

Comparison of Low-Density Lipoprotein Size by Polyacrylamide Tube Gel Electrophoresis and Polyacrylamide Gradient Gel Electrophoresis

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Abstract

We evaluated a low-density lipoprotein (LDL) subfraction separation method using polyacrylamide tube gel electrophoresis (PTGE) and compared it with the reference method, polyacrylamide gradient gel electrophoresis (PGGE-REF).

Excellent intra-assay and interassay coefficients of variation were obtained (<4%) for PTGE. For 102 subjects, LDL subclasses correlated most significantly with triglyceride (TG) level, high-density lipoprotein (HDL) cholesterol level, total cholesterol/HDL cholesterol ratio, and non-HDL cholesterol level ($P < .05$). The distribution of large LDL (76%) was predominant for subjects with low TG levels (<150 mg/dL [1.69 mmol/L]), while distribution of small LDL (79%) was predominant for subjects with high TG levels (>200 mg/dL [2.26 mmol/L]). Excellent agreement between the methods was observed (weighted kappa = 0.78). Of 51 samples classified as small, dense LDL by PGGE-REF, none were misclassified as large LDL and 4 as intermediate LDL by PTGE (92% concordance); of 44 samples classified as large LDL by PGGE-REF, 3 were misclassified as small and 7 as intermediate by PTGE (77% concordance). The PTGE method is precise and compares favorably with PGGE-REF. It has the advantage of being simple, less expensive, and more suitable for use in the clinical laboratory.

Coronary artery disease (CAD) is a leading cause of morbidity and mortality in the Western world.¹ Increased levels of low-density lipoprotein cholesterol (LDL-C) are associated with a high incidence of CAD.² Thus, the National Cholesterol Education Program Adult Treatment Panel III recommended using LDL-C levels as the main determinant for therapy.³

Attention has focused on the association of subclasses of LDL and CAD. Heterogeneity within LDL has led to the identification of at least 2 patterns based on particle size. Pattern A is identified as large, buoyant LDL particles, and pattern B is identified as small, dense particles.⁴ Several retrospective studies have reported an association between pattern B and increased risk for CAD.⁵⁻⁷ In addition, prospective studies have shown the small, dense LDL particle pattern to be a significant predictor of CAD.^{8,9} Furthermore, it has been reported that LDL phenotyping could help predict response to lipid therapy.¹⁰ The potential mechanisms for increased atherogenicity of small, dense LDL particles include increased susceptibility to oxidation, easier penetration into the intima, impaired binding to the LDL receptor, and increased binding to proteoglycans.^{11,12}

Current methods for isolating, separating, and characterizing lipoprotein subspecies and determining LDL particle size include ultracentrifugation,^{13,14} nondenaturing polyacrylamide gradient gel electrophoresis (PGGE),¹² nuclear magnetic resonance (NMR) spectroscopy,¹⁵ and high-performance liquid chromatography.¹⁶ These methods, however, are technically demanding, labor-intensive and not applicable in a routine clinical laboratory setting. Thus, there is a need for methods for separation of LDL subfractions that would be better suited TO the clinical laboratory.

A polyacrylamide tube gel electrophoresis (PTGE) method (Lipoprint LDL System, Quantimetrix, Redondo Beach, CA) has become available for the separation of LDL subfractions. The method permits separation of LDL into 7 subfractions within 60 minutes. This method is technically simpler, less expensive, and more conducive to routine laboratory testing. In the present study, we evaluated the PTGE assay and compared it with the reference method (PGGE-REF) performed in a lipid research laboratory. Association of LDL subfractions and triglyceride (TG) levels also was assessed.

Materials and Methods

Subjects and Samples

Serum samples from 102 participants (34 men and 68 women; ages 24-87 years) were analyzed. Blood samples were obtained in a serum separator tube and centrifuged at 2,000g for 6 minutes. Each serum sample was divided into 2 aliquots. Serum from the first aliquot was analyzed for lipids and LDL subfractions using PTGE. The samples were stored at 2°C to 8°C and analyzed within 2 or 3 days of collection. The second aliquot was frozen at -80°C and sent to a reference laboratory (Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX) for measurement of LDL particle size by PGGE-REF. In addition, serum samples from 50 participants were analyzed by our laboratory and by Mayo Laboratory (Rochester, MN) using the PTGE method.

Lipid Level Measurement

Total cholesterol and TG levels were measured by standard enzymatic- colorimetric assays on an automated chemistry analyzer (AU600, Olympus Diagnostic Systems, Melville, NY), using Olympus reagent (Olympus Diagnostic Systems). High-density lipoprotein cholesterol (HDL-C) was measured by using a 2-reagent homogeneous system for selective measurement of HDL-C (Olympus Diagnostic Systems). For samples with a TG level of 400 mg/dL or less (4.52 mmol/L or less), calculated LDL-C was derived by using the Friedewald equation. For those with a TG level of more than 400 mg/dL (>4.52 mmol/L), direct LDL-C was measured on the Olympus by using LDL direct liquid select cholesterol reagent (Equal Diagnostics, Exton, PA). The intra-assay and interassay precision for all lipid measurements was less than 5%.

Polyacrylamide Tube Gel Electrophoresis

LDL subfractions were separated according to the procedure provided by the manufacturer. Briefly, 25 µL of serum and 200 µL of loading gel (containing lipid-specific dye, Sudan black B) were loaded onto each precast 3%

polyacrylamide gel tube and then mixed by inversion. The tubes were photopolymerized for 30 minutes in front of a fluorescent light source. After polymerization, the tubes underwent electrophoresis at a constant current of 3 mA per tube for approximately 60 minutes until the HDL fraction had migrated to a distance of approximately 1 cm from the bottom of the tube. The gel tubes were permitted to diffuse for 30 minutes and scanned at 610 nm on a densitometer (HYRYS, Sebia, Norcross, GA). The very-low-density lipoprotein (VLDL) band (slowest migrating) was assigned an Rf (ratio of distance moved by band relative to marker) value of zero, and the HDL band (fastest migrating) was assigned an Rf value of 1. The LDL subfraction bands migrated between the VLDL and HDL bands, and their Rf values were calculated as follows:

$$\text{Rf of LDL Subfraction} = \frac{\text{Distance Between VLDL and LDL Subfraction Bands}}{\text{Distance Between VLDL and HDL Bands}}$$

LDL subclasses were designated as small (Rf > 0.40), intermediate (Rf = 0.38-0.40), and large (Rf < 0.38) by this method.

Polyacrylamide Gradient Gel Electrophoresis Reference Method

PGGE-REF was performed as described previously.^{17,18} Briefly, LDL particles in plasma were separated using PGGE-REF and then stained for cholesterol using Sudan black B. Size distribution of stained bands was determined by laser densitometer and compared with calibrators that included carboxylated polystyrene microspheres (38.0 nm), thyroglobulin (17.0 nm), and 2 LDL lyophilized standards (27.5 and 26.6 nm). The LDL size distributions for the samples were classified independently in a blinded manner by the reference laboratory as the predominant LDL particle diameter (PPD) and median diameter (the diameter at which half of the LDL absorbance is on large particles and half on smaller particles). LDL subfractions were classified as small LDL if the diameter was less than 25.8 nm, large LDL if the diameter was more than 26.3nm, and intermediate LDL if the diameter was between 25.8 and 26.3 nm.

Interferences

Serum sample aliquots were spiked with ascorbic acid (final concentration, 1, 2, and 4mg/dL), bilirubin (final concentration, 5, 10, 20, and 40 mg/dL), and hemoglobin (final concentration 50, 100, 200, and 400 mg/dL) and analyzed for LDL subfractions using the PTGE method.

Statistical Analysis

Statistical analyses were performed using the Sigma Stat statistical package (SPSS Science, Chicago, IL). A 1-way

analysis of variance was used for parametric data and Kruskal-Wallis analysis of variance on ranks for nonparametric data. The level of significance was set at P less than .05. The weighted kappa statistic was used to evaluate agreement between the methods.

Results

Assay Precision for the PTGE Method

Intra-assay and interassay precision was assessed using 2 serum pools, one with LDL pattern A and the other with LDL pattern B. For intra-assay precision, the sample pools (TG levels, 136 mg/dL [1.54 mmol/L] and 425 mg/dL [4.80 mmol/L], respectively) were run 6 times on the same day. For interassay precision, the sample pools (TG levels of 136 mg/dL [1.54 mmol/L] and 245 mg/dL [2.77 mmol/L], respectively) were run over 5 days. The Rf values of the predominant peaks for the 2 pools were calculated. Excellent intra-assay and interassay (<4%) coefficients of variation were obtained as depicted in **Table 1**.

Interferences

No interferences were observed in LDL classification for samples spiked with ascorbic acid up to 4mg/dL, bilirubin up to 20 mg/dL, and hemoglobin up to 200 mg/dL. LDL subfraction patterns were distorted for the higher concentrations of bilirubin (>40 mg/dL) and hemoglobin (>400 mg/dL) (data not shown).

Correlation of Lipids and LDL Subclasses

The lipid profiles for the 102 subjects studied are given in **Table 2**. Participants were chosen for the study to give a wide range of TG and lipid levels, ensuring a distribution of LDL subclasses. The correlation of PTGE Rf values and PGGE-REF PPD with lipid markers is shown in **Table 3**. Good correlation was seen for TG ($r = 0.68$ and -0.72 for PTGE and PGGE-REF, respectively) and for HDL-C ($r = -0.29$ and 0.38 for PTGE and PGGE-REF, respectively). Also, significant correlations were obtained for non-HDL-C and the total cholesterol/HDL ratio ($P < .05$). Samples with

Table 1
Precision Profile for the Polyacrylamide Tube Gel Electrophoresis Method*

	Intra-assay Rf (n = 6)		Interassay Rf (n = 5)	
	Mean \pm SD	Coefficient of Variation (%)	Coefficient of Mean \pm SD	Variation (%)
Level 1	0.32 \pm 0.005	1.7	0.31 \pm 0.011	3.6
Level 2	0.45 \pm 0.014	3.1	0.44 \pm 0.008	1.9

For intra-assay precision, triglyceride values for levels 1 and 2 were 136 mg/dL (1.54 mmol/L) and 425 mg/dL (4.80 mmol/L), respectively; for interassay precision, the values were 136 mg/dL (1.54 mmol/L) and 245 mg/dL (2.77 mmol/L), respectively.

an Rf of more than 0.40 by the PTGE method were classified as small, and by the PGGE-REF, a median diameter of less than 25.8 nm was designated as small, thus explaining the inverse correlation.

Association of TG and LDL Subclasses

The distribution of LDL subclasses and TG subdivided into low, intermediate, and high ranges (<150 mg/dL [<1.69 mmol/L]; 150-199 mg/dL [1.69-2.25 mmol/L]; and >200 mg/dL [>2.26 mmol/L], respectively) is given in **Figure 1**. The distribution of large LDL was predominant for subjects with low TG levels (76% [19/25]), while the distribution of small LDL was predominant for subjects with high TG levels (79% [50/63]). For subjects with intermediate levels of TG, the large, small, and intermediate LDL subclasses were distributed equally.

Comparison of PTGE and PGGE-REF Methods

Comparison of the PTGE method with the PGGE-REF based on classification of small, intermediate, and large LDL for the 102 subjects is depicted in **Table 4**. Classification of LDL subclasses was based on the Rf value for the PTGE method and on the median diameter for PGGE-REF. For both methods, the LDL subclasses were designated as small, intermediate, or large.

LDL subclass pattern B, or small, dense LDL, was found in 50.0% of the study population, whereas 43.1% were classified as pattern A, or large, buoyant LDL by the PGGE-REF (Table 4). The PTGE method achieved an agreement

Table 2
Subject Characteristics and Lipid Profile

	Mean \pm SD	Range
Age, y	52 \pm 13.7	24-87
Male/female ratio	35/67	—
Total cholesterol, mg/dL (mmol/L)	219 \pm 60.5 (5.66 \pm 1.56)	113-563 (2.92-14.56)
Total triglycerides, mg/dL (mmol/L)	270 \pm 149.3 (3.05 \pm 1.69)	61-617 (0.69-6.97)
High-density lipoprotein cholesterol, mg/dL (mmol/L)	43 \pm 19.1 (1.11 \pm 0.49)	20-183 (0.52-4.73)
Low-density lipoprotein cholesterol, mg/dL (mmol/L)	125 \pm 49.4 (3.23 \pm 1.28)	42-452 (1.09-11.69)

Table 3
Correlation of Lipid Levels With LDL Particle Size

	PTGE-Rf		PGGE-REF-PPD	
	r*	P	r*	P
Triglyceride	0.68	< .0001	-0.72	< .0001
HDL cholesterol	-0.29	.003	0.38	< .0001
Total cholesterol	0.34	.006	-0.14	.15
LDL cholesterol	0.16	.10	0.06	.58
Non-HDL cholesterol	0.44	.02	-0.27	.007
Total cholesterol/HDL ratio	0.55	< .0001	-0.52	< .0001

HDL, high-density lipoprotein; LDL, low-density lipoprotein; PGGE-REF, polyacrylamide gradient gel electrophoresis reference method; PPD, predominant LDL particle diameter; PTGE, polyacrylamide tube gel electrophoresis.
* Pearson correlation.

of 92% concordance for classification of the small LDL subclass compared with the PGGE-REF. Of the 51 samples classified as small, dense LDL by PGGE-REF, none were misclassified as large LDL and 4 as intermediate LDL by the PTGE method (92% concordance). For the 44 samples classified as large LDL by the PGGE-REF, 3 were misclassified as small and 7 as intermediate by the PTGE method (77% concordance). In addition, excellent agreement between the 2 methods was observed using kappa statistics (weighted kappa = 0.78; 95% confidence interval, 0.68-0.87).

A comparison of the aforementioned method for assignment of LDL subclasses using Rf was made with the Mayo Laboratory computer software analysis method (the Mayo Laboratory uses the PTGE method, but results are calculated using automated software). Using the PTGE method, an additional 50 serum samples were analyzed by

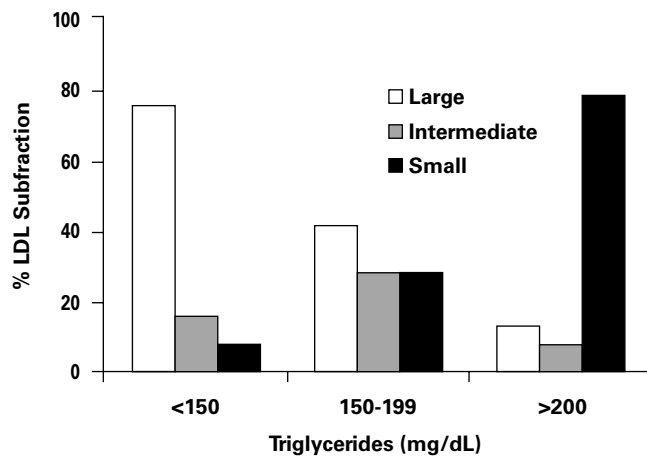


Figure 1 Effect of triglyceride levels on the low-density lipoprotein (LDL) subfraction distribution. Values are given in conventional units; for the corresponding Système International units, see the “Results” section.

the Mayo Laboratory and by us. Of the 16 large LDL classified by Mayo, 12 were classified as large and 4 as intermediate LDL by us; of the 10 intermediate LDL classified by Mayo, 6 were classified as intermediate and 4 as large by us; of the 24 small LDL classified by Mayo, 21 were classified as small and 3 as intermediate by us. There were no misclassifications of large LDL as small LDL or of small LDL as large LDL.

Discussion

There has been increasing interest in characterizing and measuring LDL subfractions. Several cross-sectional studies have reported prevalence of small, dense LDL particles among patients with CAD.^{2,6} However, this association is abolished when the data are adjusted for levels of TG and HDL-C.¹⁹ The well-documented, strong correlation among elevated TG levels, reduced HDL-C levels, and small, dense LDL^{9,20} leads to the question of whether small, dense LDL is an independent risk factor. Small LDL particles have been distinguished as a distinctive biochemical marker of inherited metabolic diseases such as dyslipidemia, hypertension, type 2 diabetes mellitus, and hypercoagulability, all of which are associated with an increased risk for CAD.²¹⁻²³

Results of large, prospective studies support the association of small, dense LDL particles and increased coronary risk independent of other lipoprotein levels.⁹ In the case-controlled Stanford Five City Project,¹⁰ a prospective, population-based study, the incidence of CAD was associated with significantly smaller LDL particle size after accounting for HDL-C, non-HDL-C, and TG levels. LDL particle size was the best predictor of CAD. The Quebec prospective cardiovascular study provided evidence suggesting that high levels of small LDL particles were associated with increased risk of subsequent development of ischemic heart disease in men, partly independent of other lipoprotein abnormality.⁹ Griffin et al⁷ also demonstrated an association of increased coronary risk and small, dense LDL particles independent of TG levels.

Currently methods for separating LDL subfractions include ultracentrifugation,¹³ high-performance liquid chromatography,¹⁶ NMR,¹⁵ precipitation,²⁴ and electrophoresis.²⁵ These methods, however, require expensive instrumentation, are labor-intensive, require experienced personnel, and are not conducive to routine laboratory testing.

Therefore, we evaluated a simpler method, the PTGE method developed by Quantimetrix. Separation of LDL subfractions into 7 bands by PTGE is based primarily on particle size. LDL subfractions 1 and 2 have been designated as large LDL and subfractions 3 through 7 as small

Table 4
Method Comparison of the LDL Subfraction Between PTGE and PGGE-REF

PGGE-REF	No. of Cases	PTGE			Concordance (%) [*]
		Small	Intermediate	Large	
Small	51	47	4	0	92
Intermediate	7	5	2	0	33
Large	44	3	7	34	77

* Weighted kappa of 0.78 (95% confidence interval, 0.68-0.87).

LDL with subsequent reduction in LDL size with increasing fraction number. The assay gave excellent precision for 2 sample pools having small (pattern B) and large (pattern A) LDL particles (<4%). Comparison of this method with the PGGE-REF was achieved by using Rf values of less than 0.38, 0.38 to 0.40, and more than 0.40 for the designation of large, intermediate, and small, dense LDL particles, respectively. The Rf values were derived experimentally to give the best agreement between the 2 methods. This method of analysis was used because it otherwise would be difficult to compare precisely the 7 LDL bands by the PTGE method with the PPD and the median diameter resulting from the PGGE-REF.

The results were promising, with 3 of 44 large LDL particles misclassified as small and 7 as intermediate by the PTGE method and no misclassifications for small LDL particles, although 4 of the 51 were designated as intermediate by the PTGE method. There was good agreement between the 2 methods, with greater than 77% concordance for small and large LDL particle classification with a weighted kappa statistic of 0.78. Thus, this method of analysis permits a simplified categorization of patients as having pattern A or pattern B, with good correlation to the PGGE-REF. Although we used serum samples as recommended by the manufacturer, this test also can be performed with plasma samples, as reported by other investigators.²⁰ Furthermore, it is recommended that LDL particle size be measured on at least 2 samples obtained 1 month apart to aid in classification.

Computer software for analysis of the bands was not available at the time of this study. Since the completion of the present study, Hoefner et al²⁰ reported a comparison of PTGE with PGGE-REF and NMR in 51 patients using computer software analysis. Our results are comparable with those reported by Hoefner et al.²⁰ The concordance between PTGE and PGGE-REF was 95% only when small and intermediate were considered as 1 group. Comparison of the PTGE with NMR showed 5 of 21 samples misclassified as small LDL particles, with a concordance of 76%. Also, we compared the PTGE method with a reference method from a lipid research laboratory and used a larger

sample to compare the 2 methods, while Hoefner et al²⁰ compared 2 commercial methods and used a smaller sample (n = 51). While we did not specifically examine lot-to-lot variability, the sample correlations were conducted with 2 lots of the PDGE kits.

We also compared our method of analysis with the computer software analysis developed by the Mayo Laboratory for the designation of LDL subclasses on 50 samples. There was excellent agreement, with no misclassifications of small LDL as large LDL particles or of large LDL as small LDL particles; however, 7 were classified as intermediate by our method. Thus, both methods would be suitable for analyses of LDL subfractions.

Several computer software analyses methods have been reported. Hoefner et al²⁰ and other investigators have used a scoring system based on average particle size distribution to describe the LDL profile. A linear scoring system developed by Campos et al⁶ and Rajman et al²⁶ is based on the relative contribution of each subfraction weighted by the fraction number (1-7); Rainwater et al¹⁸ suggested estimating the median diameter, which represents the entire LDL absorbance profile. The recently introduced linear scoring system of Quantimetrix is based on the mean LDL subfraction diameter. A logarithmic scoring system, developed by Hoefner et al,²⁰ assigns a heavier weight to smaller LDL fractions. Although additional information is provided by each of these systems, direct comparisons of these methods is hindered. Furthermore, each system has a different cut point for the classification of patterns A and B, which does not correlate precisely with that defined by the PGGE-REF. Thus, standardization of LDL subfraction analyses methods is necessary, and the methods should not be used interchangeably.

As found by many groups,^{9,20} the TG level was associated highly with LDL particle size in this study. By using 3 levels of TG (<150 mg/dL [<1.69 mmol/L]; 150-199 mg/dL [1.69-2.25 mmol/L]; and >200 mg/dL [>2.26 mmol/L]), predominantly large LDL (76%) was observed with TG levels of less than 150 mg/dL (<1.69 mmol/L) and predominantly small LDL (79%) with TG levels of more than 200 mg/dL (>2.26 mmol/L). However, for the intermediate

level of TG, large (43% [6/14]), small (29% [4/14]), and intermediate (29% [4/14]) LDL particles were present. Thus, the TG level did not predict LDL particle size in this intermediate group, and other mechanisms may be responsible for variation in LDL particle size; therefore, determining LDL subfractions for this group may be important for assessing CAD risk. Rajman et al²⁶ studied a population of patients with CAD and control subjects with TG levels less than 200 mg/dL (<2.26 mmol/L). LDL scores were significant in patients with CAD compared with the scores for control subjects, and the scores correlated with severity of CAD. One potential limitation of our study is that although we did not observe any interference when samples were spiked with ascorbic acid concentrations up to 4 mg/dL, bilirubin concentrations up to 20 mg/dL, and hemoglobin concentrations up to 200 mg/dL, the LDL subfraction patterns were distorted for the higher concentrations of bilirubin (>20 mg/dL), and this may affect test results in the rare patients with such high levels of bilirubin.

Thus, assessment of LDL subfractions may provide an additional tool to identify patients at increased risk for CAD not identified by traditional lipoprotein markers. In addition Zambon, et al²⁷ provided evidence that changes in LDL particle size predict changes in coronary stenosis independent of changes in TG and HDL-C levels. Thus, measurement of small, dense LDL particles also may be important for monitoring the therapeutic effectiveness of lipid-lowering therapy in the prevention of CAD.

Characterization of LDL subfractions by the PTGE method is precise, less expensive, and simpler and is suitable for routine use in a clinical laboratory. Designation of LDL particles into pattern A or B using a cut point of Rf (0.38-0.40) correlates well with the PGGE-REF and provides a simple and cost-effective analysis. Measurement of small, dense LDL particles is not recommended for the population at large, but should be confined to subjects with a normal lipid profile but a positive family history of CAD, in addition to measuring other evolving markers such as homocysteine and high-sensitivity C-reactive protein. Further studies are required to ensure the clinical usefulness of measuring small LDL particles as a marker of CAD.

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