Accurate Assessment of LDL Cholesterol Reduction at Levels Below 70mg/dL Has Implications in the Estimation of Efficacy for New Drugs in Development

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Introduction

- PUC is considered the "gold standard" method for LDL-C measurement (LDL-C_P),[1] but is not readily available in many laboratories because of the labor-intensive protocol requiring specialized equipment.
- A cost-effective method for calculating LDL-C was reported by Friedewald et al.[2] and has gained widespread acceptance when TG are <400 mg/dL. While the Friedewald formula was originally validated in patients with LDL-C >70 mg/dL and has proven robust and reliable above this level, its accuracy and validity for lower LDL-C levels has recently been questioned[3-4]. This may have significant implications for both combination lipid modifying therapies currently available that have demonstrated cardiovascular risk reduction directly attributable to lowering LDL-C below those levels[5], and LDL-C lowering compounds in development, that achieve very low levels.[6-7]
- An alternative formula, the Hopkins formula (LDL-C_H), has been proposed to address the shortcomings of the Friedewald formula. The Hopkins formula uses a variable TG:VLDL-C ratio (varying from 3.1 to 11.9) dependent on total cholesterol (TC), TG, and non–HDL-C levels[8] rather than the fixed ratio of TG/5 used by Friedewald. However, Hopkins has not been validated against PUC.
- Homogenous methods (LDL-C_D) are detergent based assays which are based on inhibition of measurement of cholesterol in other lipoproteins from being measured, and were originally introduced to measure LDL-C where TG >400 mg/dL or patients were non-fasting. However the performance of these assays vary by manufacturer and from reagent generation within the same manufacturer. Additionally, accuracy relative to PUC has also been shown to deteriorate in diseased (primarily dyslipidemic and cardiovascular) populations and there is no data on accuracy at low LDL-C concentrations.[9]
- We report the results of LDL-C measured by PUC as compared to LDL-C estimated by the Friedewald and Hopkins formulas and "directly" measured using a homogenous assay in 1299 samples including 961 with LDL-C ≤70 mg/dL and 896 ≤50 mg/dL.

Methodology[10]

Samples

- Serum or plasma samples were collected after an overnight fast (water only) and analyzed for TC, TG, high density lipoprotein cholesterol (HDL-C), LDL-C_P, and LDL-C_D and were evaluated for those with TG ≤400 mg/dL, resulting in total of 1299 comparisons.
- The samples were from either patients in a specialized lipid clinic or participants in clinical trials, and included pediatric patient samples. All samples were received de-identified of demographic information.

Analytical Methods

- TC, the cholesterol content of isolated fractions, and TG were measured at Medpace Reference Laboratories, Cincinnati, OH, which maintained CDC-NHLBI Lipid Standardization Program Part III throughout the period (Participant number LSP-395).[11]
- Analysis of TC and TG was by enzymatic methods on a Beckman Coulter AU Series automated chemistry analyzer with in-house developed serum calibrators directly traceable to CDC-NHLBI reference procedures.[11]
- LDL-C_P was performed using the method modified from the Lipid Research Clinics methods manual.[12] Serum or plasma was overlaid with normal saline (density 1.006 g/mL) and centrifuged (Beckman Ultracentrifuge Model # L-90K and rotor, Type 50.4) at 40,000 rpm for 18–22 hours at 10°C to separate VLDL-C in the supernatant ("top" fraction) from LDL and HDL in the infranatant or "bottom" fraction. The cholesterol concentration of the infranatant was measured. All apolipoprotein B-containing lipoproteins, VLDL-C, intermediate density lipoprotein (IDL), LDL, and Lp(a), were precipitated from serum using 50 kDa dextran sulfate with magnesium ions ($MgCl_2$),[13] and the cholesterol in the remaining HDL fraction was measured. The HDL-C concentration was subtracted from the infranatant cholesterol to provide the LDL-C_P value. VLDL-C was calculated by subtracting the "bottom" fraction cholesterol from TC. The ratio of cholesterol in VLDL to TG was calculated by VLDL-C/TG.
- Calculated LDL-C was estimated from the Friedewald formula[2] where: LDL-C_F = TC – (HDL-C + TG/5) and from the Hopkins formula where: LDL-C_H = TC – (HDL-C + TG/ adjustable factor mg/dL); the adjustable factor was determined as the strata-specific median TG:VLDL-C ratio.[8]
- LDL-C_D was measured by a homogeneous enzymatic assay using Roche C.f.a.s. Lipid Calibrator and LDL-C plus 2nd generation reagent (both traceable to the Cholesterol Reference Method Laboratory Network accuracy base for LDL-C) on a Beckman Coulter AU Series automated chemistry analyzer.

Statistical methods

- Summary statistics, mean (standard deviation [SD]) values for continuous variables, and numbers of patients and percentages for categorical variables were calculated on measured and calculated lipid parameters.
- Subgroup analyses based on the differences between LDL-C_F, LDL-C_H, LDL-C_D as compared to LDL-C_P for each sample were performed based on LDL-C_F and TG levels at selected cut-points. Similar analysis was done for VLDL-C/TG ratio.
- The percent difference for each of the measurement methods from PUC at LDL-C ≤100 mg/dL are presented in difference plots.

Results

- Overall results for the 1,299 samples are shown in Table 1. LDL-C_P ranged from 2 - 453 mg/dL. The ranges for the other measurement methods were similar; 0 – 449 mg/dL by Friedewald, 1 - 446 mg/dL by Hopkins, and 7 – 369 mg/dL by the direct method. This corresponded to an overall difference (mean ± SD) of -18.9 ± 19.34%, -9.3 ± 17.83%, and -0.8 ± 21.91% for Friedewald, Hopkins, and the direct method, respectively. TG ranged from 28 to 394 mg/dL.
- Assessment based on selected PUC LDL-C cut-points (Table 2) resulted in 947 results ≤70 mg/dL, 860 ≤50 mg/dL and 322 ≤25 mg/dL.
- The Friedewald formula underestimated LDL-C as compared to LDL-C_P at all LDL-C cut-points (Table 3). LDL-C_F showed a minimal difference of -3.4% when LDL-C was between 101-200 mg/dL. As values decreased below 100 mg/dL, the difference between Friedewald and PUC progressively increased to 6.9% between 100 and 71 mg/dL, 14.3% between 70 and 51 mg/dL, 20.9% between 50 and 26 mg/dL and 32.9% at 25 mg/dL or below (Figure 1). Within each LDL-C cut-point the difference between Friedewald and PUC increases for every 100 mg/dL rise in TG, especially at LDL-C below 50 and 25 mg/dL (Figure 2).
- Overall, the Hopkins method underestimated LDL-C as compared to PUC at all LDL-C cut points (Table 4), though to a lesser degree than as estimated by Friedewald. The underestimation using LDL-C_H increased as LDL-C levels decreased; 2.2% between 100 and 71 mg/dL, 2.3% between 70 and 51 mg/dL, 9.3% between 50 and 26 mg/dL and 19.7% at 25 mg/dL or below (Figure 3). For TG levels ≤200 mg/dL, Hopkins underestimated LDL-C at all LDL-C cut points (overall mean difference 15.5% for TG ≤100 mg/dL, 8.2% for TG 101 to 200 mg/dL) and overestimated LDL-C when TG levels were ≥201 mg/dL (overall mean difference 6.6% for TG 201 to 300 mg/dL, 20.3% for TG 301 to 400 mg/dL), shown in Figure 4. As compared to PUC, LDL-C measured with the "direct" method was accurate (Table 5) overall with a % difference of – 0.8 (p = 0.17). However, the differences at all LDL-C cut-points were statistically significant with underestimation of LDL-C as compared to PUC; 3.7% between 101 and 200 mg/dL, 2.7% between 100 and 71 mg/dL, 4.1% between 70 and 51 mg/dL, and 4.3% between 50 and 26 mg/dL (Figure 5). When LDL-C was ≤25 mg/dL, the direct method overestimated LDL-C by 8.8%. The direct method was more consistent across increasing TG levels (Figure 6).

Table 1: Summary Statistics for Lipid Parameters

Lipid Parameter (units)	N	Mean	SD	Min	Max
TC (mg/dL)	1299	126.7	57.10	51	515
HDL-C (mg/dL)	1299	48.9	14.17	18	124
TG (mg/dL)	1299	123.6	64.98	28	394
Calculated LDL-C_F by Friedewald (mg/dL)	1299	53.1	53.53	0	449
Calculated LDL-C_H by Hopkins (mg/dL)	1299	56.6	53.06	1	446
LDL-C_P by preparative ultracentrifugation (mg/dL)	1299	59.5	52.67	2	453
"Direct" LDL-C_D (mg/dL)	1289	57.1	49.00	7	369
% Difference Friedewald[a]	1299	-18.9	19.34	-100	100
% Difference Hopkins[b]	1299	-9.3	17.83	-90	150
% Difference "Direct"[c]	1289	-0.8	21.91	-63	450
VLDL-C[d] (mg/dL)	1299	18.3	11.48	2	73
VLDL-C/TG	1299	0.146	0.0434	0.028	0.433

a % difference = 100*(LDL-C_F – LDL-C_P)/ LDL-C_P
b % difference = 100*(LDL-C_H – LDL-C_P)/ LDL-C_P
c % difference = 100*(LDL-C_D – LDL-C_P)/LDL-C_P
d VLDL-C = TC – HDL-C – LDL-C_P

Table 2: Summary Statistics of LDL-C_D, LDL-C_F, and LDL-C_H and LDL-C_P by LDL-C_P Categories

LDL-C_P		LDL-C_P (mg/dL)	LDL-C_D (mg/dL)	% Difference[a]		LDL-C_F (mg/dL)	% Difference[b]		LDL-C_H (mg/dL)	% Difference[c]	
(mg/dL)	N	Mean (SD)	Mean (SD)	Mean (SD)	p-value	Mean (SD)	Mean (SD)	p-value	Mean (SD)	Mean (SD)	p-value
≤25	322	18.1 (4.85)	18.9 (5.17)(N=319)	8.8 (37.08)	<.0001	12.3 (5.67)	-32.9 (24.75)	<.0001	14.6 (5.88)	-19.7 (24.61)	<.0001
26-50	538	36.0 (6.65)	34.3 (7.33)(N=535)	-4.3 (13.26)	<.0001	28.5 (7.25)	-20.9 (14.69)	<.0001	32.8 (8.37)	-9.3 (15.60)	<.0001
51-70	87	59.5 (6.08)	57.0 (8.65)(N=87)	-4.1 (11.71)	0.0016	50.9 (9.88)	-14.3 (14.25)	<.0001	58.1 (9.29)	-2.3 (12.58)	0.0875
71-100	76	86.2 (8.78)	83.6 (11.55) (N=74)	-2.7 (10.18)	0.0253	80.2 (9.88)	-6.9 (6.40)	<.0001	84.2 (10.08)	-2.2 (8.56)	0.0317
101-200	258	138.0 (24.86)	132.9 (28.14)(N=258)	-3.7 (10.46)	<.0001	133.4 (25.10)	-3.4 (5.13)	<.0001	135.8 (24.33)	-1.4 (5.71)	0.0001
>200	18	267.5 (88.18)	235.6 (57.59)(N=16)	-3.5 (5.34)	0.0190	261.9 (90.02)	-2.4 (3.15)	0.0051	261.4 (88.37)	-2.4 (2.83)	0.0019
≤50	860	29.3 (10.57)	28.6 (9.99)(N=854)	0.6 (25.75)	0.5097	22.4 (10.34)	-25.4 (19.94)	<.0001	26.0 (11.57)	-13.2 (20.10)	<.0001
≤70	947	32.1 (13.44)	31.2 (12.85)(N=941)	0.1 (24.82)	0.8546	25.1 (13.18)	-24.4 (19.74)	<.0001	28.9 (14.68)	-12.2 (19.78)	<.0001
≤100	1023	36.1 (19.34)	35.0 (18.67)(N=1015)	-0.1 (24.06)	0.9371	29.2 (19.43)	-23.1 (19.62)	<.0001	33.0 (20.42)	-11.4 (19.35)	<.0001

a % difference = 100*(LDL-C_D – LDL-C_P)/ LDL-C_P
b % difference = 100*(LDL-C_F – LDL-C_P)/ LDL-C_P
c % difference = 100*(LDL-C_H – LDL-C_P)/LDL-C_P
p-values are from a one sample t-test performed on % difference
Note: *Overall N=1289 for direct LDL and N=1299 for other parameters.*

Table 3: Summary Statistics for *% Difference* of Calculated LDL-C_F by *Friedewald* and LDL-C_P

LDL-C_P	TG Level (mg/dL) ≤100		101-200		201-300		301-400		Overall		
(mg/dL)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	p-value
≤25	184	-22.9 (21.50)	125	-43.9 (20.84)	12	-66.2 (26.05)	1	-95.7 (.)	322	-32.9 (24.75)	<.0001
26-50	251	-12.8 (9.97)	228	-26.2 (12.37)	53	-34.9 (19.27)	6	-35.9 (21.78)	538	-20.9 (14.69)	<.0001
51-70	42	-6.7 (5.87)	21	-12.5 (9.70)	16	-27.4 (13.56)	8	-32.9 (22.00)	87	-14.3 (14.25)	<.0001
71-100	33	-4.8 (5.34)	29	-7.3 (6.67)	12	-11.2 (6.26)	2	-9.4 (9.63)	76	-6.9 (6.40)	<.0001
101-200	88	-2.3 (3.26)	114	-2.9 (4.44)	42	-6.2 (6.95)	14	-5.1 (9.38)	258	-3.4 (5.13)	<.0001
>200	9	-1.7 (2.92)	5	-2.3 (3.64)	3	-3.0 (3.06)	1	-7.2 (.)	18	-2.4 (3.15)	0.0051
Overall	607	-13.3 (15.52)	522	-23.5 (20.00)	138	-25.3 (23.58)	32	-21.0 (24.77)	1299	-18.9 (19.34)	<.0001
p-value		<.0001		<.0001		<.0001		<.0001			

Note: % difference = 100*(calculated LDL-C_F – LDL-C_P)/LDL-C_P
P-values are from a one sample t-test performed on % difference.

Table 4: Summary Statistics for *% Difference* of Calculated LDL-C_H by *Hopkins* and LDL-C_P

LDL-C_P	TG Level (mg/dL) ≤100		101-200		201-300		301-400		Overall		
(mg/dL)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	p-value
≤25	184	-26.2 (24.50)	125	-14.1 (19.94)	12	15.5 (20.91)	1	43.5 (.)	322	-19.7 (24.61)	<.0001
26-50	251	-14.6 (9.79)	228	-9.8 (12.02)	53	11.1 (18.81)	6	53.3 (24.55)	538	-9.3 (15.60)	<.0001
51-70	42	-7.8 (6.55)	21	-2.6 (9.56)	16	3.2 (11.64)	8	15.9 (22.59)	87	-2.3 (12.58)	0.0875
71-100	33	-6.4 (5.54)	29	-2.1 (7.02)	12	5.6 (8.04)	2	19.5 (13.40)	76	-2.2 (8.56)	0.0317
101-200	88	-3.9 (3.16)	114	-1.4 (4.87)	42	0.5 (5.85)	14	8.8 (9.79)	258	-1.4 (5.71)	0.0001
>200	9	-2.7 (2.82)	5	-2.5 (3.68)	3	-1.2 (2.49)	1	-2.9 (.)	18	-2.4 (2.83)	0.0019
Overall	607	-15.5 (17.10)	522	-8.2 (13.86)	138	6.6 (15.12)	32	20.3 (23.84)	1299	-9.3 (17.83)	<.0001
p-value		<.0001		<.0001		<.0001					

Note: % difference = 100*(calculated LDL-C_H – LDL-C_P)/LDL-C_P
P-values are from a one sample t-test performed on % difference.

Table 5: Summary Statistics for *% Difference* of Calculated LDL-C_D by *Direct Method* and LDL-C_P

LDL-C_P	TG Level (mg/dL) ≤100		101-200		201-300		301-400		Overall		
(mg/dL)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	p-value
≤25	182	7.5 (41.56)	124	10.1 (30.07)	12	16.4 (33.32)	1	0.0 (N/A)	319	8.8 (37.08)	<.0001
26-50	250	-5.9 (12.20)	227	-4.8 (13.52)	52	3.7 (13.44)	6	12.9 (9.79)	535	-4.3 (13.26)	<.0001
51-70	42	-4.9 (9.85)	21	-1.8 (13.26)	16	-5.5 (12.13)	8	-3.1 (16.52)	87	-4.1 (11.71)	0.0016
71-100	32	-5.3 (8.68)	28	-3.1 (11.03)	12	4.1 (9.85)	2	3.3 (6.47)	74	-2.7 (10.18)	0.0253
101-200	88	-5.2 (8.63)	114	-3.5 (9.94)	42	-3.6 (14.30)	14	3.2 (9.56)	258	-3.7 (10.46)	<.0001
>200	7	-4.7 (3.81)	5	-4.3 (8.26)	3	-0.7 (2.76)	1	0.5 (N/A)	16	-3.5 (5.34)	0.0190
Overall	601	-1.6 (25.31)	519	-0.8 (19.16)	137	1.4 (16.71)	32	3.2 (12.08)	1289	-0.8 (21.91)	0.1699
p-value		0.1130		0.3644		0.3168		0.1386			

Note: % difference = 100*(calculated LDL-C_D – LDL-C_P)/LDL-C_P
P-values are from a one sample t-test performed on % difference.

Figure 1: Difference plot for LDL-C ≤100 by Friedewald and Preparative Ultracentrifugation

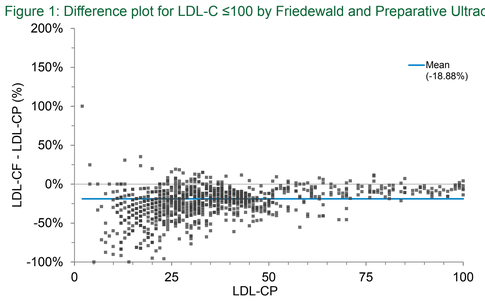


Figure 2: % Difference (mean +/-SE) in LDL-C (Friedewald) by TG level

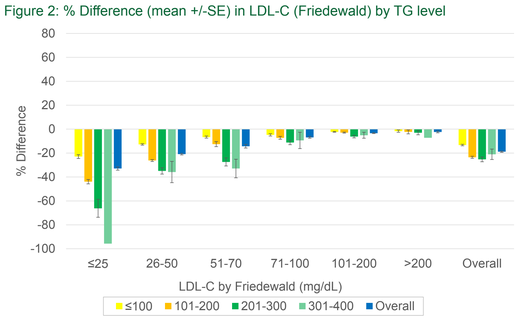


Figure 3: Difference plot for LDL-C ≤100 by Hopkins and Preparative Ultracentrifugation

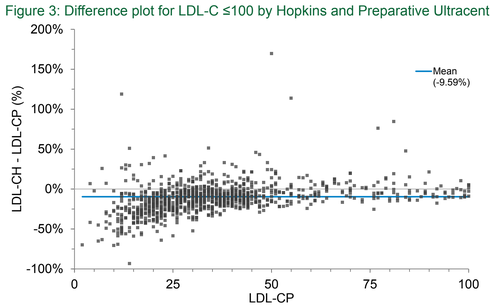


Figure 4: % Difference (mean +/-SE) in LDL-C (Hopkins) by TG level

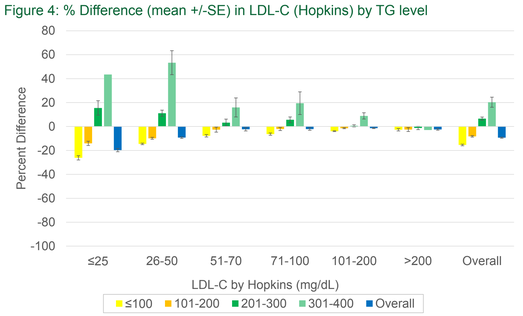


Figure 5: Difference plot for LDL-C ≤100 by Direct measurement and Preparative Ultracentrifugation

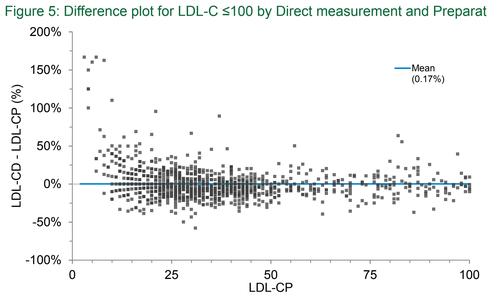
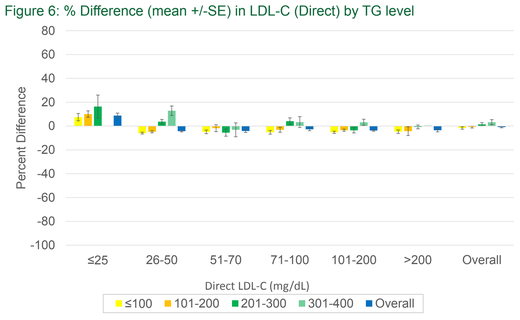


Figure 6: % Difference (mean +/-SE) in LDL-C (Direct) by TG level



Conclusions

- Compared to PUC, both calculated LDL-C methods and direct measurement methods underestimated LDL-C at pre-specified cut-points. While the direct method remained relatively stable, the calculated methods produced estimates that were progressively low as LDL-C decreased below 100 mg/dL.
- As determined by the Friedewald formula, increasing TG levels result in greater calculation error when LDL-C ≤100 mg/dL, reaching bias levels as high as 65% when LDL-C ≤25 mg/dL and TG >200mg/dL.
- At TG levels ≤200 mg/dL, the Hopkins formula also underestimates LDL-C, though not to the extent of Friedewald. However, Hopkins overestimates LDL-C when triglycerides are >200 mg/dL.
- Overall, the "direct" homogenous method for measuring LDL-C was more reliable and did not show increasing differences with various TG cut-points. However, this finding cannot be applied to other direct measurement methods as their performance has been reported to vary.
- For drugs in development, accurate measurement of key efficacy parameters, such as LDL-C, is of paramount importance to assess response to drug. Underestimation of LDL-C may lead to overestimation of treatment effect. Correct clinical trial design is essential for regulatory approval.
- Recent work demonstrating additional clinical benefit with improved cardiovascular outcomes when LDL-C levels are reduced below previous targets with combination lipid modifying therapies[5] suggests that clinicians should exercise caution when interpreting calculated laboratory values of LDL- C, as under or overestimation of LDL-C levels can lead to erroneous treatment decisions.

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