Clinical Significance of Low Density Lipoprotein Cholesterol Measurements: Direct Versus Indirect Method

Manushri Sharma¹, HS Batra², Mithu Banerjee³, Arabinda Mohan Bhattarai⁴, Deeptika Agarwal²

¹Department of Biochemistry, LHMC, New Delhi, India; ²Department of Biochemistry, AFMC, Pune, India; ³Military Hospital, Udampur, India; ⁴Department of Biochemistry, NAIHS, Kathmandu, Nepal.

ABSTRACT

Introduction: Estimation of low density lipoprotein cholesterol (LDL-C) is crucial in management of coronary artery disease patients. The management of dyslipidemia is largely based on the concentration of LDL-C. The objective of this study was to compare direct measurement of LDL-C determined by a homogenous method with LDL-C estimation done by Friedewald formula (FF) in heterogeneous populations.

Methods: In this cross sectional study, we measured LDL-C by homogenous method (D-LDL-C) in 1,000 fasting samples & compared with FF (F–LDL-C) used for calculation of LDL-C. The measurements of total cholesterol (TC) and triglycerides (TG) were performed using traditional enzymatic methods. The measurements of high density lipoprotein cholesterol (HDL-C) and LDL-C were performed using direct methods with precipitation, and the estimation of the LDL-C fraction was calculated using the FF. Results: Correlation analysis shows that the two methods had significant correlation (p<0.0001). However the FF had positive bias in regard to direct method with TG levels ≤150 mg/dL. No bias was observed between the methods for TG levels from 151-200 mg/dL & from 201-300 mg/dL. Whereas, TG levels from 301-400 mg/dL shows negative bias by FF. Conclusion: The Friedewald’s formula does not shows homogenous performance for estimating LDL-C levels in samples with different TG levels as compared with that of direct method.

Keywords: total cholesterol; triglycerides; LDL-C; direct LDL; friedewald formula.

INTRODUCTION

Coronary artery disease (CAD) accounts for the greatest number of deaths of adult individuals worldwide¹. The management of dyslipidemia is largely based on the concentration of low density lipoprotein cholesterol (LDL-C)². The concentration of LDL-C is one of the strongest markers of atherosclerosis and predictor for assessing the risk for coronary heart disease (CHD). A strong positive correlation between increased LDL-C and CHD has been well documented from various epidemiological and clinical studies ³-⁶. According to the National Cholesterol Education Program (NCEP), Adult Treatment Panel, LDL-C concentration is the primary basis for treatment and appropriate patient’s classification in risk categories⁷. Separation of
lipoprotein by combining ultracentrifugation with precipitation- ‘β quantification’ is considered gold standard for measuring LDL-C. Although ‘β quantification’ is method of choice, this process is not readily suited for routine use, as it is labor intensive, time consuming, & requires expensive instruments.

In 1972, Friedewald et al. published a landmark report describing a formula to estimate LDL-C as an alternative to tedious ultra centrifugation. 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. Although this estimation formula correlates highly with beta quantification, it has certain limitations: it is not valid for samples with chylomicrons, with TG more than 400 mg/dl or in patients with dysbetalipoproteinemia. 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). The NCEP working group on lipoprotein measurements has recommended that the LDL-C concentration be determined with a total analytical error not exceeding ±12% to guarantee correct patient classification into NCEP risk categories. It is difficult to obtain this analytical quality with FF because each component’s analytical error is added.

Homogenous assays, developed in 1998 in an effort to overcome the limitations existing with both beta quantification and the FF, represent the third generation of LDL-C measurements. These homogenous direct methods use various physicochemical combinations of surfactants, polymeric complexes, and specific binding molecules to selectively measure cholesterol from LDL fraction. There are five commercially available homogenous assays for LDL-C estimation and each of these has been certified by the Cholesterol Reference Method Laboratory Network of the Centers for Disease control and Prevention. 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 Friedewald calculation.

Recently, several homogeneous methods have been developed by different manufacturers for the direct measurement of LDL-C levels, expecting that the NCEP criteria are met, as well as that the medical community’s need to prevent coronary artery disease and myocardial infarction are fulfilled. These methods seem to be better than the previous ones that use selective chemical precipitation or immunoprecipitation, which are laborious and have a significant bias as compared with the reference method. However, mainly due to the costs of the reagents, their use in clinical laboratories has not been largely disseminated, resulting in scarcity of data about the performance and validation of those methods.

This cross sectional study was aimed at assessing the performance of a direct homogeneous method for measuring LDL-C and comparing it with the estimation of LDL-C levels using the FF, analyzing a large sample.
obtained over 2 years in a tertiary care NABL accredited hospital laboratory.

**METHODS**

Data were collected from adult patients and healthy individuals those who reported for routine medical examination from June 2012 to June 2013. Patients with incomplete lipid profile & TG level above 400 mg/dL were excluded. Blood samples were collected after a 12 to 14 hour fast in vacutainers without anticoagulant & centrifuged to harvest serum. The serum was separated and the assays were performed on the same day of sample collection.

The measurements of the TG, TC, LDL & HDL were done by using an Erba XL 600 & Erba reagents. The LDL-C measurement with the homogeneous method was performed with the reagent LDL direct, liquid stable reagent (Erba), according to the specifications of the manufacturer. The method is based on the selective protection of LDL-C with the addition of reagent 1 [MES buffer (pH 6.5) 50 mmol/L, polyvinylsulfonic acid (PVS) 50 mg/L, polyethyleneglycomethylether (PEGME) 30 ml/L, MgCl₂, detergent, 4-aminoantipyrine 0.9 gm/L, Cholesterol esterase 5 kU/L, Cholesterol oxidase 20 kU/L, peroxidase 5 kU/L]. The cholesterol of the other lipoproteins is processed by cholesterol oxidase, and the hydrogen peroxide formed is broken down by catalase. After 5 minutes, with the addition of reagent 2 [MES buffer (pH 6.5) 50 mmol/L, detergent, TODB N, N-Bis (4-sulfobutyl) - 3-methylaniline) 3 mmol, polyvinylsulfonic acid (PVS) 50 mg/L, polyethyleneglycomethylether (PEGME) 30 ml/L, MgCl₂, detergent, 4-aminoantipyrine 0.9 g/L, detergent 0.5%]. All reagents are stable fluids. According to the manufacturer, no interference occurs with TG levels up to 1000 mg/dL, bilirubin up to 30 mg/dL, hemoglobin up to 1000 mg/dL, or ascorbic acid up to 10 mmol/L. For samples with triglyceride levels < 400 mg/dL, the LDL-C level was estimated using the Friedewald formula: LDL-C = TC – HDL-C – (TG/5).

The HDL-C measurement was performed using a homogeneous method with precipitation with the HDL-Direct, liquid stable reagent (Erba). The method is based on the formation of immunocomplexes of LDL and VLDL lipoproteins and chylomicrons with human anti-β lipoprotein antibodies after the addition of reagent 1 [MES buffer (pH 6.5) 6.5 mmol/L, N, N-Bis (4-sulfobutyl)-3-methylaniline) 3 mmol, polyvinylsulfonic acid (PVS) 50 mg/L, polyethyleneglycomethylether (PEGME) 30 ml/L, MgCl₂ 2 mmol, detergent, EDTA]. Then, enzymatic processing of HDL-C occurred with the addition, after 5 minutes, of reagent 2 [MES buffer (pH 6.5) 50 mmol, cholesterol esterase 5 kU/L, cholesterol oxidase 20 kU/L, peroxidase 5 kU/L, 4-aminoantipyrine 0.9 g/L, detergent 0.5%]. All reagents are stable fluids. According to the manufacturer, no interference occurs with TG levels up to 1000 mg/dL, bilirubin up to 30 mg/dL, hemoglobin up to 1000 mg/dL, or ascorbic acid up to 10 mmol.

The statistical analysis of the data was done by calculating mean ± SD. Student t-test & pearson correlation analysis were done to assess significant difference and correlation in LDL-C values obtained by calculation and direct measurement. 95% confidence interval (CI) were also calculated and the p value of <0.05 was considered statistically significant. The statistical analysis of the results was performed with the aid of GraphPad InStat and GraphPad Prism software [demo version] (San Diego, CA, USA). The Mean, standard
deviation, student t-test and 95% CI were calculated with the aid of Excel software (Microsoft).

**RESULTS**

Out of 1,000 lipid profiles 907 were assessed and 93 (9.3%) were excluded because they had TG level more than 400 mg/dL. Out of 1,000 patients 546 (54.6%) were males, and 454 (45.4%) were females. Student t-test was done it showed significant statistical difference (p value <0.0001) between direct and calculated LDL-C level (table 1). A strong correlation was found between direct LDL-C and calculated LDL-C (F)(table 1& fig. 1).

Correlation analysis shows that the two methods had significant correlation coefficients (p<0.0001). However, the Friedewald’s formula had positive bias in regard to direct method with TG levels ≤150 mg/dL. No bias was observed between the methods for TG levels from 151-200 mg/dL and from 201-300 mg/dL. Whereas, TG levels from 301-400 mg/dL shows negative bias by Friedewald’s formula (table 2). As TG levels increased (>300 mg/dL) the absolute difference between the two methods also increased; statistically significant difference existed with different levels of TG (p<0.0001) (table 2 & fig. 2).

The mean ±standard deviation of total cholesterol, LDL-C (direct), LDL-C (Friedewald), according to triglyceride levels are shown in table 3.

**DISCUSSION**

This study aimed at assessing the performance of homogenous method for direct LDL-C measurement, as compared with LDL-C estimated by Friedewald formula. Although correlation exists between the direct and calculated LDL-C values of study subjects but the direct measurement of LDL-C & calculated LDL-C (F) determined by FF are not capable of providing identical results, this is reported by other authors as well 1,19-26.

Abu hena et al. stated that calculated LDL-C determined by Friedewald formula underestimated the LDL-C level when compared with homogenous direct assay. This difference broadens with increase TG levels2. In our study FF shows positive bias with TG level less than 150 mg/dL. However no bias was observed between the methods for TG levels ranging from 151-200 mg/dL & from 201-300 mg/dL. On the other hand, for TG levels ranging from 301-400 mg/dL, this bias of the FF became negative.

<table>
<thead>
<tr>
<th>Table 1: Paired sample statistics &amp; correlation (n = 907)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean ± SD</strong></td>
</tr>
<tr>
<td>LDL-C-D</td>
</tr>
<tr>
<td>LDL-C-F</td>
</tr>
</tbody>
</table>
Most studies of compliance with NCEP goals and CHD risk reduction have used the FF rather than direct measurement of LDL-C\(^3,27,28,29\). An exception is the heart protection study, which directly measured LDL-C in more than 20,000 adults aged 40-80 years with...

![Table 2: Paired sample statistics, correlation and p values in different levels of TG (LDLC-D and LDLC-F)](image)

<table>
<thead>
<tr>
<th>TG levels (mg/dL)</th>
<th>LDLC-D Mean ± SD</th>
<th>LDLC-F Mean ± SD</th>
<th>Correlation (r)</th>
<th>95% CI</th>
<th>t-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 150 (n = 207)</td>
<td>80 ± 24</td>
<td>93 ± 27</td>
<td>0.816</td>
<td>0.765 to 0.857</td>
<td>0.15</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>151 – 200 (n = 82)</td>
<td>100 ± 28</td>
<td>97 ± 38</td>
<td>0.886</td>
<td>0.828 to 0.925</td>
<td>0.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>201 – 300 (n = 453)</td>
<td>118 ± 35</td>
<td>107 ± 41</td>
<td>0.875</td>
<td>0.852 to 0.895</td>
<td>2.43</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>301 – 400 (n = 165)</td>
<td>122 ± 33</td>
<td>87 ± 43</td>
<td>0.904</td>
<td>0.872 to 0.929</td>
<td>7.05</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

![Figure 2: Correlation of LDL-D with Friedwald formula (LDL-F) in different levels of TG](image)
coronary disease, other occlusive disease, or diabetes\textsuperscript{30,31}. Primary outcomes of this randomized study were mortality and fatal or non-fatal vascular events. Results demonstrated a 25\% reduction in vascular disease risk when lowering direct LDL-C from 116 mg/dL to <77 mg/dL, implying the need for more aggressive treatment than currently recommended.

An additional method of assessing CHD risk nuclear magnetic resonance may be available in near future and shows promise for routine measurement of lipoprotein levels. This method quantifies lipoprotein by subclasses based on size. It is not influenced by variability in cholesterol composition. Currently, outcome data are not available for this method of measurements. However frozen plasma samples from ongoing or complete clinical trials will be analyzed by this approach to determine if it improves prediction of coronary artery disease outcomes\textsuperscript{32}.

In this cross sectional study, lipid profiles were collected over a period of one year to minimize cofounding variables. Samples for lipid profiles were collected in a minimum 12 hours fasting state. It is important to note that a non-fasting state can increase the TG levels and potentially underestimated the value of calculated LDL-C, whereas direct method is not limited by timing of food ingestion. Although the study had sufficiently large sample size to determine the difference between direct vs calculated method, it was not of sufficient duration to evaluate CHD related outcomes.

**CONCLUSION**

The Friedewald formula did not have a homogeneous performance for estimating LDL-C levels in samples with different TG levels as compared with that of the direct method, which could launch doubts on patient’s classification on the risk of developing CHD. In applying the NCEP ATP III guidelines in patient management, clinicians should be aware of the circulatory heterogeneity of LDL-C particles and the potential limitations of the calculation formula. Avoidance of calculated LDL-C especially if TG>300 mg/dl in clinical practice should be cautiously considered.

**REFERENCES**


