High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease

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Abstract High density lipoprotein (HDL) subfractions (2b, 2a, 3a, 3b, and 3c) separated by gradient gel electrophoresis (GGE) and defined by Gaussian summation analysis, and the compositions of HDL_2 and HDL_3, separated by preparative ultracentrifugation, were studied in four groups of men with or without non-insulin-dependent diabetes mellitus (NIDDM) and coronary artery disease (CAD): group 1 (DM+CAD+, n = 50); group 2 (DM−CAD+, n = 50); group 3 (DM+CAD−, n = 50); and group 4 (DM−CAD−, n = 31). HDL GGE subfraction distributions, available in 125 subjects, were not significantly different among the groups. In contrast, dividing the whole study population into quartiles of serum triglyceride (TG) concentration showed that high TG levels were significantly associated with low HDL_{2b} and high HDL_{3b} concentrations. In a multivariate linear regression model, postheparin plasma hepatic lipase (HL) activity, and fasting serum insulin and TG concentrations were all associated independently and inversely with low HDL_{2b}, but lipoprotein lipase or cholesteryl ester transfer protein activities were not correlated with HDL_{2b} concentrations. Group 1 tended to have the smallest mean particle sizes in the HDL subfractions, significantly ($P < 0.03$, CAD vs. non-CAD) for HDL_{2b} and for HDL_{2a}. These differences were independent of TG, insulin and HL, but lost their significance when adjusted for β-blocker therapy. Both HDL_2 and HDL_3 particles in group 1 were significantly depleted of unesterified cholesterol, and their HDL_2 was TG-enriched ($P = 0.053$). A high HL activity, hyperinsulinemia and hypertriglyceridemia are independently associated with low levels of HDL_{2b} and generally small HDL particle size. HDL particles in subjects with NIDDM and CAD are small-sized and have a low free cholesterol content. Both these characteristics may be markers of impaired reverse cholesterol transport.—**Syvänne, M., M. Ahola, S. Lahdenperä, J. Kahri, T. Kuusi, K. S. Virtanen, and M-R. Taskinen.** High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. *J. Lipid Res.* 1995. **36:** 573–582.

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Several studies have demonstrated an inverse relation between the risk of coronary artery disease (CAD) and the concentration of high density lipoprotein (HDL) cholesterol (1–5). As low HDL levels constitute one typical feature of the lipoprotein profile in non-insulin-dependent diabetes mellitus (NIDDM) (6, 7), it is reasonable to hypothesize that abnormalities in HDL might contribute to the well-established high risk of atherosclerosis in NIDDM (8). Indeed, low HDL cholesterol has been found to be predictive of CAD events in a prospective study of NIDDM patients (9). However, in another study, the predictive value of HDL cholesterol was relatively weak (10). The question therefore arises, might more detailed characterization of HDL improve the power to assess CAD risk in NIDDM.

Traditionally, HDL has been separated into two subfractions (HDL_2 and HDL_3) on the basis of flotation rate (11) in the analytical or preparative ultracentrifuge. Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) (12) is a technique that can separate HDL into subfractions differing primarily with respect to particle size. Five subfractions (in decreasing particle diameter 2b, 2a, 3a, 3b, and 3c) can be identified on the basis of the migration distance of the lipoproteins in a gel containing an increasing concentration of polyacrylamide. According to preliminary data, the concentration of the largest particles is reduced and there is a general shift toward smaller particles in NIDDM (13). Few studies have investigated HDL subfraction distribution in CAD by GGE, but the available data suggest that CAD patients (14–16) or those with an atherogenic lipid profile (17) have subnormal levels of the largest subfractions and generally smaller

Abbreviations: CAD, coronary artery disease; HDL, high density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; GGE, gradient gel electrophoresis; TG, triglyceride; CETP, cholesteryl ester transfer protein; BMI, body-mass index; LPL, lipoprotein lipase; HL, hepatic lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; apo, apolipoprotein; SRC, standardized regression coefficient.

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particle diameters within the HDL spectrum (18) in comparison with healthy controls. However, these studies did not analyze in detail the putative metabolic determinants of HDL subfraction distribution, such as the serum triglyceride (TG) concentration, hyperinsulinemia, and the activities of the lipolytic enzymes or cholesteryl ester transfer protein (CETP).

In the present study we investigated HDL subfraction distribution and particle size in men with NIDDM and CAD. These patients were compared with three control groups: one with CAD but no NIDDM; one with NIDDM but no CAD; and a group of healthy subjects. We also studied the compositions of HDL_2 and HDL_3. Moreover, we explored the metabolic features underlying HDL particle size and composition in this population.

METHODS

Subjects

Fifty consecutive NIDDM men who had undergone a clinically indicated elective coronary angiography at our hospital and showed significant coronary stenoses constituted group 1 (DM+CAD+). Three control groups were selected to have a similar age and body-mass index (BMI) distribution as group 1. The men in group 2 (DM−CAD+, n = 50) also had angiographically assessed CAD but no diabetes. Group 3 (DM+CAD−, n = 50) consisted of NIDDM patients with no history or symptoms of CAD. Ischemic heart disease in these subjects was further ruled out by exercise thallium-201 tomography. The men in group 4 (DM−CAD−, n = 31) had no known diseases, were not taking any regular medications, and all of them had a normal exercise ECG. None of the participants had hepatic, kidney, or thyroid disease, or were on lipid-lowering drugs. Most CAD patients had severe angina pectoris but were all in a stable state at the time of the studies.

The known duration of diabetes was 7.6 ± 5.4 years in group 1 and 7.4 ± 6.3 years in group 3. In both NIDDM groups, 12 patients were treated by diet only. Thirty (group 1) and 32 (group 3) were on oral hypoglycemic therapy, and 8 and 6, respectively, took insulin either alone or in combination with oral agents. Thirty subjects (group 1), 22 (group 2), 19 (group 3), and by selection none in group 4 had a history of hypertension. Forty-one subjects in group 1, 40 in group 2, six in group 3, and none in group 4 were on β-blocker therapy. In groups 1 to 4, 7, 8, 8, and 7 subjects were current, and 31, 33, 27, and 16 subjects were ex-smokers, respectively. Reported alcohol consumption did not significantly differ among the groups. Other selected characteristics of the study groups are outlined in **Table 1.**

All subjects gave their informed consent to participate in the study. The protocol was approved by the Ethical Committee of the First Department of Medicine, University of Helsinki.

TABLE 1. Characteristics of the study groups

	Group 1 DM+CAD+ n = 50	Group 2 DM−CAD+ n = 50	Group 3 DM+CAD− n = 50	Group 4 DM−CAD− n = 31	*P* CAD+ vs. CAD−	*P* DM+ vs. DM−	*P* CAD*DM Interaction
Age (years)	55 ± 6	55 ± 6	55 ± 7	53 ± 5	0.435	0.366	0.388
BMI (kg/m²)	29 ± 3	29 ± 4	29 ± 4	29 ± 3	0.491	0.814	0.987
HbA_{1c} (%)	7.6 ± 1.7	5.4 ± 0.4	7.1 ± 1.4	5.0 ± 0.4	0.012	<0.001	0.695
Serum insulin (mU/l)	14.6 ± 9.6	10.1 ± 5.9	12.3 ± 8.7	6.8 ± 3.4	0.017	<0.001	0.665
Serum triglyceride (mmol/l)	2.3 ± 1.1	2.0 ± 1.1	2.0 ± 1.3	1.6 ± 0.6	0.028	0.044	0.855
Serum cholesterol (mmol/l)	5.3 ± 0.9	5.7 ± 0.9	5.1 ± 0.9	5.8 ± 1.2	0.763	0.001	0.329
VLDL cholesterol (mmol/l)	0.73 ± 0.49	0.64 ± 0.47	0.63 ± 0.44	0.51 ± 0.27	0.079	0.113	0.827
IDL cholesterol (mmol/l)	0.27 ± 0.15	0.26 ± 0.17	0.24 ± 0.11	0.26 ± 0.13	0.533	0.878	0.628
LDL cholesterol (mmol/l)	3.2 ± 0.79	3.6 ± 0.77	3.1 ± 0.77	3.8 ± 1.04	0.863	<0.001	0.216
HDL cholesterol (mmol/l)	1.08 ± 0.25	1.19 ± 0.26	1.16 ± 0.32	1.24 ± 0.27	0.128	0.010	0.776
HDL_2 cholesterol (mmol/l)	0.52 ± 0.24	0.60 ± 0.22	0.59 ± 0.27	0.61 ± 0.23	0.294	0.065	0.384
HDL_3 cholesterol (mmol/l)	0.51 ± 0.09	0.55 ± 0.10	0.56 ± 0.11	0.63 ± 0.12	<0.001	0.001	0.310
HDL_2 protein (mg/dl)	45 ± 18	55 ± 19	55 ± 22	52 ± 18	0.209	0.244	0.041
HDL_3 protein (mg/dl)	86 ± 21	93 ± 16	90 ± 15	99 ± 17	0.077	0.004	0.788
ApoA-I (mg/dl)	104 ± 22	114 ± 17	121 ± 23	131 ± 28	<0.001	0.003	0.595
ApoA-II (mg/dl)	29 ± 6	34 ± 7	36 ± 7	38 ± 7	<0.001	<0.001	0.066
LPL activity	23 ± 6	25 ± 8	25 ± 8	26 ± 7	0.114	0.198	0.825
HL activity	41 ± 15	38 ± 18	35 ± 16	36 ± 16	0.103	0.668	0.310
CETP activity	1.09 ± 0.19	1.09 ± 0.19	1.09 ± 0.30	1.19 ± 0.26	0.171	0.180	0.129

BMI, body-mass index; HbA_{1c}, glycosylated hemoglobin A_{1c} (normal range, 4 to 6%); HDL, high density lipoprotein; Apo, apolipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; CETP, cholesteryl ester transfer protein. Values are mean ± SD. LPL and HL activities are in μmol free fatty acids • ml^{-1} • h^{-1}. CETP activity is in arbitrary units. HDL_2 and HDL_3 protein data were available in 47 (group 1), 49 (group 2), 49 (group 3), and 30 (group 4) subjects. CETP data were available in 45, 47, 44, and 30 subjects, respectively.

Laboratory measurements

Blood samples were obtained in the morning after an overnight fast. Postheparin plasma lipoprotein lipase (LPL) and hepatic lipase (HL) activities were determined by an immunochemical method (19) in plasma samples obtained 5 and 15 min after a bolus injection of heparin (100 IU per kg body weight). Lipoprotein fractions were separated from fresh fasting sera by sequential flotation in an ultracentrifuge (Beckman L 8-70, Beckman Inc., Palo Alto, CA) using a Ti 50.3 Beckman rotor as previously described in detail (20). Briefly, chylomicrons were isolated by centrifugation for 30 min at 18,000 rpm at 4°C. Thereafter, very low density lipoprotein (VLDL) was isolated at d 1.006 g/ml (18 h, 38,000 rpm), intermediate density lipoprotein at d 1.019 (24 h, 38,000 rpm), low density lipoprotein (LDL) at d 1.063 (24 h, 38,000 rpm), HDL_2 at d 1.125 (48 h, 38,000 rpm), and HDL_3 at d 1.210 (48 h, 38,000 rpm). Cholesterol, free (non-esterified) cholesterol, TG, and phospholipids were measured in serum and in the lipoprotein fractions by automated enzymatic methods (20, 21) and lipoprotein protein concentrations as described (22). Interassay coefficients of variation in our laboratory at concentrations typically found in unfractionated serum are: TG 2.7%, cholesterol 1.9%, free cholesterol 2.0%, phospholipids 1.7%. In concentrations measured in the lipoprotein fractions, the corresponding coefficients are: TG 3.4%, cholesterol 2.5%, free cholesterol 4.0%, phospholipids 2.5%, and protein 4.7%. Cholesteryl ester concentrations were calculated as the difference between total and free cholesterol. The compositions of HDL_2 and HDL_3 were calculated as mass percentage of free and esterified cholesterol, TG, phospholipid, and protein. Plasma apolipoprotein (apo) A-I and apoA-II concentrations were measured by immunoturbidimetry using commercially available kits (Boehringer Mannheim, Germany). CETP activity was determined by the method of Groener, Pelton, and Kostner (23). Briefly, 100 μl of plasma and 200 μl of polyethylene glycol (95 g/l, molecular weight 20,000) were mixed and centrifuged at 2,000 *g* for 15 min at 4°C to precipitate the apoB-containing lipoproteins. Thirty μl of the resulting supernate (HDL fraction containing CETP) was used in the incubation which included [^{14}C]cholesteryl ester-labeled LDL (250 nmol total cholesterol), unlabeled HDL (100 nmol total cholesterol), 0.7 μM DTNB, 17.5 μM of phosphate buffer at pH 7.4 in a total incubation volume of 355 μl. The incubation was performed at 37°C for 16 h. After the incubation, the tubes were placed on ice and 150 μl of 80 g/l bovine serum albumin and 50 μl of unlabeled LDL (total cholesterol 1 μM) were added. Thereafter 55 μl of an equivolume mixture of dextran sulfate (20 g/l) and 2 M magnesium chloride was added and the tubes were kept on ice for 20 min, after which the precipitated lipoproteins were pelleted by centrifugation at 2,000 *g* for 20 min at 4°C. The radioactivity of the supernatant was counted, which represented the cholesteryl ester transferred to HDL by the CETP of the plasma samples. The data obtained by this method have been shown to correlate closely with CETP mass (24). Serum-free insulin concentrations were measured by radioimmunoassay using the Phadeseph Insulin RIA kit (Pharmacia, Uppsala, Sweden) after precipitation with polyethylene glycol.

HDL GGE was performed by the method of Nichols, Krauss, and Musliner (25) as described (26). Briefly, 100 μl of the d < 1.21 serum fraction separated by ultracentrifugation was mixed with 25 μl of 40% sucrose, and 10 μl of the sample was electrophoresed on nondenaturing polyacrylamide 4/30 gradient gels (Pharmacia, Uppsala, Sweden) in Gel Electrophoresis Apparatus GE 2/4 (Pharmacia) for 2,000 Vh. After fixation, staining (0.04% Coomassie G-250 brilliant blue) and destaining, the gels were scanned at 595 nm with Molecular Dynamics (Sunnyvale, CA) Personal Densitometer using a 100 μm pixel size and 8-bit signal resolution. Particle diameters of the HDL subfractions were assessed by comparing the mobility of the sample with the mobility of calibration proteins (HMW, Pharmacia). Because manufacturing of the gradient gels was discontinued during the present study, HDL GGE could be performed on samples from 125 patients.

The HDL GGE subfractions were initially defined by two methods. First, we used fixed cut-points (25) to separate five HDL subfractions (HDL_{2b}, 12.9–9.7 nm; HDL_{2a}, 9.7–8.8 nm; HDL_{3a}, 8.8–8.2 nm; HDL_{3b}, 8.2–7.8 nm; HDL_{3c}, 7.8–7.2 nm). However, we found that these cut-points were arbitrary and often did not coincide with the actual HDL subpopulations observed (**Fig. 1**). Therefore, we used Gaussian summation analysis to separate the five HDL subpopulations, as described by Verdery et al. (27). This method uses an interactive computer program that allows the operator to model the scan into five Gaussian-shaped curves. The software integrates the scan area under the Gaussian subfractions, and the output defines the percentage of the area for each subfraction of the total scan area. Absolute protein concentrations in the subfractions are calculated as percentages of total HDL protein. In addition, the mean particle size of each subfraction is given. Moreover, we calculated the cholesterol concentration of each subfraction on the basis of their chromogenicities and total HDL cholesterol (27). All subfraction analyses were performed by an operator blinded to the clinical and lipoprotein characteristics of the patients.

Statistical analyses

The data are presented as mean ± SD. Variables with skew distributions were transformed into logarithms before statistical comparisons. The between-group differences were evaluated by an analysis of variance (ANOVA)

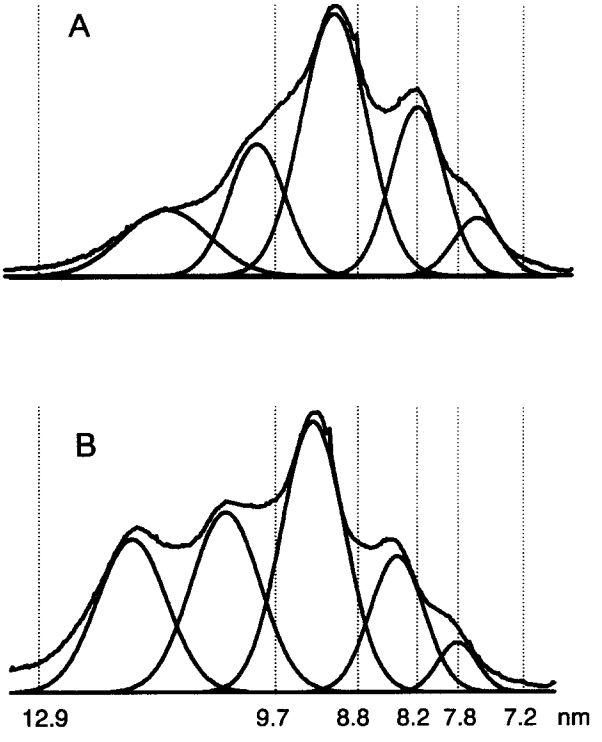

Fig. 1. Graphs illustrate Gaussian separation of high density lipoprotein (HDL) subfractions. The continuous line at the top of each panel is the original scanline, depicting relative protein concentration (ordinate) as a function of migration distance (abscissa). Thick bell-shaped lines represent Gaussian fitting of the scaline, defining the HDL subfractions, from left to right HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, and HDL_{3c}. Vertical dotted lines are the fixed cut-points according to Nichols et al. in ref. 25. These cut-points do not coincide with the Gaussian subfractions in these two individuals, as was the case in most subjects of the present study. Panel A: This subject has a relatively small fraction of HDL protein in the 2b subclass. Conversely, the subject depicted in panel B has a high relative protein concentration within HDL_{2b}.

in a two-by-two factorial design (28), the factors being the presence or absence of CAD and NIDDM. The possibility of an interaction between the two factors was also tested. If there is no evidence of a significant interaction, then a difference with respect to one factor (e.g., CAD) is independent of the other (NIDDM). Pearson's coefficients were calculated to study correlations.

Analyses of covariance were performed to assess the independence of between-group contrasts (28). The following covariates were evaluated: fasting serum TG concentration (log-transformed), fasting serum insulin concentration (logged), LPL activity (logged), HL activity (logged), CETP activity, therapy of diabetes (diet only, oral, or insulin with or without concomitant oral therapy), current smoking (yes or no), current alcohol use (yes or no), and therapy with β-blocking agents (yes or no).

Multivariate linear regression was used to study the determinants of HDL_{2b} and HDL_{3b} protein concentrations. All the covariates used in the covariance analyses were first entered as independent variables. The final model consisted of the significant or nearly significant predictors in the preliminary model.

RESULTS

Serum TG concentrations were highest in group 1 (DM+CAD+), and significantly related to the presence of both NIDDM and CAD (Table 1). Differences in HDL and HDL_2 cholesterol levels were small and significant only with respect to NIDDM. Likewise, protein concentrations in HDL_2 and HDL_3 were not predictors of CAD in this population, whereas low HDL_3 cholesterol levels were significant markers of both CAD and NIDDM. ApoA-I and A-II concentrations were clearly lower in group 1 than in any of the three other groups (Table 1).

Distribution of protein and mean particle size in HDL subfractions

Data on HDL GGE subfractions were available in 125 study subjects (**Table 2**). There were no consistent differences among the study groups in the percentage distribution of protein or the calculated protein concentrations in the GGE subfractions. The same was true for cholesterol concentrations calculated on the basis of the chromogenicities of the subfractions (data not shown). In contrast, the mean particle sizes of the five HDL subfractions were consistently smallest in group 1 (DM+CAD+), and a significant marker of CAD in fractions 2b and 2a, and borderline in 3a (**Table 3**).

As the mean particle size of HDL_{2b} appeared to be the most powerful of all GGE parameters to separate the groups, we performed analyses of covariance to test the independence of this association. The contrast between subjects with or without CAD seemed to be relatively independent of serum TG and insulin concentration, postheparin plasma lipolytic activities, CETP activity, diabetes therapy regimen, smoking, and alcohol consumption (data not shown). In contrast, the difference between CAD and non-CAD subjects was abolished when adjusted for the use of β-blockers (unadjusted $P = 0.011$, adjusted $P = 0.258$).

Correlations between HDL subfractions and other lipoprotein variables

The protein concentration in HDL_2 separated by ultracentrifugation was significantly related to the three largest GGE subfractions 2b, 2a, and 3a (**Table 4**). HDL_3 protein correlated with fractions 2a, 3a, and 3b. Thus, the GGE subfractions 2b and 3b appear to float with HDL_2 and HDL_3, respectively, whereas there is overlap in subfractions 2a and 3a with the traditional HDL subclasses. ApoA-I was correlated with the larger and apoA-II mainly with the smaller GGE subfractions (Table 4). Serum TG

TABLE 2. Percent distribution and concentration of protein in high density lipoprotein subfractions separated by gradient gel electrophoresis and analyzed by Gaussian summation analysis in the study groups

Fraction	Group 1 DM + CAD + n = 27	Group 2 DM − CAD + n = 36	Group 3 DM + CAD − n = 40	Group 4 DM − CAD − n = 22	*P* CAD + vs. CAD −	*P* DM + vs. DM −	*P* CAD*DM Interaction
2b							
Percent	10.1 ± 4.0	12.9 ± 6.4	11.5 ± 5.7	12.0 ± 6.6	0.871	0.243	0.372
Protein	13.2 ± 7.1	19.0 ± 10.3	17.0 ± 10.3	18.5 ± 11.9	0.417	0.056	0.179
2a							
Percent	17.4 ± 8.9	14.8 ± 6.5	16.4 ± 8.5	12.6 ± 4.3	0.228	0.020	0.678
Protein	23.7 ± 14.4	21.7 ± 10.3	23.9 ± 13.1	19.3 ± 8.4	0.700	0.259	0.578
3a							
Percent	40.0 ± 4.2	39.4 ± 9.4	39.4 ± 9.4	42.5 ± 7.7	0.425	0.386	0.219
Protein	53.0 ± 13.6	57.7 ± 20.1	57.2 ± 15.7	62.9 ± 10.3	0.113	0.077	0.858
3b							
Percent	26.9 ± 9.9	27.5 ± 11.0	28.0 ± 10.1	27.7 ± 7.3	0.734	0.948	0.785
Protein	36.0 ± 14.4	39.0 ± 15.5	39.2 ± 13.1	41.3 ± 12.8	0.291	0.320	0.870
3c							
Percent	5.3 ± 2.8	5.3 ± 2.7	4.7 ± 1.9	5.3 ± 4.0	0.567	0.647	0.555
Protein	6.8 ± 3.4	7.4 ± 3.3	6.8 ± 3.0	7.7 ± 5.2	0.808	0.247	0.788

Values are percent of total HDL protein or protein concentration in mg/dl, mean ± SD.

was inversely related to HDL_{2b} and directly to HDL_{3b}. HL activity had a moderate inverse correlation with HDL_{2b}, but LPL activity did not correlate with any of the GGE subfractions. These correlations were essentially similar when calculated separately within subgroups of subjects with or without NIDDM and CAD (data not shown).

Serum triglyceride concentration as a determinant of HDL subfraction distribution

We divided the whole study population of 181 men into quartiles according to fasting serum TG levels. The cut-points were 1.35, 1.71, and 2.31 mmol/l, and the mean ± SD TG concentrations of the quartiles were: Q_1, 1.03 ± 0.19; Q_2, 1.55 ± 0.10; Q_3, 2.01 ± 0.18; Q_4, 3.46 ± 1.27 mmol/l. **Table 5** shows that the proportion and protein concentration of HDL_{2b} were significantly larger and those of HDL_{3b} smaller in the bottom versus the top TG quartile. Likewise, the TG level was an important determinant of the mean particle size of all HDL subfractions, hypertriglyceridemia being associated with a small particle size (**Table 6**).

Multivariate analyses

To obtain a more complete understanding of the determinants of HDL subfraction distribution, we performed stepwise multivariate regression analyses with HDL_{2b} (log-transformed) and HDL_{3b} protein concentrations as dependent variables, and the independent variables listed in Methods.

HDL_{2b} (n = 124) was significantly and independently predicted by HL (standardized regression coefficient [SRC] −0.365, $P < 0.001$), insulin (SRC −0.245, $P = 0.003$), and TG (SRC −0.170, $P = 0.039$). The adjusted r^2 for this model was 0.255, i.e., it explained about 25% of the variance in HDL_{2b} protein concentration. The other variables were not related to HDL_{2b} concentrations and were removed from the final model. Excluding the insulin-treated NIDDM patients did not alter these associations. To evaluate whether pooling the four study groups might have distorted the results, we re-estimated the model for subjects with or without NIDDM and those with or without CAD. The results did not materially differ in these subgroups compared with the

TABLE 3. Mean particle diameters of high density lipoprotein subfractions in the study groups

Fraction	Group 1 DM + CAD + n = 27	Group 2 DM − CAD + n = 36	Group 3 DM + CAD − n = 40	Group 4 DM − CAD − n = 22	*P* CAD + vs. CAD −	*P* DM + vs. DM −	*P* CAD*DM Interaction
2b	11.02 ± 0.43	11.14 ± 0.38	11.29 ± 0.53	11.28 ± 0.30	0.011	0.502	0.394
2a	10.10 ± 0.32	10.14 ± 0.30	10.28 ± 0.47	10.26 ± 0.30	0.029	0.853	0.611
3a	9.19 ± 0.26	9.24 ± 0.22	9.38 ± 0.39	9.30 ± 0.29	0.053	0.939	0.336
3b	8.34 ± 0.20	8.38 ± 0.179	8.45 ± 0.32	8.40 ± 0.26	0.167	0.987	0.327
3c	7.74 ± 0.16	7.78 ± 0.20	7.82 ± 0.26	7.80 ± 0.22	0.176	0.734	0.444

Values are nm, mean ± SD.

TABLE 4. Pearson's correlation coefficients between protein concentrations in high density lipoprotein subfractions and other selected variables

Fraction	HDL_2 Protein[a]	HDL_3 Protein[a]	ApoA-I	ApoA-II	Log TG	LPL	HL
Log 2b	0.587[c]	0.126	0.378[c]	0.164	−0.293[c]	0.107	−0.388[c]
Log 2a	0.393[c]	0.281[c]	0.212[b]	0.132	−0.026	0.071	−0.049
3a	0.431[c]	0.496[c]	0.395[c]	0.332[c]	−0.078	0.059	0.021
3b	−0.019	0.340[c]	0.159	0.296[c]	0.259[b]	0.047	0.173
3c	0.165	0.086	−0.004	0.003	−0.096	−0.038	−0.111

HDL, high density lipoprotein; Apo, apolipoprotein; TG, triglyceride; LPL and HL, postheparin plasma lipoprotein lipase and hepatic lipase activities.
[a] HDL_2 and HDL_3 fractions separated by ultracentrifugation.
[b] $P < 0.05$; [c] $P \leq 0.001$.

pooled data. Thus, the SRCs for HL varied from −0.285 to −0.440, those for insulin from −0.206 to −0.277, and those for TG from −0.144 to −0.225.

In similar analyses with HDL_{3b} protein concentration as the dependent variable (n = 112), TG (SRC 0.292, $P = 0.002$), β-blocker therapy (SRC −0.229, $P = 0.014$), CETP activity (SRC 0.178, $P = 0.049$), and HL (SRC 0.164, $P = 0.072$) were selected as independent predictors. The adjusted r^2 for this model was 0.145. The relations between HDL_{3b} on the one hand and TG and β-blocker use on the other were consistent regardless of CAD and NIDDM (data not shown), but those with CETP and HL appeared to apply only to subjects without CAD.

In similar multivariate analyses of the mean particle sizes of the HDL subfractions (data not shown), HL activity had a significant inverse relation to all subfractions. In addition, TG was a significant independent negative predictor of the particle sizes of the three largest GGE subfractions 2b, 2a, and 3a. LPL activity correlated positively with the mean size of HDL_{2b}. The sizes of the two smallest subfractions, 3b and 3c, were negatively related to serum insulin concentration, in addition to HL activity. All of these associations were independent of the presence or absence of either NIDDM or CAD.

HDL composition

Of the ultracentrifugically separated HDL subfractions, HDL_2 was enriched with TG in group 1 (DM+CAD+, 8.4 ± 3.5% of total lipoprotein mass) as compared with the healthy controls (6.9 ± 2.7%), the other two groups falling in between (group 2, 7.1 ± 2.6%; group 3, 7.8 ± 3.6%). However, overall, TG enrichment of HDL_2 was only borderline significant with respect to the presence or absence of NIDDM ($P = 0.053$), and not significant for the CAD factor. Both HDL_2 (group 1, 3.9 ± 1.1%; group 2, 4.0 ± 1.0%; group 3, 4.2 ± 0.8%; group 4, 4.4 ± 1.0%) and HDL_3 (2.0 ± 0.7%, 2.1 ± 0.9%, 2.2 ± 0.5%, 2.4 ± 0.4%, respectively) in the

TABLE 5. Percent distribution and concentration of protein in high density lipoprotein subfractions in quartiles of fasting triglyceride concentration

Fraction	Q_1[a] n = 39	Q_2 n = 33	Q_3 n = 25	Q_4 n = 28
2b				
Percent	14.8 ± 7.2	12.0 ± 4.4	9.5 ± 4.0	9.1 ± 4.3[b]
Protein	21.9 ± 13.0	16.9 ± 6.7	14.4 ± 9.3	12.6 ± 6.4[b]
2a				
Percent	15.5 ± 6.1	16.3 ± 8.2	15.0 ± 7.9	15.2 ± 8.8
Protein	22.9 ± 11.3	23.4 ± 12.0	21.8 ± 12.9	21.0 ± 12.4
3a				
Percent	39.9 ± 7.0	42.1 ± 8.1	39.1 ± 4.9	38.6 ± 11.6
Protein	57.4 ± 13.1	61.1 ± 17.6	56.7 ± 14.1	53.9 ± 19.2
3b				
Percent	24.6 ± 9.6	24.5 ± 7.9	29.7 ± 9.4	32.3 ± 10.4[b]
Protein	34.5 ± 12.4	34.7 ± 11.5	44.9 ± 14.5	44.3 ± 15.1[b]
3c				
Percent	5.2 ± 3.4	5.1 ± 2.7	5.3 ± 2.5	4.8 ± 2.2
Protein	7.3 ± 4.2	7.1 ± 3.8	7.5 ± 3.5	6.6 ± 2.7

Values are percent of total HDL protein or protein concentration in mg/dl, mean ± SD.
[a] Q_1 to Q_4 indicate quartiles of fasting serum triglyceride concentration in the entire study population (n = 181). High density lipoprotein (HDL) subfraction data were available in 125 subjects.
[b] Significant (ANOVA $P \leq 0.001$) trend across quartiles.

TABLE 6. Mean particle sizes of high density lipoprotein subfractions in quartiles of fasting triglyceride concentration

Fraction	Q_1 n = 39	Q_2 n = 33	Q_3 n = 25	Q_4 n = 28
2b	11.43 ± 0.47	11.14 ± 0.35	11.15 ± 0.33	10.93 ± 0.45[a]
2a	10.39 ± 0.41	10.14 ± 0.32	10.14 ± 0.25	10.04 ± 0.37[a]
3a	9.44 ± 0.37	9.21 ± 0.27	9.23 ± 0.21	9.16 ± 0.25[a]
3b	8.51 ± 0.34	8.34 ± 0.20	8.37 ± 0.17	8.34 ± 0.16[b]
3c	7.89 ± 0.29	7.74 ± 0.20	7.75 ± 0.14	7.74 ± 0.12[b]

Values are nm, mean ± SD.
[a] $P \leq 0.001$; [b] $P < 0.01$, ANOVA trend across quartiles.

DM+CAD+ group were depleted of free cholesterol. These differences were significant regarding CAD (HDL_2, $P = 0.038$; HDL_3, $P = 0.032$) but not NIDDM. Otherwise, there were no consistent differences in HDL subfraction composition between the study groups.

When the study subjects were divided into TG quartiles rather than by NIDDM and CAD, a strong TG enrichment of both HDL_2 and HDL_3 was apparent in the top TG quartile, as expected (**Table 7**). We further analyzed the determinants of TG-enrichment of the HDL subfractions by multivariate linear regression models. Using the percentage of TG of the lipoprotein mass as the dependent variable we found, not unexpectedly, that the serum TG concentration (logged) was the most powerful predictor (SRC 0.603, $P < 0.001$ for HDL_2; SRC 0.528, $P < 0.001$ for HDL_3). However, the log of serum insulin concentration (SRC 0.156, $P = 0.020$ for HDL_2; SRC 0.133, $P = 0.064$ for HDL_3) and HL activity (SRC −0.136, $P = 0.038$; SRC −0.246, $P = 0.001$, respectively) were also independently related to the degree of TG enrichment of the HDL subfractions. Notably, HL was inversely related to TG-enrichment, consistent with its role as a TG hydrolase of these particles. Finally, we found that the cholesteryl ester content of HDL_2 was inversely related to the log of serum TG (SRC −0.256, $P = 0.002$) and CETP activity (SRC −0.194, $P = 0.018$).

DISCUSSION

In this study HDL subfractions, separated by their migration distances on polyacrylamide gradient gels, mainly reflecting particle size, were not efficient markers of CAD in men with NIDDM. On the other hand, when all study subjects were pooled and reclassified according to their TG levels, marked differences in HDL subclass distributions were apparent. The importance of TG concentrations for HDL size distribution has been previously demonstrated by Chang, Hopkins, and Barter (29) who showed that in subjects with fasting TG values above approximately 1.5 mmol/l the largest HDL subfractions were virtually absent. Thus, an abnormal HDL subclass distribution seems to be one of the adverse metabolic consequences of hypertriglyceridemia, as recently discussed

TABLE 7. Compositions of the HDL_2 and HDL_3 subfractions in the quartiles of fasting triglyceride concentration

	Q_1[a] n = 40	Q_2 n = 36	Q_3 n = 41	Q_4 n = 35
HDL_2				
Triglyceride	5.8 ± 2.1	6.9 ± 2.2	7.8 ± 2.9	10.1 ± 3.6[b]
Free cholesterol	4.4 ± 1.1	4.0 ± 0.8	4.0 ± 1.1	3.9 ± 0.9
Cholesteryl ester	23.6 ± 2.7	23.2 ± 3.0	22.3 ± 3.7	21.8 ± 3.0[c]
Phospholipid	26.1 ± 3.8	25.2 ± 2.6	23.6 ± 3.7	22.8 ± 3.7[b]
Protein	40.1 ± 3.7	40.6 ± 4.2	42.3 ± 6.3	41.3 ± 4.2
HDL_3				
Triglyceride	3.8 ± 1.6	4.5 ± 1.9	4.6 ± 1.8	6.4 ± 2.3[b]
Free cholesterol	2.4 ± 0.9	2.0 ± 0.6	2.2 ± 0.6	2.0 ± 0.5[d]
Cholesteryl ester	18.6 ± 2.3	18.4 ± 1.8	17.7 ± 1.9	16.5 ± 1.8[b]
Phospholipid	21.1 ± 2.0	21.0 ± 2.3	21.5 ± 2.3	20.8 ± 2.3
Protein	54.2 ± 2.5	54.1 ± 2.7	54.0 ± 2.4	54.2 ± 3.5

Values are percentages of high density lipoprotein (HDL) subfraction mass, mean ± SD.
[a] Q_1 to Q_4 indicate quartiles of fasting serum triglyceride concentration in the entire study population (n = 181). Compositional data were available in 152 subjects.
[b] $P < 0.001$; [c] $P = 0.057$; [d] $P < 0.05$, ANOVA trend across quartiles.

by Grundy and Vega (30). Of note, the change in HDL size and composition is apparent already at quite moderately elevated TG levels, in fact within the conventional normal range. As recently shown by Austin and colleagues (31), similar mild TG elevations radically alter LDL particle size distribution also.

Our data are in good concordance with those of Johansson and colleagues (14) who found no differences in HDL subfractions between mainly nondiabetic, normolipidemic CAD patients and controls, but did find low HDL_{2b} and high HDL_{3b} levels in hypertriglyceridemic CAD patients. Katzel and coworkers (15) and Wilson et al. (16) reported low HDL_{2b} percentages in men with silent myocardial ischemia and myocardial infarction, respectively. However, neither of these studies presented multivariate analyses. Thus, in these two studies it remained unclear whether the HDL subfraction distribution had independent power to distinguish CAD patients from controls regardless of TG levels and other potential confounders.

It is of interest that, in addition to the serum TG concentration, HL activity and the fasting serum insulin concentration were independent negative predictors of HDL_{2b} levels. In hypertriglyceridemia, CETP-mediated exchange of core lipids between HDL and VLDL results in TG-enrichment of HDL (32), demonstrated also by our compositional data. As the excess TG is hydrolyzed by HL (33), the particle size decreases and the concentration of the large HDL_{2b} subclass falls. Insulin-resistant states and the metabolic consequences of insulin resistance are associated with increased VLDL synthesis (34, 35) and high HL activity (15, 36). Therefore, hyperinsulinemia, which reflects insulin resistance, may partially underlie both hypertriglyceridemia and enhanced HL activity.

However, the fact that TG, HL, and insulin were independently associated with low HDL_{2b} levels implies that other mechanisms may also be involved. One possibility is that the stimulating effect of insulin on adipose tissue LPL activity (37–39) might be blunted in insulin resistance. An efficient lipolysis of the TG-rich lipoproteins adds lipid constituents to HDL particles which consequently increase in size. In contrast, a low LPL activity, a characteristic of NIDDM (38), would decrease the formation of HDL_{2b}. That LPL was not a significant predictor of HDL_{2b} in our multivariate analysis does not rule out this metabolic link. It is conceivable that the high prevalence of insulin resistance or some other metabolic characteristic may distort the relationship between HDL_{2b} and LPL in our study population consisting largely of NIDDM patients, as Johansson et al. (40) found a significant positive correlation between these variables in a mainly non-diabetic population. A subgroup analysis of our study lends tentative support to this idea: there was no relationship between HDL_{2b} and LPL in groups 1, 2, or 3, but there was a moderate positive relation within group 4, the least insulin resistant of our study groups ($r = 0.305$), although this correlation was not statistically significant ($P = 0.167$) because of the small number of subjects (n = 22).

When HDL subfractions are separated by Gaussian summation analysis (27), the mean particle size of each fraction will vary between individuals. Therefore it was possible to compare the particle sizes of the HDL subpopulations between our study groups. In the DM+CAD+ group the average sizes of all five subfractions, especially HDL_{2b}, HDL_{2a}, and HDL_{3a}, were smaller than those of the non-CAD groups (Table 3). Although the impact of serum TG concentration on HDL particle size appeared to be larger than that of NIDDM and CAD (Table 6), the between-group difference did not disappear when adjusted for TG levels or several other covariates. Our data are in agreement with those of Cheung et al. (18) who reported a general shift toward smaller particle sizes in CAD patients. Because small particles are lipid-poor compared with larger particles, the implication is that HDLs are lipid-depleted in CAD and thus presumably less efficient vehicles of reverse cholesterol transport (41). However, adjustment for β-blocker therapy rendered the between-group difference with respect to CAD nonsignificant, suggesting that a small HDL particle size may be a secondary phenomenon, not necessarily a characteristic of CAD per se. A prospective study would be needed to evaluate the predictive value of HDL particle size.

When comparing the compositions of HDL_2 and HDL_3 in group 1 (DM+CAD+) to those in the non-CAD groups, we found a reduced free cholesterol content and TG-enrichment, although the latter was of borderline statistical significance. Likewise, Lane and colleagues (42) found that HDL_2 in women with NIDDM was depleted of free cholesterol and HDL_3 was enriched in TG. Ahnadi and coworkers (43) found similar compositional abnormalities in NIDDM subjects and showed that they were associated with decreased transfer of cholesteryl ester from HDL to acceptor particles. Thus, a low free cholesterol and a high TG content of HDL particles in NIDDM patients with CAD may be another marker of impaired reverse cholesterol transport in this group. Again, hypertriglyceridemia may be the most important mechanism underlying HDL compositional changes, as demonstrated previously by Deckelbaum et al. (44). In agreement with the present data (Table 7) they found that increasing TG levels were associated with enrichment in TG and depletion in phospholipids, esterified and free cholesterol of HDL particles.

To summarize, the HDL particles in men with NIDDM and CAD appear to be characterized by small size, TG enrichment, and depletion of free cholesterol. Of these characteristics, TG content seems to be related to the presence of NIDDM regardless of CAD, and con-

versely, particle size and free cholesterol content are predicted by CAD independent of NIDDM. These alterations may reflect impaired reverse cholesterol transport in subjects with NIDDM and CAD, but they appear to be largely secondary to factors such as treatment with β-blockers and metabolic features such as insulin resistance, hypertriglyceridemia, and the activities of the lipolytic enzymes, especially HL. HDL subfraction distribution, assessed by Gaussian modeling of the subpopulations separated by nondenaturing polyacrylamide gradient gel electrophoresis, was neither a marker of CAD in men with NIDDM nor a marker of NIDDM as such. Postheparin plasma HL activity and fasting serum insulin and TG concentrations were the main determinants of HDL subfraction distribution in our study population.

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