Comparison of An *App* Based Low Density Lipoprotein Cholesterol (LDL-C) Estimation with Direct Assay and Friedewald Formula in Indian Population

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Abstract

Introduction: Accurate measurements of Low Density Lipoprotein Cholesterol (LDL-C) are imperative in classifying individuals with risk of cardiovascular Disease (CVD). Guidelines around the world advocate aggressive LDL-C lowering therapies. A novel equation, Martin Hopkins Equation [LDL-C (MH)], has been proposed to be an alternate to provide precise LDL-C estimates to initiate and/or adjust drug dosage.

Method: Lipid profile data of 739 adults were measured by enzymatic colorimetric methods. Calculated LDL-C by Friedewald formula, LDL-C (FF) and LDL-C (MH) was computed from ldlcalculator.com/ smartphone application.

Results: The sensitivity and specificity of LDL-C (MH) was 92.3% and 87.8% respectively. On Receiver Operating Characteristics Curve (ROC) analysis, Area under Curve (AUC) was 0.97 ($p<0.001$). Bland Altman plots revealed a minimal positive bias of 1.2 for LDL-C (MH).

Conclusion: The findings are suggestive of better estimates of LDL-C (MH) when compared to LDL-C (FF). A diagnostic laboratory could report LDL-C for absolutely *FREE* in the future. A new horizon in LDL-C estimation!!!

Keywords: *Low Density Lipoprotein Cholesterol, Cardiovascular Disease, Friedewald Formula, Martin Hopkins Equation, ROC.*

Introduction

Circulating Low Density Lipoprotein Cholesterol (LDL-C) has been considered as the main culprit and key contributor to plaque formation and atherosclerotic cardiovascular disease (ASCVD).[1] LDL-C cut points are considered as primary treatment goals to reduce risk of major cardiovascular events and mortality.[2-3] Thus its accurate estimations is the need of the hour to enable tailoring of therapies [(statins and non-statin based options like ezetemibe, proprotein convertase subtilisin/ kexin type 9(PCSK-9 inhibitors)] to reduce LDL-C levels which leads to improved patient care and outcome.[3]

The gold standard in estimation of LDL-C, β-quantification, is resource expensive and time-consuming. [4] Hence other scalable alternatives were developed. The analysis can be done either by **Direct estimation [LDL-C (Direct)]** which are detergent based assays or calculated using the defacto clinical standard, **Friedewald formula [LDL-C (FF)]** that considers a fixed factor of 5 to provide Very Low Density Lipoprotein Cholesterol (VLDL-C) values. [5]

Here we explore the plausibility of usage of a Novel equation to estimate LDL-C that utilizes an adjustable factor for Triglycerides (TGL)/VLDL-C ratio. With increasing incidence of high TGL states like Diabetes mellitus, Obesity, Insulin resistance, it would affect the variance of TGL/VLDL-C ratio, hence LDL-C

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measures could be inaccurate. [6] The recent AHA/ACC 2018 Guidelines have approved the usage of an alternate method of LDL-C estimation [**Martin- Hopkins equation**: LDL-C (MH)]. [2]

In an era of digital/mobile health with an App being developed for the same, ***LDL CALCULATOR,*** clinicians and patients can easily avail LDL-C values at the click of a few buttons from a lipid profile. In this age of precision medicine an attempt has to be made to begin looking beyond LDL-C (FF) and Homogenous assays for accurate LDL-C estimates enabling the correct classification of risk of CVD in all patients.

Objectives:

1) To determine underestimation/overestimation of LDL-C when calculated by using the formulae [LDL-C(FF) and LDL-C(MH)] compared with LDL-C by direct homogenous assay [LDL-C(Direct)], assuming the assay to be accurate.

2) To determine which of the calculated formulae shows maximum correlation with LDL-C (Direct) at various TGL levels.

Materials and Method

A) Data Collection:

A **cross- sectional comparative study** was conducted by the Department of Biochemistry, Ramaiah Medical College, Bengaluru and data was collected during the period May 2015 to July 2015(Power of study is 80%). The lipid profile parameters[Total Cholesterol(TC),High Density Lipoprotein Cholesterol(HDL-C),LDL-C(Direct) and TGL] were measured by enzymatic colorimetric methods on fasting venous samples on a fully automated Cobas® 6000 analyzer (Roche Diagnostics, Basel, Switzerland) at Ramaiah Hospital Laboratory. The data of 753 adults (≥18 years) was collected and those of children and pregnant women were excluded in the context of primary hyperlipoproteinemia and hemodilution respectively.

B) Calculated LDL-C:

a) FRIEDEWALD FORMULA:

LDL-C is almost always measured using LDL-C(FF) and is given as **TC-HDL-C-(TGL/5)**.[5] It begins underestimating LDL-C in patients with TGL levels as low as 150 mg/dl leading to reduced treatment of such patients and under-classification of risk of CVD. [6]

b) MARTIN HOPKINS EQUATION:

It was derived and validated by Martin et al using a large sample of lipid profiles (n=13,50,908),3015 times larger than the original Friedewald database, inclusive of fasting and non- fasting samples. The methods employed for TC was ultracentrifugation and TGL was directly measured. [7]

It features an adjustable factor for TGL/VLDL-C ratio based on the patient's Non- High Density Lipoprotein Cholesterol (Non-HDL-C) and TGL estimates. The approach consists of 6 levels of stratifying Non-HDL-C from <100 mg/dl to >220 mg/dl and TGL into 30 ranges from 7-49 mg/dl to 400-13975 mg/dl in a 180 cell table. [7]

LDL-C (MH) = TC-HDL-C-(TGL/adjustable factor)

= Non-HDL-C-(TGL/adjustable factor)

The factor ranges from 3.1-11.9 and are personalized to the specific lipid panel. It was found to be more accurate in comparison to LDL-C (FF), particularly when classifying LDL-C levels <70 mg/dl in the presence of high TGL levels. [6-9]

It is available as an online calculator on **ldlcalculator.com** or also as a **smartphone application** downloadable on Google Play or Apple play store.

C) STATISTICAL ANALYSIS OF DATA:

Statistical analyses were performed on Microsoft Excel and Medcalc software (version 19.0.5). The distribution of continuous variables were described as means with standard deviations **(mean ± SD)** and compared using **Student t-test.** Correlation between various methods of LDL- C was assessed by **Pearson's correlation**. Agreement between two measurements was tested by calculating systematic errors (Bias), and 95% limits of agreement (LOA) as Bias±2SD by **Bland-Altman plots**. The level of statistical significance was established at $p < 0.05$.

The risk of CVD was decided on the basis of LDL-C cut-points in accordance to NCEP –ATP III Criteria, i.e. <100 mg/dl (no risk of CVD) and ≥100 mg/dl (with risk of CVD). The sensitivity and specificity for LDL-C by the estimated formulae was calculated.

Receiver operating characteristics curve (ROC) and **Area under curve** (AUC) was used to evaluate the ability of the formulae to discriminate diseased cases from normal subjects.

Results

A total of 753 subjects aged between 18-93 years, of which 449(60.7%) were males and 290(39.2%) females were included in the study. Among them 14 patients had TGL> 400 mg/dl, the remaining 739 individuals were considered as the study cohort in final analysis.

The analysis at a cut- off point of LDL-C 100 mg/dl, showed sensitivity and specificity of LDL-C (MH) as 92.3% and 87.8% respectively. Odds ratio (OR) was found to be 87.22. On ROC analysis (Figure 3) the AUC was 0.97($p<0.001$). Also the sensitivity and specificity of LDL-C (MH) relative to LDL-C (FF) was 90.2% and 99.7% respectively and AUC was 0.99 ($p<0.001$). The overall mean LDL-C (Direct), LDL-C (FF) and LDL-C (MH) were 108.8±40.8, 103.5±37.5, 107.7±36.7 mg/dl respectively. On an overall basis, a minimal difference of 1.1±4.1 mg/dl was noted between LDL-C (Direct) and LDL-C (MH) values.

Gender wise comparison with and without risk of CVD of all measures of LDL-C:

There exists a significant difference ($p <0.001$) in the LDL-C estimated by both the formulae in both genders with and without risk of CVD as shown in Table 1. Also when LDL-C (Direct) was <100 mg/dl, in both genders overestimation by LDL-C (MH) was noted.

Effect of TGL on measurements of LDL-C (FF) and LDL-C (MH):

The consequences of serum TGL concentrations on the divergence of LDL-C (FF) and LDL-C (MH) values from LDL-C (Direct) was assessed. In Table 2, the study cohort was stratified into four groups on the basis of TGL levels and a measure of comparison of means of LDL-C calculated by the formulae against LDL-C (Direct) was attempted. At high TGL levels (Group III and Group IV) overestimation of LDL-C (MH) is observed though statistical significance could not be proved (Group III: $p= 0.59$, Group IV: $p= 0.42$) while underestimation was noted in LDL-C (FF) in all the groups. A strong correlation was also noted for LDL-C (MH).

Concordance in Guideline classification:

The concordance of values between LDL-C (Direct) and estimated LDL-C was analysed using NCEP-ATP III criteria. The study population was again assorted into 5 categories with respect to LDL-C (Direct) values. In Table 3, LDL-C (MH) showed a superior concordance with LDL-C (Direct) when compared with LDL-C (FF) across all the categories, also reaching statistical significance. With increasing values of LDL-C (Direct), the ability of LDL-C (FF) to correctly identify the patient population reduces but the same was not observed when LDL-C (MH) was used.

Correlation and Bland Altman plot between LDL-C (Direct) and calculated LDL-C:

Linear regression analysis between LDL-C (Direct) and estimated LDL-C revealed a strong, positive and similar correlation. The correlation coefficients were 0.87 ($p=1$) for LDL-C (MH) and 0.87 ($p <0.05$) for LDL-C (FF). (Figure 1) To find an agreement between LDL-C (Direct) and estimated LDL-C, the Bland Altman plots were used. (Figure 2) It shows a minimal positive bias of 1.2 for LDL-C (MH) and 5.3 for LDL-C (FF).

Table 1: Comparison of parameters between both genders with and without risk of CVD as defined by LDL-C cut points in accordance to NCEP-ATP III Criteria

Parameters	Males			Females		
	LDL-C (Direct)≥ 100 mg/dl (n=240) (With risk of CVD)	LDL-C (Direct)< 100 mg/dl (n=209) (Without risk of CVD)	p-value*	LDL-C (Direct)≥ 100 mg/dl (n=185) (With risk of CVD)	LDL-C (Direct)< 100 mg/dl (n=105) (Without risk of CVD)	p-value*
Age(years)	51.2±15.1	52.8±16.1	0.25	51.5±13.6	51.3±15.3	0.89
TC(mg/dl)	200.6±32.5	131.9±25.9	<0.001	205.5±31.4	138.9±22.7	<0.001

Cont... **Table 1: Comparison of parameters between both genders with and without risk of CVD as defined by LDL-C cut points in accordance to NCEP-ATP III Criteria**

TGL(mg/dl)	162.9±72.7	137.5±74	<0.001	153.3±68.1	130.3±74.8	0.05
HDL-C(mg/dl)	41.6±12.2	34.3±15.1	<0.001	45.8±13.2	40±16.4	<0.001
LDL-C (Direct) (mg/dl)	136.1±27.8	69.7±21.7	<0.001	137.2±27.1	74.2±19.4	<0.001
LDL-C(MH) (mg/dl)	130.6±27.4	75.1±20.2	<0.001	132.2±26.7	77±19.4	<0.001
LDL-C(FF) (mg/dl)	126.4±29	70.2±20.4	<0.001	129±27.5	72.9±18.7	<0.001

****student's't' test, p-value <0.05 is significant, p-value <0.001 is highly significant.***

Table 2: Comparison of mean difference and measure of comparison of means between various methods of LDL-C on the basis of different levels of TGL

LDL-C(mg/dl)	**TGL LEVELS(mg/dl)**			
	GROUP I : TGL<100 (N=217)	**GROUP II: TGL 100-199 (N=369)**	**GROUP III: 200-299 (N=118)**	**GROUP IV: 300-399 (N=35)**
LDL-C(Direct)	93.4±34.3	116.5±40.9	113.4±45.8	108.7±34.8
LDL-C(MH)	91.2±33.3	113.9±36.3	116.3±36.8	114.7±26.9
Difference in mean	2.2	2.6	-3.1	-6.0
p-value*/r value#	0.48/0.94	0.38/0.94	0.59/0.91	0.42/0.86
LDL-C(FF)	92.5±33.6	110.7±37.8	104.3±40.3	94.2±30.9
Difference in mean	0.9	5.8	9.1	14.5
p-value*/r value#	0.77/0.95	0.05/0.94	0.11/0.91	0.07/0.86

****student's't' test, p-value <0.05 is significant, p-value <0.001 is highly significant.***

r value =Pearson's correlation coefficient.

Table 3: Proportion of concordance between LDL-C (Direct) and estimated LDL-C on the basis of stratification of LDL-C cut points as per NCEP-ATP III Criteria

LDL-C (Direct)	LDL-C(MH)			LDL-C(FF)		
(mg/dl)	Concordance	%	p- value*	Concordance	%	p- value*
<100	282/314	89.8	<0.05	299/314	95.2	0.92
100-129	141/197	71.6	<0.05	127/197	64.4	<0.001
130-159	95/148	64.2	<0.001	88/148	59.4	<0.001
160-189	31/57	54.4	<0.001	26/57	45.6	<0.001
≥ 190	13/23	56.5	0.09	10/23	43.5	0.06

student's 't' test, p-value <0.05 is significant, p-value <0.001 is highly significant.

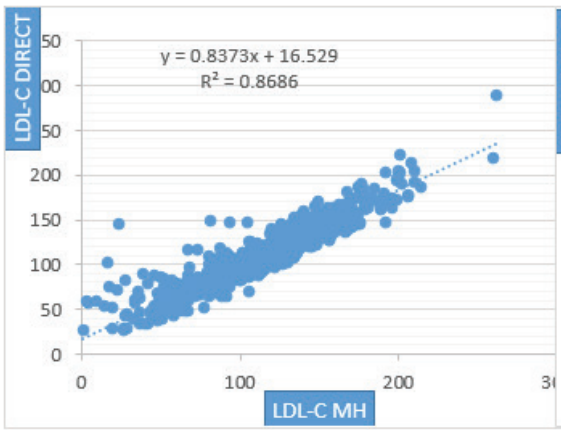

Figure 1(a): Scatter plot of LDL-C (Direct) and LDL-C (MH) shows a positive correlation of 0.86

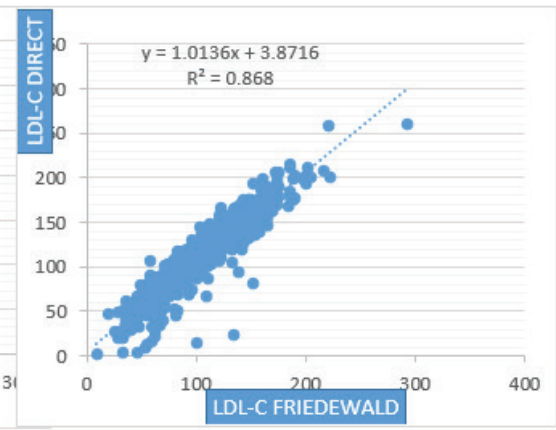

Figure 1(b): Scatter plot of LDL-C (Direct) and LDL-C (FF) shows a positive correlation of 0.86

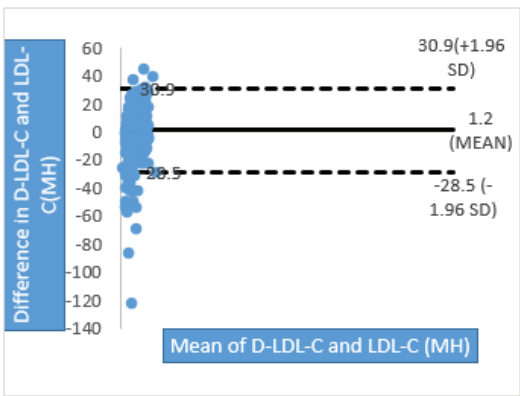

Figure 2(a): Bland Altman plot for LDL-C (Direct) and LDL-C(MH) shows a minimal bias of 1.2

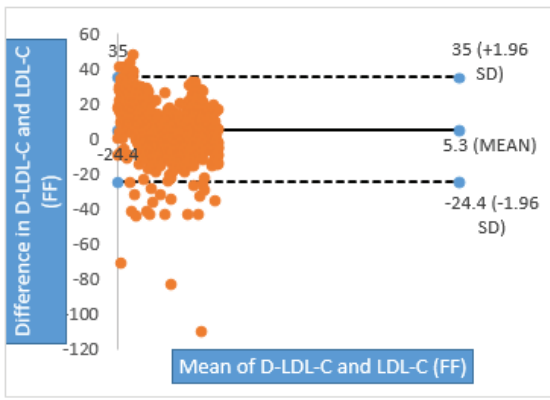

Figure 2(b): Bland Altman plot for LDL-C (Direct) and LDL-C(FF) shows a bias of 5.3

Figure 3: ROC for LDL-C (MH) at cut-off point of LDL-C 100 mg/dl (risk of CVD)

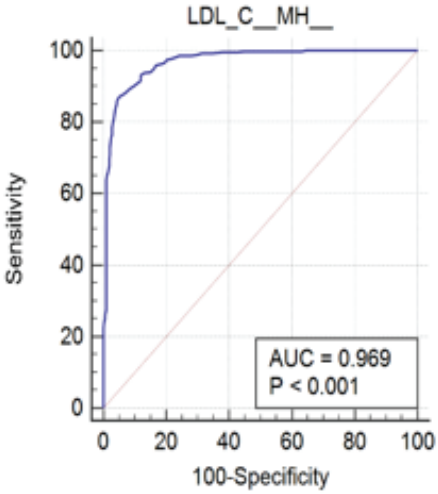

Figure 3(a): LDL-C (Direct) and LDL-C (MH)

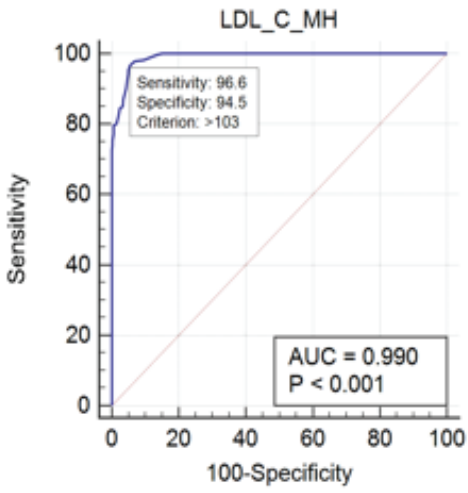

Figure 3(b): LDL-C (MH) and LDL-C (FF)

Discussion

Why is it time to look beyond the classic Friedwald formula that has been historically tolerated for five decades? The answer is Statins and PCSK-9 inhibitor therapies for hypercholesterolemia which are extremely effective at lowering LDL-C and its values are required by clinicians. As these drugs have undesirable side effects, inappropriate use is not encouraged, and thus the need for an extremely accurate LDL-C estimate is acute. Direct LDL-C offers relatively better accuracy but are costlier than a ***"FREE"*** calculation.

In 2013 a team led by Dr. Martin S Seth at John Hopkins University developed the equation which basically customizes the denominator through the 180-cell approach. Its beauty lies in the fact that using the information from a standard Lipid profile an accurate and FREE measure of LDL-C is obtainable. Besides, it obviates the need for a fasting sample thus attaining more patient compliance. [6]

This study was undertaken to assess the validity of LDL-C (MH) and LDL-C (FF) in comparison to LDL-C (Direct) assay. To the best of the author's understanding the equation has not been studied in the Indian population. This study demonstrated a superior performance of LDL-C (MH) at low LDL-C (<100 mg/dl). LDL-C (MH) tends to overestimate the LDL-C significantly ($p<0.001$) when compared to LDL-C (Direct) and LDL-C (FF). Thus it was capable of identifying individuals with high risk of CVD.

With an increase in TGL concentrations, the difference between LDL-C (Direct) and LDL-C (FF) increased but LDL-C (MH) values were independent of TGL levels. This outcome was comparable to studies by Choi *et al* [10], Decordova *et al*[11] and Martins J *et al.* [12] A strong, positive correlation ($r=0.87$) was noted for LDL-C (MH). The Bland Altman plot displayed a minimal positive bias of 1.2 for LDL-C (MH), similar to the study by Saiedullah *et al.* [13] but LDL-C (FF) showed a larger positive bias of 5.3. This can be indicative of a possibility of usage of LDL-C (MH) to obtain accurate measures in place of LDL-C (Direct) and LDL-C (FF). LDL-C (MH) showed a higher concordance with LDL-C (Direct) when compared to LDL-C (FF) across all the categories when stratified on basis of LDL-C cut points in accordance to NCEP- ATP III criteria, which was statistically significant ($p <0.001$). LDL-C (MH) showed a greater overall concordance of 62.5% in comparison to 60.9% by LDL-C (FF) which is in agreement to studies by Martin S *et al* [6] and by Lee J *et al.* [14] The sensitivity, specificity and ROC analysis as described in Results section emphasizes the strong association of LDL-C (MH) to correctly identify all individuals who are at higher risk of CVD at a cut point of LDL-C ≥100 mg/dl in comparison to both LDL-C (Direct) and LDL-C

(FF).

In our country with a burdening population of high TGL states and an increased risk of CVD, it would be thoughtful for us to move away from Friedewald formula and adopt the novel equation to prevent misclassification of risk of CVD. With diagnostic laboratories most often running on shoe string budgets LDL-C (MH) offers a cost effective and accurate solution. To routinely report LDL-C (MH), will require a re-programming of the Laboratory Information Systems (LIS) which will initially prove to be costly and daunting, but with time it will mitigate the need for Direct LDL-C assays. There could be a day in the future when a laboratory could report LDL-C for absolutely free, without costing any money to the patient for the same!

Limitations of this Study:

1) It requires verification on a large sample size of Indian population as it was originally derived and validated on the American population.

2) Difference in methodology of cholesterol estimations (Ultracentrifugation versus direct homogenous assay).

Conclusion

The novel approach can thus provide precise LDL-C levels without associated costs and inaccuracies inherent to other established methods of LDL-C measurement.

Acknowledgement: We thank Ramaiah Medical College and Hospital, Bengaluru for supporting this study.

Institutional ethics committee clearance was obtained.

Sources of Funding: Not applicable.

Conflict of Interest: The authors have declared no conflict of interest.

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