Analysis of high density lipoproteins by a modified gradient gel electrophoresis method

Zhengling Li, Judith R. McNamara, Jose M. Ordovas, and Ernst J. Schaefer[1]

Lipid Metabolism Laboratory, USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111

Abstract   A high resolution electrophoretic method has been developed to separate plasma high density lipoprotein (HDL) particles by size using 4–30% polyacrylamide agarose (PAA) gradient gels, Sudan black B staining, and laser densitometry. Fourteen distinct HDL bands were observed with HDL-1 being designated as the largest particle and HDL-14 as the smallest particle. HDL-1 was similar in size to ferritin (Stokes diameter 12.2 nm), HDL-8 to catalase (9.2 nm), and HDL-13 to lactate dehydrogenase (8.1 nm). HDL-1 to HDL-7 were found within the density range of HDL_{2b}, (d 1.063–1.10 g/ml), HDL-8 to HDL-10 within HDL_{2a} (d 1.10–1.125 g/ml), and HDL-11 to HDL-14 within HDL_3 (d 1.125–1.21 g/ml). On immunoblotting, apolipoprotein A-I (apoA-I) was found in all HDL bands examined, being most prominent in HDL-6, 11, and 12. ApoA-II was not detected in HDL bands 1–5, but was present in all other HDL bands and was most prominent in HDL-9. ApoE was detected mainly in HDL bands 1–7, and was observed in only trace amounts in other bands. Lp A-I isolated by immunoaffinity column chromatography from the plasma of five subjects contained five subspecies (HDL-5, 6, and 11–13), while Lp A-I/A-II also had five subspecies (HDL-8, 9, and 11–13) in these subjects. In normal subjects (n = 57) four or five HDL bands were generally observed, with HDL-9, 11, and 12 being the most frequently observed. Mean HDL particle score (method of sizing based on scanning densitometry, where low score indicates large size and high score indicates small size) was significantly correlated ($P < 0.001$) with the concentrations of HDL cholesterol ($r = -0.796$), HDL free cholesterol ($r = -0.780$), HDL cholesteryl ester ($r = -0.683$), HDL phospholipid ($r = -0.663$), HDL apoA-I ($r = -0.577$), and HDL protein ($r = -0.459$), but not with HDL triglyceride ($r = 0.069$). In addition, HDL particle score was significantly correlated ($P < 0.05$) with HDL total mass ($r = -0.649$), HDL free cholesterol content (% of total mass, $r = -0.608$), HDL triglyceride content ($r = 0.415$), HDL phospholipid content ($r = -0.359$), and HDL protein content ($r = 0.295$), but not with HDL cholesteryl ester content ($r = -0.219$) or HDL apoA-I content ($r = 0.183$). Stepwise multiple regression analysis results indicated that HDL free cholesterol level (HDL_{FC}) and content (%HDL_{FC}) were the most important factors associated with HDL particle score. ■ This methodology can be used to assess the effect of diet and drug therapy on HDL particle size as well as the relationship of this parameter with heart disease risk in population studies.— Li, Z., J. R. McNamara, J. M. Ordovas, and E. J. Schaefer. Analysis of high density lipoproteins by a modified gradient gel electrophoresis method. *J. Lipid Res.* 1994. **35:** 1698–1711.

Supplementary key words high density lipoprotein subspecies • HDL cholesterol

Epidemiological studies have shown that a decreased HDL cholesterol level is associated with an increased risk of coronary heart disease (CHD) (1–5). HDL particles, especially HDL_2 and Lp A-I particles, promote cholesterol efflux from cells (6–9). The interrelationships among HDL particle size, HDL function, and HDL lipid content have not been well defined. Moreover, simple and precise methods for studying the heterogeneous nature of HDL particles within populations have not been readily available. Several methods can be used to separate HDL particles, such as ultracentrifugation, precipitation, immunoaffinity chromatography, and various types of electrophoresis. In general, these methods are time-consuming, require special instruments, are costly, and may require specific antibodies. In addition, HDL particles have been found to be altered during some of these procedures. Therefore, the methods to date to examine HDL heterogeneity have not been ideal for population studies. Gradient gel electrophoresis is a relatively simple and precise method to examine HDL subspecies and five subpopulations of HDL have previously been reported (10). Such studies have required prior separation of HDL from plasma by ultracentrifugation or precipitation, and then identification of HDL subspecies by 4–30% polyacrylamide agarose (PAA) gels with Coomassie blue staining (10–12). This latter analysis allows for the separation of HDL subspecies based on particle size.

The aims of the present study were to modify the 4–30% PAA electrophoretic method so that plasma could be used to study HDL particle size instead of HDL fractions, to improve resolution of HDL subspecies by altering electrophoresis and staining conditions, and to examine the associations of HDL particle size with HDL composition.

MATERIALS AND METHOD

Study subjects

Subjects studied included 26 males and 31 females in good health, (n = 57, mean age: 51 ± 11 years with a range of 26 to 72) who were on ad libitum diet, and were not taking any medications known to affect plasma lipids. These subjects had no secondary causes of lipid abnormalities (diabetes, liver disease, or kidney disease) and were selected to have a wide range of triglyceride values. In order to assess HDL particle size changes before and after feeding, three additional subjects had blood samples drawn at 0, 5, 8, 10, and 24 h during a diet study, in which subjects were consuming an average isocaloric American diet (36% of calories as fat, 15% protein, 49% carbohydrate, 140 g of cholesterol/1000 calories, 15% saturated fat, 15% monounsaturated fat, and 6% polyunsaturated fat). Meals were given shortly after the 0-h time point, at 4 h and at 9 h, and a snack was given at 12 h. The 0-h time point blood sample was obtained after a 12-h overnight fast.

Plasma and HDL separation

All subjects had blood drawn in 0.15% EDTA tube after a 12–14 h fast except three subjects during the diet study (see above). Plasma was separated by centrifugation [2500 rpm(1000 g), 4°C, 20 min]. The HDL fraction was obtained after precipitation with dextran–sulfate–Mg^{2+} following the method of Warnick, Benderson, and Albers (13). HDL (d 1.063–1.21 g/ml) was also isolated from the plasma of all subjects by sequential ultracentrifugation (39,000 rpm, 4°C, 18–48 h), while HDL density subspecies from three subjects with varying sizes of HDL particles were isolated by sequential ultracentrifugation as HDL_{2b} (d 1.063–1.10 g/ml), HDL_{2a} (1.10–1.125 g/ml), HDL_{3a} (1.125–1.147 g/ml), HDL_{3b} (1.147–1.167 g/ml), and HDL_{3c} (1.167–1.21 g/ml). Potassium bromide was used to adjust the density (14).

Non-denaturing gradient gel electrophoresis

Electrophoresis equipment and supply. Electrophoresis was performed on a Pharmacia GE 2/4 electrophoresis unit (Pharmacia, Piscataway, NJ) that was attached to a 2209 Multitemp circulating pump (Pharmacia) and an LKB 2197 power supply (LKB Instruments Inc., Paramus, NJ). Non-denaturing 4–30% polyacrylamide agarose (PAA) gradient gels and loading applicators were obtained from Pharmacia (Piscataway, NJ). The gels were rocked during staining and destaining on an Incu-shaker 357 (Lab-Line Instruments Inc., Melrose Park, IL). Scanning and peak integration were performed with an LKB Ultrascan XL laser densitometer interfaced with an AT&T personal computer (LKB), a Canon PJ-108A printer (LKB), and GSXL software.

The 4–30% PAA Gels used in this study were obtained from Pharmacia. However, these gels are no longer commercially available. We have tested the 4–30% PAA gels obtained from Isolab (Akron, OH). With these gels we did not obtain sufficient resolution for our studies. However, efforts are underway by this manufacturer to rectify this problem. We have recently tested 4–30% PAA gels prepared in Dr. David Rainwater's laboratory (San Antonio, TX) as previously described (15), and these gels provided us with results that were very similar to those obtained with the Pharmacia gels. These gels were used for experiments shown in Figure 5. The GE 2/4 electrophoresis unit required for this method is also no longer available from Pharmacia, but is available from Taylor Plastic (San Antonio, TX) or CBS Scientific (Solana Beach, CA).

Buffer preparation. The buffer was prepared by combining 0.1018 M Tris-base, 0.0678 M boric acid, and 0.0025 M Na_2 EDTA to a final pH of 8.57. This buffer is stable for up to 6 months. The buffer must be precooled at 2°C for at least 30 min before using.

Sample storage and preparation. EDTA plasma, plasma stored at −80°C for 6 months, serum, HDL supernate, and HDL fractions separated by sequential ultracentrifugation were run using the PAA gel system. Samples (60 μl of plasma or HDL fraction, at twice its original concentration) were mixed with a 40% sucrose solution containing 0.1% bromophenol blue (20 μl) 3:1 (v/v) before loading to increase the specimen density and to provide a visual marker during loading and electrophoresis. In order to examine the effect of HDL concentration on HDL particle mobility (R_f) and percentage concentration (% area), samples were also run under standard dilution condition (3:1), as well being further diluted with normal saline at dilutions of 3:2, 3:3.7, 3:7, and 3:17.

Electrophoretic conditions and sample loading. The apparatus was filled with 3.5 l of fresh buffer, and gels were inserted and equilibrated for 20 min at 8°C at 160 V. After adding sample to each lane, samples were subjected to pre-electrophoresis at 75 V for 20 min, and then electrophoresis with a setting of 400 V (actual readings were 200 V and 250 Amps) for 24 h. A multiple loading technique was used to increase the concentration of HDL particles. A 15-μl aliquot of each prepared sample (plasma, sucrose solution) was loaded prior to pre-electrophoresis (75 V, 20 min). After 20 min of pre-electrophoresis and 1 h of electrophoresis at 400 V, a second 15-μl aliquot was added. A final 15-μl aliquot was loaded at the end of another hour of electrophoresis, for a total of 45 μl. Control plasma samples containing HDL bands 6, 9, 11, and 12 were run in the lanes furthest to the left and right of each gel to assist in identifying HDL bands and to control for run-to-run variability.

Gel staining and scanning. Sudan black B stain (Fisher, Milwaukee, WI) was used to visualize lipoprotein particles. The preparation of staining and destaining solution has been reported previously (16, 17). Gels were stained for 20 h with gentle rocking, destained in a 50% solution of ethylene glycol monoethyl ether in distilled H_2O for 2 days, and stored in water for at least 2 days to restore size and shape. The gels were then scanned at 633 nm with an LKB laser densitometer, using GSXL software which allows for the integration of HDL bands to obtain peak position and percentage area (16, 17). This method assumes identical chromogenicity of all HDL bands following Sudan black B staining.

HDL band nomenclature and identification. High molecular mass protein standards, thyroglobulin (669 kDa, Stokes diameter 17 nm), ferritin (440 kDa, 12.2 nm), catalase (232 kDa, 9.2 nm), lactate dehydrogenase (140 kDa, 8.1 nm), and albumin (67 kDa, 7.1 nm) (Pharmacia) were used to obtain a standard curve on 4–30% PAA gels, using the same electrophoretic procedure as for plasma, but stained with Coomassie blue and destained in a 2:1:6 solution of methanol–acetic acid–distilled water (v/v/v). We assigned a reference factor (R_f) for each HDL band mobility as follows:

$$R_f = \frac{\text{migration distance of HDL band}}{\text{migration distance of albumin}}$$

Albumin was used as the reference protein (R_f of albumin = 100%).

The Stokes diameter in nm (Y) of individual HDL bands was calculated using the equation shown below, where X is the previously designated R_f value of the particle. This equation was generated using the software package RS/1, to fit the standard curve with a 3rd degree function.

$$Y = -50.28 * X^3 + 126.02 * X^2 - 109.05 * X + 40.40$$

For example, if the migration distance for an HDL band was 40 mm from the top of the gel, and the migration distance for albumin was 70 mm, this HDL band would have an R_f of 0.57 (40/70 = 0.57). The Stokes diameter of this HDL particle would then be:

$$Y = -50.28 * 0.57^3 + 126.02 * 0.57^2 - 109.05 * 0.57 + 40.40 = 9.87 \text{ nm}$$

HDL band integration. We calculated a weighted HDL particle score for each subject by multiplying each band number by its fractional area, according to the following equation:

$$\text{weighted HDL score} = \sum_{i=1}^{n=14} (i * X_i)$$

Where i is the designation of the HDL band, X is the fraction of total area for that band. For example, if subject A had four HDL bands, HDL-6, HDL-9, HDL-11, and HDL-12, and the fractional area for each band was 0.30, 0.30, 0.25, and 0.15, then the weighted HDL particle score of subject A would be 9.05 as calculated:

$$6*0.3 + 9*0.3 + 11*0.25 + 12*0.15 = 9.05$$

The weighted HDL score combines the HDL size distribution and the percentage concentration of each HDL band. Because the largest HDL particle is designated HDL-1 and the smallest HDL-14, subjects with smaller HDL particles have higher weighted HDL scores.

Lipid measurements

Plasma total cholesterol (TC), triglyceride (TG), HDL cholesterol (HDL-C, after dextran sulfate–Mg^{2+} precipitation), and the HDL total cholesterol (HDL_{TC}), HDL free cholesterol (HDL_{FC}), HDL triglyceride (HDL_{TG}), and HDL phospholipid (HDL_{PL}) within the HDL region (d 1.063–1.21 g/ml) were measured enzymatically (Abbott Diagnostics, Dallas, TX) on an Abbott Diagnostics ABA-200 bichromatic analyzer (18). Phospholipid reagent was obtained from WAKO Pure Chemical Industries, Richmond VA.

LDL cholesterol was calculated by the method of Friedewald, Levy, and Fredrickson (19), except for subjects who had triglyceride values >400 mg/dl. In these latter subjects, the LDL cholesterol level was determined by direct measurement after ultracentrifugation as follows: LDL-C = cholesterol$_{1.006B \text{ infranate}}$ – HDL-C). Compositional data for the HDL density fraction (1.063–1.21 g/ml) were adjusted by using the HDL cholesterol values obtained by precipitation as a reference to account for losses during ultracentrifugation. All lipid analyses were standardized through the Centers for Disease Control (CDC)-National Heart, Lung, and Blood Institute Lipid Standardization Program (Atlanta, GA). Our laboratory serves as part of the CDC's Cholesterol Reference Method Laboratory Network.

Lipoprotein particle separation

Lipoprotein particles containing apoA-I without apoA-II (Lp A-I), and apoA-I and A-II (Lp A-I/A-II) were isolated from the plasma of five subjects by immunoaffinity column chromatography utilizing antibodies specific for apoA-I or apoA-II as previously described (8). These fractions were subjected to 4–30% PAA gel electrophoresis to determine HDL particle size as previously described.

Western immunoblotting method

PAA gels (4–30%) were used to separate HDL particles by size. Each HDL band was then cut out and subjected to SDS 4–22.5% polyacrylamide gradient gel electropho-

resis to separate the apolipoproteins by molecular weight as previously described (20). The apolipoproteins in each HDL band were then transferred to nitrocellulose paper, and monoclonal antibodies for apoA-I and A-II and polyclonal antibody for apoE were used to detect the presence of apolipoproteins in each HDL band (21).

LCAT and CETP inhibition testing

Fresh plasma was incubated with or without 5,5'-dithio-bis(2-nitrobenzoic acid) or DTNB, a lecithin:cholesterol acyltransferase (LCAT) inhibitor, at final concentration of 0.2 mM, at 4°C [no cholesteryl ester transfer protein (CETP) activity present] or 37°C (CETP activity present) for 5 h. Samples were then subjected to 4–30% PAA gel electrophoresis to determine whether these different incubation conditions affected HDL particle mobility (R_f) and percentage concentration (% area).

HDL protein measurement

The concentrations of apoA-I (HDL$_{apoA-I}$), apoB (HDL$_{apoB}$), and Lp[a] (HDL$_{Lp[a]}$) within HDL fractions (d 1.063–1.21 g/l) were measured by noncompetitive, enzyme-linked immunosorbent assays (ELISA) (22, 23). Samples were diluted 1:60,000 for the HDL$_{ApoA-I}$ assay, 1:200 for the HDL$_{Lp[a]}$ assay, and were not diluted for the HDL$_{apoB}$ assay (plasma samples are normally diluted 1:60,000, 1:20, and 1:3000 for apoAI, Lp[a], and apoB, respectively). Control samples were used on each plate as internal standards. Total protein within HDL fractions (HDL$_{pro}$) was measured by a modification of Lowry's method (24, 25), using bovine serum albumin (BSA) as a standard.

Statistical analysis

Statistical analyses were performed with the RS/1 software package (BBN Research Systems, Cambridge, MA) and the Statistical Analysis System (SAS) software (SAS Institute, Incorporated, Cary, NC) on a VAX 11/785 Computer (Digital Equipment Corporation, Maynard, MA). Pearson correlation coefficient analysis was used to test the statistical significance of associations of HDL particle score with other parameters. Stepwise multiple regression analysis was used to ascertain the significance of multiple variables in determining HDL particle score. The RS/1 program was used to compute the means and standard deviations, and to obtain the equations for the standard curves and for the estimation of the Stoke's diameter of each HDL band.

RESULTS

Fourteen discrete HDL bands were noted in the subjects studied (n = 57, see Table 1). When HDL subfractions were run on gradient gel electrophoresis, HDL$_{2b}$ migrated as HDL-1 to HDL-7, HDL$_{2a}$ migrated as HDL-8 to HDL-10, HDL$_{3a}$ as HDL-11, HDL$_{3b}$ as HDL-12, HDL-13, and HDL$_{3c}$ as HDL-14. The previous designations, as listed in **Table 1** are based on the studies of Blanche et al. (10). Estimated Stokes diameter and density for each HDL band are provided (see Table 1).

In addition, HDL bands were cut out from the 4–30% PAA gels, and subjected to SDS polyacrylamide gel electrophoresis to separate apolipoproteins. Immunoblotting was then carried out to determine apoA-I, A-II, and E

TABLE 1.   Identification of HDL bands

HDL Band Number	R_f	Estimated Stokes Diameter[a]	Estimated Density[b]	Previous Designation	ApoA-I[c]	ApoA-II[c]	ApoE[d]
		nm	g/ml				
HDL-1	0.44	12.46	1.063	HDL2b	+	−	+ + +
HDL-2	0.47	11.74	1.068	HDL2b	+	−	+ +
HDL-3	0.49	11.25	1.074	HDL2b	+	−	+
HDL-4	0.51	10.96	1.079	HDL2b	+ +	−	+
HDL-5	0.53	10.55	1.084	HDL2b	+ +	−	+
HDL-6	0.56	10.00	1.089	HDL2b	+ + +	+	+
HDL-7	0.59	9.57	1.095	HDL2b	+ +	+	+
HDL-8	0.62	9.24	1.100	HDL2a	+ +	+	trace
HDL-9	0.66	8.90	1.113	HDL2a	+ +	+ + +	trace
HDL-10	0.68	8.73	1.125	HDL2a	+ +	+ +	trace
HDL-11	0.71	8.53	1.147	HDL3a	+ + +	+ +	trace
HDL-12	0.75	8.30	1.167	HDL3b	+ + +	+ +	trace
HDL-13	0.76	8.14	1.190	HDL3b	+ +	+	trace
HDL-14	>0.86	<7.86	1.210	HDL3c	+ +	+	trace

[a]Based on the diameter and R_f of known standards, thyroglobulin (17.0 nm), ferritin (12.2 nm), lactate dehydrogenase (8.1 nm), and albumin (7.1 nm). Using the equation $Y = -50.28 \cdot X^3 + 126.02 \cdot X^2 - 109.05 \cdot X + 40.40$ where Y is estimated Stoke's diameter and X is the R_f of each band (see Methods for details). This equation generated from RS/1, to fit the standard curve with a 3rd degree function.
[b]Calculated from the equation of: $Y = -5.784e - 0.5 \cdot X^3 + 7.03e - 0.3 \cdot X^2 - 0.15 \cdot X + 1.95$ obtained from RS/1.
[c]Monoclonal antibodies for apoA-I, A-II were used to detect the presence of apos in each HDL band.
[d]Polyclonal antibody for apoE was used to detect the presence of apoE in each HDL band.

content in each HDL band, as described in the Methods section. These samples were obtained from six individuals and the relative contents of apolipoproteins detected from these samples were qualitatively classified after gel scanning as nondetectable ($-$), trace, 1+, 2+, or 3+. In these studies, apoA-I was detected in all HDL bands from 1 through 14, and was present as 1+ in HDL-1 through 3, 2+ in HDL-4, 5, 7, 8, 9, 10, 13, and 14, and 3+ in HDL-6, 11, and 12 (see Table 1). No apoA-II was detected in HDL-1 through 5, but was present as 1+ in HDL-6, 7, 8, 13, and 14, as 2+ in HDL-10 through 12, and as 3+ in HDL-9 (see Table 1). ApoE was present as 3+ in HDL-1, 2+ in HDL-2, 1+ in HDL-3 through 7 and as trace in HDL-8 through 14 (Table 1). These data are consistent with the concept that apoE is mainly present in large HDL particles and that apoA-II is mainly present in intermediate and small HDL particles especially in HDL-9, 10, 11, and 12, while apoA-I is present throughout the entire HDL spectrum, but is especially prominent in HDL-6, 11, and 12.

HDL particles containing apoA-I without apoA-II (Lp A-I) obtained from immunoaffinity columns were noted to have five subspecies on 4–30% PAA gels: HDL-5, 6, 11, 12, and 13 in samples from five subjects. HDL particles containing both apoA-I and apoA-II (Lp A-I/A-II) were also found to have five subspecies: HDL-8, 9, 11, 12, and 13 from the same subjects. Therefore, there is significant overlap between Lp A-I and Lp A-I/A-II with regard to particle size, and these particles cannot be separated by 4–30% PAA gel electrophoresis.
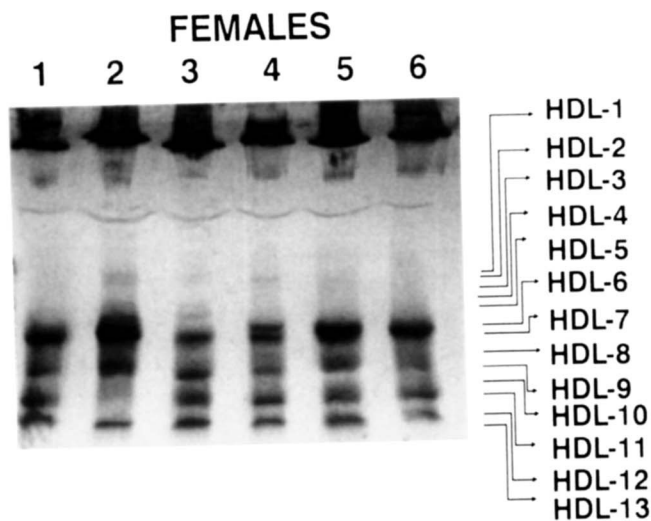
Typical 4–30% PAA gradient gels and integrations from laser scanning densitometry for four females and four males are shown in Figs. 1–4. Lanes 1 and 6 of Figs. 1 and 3 show control plasma containing HDL bands 6, 9, 11, and 12. Scans for lanes 2 to 5 shown in **Fig. 1** are provided in **Fig. 2** (females), and scans for lanes 2 to 5 shown in **Fig. 3** are provided in **Fig. 4** (males). In these analyses males had lower mean HDL cholesterol levels, smaller mean HDL particle sizes, and higher mean HDL particle scores than females. For the four females (Figs. 1 and 2), the mean HDL cholesterol level and HDL particle score were 57 ± 13 mg/dl and 8.6 ± 1.0, respectively, whereas for the four males (Figs. 3 and 4), these values were 41 ± 13 mg/dl and 9.5 ± 3.1, respectively.

In order to examine the effects of HDL concentration on HDL particle size, we have carried out dilution experiments. **Table 2** shows the HDL particle mobility (R_f) and HDL particle percentage concentration (% area) results when samples were run under standard conditions and with various dilutions. R_f values were unchanged while the % areas were changed somewhat when plasma was diluted by more than twofold (see Table 2). These data indicate that HDL concentration does not significantly affect HDL particle mobility, but it does have a modest effect on % area. With increasing dilution, the mean HDL particle score increased progressively from 8.13 to 8.58, a change of 5.5% (see Table 2). These data indicate that dilution of plasma with saline can decrease HDL particle size slightly.

An analysis of data obtained from 108 separate gel electrophoretic runs of the same control sample (separate frozen aliquots) documented that between-run coefficients of variation for HDL score, % of HDL as HDL_2, and % of HDL as HDL_3 were 0.03%, 0.06%, and 0.06%, respectively. In these analyses, the mean HDL score ($\pm$ SD) was 8.91 ± 0.35, and the mean % of HDL as HDL_2 and HDL_3 ($\pm$SD) were 57.4 ± 7.0% and 42.6 ± 7.0%, respectively. Mean R_f values ($\pm$SD) of HDL-6, HDL-9, HDL-11, and HDL-12 were 0.54 ± 0.07, 0.60 ± 0.01, 0.67 ± 0.01, and 0.72 ± 0.01, respectively.

In order to determine whether the state of feeding affects HDL particle distribution, 4–30% PAA gel electrophoresis was performed on plasma samples obtained from three individuals after an overnight fast and at 0, 5, 8, 10, and 24 h time points. Meals were given at 0, 4, 9, and 12 h. No differences were seen in the HDL banding pattern (R_f) in these samples, and no significant changes in the HDL particle percentage concentration (% area) before or after meals were noted. The mean HDL particle score ($\pm$SD) for these subjects was 11.02 ± 1.35 at 0 h, 11.02 ± 1.14 at 5 h, 11.08 ± 1.20 at 8 h, 11.12 ± 1.11 at 10 h, and 10.85 ± 1.20 at 24 h, respectively (maximal 2.4% change). In addition, when the results of electrophoresis obtained on samples (n = 3) stored at $-80°$C for 6 months were compared with the results obtained on

FEMALES

Fig. 1. HDL bands obtained from 4–30% PAA gel electrophoresis of whole plasma from four females. Lanes 1 and 6 represent control samples that contain HDL bands 6, 9, 11, and 12. The HDL cholesterol concentrations and HDL particle scores for samples from the four females were: lane 2, 76 mg/dl and 7.26; lane 3, 51 and 9.26; lane 4, 45 and 9.30; and lane 5, 56 and 8.69, respectively, with mean values of 57 ± 13 mg/dl, and 8.6 ± 1.0. Higher HDL cholesterol concentrations were associated with larger HDL sizes and lower HDL scores.
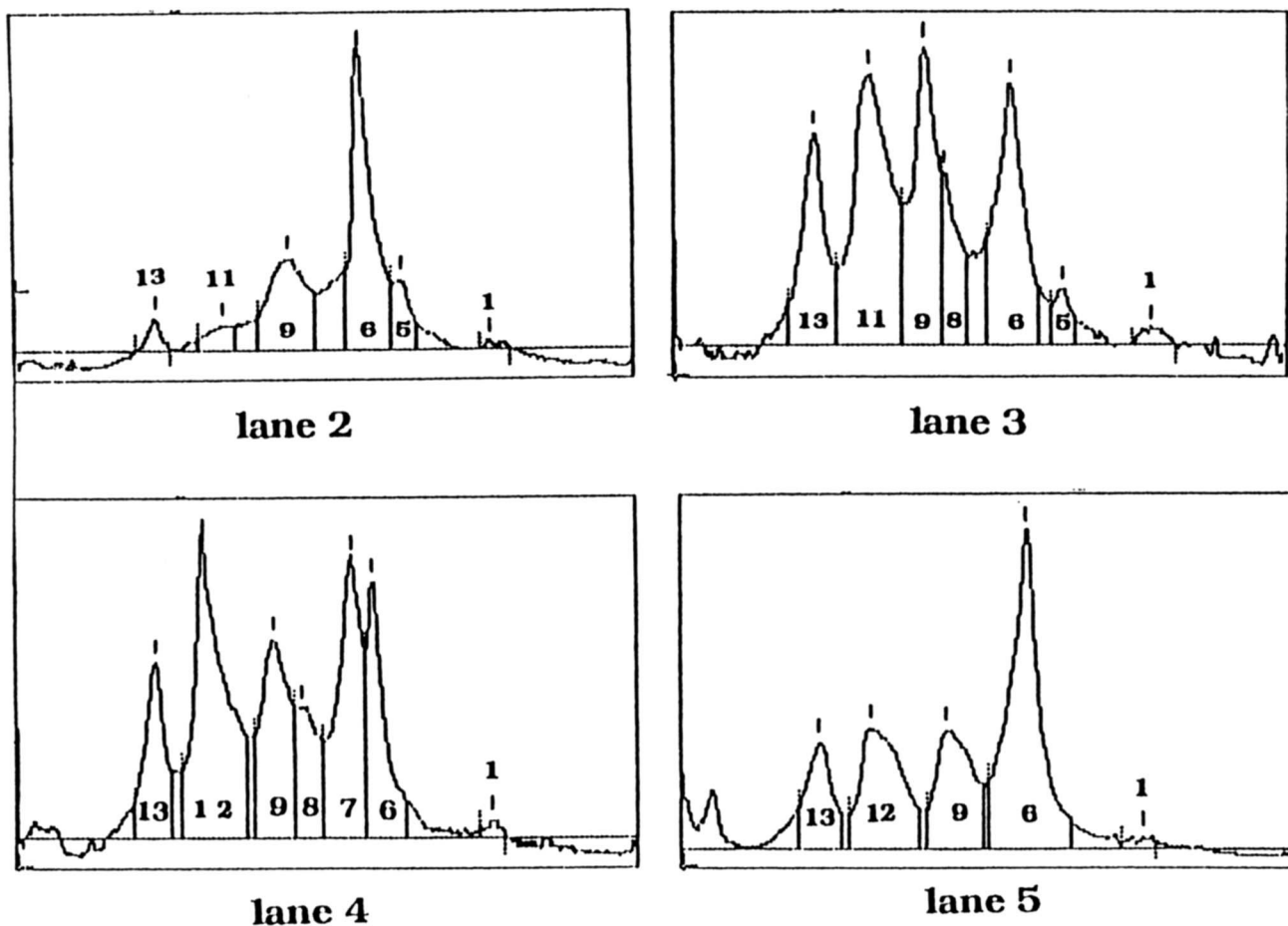
Fig. 2. Densitometric scans of plasma samples from the four females shown in Fig. 1. HDL band 1 represents the largest HDL, while HDL 14 represents the smallest HDL.

fresh plasma samples, no significant differences were observed in the HDL banding pattern or particle size with mean HDL particle scores of 9.85 ± 1.35 versus 9.93 ± 1.25 (fresh vs. frozen) in these three subjects (0.8% change, see **Fig. 5**). These data are consistent with the concept that 4–30% PAA gel analysis for HDL sizing can be carried out on frozen plasma samples, provided they have never been previously thawed, as well as on fresh samples, and that acute feeding has no significant effect on mean HDL particle score. We have previously made similar observations when assessing LDL particle size with 2–16% PAA gel electrophoresis (16).

In **Fig. 6,** the results of LCAT and CETP inhibition studies are shown. HDL particle mobility (R_f) was relatively stable, but there was some shift of HDL particle percentage concentration (% area) in these studies. The HDL particle score was 7.13 at 4°C with DTNB (lane 1 in Fig. 6), 6.92 at 37°C with DTNB (lane 2), 6.76 at 4°C (lane 3, standard conditions), and 6.82 at 37°C (lane 4). These data provide evidence that when LCAT and CETP activities are present, HDL band percentage concentration can be affected, while HDL band mobility is not
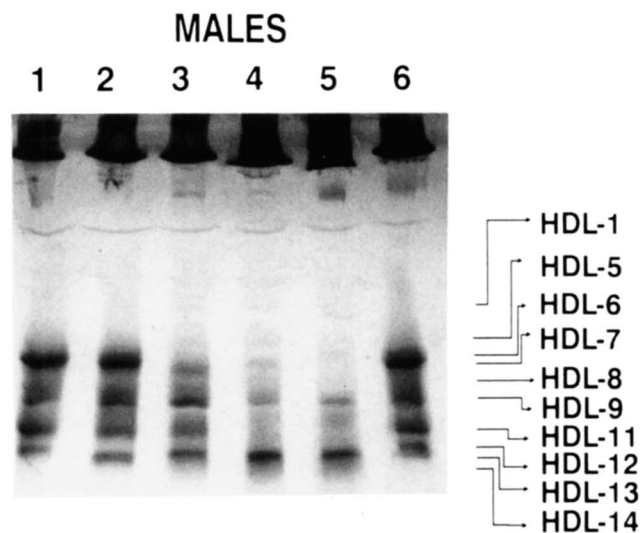

Fig. 3. HDL bands obtained from 4–30% PAA gel electrophoresis of whole plasma from four males. Lanes 1 and 6 represent controls. The HDL cholesterol concentrations and HDL particle scores were: lane 2, 58 mg/dl and 8.98; lane 3, 45 and 10.12; lane 4, 35 and 11.10; and lane 5, 27 and 11.91, respectively, with mean values of 41 ± 13 mg/dl and 9.5 ± 3.1, respectively.
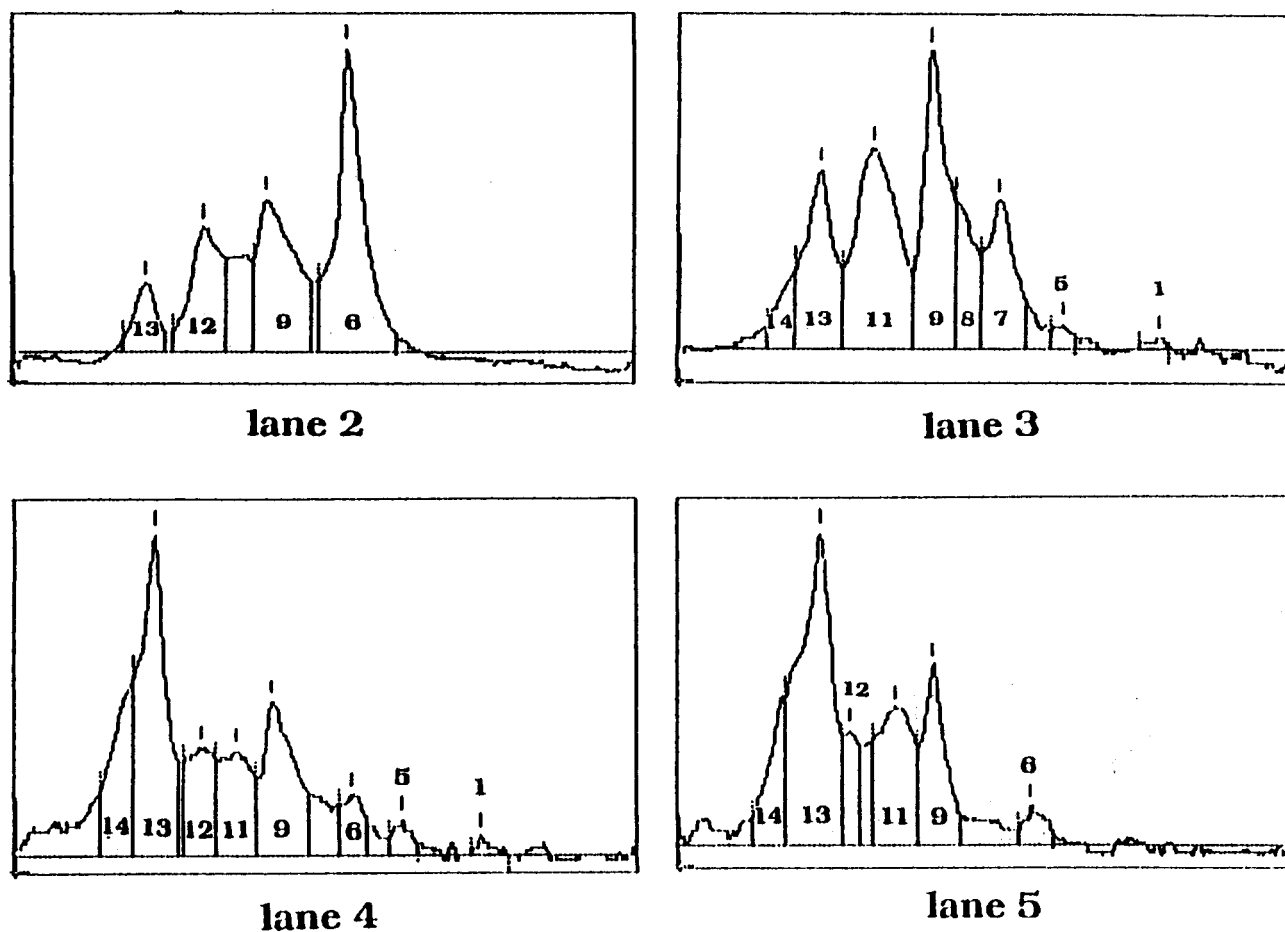
Fig. 4. Densitometric scans of plasma samples from the four males shown in Fig. 3.

affected. When gels were run under standard conditions at 4°C (with CETP inhibited), there was a 5.5% increase in HDL particle score or decrease in HDL particle size with the addition of LCAT inhibitor. The gels used in these experiments were obtained from Dr. David Rainwater's laboratory (Fig. 6).

In our view, it is best to classify subjects based on HDL particle score, which is calculated by combining the HDL size distribution and the relative staining intensity of each band, similar to what we have recommended for LDL particle size (16, 26). In **Table 3** we have grouped study subjects by HDL particle score into four categories, those

TABLE 2. R_f and % area of HDL bands with varying dilution conditions

HDL Band	3:1[a]	3:2	3:3.7	3:7	3:17	Mean ± SD
HDL-6						
R_f[b]	0.56	0.56	0.56	0.56	0.56	
% area[c]	44.8 ± 2.1	43.7 ± 0.8	40.0 ± 1.3	39.8 ± 1.9	48.0 ± 0.5	41.3 ± 2.9[d]
HDL-9						
R_f	0.65	0.65	0.65	0.65	0.65	
% area	31.8 ± 0.8	29.5 ± 1.3	30.4 ± 1.2	28.0 ± 1.0	20.5 ± 0.5	28.9 ± 2.6
HDL-11						
R_f	0.70	0.70	0.70	0.70	0.70	
% area	23.4 ± 1.3	26.8 ± 0.4	29.6 ± 2.4	32.2 ± 1.0	31.3 ± 0.1	29.8 ± 4.8
Mean HDL Particle Score[e]	8.13 ± 0.08	8.23 ± 0.02	8.39 ± 0.08	8.45 ± 0.07	8.58 ± 0.05	8.36 ± 0.2

[a]X:Y represents sample dilution condition, where X is plasma in the mixed sample, and Y represents dilution factors (saline and sucrose solution).
[b]Relative migration of HDL band (R_f of albumin = 1), see text for calculation.
[c]Mean and SD of HDL percentage concentration of three integrations in each dilution condition.
[d]Mean and SD were obtained from 15 integrations for each HDL band in all the dilutions.
[e]Mean and SD of HDL score were obtained from three integrations in each dilution condition, see text for calculation.
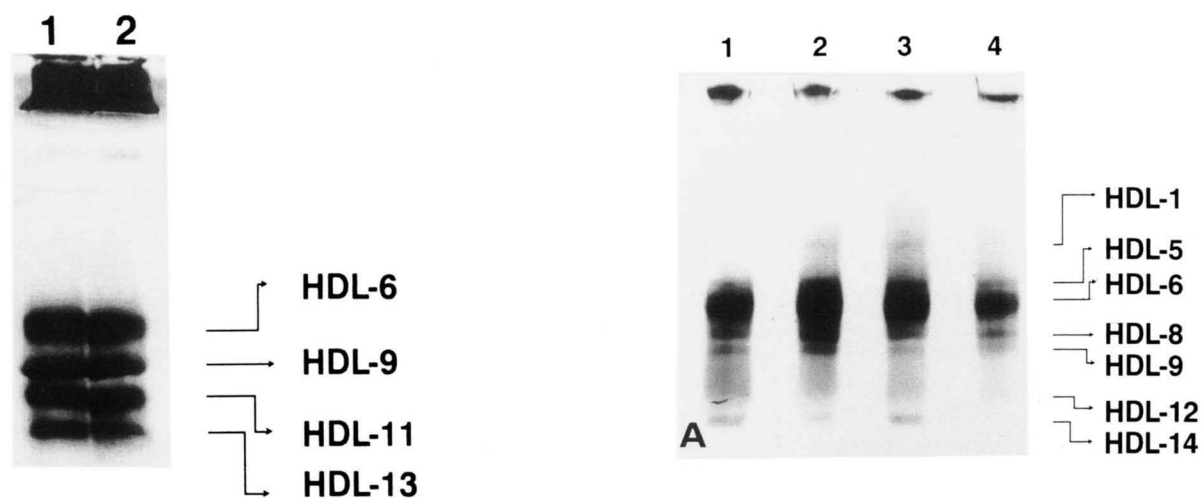
Fig. 5. HDL bands obtained from 4–30% PAA gel electrophoresis of whole plasma from one subject. Lane 1 represents fresh plasma, and lane 2 represents frozen plasma stored at −80°C for 6 months.

whose mean HDL score was less than 8 (large HDL particle group); subjects whose mean HDL particle score was 8–10.9 (intermediate HDL particle group); a third group of subjects whose mean HDL score was 11–11.9 (small HDL particle group); and those whose mean HDL score was 12–12.9 (very small HDL particle group). As can clearly be seen in Table 3, subjects within the large HDL particle group had the lowest mean triglyceride levels (70.0 mg/dl) and the highest mean HDL-C (72.4 mg/dl), and the highest mean % of HDL as HDL_2 (88.9%), while subjects in the very small HDL particle group had the highest mean triglyceride values (445.8 mg/dl), the lowest mean HDL-C levels (34.1 mg/dl), and the lowest mean % of HDL as HDL_2 (12.3%).

Data on HDL composition in these subjects grouped by HDL score category are shown in **Table 4.** In this analysis, HDL mass equals the sum of HDL_{Pro}, HDL_{PL}, HDL_{FC}, HDL_{CE}, and HDL_{TG}, measured within the density region 1.063–1.21 g/ml as isolated by sequential ultracentrifugation. All these HDL parameters have been corrected for losses due to ultracentrifugation by using the HDL cholesterol value obtained by precipitation as a reference. For each group, percentage of HDL mass as $\%HDL_{TC}$, $\%HDL_{FC}$, $\%HDL_{CE}$, $\%HDL_{TG}$, $\%HDL_{PL}$, $\%HDL_{Pro}$, are provided in Table 4. These numbers can be used to calculate the concentration of these constituents within HDL for these groups. As can be seen in this table, subjects with large HDL particles had the highest HDL_{mass} (295.0 mg/dl) and those with very small HDL particles had the lowest HDL_{mass} (162.7 mg/dl). In addition, subjects in the large HDL particle group had significantly higher $\%HDL_{FC}$ and HDL_{FC} as compared to the very small HDL particle group ($P < 0.01$). Other differences were less pronounced.
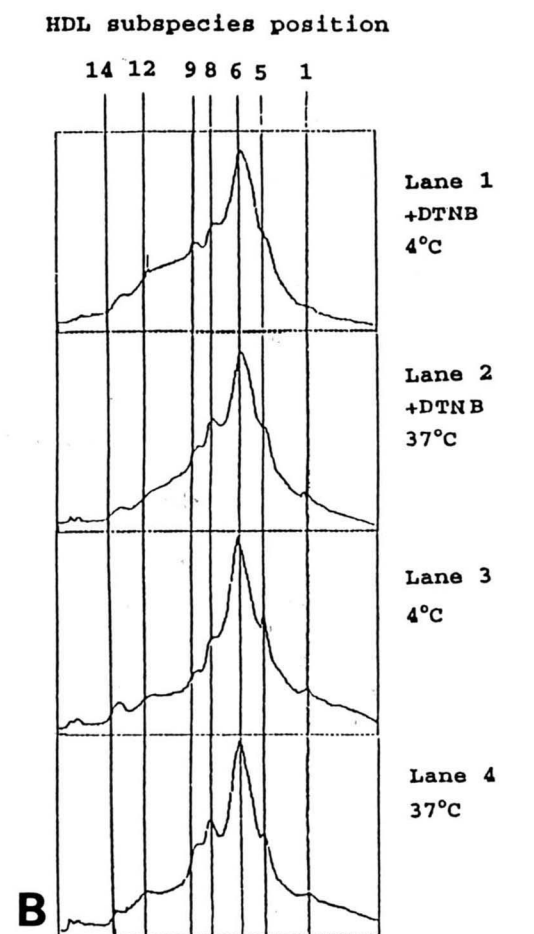

Fig. 6. A: HDL bands obtained from 4–30% PAA gel electrophoresis of plasmas from the four incubation conditions. Lane 1 represents plasma incubated with 5,5'-dithio-bis (2-nitrobenzoic acid), or DTNB at 4°C for 5 h; lane 2 represents plasma incubated with DTNB at 37°C for 5 h; lane 3 represents plasma incubated without DTNB at 4°C for 5 h; and lane 4 represents plasma incubated with DTNB at 37°C for 5 h. B: Densitometric scans for lanes 1–4 in 5A are shown.

TABLE 3. Comparison of HDL particle score with plasma lipid concentration[a]

HDL Score	n	HDL₂[b]	HDL₃[b]	TC	TG	HDL-C	LDL-C	VLDL-C
		%				*mg/dl*		
<8.0	5	88.9 ± 4.1	11.0 ± 4.1	179.2 ± 12.9	70.0 ± 7.9	72.4 ± 14.6	92.7 ± 14.6	14.80 ± 3.60
8.0–10.9	11	51.1 ± 16.7	48.9 ± 16.7	211.6 ± 18.3	274.9 ± 126.9	57.7 ± 13.4	103.6 ± 11.3	17.64 ± 7.91
11.0–11.9	24	26.0 ± 11.1	74.0 ± 11.1	205.9 ± 9.0	220.6 ± 59.1	44.2 ± 9.4	120.5 ± 5.3	40.93 ± 46.83
12.0–12.9	17	12.3 ± 8.9	87.7 ± 8.9	223.5 ± 16.5	445.8 ± 191.8	34.1 ± 7.4	121.5 ± 9.6	68.48 ± 76.79
Mean ± SD								
11.1 ± 1.4		33.6 ± 24.9	66.4 ± 24.9	208.1 ± 51.6	252.4 ± 486.9	46.8 ± 15.4	118.5 ± 33.9	42.29 ± 54.74

[a]N = 57, correlation data shown in Table 6. Values given as means ± SD.
[b]HDL₂ and HDL₃ percentage concentrations were obtained from 4–30% PAA gel.

We converted absolute lipid and protein values (mg/dl) of HDL fraction (d 1.063–1.21) to molecules of each component per HDL particle (**Table 5**). By using these chemical stoichiometries, we calculated equivalent mean particle diameters (nm) for the four HDL score groups to compare with the diameters estimated from the 4–30% PAA gel method (Table 1). Our calculations have been converted using the following values. The molecular weights of individual components were FC, 386.7; CE, 650; TG, 885; PL, 787; apoA-I, 28000; apoA-II, 17440; and amino acid, 115.2 (for calculating the lipid molecules per amino acid from measured total protein values). The molecular volumes used for each component were: FC, 0.6; CE, 1.09; TG, 1.6; PL, 1.27; apoA-I, 87.11; apoA-II, 53.98 nm³ (27, 28). The assumed ratios of apoA-I:A-II were 4:1 in the large HDL particle group, 3:1 in the intermediate and small HDL particle groups, and 2:1 in the very small HDL particle group as reported by Nichols et al. (29). The HDL particle radius was assumed to be equal to r + 2.02 nm, where r is the radius of the inner core which can be calculated from the number of molecules of triglyceride and cholesteryl ester, and 2.02 nm is the thickness of the outer layer of HDL particle composed of free cholesterol, phospholipid, and apoprotein as reported by Shen, Scanu, and Kézdy (30). We calculated the mean particle molecular weights for each group (Table

5) instead of calculating the molecular weight for HDL₂ or HDL₃ particle as others have done, and we did not take apoCs and E into account; therefore, our calculated values are slightly different than those of other investigators (27–30). However, the mean HDL particle diameter which we calculated from chemical stoichiometries in Table 5 were close to the estimated diameters that we obtained from our gel method (Table 1).

In **Table 6,** we have examined the correlations of various lipid parameters with HDL particle score. In terms of plasma lipids, the strongest correlation with HDL particle score was the correlation with HDL cholesterol concentration. Subjects with the highest HDL cholesterol level had the lowest HDL particle score, and therefore, the largest sized HDL particles. Log plasma triglyceride values and VLDL cholesterol values were significantly and positively correlated with HDL particle score, while the correlations with total cholesterol and LDL cholesterol were less striking. Stepwise analysis (**Table 7**) revealed that HDL cholesterol concentration accounted for 63% of the variability in HDL particle score, while LDL cholesterol accounted for an additional 4%. Plasma triglyceride levels were not significantly associated in the stepwise analysis after taking HDL cholesterol into account.

With regard to the concentrations of HDL lipid and protein constituents (see Table 6), the strongest correlation

TABLE 4. Comparison of HDL particle score with HDL lipoprotein composition[a]

HDL Score[b]	n = 57	HDL_MASS[c]	%HDL_TC[d]	%HDL_FC	%HDL_CE	%HDL_TG	%HDL_PL	%HDL_PRO
< 8	5	295.0 ± 66.4	24.5 ± 1.6	6.2 ± 0.5	18.3 ± 1.1	2.9 ± 0.7	30.3 ± 3.6	42.3 ± 4.7
8–10.9	11	255.9 ± 57.1	22.5 ± 3.1	4.7 ± 1.1	17.8 ± 2.4	3.7 ± 1.9	30.3 ± 4.3	43.5 ± 7.1
11–11.9	24	206.1 ± 44.7	21.5 ± 3.1	4.0 ± 1.0	17.6 ± 2.3	4.4 ± 1.9	27.3 ± 4.0	46.8 ± 6.4
12–12.9	17	162.7 ± 37.6	21.0 ± 2.0	2.0 ± 0.6	17.3 ± 1.8	5.3 ± 1.9	26.6 ± 2.4	47.1 ± 3.8
Mean ± SD								
11.08 ± 1.38		214.2 ± 58.3	21.9 ± 2.8	4.3 ± 1.2	17.6 ± 2.1	4.4 ± 1.9	28.0 ± 3.1	45.8 ± 6.0

[a]Correlation data shown in Table 6.
[b]HDL score, see Methods section for details.
[c]HDL_MASS mg/dl = HDL_TC + HDL_TG + HDL_PL + HDL_PRO (HDL fraction values were adjusted; see text for detail).
[d]%HDL_TC = cholesterol concentration in HDL fraction (d 1.063–1.21) g/ml/HDL_MASS.

TABLE 5. Estimated number of lipid molecules per HDL particle[a]

Mean HDL Score	Major HDL Bands	FC	CE	TG	PL	Assumed A-I/A-II Ratio[b]	Estimated Mean MW[c]	Estimated Mean Volume[c]	Estimated Mean Diameter[d]
							10^3 dalton	nm^3	nm
7.80 ± 0.1	6	49.08	85.94	9.94	118.63	4:1	307	692.1	9.98
9.89 ± 0.8	9	29.31	63.90	8.94	91.01	3:1	234	532.4	9.46
11.42 ± 0.3	11	22.40	58.18	10.73	76.20	3:1	218	506.2	9.38
12.32 ± 0.2	12	14.77	40.37	9.21	51.90	2:1	155	361.7	8.86

[a]These data were calculated based on the measured chemical stoichiometries from HDL fraction (d 1.063–1.21 g/ml).
[b]Assumed A-I:A-II ratios in HDL particles reported by Nichols et al. (29).
[c]Individual molecular weight and volume of each component were published by Edelstein et al. and Cheung et al. (27, 28); see text for detail.
[d]Estimated mean HDL particle diameters were calculated by using the Shen, Scanu, and Kézdy method (30); see text for detail.

with HDL score was observed with HDL_{FC}, which was inversely associated in a very significant manner with HDL particle score, and therefore positively correlated with HDL particle size. In addition, HDL_{CE} and HDL_{PL}, HDL_{apoA-I} and HDL_{Pro} concentrations were also associated inversely with HDL particle score, while $HDL_{TG(LOG)}$ was not significantly correlated (Table 6). It should be noted that by stepwise analysis, HDL_{FC} was the parameter that had the most significant effect on HDL particle score, accounting for 60% of the variability in this parameter (Table 7).

When we examined the relationship of HDL particle score with HDL composition (% of total mass), a similar

picture emerged. Here again, $\%HDL_{FC}$ was the parameter that was most highly correlated with HDL particle score in an inverse manner. Moreover, in this analysis associations of HDL score with $\%HDL_{TG}$, as well as with $\%HDL_{PL}$, were noted. $\%HDL_{Pro}$ was only weakly associated with HDL score, while $\%HDL_{CE}$ was not significantly correlated with HDL particle score (Table 6). Stepwise analysis again revealed that $\%HDL_{FC}$ produced the most significant association with HDL particle score, with $\%HDL_{apoA-I}$ having a modest additional affect. These parameters accounted for 42% of the variability in HDL particle score (see Table 7). **Figure 7** documents that HDL particle score was highly associated with HDL_{FC} concentration ($r = -0.78$) and $\%HDL_{FC}$ ($r = -0.608$).

DISCUSSION

There has been increasing interest in HDL heterogeneity because of recognition of low HDL cholesterol as a risk factor for premature CHD (1–5). It has long been recognized that HDL are a polydisperse collection of lipoprotein particles within the density region 1.063–1.21 g/ml.

TABLE 6. Correlations with HDL particle score[a]

	r	P
Plasma lipid concentrations		
Total cholesterol	0.297	0.026
Triglyceride (log)	0.537	0.0001
VLDL cholesterol (log)	0.547	0.0001
LDL cholesterol	0.247	0.068
HDL cholesterol	−0.796	0.0001
HDL lipid and protein concentrations		
HDL_{FC}[b]	−0.780	0.0001
HDL_{CE}	−0.683	0.0001
HDL_{PL}	−0.663	0.0001
$HDL_{TG(log)}$	0.069	0.611
HDL_{Pro}	−0.459	0.0004
HDL_{apoA-I}	−0.577	0.0001
HDL_{apoB}	0.202	0.140
$HDL_{Lp[a]}$	0.138	0.319
HDL_{MASS}[c]	−0.649	0.0001
HDL percentage composition (% total mass)		
$\%HDL_{FC}$[d]	−0.608	0.0001
$\%HDL_{CE}$	−0.219	0.106
$\%HDL_{PL}$	−0.359	0.0066
$\%HDL_{TG}$	0.415	0.0015
$\%HDL_{Pro}$	0.295	0.028
$\%HDL_{apoA-I}$	0.183	0.180

[a]See HDL particle score calculation in Methods section: a larger score value represents a smaller HDL size.
[b]Values were obtained from HDL fraction (1.063–1.21 g/ml).
[c]$HDL_{MASS} = HDL_{FC} + HDL_{CE} + HDL_{PL} + HDL_{TG} + HDL_{Pro}$.
[d]$\%HDL_{FC}$ = free cholesterol concentration/HDL_{mass}.

TABLE 7. Stepwise analysis of HDL particle score with plasma lipid parameters, HDL concentrations, and HDL percent compositions

Variable	Partial r^2	Model r^2	P
Model 1 (plasma lipid parameters)			
HDL-C	0.634	0.634	0.0001
LDL-C	0.039	0.673	0.0152
Model 2 (HDL constituent concentrations)			
HDL_{FC}	0.603	0.603	0.0001
Model 3 (HDL percent compositions)			
$\%HDL_{FC}$	0.364	0.364	0.0001
$\%HDL_{apoA-I}$	0.061	0.424	0.023

Equations: Model 1, HDL score = 13.4 − 0.07 HDL-C + 0.01 LDL-C; Model 2, HDL score = 13.4 − 0.21 HDL_{FC}; and Model 3, HDL score = 12.1 − 0.67 $\%HDL_{FC}$ + 4.4 $\%HDL_{apoA-I}$.
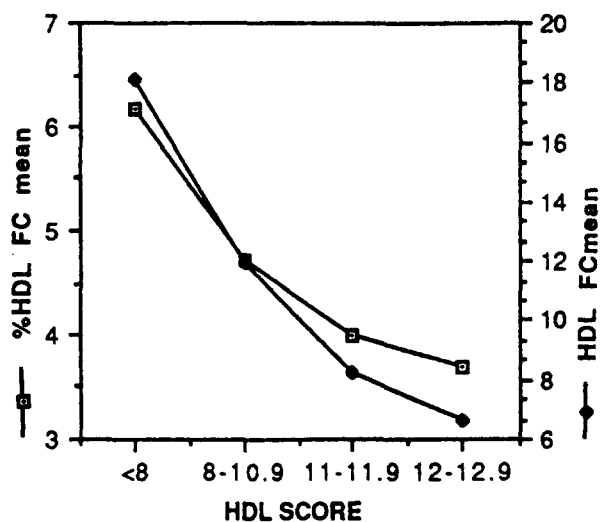
Fig. 7. The significant relationships between HDL particle score and HDL free cholesterol content in concentration ($r = -0.78$) and percent of total mass ($r = -0.608$) are shown.

When separated by agarose electrophoresis, the bulk of particles in this density region have alpha mobility. A minor prebeta band found in this density region is mainly Lp[a], but also includes an apoA-I-rich species of HDL that appears to be important for mobilization of free cholesterol from cells (9).

Cheung and co-workers (8, 28), as well as other investigators, have separated HDL particles into those containing apoA-I with apoA-II (Lp A-I/A-II) and those containing apoA-I without apoA-II (Lp A-I). By such separations, about one-third of apoA-I is found within Lp A-I particles and the remainder within Lp A-I/A-II particles. It is well known that apoA-I is the major protein of HDL, and that it can serve as the activator for LCAT, the enzyme responsible for the conversion of free cholesterol to cholesteryl ester in plasma.

In subjects in whom plasma apoA-I is not detected, a marked HDL deficiency in plasma results, and premature CHD is noted (31, 32). In contrast, when apoA-II is not present, HDL cholesterol levels appear to be normal and there is no premature CHD (33). These data suggest that apoA-I is essential for normal HDL formation. It has been proposed that Lp A-I is more important in promoting cholesterol efflux than Lp A-I/A-II, and that decreased levels of Lp A-I are a better predictor of CHD risk than decreased levels of HDL cholesterol or apoA-I only (34). However, in some studies, patients with premature CHD had reductions in both Lp A-I and Lp A-I/A-II (35).

HDL has been subdivided into various density regions, largely based on the work of researchers at the Donner Laboratories at the University of California in Berkeley. The initial studies by DeLalla, Elliott, and Gofman (36) divided HDL into HDL_2 and HDL_3, with HDL_2 having a density of 1.063–1.125 g/ml, and HDL_3 having a density of 1.125–1.21 g/ml. These early studies were based on analytical ultracentrifugation analysis. It was subsequently reported that the variable component in HDL was largely due to HDL_2. In these subsequent analyses, Anderson and Nichols (11) divided HDL into three components where HDL_2 was further subdivided into HDL_{2b}, with density 1.063–1.10 g/ml, and HDL_{2a}, 1.10–1.125 g/ml. Efforts to quantitate these HDL_2 and HDL_3 subspecies by variable precipitation have been successful and are currently being carried out in a variety of prospective studies, including the Framingham Heart Study.

Gradient gel electrophoresis has been used as a precise method of delineating lipoprotein particle size by investigators at the Donner Laboratories, as well as by our group and other investigators (10, 16, 28, 37, 38). Using 2–16% PAA gels obtained from Pharmacia, seven different sizes of LDL particles were identified, with most individuals having one major LDL band and adjacent satellite bands. Moreover, we have shown that whole plasma frozen at $-80°C$ utilizing electrophoresis and Sudan Black B staining can be useful for this purpose (16). HDL particle size has also been assessed by gradient gel electrophoresis. Blanche et al. (10) reported the presence of five HDL subspecies, classified as HDL_{2b} (the largest HDL bands), HDL_{2a} and HDL_{3a}, HDL_{3b}, and HDL_{3c} (the smallest HDL bands, see Table 1). These analyses were carried out by isolating HDL by ultracentrifugation and staining HDL protein with Coomassie blue. Ultracentrifugation has the disadvantage of decreasing the resolution of such bands on gradient gels so that a clear delineation of individual bands is not achieved.

By modifying the conditions of electrophoresis and staining with Sudan Black B, we have found that whole plasma can also be used to assess HDL particle size. This approach has been useful to more precisely resolve HDL species (Figs. 1–4). Instead of obtaining broad bands of HDL particles, we obtained discrete bands within the HDL size and density range, which allows us to better resolve HDL subspecies. However, the results obtained are similar to those reported by Blanche et al. (10). It should be noted that in our experiment we used 4800 volt-hours instead of the 3000 volt-hours reported by Blanche et al. (10). When we decreased the volt-hours in our experiments we did not obtain adequate resolution of HDL bands or sufficient migration of the HDL bands or the dye front into the gel. Nevertheless, it is possible that our method, like any isolation method, may introduce some artifact into the system and alter native HDL particles. Our method does avoid ultracentrifugation which has been shown to alter the apolipoprotein content of HDL. In addition, we detected apoE within large HDL particle, suggesting that our method does not disrupt HDL particles.

In order to examine subjects with a variety of HDL particle sizes, we analyzed HDL subspecies in subjects with varying levels of HDL cholesterol and triglyceride

concentrations. In these subjects, we noted 14 HDL subspecies on 4–30% PAA gels, with most subjects having 4–5 HDL bands; the most frequent bands were HDL-9, 11, and 12. Dilution studies revealed that HDL particle mobility (R_f) was stable, but that mean HDL score or particle size could be somewhat affected by dilution (5.5% decrease in score at maximal dilution of 3:17). The results of LCAT and CETP inhibition studies showed that LCAT and CETP activity can also affect HDL particle size with a maximal effect of about 5.5%. These data indicate that both dilution and LCAT inhibition may result in small decreases in HDL particle size.

When we examined the apoA-I, A-II, and E content in each HDL band by immunoblotting (see Table 1), apoA-I was found in all HDL bands, consistent with the concept that apoA-I is essential for HDL particle formation. Moreover, apoA-I was very prominent in HDL-6, HDL-11, and HDL-12. ApoA-II was present mainly in smaller HDL particles, and was very prominent in HDL-9. ApoE was found mainly in large HDL particles, especially HDL-1 and HDL-2.

Cheung et al. (8) reported a modified method to separate HDL subspecies by using immunoaffinity columns, in which antibodies specific for apoA-I and apoA-II were used. These investigators separated HDL subspecies into those containing apoA-I without apoA-II (Lp A-I) and those containing apoA-I with apoA-II (Lp A-I/A-II) (8). These subspecies were then subsequently run on 4–30% PAA gels and stained with Coomassie blue. Using this technology in four normal subjects, these investigators noted 11 HDL subspecies, designated as HDL 1–9 (with band 7 further divided into 7a, 7b, and 7c) in order of increasing particle size, with bands 3–7 being the major subspecies. Lp A-I/A-II were composed primarily of three subspecies of size 8.1 ± 0.1 nm, 8.9 ± 0.2 nm, and 9.5 ± 0.2 nm, with other minor HDL subspecies noted. Lp A-I in these four subjects was composed of two main subspecies of sizes 8.5 ± 0.1 nm and 10.8 ± 0.4 nm, with two minor larger subspecies and two minor smaller subspecies (28).

Using an immunoaffinity column to separate Lp A-I particles and gel filtration chromatography to measure the size of these particles, Duverger et al. (39) reported three subpopulations of Lp A-I: S-LpA-I (7.5 ± 0.3 nm), M-LpA-I (8.5 ± 0.5 nm), and L-LpA-I (10.3 ± 0.5 nm). These three subpopulations of Lp A-I were heterogeneous in size and composition, and the majority of plasma LCAT and CETP activity was found in association with the L-LpA-I particles (39). No data on Lp A-I/A-II particles were reported by these investigators.

In Lp A-I particles isolated by immunoaffinity column, we have noted five separate bands on 4–30% PAA gel in the subjects examined: HDL-5 (10.55 nm), HDL-6 (10.00 nm), HDL-11 (8.53 nm), HDL-12 (8.30 nm), and HDL-13 (8.14 nm); and five subspecies of HDL bands

from Lp A-I/A-II particles: HDL-8 (9.24 nm), HDL-9 (8.90 nm), HDL-11 (8.53 nm), HDL-12 (8.30 nm), and HDL-13 (8.14 nm). These analyses show that 4–30% PAA gradient gel electrophoresis can be used to separate HDL subspecies from isolated Lp A-I and Lp A-I/A-II particles, as well as HDL subspecies from plasma. HDL bands 11 to 13 were found to contain both Lp A-I and LpA-I/A-II particles. These data indicate that Lp A-I and Lp A-I/A-II have overlapping size, even though these particles have different apolipoprotein composition.

The estimated mean number of lipid molecules in HDL from the four HDL particle score groups (large, intermediate, small, and very small) are shown in Table 5. These data indicate significant decreases in the number of molecules of free cholesterol, cholesteryl ester, phospholipid, and apoA-I as one moves from the largest HDL group to the smallest HDL group, with no significant change in the number of triglyceride molecules. The estimated mean HDL particle size in these four groups shown in Table 5 are close to the estimated HDL particle size based on our gel method as shown in Table 1.

There was a clear relationship between HDL particle size and the composition of HDL, with HDL_{FC} emerging as the single most important determinant of HDL particle size based on concentration and percentage composition analyses. In the concentration analysis, we noted that HDL_{PL}, HDL_{CE}, HDL_{apoA-I}, and HDL_{Pro} were all associated with variation in HDL particle size. However, in the stepwise analysis, only HDL_{FC} was significant and accounted for about 60% of the variability in HDL particle size. In stepwise analysis of percentage composition, %HDL_{FC} was again the most important variable associated with HDL size (Tables 6 and 7).

In summary, this nondenaturing 4–30% PAA gel method of assessing HDL subspecies allows one to use small quantities (30 μl) of whole plasma (either fresh or frozen at −80°C) obtained from subjects after an overnight fast or in the nonfasting state to identify up to 14 discrete HDL subspecies. This methodology can be used to assess HDL particle size in population studies, as well as to determine the effects of dietary modification and lipid-lowering medication on HDL subspecies. Our data are also consistent with the concept that the absolute and relative free cholesterol content of HDL is the single most important variable associated with HDL particle size, which in turn is highly correlated with HDL mass.

REFERENCES

1. Keys, A. 1980. Alpha lipoprotein (HDL) cholesterol in the serum and risk of coronary heart disease and death. *Lancet.* **2:** 603–606.

2. Miller, G. J., and N. E. Miller. 1975. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. *Lancet.* **1:** 16–19.

3. Abbott, R. D., P. W. F. Wilson, W. B. Kannel, and W. P. Castelli. 1988. High density lipoprotein cholesterol, total cholesterol screening and myocardial infarction. The Framingham Study. *Arteriosclerosis.* **8:** 207–211.

4. Jacobs, D. R., Jr., I. L. Mebane, S. I. Bangdiwala, M. H. Criqui, and H. A. Tyroler. 1990. High density lipoprotein cholesterol as a predictor of cardiovascular mortality in men and in women: the follow-up of the Lipid Research Clinics Prevalence Study. *Am. J. Epidemiol.* **131:** 32–47.

5. Johansson, J., L. A. Carlson, C. Landou, and A. Hamsten. 1991. High density lipoproteins and coronary atherosclerosis. A strong inverse relation with the largest particles is confined to normotriglyceridemic patients. *Arterioscler. Thromb.* **11:** 174–82.

6. Eisenberg, S. 1984. High density lipoprotein metabolism. *J. Lipid Res.* **25:** 1017–1058.

7. Musliner, T. A., and R. M. Krauss. 1988. Lipoprotein subspecies and risk of coronary disease. *Clin. Chem.* **34:** B78–B83.

8. Cheung, M. C., and J. J. Albers. 1984. Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II. *J. Biol. Chem.* **259:** 12201–12209.

9. Barbaras, R., P. Puchois, J-C. Fruchart, and G. Ailhaud. 1987. Cholesterol efflux from cultured adipose cells is mediated by LpAI particles but not by LpAI:AII particles. *Biochem. Biophys. Res. Commun.* **142:** 63–69.

10. Blanche, P. J., E. L. Gong, T. M. Forte, and A. V. Nichols. 1981. Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochim. Biophys. Acta.* **665:** 408–419.

11. Anderson, D. W., and A. V. Nichols. 1978. High density lipoprotein distribution. Resolution and determination of three major components in a normal population sample. *Atherosclerosis.* **29:** 161–179.

12. Atger, V., D. Malon, M. C. Bertiere, F. N'Ciaye, and A. Girard-Globa. 1991. Cholesterol distribution between high-density-lipoprotein subfractions HDL₂ and HDL₃ determined in serum by discontinuous gradient gel electrophoresis. *Clin. Chem.* **37:** 1149–1152.

13. Warnick, G. R., J. Benderson, and J. J. Albers. 1982. Dextran-sulfate-Mg²⁺ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin. Chem.* **28:** 1379–1388.

14. Krauss, R. M., and A. V. Nichols. 1986. Metabolic interrelationships of HDL subclasses. *Adv. Exp. Med. Biol.* **201:** 17–27.

15. Rainwater, D. L., D. W. Andres, A. L. Ford, W. F. Lowe, P. J. Blanche, and R. M. Krauss. 1992. Production of polyacrylamide gradient gels for the electrophoretic resolution of lipoproteins. *J. Lipid Res.* **33:** 1876–1881.

16. McNamara, J. R., H. Campos, J. M. Ordovas, J. Peterson, P. W. F. Wilson, and E. J. Schaefer. 1987. Effect of gender, age and lipid status on low density lipoprotein subfraction distribution. Results from the Framingham Offspring Study. *Arteriosclerosis.* **7:** 483–490.

17. McNamara, J. R., H. Campos, J. M. Ordovas, P. W. F. Wilson, and E. J. Schaefer. 1988. Gradient gel electrophoretic analysis of low density lipoproteins. *Am. Biotech. Lab.* **6:** 28–33.

18. McNamara, J. R., and E. J. Schaefer. 1987. Automated enzymatic standardized lipid analyses for plasma and lipoprotein fractions. *Clin. Chim. Acta.* **166:** 1–8.

19. Friedewald, W. T., R. I. Levy, and D. S. Fredrickson. 1972. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin. Chem.* **18:** 499–502.

20. Cohn, J. S., J. R. McNamara, S. D. Cohn, J. M. Ordovas, and E. J. Schaefer. 1988. Plasma apolipoprotein changes in the triglyceride-rich lipoprotein fraction of human subjects fed a fat-rich meal. *J. Lipid Res.* **29:** 925–936.

21. Menzel, H-J., and G. Utermann. 1986. Apolipoprotein E phenotyping from serum by Western blotting. *Electrophoresis.* **7:** 492–495.

22. Ordovas, J. M., J. P. Peterson, P. Santaniello, J. S. Cohn, P. W. F. Wilson, and E. J. Schaefer. 1987. Enzyme-linked immunosorbent assay for human plasma apolipoprotein B. *J. Lipid Res.* **28:** 1216–1224.

23. Schaefer, E. J., and J. M. Ordovas. 1986. Metabolism of apolipoproteins A-I, A-II and A-IV. *Methods Enzymol.* **129:** 420–442.

24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.

25. Markwell, M. A. K., S. M. Haas, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72:** 296–303.

26. Campos, H., E. Blijlevens, J. R. McNamara, J. M. Ordovas, B. M. Posner, P. W. F. Wilson, W. P. Castelli, and E. J. Schaefer. 1992. LDL particle size distribution. Results from the Framingham Offspring Study. *Arterioscler. Thromb.* **12:** 1410–1419.

27. Edelstein, C., F. J. Kézdy, A. M. Scanu, and B. W. Shen. 1979. Apolipoproteins and the structural organization of plasma lipoproteins: human plasma high density lipoprotein-3. *J. Lipid Res.* **20:** 143–153.

28. Cheung, M. C., J. P. Segrest, J. J. Albers, J. T. Cone, C. G. Brouillette, B. H. Chung, M. Kashyap, M. A. Glasscock, and G. M. Anantharamaiah. 1987. Characterization of high density lipoprotein subspecies: structural studies by single vertical spin ultracentrifugation and immunoaffinity chromatography. *J. Lipid Res.* **28:** 913–929.

29. Nichols, A. V., P. J. Blanche, V. G. Shore, and E. L. Gong. 1988. Conversion of apolipoprotein-specific high-density lipoprotein populations during incubation of human plasma. *Biochim. Biophys. Acta.* **100:** 325–337.

30. Shen, B. W., A. M. Scanu, and F. J. Kézdy. 1977. Structure of human serum lipoproteins inferred from compositional analysis. *Proc. Natl. Acad. Sci. USA.* **74:** 837–841.

31. Schaefer, E. J., W. H. Heaton, M. G. Wetzel, and H. B. Brewer, Jr. 1982. Plasma apolipoprotein A-I absence associated with a marked reduction of high density lipoproteins and premature coronary artery disease. *Arteriosclerosis.* **2:** 16–26.

32. Ordovas, J. M., D. K. Cassidy, F. Civeira, C. L. Bisgaier, and E. J. Schaefer. 1989. Familial apolipoprotein A-I, C-III, and A-IV deficiency and premature atherosclerosis due to a deletion of a gene complex on chromosome 11. *J. Biol. Chem.* **264:** 16339–16342.

33. Deeb, S. S., K. Takata, R. L. Peng, G. Kajiyame, and J. J. Albers. 1990. A splice-junction mutation responsible for

familial apolipoprotein A-II deficiency. *Am. J. Hum. Genet.* **46:** 822–827.

34. Puchois, P., A. Kandoussi, A. P. Fievet, J. L. Fourrier, M. Bertrand, E. Koren, and J-C. Fruchart. 1987. Apolipoprotein, A-I-containing lipoproteins in coronary artery disease. *Atherosclerosis.* **68:** 35–40.

35. Genest, J. J., Jr., J. M. Bard, J-C. Fruchart, J. M. Ordovas, P. F. M. Wilson, and E. J. Schaefer. 1991. Plasma apolipoprotein A-I, A-II, B and C-III-containing particles in men with premature coronary artery disease. *Atherosclerosis.* **90:** 149–157.

36. Delalla, O. F., H. A. Elliott, and J. W. Gofman. 1954. Ultracentrifugal studies of high density serum lipoproteins in clinically healthy adults. *Am. J. Physiol.* **179:** 333–337.

37. Lamon-Fava, S., J. R. McNamara, H. W. Farber, N. S. Hill, and E. J. Schaefer. 1989. Acute changes in lipid, lipoprotein, apolipoprotein, and low-density lipoprotein particle size after an endurance triathlon. *Metabolism.* **38:** 921–925.

38. McNamara, J. R., J. L. Jenner, Z. Li, P. W. F. Wilson, and E. J. Schaefer. 1992. Change in low density lipoprotein particle size is associated with change in plasma triglyceride concentration. *Arterioscler. Thromb.* **12:** 1284–1290.

39. Duverger, N. J., D. J. Rader, P. Duchateau, G. Ailhaud, J. C. Fruchart, G. Castro, and H. B. Brewer, Jr. 1992. Composition and metabolic activity of subpopulations of lipoprotein particles containing apoA-I without apoA-II (LPA-I) from normolipidemic human subjects. *Circulation.* **86:** I–73.