Lack of Agreement of Homogeneous Assays with the Reference Method for LDL-Cholesterol May Not Indicate Unreliable Prediction of Risk for Cardiovascular Disease

To the Editor:
The recent evaluation of four homogeneous methods for LDL-cholesterol (LDL-C) by Miller et al. (1) appropriately concludes that the methods do not meet the analytical performance goals of the National Cholesterol Education Program (NCEP) because of excessive deviation from the Reference Method (RM) in the tested samples. The comparison method in this study, the accepted RM for LDL-C, was performed at the CDC, considered the “gold standard” in lipid/lipoprotein testing. Although the study was well designed and the conclusions were certainly supported by the reported observations in the context of current thought about accuracy in lipoprotein testing, an important issue is raised concerning the balance between standardization and innovation.

Standardization of results is considered to be especially important with the lipid and lipoprotein analytes because national cutpoints have been set by expert panels based on cardiovascular disease (CVD) risk relationships and population distributions from large epidemiology studies (2). The CDC, charged with standardizing the research laboratories, developed RMs using techniques derived from the early research methodologies. Subsequently, an expert panel convened by the NCEP recommended that the CDC RMs be continued for standardization of routine laboratories (3). Other analytical methods demonstrated to give acceptable agreement with the RMs can not only be reasonably assured of reliable patient classification based on the recommended cutpoints, but can also relate to the extensive base of research using standardized methods. Nevertheless, although the RMs were chosen by a consensus process, are widely accepted, and have been useful for years in standardizing both research and routine measurements, they have not been demonstrated to provide optimum selection of lipoprotein particles in predicting CVD risk. A possible unfortunate consequence of the emphasis on accuracy and standardization of results in lipoprotein measurement might be to discourage innovation in developing new and improved methods. Thus a new method, such as the homogeneous assays for LDL-C, with obvious advantages in convenience and reproducibility (4) might be rejected for lack of agreement with the RM, although one cannot rule out the possibility that such a method might be demonstrated to better predict CVD risk.

Complicating the evaluation of new methodologies is the complexity of the lipoproteins, with the defined major classes consisting of heterogeneous populations of subparticles varying in composition and physical properties as well as in contribution to CVD. Different separation techniques can select various populations of lipoprotein particles, which will certainly influence their overall association with CVD risk. The differences in the obtained lipoprotein fractions are often not identified by the nomenclature; for example, the population of particles separated by a particular chemical method might not be the same as that by a physical method, such as ultracentrifugation, although the obtained fractions are commonly designated by the same term, e.g., LDL or HDL.

Considering the separation techniques used in the LDL RM, the tedious but robust ultracentrifugation step derived from β-quantification floats any particles with a density <1.006 kg/L, by definition VLDL. This cut is justified as the background solvent density of serum, requiring no adjustment, and is in a zone relatively free of lipoprotein species in most specimens. However, lipoprotein remnants, sometimes present as catabolic products of triglyceride-rich particles and considered to be proatherogenic (5), can occur in both the VLDL range and the intermediate-density range (IDL; density, 1.006–1.019 kg/L), which together with the remainder of “narrow-cut” LDL (density, 1.019–1.063 kg/L) constitute the β-quantification and RM “broad-cut” LDL fraction. In specimens with increased remnants, e.g., in type III hyperlipoproteinemia (dysbetalipoproteinemia), the RM can fractionate a substantial portion of the proatherogenic lipoprotein remnants with VLDL rather than LDL. Because the ultracentrifugation technique separates in the middle of the remnant class, other methods using different principles may not be able to achieve equivalent fractionations.

The second fractionation in the RM, separating HDL from LDL, uses the polyanion, heparin, with a divalent cation, Mn²⁺. Because both HDL and LDL classes are heterogeneous, the specificities of the separations can vary with different reagents and conditions. Recognizing that the associations of LDL-C and HDL-cholesterol (HDL-C) with risk are opposite, the specific fractionation could have considerable effect on the overall association with CVD risk.

Of particular interest is a subclass of apolipoprotein (apo) E-rich particles at the lighter and larger end of the HDL range with properties in some respects more like those of LDL (6). Although the evidence is not conclusive, some have speculated that this particle might be proatherogenic, in contrast to most particles in the HDL range, which are considered protective. This apo E-rich HDL particle has been reported to fractionate primarily with HDL under the conditions of the heparin-Mn²⁺ RM, but can be fractionated with LDL by other separation methods (6).

Within this context, consider the agreement in separation and quantification of LDL-C reported in the study by Miller et al. (1). The largest discrepancies were observed in individuals with type III hyperlipoproteinemia, with two of the homogeneous assays substantially overestimating and one underestimating LDL-C compared with the RM, leading to the conventional conclusion that the homogeneous assays...
must be inaccurate. However, recognizing the increase in proatherogenic remnant lipoproteins in type III hyperlipoproteinemia, the overestimations may be a result of the homogeneous assays including more of the remnants with LDL, which could improve the association with CVD risk. On the other hand, the method that tends to underestimate LDL-C in type III individuals might be more specific for narrow-cut LDL in suppressing measurement of remnant lipoprotein-cholesterol.

The second major category of discrepancy by the homogeneous assays seems to be related to a general tendency to overestimate LDL-C with increasing triglycerides, indicated by the generally positive slopes on the bias plots. The overestimation might be an issue of specificity associated with increasing amounts of remnant lipoproteins, as explained previously. On the other hand, the discrepancies might be an issue of ruggedness, a lack of capacity, or interference from overloading of the reagent’s capability to suppress measurement of cholesterol in increasing amounts of VLDL and chylomicrons. The two alternatives might be distinguished by further analysis of the Miller et al. (1) comparison data. Because remnants are enriched in cholesterol relative to triglycerides, stratification of the samples by the ratio of cholesterol to triglycerides in VLDL [or the common surrogate, ratio of VLDL-cholesterol (VLDL-C) to total serum triglycerides] might distinguish samples containing more remnants from those with fewer remnants (7). Comparison of LDL-C difference plots vs total triglyceride for the subsets with the highest and the lowest ratios might indicate whether the discrepant results for each homogeneous assay are primarily a result of the inclusion of remnant lipoproteins or of interference/overloading of the suppression capability.

Similar observations can be made about the separation between LDL and HDL, especially in the fractionation of the apo E-rich HDL particle. If indeed this particle is demonstrated to be proatherogenic, then a method that includes the particle subclass with LDL might better associate with CHD risk than the LDL-C RM, with similar but inverse considerations for HDL-C methods. The study by Miller et al. (1) was not designed to address this issue, although an approximation might be obtained by examining within a subset of samples with low serum triglycerides (little VLDL-C) the LDL-C differences plotted vs RM HDL-C. Because the apo E-rich HDL fraction is more likely to be present when HDL concentrations are high, the slopes of the bias plots could be informative. In future studies, comparing the methods on a VLDL-free (density >1.006 kg/L) fraction obtained by ultracentrifugation might be useful to isolate the LDL-HDL from the LDL-VLDL separations.

The preceding observations point out additional analyses of the study data presented by Miller et al. (1) that might shed light on the nature of the separation specificities and may provide guidance for future studies of the homogeneous assays. In offering these suggestions, I wish to emphasize that no criticism is implied of either the organization or the interpretation of this excellent study, nor in the choice and use of the RMs for standardization of LDL-C and HDL-C. Standardization, essential for achieving reliable classification of patients, requires a target, and the RMs were recommended as such by experts based on sound rationale. The CDC has provided an important service in making the RMs conveniently available, facilitating national and international standardization programs. Nevertheless, even in acknowledging the importance of efforts to achieve consistent accuracy, improvements in the methodology must still be encouraged.

These observations certainly do not prove that one or more of the homogeneous assays might be better than the RM in identifying patients at risk, simply that the possibility cannot be discounted without additional study. The responsibility should be on the developer/manufacturer to demonstrate that a method, although lacking agreement with the RM, does have compensating advantages. Well-designed case-control studies might be sufficient to demonstrate the association with an accepted index of coronary heart disease risk. Provided that a method can be demonstrated to be reasonably unaffected by freezing, a convenient and cost-effective approach might be a retrospective nested case–control study on archived specimens. In addition, it is advisable that any deviations in accuracy from the accepted definitions and RMs be indicated by the nomenclature. A homogeneous assay nominally targeted on the LDL class but which in fact actually measures a population of lipoprotein particles different from that measured by the RM, e.g., in excluding or including remnant lipoproteins, should have a product name and labeling that reflects the actual specificity.

In summary, Miller et al. (1) appropriately conclude that the four homogeneous assays for LDL-C do not meet accepted criteria for total error, compromising their ability to reliably classify patients, especially those with unusual lipoprotein patterns, based on NCEP cutpoints. However, because the RM for LDL-C (as well as for HDL-C) has not been proven to optimize separation of lipoprotein particles in characterizing risk, one cannot necessarily conclude that the homogeneous methods will not be useful in predicting risk of CVD. The homogeneous assays would seem to have a sufficient advantage in convenience over existing methods to warrant undertaking additional studies to demonstrate their clinical utility in predicting CVD risk.

References
4. Nauck M, Warnick GR, Rifai N. Methods for


G. Russell Warnick
Pacific Biometrics Research Foundation
24415 SE 156 St.
Issaquah, WA 98027
E-mail grwarnick@hotmail.com

Authors of the article cited above respond:

To the Editor:
The comments by Warnick are provocative regarding the specificity of the β-quantification reference method (RM) for LDL-cholesterol (LDL-C). The RM has provided the basis for current method standardization and is founded on decades of epidemiologic data for risk evaluation of cardiovascular disease. It is interesting to consider that the RM may not optimally fractionate lipoprotein molecules in terms of association with cardiovascular disease and that newer methods based on other analytic technologies might improve the prediction of risk. Additional clinical outcome studies are necessary to establish the predictive power for clinical risk assessment of methods with different specificities for lipid subfractions.

Application of the data analysis approach suggested by Warnick to the data reported by Miller et al. (1) provides additional insight into the lipid particle specificities of the four homogeneous LDL-C assays. Stratification of the subjects’ results by the ratio of VLDL-cholesterol (VLDL-C) to total triglycerides (TGs) presumably identifies specimens enriched in VLDL remnant lipoproteins. Individuals with high ratios include those with higher concentrations of remnant lipoproteins, which have atherogenic risk potential. Table 1 shows regression analysis results for plots of the LDL-C difference between homogeneous and RM results vs TG concentration for 32 individuals with high (≥0.22) and 65 individuals with low (<0.22) VLDL-C/TG ratios (excluding 3 individuals with Fredrickson type III hyperlipidemia from the high-ratio group). The slopes indicate that the Roche method had increasing LDL-C biases with increasing TGs for high-ratio specimens and virtually no change with increasing TGs for low-ratio specimens. This observation is consistent with the assay detecting remnant lipoproteins but not being influenced by increasing abnormal amounts of intact VLDL. In contrast, the other three assays showed a similar increase in LDL response to increasing TGs in both high- and low-ratio specimens, which may be consistent with an undesired nonspecific overloading by VLDL. However, because remnant particles were not measured in these specimens, the actual method sensitivity to remnant VLDL will require additional investigation.

The impact of increasing HDL on the specificity of the homogeneous methods for LDL-C was less clear. As stated in the original report (1), the Roche method was relatively insensitive to the HDL-cholesterol (HDL-C) concentration in the specimens, whereas the other three methods showed various degrees of bias at low or high HDL-C concentrations. Warnick suggests that specimens with low TGs and higher HDL-C are more likely to include potentially atherogenic apolipoprotein E particles in the RM HDL fraction. Plots of the LDL-C difference between homogeneous and RM results vs HDL-C concentration for specimens with TGs <1500 mg/L had a tendency toward increasing negative bias at higher HDL-C for the three homogeneous methods that were sensitive to HDL-C. The Roche method was insensitive to HDL-C under these conditions. These observations are not conclusive but are consistent with a possible difference in sensitivity to the apolipoprotein E particle among the assays. However, the presence of apolipoprotein E in these specimens was not determined, and the sensitivity of the homogeneous LDL-C methods to potentially atherogenic particles in the HDL fraction requires further investigation.

Reevaluation of the data describing the performance of four homogeneous LDL-C methods vs the β-quantification RM suggests that at least one method may measure potentially atherogenic VLDL remnant

### Table 1. Regression analysis of LDL-C difference between homogeneous and RM results vs TG concentration for specimens with high (≥0.22) and low (<0.22) VLDL-C/TG ratios.

<table>
<thead>
<tr>
<th>Ratios</th>
<th>Slope</th>
<th>Intercept, %</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genzyme high</td>
<td>2.90 × 10⁻⁵</td>
<td>-0.0762</td>
<td>0.564</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genzyme low</td>
<td>2.16 × 10⁻⁵</td>
<td>-0.0629</td>
<td>0.511</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ref. Diag. high</td>
<td>1.27 × 10⁻⁵</td>
<td>-0.0774</td>
<td>0.092</td>
<td>0.091</td>
</tr>
<tr>
<td>Ref. Diag. low</td>
<td>1.41 × 10⁻⁵</td>
<td>-0.0486</td>
<td>0.177</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Roche high</td>
<td>4.46 × 10⁻⁵</td>
<td>-0.0541</td>
<td>0.647</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Roche low</td>
<td>-2.21 × 10⁻⁵</td>
<td>-0.0367</td>
<td>5.6e-5</td>
<td>0.953</td>
</tr>
<tr>
<td>Sigma high</td>
<td>6.49 × 10⁻⁵</td>
<td>-0.00969</td>
<td>0.888</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sigma low</td>
<td>6.55 × 10⁻⁵</td>
<td>-0.0386</td>
<td>0.649</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Probability that the slope equals zero for a t-test calculated as (slope/SE of the slope) for n = 2 degrees of freedom (2).

b Probability that the high-ratio and low-ratio slopes are the same for a t-test calculated as (slope₁ - slope₂)/(SE₁² + SE₂²)¹/² for (n₁ + n₂ - 4) degrees of freedom (2).

Reference Diagnostics.
lipoproteins not usually included in the LDL-C fraction. The data also suggest that some homogeneous methods may lack adequate specificity for the principal atherogenic species in the LDL fraction. Further studies of the specificity of homogeneous LDL-C methods should be performed to establish the relationship between the lipid species measured and clinical risk of atherogenic disease. Manufacturers should consider improving the assays to assure measurement of the most atherogenic lipoprotein fractions and thus improve their clinical utility for risk assessment and monitoring response to therapy.

References


W. Greg Miller¹
Parvin P. Waymack²
F. Philip Anderson¹

¹ Department of Pathology
Virginia Commonwealth University Health System
Richmond, VA 23298

² CDC
National Center for Environmental Health
Division of Laboratory Sciences
Special Activities Branch
Atlanta, GA 30341

*Author for correspondence.