariate analyses, but only in men and the combined cohort in the multivariate analyses. There were no significant gender interactions with these parameters. In general therefore, more severe retinopathy was associated with higher total triglyceride levels, lower HDL cholesterol levels, and a trend toward higher LDL cholesterol.

Tables 4 and 5 present results from the detailed lipoprotein subclass profile obtained with NMR analysis. The tables describe 947 subjects in whom the multivariate analysis was possible for all covariates. Table 4 summarizes data for the individual lipoprotein subclasses. Subclasses of VLDL, LDL, and HDL all exhibited differential associations with ETDRS score. Although large VLDL was not associated with ETDRS score, medium and, in particular, small VLDL levels were strongly and positively associated with more severe retinopathy in both genders. For LDL, significant associations of subclasses were observed in men only. Multivariate analysis revealed that the larger, relatively less atherogenic L3 subclass was inversely associated with ETDRS score, whereas medium LDL (L2) and the smaller, denser, and more atherogenic L1 subclasses were positively associated. Thus, in men, more severe retinopathy was associated with a shift in LDL particle size distribution away from (large) L3 toward (small) L1. For HDL subclasses, associations with ETDRS score were again more marked in men. Multivariate analyses showed that in men and in the combined cohort, large HDL was inversely associated with ETDRS score, whereas small HDL was positively associated with ETDRS score. In women, a similar trend was seen with large HDL only.

TABLE 5. Associations of ETDRS Scores with NMR-Determined Lipoprotein Particle Diameter and LDL Particle Concentration

NMR Particle Size and LDL Concentration	ETDRS Score			Significance		
	1–3 (237 Females; 231 Males: Total 468)	4–9 (147 Females; 255 Males: Total 402)	10–23 (35 Females; 42 Males: Total 77)	P^1	P^2	P^3
VLDL size (nm)						
Female	53.61 (1.46)	51.26 (1.60)	45.45 (3.07)	**<0.05**	**<0.02**	
Male	49.29 (1.07)	47.52 (0.75)	43.31 (1.07)	**<0.002**	**<0.0001**	
Total	51.48 (0.91)	48.89 (0.76)	44.28 (1.51)	**<0.0001**	**<0.0001**	0.08
LDL size (nm)						
Female	20.99 (0.04)	21.10 (0.04)	21.07 (0.10)	NS	NS	
Male	20.88 (0.04)	20.76 (0.04)	20.55 (0.10)	**<0.0002**	**<0.02**	
Total	20.93 (0.03)	20.88 (0.03)	20.78 (0.07)	**<0.01**	NS	**<0.02**
HDL size (nm)						
Female	9.29 (0.03)	9.19 (0.03)	9.20 (0.08)	0.06	NS	
Male	8.92 (0.03)	8.83 (0.03)	8.64 (0.05)	**<0.0001**	**<0.001**	
Total	9.11 (0.02)	8.96 (0.02)	8.90 (0.05)	**<0.0001**	**<0.01**	0.07
LDL particle concentration (nmol/L)						
Female	1384 (24.77)	1459 (30.14)	1463 (68.59)	0.06	NS	
Male	1426 (28.05)	1484 (24.11)	1665 (85.21)	**<0.002**	0.07	
Total	1405 (18.69)	1475 (18.84)	1574 (56.84)	**<0.002**	NS	NS

Data are expressed as the mean ± SE. Definitions of probabilities are as in Table 3.

Table 5 shows data for the remaining NMR-derived lipoprotein parameters: mean particle size (diameter) for VLDL, LDL, and HDL, and molar particle concentration for LDL. Consistent with the data in Table 4, significant associations with ETDRS score were found more frequently in men than in women. In men, smaller VLDL, LDL, and HDL sizes were all associated with higher ETDRS scores, whereas higher LDL particle concentration showed a borderline association with ETDRS score. In women, the only significant association was with mean VLDL particle size. Among apolipoproteins A1 and B and Lp(a), and susceptibility of LDL to oxidation, only apoB in men and in the combined cohort was significantly associated with ETDRS score in multivariate analyses (data not shown). Hard exudate scores were available for 871 subjects from our cohort, and of these, 685 were classified as having no hard exudates, 99 had mild ones, and 87 had severe ones. Hard exudate scores exhibited essentially the same associations with NMR lipoprotein subclasses as total ETDRS scores (data not shown). Hard exudate scores also exhibited the same associations as ETDRS scores, with particle size determined by NMR and with conventional lipoprotein profiles, apolipoprotein A1 and B and Lp(a) levels, and susceptibility of LDL to oxidation (data not shown). Also, reanalysis of ETDRS scores after adjustment to remove the hard exudate component had no impact on the associations found (data not shown).

DISCUSSION

Our study describes new associations between plasma lipoproteins and diabetic retinopathy. The findings complement other recent work in which we have described the associations of gender, glycemia, and nephropathy status with serum lipoproteins in the same cohort of people with type 1 diabetes.[28,29] The strengths of all these studies are the extent of clinical data available in the DCCT/EDIC and the comprehensive nature of the lipoprotein analyses, which included not only conventional lipid profiles, but also apolipoprotein assays and NMR-LSP.

In the present study, NMR-LSP in particular revealed associations between retinopathy status and lipoprotein subclasses that were previously unknown and could not be discerned from conventional lipid profiles. The severity of retinopathy was only weakly associated with conventional lipid profiles (Table 3). Specifically, there was a borderline association between severity of retinopathy and triglycerides in the entire cohort, whereas in men and in the entire cohort, retinopathy score was associated with lower HDL cholesterol. The findings are broadly consistent with those in studies by others.[3,4] In contrast, much stronger associations and gender differences were identified by the NMR-LSP (Tables 4, 5). These associations were consistent across all three measures of retinopathy: ETDRS scores, hard exudate scores, and ETDRS scores with the hard exudate component subtracted. Furthermore, the associations involved subclasses within all three major lipoprotein classes (VLDL, LDL, HDL).

Retinopathy was strongly associated with small and medium, but not large, VLDL in each gender. Accordingly, retinopathy was inversely associated with average VLDL particle size. Associations between retinopathy and levels of triglyceride-rich lipoproteins may be partly explained by prothrombotic effects of the latter on vascular cells. For example, VLDL may increase secretion of plasminogen activator inhibitor (PAI-1) by human endothelial cells.[30]

Retinopathy was also independently associated with NMR-derived LDL parameters, but only in men. Thus, in men, retinopathy was positively associated with small LDL cholesterol concentration (L1, milligrams per deciliter) and LDL particle concentration (nanomolar) and negatively associated with large-LDL-cholesterol concentration (L3, milligrams per deciliter) and average LDL particle diameter (nanometers). The opposite associations of retinopathy with large and small LDL particles can explain why there was no association with (total) LDL-cholesterol in the conventional lipid panel. The absence of these associations in women reflects a gender difference that could not be discerned from the conventional lipid profile. Small LDL is known to be more atherogenic than large LDL,[11] perhaps because it more readily crosses the endothelium and/or is more readily oxidized, and these same properties may also contribute to retinal capillary injury. In the retinal capillary, oxidized LDL is toxic to both pericytes and endothelial cells[31] and may have prothrombotic effects[32] mediated by activation of protein kinase C.[33]

Similar considerations apply to HDL. The conventional lipid profile demonstrates that in men and in the combined cohort there is a significant inverse association (borderline in women) of retinopathy with HDL cholesterol. The NMR-LSP provides more insight, demonstrating a strong negative association with the large HDL subclass in men (borderline in women), and a positive (i.e., opposite) association with small HDL in men. These positive and negative associations with HDL subclasses are obscured in the conventional lipid panel, which quantifies only total HDL cholesterol. NMR-defined small HDL corresponds to HDL_{3b} and HDL_{3c} as defined by polyacrylamide gel electrophoresis, and these particles are considered potentially proatherogenic.[34] In contrast, the negative association of retinopathy with large HDL (corresponding to HDL_2) may be attributable in part to the paraoxonase activity associated with this lipoprotein. Paraoxonase detoxifies lipid peroxidation products, is carried in association with large HDL, and is believed to have a protective role against retinopathy.[35]

We also measured levels of apoB, apoA1, and Lp(a). Retinopathy was associated with apoB (present on VLDL, LDL, IDL, and Lp(a)) in men and in the combined cohort. Again, this reflects parallels with atherosclerosis risk, because elevated apoB is an established risk factor for coronary disease. ApoA1, present on HDL, trended downward with more severe retinopathy (low ApoA1 is also a cardiovascular risk factor), but the negative association of retinopathy with ApoA1 did not reach significance either for men or women. No association of retinopathy with Lp(a) was found, in agreement with the previous findings of Maser et al.,[36] but in contrast to those of Guerci et al.[37] Ongoing analyses of Lp(a) (phenotyping, genotyping) are in progress and may yield associations. Finally, we were unable to find any association of retinopathy status with the susceptibility of isolated LDL to oxidative stress.

Retinal hard exudates are the component of diabetic retinopathy most likely to be related to plasma lipoproteins, because the exudates are lipid rich. The ETDRS showed that severity of retinal hard exudates was strongly associated with total triglycerides, total cholesterol, and LDL cholesterol.[4] Also, elevated levels of these lipids conferred increased risk for future hard exudates and subsequent visual deterioration.[38] In our study, we found associations of hard exudates with conventional lipid measures in univariate analyses, but these were lost in multivariate analyses. Again, NMR lipoprotein subclass analyses revealed associations that remained significant in the multivariate analyses. Studies using in vitro modified LDL (to simulate modified, extravasated lipoproteins such as are found in hard exudates) demonstrate adverse effects on retinal capillary pericytes and endothelial cells,[31] and so over the years, hard exudates may reflect conditions (e.g., adverse lipoprotein profile, more severe capillary leakage) that accelerate retinopathy. Hard exudates are known to improve with treatment of hyperlipidemia, but typically this improvement does not reverse visual loss, at least in the short term.[6]

The associations we observed between retinopathy status and NMR-LSP are similar to those we recently described between nephropathy (AER) and NMR-LSP.[29] It is important to note that the association between retinopathy and NMR-LSP persisted after we controlled for AER in multivariate analyses. This supports a possible role for dyslipidemia in the development of retinopathy. Whereas one may envisage nephropathy as a cause, not a consequence, of alterations in plasma lipoproteins, it seems unlikely that retinopathy, per se, would cause dyslipidemia. Another recent study from our group, again using NMR-LSP, showed that insulin resistance in the absence of diabetes is associated with a dyslipidemia similar to that we now identify as associated with retinopathy,[39] at least for subclasses of LDL and HDL. It is of interest, however, that insulin resistance was associated with large, not medium or small, VLDL, as were retinopathy and the AER. These findings suggest that insulin resistance could underlie, at least in part, the dyslipidemia associated with both retinopathy and nephropathy. Thus, insulin resistance may confer risk, not only for atherosclerosis in patients with impaired glucose tolerance or type 2 diabetes, but also for atherosclerosis and microvascular complications, including retinopathy, in patients with type 1 diabetes.

In summary, our data show that within all three major lipoprotein classes (VLDL, LDL, and HDL), diabetic retinopathy is associated with a shift in subclass distribution toward smaller-diameter particles, and with an increase in LDL particle concentration. These associations cannot be detected from conventional lipid profiles, which do not discern subclass distributions. Our findings in relation to VLDL apply to both men and women, whereas those in relation to LDL and HDL apply much more strongly to men, in multivariate analyses. Because many of the alterations in lipoprotein subclasses have been shown to confer increased cardiovascular risk, our data are also consistent with the theory that dyslipoproteinemia may act as a common risk factor for retinopathy and atherosclerosis in diabetes.[40] The different associations of retinopathy with lipoprotein parameters between men and women suggest a gender differential in risk for retinopathy, because typically men have a much less favorable lipoprotein subclass profile than women. This was also the case in the DCCT/EDIC cohort.[28] Indeed, there is evidence in the literature that diabetic men may be more susceptible than women to retinopathy,[41,42] and our data from the DCCT/EDIC cohort are in concurrence with this. When we controlled for relevant variables, men had slightly but significantly more severe retinopathy. Further prospective studies, already in progress, are needed to support or refute the possibility that lipoprotein subclass profiles contribute directly to the development of retinopathy. Future studies must also determine whether measures to modify subclass distribution, which may include use of existing insulin-sensitizing agents, may prevent or mitigate retinopathy. NMR-LSP appears to be a powerful and valuable tool with which to address these questions.

Acknowledgments

The authors thank James Otvos, Ronald Klein, Larry Hubbard, Matthew Davis, John Lachin, and Saul Genuth for helpful discussions and Karina Moller, Yanis Bellil, Lyle Walton, Leslie Eldridge, Kevin Joyce, Andrea Semler, Jenny Smith, Leslie Nichols, Marlene Brabham, and Erica Hood for technical assistance.

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APPENDIX A

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