paper on methodology

Correlations of plasma lipoproteins with LDL subfractions by particle size in men and women

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Abstract Nondenaturing gradient gel electrophoresis of plasma low density lipoprotein (LDL) has been used to identify major LDL subclasses that are influenced by genetic and other factors. In the present paper, this technique has been extended by measuring absorbance of lipid- or protein-stained gels as an index of concentration at intervals of 0.05 nm across the entire LDL particle size range (21.8–30 nm) in moderately overweight men (n = 115) and women (n = 78). When LDL absorbance levels were correlated with other lipoprotein variables, we found that the strengths of the correlations with each of triglycerides, apolipoprotein (apo) B, high density lipoprotein $(HDL)_2$, and apoA-I achieve relative maximum values for two regions within the small LDL range (21–26 nm), one within LDL-IVB (22–23.2 nm) and a second within LDL-III (24.2–25.5 nm). We also found that the increase in LDL accompanying higher triglyceride levels occurs below 25.5 nm in men and between 24.5 and 26.5 nm in women, suggesting either that triglycerides are related to different LDL subclasses in men and women, or that particle sizes of metabolically homologous LDL subclasses may differ in men and women. As compared to analytic ultracentrifuge measurements, gradient gel measurements of LDL absorbance by the procedure described here provide greater resolution of LDL subclasses but less precision in estimating LDL levels.—**Williams, P. T., K. M. Vranizan, and R. M. Krauss.** Correlations of plasma lipoproteins with LDL subfractions by particle size in men and women. *J. Lipid Res.* 1992. **33:** 765–774.

Supplementary key words gradient gel electrophoresis • very low density lipoproteins • low density lipoproteins • high density lipoproteins • sex differences

Human low density lipoproteins (LDL) comprise subpopulations of differing size particles that are distinguished by their electrophoretic mobility on nondenaturing polyacrylamide gradient gels (1, 2). When stained for lipid or protein, the gels reveal LDL particle distributions with major and minor peaks (1, 2). The frequency distribution of these peaks from population samples has been used to divide the LDL distribution into particle size intervals. These intervals include LDL-IVB (22.0–23.2 nm), LDL-IVA (23.3–24.1 nm), LDL-IIIB (24.2–24.6 nm), LDL-IIIA 24.7–25.5 nm), LDL-II (25.5–26.4 nm) LDL-I (26.0–28.5 nm), and intermediate density lipoproteins (IDL, 28.0–30.0 nm) (1, 3).

The positions of the predominant (major) and secondary (minor) LDL peaks, and the mean particle size based on the integration of all peaks have been successfully used to study the relationships of LDL to a variety of factors, including other lipoproteins (2), acute and long-term effects of exercise (4, 5), diet-induced and exercise-induced weight loss (6), menopause in women (7), estrogen therapy in postmenopausal women (8), and genetic influences on lipoprotein metabolism and coronary heart disease risk (as distinguishing criteria for the LDL subclass phenotypes A and B) (9–11). The peak position, though revealing, may not include important data contained in the total distribution. We examined this hypothesis through the densitometric measurements of absorbance by lipid-stained and protein-stained gradient gels from a population sample of men and women. Mean differences and correlation coefficients are computed for LDL levels (absorbance) at each LDL diameter value at intervals of 0.05 nm. Our objectives are: *1*) to determine sex differences and lipoprotein relationships for LDL-lipid and LDL-protein by particle size; *2*) to assess whether the LDL peak diameter fully characterizes these sex differences and lipoprotein relationships; and *3*) to compare LDL as measured by gradient gel electrophoresis and analytic ultracentrifugation.

METHODS

Subjects

We examined LDL particle size distribution in healthy nonsmoking men and women aged 25 through 49 years old. All were sedentary (exercising vigorously no more than twice per week), moderately overweight (body mass

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein.

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index between 28 and 34 kg/m² in men and between 24 and 30 kg/m² in women), free of medication that might affect lipid metabolism, nonhypertensive (blood pressure <160/95 mm Hg), and having plasma total cholesterol <260 mg/dl and plasma triglycerides <500 mg/dl. None of the women were pregnant, lactating, or using oral contraceptives.

Laboratory methods

All participants reported to our clinic in the morning, having abstained for 12–16 h from all food and any vigorous activity. Venous blood was collected through a butterfly catheter into a syringe, with the subject in a supine position for less than 10 min. The blood was immediately transferred into tubes containing sodium EDTA (1 mg/l). Plasma total cholesterol and triglyceride concentrations were measured by enzymatic methods (Abbott ABA 200 instrument, Abbott Diagnostics, North Chicago, IL) (12, 13); plasma concentrations of high density lipoprotein (HDL) cholesterol were determined by dextran sulfate–magnesium precipitation, followed by enzymatic determination of cholesterol (14). These measurements were consistently in control as monitored by the Lipid Standardization Program of the Center for Disease Control, Atlanta, GA, and the National Heart, Lung, and Blood Institute, Bethesda, MD. HDL_3 cholesterol was determined by a dextran sulfate–magnesium precipitation method and HDL_2 cholesterol was calculated as the difference between HDL cholesterol and HDL_3 cholesterol (15). LDL cholesterol concentrations were calculated from the equation by Friedewald, Levy, and Fredrickson (16). Plasma apolipoprotein A-I and B concentrations were determined by rate immunonephelometry (Beckman ARRAY) with the same quality control procedures as applied to lipoprotein assays (17). The coefficients of variation were 3% or less for total cholesterol, triglycerides, and HDL cholesterol and less than 10% for apoA-I and B.

Electrophoresis of LDL in whole plasma and the d < 1.063 g/ml fraction was performed on a Pharmacia Electrophoresis Apparatus (GE 4-II Pharmacia, Piscataway, NJ) using slab gradient gels (PAA 2/16, Pharmacia) (1, 3). Lipid staining was performed by incubating the gels overnight in a 50–60°C oven in a solution of 945 ml of 95% ethanol, 555 ml distilled water and 0.61 g Oil Red O stain. Protein-stained gels were obtained by agitating the gels in 50–75 ml solution of 0.04% Coomassie G-250 and 3.5% perchloric acid after fixing the protein in 10% sulfosalicylic acid for 1 h.

The lipid- or protein-stained gradient gels for LDL were scanned with a model RFT densitometer (Transidyne Corp., Ann Arbor, MI) at wave lengths of 555 nm for lipid-stained gels, and 596 nm for protein-stained gels (3). A mixture of four globular proteins (HMW Calibration Kit, Pharmacia, Piscataway, NJ) was run on the central lane to calibrate for particle size. Latex beads were added to the high molecular weight standard to determine particle diameter. The migration distances (R_f) were measured from the beginning of the gel. A computer file of absorbancy versus migration distance was obtained at 1000 points along the gradient gel. Calculus (transformation of variables) was then used to transform the LDL distribution from the migration distance scale to the particle diameter scale (18). The height of the distribution, as measured by absorbance at each diameter value, was then determined by interpolation for each 0.05-nm value between 21.8 and 30 nm. The coefficients of variations for LDL absorbance, measured at the peak, were 15.6% when stained for lipid and 17.6% when stained for protein. For each individual, we also used integration to calculate the LDL mean diameter from the distributions of the lipid- and protein-stained gels.

Electrophoresis of HDL in the ultracentrifuged d ≤ 1.20 g/ml fraction was also done using slab gradient gels (PAA 4/30, Pharmacia) as described by Blanche et al. (19). The protein-stained gradient gels were scanned at a wave length of 603 nm. The HDL distributions were converted from the migration distance scale to the particle diameter scale (18). Integration was used to calculate HDL average diameter.

Analytic ultracentrifugation was used to measure concentrations of total lipoprotein mass for 15 HDL flotation intervals ($F_{1.20}0$–9), 11 LDL flotation intervals (S_f0–12), and four IDL flotation intervals (S_f12–20) (20). These were combined into HDL_3 ($F_{1.20}0$–3.5), HDL_2 ($F_{1.20}3.5$–9), small LDL (S_f0–7), large LDL (S_f7–12), IDL (S_f12–20), and VLDL mass concentrations (S_f20–400).

Statistical analysis

We used Pearson correlation coefficients (r) and t-tests to assess the relationships of LDL absorbance to plasma lipoprotein concentrations. The absorbance of lipid- and protein-stained polyacrylamide gradient gels were used as an index of mass concentrations of LDL at intervals of 0.05 nm across the entire LDL particle size range (21.8–30 nm). For example, at each diameter, LDL absorbance and triglyceride levels were correlated across the sample of 115 men to yield a correlation coefficient at that diameter value. The calculation was repeated for each increment of 0.05 nm. The correlations were then plotted as a function of particle diameter. Similarly, the calculation of differences, e.g., between men and women, were computed by subtracting the mean absorbance of the two groups at each diameter, and plotting the set of differences as a function of LDL diameter.

Partial correlations and analysis of covariance were used to adjust the correlations and mean differences for LDL peak diameter. Mean differences and significance levels are accurately plotted so that the values may be taken from the figures. The subclass intervals are pre-

sented on these graphs for reference (1). We have split the differences for overlapping subclasses. The intervals are approximate and may change with further study. For ease of exposition, however, we identify mean differences and correlations with a particular subclass when significance at $P \leq 0.01$ is achieved in at least one-half of its diameter range.

RESULTS

The 115 men and 78 women differed significantly in their plasma concentrations of total cholesterol (men vs. women: 209.3 ± 33.6 vs. 192.0 ± 28.7 mg/dl), triglycerides (128.0 ± 74.0 vs. 74.2 ± 38.8 mg/dl); HDL_2 mass (23.5 ± 24.3 vs. 79.9 ± 48.8 mg/dl); HDL_3 mass (192.3 ± 35.8 vs. 210.7 ± 33.9 mg/dl); HDL_2 cholesterol (7.9 ± 8.1 vs. 21.2 ± 11.8 mg/dl); HDL_3 cholesterol (34.9 ± 6.6 vs. 37.2 ± 7.3 mg/dl); apolipoprotein A-I (122.6 ± 20.7 vs. 138.5 ± 25.8 mg/dl); small LDL mass (197.3 ± 68.0 vs. 134.5 ± 51.9 mg/dl); large LDL mass (111.4 ± 40.4 vs. 130.7 ± 37.1 mg/dl); IDL mass (30.7 ± 17.9 vs. 17.6 ± 14.9 mg/dl); VLDL mass (107.0 ± 75.9 vs. 48.2 ± 45.8 mg/dl); apolipoprotein B (86.4 ± 20.0 vs. 69.0 ± 15.9 mg/dl), LDL peak flotation rate (5.6 ± 1.2 vs. 6.8 ± 1.1 S_f). As compared to women, the average diameter for the individual distributions was lower in men for HDL stained for protein (8.58 ± 0.20 vs. 8.94 ± 0.25 nm) and LDL stained for lipid (26.66 ± 0.63 vs. 26.90 ± 0.43 nm) and protein (26.40 ± 0.68 vs. 26.72 ± 0.52).

Correlations of LDL peak particle diameter (gradient gel) with plasma lipids, lipoproteins, and LDL peak flotation rate (analytic ultracentrifuge)

Table 1 shows correlations of LDL peak particle diameters with selected lipoprotein measurements. Lower peak diameter measurements in both the uncentrifuged plasma (lipid-stained) and the $d \leq 1.063$ g/ml centrifugation fraction (protein-stained) were associated with higher concentrations of triglyceride, small LDL mass, IDL mass, VLDL mass, and apolipoprotein B, and lower concentrations of HDL_2 cholesterol, HDL_2 mass, and large LDL mass. LDL peak diameter correlated positively with HDL_3 cholesterol and HDL_3 mass in men, and positively with HDL average diameter in both men and women. Lipid-stained and protein-stained gels both yielded peak diameters that correlated strongly with the LDL peak flotation rate (from analytic ultracentrifugation). The HDL average diameter correlated positively with LDL average diameter for both lipid-stained (men: $r = 0.53$; women: $r = 0.48$) and protein-stained gels (men: $r = 0.58$; women: $r = 0.30$). The LDL peak diameters of the lipid-stained and protein-stained gradient gels were more strongly correlated with each other in men ($r = 0.76$) than women ($r = 0.54$).

LDL absorbance (gradient gel)

Fig. 1 compares the mean LDL absorbance at each diameter between men and women. We computed the means by averaging the absorbance for individual gels at each diameter value, and the differences by subtraction of

TABLE 1. Correlations of protein- and lipid-stained LDL peak diameter and LDL peak flotation rate versus lipoproteins and apolipoproteins in a cross-sectional sample of 115 men and 78 women

	Men			Women		
LDL Peak	Protein-Stained Diameter	Lipid-Stained Diameter	Flotation Rate	Protein-Stained Diameter	Lipid-Stained Diameter	Flotation Rate
Triglycerides	−0.68[a]	−0.71[a]	−0.72[a]	−0.61[a]	−0.65[a]	−0.75[a]
LDL cholesterol	−0.19	−0.19	−0.35[a]	−0.28	−0.39[a]	−0.47[a]
HDL_2 cholesterol	0.59[a]	0.53[a]	0.64[a]	0.63[a]	0.52[a]	0.66[a]
HDL_3 cholesterol	0.31[a]	0.36[a]	0.30[a]	0.06	0.35[a]	0.22
HDL_2 mass	0.54[a]	0.56[a]	0.68[a]	0.53[a]	0.60[a]	0.73[a]
HDL_3 mass	0.29[a]	0.28[a]	0.35[a]	0.01	0.09	−0.01
HDL average diameter	0.70[a]	0.65[a]	0.74[a]	0.63[a]	0.64[a]	0.77[a]
ApoA-I	0.40[a]	0.45[a]	0.49[a]	0.23	0.42[a]	0.46[a]
Small LDL-mass	−0.55[a]	−0.58[a]	−0.78[a]	−0.65[a]	−0.61[a]	−0.81[a]
Large LDL-mass	0.67[a]	0.63[a]	0.61[a]	0.46[a]	0.36[a]	0.39[a]
IDL mass	−0.52[a]	−0.54[a]	−0.54[a]	−0.45[a]	−0.48[a]	−0.61[a]
VLDL mass	−0.71[a]	−0.72[a]	−0.77[a]	−0.58[a]	−0.60[a]	−0.77[a]
ApoB	−0.47[a]	−0.47[a]	−0.67[a]	−0.54[a]	−0.50[a]	−0.68[a]
LDL peak flotation rate	0.80[a]	0.81[a]	1.00	0.79[a]	0.75[a]	1.00

[a] Significantly different from zero at $P < 0.01$. LDL peak diameters are determined from gradient gel electrophoresis and LDL peak flotation rates are determined by analytic ultracentrifugation.

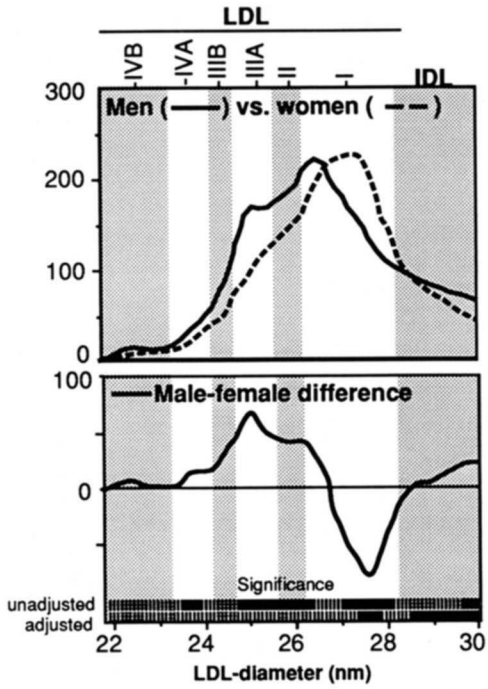

Fig. 1. Mean levels of lipid-stained LDL absorbance at 603 nm in 115 men and 78 women (top) and the mean difference between men and women (bottom). The bar at the bottom of the difference plot designates the diameter values having significant (solid) and nonsignificant (dashed) differences at $P \leq 0.01$. Significance levels are also given for the mean differences adjusted for LDL-peak diameter. Shading is used to separate the LDL and IDL subclass intervals designated by Krauss et al. (1).

the means. Significant differences (i.e., $P \leq 0.01$) from two-sample *t*-tests are shown at the bottom. Both the lipid-stained gels (displayed) and protein-stained gels (not displayed) showed that men had higher LDL-IIIA and LDL-II absorbance and lower absorbance within LDL-I.

Table 2 summarizes the relationships of lipid-stained and protein-stained LDL absorbance to triglycerides, LDL cholesterol, HDL_2, HDL_3, apoA-I and B, IDL and VLDL mass, and LDL peak flotation rate. There is general agreement between the lipid- and protein-stained LDL; however, the lipid stain may be more sensitive than protein stain for detecting LDL lipoprotein relationships. There are 17 cases where the correlation is significant for lipid-stained LDL but not protein-stained LDL and only 4 cases where the opposite is true. The table shows that for men: *1*) lipid-stained LDL-IVB and LDL-III correlate positively with triglycerides, apoB, IDL, and VLDL mass concentrations, and negatively with HDL_2 and apoA-I; *2*) lipid-stained and protein-stained LDL-II exhibit positive correlations with LDL cholesterol; *3*) lipid-stained LDL-II also exhibits positive correlation with apoB and negative correlations with HDL_3 cholesterol and HDL_2 mass whereas protein-stained LDL-II does not; *4*) triglycerides and VLDL mass both correlate positively with LDL-IVA and negatively with LDL-I; and *5*) LDL peak flotation rate correlates negatively with LDL-IV and III and positively with lipid-stained LDL-II. In women, LDL-IIIA and LDL-II correlate positively with

TABLE 2. Summary of the relationships of lipid-stained and protein-stained LDL to plasma lipid, lipoprotein, and apolipoprotein levels in a cross-sectional sample of 115 men and 78 women

	LDL-IVB	LDL-IVA	LDL-IIIB	LDL-IIIA	LDL-II	LDL-I	IDL
Men (lipid-stained/protein-stained)							
Triglycerides	+ / +	+ / +	+ / +	+ / +	0/0	– / –	0/0
LDL cholesterol	0/0	0/0	0/0	+ / +	+ / +	0/0	+ /0
HDL_2 cholesterol	– /0	0/0	– / –	– / –	0/0	0/ +	0/ +
HDL_3 cholesterol	0/0	0/0	– / –	0/0	– /0	0/0	+ /0
HDL_2 mass	– /0	0/0	– / –	– / –	– /0	0/0	0/0
HDL_3 mass	0/0	0/0	– / –	0/0	0/0	0/0	0/0
ApoA-I	– /0	– /0	– / –	– /0	0/0	0/0	0/0
IDL mass	+ /0	0/0	+ /0	+ / +	0/0	0/0	0/0
VLDL mass	+ / +	+ / +	+ / +	+ / +	0/0	– / –	0/0
ApoB	+ /0	+ /0	+ / +	+ / +	+ /0	0/0	0/0
LDL peak flotation rate	– / –	– / –	– / –	– / –	+ /0	0/ +	0/0
Women (lipid-stained/protein-stained)							
Triglycerides	0/0	0/0	0/0	+ / +	+ / +	0/0	0/0
LDL cholesterol	0/0	0/0	0/0	0/0	+ / +	0/0	0/0
HDL_2 cholesterol	0/0	0/0	0/0	– / –	– / –	0/0	0/0
HDL_3 cholesterol	0/0	0/0	0/0	0/0	0/0	0/0	0/0
HDL_2 mass	0/0	0/0	0/0	– /0	– / –	0/0	0/0
HDL_3 mass	0/0	0/0	0/0	0/0	0/0	0/0	0/0
ApoA-I	0/0	0/0	0/0	0/0	0/0	0/0	0/0
IDL mass	0/0	0/0	0/0	+ / +	+ / +	0/0	0/0
VLDL mass	0/0	0/0	0/0	+ / +	+ / +	0/0	0/ +
ApoB	0/0	0/0	0/0	+ / +	+ / +	0/0	0/0
LDL peak flotation rate	– /0	0/0	0/0	– / –	– / –	0/0	0/0

(+)Designates a significant positive correlation ($P \leq 0.01$) for at least one-half of the LDL subclass; (–) designates a significant negative correlation ($P \leq 0.01$) for at least one-half of the LDL subclass; (0) designates that the correlations are not significant for at least one-half of the subclass.

triglycerides, apoB, IDL and VLDL mass, and negatively with HDL_2 and LDL peak flotation rate.

Fig. 2 and **Fig. 3** display the correlations between selected lipoprotein measurements and lipid-stained LDL by particle diameter. The graphs show that the subclass intervals of Table 2 only approximate the regions of positive and negative correlation. In men, the graphs reveal that the strength of the correlations with smaller LDL (21–25.5 nm) have two relative maxima: one within LDL-IVB and one within LDL-III. In women, LDL absorbance correlates with triglycerides, apoB, and IDL; however, the domain of positive correlations is more restricted than in men and for particles larger than 24 nm the positive correlation appears to be shifted about 1 nm to the right. Women's LDL-IIIB shows no significant relationship to triglycerides, apoB, IDL mass, HDL_2 mass, and HDL_3 mass despite the significant correlation between men's LDL-IIIB and these variables.

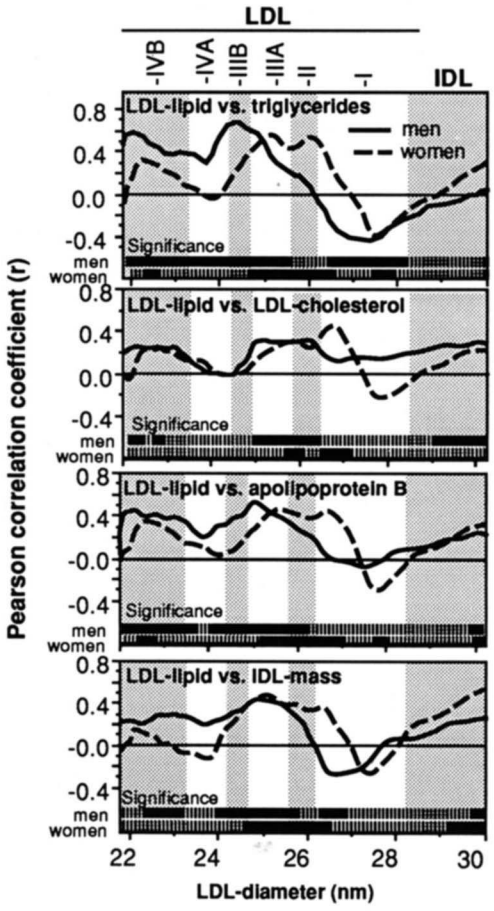

Fig. 2. Correlation coefficients for absorbance of lipid-stained LDL with plasma triglyceride, LDL cholesterol, apoB, and IDL mass concentrations in 115 men and 78 women. The bar at the bottom of each graph designates the diameter values having significant (solid) and nonsignificant (dashed) correlations at $P \leq 0.01$. The calculations are performed by correlating each persons' lipoprotein value with their absorbance of lipid-stained LDL. This is repeated for all 165 diameter values.

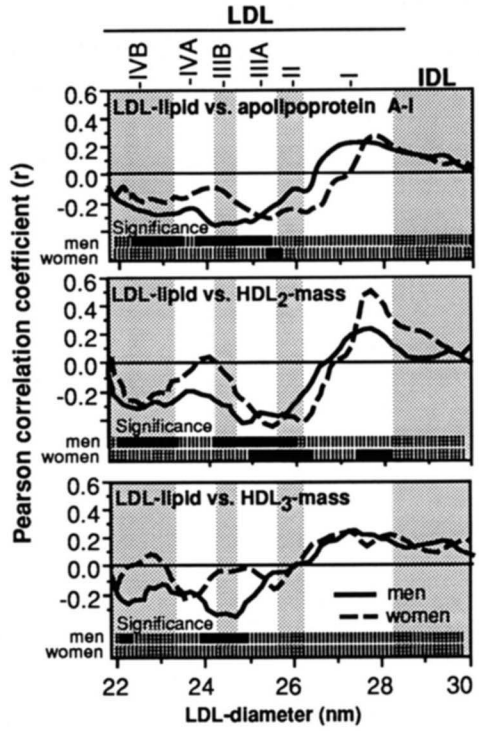

Fig. 3. Correlation coefficients for absorbance of lipid-stained LDL with plasma apoA-I, HDL_2 mass, and HDL_3 mass in 115 men and 78 women. The bar at the bottom of each graph designates the diameter values having significant (solid) and nonsignificant (dashed) correlations at $P \leq 0.01$.

Fig. 4 compares the mean levels for lipid-stained LDL for men with triglycerides above and below 150 mg/dl. The men with high triglyceride levels have significantly higher LDL-IVB and LDL-III and significantly lower LDL-I.

Correlations between LDL and HDL absorbance (gradient gel)

Blanche et al. (19) proposed five HDL subclasses based on their electrophoretic mobility: HDL_{3c} (7.2–7.8 nm), HDL_{3b} (7.8–8.2 nm), HDL_{3a} (8.2–8.8 nm), HDL_{2a} (8.8–9.7 nm), and HDL_{2b} (9.7–12 nm.) Their correlations with lipid-stained LDL are displayed in the contour plots of **Fig. 5** for women, and **Fig. 6** for men. Contours designate levels of constant positive correlation (solid lines) or negative correlation (dashed lines). This is illustrated in the example at the bottom of Fig. 5, which plots the correlation of LDL absorbance versus HDL absorbance at an HDL diameter of 7.9 nm. Vertical lines show the correspondence between the contour lines (above) and the correlation coefficients (below). Shading indicates those regions that are significantly correlated at $P \leq 0.01$.

Absorbance within LDL-I correlates positively with HDL_{2a} and HDL_{2b} and negatively with HDL_{3b} and

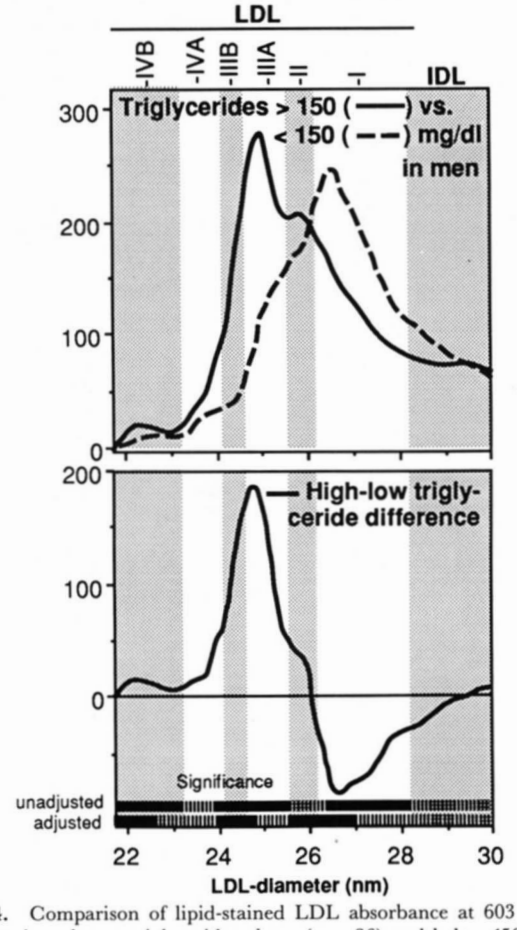

Fig. 4. Comparison of lipid-stained LDL absorbance at 603 nm in men having plasma triglycerides above (n = 36) and below 150 mg/dl (n = 79). The bar at the bottom of the difference plot designates the diameter values having significant (solid) and nonsignificant (dashed) differences at $P \leq 0.01$. Significance levels are also given for the mean differences adjusted for LDL-peak diameter. Only four women had triglycerides ≥150 mg/dl.

larger HDL_{3c}. LDL-IIIA and LDL-II absorbance correlate negatively with HDL_{2b} and positively with HDL_{3b}. In men, but not women, LDL-IIIB and LDL-IVB correlate negatively with HDL_{2b} and HDL_{2a}, and LDL-IVB correlates positively with HDL_{3b}. **Fig. 7** shows that men who have a predominant HDL_{3b} peak have significantly higher absorbance within LDL-IVB and LDL-III and significantly lower LDL-I absorbance than men with a predominant HDL_{3a} peak.

Adjustment of LDL absorbance for LDL peak diameter

Analysis of covariance was used to determine whether mean differences in absorbance in Figs. 1, 4, and 7 are significant when adjusted for LDL peak diameter. The significance levels for the adjusted mean differences in LDL lipid absorbance are given at the bottom of each plot. When adjusted, the men with high triglyceride levels had higher absorbance within LDL-IVB and LDL-IIIB and lower absorbance within LDL-II and LDL-I than men with low triglyceride levels. Adjustment eliminated the LDL-IVA, LDL-IIIA, and LDL-II differences between men and women and the LDL-IVB difference between men with predominant HDL_{3a} and HDL_{3b} peaks.

Partial correlations (not displayed) were used to adjust the correlations of Figs. 2 and 3 for LDL peak diameter. The results were as follows. In men: triglycerides correlated with LDL absorbance between 21.8–22.4 nm, 23.9–24.7 nm, 26.1–27.3 nm, and 28.5–29.6 nm; apoB correlated with absorbances between 21.9–22.6 nm, 24.7–25.1 nm, and 27.6–30 nm; IDL mass correlated with absorbance between 26.4–26.6 nm and 27.6–30 nm; and HDL_2 mass correlated with absorbance between 28.1–29.3 nm. In women: triglycerides correlated with LDL absorbance between 28.3–30 nm; apoB correlated with absorbances between 26.4–26.7 nm and 28.3–30.0 nm; and IDL mass correlated with absorbance between 28.1–30 nm. Adjustment for LDL peak diameter does not eliminate the tendency for the positive correlations of LDL absorbance with triglycerides, IDL mass, and VLDL mass to be shifted towards larger diameter values in women as compared to men.

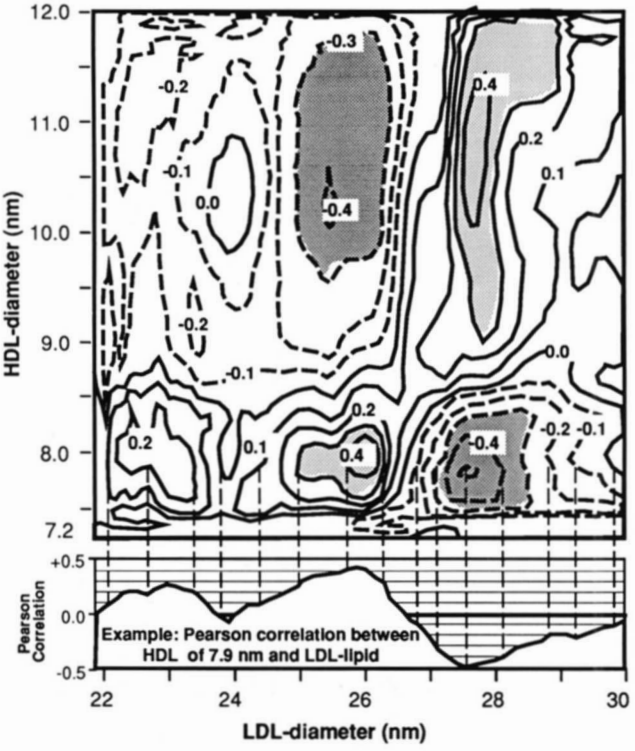

Fig. 5. Contour plot of the correlations between absorbance of lipid-stained LDL and protein-stained HDL in 78 women. Contours designate levels of constant positive correlation (solid lines) or negative correlation (dashed lines). This is illustrated in the example at the bottom of the figure, which plots the correlation of LDL absorbance versus HDL absorbance at an HDL diameter of 7.9 nm. Vertical lines show the correspondence between the contour lines and the correlation coefficients. Shading indicates those regions that are significantly correlated at $P \leq 0.01$.

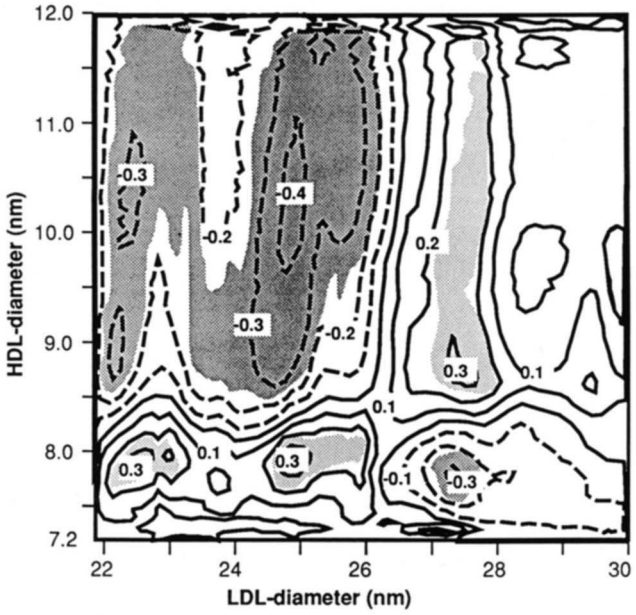

Fig. 6. Contour plot of the correlations between absorbance of lipid-stained LDL and protein-stained HDL in 115 men.

Correlations of LDL and IDL mass concentrations and plasma lipoproteins (analytic ultracentrifuge)

Table 3 displays the correlation coefficients between plasma lipoprotein and LDL mass concentrations by flotation intervals. Consistent with the correlations of Figs. 2 and 3: *1*) in men, but not women, LDL of S_f0–2 was significantly correlated with triglycerides, apoB, and apoA-I; *2*) significant correlations between the womens' LDL mass and their triglycerides, apoB, IDL mass, apoA-I, and HDL_2 mass were shifted about 1 flotation unit higher than the mens'. In general, larger coefficients were obtained when lipoprotein and apolipoprotein concentrations were correlated with LDL mass than with LDL absorbance on lipid-stained or protein-stained gels. In both men and women, apoB and IDL mass correlated significantly with plasma mass concentrations of S_f10–12 (Table 3) and portions of the IDL on lipid-stained gels (Fig. 2). In women, plasma IDL mass concentrations also correlated with protein-stained absorbance between 29 and 30 nm (not displayed).

Correspondence between gradient gel electrophoresis and analytic ultracentrifugation

Fig. 8 examines the relationship between gradient gel electrophoresis and analytic ultracentrifugation. For the individual flotation intervals S_f0–1, S_f1–2, ...S_f12–14, we correlated mass concentrations with lipid-stained LDL absorbance for all diameter values between 21.8 and 32 nm. The diameter having the highest correlation was plotted against the midpoint of the flotation interval. Although the correlation is expected to be highest for the diameter having the highest concentrations of lipoprotein mass from the flotation interval, the maximum value may be affected by measurement error and the interdependence among subclasses. A quadratic regression curve fitted through the points provides one estimate of the correspondence between flotation rate and diameter. The curve is monotonic between $S_f1.5$ and 13, and has the following formula:

$$\text{diameter} = 23.78 + 0.18 \times \text{flotation rate} + 0.03 \times \text{flotation rate}^2$$

The graph suggests that relationships involving IDL mass concentrations (S_f12–20) may be primarily reflected in lipid-stained absorbance of particle diameters above 30 nm.

DISCUSSION

Analytic ultracentrifugation measures plasma lipoprotein mass concentrations over the domain of LDL flotation rates. The method is laborious and is often impractical for large studies. Our objective has been to assess

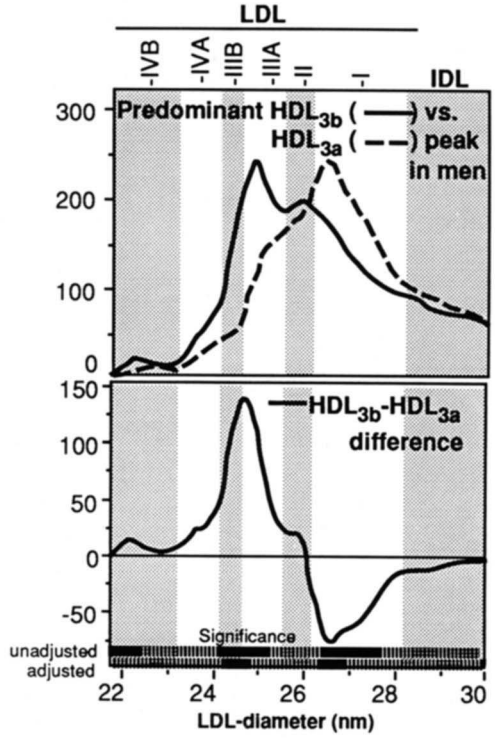

Fig. 7. Comparison of lipid-stained LDL absorbance at 603 nm in men having a predominant HDL_{3b} peak (n = 34) and those having a predominant HDL_{3a} peak (n = 81). The bar at the bottom of the difference plot designates the diameter values having significant (solid) and nonsignificant (dashed) differences at $P \leq 0.01$. Significance levels are also given for the mean differences adjusted for LDL-peak diameter. No women in our sample had a predominant HDL_{3b} peak.

TABLE 3. Pearson correlation coefficients between the analytic ultracentrifuge measurements of mass concentrations of individual LDL flotation intervals and apoA-I and B, triglycerides, HDL , IDL, and VLDL in men and women

	Triglycerides	Apolipoprotein B	IDL Mass	Apolipoprotein A-I	HDL_2 Mass	HDL_3 Mass
Men						
S_f 0–1	0.33[a]	0.30[a]	0.07	−0.26[a]	−0.20	−0.25[a]
S_f 1–2	0.63[a]	0.50[a]	0.30[a]	−0.34[a]	−0.28[a]	−0.34[a]
S_f 2–3	0.75[a]	0.61[a]	0.42[a]	−0.38[a]	−0.37[a]	−0.36[a]
S_f 3–4	0.75[a]	0.71[a]	0.53[a]	−0.38[a]	−0.48[a]	−0.30[a]
S_f 4–5	0.56[a]	0.76[a]	0.54[a]	−0.27[a]	−0.51[a]	−0.17
S_f 5–6	0.04	0.57[a]	0.28[a]	0.08	−0.27[a]	0.12
S_f 6–7	−0.41[a]	0.13	−0.15	0.39[a]	0.18	0.34[a]
S_f 7–8	−0.56[a]	−0.12	−0.32[a]	0.45[a]	0.46[a]	0.39[a]
S_f 8–9	−0.57[a]	−0.23	−0.32[a]	0.43[a]	0.61[a]	0.35[a]
S_f 9–10	−0.47[a]	−0.11	−0.09	0.41[a]	0.62[a]	0.35[a]
S_f 10–12	0.01	0.46[a]	0.67[a]	0.18	−0.08	0.26[a]
Women						
S_f 0–1	−0.16	−0.12	−0.08	0.07	0.09	0.01
S_f 1–2	0.00	0.05	−0.05	−0.01	−0.02	−0.02
S_f 2–3	0.27	0.34[a]	0.13	−0.17	−0.29	−0.04
S_f 3–4	0.45[a]	0.54[a]	0.35[a]	−0.29	−0.47[a]	−0.05
S_f 4–5	0.58[a]	0.70[a]	0.48[a]	−0.33[a]	−0.60[a]	0.03
S_f 5–6	0.55[a]	0.77[a]	0.49[a]	−0.30[a]	−0.60[a]	0.17
S_f 6–7	0.27	0.65[a]	0.31[a]	−0.18	−0.40[a]	0.28
S_f 7–8	−0.25	0.28	−0.01	0.04	0.11	0.27
S_f 8–9	−0.53[a]	−0.05	−0.18	0.23	0.55[a]	0.14
S_f 9–10	−0.41[a]	−0.04	−0.02	0.26	0.65[a]	0.11
S_f 10–12	0.23	0.47[a]	0.76[a]	0.01	0.12	0.19

[a]Correlation significantly different from zero at $P < 0.01$.

whether gradient gel electrophoresis can provide quantitative LDL measurements over the domain of LDL particle sizes. Fig. 8 suggests there is a close correspondence between flotation rate and particle diameter.

Gradient gel electrophoresis versus analytic ultracentrifugation

There are aspects of LDL heterogeneity that are evident by gradient gel electrophoresis and not by analytic ultracentrifugation. Table 3 shows that LDL within S_f 0–7 correlates positively with triglycerides, apoB, and IDL mass and negatively with HDL_2 mass and apoA-I. The correlations attain a single maximum within this range. In contrast, the strength of the correlations of these lipoproteins with protein-stained or lipid-stained LDL within the 21–26 nm range appears to contain two relative maxima in men, one between 22.4 and 22.7 nm, the other between 24.4 and 25.2 nm (Figs. 2 and 3). These intervals correspond to the LDL-IVB and LDL-III subspecies we have identified previously (1). Thus a significant advantage of gradient gel electrophoresis is its ability to reveal relationships and group differences in LDL-IVB. We found that LDL-IVB absorbance correlated positively with triglyceride, apoB, IDL mass, and HDL_{3b} protein levels and negatively with apoA-I, HDL_2 mass, HDL_{3a} protein, HDL_{2a} protein, and HDL_{2b} protein. The strong correlations within this region are unexpected given that it represents a very small percentage of the total LDL mass (Fig. 1).

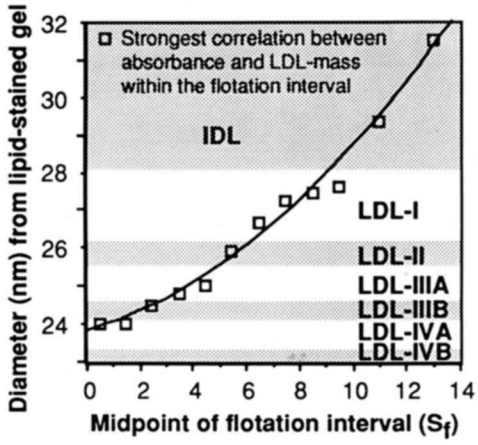

Fig. 8. Correspondence between LDL flotation rate (analytic ultracentrifuge) and lipid-stained LDL particle diameter (gradient gel electrophoresis) in 115 men. For the individual flotation intervals S_f0–1, S_f1–2, ...S_f12–14, the diameter of the lipid-stained LDL having the highest correlation with lipoprotein mass was plotted against the midpoint of the flotation interval.

Gradient gel electrophoresis of LDL may be less precise, however, than analytic ultracentrifugation. Table 1 shows that plasma lipoprotein concentrations often correlated more strongly with measurements of the LDL peak by analytic ultracentrifugation (peak flotation rate) than by gradient gel electrophoresis (peak diameter). They also correlated more strongly with measurements of amplitude by analytic ultracentrifugation (mass concentration, Table 3) than by gradient gel electrophoresis (absorbance, Fig. 2 and 3).

We have previously used contour plots to examine the interrelationships among levels of IDL, LDL, and HDL subclasses in terms of their individual flotation intervals (21), LDL individual flotation intervals of S_f3–6 and IDL of S_f12–20 correlated positively with HDL flotation intervals of $F_{1.20}0$–1.5 and negatively with $F_{1.20}2.5$–8. Larger LDL of 7–10 also correlated positively with $F_{1.20}2.5$–8. These relationships are also apparent for LDL and HDL absorbance (Fig. 6) when the following correspondences are drawn: $F_{1.20}0$–1.5 with 7.6–8.2 nm (HDL_{3b} and larger HDL_{3c}); $F_{1.20}2.5$–8 with 8.5–12 nm (primarily HDL_{2a} and HDL_{2b}); S_f3–6 with 24–26.5 nm (LDL-III and LDL-II); S_f7–10 and 26.5–28 (LDL-I). The contour plots for analytic ultracentrifuge measurements do not reveal one important set of relationships. Fig. 6 shows that LDL-IVB (22–23.2 nm) correlates positively with HDL_{3b} (7.6–8.2 nm) and negatively with HDL_{3a}, HDL_{2a}, and HDL_{2b} (8.5–12 nm).

Sex differences and LDL subclasses

There is a consistent male–female difference for correlations between LDL absorbance and lipoprotein concentrations. As compared to men, the positive correlation between triglycerides and LDL absorbance in women is shifted towards larger diameter values (i.e., shifted to the right). The increase in LDL accompanying higher triglyceride levels is restricted to particles with diameters less than 25.5 nm in men whereas in women the increase involves particles with diameters between 24.5 and 26.5 nm. Correspondingly, triglyceride levels correlate positively with LDL mass between S_f0–5 in men and between S_f3–6 in women. This shift is also apparent when lipid-stained LDL is correlated with HDL_2 mass, IDL mass, and apoB (Figs. 2 and 3).

These analyses raise the questions as to whether LDL subclasses in men and women are better defined by particle size or by their metabolic or functional characteristics. For example, delimiting LDL subclasses by size suggests that variations in triglycerides are associated with different LDL subclasses in men versus women. However, the observed relationship of LDL particle size with triglycerides and HDL_2 leads to the alternate interpretation that these relationships define particle subclasses with common metabolic function but differing size distribution in men versus women. It remains to be determined whether these differences might be related to differing physicochemical characteristics of LDL in men and women.

The shift could also arise because the men express a recently described LDL phenotype B pattern at a younger age than do women (10). The LDL phenotype B appears to be genetically inherited through a dominant major gene with age-dependent penetrance (10). Phenotype B individuals tend to have a peak diameter <25.5 nm (predominance of LDL-III and less commonly LDL-IV) and a distribution that is skewed towards larger diameter particles, whereas phenotype A individuals tend to have a peak diameter ≥ 25.5 nm and skewness towards smaller LDL (11). As compared to A, phenotype B individuals will tend to have higher triglycerides and apoB and lower HDL cholesterol and apoA concentrations (9). In this regard, their plasma lipoprotein profiles are consistent with the lipoprotein relationships associated with smaller LDL (i.e., high levels of LDL-IVA and LDL-III, see Table 2). In women, the B phenotype is primarily expressed after menopause (10). The phenotype B is expected to be incompletely expressed in our sample of women because they were all premenopausal.

Summary

We believe that analysis of the total LDL particle size spectrum is a useful approach to studying lipoprotein heterogeneity. Statistical analysis of the absorbance of LDL stained for protein or lipid at each diameter value invokes no assumptions as to the range or shape of the component distributions. The correlations using gradient gel electrophoresis are weaker, but otherwise comparable to those obtained for analytic ultracentrifuge measurements of plasma LDL mass concentrations. The analysis of LDL lipid by Oil Red O stain in whole plasma yielded correlations and group differences involving particle size distributions comparable to those obtained by analyzing LDL protein by Coomassie G-250 stain in the $d < 1.063$ g/ml plasma fraction. Staining whole plasma for lipid eliminates the need to separate lipoprotein subfractions by ultracentrifugation.

Even though the chromogenic conversion from absorbance to plasma concentration is unknown, the correlations for LDL lipid and LDL protein concentrations will be identical to those calculated for absorbance if absorbance at any given LDL diameter increases linearly with plasma concentration, i.e., when the absorbance to lipid conversion involves the addition and/or multiplication of numerical constants. The linear conversion may even be different at each diameter. The *t*-test for LDL concentrations will also be identical to those calculated from absorbance. McNamara et al. (2) examined the possibility of concentration-dependent chromogenicity by sequentially diluting plasma samples and then comparing the area of the LDL band. Their procedure also used a lipid stain of whole plasma (Sudan black B stain). Over 98% of the variance in band area was explained by a linear regression of the plasma dilution ratio versus area (our analysis of their Table 1 data). The assumption of a linear conversion from absorbance to plasma concentration may be further relaxed when Spearman's correlations and Wilcoxon two-sample sign rank tests are used. Nonparametric statistical tests of LDL absorbance will be identical to those based on the unknown plasma concentrations provided that ordering the gels from lowest to highest absorbance is the same as ordering the gels from lowest to highest plasma

concentrations (i.e., monotonic transformations). This does not prove a direct correspondence between absorbance and concentrations, however, because other factors may affect the affinity of stain to LDL. Our results suggest that, as compared to analytic ultracentrifuge measurements of LDL mass, gradient gel measurements of LDL absorbance as described here provide greater resolution of LDL subclasses but less precision in estimating LDL levels.

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