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Original Article

Biochemistry Section

Comparison between Values of Low Density Lipoprotein Cholesterol as Estimated by Direct Enzymatic Method with Calculated Methods Applying Friedewald's Equation and Novel's Equation: A Cross-sectional Study

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ABSTRACT

Introduction: Lipid profile is routinely used as a screening test to identify the risk of Cardiovascular Diseases (CVD). Elevated Low Density Lipoprotein cholesterol (LDL-c) is an important modifiable risk factor of atherosclerotic CVD. The LDL-c lowering strategy is a known recommendation for the prevention and treatment of CVD. The gold standard method of LDL-c estimation is β-quantification by ultra centrifugation. Other methods include Direct LDL-c measurement (D-LDL-c) using enzymatic assay which is tedious, time consuming and expensive. Hence, calculated method using Friedewald's equation (F-LDL-c) is routinely used in clinical laboratories in India.

Aim: To compare LDL-c values as estimated by direct enzymatic method with LDL-c values obtained by Friedewald's equation and Novel's equation, and, also to assess the effects of LDL-c values obtained by both the methods towards the risk stratification of CVD.

Materials and Methods: A cross-sectional study, was conducted in the Central Diagnostic Laboratory of Mandya Institute of Medical Sciences, Mandya, Karnataka, India, for a duration of three months from July to September 2020, where, 600 subjects, aged 20-75 years, visiting for routine lipid profile estimation were included. LDL-c was estimated by direct enzymatic method (D-LDL-c) and calculated methods using Friedewald's {F-LDL-c=TC-HDL-c-(TG/5)} and Novel's equation {N-LDL-c=TC-HDL-c-(TG/Adjustable factor)}. Values obtained by calculated methods were compared with D-LDL-c values. The LDL-c values obtained were compared at different ranges of Total Cholesterol (TC), Triglycerides (TG) and High-Density Lipoprotein cholesterol (HDL-c). The association between direct and calculated LDL-c values were analysed by Pearson's correlation. Receiver Operator Characteristic Curve (ROC) analysis was done to predict the better diagnostic method among the calculated methods of LDL-c.

Results: The mean±SD of D-LDL-c (115.68±36.94 mg/dL) was high compared to F-LDL-c (106.95±33.48 mg/dL) and N-LDL-c (110.78±32.58 mg/dL). The mean difference between D-LDL-c and N-LDL-c (4.9±4.36 mg/dL) was low compared to F-LDL-c (8.75±3.46 mg/dL). Significant positive correlation was observed between D-LDL-c vs F-LDL-c (r=0.96; p=<0.001) and D-LDL-c vs N-LDLc (r=0.97; p=<0.001). The ROC showed maximum AUC value for N-LDL-c than F-LDL-c at a cut-off value of 100 mg/dL. LDL-c as estimated by Novel's and Friedewald's equation led to approximately 5% and 10% less patients being subjects for lipid lowering therapy respectively as compared to D-LDL-c.

Conclusion: In conclusion, the use of Novel's equation for LDL-c estimation instead of Friedewald's equation could be associated with the small net increase in lipid lowering agent eligible population for primary prevention of atherosclerotic CVD. Replacement of Friedewald's equation by Novel's equation would enable for the improved accuracy of LDL-c estimation especially at higher levels of TC, TG and lower levels of HDL-c.

Keywords: Cardiovascular disease risk, Dyslipidemia, Lipid profile, Obesity

INTRODUCTION

The CVD are the leading cause of morbidity and mortality worldwide [1]. The major pathological conditions associated are arrhythmia, Ischaemic Heart Disease (IHD), cardiomyopathy, thromboembolic phenomenon, hypertensive heart disease, cerebrovascular disease and congenital heart disease [2]. According to World Health Organisation (WHO), CVD holds the first position among the top 10 causes of deaths in low-middle-income countries [3]. In the year 2016, the rate of mortality due to CVD was accounted to 17.9 million, which contributed to 31% of all the deaths globally [3]. In past two decades, the prevalence and mortality of CVD in India and other South Asian countries has increased at an alarming rate [4]. There has been a four-fold rise of CVD prevalence in India over past 40 years [3]. This increased CVD prevalence was contributed by various risk factors such as type-2 diabetes mellitus, hypertension, atherogenic dyslipidemia, smoking, alcoholism, central obesity, physical inactivity, rapid urbanisation and change in lifestyle [4].

Dyslipidemia is a widely accepted risk factor for CVD and it is mainly characterised by the presence of lipid triad i.e., increased levels of TC, LDL-c and TG levels or decreased HDL-c levels [5]. Elevated LDL-c is a known biomarker, with a high predictive value for the development of atherosclerotic CVD and stroke. Plasma concentration of LDL-c shows a direct relationship towards the commencement of atherosclerotic risk. Extracellular deposition of small lipid droplets and vesicles derived from LDL-c is the major factor towards the causation of atherosclerotic CVD. Hence, maintaining the LDL-c levels within the desirable limit is recommended towards the diagnosis, prevention and management of CVD [6,7]. The National Cholesterol Education Programme (NCEP) Adult Treatment Panel III (ATPIII) recommend treating patients to risk stratified LDL-c

target levels such as, <70 mg/dL, <100 mg/dL, <130 mg/dL and <160 mg/dL as very high risk, high risk, intermediate and low risk, respectively [8]. Thus, estimation of accurate LDL-c value is crucial in diagnosis, prevention and management of CVD.

The gold standard method for LDL-c estimation is β-quantification. This method is laborious, time consuming, inconvenient, requires ultracentrifugation and large volume of serum sample. Hence, β-quantification method is not suited for routine use in most of the clinical laboratories. Other method includes, D-LDL-c using homogenous assay. The diagnostic measurement of D-LDL-c is limited by high cost of these assays and requirement of expensive equipments [6]. Numerous calculation methods are available for LDL-c estimation such as Novel's method [9], Anandaraja's method [10], Hattori's method [11] etc., routinely, most clinical laboratories estimate LDL-c using Friedewald's equation [12].

In the year 1972, Friedewald WT et al., derived F-LDL-c based on the analysis of 448 subjects. Friedewald's equation is the commonly employed method for the estimation of LDL-c in most of the clinical laboratories and for large population studies. The LDL-c levels by Friedewald's method is calculated by using the equation {F-LDL-c=TC-HDL-c-(TG/5)}. This equation uses a fixed factor of 5 for the ratio of TG to VLDL-c. The F-LDL-c does not address inter-individual variability in TG:VLDL-c ratios. The Friedewald's equation inaccurately measures LDL-c at TG levels ≥400 mg/dL. This is the major demerit of F-LDL-c equation towards LDL-c estimation. The F-LDL-c is less reliable in patients with history of type-III hyperlipoproteinemia or dysbetalipoprotenemia which is characterised by the accumulation of lipoproteins with an increased proportion of cholesterol relative to TG. It is also less accurate in non fasting serum sample, since chylomicronaemia in non fasting serum sample leads to overestimation of VLDL-c and thus underestimation of LDL-c [13]. The utilisation of this formula is not recommended for patients with history of type-2 diabetes mellitus, nephrotic syndrome, end stage renal disease and chronic alcoholic liver diseases, because in these conditions the TG: VLDL-c ratio is altered [14]. Inspite of these limitations associated with Friedewald's equation, it remains the commonly incorporated method of LDL-c estimation in most of the clinical laboratories [13].

The Novel's equation (N-LDL-c) for LDL-c estimation was derived by Martin SS et al., on 13,50,908 subjects. The N-LDL-c is calculated by using the equation {N-LDL-c=TC-HDL-c (TG/Adjustable factor)}. Thus, N-LDL-c uses an adjustable factor for the TG: VLDL-c ratio based on TG and non HDL-c values. The adjustable factor was generated using a two-dimensional table of different median values of TG/VLDL-c against different combination of TG and non HDL-c range. A 180-cell table of strata specific median TG: VLDL-c values were obtained and applied in validation data for the adjustable factor and was found to be 5.2 with the interquartile range of 4.5-6.0. Martin SS et al., also generated LDL-c calculator for the ease of LDL-c calculation and it is made available on the website- http:www.ldlcalculator.com [9]. However, many studies have reported that this novel's equation has no clear benefit over Friedewald's calculation [9,15]. Many studies have been conducted to compare the direct method of LDL-c estimation with the calculated methods and have shown conflicting results [15-17].

Studies on Indian population have been conducted to compare the LDL-c concentration obtained by calculation methods by using Friedewald's and Anandaraja's formula [15-17]. But there are very few studies documented on Indian population for comparison of LDL-c concentration obtained by Friedewald's and Novel's equation. Before incorporating any new method for routine clinical use, it is required to substantiate and validate the method in independent population comprising of various races. Hence, this study was conducted to compare the LDL-c values as estimated by direct method and the calculated methods using Friedewald's equation and Novel's equation among Indian population. Thus, this study might enable proper risk stratification, early diagnosis of CVD and initiation of appropriate treatment for high-risk study subjects at the earliest.

Study Objectives

- To compare LDL-c values as estimated by direct enzymatic method with LDL-c values obtained by Friedewald's equation and Novel's equation.
- To assess the effects of LDL-c values obtained by both the methods towards the risk stratification of CVD.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Clinical Biochemistry Section, Central Diagnostic Laboratory, Mandya Institute of Medical Sciences, Mandya, Karnataka, India. The study was conducted for a period of three months from July to September 2020. The study was initiated after obtaining ethical clearance from the Institutional Scientific committee and Institutional Ethics Committee: No-MIMS/IEC/2020/410 dated 10/06/2020.

Considering the standard deviation for LDL-c from a previous study, the minimum sample size for our study was estimated as 400 [18]. Considering the period of the study 600 subjects of age 20-75 years visiting biochemistry laboratory for routine fasting lipid profile estimation were enrolled for the study.

Study Procedure

Under all the aseptic precautions, 3 mL of venous blood was drawn into a plain tube after confirming 10-12 hours of overnight fasting for the estimation of lipid profile parameters. These tubes were allowed to stand for about 10-15 minutes and subjected to centrifugation at 3500 rpm for 15-20 minutes. The serum was subsequently analysed on fully automated Abbott architect analyser. After ensuring both internal quality control (Randox level-2 and level-3) and external quality control checks (CMC EQUAS), the samples were processed. Lipid profile parameters such as TC was estimated by Cholesterol Oxidase Peroxidase (CHOD-POD) method, TG by Glycerol phosphate peroxidase-Phenol 4-Amino antipyrine method (GPO PAP) and HDL-c by the action of cholesterol oxidase. Subjects with TG value ≥400 mg/dL were excluded from the study.

The LDL-c was estimated by direct enzymatic method and calculated methods using Friedewald's and Novel's equation. Friedewald's equation is given by the formula; F-LDL-c=TC-HDL-c-(TG/5), the factor TG/5 is the VLDL-c concentration based on average ratio of TG to cholesterol in VLDL-c [13]. Novel's equation is given by the formula; N-LDL-c=TC-HDL-c- (TG/Adjustable factor) [9]. The LDL-c values obtained by these methods were compared at different ranges of TC, HDL-c and TG.

STATISTICAL ANALYSIS

Data were entered into Microsoft Excel worksheets and were analysed using statistical software- IBM Statistical Package for the Social Sciences (SPSS) 22.0 and R environment ver.3.2.2. Descriptive and inferential statistical analysis was used for Mean±Standard Deviation. The strength of association between the studies variables were analysed by using Pearson's correlation. Correlation co-efficient ranging between -1 to 1; -1 being the perfect negative correlation, 0 is the no correlation and 1 means perfect positive correlation. ROC analysis was done to predict the better diagnostic method among the calculated methods of LDL-c such as F-LDL-c and N-LDL-c. Probability value (p) of <0.05 was considered as statistically significant.

RESULTS

The present study included 600 study subjects visiting the Clinical Biochemistry section, Central Diagnostic Laboratory, Mandya Institute of Medical Sciences, Mandya, Karnataka, India, for their fasting

serum lipid profile estimation. Out of which, 295 were males and 305 were females. The baseline characteristics of the study subjects are depicted in [Table/Fig-1] using descriptive statistics. The mean age of the study subjects was 45.68±12.79 years. The mean±SD of D-LDL-c (115.68±36.94 mg/dL) was high compared to F-LDL-c (106.95±33.48 mg/dL) and N-LDL-c (110.78±32.58 mg/dL).

Variables	Mean±Standard deviation
Males, n (%)	295 (49.2%)
Females, n (%)	305 (50.8%)
Age (years)	45.68±12.79
TC (mg/dL)	176.46±39.95
TG (mg/dL)	160.99±73.19
HDL-c (mg/dL)	38.53±9.82
D-LDL-c (mg/dL)	115.68±36.94
F-LDL-c (mg/dL)	106.95±33.48*
N-LDL-c (mg/dL)	110.78±32.58*

[Table/Fig-1]: The baseline characteristics of the subjects.
*Moderately significant (p-value: 0.01<p ≤0.05)

According to NCEP ATP-III guidelines, LDL-c levels of ≥160 mg/dL, ≥130 mg/dL and ≥100 mg/dL are considered as the treatment goals for patients with low risk, moderate risk and high risk respectively. The comparison of risk stratification of patients on the basis of LDL-c values using direct method and the calculated methods such as Friedewald's and Novel's equation lead to approximately 10% and 5% lesser number of patients being the candidates for lipid lowering drug therapy as shown in [Table/Fig-2].

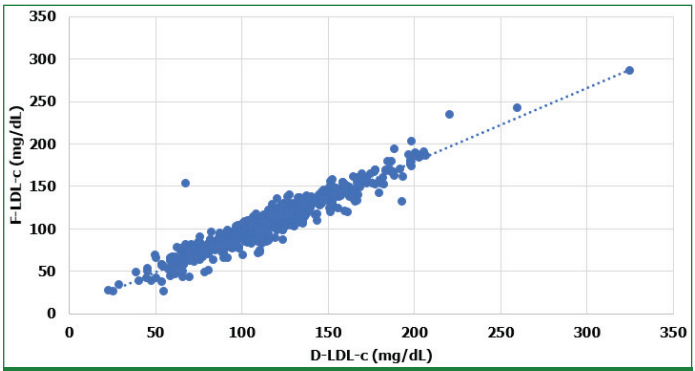
LDL-c (mg/dL)	No. of patients by D-LDL-c	No. of patients by F-LDL-c	No. of patients by N-LDL-c
≥100	384 (64%)	320 (53.33%)	362 (60.33%)
≥130	189 (31.5%)	130 (21.6%)	151 (25.16%)
≥160	73 (12.16%)	34 (5.66%)	44 (7.33%)

[Table/Fig-2]: Risk stratification of patients on the basis of LDL- c values by NCEP-ATP-III guidelines.

The strength of association between direct and calculated LDL-c values were analysed by using Pearson's correlation. [Table/Fig-3] shows the significant positive correlation between D-LDL-c vs. F-LDL-c (r=0.96; p=<0.001) and D-LDL-c vs. N-LDL-c (r=0.97; p=<0.001). [Table/Fig-4,5] shows, the scatter plots between D-LDL-c and calculated methods of LDL-c estimation.

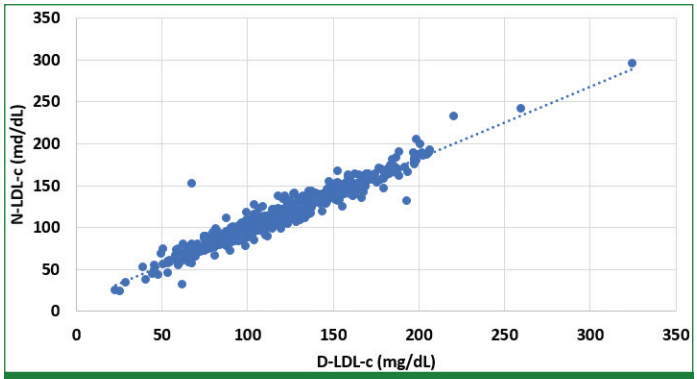
Pair	r-value	p-value
D-LDL-c vs F-LDL-c	0.96	<0.001**
D-LDL-c vs N-LDL-c	0.97	<0.001**

[Table/Fig-3]: Pearson's correlation between D-LDL-c and calculated methods among study subjects (N=600).
**Strongly significant (p-value ≤0.01)



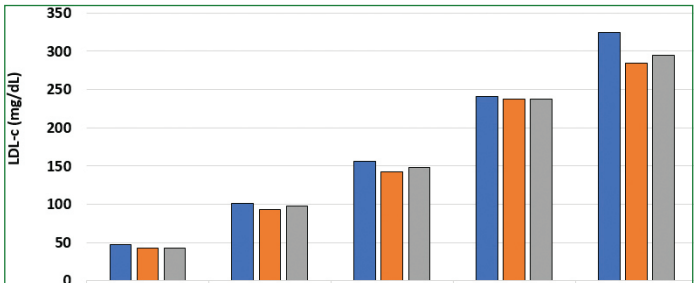
[Table/Fig-4]: Scatter plot between D-LDL-c and F-LDL-c (N=600).

Our study showed underestimation of LDL-c by calculation methods at all the ranges of TC. There was a statistically significant difference



[Table/Fig-5]: Scatter plot between D-LDL-c and N-LDL-c (N=600).

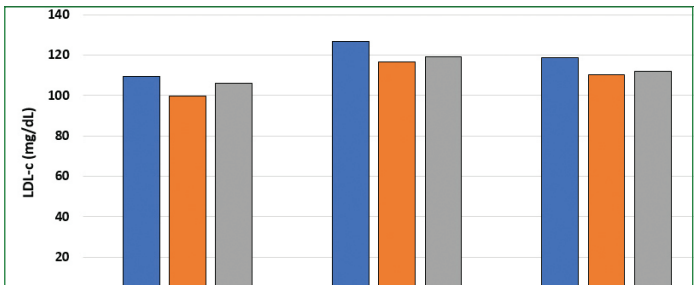
(p≤0.05) between D-LDL-c values vs. F-LDL-c and N-LDL-c values at 100-199 mg/dL and 200-299 mg/dL of TC range as shown in [Table/Fig-6].



TC (mg/dl)	<100	100-199.99	200-299.99	300-399.99	≥400
D-LDL-c	46.91	101.17	156.15	240.5	325
F-LDL-c	43.15	92.72	142.48	237.75	285
N-LDL-c	42.75	97.66	147.65	237.12	295.25

[Table/Fig-6]: Comparison of mean values of LDL-c at different TC range.

[Table/Fig-7] depicts underestimation of LDL-c values as estimated by calculated methods at all the ranges of HDL-c. But a statistically significant difference (p ≤0.05) was witnessed at the HDL-c values of <40 mg/dL and between 40-60 mg/dL for LDL-c values obtained by Friedewald's equation.

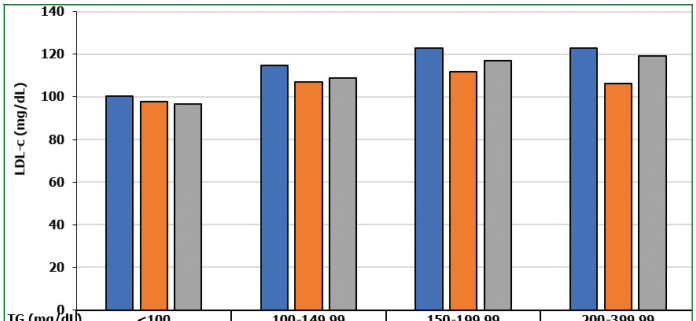


HDL(mg/dL)	<40	40-60	>60
DLDL-c	109.48	126.71	118.6
F-LDL-c	99.75	116.7	110.21
N-LDL-c	105.99	119.37	111.9

[Table/Fig-7]: Comparison of mean values of LDL-c at different HDL-c range.

There was an underestimation of LDL-c by calculated methods at all the ranges of TG. But this difference was statistically significant (p≤0.05) for LDL-c obtained by Friedewald's equation at TG ranges of 100-149 mg/dL, 150-199 mg/dL and 200-399 mg/dL as shown in [Table/Fig-8].

Receiver Operating Characteristic curve (ROC) analysis was done to predict the better diagnostic tool among the calculated methods of LDL-c by taking 100 mg/dL as the cut-off. As seen in [Table/Fig-9,10], it was found that, the Area Under the Curve (AUC) for Friedewald's equation and Novel's equation was 0.974 and 0.985, respectively. Among the calculated methods of LDL-c estimation, the AUC for N-LDL-c was more compared to F-LDL-c. In our study N-LDL-c emerged as a better biomarker towards the prediction of CVD risk.

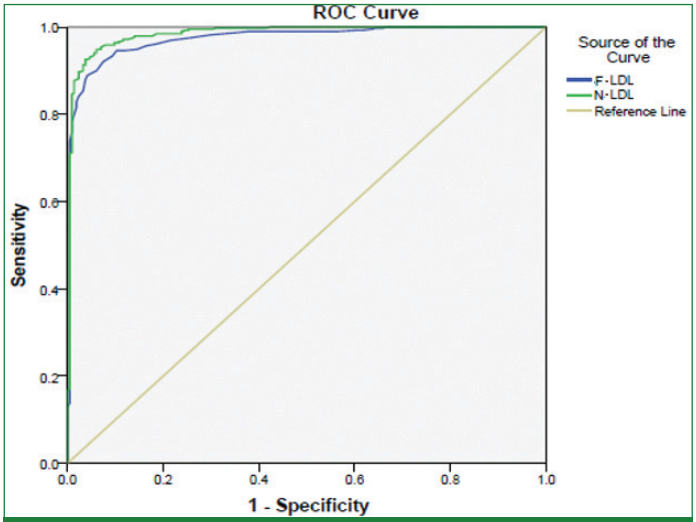


TG (mg/dL)	<100	100-149.99	150-199.99	200-399.99
D-LDL-c	100.24	114.77	123.02	122.93
F-LDL-c	97.91	106.86	111.66	106.13
N-LDL-c	96.75	108.73	116.91	119.29

[Table/Fig-8]: Comparison of mean values of LDL-c at different TG range.

Variables	Sensitivity	Specificity	AUC	SE	p-value
F-LDL	93.73	89.15	0.974	0.011	<0.001**
N-LDL	91.51	92.25	0.985	0.019	<0.001**

[Table/Fig-9]: ROC curve analysis of the different methods of LDL-c estimation. **Strongly significant (p-value ≤0.01)



[Table/Fig-10]: ROC curve analysis for different methods of LDL-c.

DISCUSSION

The CVDs are the diseases of heart and the vascular system. CVD is mainly characterised by impairment of cardiac functioning due to inadequate blood supply and atherosclerotic obstruction in the blood vessels [19]. Atherosclerosis is a progressive inflammatory disorder of the arterial wall which is characterised by focal deposition of lipid-rich atheroma that remain clinically unnoticed till they become large enough to cause tissue perfusion, ulceration and disruption [20].

Increased LDL-c concentration is a well-known atherogenic risk factor. Changing life style and reducing cardiovascular risk factors are well known measures for the prevention of CVD. The LDL-c is a major modifiable cardiovascular risk factor. The concentrations of LDL-c are considered as the primary target towards the diagnosis and treatment of hyperlipidaemic subjects. Hence, monitoring of LDL-c values has a prominent role in the CVD management. CVD risk stratification is dependent on LDL-c values as it enables in commencement of preventive modalities, treatment strategies and monitoring the associated comorbidities at the earliest. The commonest problem encountered in most of the laboratories is the accurate estimation of LDL-c values [9].

The gold standard or the reference method of the LDL-c estimation is beta quantification. It is not used for routine testing at the clinical laboratories as it is expensive, laborious and requires skilled personnel [17]. Hence, to combat these limitations associated with the gold standard method, the direct LDL-c estimation was developed and implemented as an alternative method. The direct method of LDL-c estimation is expensive and time consuming. As a result, many calculated methods were incorporated for the ease of LDL-c calculation considering the values of other lipid profile parameters [13].

Among the calculated methods of LDL-c estimation, Friedewald's equation is commonly used in most of the laboratories inspite of its known limitations. Friedewald's equation is not applicable on non fasting blood samples, when the serum TG values are ≥400 mg/dL and in subjects with the history of type-III hyperlipoproteinemia or dysbetalipoprotenemia. Inter-individual variability is not addressed since a fixed factor of 5 is used as a divisor for TG in Friedewald's equation [17]. In Novel's equation, based on the strata of TG and non HDL-c values, a 180-cell table of strata specific median of TG:VLDL-c values were obtained and applied in validation data for the adjustable factor. The adjustable factor ranging from 3-12 is known to provide accurate LDL-c values than the Friedewald's equation [9].

The current study mainly aimed at the comparison of LDL-c values as estimated by direct enzymatic method with the LDL-c values as estimated by calculated methods using Friedewald's and Novel's equation. In our study, it was observed that, the LDL-c values as calculated by Friedewald's equation and Novel's equation was lower compared to LDL-c values obtained by direct enzymatic method. Friedewald's and Novel's equation lead to approximately 10% and 5% less patients being the candidates for lipid lowering drug therapy which indicates that, when Novel's equation is used instead of Friedewald's equation would result in a small net increase in population eligible for lipid lowering drug therapy. Similar observations were seen in a study done by Shin D et al., among American population, which concluded that, the use of Novel's equation for estimating LDL-c instead of Friedewald's equation could result in a small net increase in the statin eligible population for primary prevention of CVD [7].

The calculated LDL-c values obtained by Friedewald's ($r=0.96$; $p\leq0.001$) and Novel's equation ($r=0.97$; $p<0.001$) showed a very good correlation with the D-LDL-c values. But the calculation methods underestimated LDL-c values compared to direct enzymatic method of LDL-c estimation. This may lead to delay in initiation of lipid lowering drug therapy in high-risk patients. Our results are in concurrence with the studies done by Krishnaveni P and Gowda VMN, and Gupta S et al., in which, Friedewald's and Anandaraja's formula was used for LDL-c calculation. Their study also showed higher D-LDL-c values than that obtained by calculation using the formulas and a statistically significant positive correlation was witnessed between D-LDL-c and calculated methods of LDL-c estimation [6,15].

In our study, it was observed that, F-LDL-c and N-LDL-c values were lower than the D-LDL-c values at different levels of TC, TG and lower ranges of HDL-c. This difference was statistically significant at TC levels of 100-199 mg/dL and 200-299 mg/dL, at HDL-c levels <40 mg/dL and at TG levels of 200-399 mg/dL. Calculated methods underestimated LDL-c at higher ranges of TC, TG and lower levels of HDL-c which may lead to delay in initiation of therapeutical intervention in high-risk patients. Similar observations were made in a study conducted by Kannan S et al., where they observed that, Friedewald's equation underestimated LDL-c at higher levels of TG [17].

The ROC curve analysis of our study showed, the AUC was maximum for N-LDL-c (0.985) than F-LDL-c (0.974) at a cut-off value of 100 mg/dL of LDL-c. This signifies that, Novel's equation would enable improved accuracy for calculation of LDL-c values compared to Friedewald's equation. Similar findings were observed in a study conducted on Ghanaian population by Ephraim RKD et al., in which the AUC for N-LDL-c was more compared to F-LDL-c [18].

Limitation(s)

The limitations associated with the study are, small sample size and the fact that the gold standard method of LDL-c estimation i.e.,

β-quantification method was not used as the reference method. The validity of Novel's equation in patients with lipid lowering therapy and TG ≥400 mg/dL needs to be evaluated further.

CONCLUSION(S)

To conclude, the use of Novel's equation for LDL-c estimation instead of Friedewald's equation could be associated with the small net increase in lipid lowering agent eligible population for primary prevention of atherosclerotic CVD. Replacement of Friedewald's equation by Novel's equation would enable for the improved accuracy of LDL-c estimation especially at higher levels of TC, TG and lower levels of HDL-c. Novel's equation yielded a better diagnostic accuracy compared to F-LDL-c and thus could serve as a substitute for D-LDL-c estimation especially at higher levels of TC and TG in the routine clinical practice for the better risk stratification for CVD.

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