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

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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 worldwide[1]. 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 categories[7]. Separation of

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lipoprotein by combining ultracentrifugation with precipitation- 'β quantification' is considered gold standard for measuring LDL-C[8]. 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[8,9].

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[10]. 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)[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% to guarantee correct patient classification into NCEP risk categories[12]. It is difficult to obtain this analytical quality with FF because each component's analytical error is added[13].

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[14]. These homogenous direct methods use various physicochemical combinations of surfactants, polymeric complexes, and specific binding molecules to selectively measure cholesterol from LDL fraction[15]. 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[16]. 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[16-18].

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[11,19]. 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, $MgCl_2$, 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/L], LDL-C is released for enzymatic processing and development by the Trinder reaction. All reagents are stable fluids. According to the manufacturer, no interference occurs with triglyceride levels up to 1,000 mg/dL, bilirubin up to 40 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, $MgCl_2$ 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

Table 1: Paired sample statistics & correlation (n = 907)

	Mean ± SD	Correlation (r)	95% CI	t-test	P value
LDLC-D LDLC-F	108 ± 36 92 ± 39	0.815	0.792 – 0.836	1.90	<0.0001

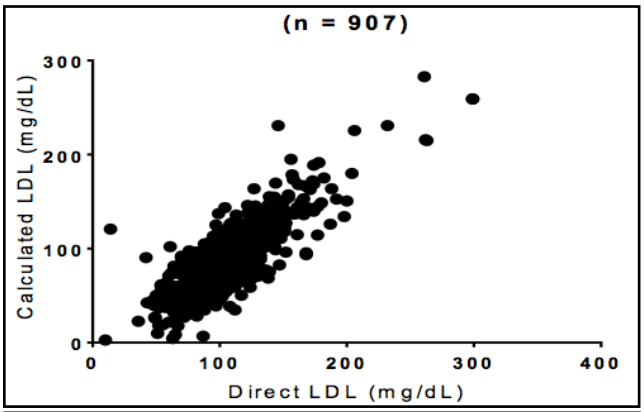

Figure 1: Correlation of LDL-D with Friedwald formula (LDL-F) [n = 907; r = 0.815; p = <0.0001]

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

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

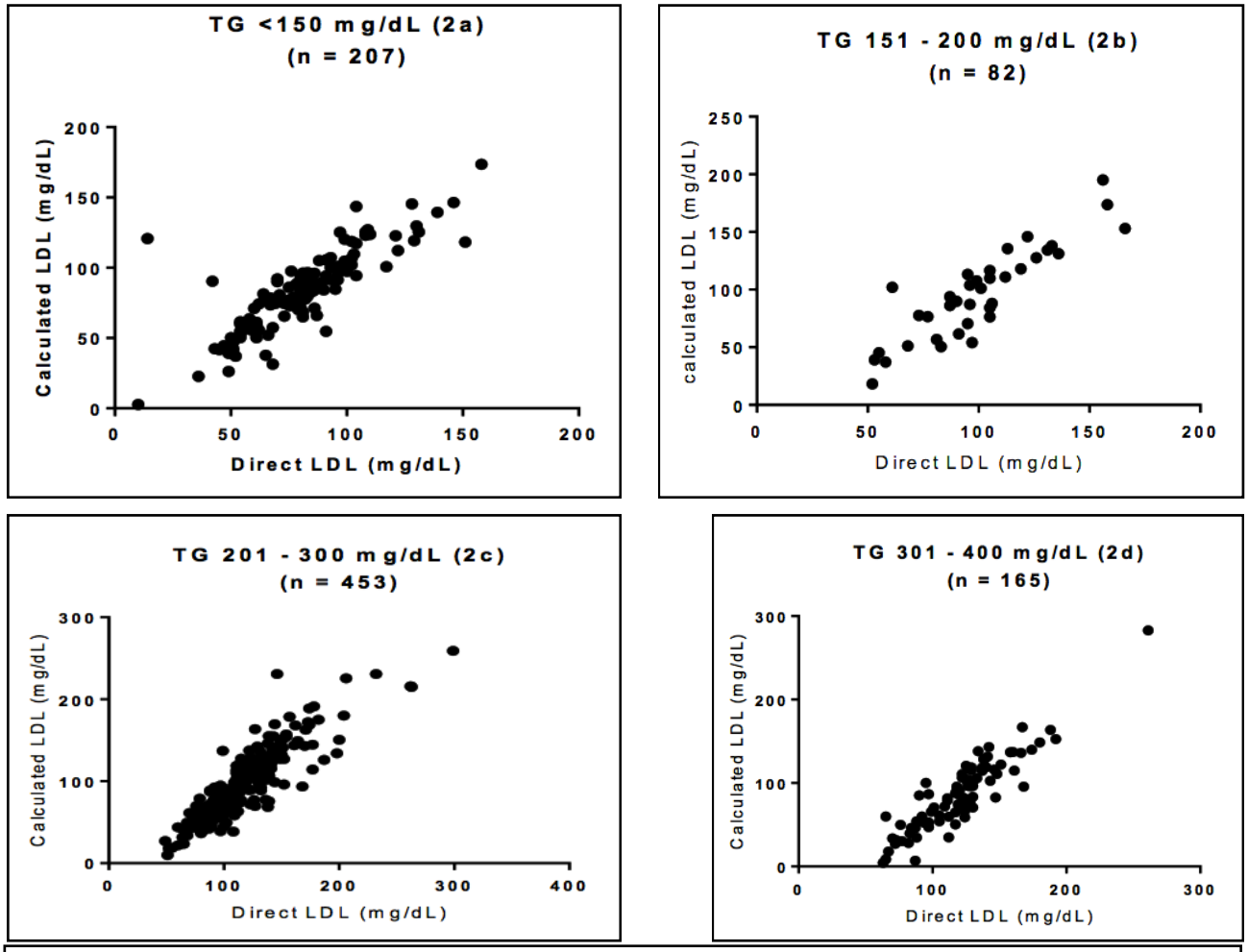

Figure 2: Correlation of LDL-D with Friedwald formula (LDL-F) in different levels of TG

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 3: Summary of the measurements of total cholesterol, LDL-C (direct), LDL-C (Friedewald) according to triglyceride levels, presented as mean ± standard deviation

Triglyceride (mg/dl)	Total cholesterol	LDL-C (D)	LDL-C (F)*
<150 (n = 207)	150±37	80±24	93±27
151-200 (n = 82)	178±43	100±28	97±38
201-300 (n = 453)	186±47	118±35	107±41
301-400 (n = 165)	196±49	122±33	87±43

coronary disease, other occlusive disease, or diabetes[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 then 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[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

1. Caio Maurício MC, Carlos RS, Iara DJ, Comparison of LDL-Cholesterol Direct Measurement with the Estimate Using the Friedewald Formula in a Sample of 10,664 Patients. Arquivos Brasileiros de Cardiologia - Volume 83, Nº 6, Dezembro 2004. http://www.scielo.br/pdf/abc/v83n6/en_a06v83n6.
2. Abu Hena MK, Mosharref H, Shahanara C, Nasir Uddin M. A comparison of calculated with direct measurement of low density lipoprotein cholesterol Level.

JCMCTA 2009; 20 (2): 19-23. http://www.banglajol.info/index.php/JCMCTA/article/download/5621/4412

3. Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet. 1994;344:1383-9. http:// DOI: 10.1016/j.atherosclerosissup.2004.08.027 ·PMid: 7968073

4. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group: prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. N Engl J Med. 1998;339:1349-57. http://dx.doi.org/10.1056/NEJM199811053391902 PMid:9841303

5. Ridker PM, Stampfer MJ, Rifai N. Novel risk factors for systemic atherosclerosis. J Am Med Assoc. 2001;285:2481-5. http://dx.doi.org/10.1001/jama.285.19.2481

6. Keevil JG, Cullen MW, Gangnon R, McBride PE, Stein JH. Implications of cardiac risk and low density lipoprotein cholesterol distributions in the United States for the diagnosis and treatment of dyslipidemia: data from National Health and Nutrition Examination Survey 1999 to 2002. Circulation. 2007; 115:1363-70. http://dx.doi.org/10.1161/CIRCULATIONAHA.106.645473 PMid: 17353444

7. National Cholesterol Education Program (NCEP) Expert panel. Third report of the National Cholesterol Education Program (NCEP) Expert panel on detection, evaluation and treatment of high cholesterol in adults (Adult Treatment Panel III) final report. Circulation. 2002;106:3143-421. PMid:12485966

8. Hirany S, Li D, Jialal I. A more valid measurement of LDL-C in diabetic patients. Am J Med 1997; 102: 48-53. http://dx.doi.org/10.1016/S0002-9343(96)00375-0

9. Marniemi J, Maki J, Maatela J, Jarvisalo J, Impivaara O. Poor applicability of the Friedewald formula in the assessment of serum LDL-C for clinical purposes. Clin Biochem 1995; 28; 285-9. http://dx.doi.org/10.1016/0009-9120(94)00095-D

10. 10. Friedewald WT, Levy Rl, Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem. 1972; 18:499-502. PMid:4337382

11. 11. McNamara JR, Cohn JS, Wilson PW, Schaefer EJ. Calculated values for low density lipoprotein cholesterol in the assessment of lipid abnormalities and coronary disease risk. Clin Chem. 1990; 36:36-42. PMid:2297935

12. 12. Bachorik PS, Ross JW. National Education Program recommendations for measurements of low density lipoprotein cholesterol: executive summary. National Cholesterol Education Program Working Group on Lipoprotein Measurements. Clin Chem. 1995;41:1414-20. PMid:7586510

13. 13. Turkalp I, Cil Z, Ozkazanc, D. Analytical performance of a direct assay for LDL-cholesterol: a comparative assessment versus Friedewald's formula

original investigation. Anadolu Kardiyol Derg. 2005;5:13-7. PMid:15755695/http://www.journalagent.com/z4/download_fulltext.asp?pdir=anatoljcardiol&plng=eng&un=AJC-62282

14. Kamal AHM, Hossain M, Chowdhury S, Mahmud NU. A comparison of calculated with direct measurement of low density lipoprotein cholesterol level. JCMCTA. 2009;20:19-23. http://www.banglajol.info/index.php/JCMCTA/article/download/5621/4412
15. Miller WG, Waymack PP, Anderson FP, Ethridge SF, Jayne EC. Performance of four homogenous direct methods for LDL-cholesterol. Clin Chem. 2002;48:489-98. PMid:11861439
16. Nauck M, Warnick GR, Rifai N. Methods for measurement of LDL-cholesterol: a critical assessment of direct measurement by homogenous assays versus calculation. Clin Chem. 2002;48: 236-54. PMid: 118050041
17. Sakaue T, Hirano T, Yoshino G, Sakai K, Takeuchi H, Adachi M. Reactions of direct LDL cholesterol assays with pure LDL fraction and LDL: comparison of three homogenous methods. Clin Chim Acta. 2000;295:97-106. http://dx.doi.org/10.1016/S0009-8981(00)00200-X
18. Sahu S, Chawla R, Uppal B. Comparison of two methods of estimation of low density lipoprotein cholesterol. The direct versus Friedewald estimation. Ind J Clin Biochem. 2005;20:54-61. http://dx.doi.org/10.1007/BF02867401 PMid:23105534
19. Harris N, Neufeld E, Newburger JW et al. Analytical performance and clinical utility of a direct LDL-cholesterol assay in a hyperlipidemic pediatric population. Clin.Chem. 1996; 42:1182-88. PMid: 8697574
20. Rifai N, Iannoti E, DeAngelis K et al. Analytical and clinical performance of a homogeneous enzymatic LDL-cholesterol assay compared with ultracentrifugation dextran sulfate-Mg2+ method. Clin. Chem. 1998; 44:1242-50. http://citeseerx.ist.psu.edu/doi=10.1.1.602.8210. PMid:9625048
21. Nauck M, Graziani MS, Bruton D et al. Analytical and clinical performance of a detergent-based homogeneous LDL-cholesterol assay: a multicenter evaluation. Clin. Chem. 2000; 46:506-14. http://citeseerx.ist.psu.edu/doi=10.1.1.522.996.PMid:10759474
22. Esteban-Salán M, Guimón-Berdesi A, de la Viuda-Unzueta JM et al. Analytical andclinical evaluation of two homogeneous assays for LDL-cholesterol in hyperlipidemicpatients. Clin. Chem. 2000; 46:1121-31. http://citeseerx.ist.psu.edu doi=10.1.1.544.8577. PMid:10926892
23. Yu HH, Ginsbrug GS, Harris N et al. Evaluation and clinical application of a directlow density lipoprotein cholesterol assay in normolipemic and hyperlipidemicadults. Am. J. Cardiol 1997; 80:1295-9.http://dx.doi.org/10.1016/S0002-9149(97)00668-1
24. Sheikh M, Miller NE. Evaluation of commercial reagent for precipitating humanserum low density lipoprotein. Clin. Chem. Acta 1985; 152:213-7 http://dx.doi.org/10.1016/0009-8981(85)90194-9

25. Demacker PN, Hijmans AG, Brenninkmeijer BJ et al. Five methods for determininglow density lipoprotein cholesterol compared. Clin. Chem. 1984; 30:1797-1800. PMid:6593139

26. Assmann G, Jabs HU, Nolte W et al. Precipitation of LDL with sulphopolyanions:a comparison of two methods for LDL cholesterol determination. J. Clin. Chem.Clin. Biochem. 1984; 22:781-5. http://dx.DOI: 10.1515/cclm.1984.22.11.781. PMid: 6527097

27. Downs JR, Clearfield M, Weis S, et al, for the AFCAPS/TexCAPS research group. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels, results of AFCAPS/TexCAPS. JAMA 1998;279:1615-22. http://dx.doi.org/ 10.1001/jama.279.20.1615 PMid:9613910

28. ALLHAT officers and coordinators for the ALLHAT collaborative research group. Major cardiovascular events in hypertensive patients randomized to doxazocin vs chlorthalidone: the antihypertensive and lipid lowering treatment to prevent heart attack trial (ALLHAT), JAMA 2000;283:1967-75. http://dx.doi.org/10.1001/jama. 283.15.1967 PMid:10789664

29. Bezafibrate infraction prevention (BIP) study group. Secondary prevention by raising HDL cholesterol reducing triglycerides in patients with coronary artery disease. Circulation 2000;102:21-7. http://dx.doi.org/10.1161/01.CIR.102.1.21 PMid:10880410.

30. National Cholesterol Education Program (NCEP) Expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel III). Summary of the third report of the National Cholesterol Education Program (NCEP) Expert panel on detection, evaluation and treatment of high blood cholesterol in adults. JAMA 2001;285 (19):2486-97. http://dx.doi.org/ 10.1001/jama.285.19.2486 PMid:11368702

31. Heart protection study collaborative group. MRC/BHF heart protection study of cholesterol lowering with simvastatin in 20,536 high risk individuals: a randomized placebo-controlled trial. Lancet 2002;360:7-22. http://dx.doi.org/10.1016/ S0140-6736(02)09327-3

32. Otvos JD, Elias J, Cromwell WC. Measurement issues related to lipoprotein heterogeneity. Am J Cardiol 2002:90 (suppl):22-9. http://dx.doi.org/10.1016/ S0002-9149(02)02632-2.