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Comparison of Friedewald's Formula and Martin's Method with Direct Assay of Serum LDL-Cholesterol

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Abstract: Serum levels of low-density lipoprotein cholesterol (LDL-C) dictate not only diagnosis but also risk classification and treatment of cardiovascular diseases. Owing to the cost-effectiveness, Friedewald formula (FF) is primarily preferred and adopted method to assess serum LDL-C levels especially when triglycerides (TG) <400 mg/dL. FF employs a fixed factor of 5 for the ratio of TG to very low density lipoprotein cholesterol (TG:VLDL-C). Martin *et. al.,* in their recent study proposed an adjustable factor using N-strata-specific median TG:VLDL-C ratios. They constructed 180-cell table based on non-High-density lipoprotein cholesterol (non-HDL-C) and TG levels. In the present cross-sectional study, the performance of LDL-C measurements based on FF (LDL-C_F) and novel method (LDL-C_N) in comparison to direct homogenous assay (LDL-C_D) was evaluated. A total of 480 lipid profile data with serum TG <400 mg/dL were procured. The efficacy of both LDL-C_F and LDL-C_N measurements were evaluated under broader as well as in constricted ranges of TG. The entire lipid profile data was pooled under Group I comprising broader range of TG i.e., TG <400 mg/dL. The data of Group I were further stratified into subsequent three groups with constricted range of TG. Hence, Group II (TG<100 mg/dL), Group III (TG=100-199 mg/dL), Group IV (TG=200-399 mg/dL) comprised 153, 170 and 147 samples, respectively. Bland-Altman (B&A) and Lin's concordance correlation coefficient (CCC, ρ_c) analysis were used as statistical tools. Difference plot of B&A analysis indicated narrow 95% limits of agreement (LOA) as bias±2 standard deviations with low percentage error for LDL-C_N in contrast to LDL-C_F at all ranges of TG. Although both formula based methods were in substantial strength-of-agreement with LDL-C_D yet LDL-C_N expressed marginally higher ρ_c (95%CI) with precision and accuracy in all Groups. Hence, in corroboration with earlier studies, our study also further supports the efficacy of LDL-C_N in comparison to LDL-C_F.
Keywords: Friedewald's formula (FF), Total cholesterol (TC), Triglyceride (TG), Low-density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C).

INTRODUCTION

Low-density lipoprotein cholesterol (LDL-C) is a longstanding independent modifiable risk factor for cardiovascular disease [1]. It is the primary target for diagnosis, risk classification and treatment of cardiovascular disease in both national and international clinical practice guidelines [2]. Therefore, the precision and accuracy of serum LDL-C estimation is of utmost clinical relevance. Beta (β) quantification based on ultracentrifugation technique is the gold standard/ reference method yielding the precise and accurate values [3]. However, apart from costly instrumentation; tedious and time consuming analytical process with the requirement of large sample volumes imposed a limitation for its establishment as a routine diagnostic tool. Hence, there exists the necessity for alternative simple and cost effective method for the quantification of serum LDL-C.

Homogeneous assays emerged as one of the promising and feasible alternative for direct LDL-C (LDL-C_D) estimation [4,5]. Among the conventional lipid profile parameters, relatively costlier LDL-C_D assay widens the financial burden on patient. Hence, its establishment as a routine lipid profile parameter in most of the Indian laboratories remains ambiguous. Most of these clinical laboratories adopted the Friedewald equation obviating the need of expensive ultracentrifuge dependent β-quantification and homogenous assays. Friedewald proposed (1972) formula estimates LDL-C as total cholesterol (TC) minus high density lipoprotein cholesterol (HDL-C) minus triglycerides (TG)/5 in milligrams per deciliter (mg/dL) [6]. The utility of this formula is not

recommended for hypertriglyceridemia, type III hyperlipidemia, low LDL-C concentration and secondary hyperlipidemias (observed in type II diabetes mellitus, nephrotic syndrome, chronic alcoholics and patients on hormone replacement therapy) [7,8].

Friedewald's formula (FF) was built on an assumption that the ratio of TG:VLDL-C remains constant as 5:1 under fasting conditions. Undoubtedly this ratio is influenced with the presence of chylomicrons and chylomicron remnant in non-fasting samples. Hence, fasting sample is mandatory for Friedewald LDL-C (LDL-C_F) estimation. Moreover, fixed factor of 5 for every individual even in fasting conditions compromising the variance in the TG:VLDL-C across the range of TG and non-HDL-C might be prone to erroneous values. In majority of these reports, the interindividual variance in the TG: VLDL-C ratio was not addressed. In one of the recent study, Martin *et. al.*, proposed an adjustable factor derived as N-strata-specific median TG: VLDL-C ratio based on TG and non-HDL-C to measure the LDL-C (LDL-C_N) [9]. They built 180-cell table using a large cohort of United States patients. The implementation of adjustable factor demonstrated the accommodation of interindividual variance.

However, before contemplating any novel method to routine clinical use, verification and validation using other laboratory techniques and in independent population comprising various races is mandatory. Based on the available literature [10], this is the first validation study of Martin's method on Indian population. In view of the above facts, this study was undertaken as an effort to evaluate the performance of LDL-C_N and LDL-C_F in comparison to LDL-C_D.

MATERIALS AND METHODS

A cross-sectional study was conducted from January 2017 to June 2017 in the clinical biochemistry laboratory, Karpagam Faculty of Medical Sciences & Research, Coimbatore. Institutional ethics committee clearance was obtained. Only the subjects with age >18 years were recruited in this study. In one of the earlier studies, correlation coefficient r = 0.976 was reported between LDL-C_D and LDL-C_F [11]. In order to achieve similar result with 90% power, 95% confidence level and population coefficient as 0.96; a minimum sample size of 159 was estimated. In the present study, a total of 480 lipid profile data records were procured from the patient's whose laboratory investigations involved lipid profile analysis. Demographic data was extracted from each subject prior to the blood sample collection. Fasting venous blood samples from the antecubital vein were drawn in tubes without anticoagulant. The samples were allowed to clot at room temperature. Subsequently they were centrifuged at 3000 rpm for 15 minutes and then serum was separated. Complete lipid profile analysis comprised estimation of TC, TG, HDL-C, and LDL-C_D. Serum concentrations of the lipid profile parameters were analyzed on EM 360 Clinical Chemistry Analyzer, (TransAsia Bio-Medicals Ltd, Mumbai, 400 072) using Erba Mannheim XL System Packs. The entire process of sample collection, processing and analysis were strictly carried out under aseptic conditions as per standard laboratory protocols. After acquiring the lipid profile report of each participant, non-HDL-C and formula based LDL-C was calculated. Non-HDL-C was derived by subtracting HDL-C from TC. LDL-C using FF (LDL-C_F) was calculated as [non-HDL-C]-[TG/5] [6]. And 180-cell based novel method (LDL-C_N) was computed as [non-HDL-C]-[TG/AF] where AF is an adjustable factor extracted from 180-cell table constructed by Martin *et al.* [9].

The exclusion criteria comprise patients diagnosed with cancer, myocardial infarction, stroke, heart failure and on lipid lowering drugs. The participants who were pregnant or possibility of being pregnant on the day of registration was also not included.

STATISTICAL ANALYSIS OF DATA

The lipid profile data procured in the present analysis were analyzed using Microsoft Excel sheet 2016, Statistical Package for Social Science (SPSS) version 24 software (Chicago, IL, USA) and MedCalc software Trial version 18 (MedCalc, Ostend, Belgium). Normal distribution of the data was analyzed using Kolmogorov-Smirnov with Lilliefors significance correction, skewness and kurtosis. Normally distributed continuous variables were presented as means with standard deviation. Not normally distributed variables were described as a median with an interquartile range. Categorical variables were reported as observed numbers and percentages. Pearson's correlation (ρ) analysis was performed only to assess the linearity between the LDL-C_D and formulae based estimates. Bland-Altman (B&A) plot and Lin's concordance correlation coefficient (CCC, ρ_c) analysis were employed as statistical tools to evaluate the performance of the calculated formulae in comparison to LDL-C_D. B&A plot analysis was utilized to calculate systematic errors (bias), and 95% limits of agreement (LOA) as bias ±2 standard deviations [12,13]. Normality of the difference plot was computed using Shapiro-Wilk (SW) test. Original data were subjected to square root transformation if difference plot was not normally distributed and back transformed to original values in B&A plots. The percentage error (PE) was computed dividing the limits of agreement of each B&A plot by mean value of the LDL-C_D [13]. Lin's CCC was used to quantify the strength-of-agreement with 95% Confidence Interval (95%CI) between the values derived from formulae and D-LDL-C estimations [14,15]. It not only quantifies closeness of observations on the regression line i.e., precision (Pearson's correlation, ρ), but also closeness of regression line to the 45^0 line-of-identity through origin

(Bias correction factor, C_b). Strength-of-agreement of CCC was characterized using McBride guidelines [16].

RESULTS

The general characteristic features of the subjects involved in our method comparison study were presented in Table 1. A total of 480 lipid profile records were procured from the active participants. The median age was 48 years with an interquartile range of 40 – 55 years. Among them males comprised 268 (55.8%) and females were 212 (44.2%). The body mass index was 24.8 ± 4.1 kg/m^2. TC ranged from 152 – 258 mg/dL with a mean of 205 mg/dL. The computed median with an interquartile range of TG and HDL-C were 95 mg/dL, 141 – 211 mg/dL and 38 mg/dL, 32 – 45 mg/dL; respectively. LDL-C_D exhibited a range of 86 – 180 mg/dL with a mean of 133 mg/dL. As apparent from Figure 1, both LDL-C_F and LDL-C_N showed statistically significant linear relationship with LDL-C_D with a Pearson correlation coefficient (r) of 0.986 and 0.990, respectively.

Table-1: General characteristics of study participant (480)

Variables	Total (n=480)
Age, years	48 [40 – 55]
Sex, n(%)	
Male	268 (55.8%)
Female	212 (44.2%)
Body mass index (BMI), Kg/m^2	24.8 ± 4.1
Total cholesterol (TC), mg/dL	205 ± 53
Triglycerides (TG), mg/dL	95 [141 – 211]
High Density Lipoprotein-Cholesterol (HDL-C), mg/dL	38 [32 – 45]
Low Density Lipoprotein-Cholesterol (LDL-C), mg/dL	
Directly-measured (LDL-C_D), mg/dL	133 ± 47
Friedewald (LDL-C_F), mg/dL	134 ± 49
180-cell based novel method (LDL-C_N), mg/dL	137 ± 48

Normally distributed data was expressed as mean ± standard deviation; non-normal distributed data was expressed as median [interquartile range] and number (percent) for categorical variables.

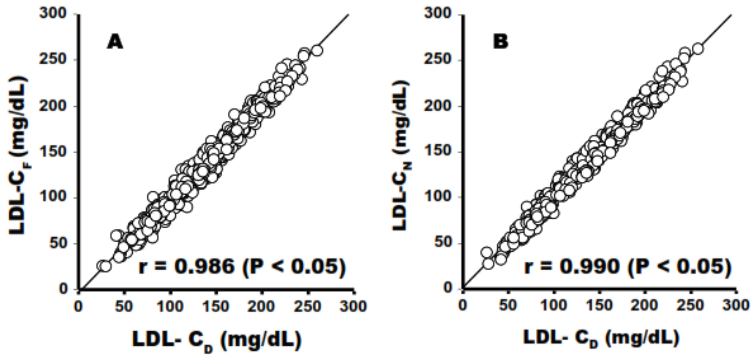

Fig-1: Scattered plot: A. LDL-C_F vs LDL-C_D; and B. LDL-C_N vs LDL-C_D

The entire lipid profile data was segregated into four groups based on TG levels: Group I (TG <400 mg/dL), Group II (TG < 100 mg/dL), Group III (TG = 100 – 199 mg/dL), and Group IV (TG = 200 – 399 mg/dL) with a sample size of 480, 153, 170 and 147, respectively. The details of each "Group" were provided in Table 2. As a conventional approach (apparent from most of the earlier studies), first entire lipid profile data was pooled and analyzed under Group I. The same pooled data of Group I was further segregated into three groups (Group II, III & IV) with an objective to understand whether LDL-C_F and LDL-C_N measurements against LDL-C_D in constricted ranges of TG were in accordance with their respective statistical outputs of Group I. In the first step of analysis, to examine the differences between the formulae based measurements against LDL-C_D of each "Group", B&A plots were constructed.

Table-2: Details of segregated lipid profile data into groups based on triglyceride strata

Group (TG levels)	Sample size	LDL-C estimates	Mean ± Standard deviation
Group I (<400 mg/dL)	480	LDL-C_D	133 ± 47
		LDL-C_F	134 ± 49
		LDL-C_N	137 ± 48
Group II (<100 mg/dL)	153	LDL-C_D	130 ± 52
		LDL-C_F	132 ± 52
		LDL-C_N	130 ± 52
Group III (100 – 199 mg/dL)	170	LDL-C_D	135 ± 52
		LDL-C_F	138 ± 57
		LDL-C_N	140 ± 55
Group IV (200 – 399 mg/dL)	157	LDL-C_D	135 ± 35
		LDL-C_F	131 ± 36
		LDL-C_N	139 ± 34

B&A plot provided the bias and 95% LOA with PE between the direct and formula based methods of LDL-C. In B&A analysis of entire pooled data (Group I), both the formulas underestimated the LDL-C. LDL-C_F expressed lowest negative bias of -0.5 and LOA ranging from -16.5 to 15.6 with a PE of 24.0% (Figure 2A). Whereas, LDL-C_N had -3.5 bias, -16.9 to 9.9 LOA and 20.0% as PE (Figure 2B). Although LDL-C_F expressed relatively lowest negative bias but the LOA with PE were minimal in LDL-C_N. At the low end of TG range (Group II), among the scattered plots of both methods (Figure 2C & 2D), LDL-C_F showed protracted negative bias (-1.7), LOA (-11.4 to 8.0) and PE (14.8%) in comparison to LDL-C_N (bias: -0.4; LOA: -9.4 to 8.5 and PE: 13.7%). In the mid-range of TG i.e., Group III analysis revealed features analogous to Group I. LDL-C_F had a relatively lower bias of -3.3 but broader LOA i.e., -20.6 to 13.9 and increased PE of 25.5% (Figure 2E). LDL-C_N indicated -5.1 bias, -19.7 to 9.6 LOA and 21.7% as PE (Figure 2F). As evident from Figure 2E, most of the differences were above the line of equality especially when LDL-C levels were < 100 mg/dL. Thereafter (LDL-C > 100 mg/dL) there was gradual and intense drifting of underestimations as a negative trend. Though the negative trend was apparent in LDL-C_N vs LDL-C_D plots (Figure 2F) but there was predominance of underestimation across the X-axis. In the subsequent analysis i.e., at the high end of the TG range (Group IV) both methods showed contrasting features (Figure 2G & 2H). LDL-C_N in lines of earlier groups underestimated with a negative bias of -4.8 whereas LDL-C_F overestimated with a positive bias of 3.9. Even in this range also, LOA (-18.5 to 9.0) and PE (19.9%) of LDL-C_N remained confined in comparison to LDL-C_F (LOA: -12.5 to 20.3 & PE: 24.2%). The normal distribution of difference plot in B&A analysis between formula based estimations and LDL-C_D with respect to each "Group" was presented in Figure 3 (A-H). In our analysis, Shapiro-Wilk (SW) test with p value > 0.05 was considered as normal distribution plot. Accumulating data of evidence demonstrated the efficiency of B&A plots only in quantifying LOA (95% of the differences between formula based methods and LDL-C_D) with bias and percentage error but not in assessing degree of concordance [12,13]. In view of that, as a second step of analysis, Lin's CCC analysis of each group was carried out to quantify the degree of agreement between formula based method and LDL-C_N [14-16].

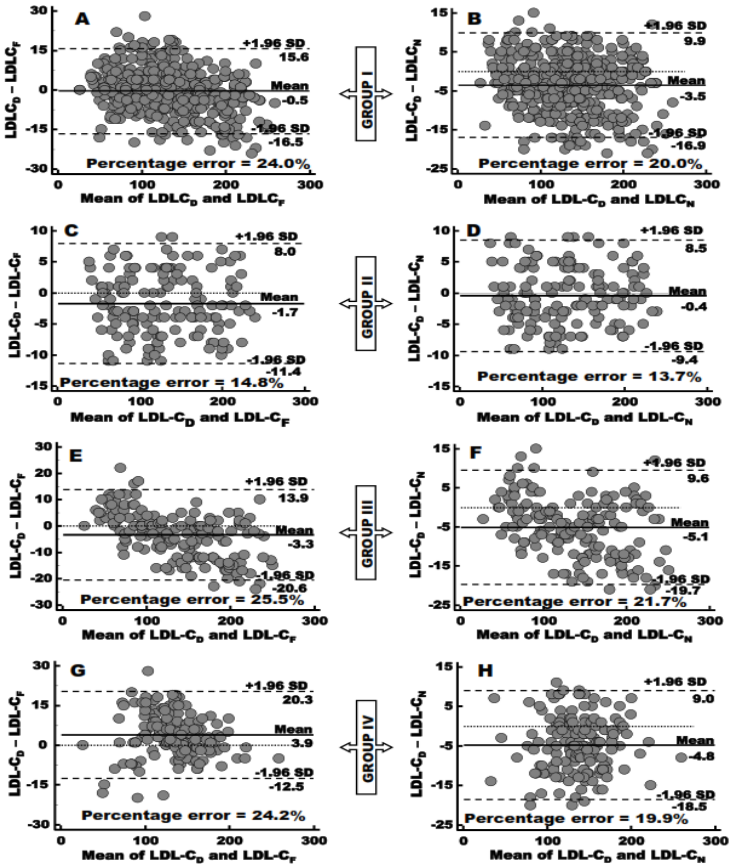

Fig-2: Bland-Altman plots of Friedewald's formula (LDL-C$_F$) and Novel method (LDL-C$_N$) against Direct (LDL-C$_D$) assay of each group. Measurements of both X- and Y-axis in all plots were carried out in mg/dL.

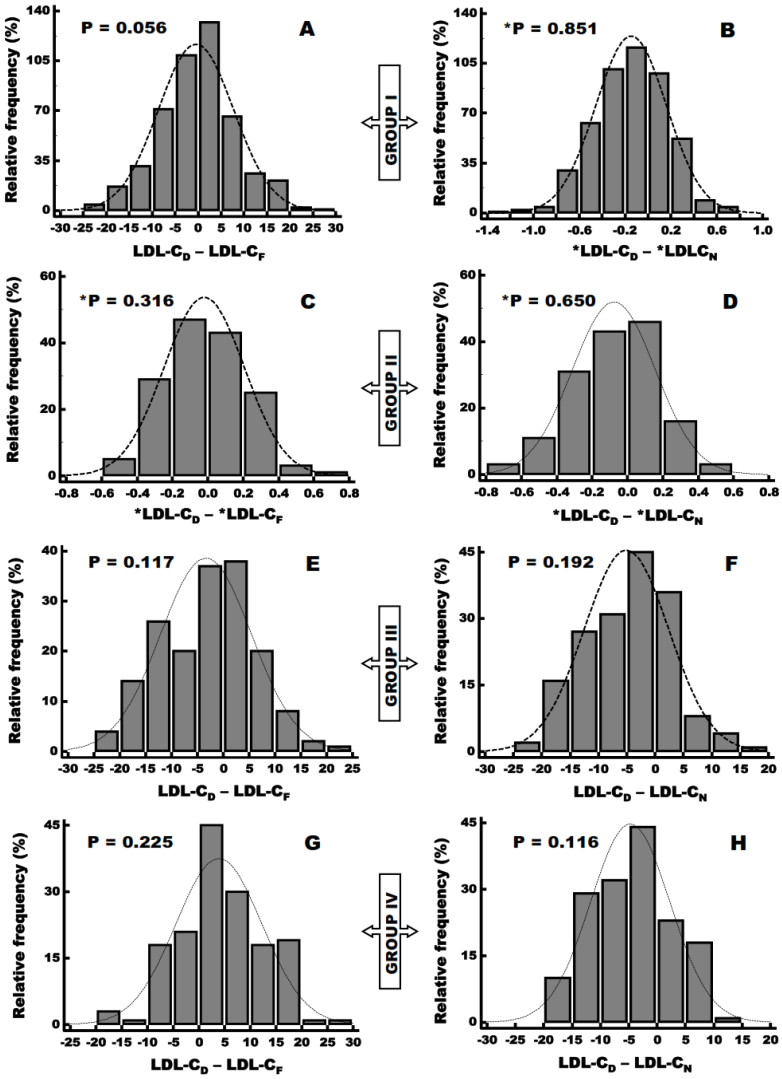

Fig-3: Distribution plot of differences between FF (LDL-C$_F$) and Novel method (LDL-C$_N$) against Direct (LDL-C$_D$) assay of each group. Dotted line represents normal distribution. P value in each plot was computed from Shapiro-Wilk test. *indicates square root transformed original data of respective LDL-C measurements

Lin's CCC (ρ_c) analysis not only elucidated the degree of concordance with 95% CI but also estimated precision (ρ) and accuracy (C_b) of agreement between two measurements. Scattered plot of formulae based measurements (LDL-C_F & LDL-C_N) against LDL-C_D of each "Group" were presented in Figure 4 (A-H). The computed measurements of the respective scattered plot of each "Group" in terms of ρ_c (95% CI), ρ and C_b were tabulated in Table 3. In our analysis, both LDL-C_F and LDL-C_N measurements revealed substantial strength-of-agreement with LDL-C_D estimations in all groups (Group I-IV). Even in CCC analysis not only the relative dominance of LDL-C_N measurements in each group was evident but also had highest ρ_c (0.9961; 0.9947-0.9972) with ρ (0.9962) and C_b (1.0000) when TG<100mg/dL. The impact of increasing TG concentration (Group II-IV) with gradual declination of ρ_c in both formula based methods was also apparent despite of their substantial strength-of-agreement with LDL-C_D. Our observations were in lines of earlier studies [17-20]. Among the formula based methods, based on the output of CCC analysis, LDL-C_N exhibits relative dominance in terms of ρ_c estimations with precision and accuracy.

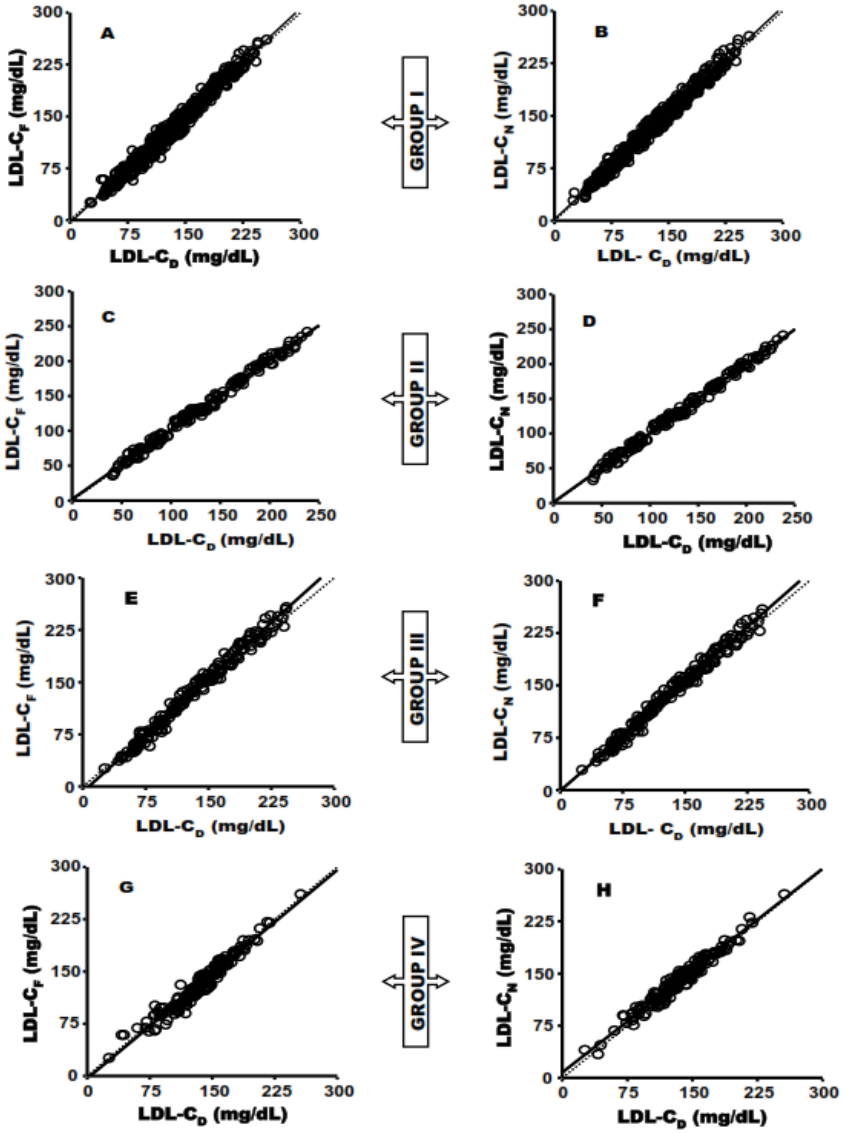

Fig-4: Scattered plot: Lin's concordance coefficient of Friedewald's formula (LDL-C_F) and Novel method (LDL-C_N) against Direct (LDL-C_D) assay of each group. Dotted lines represent 45^0 line-of-equality and thick line indicates regression line of LDL-C measurements

Table-3: Details of Lin's concordance correlation coefficient measurements

Group	Scatter plot (Figure 4)	CCC (95% CI)	Precision (ρ)	Accuracy (C_b)
Group I	A. LDL-C_F Vs LDL-C_D	0.9857 (0.9831 – 0.9880)	0.9868	0.9989
	B. LDL-C_N Vs LDL-C_D	0.9873 (0.9849 – 0.9893)	0.9902	0.9971
Group II	C. LDL-C_F Vs LDL-C_D	0.9951 (0.9932 – 0.9964)	0.9956	0.9995
	D. LDL-C_N Vs LDL-C_D	0.9961 (0.9947 – 0.9972)	0.9962	1.0000
Group III	E. LDL-C_F Vs LDL-C_D	0.9855 (0.9813 – 0.9888)	0.9911	0.9943
	F. LDL-C_N Vs LDL-C_D	0.9861 (0.9816 – 0.9895)	0.9916	0.9944
Group IV	G. LDL-C_F Vs LDL-C_D	0.9666 (0.9548 – 0.9754)	0.9727	0.9937
	H. LDL-C_N Vs LDL-C_D	0.9710 (0.9609 – 0.9786)	0.9800	0.9908

CCC (95% CI): Lin's Concordance Correlation Coefficient (95% Confidence Interval).

DISCUSSION

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) strongly recommended serum LDL-C levels <100 mg/dL as optimal [1,2]. The popularity of LDL-C as strong atherogenic marker insists its accurate estimation [1]. Especially in developing countries like India, FF has well established as routine lipid profile parameter in clinical practice as well as in health screenings [6,10]. Several modified formulae were proposed periodically as an improved alternative. The ambiguity raised from evaluation studies of these modified FF hindered their

evolution as a potential alternative for FF [17]. However, as already discussed in the previous sections, Martin method was demonstrated to possess inherent attributes to be a strong alternative for FF. Martin's method i.e., LDL-C_N was calculated using the N-strata-specific median TG:VLDL ratios based on non-HDL-C and TG in 180-cell table [9]. Therefore, in our cross sectional study, we compared LDL-C_F and LDL-C_N against LDL-C_D in our tertiary health care setup.

It was evident from our B&A analysis, interpretations concluded from difference plot of Group I (Figure 2A & 2B) could be presumed as cumulative effect of their respective plots in the remaining three groups (Group II – IV). FF mostly underestimated LDL-C except at the higher end of TG (TG = 200-399 mg/dL). On the other hand, novel method underestimated LDL-C in both broader and constricted ranges of TG. B&A plot evidenced much precise and narrow confidence limits with low PE for LDL-C_N vs LDL-C_D in comparison to LDL-C_F vs LDL-C_D in each group. Even Lin' CCC interpretations were also in the same lines. Hence LDL-C_N measurements were more reliable in comparison to FF. Narrowest LOA with minimal bias and lowest PE in B&A plot and highest degree of concordance with 95% CI in CCC (ρ_c) of LDL-C_N vs LDL-C_D in Group II indicated the best performance of novel method when TG <100 mg/dL. Barring the negative drift influenced B&A plot of either method in Group III (TG = 100 – 199 mg/dL), the impact of increasing TG on the measurements of LDL-C_F and LDL-C_N was also apparent in our comparative study analysis. All these observations were in corroboration with earlier studies [17-20]. Hence, in our cross-sectional study with limited sample size, 180-cell based novel method with marginal outperformance exhibited the feasibility as an improved alternative cost-effective tool in comparison to FF to measure LDL-C.

The limitation of our study comprises: β-quantification of LDL-C is not used as gold standard method, lack of considering influence of co-morbidities of each subject on LDL-C_D estimations and a bias in the participant selection based on exclusion criteria.

CONCLUSION

In our comparative study, intragroup analysis of B&A plots indicated predominance of relatively precise and narrow 95% confidence LOA as bias ±2 standard deviations and low percentage error in scattered plots of LDL-C_N Vs LDL-C_D only. Even relatively higher CCC (95% CI) of LDL-C_N Vs LDL-C_D analysis in each group, despite of existence of substantial strength-of-agreement between formula based method and direct assay, further substantiated reliability of novel method's measurements. Moreover, both B&A and CCC (95% CI) analysis also evidenced minimally influenced LDL-C_F and LDL-C_N measurements when TG <100 mg/dL. Among them, novel method not only exhibited narrowest 95% confidence LOA as bias ±2 standard deviations with lowest percentage error in B&A analysis but also had highest CCC (95% CI) with precision and accuracy. This feature remained unique in comparison to statistical outputs of novel method analysis in other ranges of TG. Hence, novel method outperforms when TG <100 mg/dL in comparison to remaining ranges of TG. The impact of increasing TG on the LDL-C measurements was evident in both statistical analyses (B&A and CCC analysis) of either method (FF and novel method) even in our study except for the negative drift influenced B&A plots of Group III (TG = 100 – 199 mg/dL). Hence our study also further supports the efficacy of 180-cell based novel method as a feasible alternative for FF in LDL-C measurements. However, further studies are warranted with increased sample size and robust statistical tools under different conditions to evaluate the performance of Martin's method in broader as well as constricted ranges of TG.

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