Phenotyping of LDL using Polyacrylamide Gel Tube Electrophoresis (PGTE, Quantimetrix) and its comparison with Non denaturant gel Electophoresis (NDGGE)

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Abstract

Several retrospective and prospective studies have reported an association between small dense LDL and increased risk of coronary artery disease. Current methodologies for LDL subfraction require expensive instrumentation, are labor intensive, and unsuitable for routine clinical use. We have therefore evaluated a LDL subfraction separation method using polyacrylamide gel tube electrophoreses (PGTE) developed by Quantimetrix and compared it with the reference non-denaturing gradient gel electrophoreses method (NDGGE).

Excellent itra- and interassay coefficient of variation were obtained (<4%) for the PGTE method. For 102 subjects, LDL subclasses correlated significantly with TG and HDL-Cholesterol ($p<0.001$). The distribution of large LDL (48%) was predominant for subjects with low TG (TG < 150 mg/dL) whilst distribution of small LDL (89%) was predominant for subjects with high TG levels (> 250 mg/dL). Excellent agreement between the two methods was observed for the 102 subjects (Kappa = 0.91). Of the 37 samples classified as small dense LDL by NDGGE only 1 was misclassified as large LDL and 2 as intermediate LDL (92% concordance), for the 46 samples classified as large LDL by NDGGE, 3 were classified as intermediate (93% concordance).

On the basis of these findings the PGTE method is precise and compare favorably with the reference NDGGE method. It also has the advantage of being a simpler and less expensive method and more suitable for clinical testing.

Introduction

Coronary artery disease (CAD) is one of the leading causes of morbidity and mortality in the western world[1]. Increased levels of low density lipoprotein cholesterol (LDL-C) are associated with high incidence of CAD.[2] Thus, the National Cholesterol Education Program (NCEP), Adult Treatment Panel III (ATPIII) has recommended LDL-C levels as the main determinant for therapy.[3] Recently, attention has been focused on the association of subclasses of LDL and CAD.

Heterogeneity within LDL has led to the identification of two 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, the small dense LDL particles, and increased risk for CAD.[5-7] In addition, prospective studies have shown small dense LDL particles to be a significant predictor of subsequent 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 include increased susceptibility to oxidation, easier penetration into the intima, impaired binding to the LDL receptor.[11-12]

Current methodologies for isolating, separating, characterizing lipoprotein subspecies and determining LDL particle size include ultracentrifugation[13], non-denaturing gradient gel electrophoresis[14], nuclear magnetic resonance (NMR) spectroscopy[15] and HPLC[16]. These methods however, are technically demanding, labor intensive and not applicable in a routine clinical laboratory setting. Thus, there is a need for development of methods for separation of LDL subfractions that would be better suited to routine laboratory testing.

Recently a polyacrylamide gel tube electrophoresis (PGTE) method developed by Quantimetrix (Lipoprint™ LDL System, Quantimetrix, Redondo Beach, CA) has become available for separation of LDL subfractions. The method allows for separation of LDL into seven subfractions within 60 minutes. This method is technically simpler, less expensive, and more conducive to routine laboratory testing. In the present study, we have evaluated the PGTE assay and compared this assay with the reference method using

non-denaturing gradient gel electrophoresis (NDGGE). Association of LDL subfractions and triglyceride levels was also assessed.

Materials and Methods:

Subjects:

Serum samples from 102 participants (34 male and 68 female; ages 24-87 years) were analyzed. Blood was collected in a serum separator tube and centrifuged at 2000g for 6 minutes. Each serum sample was divided into two aliquots. Serum from the first aliquot was analyzed for lipids and LDL subfractions using PGTE. The samples were stored at 2-8 ^{0}C and analyzed within 2-3 days of collection. The second aliquot was frozen at –80°C and sent to a reference lab (Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX) for measurement of LDL particle size by NDGGE. According to Rainwater, 2000[17], the sample can be used after being stored in single use aliquots.

Lipids measurement:

Total cholesterol and triglyceride (TG) were measured by standard enzymatic-colorimetric assays on an automated chemistry analyzer (Olympus, AU600), using Olympus reagent (Olympus Diagnostics, N.Y.). HDL–cholesterol was measured using a two reagent homogeneous system for selective measurement of HDL-C (Olympus Diagnostics, N.Y.). For samples with TG$\leq$ 400 mg/dL, calculated LDL-cholesterol was derived using the Friedewald equation. For those with TG > 400 mg/dL, direct LDL-C was measured on Olympus, using LDL direct liquid select^{tm} cholesterol reagent (Equal Diagnostics, PA).

Polyacrylamide Gel Tube Electrophoresis (PGTE):

LDL subfractions were separated according to the procedure provided by the manufacturer. Briefly, 25 ul of serum and 200 ul 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 were electrophoresed at a constant current of 3mA 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 allowed to diffuse for 30 minutes and scanned at 610 nm on a densitometer (Sebia, HYRYS, NorCross, GA). The VLDL band (slowest migrating) was assigned a Rf value of zero and the HDL band (fastest migrating) was assigned a Rf value of one. The LDL subfraction bands migrated between the VLDL and HDL bands and their Rf values were calculated as follows:

$$\text{Rf of LDL subfraction} = \frac{\text{distance between VLDL and LDL subfraction bands}}{\text{distance between VLDL and HDL bands}}$$

Non-denaturing gradient gel electrophoresis (NDGGE) reference method:

NDGGE was performed as described previously (Rainwater et al.[18]). Briefly LDL particles in plasma were separated using NDGGE then stained for cholesterol using Sudan Black B. Size distribution of stained bands was determined by laser densitometer and compared to calibrators which included carboxylated polystyrene microspheres (38.0 nm), thyroglubulin (17.0 nm) and two LDL bands in the lyophilized standard (27.5 nm and 26.6 nm). The LDL size distributions were classified by the reference laboratory as the predominant LDL particle diameter (PPD) and median diameter (the diameter where half of the LDL absorbance is on large particles and half on smaller particles). The classification of small, large and intermediate LDL subclasses was made as follows: If both the PPD and the median diameter were > 26.3 nm then large buoyant LDL particles were predominantly present; if the both the PPD and median diameter were < 25.8nm then small dense LDL particles were predominantly present; and if the PPD was > 26.3nm but the median diameter was < 25.8 nm then both small and large LDL were present and classified as intermediate (the same would hold true if the PPD was < 25.8 nm but the median diameter was > 26.3 nm).

Statistical Analysis:

Statistical analyses were performed using Sigma Stat statistical package. A one way ANOVA was used for parametric data and Kruskall-Wallis ANOVA on ranks for nonparametric data, level of significance was set at $p<0.05$. The weighted Kappa statistic was used to evaluate the agreement between the two methods.

Results:

Assay precision for PGTE method

Intra- and inter-assay precision was assessed using two serum pools with LDL patterns A and B respectively. For intra-assay precision, the samples pools (TG levels of 136 mg/dL and 425 mg/dL respectively) were run 6 times on the same day. For inter-assay precision, the pools (TG levels of 136 mg/dL and 245 mg/dL respectively) were run over 5 days. The Rf values of the predominant peaks for the two pools were calculated. Excellent intra- (<2%) and inter-assay (<4%) CVs were obtained as depicted in Table 1.

Correlation of Lipids and LDL subclasses

The lipid profile for the 102 subjects studied is presented 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 between LDL subclasses determined by the PGTE method and lipid markers is shown in Table 3. Significant positive correlation was seen for TG ($p<0.001$) and negative correlation for HDL-cholesterol ($p<0.001$) when comparing LDL subclasses from large to small LDL. No significant differences were observed between LDL subclasses and total cholesterol or LDL cholesterol ($p=0.51$ and $p=0.71$, respectively).

Association of Triglyceride and LDL Subclasses

The distribution between LDL subclasses and TG subdivided into low, intermediate, and high ranges (TG < 150mg/dL, TG = 150-250 mg/dL and TG > 250 mg/dL respectively) is shown in Fig 1. The distribution of large LDL was predominant for subjects with low

TG levels (48%) whilst the distribution of small LDL was predominant for subjects with high TG levels (89%). For subjects with intermediate levels of TG, a predominance of intermediate (both small and large, 55%) LDL subclass was present.

Comparison of PGTE and NDGGE method

Comparison of the PGTE method to the NDGGE method based on classification of small, intermediate and large LDL for the 102 subjects is depicted in Table 4. For appropriate assignment of small, intermediate and large LDL subclasses for PGTE method, a Rf value of 0.33 was employed. A predominant peak with a Rf < 0.33 was assigned as large LDL, a predominant peak with Rf > 0.33 was assigned as small LDL, and peaks with Rfs both > 0.33 and < 0.33 were assigned as intermediate with both small and large LDL present for one individual.

LDL subclass pattern B or small dense LDL was found in 36% of the study population whereas 45% were classified as pattern A or large buoyant LDL by the NDGGE reference method (Table 4). The PGTE method achieved an agreement of greater than 92% concordance for small and large LDL subclasses when compared to the NDGGE reference method. Of the 37 samples classified as small dense LDL by NDGGE, only 1 was misclassified as large LDL and 2 as intermediate LDL by the PGTE method (92% concordance). For the 46 samples classified as large LDL by NDGGE, none were misclassified as small and 3 were classified as intermediate by PGTE method (93% concordance). In addition excellent agreement between the two methods was observed using kappa statistics (Kappa = 0.91; 95% CI 0.85-0.97).

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 CAD patients[2,6]. However this association is abolished when the data is adjusted for TG and HDL-C[19]. A strong correlation between elevated TG, reduced HDL-C, and small dense LDL is well documented[9,20] leading to the question of whether small dense LDL is an independent risk factor. Small LDL particles have been distinguish as a

distinctive biochemical marker of an inherited metabolic disease such as dyslipidemia, hypertension, insulin resistance diabetic, hypercoagulability all of which are associated with an increased risk for CAD[21-23].

Recently, 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[9], a prospective population based study, 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 shown to be the best predictor of CAD. The Quebec prospective based cardiovascular study provided evidence suggesting high levels of small LDL particles were associated with increased risk of subsequently developing Ischemic heart disease (IHD) in men partly independent of other lipoprotein abnormality. Griffin et al[7] also demonstrated an association of increased coronary risk and small dense LDL particles independent of TG levels.

Currently methodologies for separating LDL subfractions Ultracentrifugation[13], HPLC[16] ,Nuclear magnetic resonance[15], precipitation technique[24], and electrophorsis[25]. These methods, however, require expensive instrumentation, are labor intensive, required experienced personnel, and are not conductive to routine laboratory testing.

Therefore, we have undertaken evaluation of a simpler method, the PGTE method developed by Quantimetrix. Separation of LDL subfractions into seven bands by PGTE is based primarily on particle size. LDL fraction #1-2 have been designated as large LDL and #3-7 as small LDL with subsequent reduction in LDL size with increasing fraction number. The assay gave excellent precision for two sample pools having small (pattern B) and large (pattern A) LDL (<4%). Comparison of this method with the reference NDGGE was achieved by employing a Rf cut-point of 0.33 for designation of large, small and intermediate LDL. The Rf cut-point was experimentally derived to give the best agreement between the two methods. This method of analysis was used since it would otherwise be difficult to compare precisely the seven LDL bands by PGTE LDL method to the PPD and median diameter resulted by NDGGE method. The results were promising with only 1 of 37 small dense LDL particles misclassified as large and no misclassifications for large LDL although 3 of the 46 were designated as intermediate by the PGTE method. The agreement between the two methods was excellent with greater

than 92% concordance for small and large LDL classification and Kappa statistic of 0.91. Thus, this method of analysis allows for a simplified categorization of patients into pattern A or pattern B with good correlation to the NDGGE method. Computer software for analysis of the bands was not available at the time of this study. Since the completion of this study, Hoefner et al[20] have reported a comparison of PGTE with NDGGE and NMR in 51 patients. Our results show a better correlation with the NDGGE than those reported by Hoefner et al. The concordance between PGTE and NDGGE was 95% only when considering both small and intermediate as one group. Comparison of the PGTE with NMR showed 5 of 21 samples misclassified as small LDL with a concordance of 76% whilst one was misclassified as small when compared to NDGGE. The concordance between PGTE and NDGGE was 95%.

Hoefner and other investigators also have used a scoring system based on average particle size distribution to describe the LDL profile. A linear scoring system developed by Campos et al[6] and Rajman et al[26] is based on the relative contribution of each subfraction weighted by the fraction number (1-7) whereas Quantimetrix's linear scoring system (recently introduced) is based on mean LDL subfraction diameter. A logarithmic scoring system has been developed by Hoefner et al[20]. which assigns heavier weight to the 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 reference NDGGE method. Thus standardization of LDL subfraction methods is necessary and the methods should not be used interchangeably.

As has been reported previously by many groups[9,20], TG was highly associated with LDL particle size in this study. Using three levels of TG (<150 mg/dL, 150-250 mg/dL, and >250 mg/dL), predominantly large LDL (48%) was observed with TG < 150 mg/dL and predominantly small LDL(89%) with TG > 250 mg/dL. However, for the intermediate level of TG large (39%), small (10%), and intermediate (55%) LDL were present. Thus, TG did not predict LDL size in this intermediate group and other mechanisms may be responsible for variation in LDL size and therefore, determining LDL subfractions for

this group may be important in assessing CAD risk. Rajman et al[26], studied a population of CAD patients and controls with TG < 200 mg/dl. LDL scores were highly significant in CAD patients compared to controls and the scores correlated with severity of CAD. In addition Zambon, et al.[27], provide evidence that changes in LDL size predicts changes in coronary stenosis independent of changes in TG and HDL-C. Thus, measurement of small dense LDL may be important in therapeutically monitoring effectiveness of lipid lowering therapy in prevention of CHD. Also, assessment of LDL subfractions may provide an additional tool to identify patients at increased risk for CAD not identified by traditional lipoprotein markers. Especially, it was found that some patient with normal LDL-C and still are at high risk of developing IHD. Furthermore, Multivariate and subgroup analysis indicated that small dense LDL-C particles predicted the rate of IHD independent of LDL-C, TG, HDL-C, apolipoprotein B and cholesterol/HDL ratio[28].

In conclusion, characterization of LDL subfractions by PGTE method is precise, less expensive and a simpler method suitable for routine use in a clinical laboratory. Designation of LDL into pattern A or B using a cut-point of Rf = 0.33 correlates well with the reference NDGGE method and provides for a simple and cost effective analysis. Further studies will be required to assure the clinical utilities of measuring small LDL as a CAD marker.

Table 1: Precision profile for PGTE method

	Intra-assay (n = 6) Rf Mean ± SD	CV%	Interassay (n = 5) Rf Mean ± SD	CV%
Level 1	0.315 ± 0.005	1.74	0.31 ± 0.011	3.63
Level 2	0.75 ± 0.014	1.83	0.44 ± 0.008	1.91

Table 2. Subject Characteristics and Lipid Profile

	Mean±SD	Range
Age, years	52 ± 13.7	24-87
Total Chol, mg/dL	219 ± 60.5	113-563
TG, mg/dL	270 ± 149.3	61-617
HDL, mg/dL	43 ± 19.1	20-183
LDL, mg/dL	125 ± 49.4	42-452
Male/Female ratio:	34/68	

Table 3. The distribution of lipids levels according to LDL subfraction

	Large	Int	Small	p
Chol	**212±68**	**219±52**	**228±54**	0.51
TG	**168±70**	**268±131**	**398±133**	<0.001
HDL	**49±15**	**38±7**	**34±6**	<0.001
LDL	**127±61**	**128±34**	**121±41**	0.71

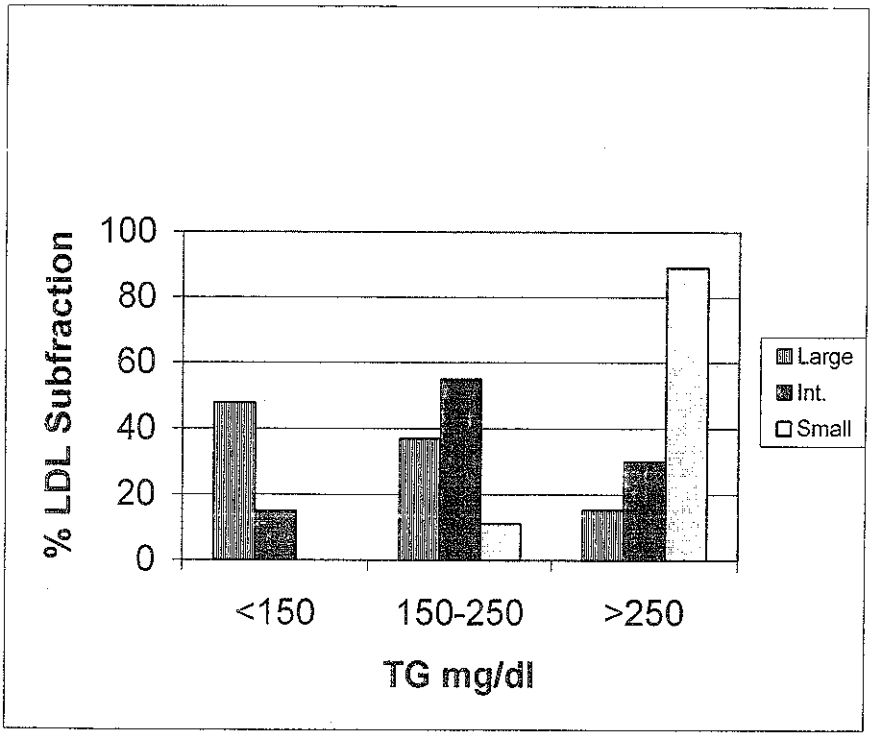
p: One way ANOVA for parametric data (Chol and LDL-C) and Kruskall Wallis ANOVA on ranks for non parametric data (TG and HDL-C)

Table 4. Method Comparison of LDL Subfraction between PGTE and NDGGE

		Quantimetrix			
GGE	n	Small	Int.	Large	Concordance*
Small	37	34	2	1	92%
Intermediate	19	0	16	3	84%
Large	46	0	3	43	93%

* Weighted Kappa of 0.9068 with 95% Confidence limits of 0.85- 0.97.

Fig 1. Effect of Triglyceride levels on LDL subfraction distribution.



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