Can calculated SdLDL serve as a substitute for estimated SdLDL?

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ABSTRACT

Background: SdLDL is the atherogenic component of LDL. Though it is a predictive biomarker for coronary artery disease, lack of standardisation, complexity and cost of analytical techniques has prevented SdLDL from being routinely estimated in clinical practice. Methods available for estimation include Ultracentrifugation, NMR, HPLC and the enzymatic method. Srisawasdi et al. 2011 developed a formula for estimation of SdLDL using commonly estimated lipid parameters. The formula was: *SdLDL (mg/dl) = 0.580(non HDL- Cholesterol) + 0.407(direct LDL- Cholesterol) – 0.719(calculated LDL- Cholesterol) – 12.05.* Mohan et al. 2005 proposed that Triglyceride/ HDL ratio of 3.0 had optimum sensitivity for predicting elevated SdLDL, hence TG/HDL ratio could be used to predict SdLDL levels instead of directly estimating SdLDL. **Aims and Objectives:** The study was to determine the correlation between SdLDL estimated using the enzymatic method and between that calculated using Srisawasdi's formula, and Mohan et al.'s proposed TG/HDL ratio. **Materials and Methods:** This a retrospective study done on 374 samples for which total cholesterol, HDL, LDL and triglycerides were estimated using routine methods and SdLDL by the enzymatic method. SdLDL was calculated using Srisawasdi's formula and correlation was determined between estimated and calculated SdLDL. We also determined the correlation between the estimated SdLDL and TG/HDL ratio, and with Non HDL. **Results:** A highly significant and positive correlation was found between estimated and calculated SdLDL (r = 0.74, p < 0.001), and between estimated SdLDL and non HDL (r = 0.721, p = < 0.001). The correlation between SdLDL and TG/HDL ratio was positive but poor (r = 0.353). **Conclusions:** Calculated SdLDL may be used as a substitute for estimated SdLDL. Further studies on a larger population are required before use of calculated SdLDL can be implemented in routine clinical practice.

Key words: Calculated SdLDL; Measured SdLDL; Non HDL; Lipid profile; TG HDL ratio

INTRODUCTION

Hyperlipidemias are long known to be a major contributing factor in the development of CAD. Lipid parameters, in particular LDL-C are routinely used for risk assessment in CAD patients. However it has been found that LDL is not elevated in all those patients suffering from CAD.[1] Moreover, though lipid lowering agents are widely used in clinical practice as a treatment component in CAD, the risk reduction after lipid lowering therapy has been found to be not more than 30% in most of the clinical studies.[2] These findings led to the search of risk factors in addition to those previously known which could contribute to the

development of atherosclerosis and CAD, i.e. the 'Beyond Cholesterol' concept. Further studies started pointing towards the evidence that it is in particular the small dense component of LDL (SdLDL) which is responsible for its atherogenic property.[3]

LDL particles exist as two major sub-types; pattern A(Density 1.019-1.044 gm/ml), with a higher proportion of large buoyant LDL (lbLDL) particles, and pattern B, (density 1.044-1.060 gm/ml), which consists of mainly smaller denser LDL (SdLDL) particles.[4] Properties of SdLDL like higher penetration into the arterial wall, lower binding affinity for the LDL receptor, prolonged plasma

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half-life and lower resistance to oxidative stress account for its higher atherogenic potential as compared to pattern A. Lower resistance to oxidative stress leads to increased generation of oxidised LDL. Oxidised LDL by passes the normal feedback control of receptor mediated LDL uptake, and is instead internalized by macrophages. Accumulation of cholesterol and cholesterol esters within macrophages converts them to foam cells, which in turn promote the early stages of atherosclerosis.[5]

According to the National Cholesterol Education Programme Panel III; SdLDL, along with lipoprotein remnants and low HDL-C are components of the atherogenic lipid triad, which as a whole is a risk factor for CAD. Presence of SdLDL is used as an indicator for the presence of atherogenic dyslipidemia and metabolic syndrome. Modifications in lipid lowering therapy may be required if SdLDL presence is accompanied by presence of low HDL- C and high Triglycerides.[6]

Various techniques have been in use to assess the SdLDL levels in serum; including Analytical Ultracentrifugation, GGE under non denaturing conditions, Nuclear Magnetic Resonance (NMR), HPLC, Dynamic Light Scattering and Ion Mobility Analysis.[7] The problem with all these techniques was that they were time consuming and not very cost effective for routine clinical use. Hirano et al were the first to develop a simple homogenous assay for SdLDL assessment. A two-step reaction was used which separates SdLDL from large buoyant LDL and then estimates its level.[8] But cost affectivity again remained the hindering factor. Moreover, there has been wide variation in the results obtained through different techniques. It is hence difficult to decide which of the above is appropriate for routine clinical use.

The utility of the Friedewald formula in estimating LDL levels is well known for its convenience and accuracy. Efforts have been made for developing a similar formula which could be used to estimate SdLDL levels and be implemented in clinical practice as a regular screening tool. Srisawasdi et al using regression analysis developed an equation for estimation of SdLDL levels using routine lipid parameters i.e. Non HDL-C, direct LDL-C and calculated LDL-C.[9] Mohan et al separated SdLDL sub fractions using LDL Lipoprint system and found that Triglyceride/ HDL ratio of 3.0 had optimum sensitivity for predicting elevated SdLDL levels.[10]

In this study, a retrospective analysis was done on samples previously analysed for lipid profile and SdLDL using the enzymatic method. Samples had been collected from pregnant women in the middle of each trimester and post-partum, and their lipid profile and SdLDL levels were analysed. Pregnancy is a physiological state known to be associated with significant hypertriglyceridemia and LDL sub fraction redistribution. The data obtained has been used in this study since hyperlipidemia of pregnancy was found to be associated with significant elevation of SdLDL.[11] The clinical importance of SdLDL is mainly as a cardiac biomarker; elevated SdLDL being associated with increased atherogenic risk. Since in our previous study, we found elevated SdLDL levels in pregnancy, we calculated SdLDL for the same subjects using Srisawasdi's formula, and also the TG/HDL ratio. Our objective here was to compare the SdLDL levels in both lower and higher ranges obtained using direct enzymatic analysis, and by the above equations, and to evaluate the correlation between the above. We also evaluated the correlation between levels of SdLDL Cholesterol determined by the enzymatic method and non HDL Cholesterol to determine whether non HDL alone could suffice as a predictor for high SdLDL values.

MATERIALS AND METHODS

This is a retrospective study using data obtained from our previous study on lipid profile and SdLDL in normal pregnancy and post-partum. The study was pre-approved by the institutional ethics committee. Women attending the ante natal OPD from early pregnancy had been included in the study and were followed up throughout pregnancy and once post-partum. Those with previous history of Diabetes, Hypertension, Thyroid disorders or known cardiac conditions were excluded from the study. Those who developed gestational diabetes or hypertension during the course of pregnancy were also excluded. One hundred twenty three women had been included in the study. Samples were collected four times from each subject, once in the middle of each trimester and the fourth sample once 3-4 months after childbirth.

The patients were asked to come after 10-12 hours of fasting. Samples were collected at the same time they came for their fasting glucose estimation. The samples were collected in non EDTA vials. Serum was separated and stored in aliquots at 4°C to be processed within 7 days. Total Cholesterol (TC), HDL-C, LDL-C and Triglycerides (TG) estimation was done using ROCHE kits on Modular P-800 auto-analyzer. Non HDL was calculated as Non HDL (mg/dL) = TC-HDL-C.

SdLDL was estimated on Hitachi 902 auto-analyzer using the SdLDL kit provided by Randox. The assay was performed according to the instructions of the manufacturer. It consists of a two-step reaction using 5 μL of serum. The first reaction involves degradation of non SdLDL lipoproteins and enzymatic degradation

of released cholesterol. In the second reaction cholesterol from SdLDL is released and subjected to an enzymatic reaction leading to the formation of purple red coloured complex. Readings were taken at 600nm. This method has a detection limit of 1mg /dl and linearity up to 100mg/dl. Correlation coefficient (r) of this method vs. the standard method, ultra centrifugation is 0.954. Quality was checked by running of a control along with each batch.

SdLDL levels in the above patients were then determined by the equation suggested by Srisawasdi et al., i.e. SdLDL(mg/dl) = 0.580(non HDL- Cholesterol) + 0.407(direct LDL- Cholesterol) − 0.719(calculated LDL-Cholesterol) − 12.05. TG/HDL ratio was determined as proposed by Mohan et al.

Statistical analysis was done using SPSS software version 16. All values were expressed as mean and standard deviation. Bivariate analysis was used for the assessment of correlations between the variables, namely estimated and calculated SdLDL levels, and also with TG/HDL ratio. According to the levels of SdLDL obtained by the enzymatic method, the samples were then divided into 3 groups; Group A: SdLDL less than 10mg/dl; Group B: SdLDL = 10- 24 mg/dl and Group C: SdLDL more than 24 mg/dl. The cut of value of 10mg/dl was chosen for Group A because in previous studies a cut of value of 10mg/dl has been found to be associated with CAD.[12] Correlation was then analysed individually in each of the groups to determine whether the correlation among calculated and estimated SdLDL varies among different ranges.

Finally we evaluated the correlation between levels of SdLDL Cholesterol determined by the enzymatic method and non HDL Cholesterol to determine whether non HDL alone could suffice as a predictor for high SdLDL values.

RESULTS

Out of 123 women recruited in the study, 9 developed hypertension, and 2 developed gestational diabetes, and 12 were lost to follow up. So in total we had 100 subjects, for whom the samples had been collected four times; so in total we had 400 samples. Among those, in 26 samples, the Triglyceride values exceeded 399mg/dl. Since Srisawasdi et al.'s proposed formula is dependent on calculated LDL (Friedewald formula), which is not valid if TG values exceed 399 mg/dl, they too had to be excluded from the study. Hence final analysis was done retrospectively on a total 374 samples.

Biochemical characteristics of the subjects are summarized in Table 1 and Figure 1. Mean age of the subjects was 24.6

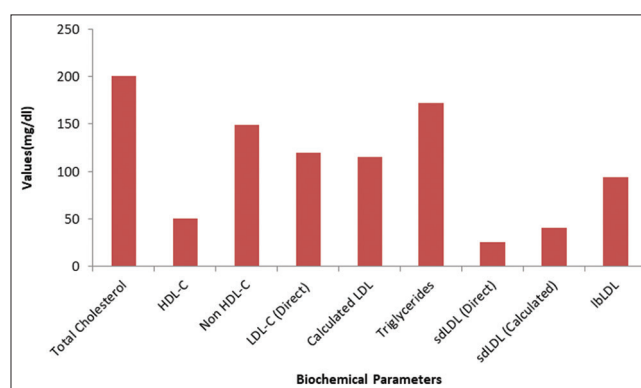| Table 1: Biochemical characteristics of the study population	
Parameter	**Result±SD**
Age (Years)	24.63±2.68
Total Cholesterol (mg/dl)	200.06±54.24
HDL-C (mg/dl)	50.73±11.81
Non HDL-C (mg/dl)	149.32±50.43
LDL-C (Direct) (mg/dl)	119.21±45.09
Calculated LDL(mg/dl)	114.90±42.12
Triglycerides (mg/dl)	172.09±76.77
SdLDL (Direct) (mg/dl)	25.11±16.16
SdLDL (Calculated) (mg/dl)	40.46±18.68
lbLDL (mg/dl)	94.10±35.83
TG/HDL Ratio	3.57±1.92

Figure 1: Biochemical characteristics of the study population

± 2.6 years. All were pregnant women in different stages of pregnancy and post-partum. The TC, HDL-C, dLDL-C, TG and SdLDL values obtained ranged from 92 -426, 23-82, 44-333, 40-390 and 3-94 mg/dl respectively.

Figure 2 shows the association between the estimated (x) and calculated SdLDL(y) obtained from all study samples. From the scatter plot the least squares regression analysis yielded the equation y (mg/dl) = 0.873x +18.53(95% confidence interval 0.796-0.950 for slope), and $R^2 = 0.570$. Correlation coefficient (r) was 0.75, suggesting a highly significant strong positive relationship (p value< 0.001) (Table 2).

Correlation analysis was then done separately on three groups divided on the basis of their SdLDL levels; Group A with SdLDL less than 10mg/dl; Group B: SdLDL = 10- 24 mg/dl and Group C: SdLDL more than 24 mg/dl. The r values obtained are represented in Table 2.

We also performed a correlation analysis between measured SdLDL and TG/HDL ratio, and between measured SdLDL and non HDL levels. Non HDL showed a good positive correlation with measured SdLDL (r= 0.721, p value < 0.001). Regression analysis yielded the equation y (Non HDL) (mg/dl) = 2.251x (SdLDL) (mg/dl) +92.78.; $R^2 = 0.520$. (Figure 3). The TG/ HDL ratio however had

Table 2: Correlation between estimated and calculated SdLDL group wise and as a whole

Study population	Whole study Group (SdLDL = 3- 94 mg/dl) n=374	Group A (SdLDL<10mg/dl) n=48	Group B (SdLDL = 10- 24 mg/dl) n=168	Group C (SdLDL >24 mg/dl) n=158
Correlation with calculated SdLDL (r)	0.74	0.036	0.417	0.647
p value	<0.001*	0.805	<0.001*	<0.001*

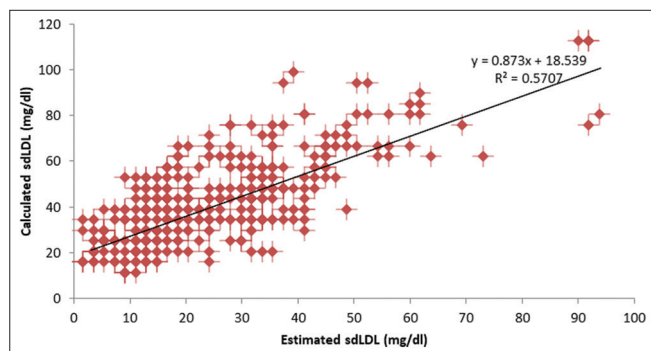*Significant if p<0.05

Figure 2: Linear regression of the estimated (x) and calculated (y) SdLDL (mg/dL), y = 0.873x +18.53; R^2 = 0.570; n= 374
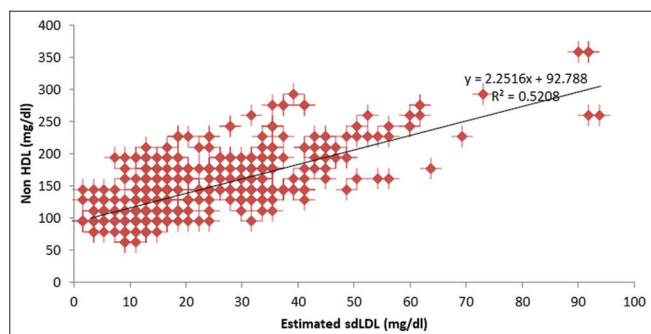

Figure 3: Linear regression of estimated SdLDL (mg/dl) (x) and Non HDL (mg/dl) (y), y = 2.251x +92.78; R^2 = 0.520; n= 374

a positive but weak correlation (r= 0.353, p value < 0.001) with measured SdLDL (Table 3).

DISCUSSION

Small dense LDL is now well established as a prospective biomarker for prediction of cardiovascular disease.[13] Various case control and prospective studies have shown that SdLDL levels are high in patients at high risk of cardiovascular events, including those with Type 2 diabetes and metabolic syndrome. The Framingham Offspring Study found that SdLDL levels were higher in women with CHD, even though their mean LDL-C levels were not significantly higher than those without CAD.[14] Nishikura et al in a prospective cohort study concluded that SdLDL is a promising biomarker to predict future cardiovascular events in secondary prevention of stable CAD.[13] Besides, increased SdLDL is a significant predicting factor for

Table 3: Correlation between estimated and calculated SdLDL, Non HDL and TG/HDL ratio

Parameter	Non HDL		TG/HDL Ratio	
	r	p Value	r	p Value
Estimated SdLDL	0.721	<0.001*	0.353	<0.001*

*Significant if p<0.05

onset of AIS (Acute Ischemic Stroke). Mortality rate of patients with early onset AIS was also higher in those having significantly higher levels of SdLDL.

In spite of its significance, a major obstacle in the use of SdLDL in routine clinical practice is the lack of a simple, cost effective method of estimation. The methods currently used for analysis include Ultracentrifugation, Gradient Gel Electrophoresis, NMR and Ion Mobility Analysis. The problems with these methods are cost, complexity and long duration of analysis, along with the need of complex equipment. Hirano et al developed an enzyme based analytic method. However none of the above has been established till yet as a gold standard method.

Srisawasdi et al. conducted a study on 297 patients attending the Medicine outpatient clinic. They studied their biochemical parameters, and based on that developed a formula for calculation of SdLDL levels using routine lipid parameters. Their equation [SdLDL(mg/dl) = 0.580(non HDL- Cholesterol) + 0.407(direct LDL- Cholesterol) – 0.719(calculated LDL- Cholesterol) – 12.05] was developed on the hypothesis that inaccuracy of calculated LDL-C was related linearly to TG and HDL-C levels, which in turn is associated with high SdLDL levels.[9] We compared the SdLDL levels obtained from the enzymatic method to those obtained from Srisawasdi's formula and found a strong positive correlation of 0.74.

The samples were then divided into three groups based on their SdLDL levels; Group A: SdLDL less than 10mg/dl; Group B: SdLDL = 10- 24 mg/dl and Group C: SdLDL more than 24 mg/dl. The cut of value of 10mg/dl was chosen for Group A because in previous studies a cut of value of 10mg/dl has been found to be associated with CAD.[12] We found no correlation in the lower ranges of SdLDL (0.036), and medium to strong positive correlation with calculated SdLDL in higher ranges (0.417 and 0.647).

This variation might be explained by the small sample size obtained when the whole study population was divided into subgroups. Group A had only 48 subjects; it is difficult to comment about the correlation with such a small sample size. However, better results were obtained when SdLDL values were more than 10 mg/dl, the cut off value for high risk.

We also found a strong positive correlation (0.72, R^2=0.52) between measured SdLDL and non HDL. This is in accordance with the findings of Srisawasdi et al. who got a correlation of r = 0.866. Non HDL is measured by subtracting HDL from total cholesterol. It includes all lipid components other than HDL, namely LDL, IDL, VLDL, lipoprotein (a) and lipoprotein remnants. Experimental evidence suggests an atherogenic role of all these ApoB containing lipoproteins as a whole. It has been hypothesized that since HDL is the only good part of cholesterol, measuring all the lipid components other that HDL can suffice as a simple alternative for cardiovascular risk assessment with better predictive value than LDL-C. Besides, non HDL has an additional advantage that it can be measured in both the fasting and post- prandial state, and that the assessment is simple and valid even if the TG values exceed 399mg/dl.[15]

Mohan et al studied the association of SdLDL with CAD and diabetes in Asian Indians. They estimated SdLDL levels using electrophoresis with 3% polyacrylamide tube gel with Lipoprint LDL System. They constructed ROC curves to predict higher levels of SdLDL (>9.0mg/dl), and found that TG/HDL ratio of >3.0 had optimum sensitivity (80%) and specificity (78%) of predicting high SdLDL values. They concluded that TG/HDL ratio of >3.0 could serve as a surrogate marker for predicting elevated SdLDL [10]. We determined the correlation between SdLDL determined by the enzymatic method with the TG/HDL ratio as proposed by Mohan et al. Though positive and significant, the correlation however was found to be weak (r= 0.353), whereas Mohan et al had found a strong positive correlation (r=0.728). This variation maybe explained by differences in the method used for SdLDL quantification.

The importance of SdLDL as a cardiac biomarker is now well established.[3] The challenge that still remains is the development and selection of a gold standard method of analysis. SdLDL estimation can then probably be included among the lipid profile parameters. Until such a method is developed, the use of Srisawasdi's formula for SdLDL estimation can be thought of. The formula has a good positive correlation with the enzymatic method developed by Hattori et al. Though not absolutely accurate, the utility of this formula lies in the fact that it is simple, cost effective, and that the calculation is based on parameters routinely assessed in the lipid profile; so no additional cost will be incurred. In addition, calculated SdLDL was very strongly correlated to non HDL which is considered by some to be a treatment target. Also in patients already diagnosed with CAD, lipid parameters are normally assessed in their follow up for secondary prevention. Calculated SdLDL if included in their lipid profile will give additional information regarding the response to lipid lowering therapy.

The limitation of this study was the sample size. Our sample size was 374. Studies are required on a larger population with wide range of SdLDL levels to determine the correlation between estimated and calculated SdLDL in different ranges. Secondly our study was a retrospective study done on pregnant women. Pregnancy is known to cause a temporary shift in the LDL distribution towards the smaller denser type. We did the study on these samples because our aim was to determine the correlation between calculated and estimated SdLDL in both lower and higher levels of SdLDL, and in this population we had the entire range. However since the clinical implication of SdLDL estimation is CAD risk assessment and prevention, studies on a similar population with long term follow up are desirable.

It is hence concluded that calculated SdLDL may serve as a substitute for estimated SdLDL. However, further studies on a larger population are required before it can be used as a clinical tool in regular practice.

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BS-Study design, data collection, statistical analysis and interpretation, and preparation of the manuscript.

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