

Comparison of Direct and Indirect Measurement of LDL-C in HIV-Infected Individuals: ACTG 5087

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Background: Hypertriglyceridemia is common in HIV-infected individuals on antiretroviral therapy. Triglyceride (TG) levels >400 mg/dL interfere with the accurate determination of low-density lipoproteins (LDL-C) by the Friedewald equation, making it difficult to assess coronary heart disease risk. **Objective:** The objective of this study is to compare the agreement of the direct LDL-C assay and the Friedewald equation with a reference ultracentrifugation method in the estimation of LDL-C concentrations. **Method:** Samples from ACTG 5087 were assayed by ultracentrifugation and a direct enzymatic assay and calculated using the Friedewald equation. **Results:** In subjects with TG <400 mg/dL ($n = 271$), 90% of the direct LDL-C values and Friedewald calculations were within 30 mg/dL and 32 mg/dL of the ultracentrifugation values, respectively. With TG ≥ 400 mg/dL ($n = 186$), 90% of the direct assay and Friedewald observations were within 68 mg/dL and 120 mg/dL of the ultracentrifugation results, respectively. Only 27% of the LDL-C values were within 15 mg/dL of the ultracentrifugation LDL-C results for direct assay and 16.3% for the Friedewald equation. **Conclusion:** The direct LDL-C assay and the calculated LDL-C values did not display adequate agreement with the reference ultracentrifugation method. In subjects with TG >400 mg/dL, the direct assay overestimates the actual LDL-C whereas the Friedewald calculation underestimates the actual LDL. Clinical usage of these methods may lead to misclassification of the severity of dyslipidemia, resulting in improper management. **Key words:** *low-density lipoproteins, hypertriglyceridemia, Friedewald equation, coronary heart disease*

Several cohort studies suggest that HIV-infected individuals may be at higher risk for the development of coronary heart disease (CHD).^{1,2} The type of dyslipidemia most often encountered among persons with HIV resembles that seen in persons with diabetes mellitus. This dyslipidemia is characterized by high triglycerides (TG), low high-density lipoprotein (HDL-C), and elevated or normal low-density lipoprotein (LDL-C).³⁻⁶ Hypertriglyceridemia is common in HIV-infected individuals on highly active antiretroviral therapy (HAART) and is an independent risk factor for CHD.⁷⁻¹⁰ Because the risk of a myocardial infarction is most closely linked to elevated LDL-C values, it is important to be able to accurately determine LDL-C in persons with hypertriglyceridemia. Unfortunately, LDL-C values that are calculated using the estimation method derived by Friedewald et al.¹¹ are inaccurate when TG exceeds 400 mg/dL. Therefore, LDL-C values in patients with elevated TG must traditionally be measured directly by ultracentrifugation. This method is time consuming,

requires significant technical skill, and is costly. A direct enzymatic LDL-C assay (Genzyme Diagnostics; Cambridge, Massachusetts, USA) has been developed for this purpose. This direct assay requires less laboratory time and technical skills and is significantly lower in cost than the ultracentrifugation method. However there have been few comparisons of this assay to standard reference methods in patients with high triglycerides, and there has been no evaluation of this assay in HIV-infected persons. In addition, the applicability of the estimation method proposed by Friedewald has not been evaluated in the dyslipidemia observed in persons with HIV infection. Development of more

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cost-effective methods of LDL-C measurement would be useful in both clinical trials and practice to identify patients with elevated LDL-C. Therefore, we sought to evaluate the precision of a direct enzymatic assay and Friedewald equation in comparison to a reference ultracentrifugation method of LDL-C measurement in HIV-infected persons with and without dyslipidemia who were being screened and enrolled in a trial of lipid-lowering therapy.

METHOD

Objective

The objective of this analysis is to describe and compare the agreement of the direct enzyme (Genzyme®) assay run on the Hitachi 917 and the Friedewald equation with lipoprotein fractionation by ultracentrifugation in the quantitation of LDL-C.

Design

ACTG 5087 was a randomized, open-label, comparative 48-week clinical trial to determine whether fenofibrate was noninferior to pravastatin with respect to the management of HIV-related hyperlipidemia.¹² All screening and week 12 (overnight fasting) lipid profiles collected in ACTG 5087 were shipped to Quest Diagnostics, Inc. (San Juan Capistrano, California, USA) and assayed by ultracentrifugation (i.e., the reference method). Samples with a sufficient amount of excess serum also had the direct assay performed. LDL-C estimates using Friedewald were calculated from the total cholesterol, TG, and HDL-C values determined by standard techniques on the Roche Hitachi 917 (Roche Diagnostics, Basel Switzerland, and Hitachi Ltd., Tokyo, Japan). LDL-C from ultracentrifugation was determined after ultracentrifugation using a TL120 tabletop ultracentrifuge. HDL-C and LDL-C were isolated together by spinning them into a low salt solution (0.9% NaCl). HDL-C alone was obtained by centrifugation into a high salt solution (16.7% NaCl). LDL-C was calculated by subtracting the HDL-C (16.7% bottom) from the LDL-C + HDL-C (0.9% bottom).

The direct and calculated LDL-C values were evaluated for "agreement" with ultracentrifugation. In addition, a comparison among the three methods for determination of subject eligibility for

ACTG 5087 (LDL-C >130 mg/dL) was performed. Finally, week 12 data were examined to investigate whether the LDL-C measurement methods classify subjects similarly with respect to the primary study endpoint for ACTG 5087. The primary study endpoint defined an adequate response to therapy as subjects having LDL-C \leq 100 mg/dL for individuals with two or more cardiovascular (CV) risk factors and an LDL-C <130 mg/dL for individuals with fewer than two CV risk factors. Data were also classified into the National Cholesterol Education Panel (NCEP) categories (<100, 100–129, 130–159, 160–189, 190 and above) to determine whether the measurement methods similarly classify subjects with respect to NCEP classification.⁸

Definitions and Statistical Analyses

Agreement for direct and calculated was defined as having 90% of the direct and calculated values within 15 units of the respective ultracentrifugation reference values. Fifteen units were chosen based upon the NCEP classification system and acceptable variability of individual assay results.

The *Total Deviation Index [TDI (0.90)]* is a measure that indicates that 90% of the paired observations (direct with reference or calculated with reference) are within the identified value of the statistic.¹³

The *Coverage Probability [CP (15)]* is a measure (bounded between 0 and 1) that indicates the percentage of direct (or calculated) observations that are within 15 units of their respective target reference values.¹³

The *Concordance Correlation Coefficient (CCC)* is a measure of agreement that takes a value of 1 indicating perfect agreement, a value of -1 indicating perfect disagreement, and a value of 0 indicating no agreement.¹³

There are two possible sources of a lack of agreement: (1) large within-sample variation (imprecision), and (2) a scale shift in marginal distributions (inaccuracy, i.e., a calibration problem). *Accuracy* and *precision* measures are defined such that 0 represents no agreement and 1 represents perfect agreement.

Graphical techniques¹⁴ are used to display the estimated error of the direct assay and the Friedewald equation as a function of TG. Analyses were stratified by TG level (<400 vs. \geq 400 mg/dL). Sensitivity, specificity, and predictive values were used to evaluate categorical classification.

RESULTS

When TG ≥ 400 mg/dL, the direct assay is biased and overestimates the “true” LDL-C as measured by ultracentrifugation (**Figure 1A**). The bias becomes greater with increasing TG. The variability of the error also appears to increase as TG increases.

Friedewald calculation is also biased and underestimates the true LDL-C (**Figure 1B**). The bias becomes greater with increasing TG. The variability of the error also increases with increasing TG.

Table 1 summarizes agreement statistics for the direct assay and the Friedewald calculation by TG level. When TG < 400 mg/dL, 90% of observations

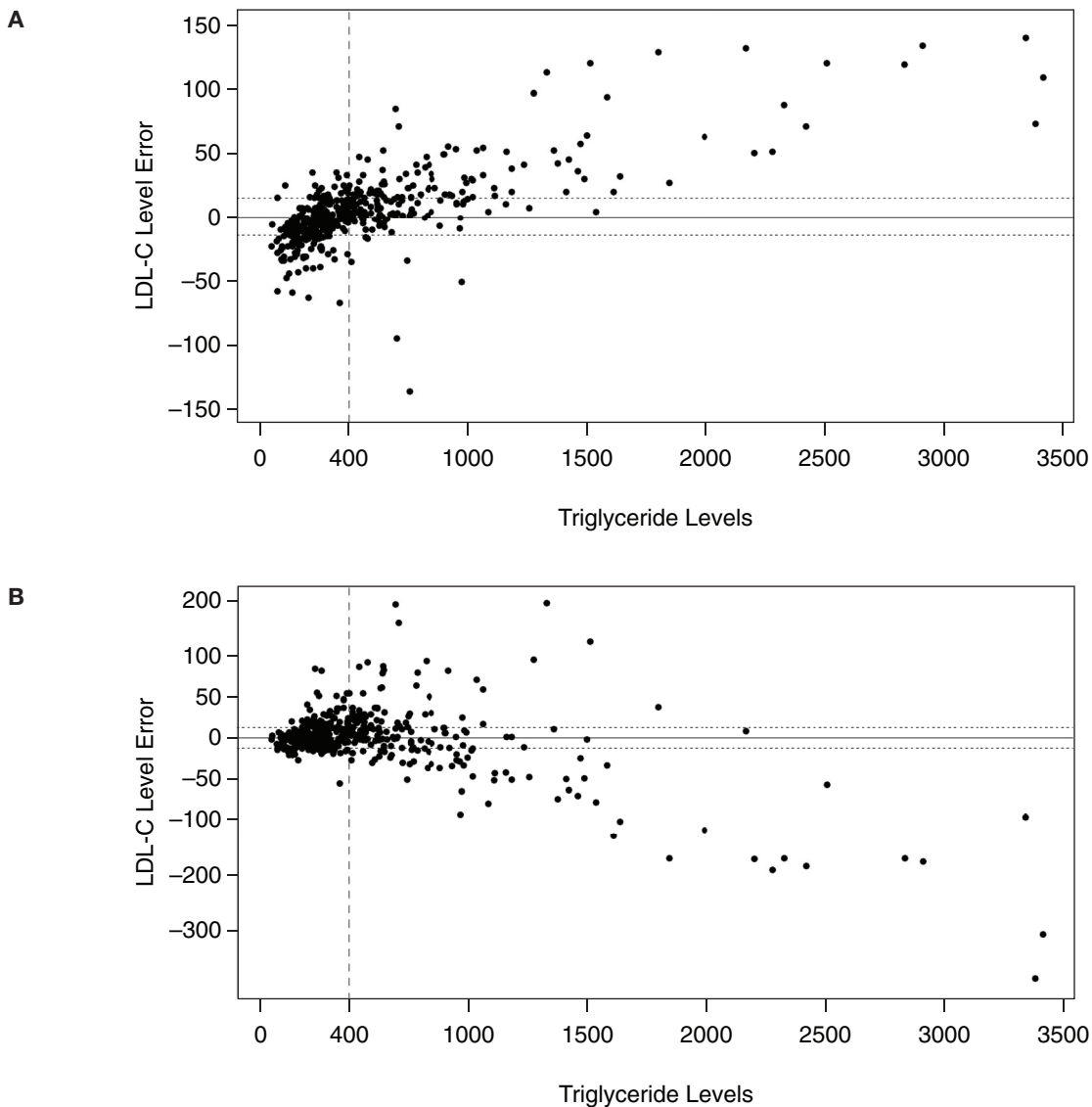


Figure 1. Plots of LDL-C measurement error levels when comparing standard ultracentrifugation method to (A) direct assay and (B) Friedewald calculation methods. Y-axis indicates the absolute difference in assay methods where ultracentrifugation is the standard. For example, zero would mean that standard assay agrees with alternate method. Horizontal dashed lines above and below zero indicate ± 15 mg/dL.

Table 1. Agreement statistics for the direct assay and Friedewald equation versus ultracentrifugation method.

Triglycerides	Direct assay		Friedewald equation	
	Estimate	95% CL	Estimate	95% CL
<400 mg/dL				
<i>n</i> = 271				
Concordance Correlation Coefficient	0.902	0.887	0.892	0.875
Precision	0.923	0.911	0.896	0.879
Accuracy	0.977	0.968	0.996	0.989
TDI (0.90)	29.51	31.53	32.44	34.81
CP (15)	0.598	0.567	0.552	0.519
>400 mg/dL				
<i>n</i> = 186				
Concordance Correlation Coefficient	0.557	0.490	0.492	0.445
Precision	0.640	0.571	0.650	0.582
Accuracy	0.870	0.826	0.758	0.717
TDI (0.90)	68.14	73.53	119.57	130.25
CP (15)	0.270	0.248	0.163	0.149

Note: TDI = Total Deviation Index: a measure that indicates that 90% of the paired observations (direct assay or Friedewald equation compared to ultracentrifugation) are within the identified value of the statistic. Thus, for example, 90% of measured values were within 29.51 mg/dL by direct assay measurement compared to ultracentrifugation when the triglyceride (TG) level was <400 mg/dL. CP = Coverage Probability: a measure (bounded between 0 and 1) that indicates the percentage of direct assay or Friedewald equation observations that are within 15 mg/dL of their respective target ultracentrifugation values. Thus, for example, nearly 60% of direct assay measures are ± 15 mg/dL of ultracentrifugation values when the TG level is <400 mg/dL. The Concordance Correlation Coefficient is a measure of agreement that takes a value of 1 indicating perfect agreement, a value of -1 indicating perfect disagreement, and a value of 0 indicating no agreement. There are two possible sources of a lack of agreement: (1) large within-sample variation (imprecision), and (2) from a scale shift in marginal distributions (inaccuracy, i.e., a calibration problem). *Accuracy* and *precision* measures are defined such that 0 represents no agreement and 1 represents perfect agreement. CL = Confidence Limit: the boundary of a 95% confidence interval (i.e., the lower limit when measuring accuracy, precision, CCC, and CP; the upper limit when measuring TDI).

are within 30 units of reference and 60% of observations are within 15 units of reference by the direct assay. By Friedewald, 90% of observations are within 32 units of reference and 55% of observations are within 15 units of reference. When TG ≥ 400 mg/dL, 90% of observations are within 68 units of reference and 27% of observations are within 15 units of reference by the direct assay. By Friedewald, 90% of observations are within 120 units of reference and 16% of observations are within 15 units of reference.

The direct assay correctly classifies 62% into NCEP categories for TG <400, while Friedewald

correctly classifies 67%. When TG is ≥ 400 , the direct assay correctly classifies 57%, while Friedewald correctly classifies 54% (**Table 2**).

CONCLUSION

Measurement of LDL-C by the direct enzymatic (Genzyme[®]) method or estimation by the Friedewald equation in persons with HIV infection and hypertriglyceridemia does not agree with the reference gold standard method of ultracentrifugation. Clinical trials should not utilize the direct enzymatic assay and Friedewald methods particularly

Table 2. Classification results at time of eligibility, response to treatment, NCEP categories, and low-density lipoprotein (LDL-C) above or below 160 mg/dL by the direct assay and Friedewald equation compared to ultracentrifugation method

Triglycerides	Direct assay	Friedewald equation
<400 mg/dL		
Eligibility for study		
<i>n</i>	271	271
Sensitivity	90.00%	93.16%
Specificity	87.65%	74.07%
PPV	94.48%	89.39%
NPV	78.89%	82.19%
Response determination		
<i>n</i>	114	114
Sensitivity	100%	87.50%
Specificity	93.88%	96.94%
PPV	72.73%	82.35%
NPV	100.00%	97.94%
NCEP category		
% Correct	62.36%	67.16%
LDL <160 mg/dL		
% Correct	94.70%	86.75%
LDL >160 mg/dL	75.83%	90.00%
>400 mg/dL		
Eligibility for study		
<i>n</i>	186	186
Sensitivity	90.90%	75.00%
Specificity	73.94%	78.87%
PPV	51.95%	52.38%
NPV	96.33%	91.06%
Response determination		
<i>n</i>	31	31
Sensitivity	62.50%	75.00%
Specificity	95.65%	91.30%
PPV	83.33%	75.00%
NPV	88.00%	91.30%
NCEP category		
% Correct	56.99%	53.76%
LDL <160 mg/dL		
% Correct	93.57%	86.55%
LDL >160 mg/dL	86.67%	93.33%

Note: PPV = positive predictive value; NPV = negative predictive value; NCEP = National Cholesterol Education Project.

when TG values are greater than 400 mg/dL. Clinical usage of these methods may lead to misclassification of the severity of dyslipidemia.

DISCUSSION

The direct assay and the Friedewald equation did not display adequate agreement with the reference method (i.e., ultracentrifugation) when LDL-C was measured in HIV-infected subjects. Both have a bias that increases with increasing TG. The lack of agreement of the direct assay and Friedewald equation with ultracentrifugation is due to a lack of precision (a variance reduction problem) rather than a lack of accuracy (calibration problem) when TG is <400 mg/dL. Thus efforts to develop a "correction factor" to adjust measurements for bias are not likely to be successful. Both accuracy and precision measures are problematic for both methods when TG \geq 400 mg/dL. The level of agreement of the direct assay with ultracentrifugation versus the Friedewald equation with ultracentrifugation is comparable. Use of the direct assay appears to provide little benefit over that of the Friedewald equation when LDL-C is measured in HIV-infected subjects.

When we compared the Friedewald and direct assay results to the ultracentrifugation results in the ACTG 5087 main study, we found that we would have erroneously misclassified subjects according to NCEP categories by 43%–46% with either method when the TG level is >400 mg/dL and by 33%–38% when the TG level is <400 mg/dL. This has significant implications when conducting clinical trials where decision points are made in regard to treatment and determination of statistical significance of efficacy. The findings of our current study are in agreement with those of Miller et al. who compared four different direct measurements of LDL-C assays.¹⁵ They concluded that the Genzyme[®] assay was no better than Friedewald at TG levels <300–400 mg/dL and noted a progressively poor performance as the TG increased. Their study was limited by the small number of only three samples that contained TG >600 mg/dL. In comparison, our study had 99 subjects with TG >600 mg/dL. Three other studies that noted a general acceptance of the direct assays compared with a reference standard also failed to have samples with excessive TG.^{16–18} In ACTG 5087, the median TG at baseline was 325 mg/dL with values ranging up to

more than 3000 mg/dL.¹² Similarly, another ACTG study evaluating the efficacy of fenofibrate and fish oil had median TG of 667 mg/dL at entry, again with TG up to 3000 mg/dL.¹⁹ Given the frequency of severe hypertriglyceridemia in the HIV-infected population, these direct LDL-C assays provide limited utility. This inability to estimate LDL-C accurately would be particularly problematic in clinical trials.

These results of the current study may also have significant impact on clinical practice in HIV-infected patients with hypertriglyceridemia. The NCEP Adult Treatment Panel-III and Infectious Disease Society of America guidelines recommend therapy targeted toward lowering TG when the TG >500 mg/dL.^{8,20} Once the TG is below 400 mg/dL, calculation of the LDL-C using the Friedewald equation is routinely performed by most laboratories. If the LDL-C is elevated above the NCEP goal, initiation of statin therapy is recommended. Therapeutic decisions are further complicated in persons with HIV, because it is not uncommon to observe high TG levels (> 400 mg/dL) even after various lipid-lowering modalities are used.^{12,19} If the TG remains above 400 mg/dL, the Friedewald calculation could falsely estimate the LDL-C to be at goal and patients would not be offered treatment directed at lowering LDL-C when, in fact, it would be indicated. Conversely, if one used a direct assay, the LDL-C may overestimate the LDL-C and patients could be offered LDL-C-lowering therapy when it is not indicated, potentially putting patients at risks for side effects, drug interactions, and excessive costs.

There are several explanations why the Friedewald equation and the direct assay are not in agreement with the ultracentrifugation assay. The Friedewald equation was derived in populations with familial hyperlipidemia.¹¹ It is likely that the lipid content in these disorders differs from that observed in HIV-infected persons taking HAART. For example, protease inhibitors induce fatty acid synthesis and cholesterol production leading to very high levels of very low-density lipoprotein (VLDL-C) and intermediate-density lipoprotein (IDL) with concomitant decreases in HDL-C.^{4,21-23} The bias observed with the direct assay is unknown. We suspect the increased bias of the direct assay may be due to the altered proportions of IDL and lipoprotein (a), β -VLDL-C, and other intermediate lipoproteins, however we were unable to construct

a formula that would allow us to correct for these particles. Further investigation will be required to determine the precise mechanisms involved.

In keeping with NCEP guidelines, it seems reasonable to offer a TG-lowering agent first when the TGs are above 500 mg/dL. One should keep in mind that, as the TG lowers, there is a shift in the lipoprotein metabolism and the LDL-C may increase. If the TG remains above 400 mg/dL despite lipid-lowering therapy, it may be reasonable to check the LDL-C by ultracentrifugation. If the LDL-C is above clinical goal, therapy with a statin, particularly one with significant TG-lowering activity would be recommended. Additionally one may consider evaluating LDL-C by particle size to look at its atherogenic potential. However, actual levels of LDL-C require methods such as ultracentrifugation to determine the risk of the development of CHD. Alternatively, one could measure other markers to determine cardiac risk, such as apolipoproteins and their ratio, non-HDL-C, and C-reactive proteins. Finally, all interventions must be carefully monitored in the setting of HIV-infected individuals on HAART given the significant drug interactions between the antiretroviral therapy and statins.²⁴⁻²⁹ Thus, we recommend caution in using the Friedewald equation or direct enzymatic assay in clinical trials or clinical practice to determine interventions for dyslipidemia and to assess CHD risk in persons with HIV-associated lipid disorders.

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