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Evaluation and Importance of Different Formulas for Low Density Lipoprotein Cholesterol Calculation

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Abstract: Dyslipidemia is one of the major modifiable risk factor. Low density lipoprotein cholesterol (LDL-C) acts as the primary lipid agent for CAD risk prediction and therapeutic target, emphasizing the importance of accuracy and precision of LDL-C estimation. The aim of this study is to compare the results obtained by direct homogenous assay for LDL-C to those obtained by Friedewald's formula and Anandraja's formula in north Indian population catered to our Hospital and their correlation. Lipid profile reports of 500 patients above 18 years (TG<400mg/dl) were anlaysed. LDL-C estimation was done by homogenous assay and also calculated using the Friedewald's Formula and Anandaraja's Formula along with their correlation studies. According to Friedwald formula and Anandraja's formula 9.8% and 6.4% patients were classified under low risk category respectively but showed a significant positive correlation of 0.95 and 0.91 between direct LDL and FFLDL and AFLDL. Calculated LDL-C results obtained by Friedewald's and Anandaraja's formulas show very good correlation with the measured LDL-C but underestimate risk of heart disease when compared to direct LDL cholesterol. LDL-C is considered as the primary basis for diagnosis, treatment and risk classification of patients with hyperlipidemia and it is imperative to validate all these formulas normal healthy and diseased in large populations for a definitive concluding remark.
Keywords: Friedewald's Formula, Anandaraja's Formula, Low density lipoprotein cholesterol

INTRODUCTION

Coronary heart disease (CHD) one of the cardiovascular disease is epidemic in India. 23% of total and 32% of adult deaths in 2010-2013 are due to CHD which has been reported by The Registrar General of India. The World Health Organization (WHO) and Global Burden of Disease Study also have highlighted increasing trends in years of life lost (YLLs) and disability-adjusted life years (DALYs) from CHD in India. CHD prevalence has increased over the last 60 years, from 1% to 9%-10% in urban populations reflecting its increased prevalence. The important risk factors for CHD are dyslipidemias, smoking, diabetes, hypertension, abdominal obesity, psychosocial stress, unhealthy diet, and physical inactivity. Hence, suitable preventive strategies are required to combat this epidemic [1].

Dyslipidemia is one of the major modifiable risk factor. The National Cholesterol Education Programme's (NCEP) Adult Treatment Panel III (ATP III) recommended low density lipoprotein cholesterol (LDL-C) as the primary lipid agent for CAD risk prediction and therapeutic target, emphasizing the importance of accuracy and precision of LDL-C estimation [2].

A strong positive correlation between increased LDL-C and CHD has been well documented from various epidemiological and clinical studies [3–6]. The reference method for determining LDL-C is b-quantification [7]. It requires ultracentrifugation, uses large volumes of samples and is a time consuming and expensive technique. Therefore, this method is not suitable for routine laboratory testing [8].

In 1972, Friedwald et al. published a landmark report describing a formula to estimate LDL-C as an alternative to tedious ultra-centrifugation. The formula is LDLC=TC-HDLC-TG/5

Because VLDL (very low-density lipoprotein) carries most of the circulating triglycerides (TG), VLDL-C can be estimated reasonably well from the

measured TG divided by 5 for mg/dl units. LDL-C is then calculated as total cholesterol (TC) minus high density lipoprotein cholesterol (HDL-C) minus estimated VLDL-C [9].

Although this estimation formula correlates highly with beta quantification, it has certain limitations: it is not valid for samples with chylomicrons, with TG>400 mg/dl or in patients with dysbetalipoprotenemia. This formula assumes the ratio of total TG to VLDL-C to be constant in all samples. The formula will overestimate VLDL-C and underestimate LDL-C as a consequence if TG rich chylomicrons and chylomicron remnants are present in the serum sample (hence the requirement for a fasting sample) [10].

The use of this formula is not recommended for type 2 diabetes, nephrotic syndrome and chronic alcoholic patients because accompanying abnormalities in lipoprotein composition render the underlying assumptions invalid for assessment of cardiovascular risk in these patients and thus leading to erroneous results even when TG levels are between 200 and 400 mg/dl [11]. The NCEP working group on lipoprotein measurements has recommended that the LDL-C concentration be determined with a total analytical error not exceeding ±12% (≤4% imprecision and ≤4% inaccuracy) to guarantee correct patient classification into NCEP risk categories [12]. It is difficult to obtain this analytical quality with Friedewald's formula (FF) because each component's analytical error is added [7].

Homogenous assays, developed in 1998 in an effort to overcome the limitations existing with both beta quantification and the Friedewald formula, represent the third generation of LDL-C measurements [13]. These homogenous direct methods use various physicochemical combinations of surfactants, polymeric complexes, and specific binding molecules to selectively measure cholesterol from LDL fraction [14]. But these methods are not routinely used in most of the Indian laboratories as they are expensive which increase the cost of lipid profile estimation. Moreover, many studies done to compare the direct methods with FF have shown to give the results comparable to the Friedwald calculation [15-17].

Many modifications of FF have also been reported, claiming better accuracy and precision than FF [18, 19].

To overcome these limitations, several modifications in this formula have been suggested. Anandaraja *et al*., [19] formula LDLC = 0.9TC -0.9 TG/5 -28. In this formula only TC and TG were used. However, they have not included serum having TG > 350mg/dl. The new formula appeared to be more accurate than Friedewald's formula in Indian population. However, Shalini *et al*., [20] reported that Friedewald's (FF) formula was better in agreement with measured LDLC(Direct homogeneous method) than Anandaraja's formula in Indian subjects. Interestingly, this new formula was found to be working well in Brazilian [21] and Greek population [22].

This study was aimed to compare two different calculated methods (FF and Anandaraja formula) with direct homogeneous assay to assess their validity, suggest most precise, accurate and suitable method for LDL-C estimation in clinical labs and to assess whether different methods affect the classification of patients for CAD risk.

In spite of the technical disadvantages of FF, it is difficult to displace it from clinical practice unless a method with clear advantages in performance and overall cost effectiveness is developed. The aim of this study is to compare the results obtained by direct homogenous assay for LDL-C to those obtained by Friedewald's formula and Anandraja's formula in north Indian population catered to our Hospital.

MATERIALS AND METHODS

This was a comparative study for the estimation of LDL-C using two different formulas and direct estimation by a homogenous assay. Data was collected for the lipid profile samples received in the lab of a tertiary care hospital VMMC and SJH and included patients of at least 18 years of age. Lipid profiles with TG >400 mg/dl were also excluded. The serum samples were obtained by withdrawing 3 ml of venous blood after 10–12 h of overnight fasting and collected in plain vials. The serum was separated by centrifugation and analyzed on ADVIA 2400 autoanalyser.

Serum total cholesterol (TC) was measured by enzymatic endpoint method with a coefficient of variation (CV) of 3.1%. Serum triglyceride (TG) was measured by enzymatic method with a CV of 3.6%. Serum high density lipoprotein cholesterol (HDL-C) was measured by direct homogeneous assay with a CV of 5.6%. Serum low density lipoprotein cholesterol (LDL-C) was measured by direct homogeneous assay with a CV of 4.9%. All biochemical lipid analysis was done on ADVIA 2400 chemistry auto analyzer by using ADVIA Chemistry, Siemens Kits. LDL-C levels were also calculated by Friedewald's formula (FF); LDL-C = TC - (HDL-C +TG/5). TC, TG, LDLC, HDLC were measured enzymatically by enzymatic methods using reagent kits obtained from SIEMENS.

The Triglycerides (TRIG) estimation method was based on the Fossati three-step enzymatic reaction with a Trinder endpoint. The triglycerides are converted to glycerol and free fatty acids by lipase. The glycerol is then converted to glycerol-3-phosphate by glycerol kinase followed by its conversion by glycerol-3-phosphate-oxidase to hydrogen peroxide. A colored

complex is formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The absorbance of the complex is measured as an endpoint reaction at 505/694 nm.

The Cholesterol_2 (CHOL_2) method is based on an enzymatic method using cholesterol esterase and cholesterol oxidase conversion followed by a Trinder endpoint. The cholesterol esters are hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. The cholesterol is converted to cholest-4-en-3-one by cholesterol oxidase in the presence of oxygen to form hydrogen peroxide. A colored complex is formed from hydrogen peroxide, 4-aminoantipyrine and phenol under the catalytic influence of peroxidase. The absorbance of the complex is measured as an endpoint reaction at 505/694 nm.

The LDL Cholesterol Direct (DLDL) method measures LDL cholesterol in serum and plasma. The first step of the reaction eliminates cholesterol associated with lipoproteins other than low-density lipoprotein. A selective surfactant releases cholesterol preferentially from non-LDL particles. Hydrogen peroxide produced by cholesterol esterase and cholesterol oxidase in the first step is eliminated by catalase. Another surfactant releases cholesterol from the low-density lipoprotein. Azide inhibits the catalase. Hydrogen peroxide generated by cholesterol esterase and cholesterol oxidase is quantified using a Trinder endpoint.

The Direct-HDL Cholesterol (D-HDL) method measures HDL cholesterol in serum and plasma without prior separation, based on procedures developed by Izawa, Okada, and Matsui.1 Cholesterol from non-HDL particles is released and eliminated in the first step of the reaction. Cholesterol in HDL particles is released in the second step by detergent and the HDL cholesterol measured by a Trinder reaction

TG and TC was calibrated using general chemistry calibrator provided by SIEMENS. Lypocheck assayed chemistry control (LOT: 26401) Level 1 and (LOT: 26402) 2 control sera (BIORAD) were used as quality control for these parameters. HDL/LDL lyophilized cholesterol calibrator (LOT: 324635, ADVIA Chemistry) and TG (chemistry calibrator, LOT: 680725A, ADVIA Chemistry) was used for the calibration of HDL-C and LDL-C and TG.

Statistical Analysis

The results were expressed as mean ± SD. The comparisons between groups were done using t test using Graph pad prism v6. Student t test and Pearson's correlation was used for comparing the differences in LDL-C concentrations. The level of significance was taken as $p < 0.05$. Bland–Altman graphical plots were used in order to measure or analyse the degree of agreement between the direct LDL-C assay method and formulae for LDL-C calculation.

RESULTS

A total of 500 lipid profiles were assessed. 342 (68.4%) samples were received from the male patients and 158 (31.6%) were from females. The mean age of the patients was 48.8 ± 14.2 years in males and 42.1±4.6 in females. (Table1)

Mean TG was 145.7±87.6 mg/dl and maximum no of patients 282(56.4%) had TG level less than 200mg/dl (Table 1, 2). Mean Cholesterol level was 175± 40.04 mg/dl with 271(54.2%) patients had cholesterol level greater then 200mg/dl (Table1, 3). Mean HDL cholesterol level was 53.5 ±12.39 mg/dl and 283(56.6%) patients had HDL level less than 50mg/dl (Table 1,4). Mean LDL cholesterol obtained by direct homogenous assay level was 108.4 ±34.2 mg/dl, by Friedwald formula was 95.8 ± 32.5mg/dl, Anandraja's formula 97.4±32.73mg/dl respectively (Table 1). Both of the formulas underestimated the LDL cholesterol level compared to direct homogenous assay. Friedwald formula underestimated the level of LDL cholesterol at all values of TG, Cholesterol and HDL (Table -2, 3, 4). The difference was maximum at TG value 300-400mg/dl (19.5%difference) Table 2, Cholesterol >200mg/dl (11.7% difference) Table 3. HDL<40mg/dl (difference 12.6%) Table-4.

Further on applying Anandraja's formula there was underestimation of LDL cholesterol values compared to direct LDL cholesterol values. The difference was maximum at TG value 300-400mg/dl (22.2% difference) Table-2, Cholesterol<100mg/dl (22%difference) Table-3, HDL<40mg/dl(19.7% difference, Table-4. However, at HDL level >60mg/dl there was overestimation of LDL cholesterol.

Subjects were divided into two categories taking NCEP criteria of 130 mg/dl LDL cholesterol as cut off. It was seen that more number of subjects were classified into lower risk category ([130 mg/dl) by using calculated LDL measurement than by direct LDL. According to Friedwald formula and Anandraja's formula 9.8% and 6.4% patients were classified under low risk category respectively (Table-5).

Further our study showed strong positive correlations between dLDLC and all calculated LDLC (FFLDL and DLDL, $r=0.95$, $p<0.005$) Figure-1a, Anandaraja's formula LDL and DLDL($r=0.91$,$p<0.001$) Figure 1b. The calculated LDLC showed a negative bias on Bland–Altman graphs, FFLDL (bias 12.4 with a mean difference ± SD 8.3-33) Figure-2a and Anandaraja's formula had bias 11.3 with a mean difference ± SD of -17.4-40, Figure-2b, Table-6.

Table-1: Mean baseline values of study population

Age (years)	48.8 ± 14.2 years (males) ,42.1±4.6 years(females)

Sex	342 (68.4%, Male),158 (31.6%, Female)
Triglyceride level (mg/dL)	145.7±87.6mg/dL
Total cholesterol level (mg/dL)	175± 40.04 mg/dL
HDL cholesterol level (mg/dL)	53.5 ±12.39 mg/dL
Direct LDL cholesterol (mg/dL)	108.4 ±34.2 mg/dL
Friedewald's calculation (mg/dL)	95.8 ± 32.5mg/dL
Anandaraja's calculation (mg/dL)	97.4±32.73mg/dL

Table-2: Classification of subjects showing difference between direct and calculated LDL cholesterol level depending on Triglyceride level

TG (mg/dL)	TG(mg/dL)	DLDLmg/dL (mean±SD)	FFLDLmg/L (mean±SD)	%FF(Diff)	AFLDL (mg/dL) (mean±SD)	%AF(Diff)
<100 (n=53)	77.7 ±9.8	94.6±8.7	87.5±8.9	7.5	91.7±9.5	3.1
101-200 (n=282)	174.1±11.2	97.8±10.7	86.8±10.5	10	85.7±10.8	12.0
201-300 (n=140)	232.9±12.1	114.6±12.8	97.3±12.2	15.6	93.8±12.1	18.1
301-400 (n=25)	342.5±4.2	115.9±14.7	93.3±5.9	19.5	90.1±7.7	22.2

n= No of Patients TG =Triglyceride, DLDL= Direct homogenous assay Low density lipoprotein Cholesterol, FFLDL= Friedewald's formula LDL cholesterol, %FF (Diff)= difference between DLDL and FFLDL Cholesterol, AFLDL= Anandaraja's Formula LDL cholesterol, %AF (Diff)= Difference between DLDL and AFLDL Cholesterol

Table-3: Classification of subjects showing difference between direct and calculated LDL cholesterol level depending on total cholesterol level

Chol (mg/dL)	Chol (mg/dL)	DLDL mg/dL (mean±SD)	FFLDLmg/dL (mean±SD)	%FF(Diff)	AFLDLmg/dL (mean±SD)	%AF(Diff)
<100 (n=70)	89.5±10.3	47.8±12.1	42.6±12.9	10.3	37.3±11.9	22.0
101-200 (n=158)	155.9±9.8	99.4±10.2	88.5±14.1	11.1	88.0±12.9	11.5
>200 (n=272)	224.3±14.1	153.4±12.8	135.5±12.7	11.7	138.5±12.4	9.7

n= No of Patients Chol= Cholesterol, DLDL= Direct homogenous assay Low density lipoprotein Cholesterol, FFLDL= Friedewald's formula LDL cholesterol, %FF (Diff)= difference between DLDL and FFLDL Cholesterol, AFLDL= Anandaraja's Formula LDL cholesterol, %AF (Diff)= Difference between DLDL and AFLDL Cholesterol

Table-4: Classification of subjects showing difference between direct and calculated LDL cholesterol level depending on HDL cholesterol level a

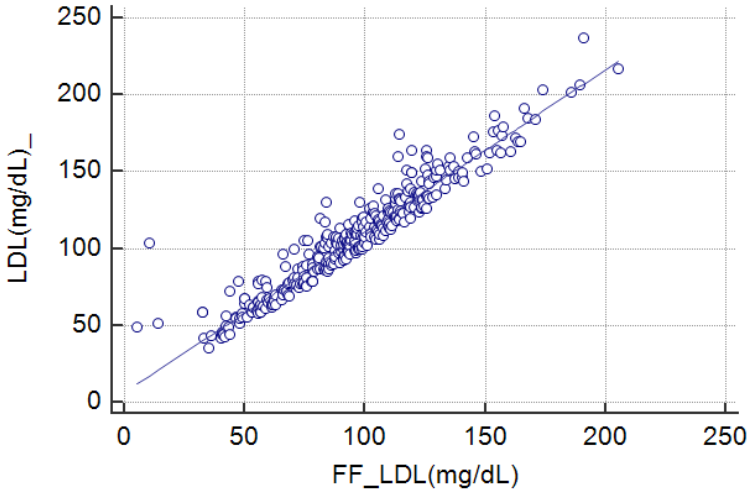
HDL mg/dL	HDL mg/dL	LDL(mg/dl) (mean±SD	FFLDL(mg/dl) (mean±SD	%FF(Diff)	AFLDL(mg/dl) (mean±SD	%AF(Diff)
<40 (n=193)	32.8±9.2	100.7±10.2	88.4±7.3	12.6	81.0±12.1	19.7
40-60 (n=283)	47.7±8.5	113.7±8.3	101.8±10.4	10.1	106.5±6.1	7.2
>60 (n=24)	68.6±2.1	128.5±9.4	117.4±5.4	8.1	139.4±7.1	-10.9

n= No of Patients HDL= HDL cholesterol, DLDL= Direct homogenous assay Low density lipoprotein Cholesterol, FFLDL= Friedewald's LDL cholesterol, %FF (Diff)= difference between DLDL and FFLDL Cholesterol, AFLDL= Anandaraja's Formula LDL cholesterol, %AF (Diff)= Difference between DLDL and AFLDL Cholesterol

Table-5: Classification of subjects taking 130 mg/dl LDL-C as cutoff level as per NCEP criteria

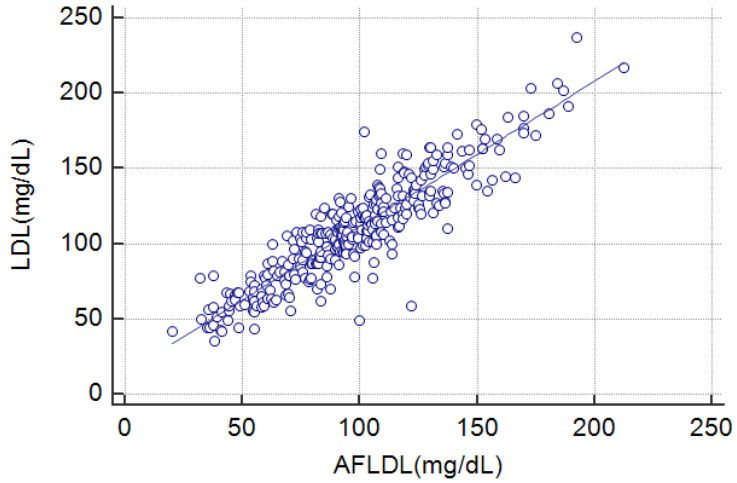
LDL-C mg/dL	D-LDL	FFLDL	AFLDL	Diff (LDL and FFLDL)	Diff (LDL and AFLDL)	p

<130 (Low Risk)	268(53.6%)	317(63.4%)	300(60%)	9.8%	6.4%	<0.05
>130(High Risk)	232(46.4%)	183(36.6%)	200(40%)			<0.05



Sample size	500
Correlation coefficient r	0.9570
Significance level	P<0.0001

Fig-1a: Comparison of F-LDL-C vs. D-LDL-C. Scatter plot of F-LDL-C against directly measured LDL-C



Sample size	500
Correlation coefficient r	0.9069
Significance level	P<0.0001

Fig-1b: Comparison of F-LDL-C vs. D-LDL-C. Scatter plot of AF-LDL-C against directly measured LDL-C

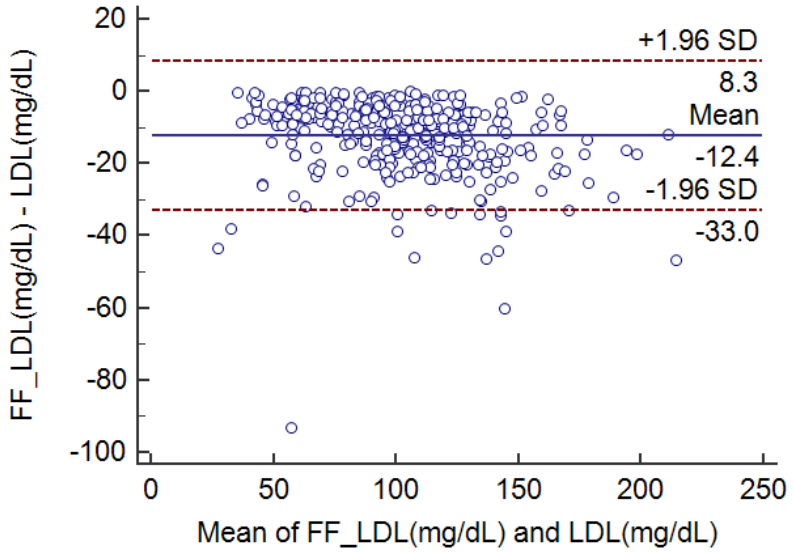


Fig-2a: Bland–Altman plot for LDL-C estimated directly and by Friedewald's calculation

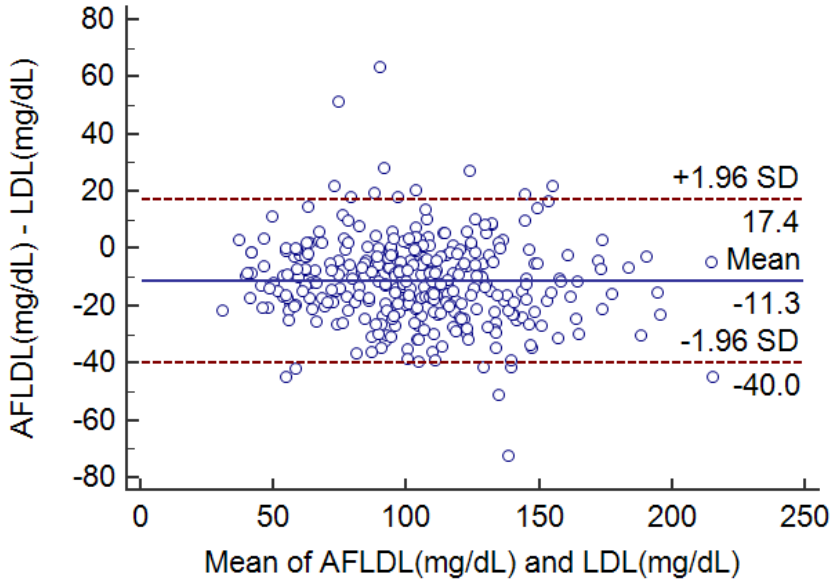


Fig-2b: Bland–Altman plot for LDL-C estimated directly and by Anandaraja's calculation

Table-6: Pearson's correlation and Bland–Altman comparison between LDL-C estimated directly and by Friedwald and Anandaraja's calculation

	Mean+SD	SD	SEM	N	Spearmans correlation		BLANDMANN ALTMANN	
					r	p	Mean difference	Mean −1.96 SD to mean +1.96 SD
LDL	109.2	34.5	1.8	500			0	
FF LDL	97.34	31.1	1.6	500	0.95	<0.0001	-12.4	8.3-33.0
AF	97.69	32.6	1.7	500	0.91	<0.0001	-11.3	-17.4-40

DISCUSSION

Treatments of lipid abnormalities are largely based on the concentrations of LDL-C. Augmenting accurate determination of LDL-C in order to initiate dietary adjustments, drug therapy and to monitor their effects in patients at risk of CHD.

Beta quantification, which is the reference method [7] for LDL-C estimation is time consuming and expensive and is not suitable for routine laboratory testing [23]. Homogenous methods developed during last few years are expensive and have failed to show clear advantages in terms of performance when compared to Friedewald's calculation [14–16]. But FF has its well-known limitations [11, 23, 26]. Several

efforts made in last few decades to derive more accurate formulas for LDL-C calculation than the widely used Friedewald's formula [27-31] to study the difference in LDL c values resulting from diversity in terms of study populations and/or pathologies [26, 32, 33]. Anandaraja *et al*., [19] had described a new formula for calculation of LDL-C and confirmed a reduction in false overestimation of LDL-C compared with FF in Indian population of validated its accuracy in 1008 Indian patients.

The present study was designed to evaluate the performance and compare between direct and calculated LDLc calculated formula in a group of Indian patients.

We have found directly measured LDL-C to be higher than that obtained by calculation using both the formulas. The only exception was higher A-LDL-C results compared to the measured LDL-C when HDL-C levels were > 60 mg/dl. In our study %DLDL-C for FF formula was higher at -12.4% compared to that for Anandaraja's at -11.3% (Table-1). Other studies by Kamal *et al*., [13], Kamazeki *et al*., Vujovic *et al*., [18] have reported an underestimation of LDL C by Friedewald's and Anandaraja's formulas compared to direct LDL C. Vujovic *et al*., [18] have also reported higher values for D-LDL-C. They have found a percentage difference of -6.9 for F-LDL-C and -3.9% for A-LDL-C.

The present study showed a significant positive correlation of 0.95 and 0.91 between direct LDL and FFLDL and AFLDL. Other studies have reported a correlation 0.86 [34] and 0.88 [17] and 0.786 [13], respectively. In a study done in Japan, a positive correlation was found between F-LDL-C and D-LDL-C with r2 = 0.975 [35]. Anandaraja *et al*., [19] reported the Pearson's correlation of 0.97 between LDL-C measured by their formula and D-LDL-C which was better as compared to that for F-LDLC.

Vujovic *et al*., have reported a correlation of 0.89 between A-LDL-C and D-LDL-C in the study done in Serbian population [18]. Kamal *et al*., [13] have also reported a good correlation between these with r = 0.810. In the study by Agrawal *et al*., [36], comparison of F-LDL-C results with measured LDL-C during three different periods with three different homogenous assays was done. A substantial lack of agreement between direct and calculated LDL-C with higher D-LDLC values by all the methods in spite of having good correlation coefficients was reported by the authors.

Some studies have reported opposite trends with higher results with calculated LDL-C by FF as compared to measured LDL-C [17, 21]. The difference between measured and calculated LDL-C results can be significant in terms of patients' risk classification for coronary artery disease. According to NCEP ATP III, LDL-C levels of 160, 130 and 100 mg/dl are the treatment goals for low risk, moderate risk and high risk patients for CHD, respectively [37].

We have found a statistically significant difference in risk classification of patients when direct LDL-C was used instead of the calculated one (Table-6). Similar results have been reported by other authors also [13, 33, 34]. Direct measurement leads to approximately 10% and 6% more patients being candidate for lipid lowering drug therapy as compared to the use of calculated LDL-C. Use of Anandaraja's formula does not produce any significant effect on patient risk classification when compared to FF

Comparison of LDL-C results obtained by Friedwald and Anandraja's formulas at different levels of the TG, Chol and HDL indicates that at higher TG, Chol and HDL concentrations produce maximum difference in calculated LDL-C results. As TG levels increase, increase in mean difference between the results of direct and F-LDL-C has been reported in previous studies [13, 38]. Our results support this finding.

LDL-C results obtained by calculated formulas show very good correlation with the measured LDL-C but the negative bias in results is responsible for producing different results compared to the directly measured LDL-C.

CONCLUSION

Calculated LDL-C results obtained by Friedewald's and Anandaraja's formulas show very good correlation with the measured LDL-C but underestimate risk of heart disease when compared to direct LDL cholesterol. Thus, for evaluating patients with hyperlipidemia, the direct method of determining the LDL-C appears to beneficial than the calculated LDL values.

LDL-C is considered as the primary basis for diagnosis, treatment and risk classification of patients with hyperlipidemia. The different modified formulas have been validated in different population with controversial results hence it is imperative to validate all these formulas normal healthy and diseased in large populations for a definitive concluding remark.

Conflict of interest

The authors declare no conflict of interest

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