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Development of a Rapid, Quantitative Method for LDL Subfractionation with Use of the Quantimetrix Lipoprint LDL System

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Background: **Recent evidence suggests that the presence of small, dense LDL is independently associated with increased risk of developing coronary artery disease. Current methods to subfractionate LDL are time-consuming and/or technically demanding. Therefore, we have sought the development of a less complex LDL subfractionation procedure.**

Methods: **LDL subfractions were separated using the Quantimetrix Lipoprint™ LDL System. High-resolution 3% polyacrylamide gel tubes were scanned densitometrically (610 nm) with a Helena EDC system. A computerized method to identify and quantitatively score the resolved LDL subfractions was developed. Results from the Quantimetrix method were compared using 51 plasma samples with values obtained by nondenaturing gradient gel electrophoresis (NDGGE) and nuclear magnetic resonance (NMR) spectroscopy.**

Results: **LDL subfractionation scores correlated significantly ($P < 0.05$) with triglyceride, HDL-cholesterol, apolipoprotein B100, and LDL-cholesterol/apolipoprotein B100 ($r = 0.591$, -0.392, 0.454, and -0.411, respectively). For 51 samples, the Quantimetrix method classified 21 with small, 14 with intermediate, and 16 with large LDL. Of the 21 samples classified as small by Quantimetrix, 20 (95%) were classified as small (n = 18) or intermediate (n = 2) by NDGGE. All of the 16 specimens classified as large by Quantimetrix were either large (n = 14) or intermediate (n = 2) by NDGGE. LDL score was inversely correlated ($r = -0.674$; $P < 0.0001$) with LDL particle size determined by NMR spectroscopy.**

Conclusions: **A quantitative method for the assessment of LDL particle size phenotype was developed using the Quantimetrix Lipoprint LDL System. The method can be performed in less than 3 h in batch mode and is suitable for routine use in clinical laboratories.**

Increased blood concentrations of LDL have a high positive correlation with the incidence of coronary artery disease (CAD).[4] Circulating LDL particles show heterogeneity with respect to size, density, and chemical composition, differences that have led to the recognition of two distinct phenotypes: phenotype A, associated with large, buoyant LDL particles; and phenotype B, in which small and dense particles predominate *(1)*. An association between phenotype B and increased risk for CAD was first postulated in retrospective studies *(2–5)* and was later supported by prospective studies *(6, 7)*. Subsequently, it has been reported that LDL phenotyping could help predict response to lipid-lowering therapy *(8)*. Recently, the small and dense LDL particles have been recognized as a distinctive biochemical feature of an inherited condition characterized by dyslipidemia, hypertension, insulin resistance, and hypercoagulability—a milieu of metabolic abnormalities that confers an increased risk for CAD *(9, 10)*.

Despite this established role of small, dense LDL particles in cardiovascular risk assessment, current meth-

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[4] Nonstandard abbreviations: CAD, coronary artery disease; NDGGE, nondenaturing gradient gel electrophoresis; NMR, nuclear magnetic resonance; LDLSF, LDL subfractionation; AUC, area under the curve; and IDL, intermediate-density lipoprotein.

ods available for LDL subfractionation are technically demanding and not applicable in a routine clinical laboratory. These include density gradient ultracentrifugation *(11–14)*, nondenaturing gradient gel electrophoresis (NDGGE) *(15, 16)*, and nuclear magnetic resonance (NMR) spectroscopy *(17–19)*, procedures that have one or more of the disadvantages of being labor-intensive, technically demanding, expensive, and having a lengthy turnaround time.

We evaluated a polyacrylamide gel tube electrophoresis method (Lipoprint™ LDL System; Quantimetrix, Redondo Beach, CA), which requires 25 μL of plasma or serum and has a total analysis time of <3 h. Although the equipment and all necessary reagents are commercially available, only minimal recommendations regarding densitometry are provided without reliable instructions to evaluate the scan or account for lot-to-lot inconsistencies. For these reasons, we developed a densitometric scanning system to generate raw data and a program to analyze data and calculate a numerical score for the electrophoretic scan pattern of the size-fractionated LDL. The data analysis system is effective in minimizing intraindividual interpretation bias, recalibration after lot changes, and particularly in establishing subfractionation scores corresponding to LDL size phenotypes.

We applied this modified method to plasma samples from 51 patients and compared the results with a commercially available NDGGE method (Zaxis) and an NMR method (LipoMed). Correlation between LDL subfractionation (LDLSF) scores and other measured lipid markers were also assessed.

Materials and Methods

SAMPLES

Eighty-nine blood samples were collected in EDTA tubes, and plasma was isolated by centrifugation (2000*g* for 15 min). These samples were used to establish assignment of LDL particle size phenotype to LDL score ranges. Fifty-one additional samples were collected for use in method comparison studies. Plasma was stored at −70 °C until analysis. Analyses were performed within 1 month of sample collection. This study was approved by the Mayo Clinic Institutional Review Board (IRB No. 1793-97). Quantimetrix LipoPhor lyophilized lipoprotein material was used as quality-control material. This control material was analyzed with each batch of samples to establish run acceptability. The interassay mean value ± SD was 5.1 ± 0.8 (n = 25).

PROCEDURES

Quantimetrix Lipoprint LDL System. High-resolution 3% polyacrylamide gel tubes were used for electrophoresis. LDL subfractionation was performed as described in the Lipoprint LDL System product insert. Briefly, 25 μL of sample was mixed with 200 μL of liquid loading gel. The loading gel contained Sudan Black B dye to stain the lipoproteins. The resulting mixture was added to the top of precast 3% polyacrylamide gel tubes. After photopolymerization at room temperature for 30 min, samples were electrophoresed for 1 h (3 mA/gel tube). The electrophoresis was followed by "resting" the tubes in the dark for 1 h before performing the densitometry. This ambient incubation was added to increase the uniformity of the bands before scanning.

Densitometry was performed at 610 nm using a Helena EDC system (Helena Laboratories), which was improved by addition of a 12-tube holding device engineered and built by the Mayo Clinic Engineering Department. The holding device was constructed from 1/4-inch black Lucite® CP acrylic (ICI acrylics, Inc.) engineered to fit securely in the Helena EDC densitometer. Twelve slots (7.6 × 0.6 cm) were cut through the acrylic sheet to hold the gel tubes (7.7 × 0.7 cm) in place. The holding device markedly improved the consistency of gel tube scanning by securing the tube during the scanning process. Raw data generated by the densitometer and the electrophoretic scan pattern of the size-fractionated LDL were analyzed using a computer program of our design to calculate a LDL subfractionation score and assign a phenotype (A, B, or intermediate).

Computer-assisted data analysis of densitometric scans. Raw data from the densitometer were imported into a Microsoft Excel spreadsheet, and a scan was generated. Using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image), we wrote a macro that defined electrophoretic mobility (R_f) values across the scan, divided the scan at designated R_f values, and calculated the area under the curve (AUC) for each fraction. A complete description of the R_f designation may be found in the *Results* under "Development of a LDLSF Scoring System". The computer then summed the AUCs in a weighted manner [$(LDL1_{AUC} \cdot 1) + (LDL2_{AUC} \cdot 2) + (LDL3_{AUC} \cdot 4) + (LDL4_{AUC} \cdot 8) + (LDL5_{AUC} \cdot 16) + \ldots$] and divided the sum by the total unweighted LDL_{AUC} (i.e., $LDL1_{AUC} + LDL2_{AUC} + LDL3_{AUC} + LDL4_{AUC} + \ldots$) to generate a LDL score. This scoring system produced scores ranging from as low as 1 to as high as 100 in patient samples with a great preponderance of small, dense (fast migrating) LDL in the samples we have tested to date. Division by the total unweighted LDL_{AUC} was performed to normalize the effect of various sample LDL concentrations on the LDLSF score.

LDL scores were determined in plasma samples from 89 patients. On the basis of LDL migration rates on the scan, LDL phenotypes A (predominantly large, buoyant LDL), B (predominantly small, dense LDL) *(1)*, or intermediate were assigned to score ranges as follows: LDLSF score <5.5 = phenotype A, 5.5–8.5 = intermediate, >8.5 = phenotype B. A complete description of the rationale for this phenotyping assignment is given in the *Results* under "Development of a LDLSF Scoring System".

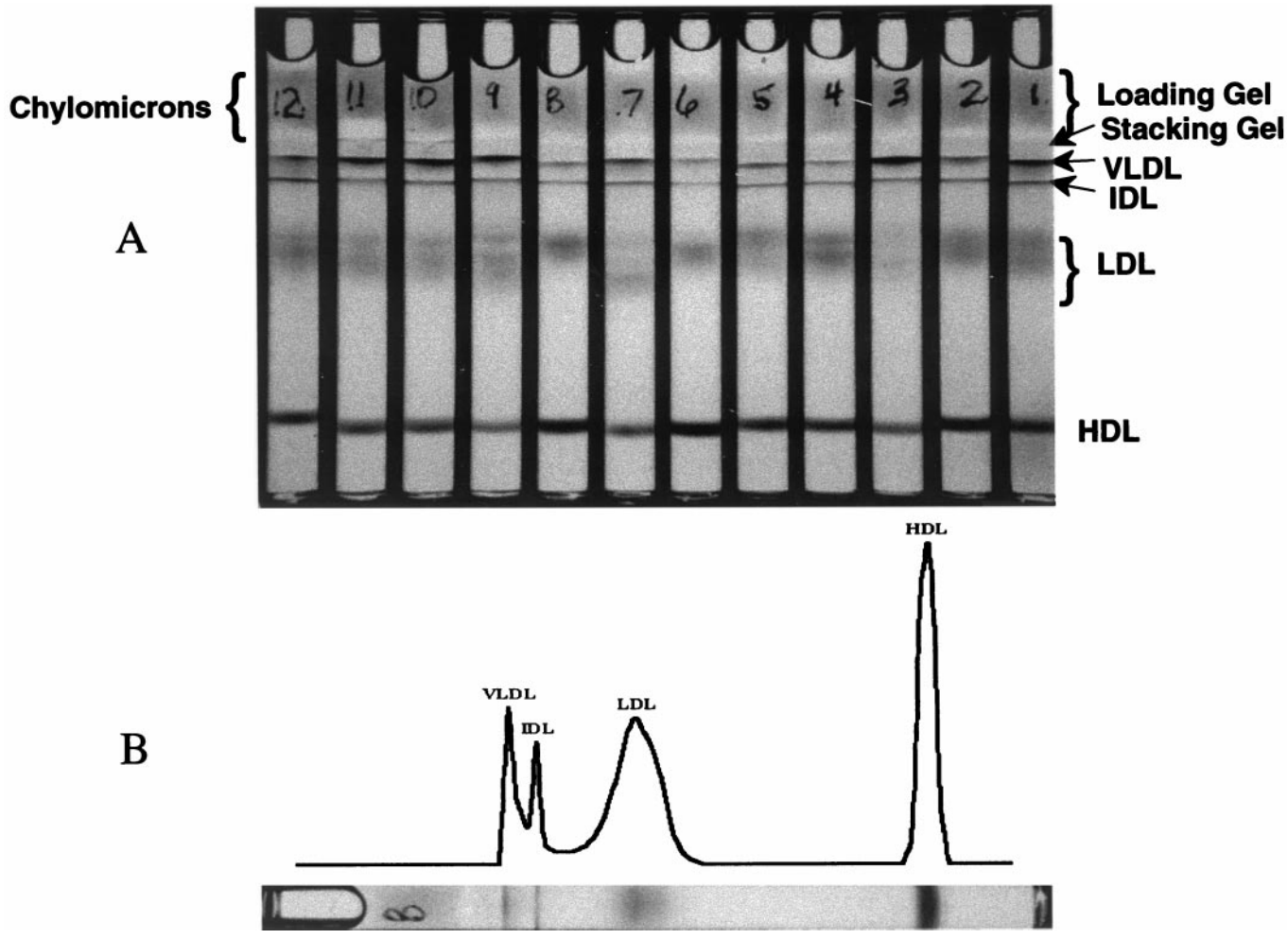

Fig. 1. Electrophoretic separation of lipoproteins on the Quantimetrix Lipoprint LDL System.

(*A*), photograph of 12 gel tubes to which sample had been added and electrophoresis performed. Electrophoretic migration was from the *top* of the tube (cathode) to the *bottom* (anode). Separation is based primarily on particle size by the sieving action of the gel. Chylomicrons remain in the loading gel, VLDL migrates slowly, and HDL migrates rapidly. The LDL particles are separated in the *middle portion* of the gel. (*B*), densitometric scan of gel tube 8.

Method comparison. A method comparison study was performed using 51 patient plasma samples to confirm proper assignment of LDLSF score to phenotype. Samples were chosen to give a wide range of LDL scores based on the Quantimetrix method and were sent to the South Bend Medical Foundation (South Bend, IN) for LDL subfractionation using NDGGE (LFS Lipogel System®; Zaxis) and to LipoMed (Raleigh, NC) for LDL subfractionation using the LipoMed NMR Lipoprofile®. Personnel performing subfraction analysis at South Bend and LipoMed were blinded to the Quantimetrix results until analysis was complete.

Zaxis LFS Lipogel System. This system uses a bifocal gradient polyacrylamide gel. The bottom half of the gel consists of an 8–27% gradient, whereas the top half is a 0.5–5% gradient. Briefly, sample was mixed with loading dye, and 4 μL was added to the preelectrophoresed gel. Electrophoresis was performed for 15–20 h, and gels were stained overnight with Sudan Black B at room temperature. After destaining, the gels were fixed in 0.35 mol/L acetic acid. The gels were scanned by densitometry, and LDL phenotype was assigned by comparison of LDL migration distance in the sample to that of plasma calibrators of known LDL phenotype. Plasma samples that were used as calibrators for the Zaxis method were sent to Berkeley HeartLab (Alameda, CA) for determination of LDL size and phenotyping by NDGGE. The samples were classified by the Berkley HeartLab method as having predominantly LDL particle sizes corresponding to the Berkley HeartLab established cutpoints for phenotypes A and B, respectively. These plasma samples were run as calibrators along with patient samples on each gel and served as the basis by which patient samples were phenotyped using the Zaxis method.

LipoMed NMR Lipoprofile. This system measures lipoproteins directly, without fractionation, in <5 min *(20)*. An automated liquid sample handler transfers plasma to the NMR instrument. Each lipoprotein particle with a given

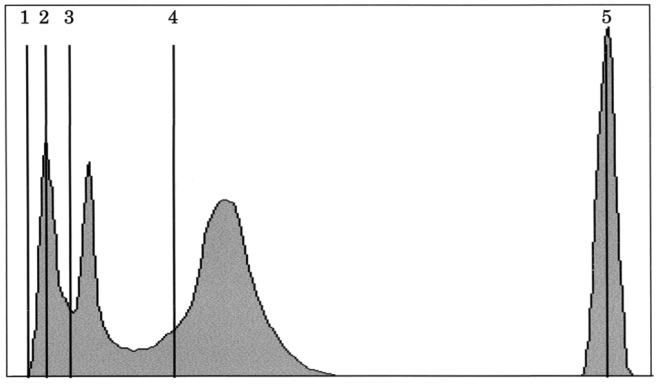

Fig. 2. Example of the computer scoring system.

To calibrate the computer scoring system, 30 phenotype A samples were used. Five points were identified on the densitometric scan of each sample, based on migration of the various lipoproteins as described in the text. Mean R_f values were used as reference points for scoring patient samples.

diameter range broadcasts a unique NMR signal, producing a NMR spectrum for the sample. Computer algorithms are used to deconvolute the spectrum, providing a direct measure of the concentrations of 15 different subclasses of VLDL, LDL, and HDL. An overall LDL size measurement is determined and used to categorize the patients phenotype as phenotype A (20.6–22.0 nm), intermediate (20.4–20.5 nm), or phenotype B (19.0–20.3 nm). This instrumentation is not currently available for purchase, and samples must be sent to LipoMed for analysis.

Lipid measurements. Total cholesterol and triglycerides were assayed on a Hitachi 717 chemistry analyzer (Roche Diagnostics) using Bayer/Technicon Omnipack and Bayer/Technicon RA/opeRA enzymatic reagents (Bayer/Technicon), respectively. HDL-cholesterol was determined using selective precipitation followed by the enzymatic/colorimetric method for measuring cholesterol described above. A solution of dextran sulfate and Ca^{2+} (1:10 solution of 40 g/L dextran sulfate in 111 g/L $CaCl_2$) was used to precipitate non-HDL lipoproteins (chylomicrons, VLDL, and LDL). HDL-cholesterol in the supernatant was measured using Technicon reagents on a Hitachi 717. Calculated values for LDL-cholesterol were derived using the Friedewald equation *(21)* as follows:

$$\text{LDL-cholesterol} = \text{Total cholesterol} - \text{HDL-cholesterol} - \text{Triglyceride}/5$$

Apolipoprotein AI and B100 were measured by automated immunoturbidimetric assays on a Hitachi 912 chemistry analyzer (Roche Diagnostics) using reagents from Diasorin Inc. Lipoprotein(a) was also measured by immunoturbidimetry using Diasorin reagents on a Hitachi 912.

ANALYTICAL PERFORMANCE

Precision. Intraassay precision was determined using plasma samples from a phenotype A patient (mean LDL score = 3.2) and a phenotype B patient (mean LDL score = 13.0) analyzed in 10 gel tubes. Interassay precision was determined using plasma from 19 patients assayed on 3 separate days over a 1-week period.

Linearity. Linearity of the method was determined by performing mixing studies of a patient serum sample containing predominantly large, buoyant LDL particles (phenotype A) with a sample containing a high proportion of small, dense LDL (phenotype B).

Stability. Fresh serum samples from 20 patients were analyzed immediately after collection and after storage at 4, −20, and −70 °C for 14 days. LDLSF scores were 2.9–15.8. Sample stability at −20 and −70 °C was also investigated using six EDTA plasma samples from phenotype B patients (LDLSF scores = 9.6–54).

Statistics. Spearman rank correlation between LDLSF score and other lipid markers as well as between LDLSF score and LDL size (nm; LipoMed) was determined using StatView (Abacus Concepts, Inc.).

Results

QUANTIMETRIX LIPOPRINT LDL SYSTEM

Fig. 1A shows 12 gel tubes after completion of the electrophoresis. Electrophoretic migration was from the top of the tube (cathode) to the bottom (anode). Visible bands correspond to lipoprotein particles stained with Sudan Black B dye before electrophoresis. Visual inspection of the LDL region showed variable patterns, e.g., tube 7 has predominantly fast-migrating LDL (small LDL), whereas the LDL in tube 8 is predominantly slow-migrating LDL (large LDL). A densitometric scan of tube 8 is shown in Fig. 1B.

To document that visual interpretation of scans is highly individual-dependent, 34 densitometric scans from randomly selected patient samples were obtained and reviewed by five separate individuals who independently assigned a phenotype to each scan. Individuals were asked to assign a phenotype (A, B, or intermediate) to each scan. No restrictions were placed as to how each individual ascribed phenotypes. All reviewing individuals had been involved with initial evaluation of the Quantimetrix method, understood the electrophoretic process, and were familiar with LDL size phenotyping. However, complete agreement was achieved for only 11 (32%) of the 34 samples. Of the 11 samples with complete agreement, 10 were phenotype A and 1 was phenotype B. For nine (26%) of the samples, opposite phenotypes (A vs B) were assigned for the same sample. This survey supported our hypothesis that a standardized method for phenotype assignment would be helpful in avoiding subjective differences in result interpretation.

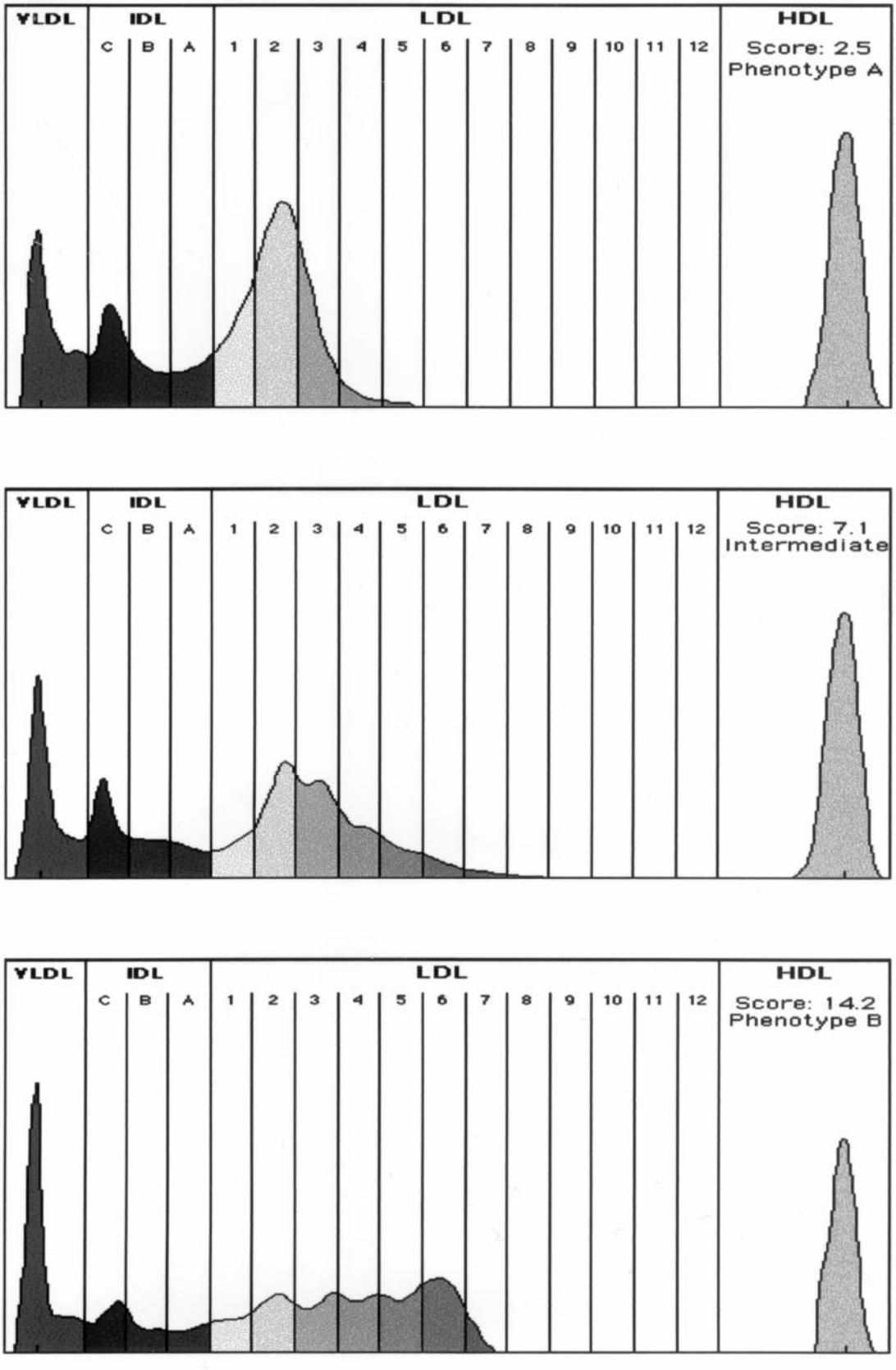

Fig. 3. Densitometric scans from phenotype A (*top*; score = 2.5), intermediate (*middle*; score = 7.1), and B (*bottom*; score = 14.2) samples.
Areas under the curve for each fraction were calculated, and the LDLSF score was generated using a *NIH Image* sample analysis macro as described in the text.

Table 1. Reagent lot-to-lot variability was addressed using a calibration macro.

Sample no.	LDLSF score: Lot 1 reagents; Lot 1 macro	Lot 2 reagents; Lot 1 macro	Lot 2 reagents; Lot 2 macro
1	3.6	2.4	3.8
2	3.8	2.3	4.2
3	4.2	2.5	4.1
4	4.6	2.8	5.2
5	4.9	2.8	5.4
6	5.2	2.5	4.8
7	5.8	2.5	4.5
8	6.2	2.8	5.0
9	6.2	2.6	4.8
10	6.8	4.2	7.1
11	8.7	4.4	8.5
12	9.2	5.0	11.1
Mean LDL score	5.77	3.07	5.71
Mean deviation, %		46.8	1.04

DEVELOPMENT OF A LDLSF SCORING SYSTEM

Using an image processing program (NIH Image), we wrote a calibration macro to set separation lines at defined points across the densitometric scan. Vertical separation lines were defined using 30 different samples with type A phenotypes, based on qualitative scan appraisal. Five vertical lines were then placed at defined points for each of the 30 samples, as depicted in Fig. 2. The lines were placed as follows: Line 1 was placed at the beginning of the VLDL peak; lines 2 and 5 were identified as reference points at the apex of the VLDL ($R_f = 0$) and HDL ($R_f = 1$) peaks, respectively. Line 3 was placed at the valley between VLDL and intermediate-density lipoprotein (IDL). Line 4 was placed at a point representing 10% of the height from the valley between IDL and LDL to the top of the LDL peak to reduce the likelihood of including small IDL particles in the LDL scoring region. Relative to the two reference points, all 30 R_f values for each of the three lines (1, 3, and 4) were averaged. The R_f means were then used to identify lipoprotein windows: the window between lines 1 and 3 was used for VLDL, the window between lines 3 and 4 was used for IDL, and the window from line 4 to line 5 was used for LDL.

The average R_f values were then entered into a second NIH Image sample analysis macro, which was designed to calculate a LDLSF score for patient samples and controls. The program dropped vertical lines at appropriate R_f locations after the technologist identified the apex of the VLDL and HDL peaks. A total of 18 lines were dropped, creating 17 fractions. The first fraction contained VLDL. The IDL window was divided into 3 equidistant fractions, and the LDL window into 12. HDL was contained in the final fraction. The program then calculated the AUC for each fraction in the LDL window and performed logarithmic transformation of the AUC data, as described in *Materials and Methods*, to produce a LDLSF score. Fig. 3 depicts the scoring process for phenotype A, intermediate, and B samples (LDLSF score = 2.5, 7.1, and 14.2, respectively). The computer system allowed a high degree of consistency and a means to alleviate interpretative bias.

Scored densitometric scans from 89 patient plasma samples were used to establish assignment of LDL particle size phenotype to LDLSF score ranges. This was accomplished as follows: If the combined area of the first 3 LDL fractions was greater than two-thirds of the total area, phenotype A was assigned. If the sum of the AUCs of fractions 1–3 was equal to or greater than one-half but less than two-thirds of the total area, an intermediate classification was made. If the sum of the AUCs of fractions 1–3 was less than one-half of the total area, phenotype B was assigned. This allowed classification of 55 patients with pattern A, 19 as intermediate, and 15 with pattern B. The LDLSF scores (mean ± SD) for the phenotype A, intermediate, and phenotype B groups were 3.7 ± 1.1, 6.6 ± 0.8, and 15.0 ± 7.2, respectively. On the basis of these results, the following phenotyping criteria were established: LDLSF score <5.5, phenotype A; score 5.5–8.5, intermediate; score >8.5, phenotype B. The SD for phenotype B overlaps with the intermediate range because of very high LDL scores associated with some phenotype B specimens.

ASSAY IMPRECISION

Patient plasma samples were used to assess the imprecision of the assay. Intraassay imprecision (n = 10) for two plasma samples gave CVs of 8.1% and 8.4% at LDL subfractionation scores of 3.2 and 13.0, respectively. For 19 patients with LDL scores ranging from 2.9 to 16.5 assayed on 3 days over a 1-week period, the mean interassay CV was 13%. Improved precision was demonstrated by analysis of each patient sample in duplicate. The intraassay CVs for patient samples analyzed 10 times

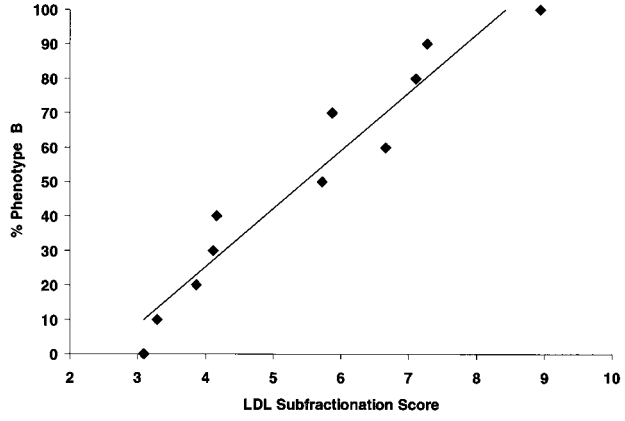

Fig. 4. Linearity of the LDLSF scoring system.

Increasing amounts of a phenotype B sample (LDLSF score = 8.9) were added to a phenotype A sample (LDLSF score = 3.1). The LDLSF score increased with increasing percentage of phenotype B sample.

Table 2. Correlation of LDL subfractionation score with other lipid markers (n=51).

Analyte	Spearman correlation coefficient (*r*)	*P*	Analyte concentration (mean ± SD), mg/L		
			Phenotype A	Intermediate	Phenotype B
Triglyceride	0.591	<0.0001	1260 ± 330	2590 ± 1130	2580 ± 1510
HDL-cholesterol	−0.392	0.006	600 ± 160	470 ± 120	450 ± 170
Apo[a] B100	0.459	0.001	920 ± 180	1210 ± 240	1160 ± 220
LDL-cholesterol/Apo B100 (no units)	−0.411	0.004	1.21 ± 0.13	1.05 ± 0.15	1.01 ± 0.22
Apo AI	−0.152	0.287	1530 ± 320	1440 ± 240	1380 ± 300
Total cholesterol	0.165	0.247	1980 ± 320	2270 ± 490	2160 ± 410
LDL-cholesterol	0.103	0.469	1130 ± 290	1280 ± 390	1210 ± 390

[a] Apo, apolipoprotein.

in duplicate were 4.6% and 4.3% at LDLSF scores of 3.4 and 13.3, respectively.

LOT-TO-LOT VARIABILITY

To determine whether variability between lots of reagents (lots included gel tubes, loading gel with stain, and electrophoresis buffer) existed, 12 patient samples with a range of LDLSF scores were assayed in duplicate by each of two lots, and the results were compared. With the first lot of reagents, LDLSF scores of 3.6–9.9 (mean = 5.8) were observed. With the second lot, scores were 2.3–5.0 (mean = 3.1). Thus, from one lot to the next, the absolute mean difference in LDL score was 46.8%. Significant lot-to-lot variability was noted when other lots were acquired and tested. This problem was addressed by acquiring 30 phenotype A samples, performing analysis, and applying the NIH Image calibration macro described above to drop lines at defined points across the scans. This established mean R_f values for the new lot. The new R_f values were then incorporated into the sample analysis macro, producing good correlation between reagent lots. When the updated macro was used to score the same 12 samples, the LDLSF scores were 3.8–11.1 (mean = 5.7) and the mean lot-to-lot difference was 1.04% (Table 1).

LINEARITY

Serum samples containing predominantly large, buoyant LDL particles (phenotype A) were mixed with samples containing a high proportion of small, dense LDL (phenotype B). The LDL scores progressed in a uniform manner, becoming larger with increasing proportions of phenotype B sample. Plotting the LDLSF score (x-axis) vs the percentage of phenotype B (y-axis) yielded the following linear equation: $y = 16.8x - 42.1$, with a correlation coefficient (r) of 0.9663 (Fig. 4).

SAMPLE TYPE AND STABILITY

EDTA plasma and serum samples were simultaneously collected from 33 volunteers. LDLSF scores ranged from 2.2 to 53.9 (EDTA) and from 2.4 to 50.5 (serum). Mean ± SD values for EDTA plasma and serum samples were 7.8 ± 9.9 and 8.4 ± 10.7, respectively. The regression equation was: serum = 1.039(plasma) + 0.252; $r = 0.9559$.

There was no discernible difference in LDL score between fresh controls (<1 week old) and those stored at 4 °C for up to 2 months. Specimen stability was assessed in 20 patient serum samples analyzed fresh and after storage for 14 days. The mean recoveries (± SD) of LDLSF scores at 2 weeks were 105% ± 16%, 69% ± 10%, and 74% ± 8.9% for samples stored at 4, −20, and −70 °C, respectively. Larger decreases (as a percentage) in the LDL score after freezing were noted for phenotype B samples. The mean recoveries of the LDLSF score in six EDTA plasma samples (phenotype B) frozen for 2 months at −20 and −70 °C were 95% ± 29% and 102% ± 15%, respectively.

INTERFERENCES

Two plasma samples were supplemented with ascorbic acid to a final concentration of 50 mg/L and with hemoglobin to a final concentration of 2000 mg/L. Neither

Table 3. Method comparison of LDLSF scores (n = 51).

Quantimetrix phenotype	n	LipoMed				Zaxis			
		Phenotype			Concordance, %	Phenotype			Concordance, %
		A	Int.[a]	B		A	Int.	B	
A	16	15	1	0	100[b]	14	2	0	100[b]
Intermediate	14	6	1	7	7[c]	3	9	2	64[c]
B	21	5	2	14	76[d]	1	15	5	95[d]

[a] Int., intermediate.
[b] Phenotype A or intermediate.
[c] Phenotype intermediate.
[d] Phenotype B or intermediate.

substance caused significant interference with LDLSF score determination (data not shown).

CORRELATION WITH OTHER LIPID MEASURES

The correlations between the LDL score and other lipid markers are shown in Table 2. The correlation was significant ($P \leq 0.05$) for triglycerides, HDL-cholesterol, apolipoprotein B100, and LDL-cholesterol/apolipoprotein B100. Statistically significant correlation was not observed between LDLSF score and total cholesterol, LDL-cholesterol, or apolipoprotein AI.

METHOD COMPARISONS

To verify appropriate assignment of LDL size phenotype to LDLSF score range, the Quantimetrix method was compared with a NDGGE method (Zaxis) and a NMR method (LipoMed). EDTA plasma samples from 51 patients, selected to give a mixture of phenotypes (A, B, and intermediate, based on LDL score) were sent for comparative analysis. Table 3 compares phenotypes assigned by each method. Quantitative analysis of LDL size was not performed with the Zaxis NDGGE method; therefore, a correlation plot could not be generated. A significant inverse correlation was observed ($r = -0.674$; $P < 0.0001$) between the LDLSF score and LDL size (nm) determined by the LipoMed method. The inverse relationship was expected because of the nature of the LDLSF scoring system, which produces higher LDL scores as particle size decreases.

Discussion

We have developed an automated, computer-assisted scoring system for routine quantitative determination of LDL subfractions in a clinical laboratory. This system is based on the Quantimetrix Lipoprint LDL System coupled with a processing method that is able to consistently score the electrophoretic patterns. This method is simple, cost-effective, and free of interindividual interpretation bias. A potential problem of lot-to-lot variability was eliminated by calibrating new lots against lot-specific average R_f values established using 30 samples from phenotype A patients.

The assay could be performed reliably on EDTA plasma or refrigerated serum, but LDL subfraction scores were not stable in frozen serum samples. Freezing serum at −20 or −70 °C decreased LDLSF scores, particularly in samples with high scores (phenotype B). For long-term storage, serum samples should not be used. The LDLSF score was demonstrated to be stable in EDTA plasma for at least 2 months when stored at −70 °C. LDL subfractionation can be performed reliably in serum samples stored refrigerated for up to 2 weeks.

The precision of the Quantimetrix method (between-run CV = 13%) was improved (mean CV = 4.5%) if samples were assayed in duplicate and reported as the average LDL subfraction score. For this reason, we run all of our routine LDL subclass analyses in duplicate and report the average score. This may be particularly important if the method is used in therapeutic studies to monitor treatment effects because duplicate measures decrease analytical variability, allowing more discrete determination of changes resulting from dietary or pharmacologic therapy.

In 51 samples used for method comparison, the LDLSF score was found to correlate significantly with triglyceride ($r = 0.59$; $P < 0.0001$) and HDL-cholesterol ($r = -0.392$; $P = 0.006$) concentrations as well as with other lipid markers (Table 2). This observation is consistent with previous reports including that of the Quebec Cardiovascular Study *(7)*, which reported correlation coefficients of −0.46 ($P < 0.001$) and 0.39 ($P < 0.001$) between LDL size (nm) and triglyceride and HDL-cholesterol concentrations, respectively. In the Quebec Cardiovascular Study *(7)*, the presence of small, dense LDL was independently associated with increased risk of developing ischemic heart disease even after adjustment for plasma triglyceride and HDL-cholesterol concentrations.

Phenotypes (A, B, or intermediate) were ascribed to LDL score ranges, and the method was compared with NDGGE and NMR methods for LDL subfractionation. The correlation of results between methods was satisfactory, indicating the viability of the Lipoprint LDL System for assessing LDL particle size phenotype. However, 5 of 21 samples classified as phenotype B by the Quantimetrix method were classified as type A by NMR. All five of these samples were classified as type B or intermediate by NDGGE. Additionally, one sample classified as type B by the Quantimetrix method was classified as type A by NDGGE. NMR classified this sample as intermediate. Adjustment of LDLSF score range did not improve the method comparison results. This comparison confirmed that appropriate assignment of LDLSF score ranges to LDL particle size phenotypes was achieved. These data indicate that although there is general agreement among the three methods, standardization of LDLSF methods is needed, and that the methods should not be used interchangeably.

In conclusion, the Quantimetrix Lipoprint LDL System combined with the automated scoring methodology developed in our laboratory enables quantitative LDL subfractionation in <3 h in batch mode; thus, it is suitable for routine use in clinical laboratories. The Quantimetrix Corporation has recently incorporated an adaptation of our scoring system into their method, is working on production of a completely automated LDL subfraction analysis system, and is seeking Food and Drug Administration approval for their product. Quantimetrix is additionally taking steps to minimize the observed lot-to-lot variability of their product. Studies are currently underway to assess the clinical utility of this method as a CAD risk marker, in predicting overt CAD, and for monitoring therapy.

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