Ratio of Dense to Buoyant LDL Subclass is Associated with LDL Density Phenotype (VLDL-5)

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Abstract: *Background*: Dense LDL phenotypes are associated with increased atherogenicity, and are commonly evaluated for the purposes of atherosclerosis research and cardiovascular risk discrimination.

Objective: To examine the ability of LDL subclasses, expressed as a ratio of dense-to-buoyant subclass, to predict LDL density phenotype.

Methods: LDL subclasses and density phenotypes were measured with vertical auto profile ultracentrifugation in 1,339,898 consecutive lipid profiles between 2009 and 2011 from a clinical sample of US adults. Logarithmic LDL density ratio (LLDR) was calculated as ratio of dense-to-buoyant LDL subclasses, $\ln[(LDL_3\text{-C} + LDL_4\text{-C}) / (LDL_1\text{-C} + LDL_2\text{-C})]$; normally distributed after log-transformation. LLDR was compared to density phenotype using ROC C-statistic with optimum sensitivity and specificity cutpoints determined.

Results: There was a strong, highly significant, monotonic increase in LLDR across progressively higher density phenotypes ($p<0.001$). Mean LLDR for Phenotype A was 0.122 (95% CI 0.121-0.123), Phenotype A/B was 0.751 (95% CI 0.750-0.752), and Phenotype B was 1.336 (95% CI 1.335-1.338). ROC analysis showed a strong association of LLDR with phenotype A, C=0.915 (0.914-0.915), $p<10^{-4}$, optimum cutpoint <0.398, sensitivity 72%, specificity 95%; and phenotype B, C= 0.923 (0.923-0.924), $p<10^{-4}$ optimum cutpoint >0.905, sensitivity 81%, specificity 86%. There was also a positive correlation between LLDR and LDL Max Time (R^2=0.802).

Conclusion: LLDR is a convenient, easily calculated, and continuous variable that is strongly associated with LDL density phenotype and LDL Max Time. Further research is needed to investigate the relationship between lipoprotein density and size, and whether LLDR provides more cardiovascular risk discrimination than LDL density phenotype.

Keywords: LDL cholesterol, lipids, hyperlipidemia, atherosclerosis, ultracentrifugation.

INTRODUCTION

Cardiovascular disease has remained the number one cause of death in the United States, currently accounting for nearly one in three deaths annually [1,2]. Over the past few decades, a wealth of laboratory and epidemiologic data has demonstrated clear associations between serum cholesterol levels and cardiovascular disease outcomes [3-6]. In particular, serum low-density lipoprotein (LDL) is thought to play a causal role in artery wall inflammation and atherogenesis [7]. Proteoglycan constituents of arterial walls are thought to bind LDL, subsequently initiating a process of oxidative modification, local inflammation, and a pathway towards atherosclerosis. Various fractions of LDL may interact differently with the proteoglycans within the arterial wall. In particular, small, dense subclasses of LDL are thought to bind to proteoglycan more readily than larger, buoyant subclasses, and are accordingly seen more frequently in patients at increased risk for cardiovascular disease [7-9].

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Krauss, *et al*. first characterized small, dense LDL in serum using gradient polyacrylamide gels and labeled specimens with a predominance of this subclass as "LDL phenotype B" [10,11]. Since then, several prospective and case-control studies have observed an association between phenotype B and increased risk of cardiovascular disease events [12-14]. Accordingly, serum LDL particle density and size are often measured for purposes of atherosclerosis and coronary heart disease research. The overall size and density of LDL particles is commonly characterized by the position of the modal LDL density distribution peak on serum lipid ultracentrifugation. The relative position of this peak (LDL Max Time) is used to categorize LDL density phenotype pattern as characteristic of normal, buoyant LDL (phenotype A), intermediate density LDL (phenotype A/B), or small, dense LDL (phenotype B).

In this study, we obtained LDL subclasses by ultracentrifugation, expressed them as a logarithmic ratio of concentration of dense-to-buoyant LDL subclasses, and found the ratio to be associated with LDL modal density phenotype and LDL Max Time. We propose the use of this log transformed ratio as a convenient, normally distributed,

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continuous variable quantifying LDL modal density phenotype for research purposes.

METHODS

Venous blood was obtained from 1,339,898 consecutive primary and secondary prevention US patients age 18-112 (median 59, interquartile range 49-70) from the Atherotech Clinical Laboratory between 2009 and 2011. All samples were from patients who were clinically referred for lipoprotein cholesterol measurement for primary or secondary cardiovascular disease prevention. The samples were evenly distributed by gender**.** All specimens were collected for clinical purposes in serum separator tubes (SSTs) and were then spun within four hours to separate serum after allowing clotting (all standard procedure). SSTs with separated serum were then placed on ice packs (4°C) and were shipped to the Atherotech Laboratory in Birmingham, Alabama by overnight express delivery. If specimens could not be shipped on the day of lab draw the SSTs were stored at (4°C) until they were shipped. Testing was not performed if specimens were older than seven days from the draw date. If specimens could not be shipped within seven days, phlebotomy facilities were advised to store specimens at (-70°C) until they could be shipped on dry ice. Approximately 40% of lab specimens were received on dry ice after being stored at (-70°C). Testing on all samples was then performed within 48 hours of receipt with more than 90% performed within 24 hours.

Cholesterol concentrations of lipoprotein classes and subclasses as well as LDL density phenotype were quantified by Vertical Auto Profile (VAP) (Atherotech; Birmingham, AL). The dataset of 1,339,898 samples was then retrospectively de-identified of patient information and returned to the Johns Hopkins Hospital for use in the Very Large Database of Lipids (VLDL) studies. Johns Hopkins Institutional Review Board waiver was obtained for the VLDL study since the dataset was de-identified, since blood was collected primarily for clinical use rather than for this study, and since no testing was conducted on the human subjects for the purposes of this analysis.

The VAP procedure is an accurate and reproducible method that we have described previously [15, 16]. In brief, lipoprotein classes and subclasses are first separated using a single vertical spin density gradient ultracentrifugation. A density gradient is prepared by pipetting a known amount of 1.006 g/mL saline solution followed by a known amount of plasma or serum that is diluted 40-fold with 1.21 g/mL KBr solution. The tube is then centrifuged at 65,000 rotations per minute for 45 minutes. The bottom of the tube is punctured with a needle, allowing for the contents to drain and be analyzed for cholesterol concentration using a continuous flow VAP analyzer. The drained contents are mixed with cholesterol specific enzymatic reagent and the mixture is allowed to flow through a heated narrow bore Teflon tubing where a reaction with enzymatic reagent takes place. The enzymatic reaction results in a red color which is monitored by a spectrophotometer placed at the end of the Teflon tubing. The intensity of the color is directly proportional to the cholesterol concentration of the flowing mixture.

In essence, the VAP spectrophotometer records a continuous absorbance curve corresponding to the distribution and concentration of cholesterol among different lipoproteins as they flow out of the centrifuge tube. The high-density lipoprotein (HDL) peak separates at the bottom of the tube and appears first (most dense), followed by lipoprotein a [Lp(a)], LDL, intermediate density lipoprotein (IDL), and then very low density lipoprotein (VLDL) peak. HDL peak corresponds to 0 seconds on the density gradient (since it appears first), and VLDL peak corresponds to 200 seconds on the opposite end of the spectrum. LDL Max Time is the time until peak LDL on the density gradient.

We defined **L**ogarithmic **L**DL **D**ensity **R**atio (LLDR) as: $LLDR = \ln[(LDL_3\text{-}C + LDL_4\text{-}C) / (LDL_1\text{-}C + LDL_2\text{-}C)]$ where LDL subclass density increases from least dense LDL_1 to most dense LDL_4.

Deviation of variables from Gaussian distribution was measured using the X^2 statistic. The association between modal density phenotype and LLDR was assessed using ROC analysis. Optimum sensitivity and specificity cutpoints were calculated using Youden's Index method as well as the Top Left Corner method.

LLDR was then compared to LDL Max Time in a second subset of 5,957 consecutive samples. Finally, LDL size measured by nuclear magnetic resonance (NMR) was compared to LDL Max Time and LLDR in a third subset of 372 samples. Statistical analyses were conducted using STATA v12.0 and R v2.15 software packages.

RESULTS

The distribution of dense-to-buoyant LDL ratios was not normal and had moderate right skewness. Accordingly, the ratio was log-transformed to create LLDR, which was found to be normally distributed across the 1.3 million blood samples (skewness and kurtosis values of 0, Fig. **1**). Mean LLDR in the population sample was 0.640 [95% CI (confidence interval) 0.639-0.641].

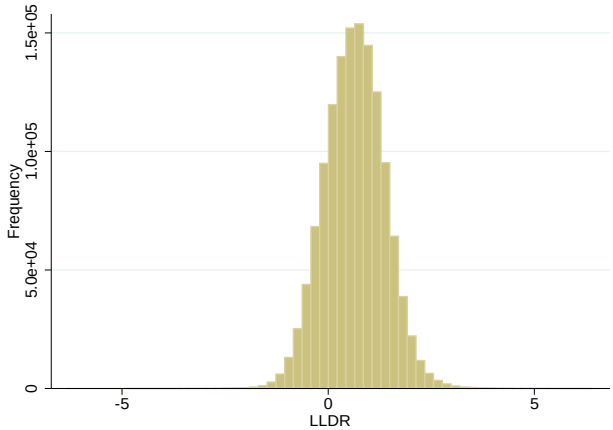

Fig. (1). Normal Distribution of LLDR Across 1,339,898 Serial Samples (Skewness and Kurtosis Values of 0)

There was a strong, highly statistically significant, monotonic increase in mean LLDR across groups with progressively higher density phenotype ($p<0.001$ by ANOVA) as seen in Table **1** and Fig. (**2**). Mean LLDR for Phenotype A was 0.122 (95% CI 0.121-0.123), for Phenotype A/B was 0.751 (95% CI 0.750-0.752), and for Phenotype B was 1.336 (95% CI 1.335-1.338).

Table 1. Mean LLDR Across LDL Modal Density Phenotypes (p<0.001 by One-Way ANOVA)

Phenotype	N (%)	Mean LLDR (95% CI)
A	640,656 (48)	0.122 (0.121-0.123)
A/B	265,044 (20)	0.751 (0.750-0.752)
B	434,198 (32)	1.336 (1.335-1.338)
Total	1,339,898 (100)	0.640 (0.639-0.641)

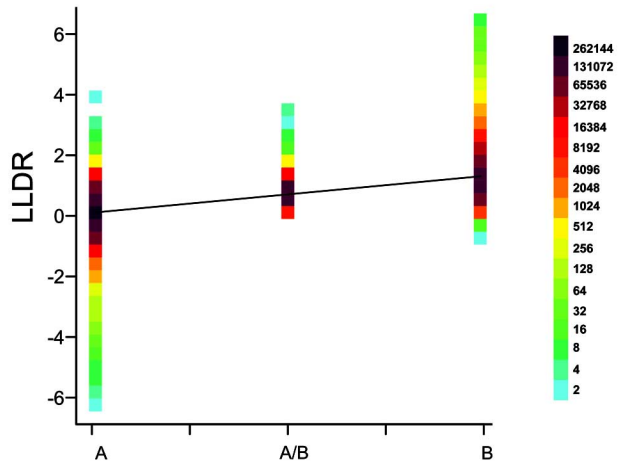

Fig. (2). Heat Plot of LLDR Across LDL Density Phenotype (R=0.73, p<0.001) Showing Increase in Mean LLDR Across Progressively Higher Density Phenotypes.

ROC plots with optimum sensitivity-specificity cutpoints for classification of subjects into LDL modal density Phenotypes A (A *vs* not A) and B (B *vs* not B) are shown in Fig. (**3**). LLDR was strongly associated with phenotype A, C=0.915 (0.914-0.915), $p<10^{-4}$; optimum LLDR cutpoint <0.398, sensitivity 72%, specificity 95%; and phenotype B, C= 0.923 (0.923-0.924), $p<10^{-4}$ optimum LLDR cutpoint >0.905, sensitivity 81%, specificity 86% (Table **2**). The slope of the cutpoint tangent line in Fig. (**3A**) was slightly greater than that of Fig. (**3B**), making the specificity for Phenotype A cutoff slightly higher than that for Phenotype B, and sensitivity slightly lower.

In a second subset of samples (N=5,957) where VAP LDL Max Time was available, LLDR was found to be correlated with LDL Max Time with R^2=0.802 (Fig. **4**). In a third subset (N=372) where LDL size was available by NMR, size was plotted against LDL Max Time. Two outlier datapoints were excluded from this subset, leaving 370 samples showing a modest, but positive correlation between LDL size and LDL Max Time as well as LDL size and LLDR (Fig. **4**).

DISCUSSION

Lipoprotein classes are not only heterogeneous, but have varying potential for atherogenic risk [17-20]. Small, dense LDL (phenotype B) has been observed to be strongly associated with cardiovascular disease risk in multiple studies [12, 13, 19, 21-23]. Accordingly, the development of simple and accurate lipoprotein subclass categorization techniques play an increasingly important role in atherosclerosis research.

Table 2. Optimum Sensitivity-Specificity Cutpoints for LDL Modal Density Phenotypes

Category	C (95% CI)	Optimum	Sensitivity (%)	Specificity (%)
		Youden's Index Method		
A, not A	0.9145 (0.9141-0.9150)	0.3976	72	95
B, not B	0.9232 (0.9228-0.9237)	0.9045	81	86
		Top Left Corner Method		
A, not A	0.9145 (0.9141-0.9150)	0.5453	79	87
B, not B	0.9232 (0.9228-0.9237)	0.8746	83	84

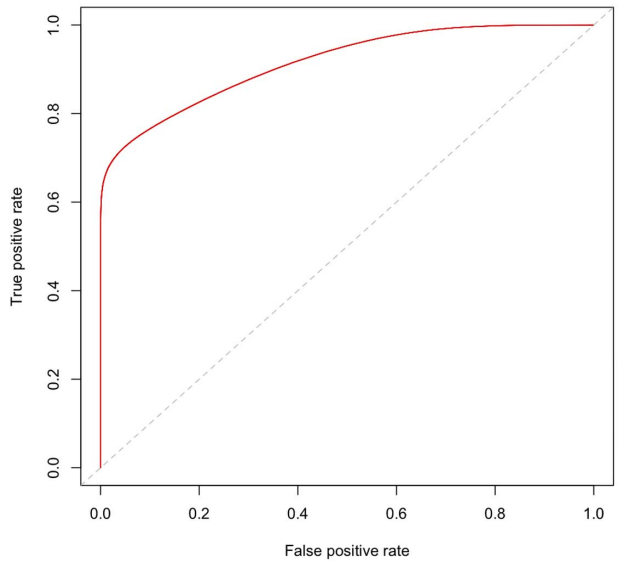

Fig. (3A). ROC Plot of LDL Modal Density Phenotype A (Area Under ROC Curve 0.9145) Showing True Positive Rate (Sensitivity) *versus* False Positive Rate (1-Specificity)

Historically, LDL cholesterol has been estimated by using multiple aliquots of serum samples to independently measure each lipoprotein class and then estimate LDL using the Friedewald formula [LDL cholesterol = total cholesterol – HDL cholesterol – (triglycerides/5)] [24]. However, this estimate is only clinically accurate when serum triglycerides are within normal limits, and often subjects the sample to multiple analyses and room for error. Two alternative methods for quantification of LDL are VAP (cholesterol content) and NMR spectroscopy (particle number) [15, 25, 26]. VAP technique allows for all lipoprotein classes to be separated by single vertical spin density-gradient ultracentrifugation. This technique provides all lipoprotein class measurements using the same serum sample in a single aliquot, is efficient, and separates subclasses by density which allows for more accurate measurement of dense LDL. NMR spectroscopy is able to measure lipoproteins based on

NMR signals of various subclasses due to differences in orientation of the phospholipid shells of the lipoprotein particles surrounding the lipid core [27].

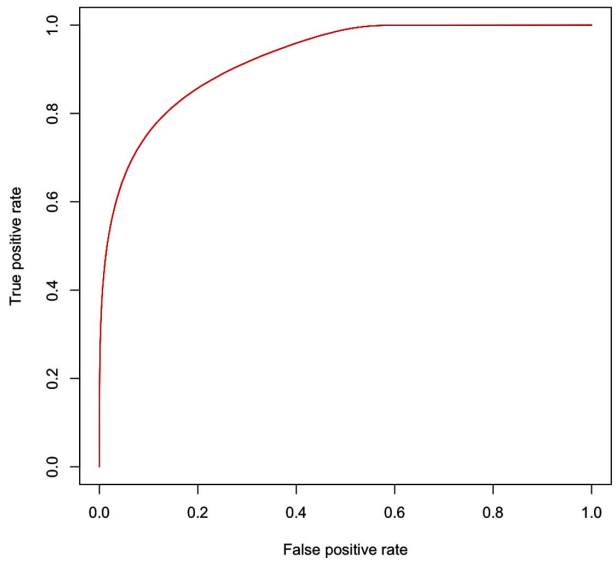

Fig. (3B). ROC Plot of LDL Modal Density Phenotype B (Area Under ROC Curve 0.9232) Showing True Positive Rate (Sensitivity) *versus* False Positive Rate (1-Specificity)

(R^2=0.4013 and 0.364, respectively, in Fig. **4**). This raises the question of whether LDL density and size are interchangeable properties. For example, it is possible that phospholipids, cholesterol content, and triglycerides all introduce heterogeneity in the density of a particle, independent of its size. A number of studies by Blake, *et al.* have observed differences between size and other LDL parameters with regards to cardiovascular risk prediction and treatment [28, 29]. Further research is needed to explore this relationship between lipoprotein density and size, and whether LLDR as a continuous measure provides more cardiovascular risk discrimination than LDL density phenotype.

CONCLUSION

LLDR was found to be a normally distributed continuous variable which 1) is easily calculated from readily available centrifugation methods, 2) reflects overall distribution of LDL particle density, and 3) is strongly associated with LDL modal density phenotype with C statistics in the range of 0.92. We propose the use of this log transformed ratio as a convenient, normally distributed, continuous variable quantifying LDL particle density phenotype for research uses. Further research is needed to investigate the relationship between lipoprotein density and size, and whether LLDR provides more cardiovascular risk discrimination than LDL density phenotype.

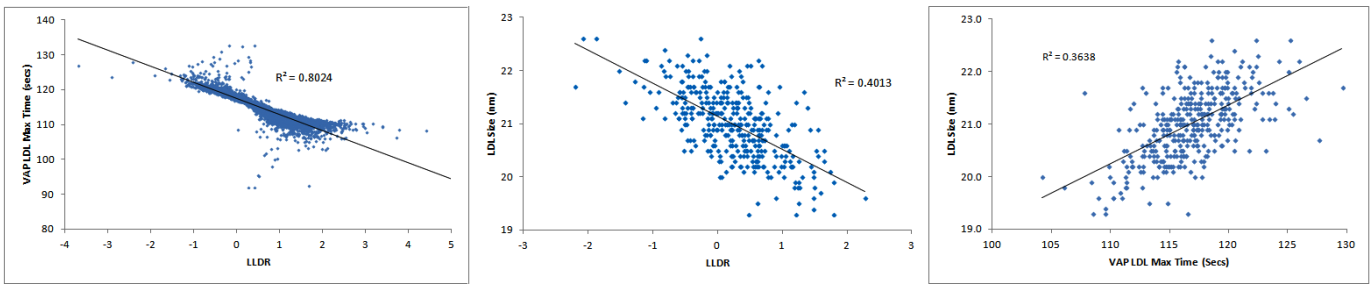

Fig. (4). Left: VAP LDL Max Time *versus* LLDR in 5,957 Consecutive Samples. Middle: LDL Size (nm) *versus* LLDR in 370 Samples. Right: LDL Size (nm) *versus* VAP LDL Max Time in 370 Samples.

After log-transforming the LDL subclasses separated by VAP in this study, we observed a normal distribution of LLDR across the 1.3 million consecutive samples. In essence, this easily-calculated continuous variable from readily available ultracentrifugation methods was shown to reflect overall distribution of dense and buoyant LDL particles. We observed a strong, highly statistically significant, monotonic increase in mean LLDR across groups with progressively higher density phenotype classification ($R=0.73$, $p<10^{-6}$).

Furthermore, LLDR was found to be correlated with LDL Max Time (R^2=0.802). We have previously shown that LDL Max Time is highly correspondent with LDL particle density [16]. The continued correlation between LLDR and LDL density phenotype *via* this second modality further supports this relationship.

When exploring the relationship between LDL size and density (by LLDR or Max Time), we observed a positive, but modest correlation between the two properties

CONFLICTS OF INTEREST

Disclosures include Krishnaji Kulkarni's employment by Atherotech, and grant support for this study from Atherotech.

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