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Estimation of Small, Dense LDL Particles Using Equations Derived From Routine Lipid Parameters as Surrogate Markers

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Abstract

Background: Small, dense LDL particles are highly atherogenic and has been recognized as an emerging coronary artery disease risk factor. But its estimation is seldom undertaken even among high risk patients as laboratory methods for its assessment are laborious and expensive. Investigators have put forth convenient equations derived from classic lipid parameters that act as surrogate markers for small, dense LDL.

Objective: Our objective was to assess small, dense LDL particles by using three equations: triglycerides/HDL-cholesterol ratio, LDL-cholesterol/LDL-apo B ratio and small, dense particles in mg/dL = 0.580 (non HDL-cholesterol) + 0.407 (direct LDL-cholesterol) – 0.719 (calculated LDL-cholesterol) – 12.05 among normal (n=62) and type 2 diabetes subjects (n=64) and evaluate the correlation between these equations.

Methods: Blood glucose, total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol and apolipo protein B were estimated biochemically and the three equations were calculated from these lipid parameters. Triglycerides/HDL-cholesterol ratio ≥ 3.0, LDL-cholesterol/LDL-apo B <1.2 were considered to predict small, dense LDL particles while the third equation provided the concentration of small, dense LDL particles in mg/dL.

Results: Fasting plasma glucose, triglycerides, LDL cholesterol and apoB were significantly higher among diabetics. Diabetics had significantly higher triglycerides/HDL-cholesterol ratio and also had higher concentrations of small, dense LDL. The two groups did not differ significantly on using LDL-cholesterol /LDL-apo B ratio. No consistent correlation was observed between these three equations.

Conclusion: Since LDL particle size is a major determinant of cardiovascular risk, validation and the establishment of the predictive value of these equations need to be done before adapting these as clinical tools.

Keywords: LDL-cholesterol/LDL-apo B ratio; Small, dense LDL; Surrogate markers; Triglycerides/HDL-cholesterol ratio

Introduction

Elevated level of low density lipoprotein-cholesterol (LDL-C) has long been established as one of the strongest risk factors for coronary artery disease (CAD) [1]. Depending on the size, chemical composition and density, there are different subclasses of LDL that can be separated by advanced techniques. Two phenotypes of LDL based on particle size have been identified – pattern A with LDL diameter > 25.5nm (large buoyant LDL or lbLDL) and pattern B with LDL diameter ≤ 25.5nm (small, dense LDL or sdLDL). Approximately 30% of the total LDL-C in blood is comprised of sdLDL in normolipidemic individuals and its proportion increases considerably in subjects with CAD depending upon the severity of disease [2]. SdLDL particles are more atherogenic than lbLDL molecules as reduced content of antioxidants and increased concentration of polyunsaturated fatty acids make them more susceptible to oxidative modification [3]. A predominance of sdLDL particles has been recognized as an emerging CAD risk factor by National Cholesterol Education Program Adult Panel III [4] and large clinical studies have proven the association of sdLDL particles with cardiovascular diseases [5,6].

In spite of its clinical significance, sdLDL particles are seldom estimated routinely even in high risk patients. Laboratory methods currently available for its assessment such as ultracentrifugation, gradient gel electrophoresis, nuclear magnetic resonance etc. are complicated, not cost effective and need elaborate equipment. Alternative methods for estimating sdLDL in the form of equations derived from classic lipid parameters have been proposed by different investigators and these could be of importance in the current clinical setting. A study conducted by Mohan et al in Indian population proposed that a triglycerides/high density lipoprotein-cholesterol (HDL-C) ratio ≥ 3.0 could serve a surrogate marker for sdLDL in this population [7]. A study by Hattori et al in a large population in Japan had suggested LDL-C /LDL-apolipoprotein B (apoB) ratio < 1.2 as a predictive marker for the presence of sdLDL particles [8]. Srisawasdi et al based on a study in Thailand population, put forth an equation from classic lipid parameters that could provide the value of sdLDL particles in milligram per deciliter (mg/dL) [9].

The incidence of type 2 diabetes, metabolic syndrome and CAD is high among Asian Indians [10-12] and increased concentrations of sdLDL have been reported among type 2 subjects of Indian subcontinent [13]. Studies in American and Japanese subjects have proven that increased concentrations of sdLDL is present among type

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2 diabetic subjects of these populations [14,15]. We conducted this study to assess sdLDL particles by using three equations: triglycerides/HDL-C ratio, LDL-C /LDL-apo B ratio and sdLDL particles in mg/dL among normal and type 2 diabetes subjects of Kerala (South India) and evaluate the correlation between these three equations.

Methods

Subjects

The study was conducted at a tertiary care university hospital after obtaining approval from Institutional Ethics Committee in accordance with the Helsinki Declaration of 1975, as revised in 1983. Normal and type 2 diabetic men, aged 35 to 65 years, not on lipid lowering therapy, attending the outpatient departments for routine health evaluation, consenting to participate in the study were considered. Subjects without prior history of diabetes, hypertension, renal or thyroid abnormalities, and who were free from history of CAD as confirmed by a normal resting 12-lead ECG and absence of inducible ischemia on stress test were included as controls (group 1). Subjects with type 2 diabetes based on World Health Organization diagnostic criteria for diabetes (fasting blood glucose levels >126mg/dL) [16], without renal or thyroid abnormalities, and without previously diagnosed CAD were included in patient group (group 2). After careful screening, 62 subjects were enrolled as controls and 64 subjects were enrolled in the diabetic group. Informed consent was obtained from the participating subjects. Subjects were interviewed on recruitment to obtain details regarding their diet and lifestyle.

Laboratory methods

Blood samples were collected after an overnight fasting from each subject; serum was separated and total cholesterol, triglycerides, LDL-C and HDL-C was measured using kits from Roche Diagnostics in Hitachi 912 auto analyzer. Fasting glucose level was estimated in plasma using kits from Roche Diagnostics in Hitachi 912 auto analyzer. Apo B was analyzed by immunoturbidimetry using kits from Daiichi Chemicals Co., Tokyo, Japan. Briefly, apo B in serum reacts with antihuman apo B antibody, causing turbidity, which was measured against blank. Apo B concentrations of the serum were calculated using a single-point calibrator. Apo B concentrations above 90 mg/dL were considered to be pathogenic [17]. Triglycerides/HDL-C ratio (henceforth referred to as equation 1) was calculated as a surrogate marker for sdLDL particles. Mohan et al proposed that a ratio ≥ 3.0 could predict the presence of sdLDL particles [7]. LDL-C /LDL-apo B ratio (henceforth referred to as equation 2) was calculated from concentrations of total cholesterol, triglycerides, HDL-C and apo B, as described by Hattori et al [8]. The equations LDL-C = 0.94total cholesterol - 0.94HDL-C - 0.19triglycerides and LDL-apoB = apoB - 0.09total cholesterol + 0.09HDL-C - 0.08triglycerides, is used for the calculation of LDL-C /LDL-apoB ratio. A lower ratio of LDL-C /LDL-apo B (<1.2) is postulated to indicate preponderance of sdLDL particles. Using the third equation (henceforth referred to as equation 3), small, dense particles in mg/dL = 0.580 (non–HDL-C) + 0.407 (direct LDL-C) – 0.719 (calculated LDL-C) – 12.05, where calculated LDL-C = Total cholesterol – HDL-C – (Triglycerides/5) was calculated [9].

Statistical analysis

Statistical analysis of data was done using IBM SPSS software 19.0. All values were expressed as mean and standard deviation. Student's t test was used to compare mean values of the parameters between groups and a p value < 0.05 was considered to be statistically significant. Pearson Correlation was used to calculate the correlation between the three equations, and also to find out the correlation between different lipid parameters and the three equations among the two groups.

Later, analyses of correlation between the equations and the biochemical parameters were carried out by considering both groups as a single population. Parameters that had moderate or strong correlation in the

univariate analysis was included in the multivariate model as independent variables. The equations were considered as dependent variable in each multivariate model, and the multivariate analyses were done after adjusting for age.

Results

The clinical characteristics of the study subjects are given in Table 1. The mean age of the controls was 49 ± 7 years and that of diabetics was 53 ± 7 years. While 14.5% among controls were tobacco users, it was 10.9% among diabetics. A tobacco user in this study is defined as a person currently smoking cigarettes or beedis or using smokeless tobacco or had done so in the past six months of enrollment. All the type 2 diabetes subjects were either on insulin therapy or on oral hypoglycemic agents. Among diabetics, 26 subjects were hypertensives and they were on antihypertensive drugs. No one in either group had total cholesterol level >300 mg/dL or triglycerides level >300 mg/dL.

Results of the biochemical and calculated estimations are included in Table 1. Fasting plasma glucose concentrations was significantly different between the two groups (p=0.0001). Total cholesterol did

Parameters	Group1 (Controls) n = 62	Group 2 (Diabetic) n = 64
Age (Years)	49±7	53±7
No: of hypertensives (n)	--	26
No: of tobacco users (n)	9	7
Plasma Fasting Glucose (mg/dL)	94±13	117±18
Total cholesterol (mg/dL)	168.16±25.64	178.81±35.49
Triglycerides (mg/dL)	125.19±37.74	161.75±36.07*
HDL-C (mg/dL)	45.08±9.13	42.53±9.64
LDL-C (mg/dL)	88.5±21.94	115.75±30.39*
Apo B (mg/dL)	85.45±17.46	95.73±17.96*
Triglycerides/HDL-C ratio	2.88±1.06	4±1.32*
LDL-C/LDL-apo B ratio	1.56±0.67	1.47±0.62
Small, dense LDL (mg/dL)	19.76±12.42	46.23±10.69*

* indicate p value <0.05 compared to control group

Table 1: Clinical and biochemical characteristics of subjects

Parameters	Triglycerides/ HDL-C	LDL-C/LDL-apo B	small, dense LDL in mg/dL	Triglycerides /HDL-C	LDL-C /LDL-apo B	small, dense LDL in mg/dL
Age	0.336	0.087	-0.071	-0.104	0.186	0.048
Fasting glucose	-0.005	-0.03	-0.035	0.496	0.029	0.008
Total cholesterol	0.409	0.592	0.18	-0.269	0.752	0.318
Triglycerides	0.813	0.344	0.251	0.779	0.015	0.321
LDL-C	0.205	0.453	0.567	-0.312	0.689	0.456
HDL-C	-0.513	-0.333	0.269	-0.674	0.163	0.268
Apo B	-0.612	-0.599	0.087	-0.571	-0.412	0.138

Weak (+/-) correlation: r = (+/-) 0.3 to 0.5, Moderate (+/-) correlation: r = (+/-) 0.5 to 0.8,
Strong (+/-) correlation: r = (+/-) 0.8 to 1.0

Table 2: Correlation (r) between parameters and the equations

LDL-C/ LDL-apo B ratio			Small, dense LDL (mg/dL)	
Controls		Diabetics	Controls	Diabetics
Triglycerides/	0.516	-0.046	0.045	0.257
HDL-C ratio				
LDL-C/			0.108	0.068
LDL-apo B ratio				

Weak (+/-) correlation: r = (+/-) 0.3 to 0.5, Moderate (+/-) correlation: r = (+/-) 0.5 to 0.8,
Strong (+/-) correlation: r = (+/-) 0.8 to 1.0

Table 3. Correlation between the three equations in both groups

not show significant difference between the two groups (p=0.055), but was higher among diabetic subjects. Similarly, HDL-C was also not significantly different between the controls and diabetics (p=0.13), though its concentration was lower among diabetics. Triglycerides level was significantly higher for the type 2 diabetes subjects compared to controls (p=0.000) and similar results were observed for LDL-C levels (p=0.000). Apo B concentrations were higher for type 2 diabetes subjects compared to the control subjects (p=0.001). Among controls, 22 subjects had hyper apo B concentrations (>90 mg/dL), while it was 36 among type 2 diabetes subjects.

Triglycerides/HDL-C ratio was ≥ 3.0 in 27 controls and 37 diabetic subjects and was found to be significantly different between the two groups (p=0.000). LDL-C/LDL-apo B ratio was below 1.2 for 19 control subjects and 26 diabetic subjects, but no significant difference was observed on comparing the two groups (p=0.46). SdLDL values calculated as per equation 3 showed that diabetic subjects had significantly higher values compared to control group (p =0.000). The number of subjects with sdLDL concentration >35 mg/dL was very high in diabetic group (2 vs. 53).

Correlation between various parameters and the three equations are given in Table 2. It was observed that age was not found to be correlated with any of the three equations for both groups, except for a weak positive correlation with equation 1 among control subjects. Fasting glucose was found to have moderate positive correlation with equation 1 among diabetics. Total cholesterol had weak positive correlation with equation 1 among controls and was moderately correlated with equation 2 in both groups. A weak positive correlation was noted between total cholesterol and equation 3, but only among diabetic subjects. A strong correlation for triglycerides with equation 1 was observed among controls and a similar result was observed among diabetics. Triglycerides showed a weak positive correlation with equation 2 among controls and with equation 3 among diabetics. LDL-C showed moderate positive correlation with equations 2 and 3 among both groups. A weak positive correlation between LDL-C and equation 1 was noticed among diabetic subjects. HDL-C showed moderate negative correlation with equation 1 in controls and diabetics and a weak negative correlation with equation 2 among controls. Apo B had moderately negative correlation with equations 1 and 2 among both groups.

Results of Pearson correlation analysis between the equations is given in Table 3. There was moderate positive correlation between equations 1 and 2 among subjects of group 1, whereas diabetic group did not show any correlation between these two equations. No correlation was observed between equations 1 and 3 and between 2 and 3 among control subjects. Among diabetic subjects, no correlation between equations 1 and 3 and between 2 and 3 were noted.

Multivariate regression analysis with equation 1 as dependent variable showed that triglycerides and HDL were significant contributors to the multivariate model and yielded the regression equation 3.257 + 0.78(triglycerides)-0.525(HDL-C), that explains 95.8% of variants in this equation. Similarly, total cholesterol and apoB were significant predictors of the multivariate model with equation 2 as dependent variable. The regression equation 0.697+0.766(cholesterol)-0.631(apoB) explains 79.9% of the variants in equation 2. Regression analysis with equation 3 as dependent variable showed that total cholesterol, triglycerides, HDL-C and LDL-C were the best predictors of sdLDL and yielded the regression equation (-13.045 - 1.181)total cholesterol + 0.604(triglycerides) + 0.362(HDL-C) + 1.578(LDL-C), and this model explains 98.2% variants in equation 3.

Discussion

Number of subjects with sdLDL was higher among type 2 diabetes subjects compared to normal subjects in this study. LDL phenotype changes from lbLDL to sdLDL in diabetic subjects [18] and the mechanism underlying this phenomenon has been established earlier. Insulin resistance associated with diabetes causes increased mobilization of free fatty acids from adipocytes into circulation. This leads to increased triglycerides synthesis in the liver utilizing these free fatty acids. Hepatic over-synthesis of triglycerides escalates the production of triglyceride-rich very low density lipoprotein, which is preferably acted upon by hepatic lipase enabling triglyceride hydrolysis

and its subsequent conversion to sdLDL [19].

Mohan et al used Receiver Operating Characteristic curve to propose triglycerides/HDL-C ratio for predicting elevated sdLDL in their study in a small population in South India. They found that this equation had fair sensitivity and specificity and its association with sdLDL was evident on regression analysis even after adjusting for other parameters. Though there are numerous studies that have used this ratio as a marker for insulin resistance [20,21] there are very few that tested for its suitability as marker for predicting sdLDL. Maruyama et al determined sdLDL by gradient gel electrophoresis in healthy, normolipidemic Japanese subjects and reported that 75 % of those with sdLDL particles had triglycerides/HDL-C ratio above 2.0 [22]. That high triglycerides and low HDL-C concentrations induce an increase in the proportion of small, dense LDL particles was observed by Dobiasova et al also [23]. This equation had strong positive correlation with triglycerides and negative correlation with HDL-C in the present study, in both univariate and multivariate models, as expected. It was also noticed that apoB was negatively correlated with this equation in univariate analysis, which disagrees with the previous literature that sdLDL concentrations were positively associated with the level of apoB [15,24]. Bowden et al have reported a moderate inverse correlation between LDL particle size and number in their study, but on end-stage renal disease subjects [25]. The correlation between apoB and equation 1 was not investigated by Mohan et al in their study; hence a comparison with their study could not be done.

Hattori et al derived equation 2 from the ultracentrifugation data obtained from 2179 subjects and suggested that this formula could be used for analyzing lipoprotein disorders both qualitatively and quantitatively. They observed a significant correlation between observed and mathematically measured values. A study in diabetic subjects by Wagner et al had reported a lower LDL-C/LDL-apoB ratio among subjects with sdLDL [26]. A previous study done in Indian women has shown that a high percentage of CAD subjects had lower LDL-C /LDL-apo B ratio compared to healthy controls, but it was not studied if the ratio had good predictive value [27]. Gazi et al on examining LDL-C/ LDL-apoB ratio in subjects with metabolic syndrome concluded that the ratio did not appear to be a sensitive marker of decreased mean LDL particle size, as against results obtained by Lipoprint LDL System and that Hattori et al derived the normal range of this ratio from a small number of controls, n=18 [28]. Furuya et al in their study in hyper lipemic and control samples reported that results of LDL particle size estimated by gradient gel electrophoresis did not agree with that of LDL-chol/LDL-apo B ratio [29]. LDL-C / LDL-apo B ratio showed positive correlation with total cholesterol and LDL-C, and negative correlation with apoB and HDL-C concentration in univariate analysis our study in both groups. Wagner et al found in their study that subjects with sdLDL had higher total cholesterol, triglycerides and apo B, and lower HDL-C levels. On multivariate analysis, the significant predictors for equation 2 were found to be total cholesterol and apo B in our study.

Srisawasdi et al developed equation 3 on the hypothesis that the inaccuracy observed while calculating LDL using Friedewald formula was related to triglycerides and HDL-C levels, which often associate with presence of sdLDL particles. They had reported a good correlation between measured and calculated sdLDL values, but had cautioned that the equation was not tested for aptness in other ethnic populations and also among patient groups with cardiovascular diseases, renal disorders, diabetes, metabolic syndrome etc. Cho Y et al in their study in Korean population opined that the equation could not be applied directly to their population, healthy or with metabolic syndrome and suggested a modified equation [30]. SdLDL value estimated by equation 3 among diabetics in this study was found to be similar to those reported in other diabetic populations, but the sdLDL concentrations among controls were lower in our population compared to controls of these studies [9,15]. Srisawasdi et al had shown that the calculated sdLDL value had weak positive correlation with triglycerides level (<200mg/dL) and strong positive correlation with non HDL-C. This equation showed weak positive correlation with triglycerides and HDL-C and moderate positive correlation with LDL-C in our study for both groups in univariate analysis, whereas total cholesterol, triglycerides LDL-C and HDL-C were significant predictors on doing multivariate analysis.

These three equations have been put forth as convenient tools for measuring sdLDL from lipid parameters without additional elaborate laboratory procedures or equipments and also without causing financial burden to the patients. It should be noted that no considerable correlation existed between the three equations in our study and the results were not consistent between the two groups studied. Investigators have earlier shown that results of different methodologies adopted for the measurement of the same parameter are not comparable [31]. It should be noted that our methodologies were not entirely similar to the original studies (e.g., immuno turbidimetry vs. latex method for estimating apoB, difference in assay kit/equipment used for measuring lipid parameters etc.) and this might have influenced the outcome. Though many investigators have used these equations as a predictive marker for sdLDL, their validation and predictive value remains to be established. Given the significance of LDL particle size determination, the suitability of these equations to each population has to be tested and the best should be adapted. Large scale studies are needed in healthy controls to establish a normal range of the equation best suited for each population before putting these to use as a clinical tool.

Our study has many important limitations. The sample size of this study was small and extrapolation of the data to the entire population may not be practical. Also dissimilar assays adopted for various biochemical estimations compared to original studies might have affected the results. It does not provide information on the predictive capability of the equations nor do indicate the equation best suited for this population and is not tested against "standard gold" methods.

Conclusion

The diabetic subjects of this study had higher small, dense LDL level compared to controls and this could be an important risk factor in this population. That the equations did not show significant correlation warrants the need for large scale studies to determine the equation appropriate to this population and its validation before using as a clinical tool.

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