Recommendations on Lipoprotein Measurement

From the Working Group on Lipoprotein Measurement

NATIONAL INSTITUTES OF HEALTH
National Heart, Lung, and Blood Institute

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# Table of Contents

**PART ONE: RECOMMENDATIONS FOR MEASUREMENT OF LOW DENSITY LIPOPROTEIN CHOLESTEROL** ................................................................. 1  
TABLE OF CONTENTS .................................................................................................................................................................................. 3  
EXECUTIVE SUMMARY .................................................................................................................................................................................. 7  
1. BACKGROUND .................................................................................................................................................................................. 17  
2. SOURCES OF VARIATION IN LDL-CHOLESTEROL CONCENTRATION ..................................................................................... 25  
3. ACCURACY OF LDL-CHOLESTEROL MEASUREMENTS ............................................................................................................. 31  
4. DIRECT METHODS FOR LDL MEASUREMENT ................................................................................................................................. 35  
5. RECOMMENDATIONS ........................................................................................................................................................................... 45  
6. CALCULATIONS .................................................................................................................................................................................. 53  
REFERENCES ......................................................................................................................................................................................... 55  

**PART TWO: RECOMMENDATIONS FOR MEASUREMENT OF HIGH DENSITY LIPOPROTEIN CHOLESTEROL** ................................................................. 63  
TABLE OF CONTENTS .................................................................................................................................................................................. 65  
EXECUTIVE SUMMARY .................................................................................................................................................................................. 67  
1. BACKGROUND .................................................................................................................................................................................. 77  
2. CONSIDERATIONS REGARDING ACCURACY IN HDL-CHOLESTEROL MEASUREMENT .............................................................................................................. 81  
3. SOURCES OF VARIATION IN HDL-CHOLESTEROL CONCENTRATION ..................................................................................... 83  
4. CRITICAL REVIEW OF HDL-CHOLESTEROL METHODS ............................................................................................................. 93  
5. CURRENT LABORATORY PERFORMANCE IN HDL-CHOLESTEROL ANALYSIS ..................................................................................... 101  
6. PERFORMANCE STANDARDS .................................................................................................................................................................. 105  
7. RECOMMENDATIONS ........................................................................................................................................................................... 109  
REFERENCES ......................................................................................................................................................................................... 115
PART THREE: RECOMMENDATIONS FOR TRIGLYCERIDE MEASUREMENT .......... 125
TABLE OF CONTENTS ..................................................................................................................... 127
EXECUTIVE SUMMARY ................................................................................................................... 129
1. BACKGROUND ............................................................................................................................ 137
2. METHODS FOR TRIGLYCERIDE MEASUREMENT ................................................................... 143
3. MAJOR ISSUES CONCERNING TRIGLYCERIDE MEASUREMENT ....................................... 147
4. TRIGLYCERIDE BLANK MEASUREMENTS ............................................................................... 153
5. TRIGLYCERIDE STANDARDS .................................................................................................... 155
6. PERFORMANCE AND STANDARDIZATION OF TRIGLYCERIDE MEASUREMENTS ... 157
REFERENCES ...................................................................................................................................... 165

APPENDIX I: GLOSSARY OF ACRONYMS .................................................................................. 171
APPENDIX II: ANALYTICAL GOALS FOR LIPOPROTEIN CHOLESTEROL AND PROCEDURE TO MEASURE INDIVIDUAL LABORATORY CONFORMANCE TO GOALS ............................................................................................................................ 173
APPENDIX III: NATIONAL REFERENCE METHOD LABORATORY NETWORK PARTICIPATING LABORATORIES ............................................................................................................................. 185
Part One:
Recommendations for Measurement of Low Density Lipoprotein Cholesterol

National Cholesterol Education Program
Working Group on Lipoprotein Measurement
Recommendations on Lipoprotein Measurement—LDL
# Table of Contents

EXECUTIVE SUMMARY ................................................. 7  
Considerations .......................................................... 7  
Sources of Variation in LDL-Cholesterol Measurement .............................. 8  
Recommendations ........................................................ 9  
  General Recommendations .............................................. 9  
  Recommendations for Manufacturers .................................. 12  
  Recommendations for Health Care Providers .......................... 12  
  Recommendations for Laboratories .................................. 14  
  Recommendations for Government Agencies and Other Professional Groups .... 15  
  Recommendations for Further Research ................................ 15  

1. BACKGROUND .......................................................... 17  
  Lipoprotein Contributors to Total Cholesterol ........................... 18  
  Major Issues Concerning LDL-Cholesterol Measurement ...................... 19  
  Beta Quantification .................................................... 19  
  The Friedewald Equation .............................................. 20  
  Potentially Atherogenic Particles ...................................... 21  

2. SOURCES OF VARIATION IN LDL-CHOLESTEROL CONCENTRATION .......... 25  
  Physiological Variation ................................................ 25  
  Analytical Variation .................................................. 25  
  Beta Quantification .................................................... 25  
  The Friedewald Equation .............................................. 27  
  Surveys of Clinical Laboratories ...................................... 27  
  Number of Specimens Required To Estimate LDL-Cholesterol .................. 29  

3. ACCURACY OF LDL-CHOLESTEROL MEASUREMENTS ........................... 31  

4. DIRECT METHODS FOR LDL MEASUREMENT ................................ 35  
  Precipitation Methods ................................................ 35  
  Electrophoretic Methods .............................................. 35
Density Gradient Ultracentrifugation and Nuclear Magnetic Resonance .......................................... 36
Recommended Methods ................................................................. 36
Standardization of LDL-Cholesterol Measurements ................................................................. 37
Criteria for Accuracy and Precision ....................................................................................... 38
Controllable Preanalytical Factors That Can Affect LDL-Cholesterol Measurements .......... 40
  Fasting Samples ........................................................................... 41
  Serum-Plasma Differences .......................................................... 43
  Posture ....................................................................................... 44
  Frozen Specimens ....................................................................... 44

5. RECOMMENDATIONS ..................................................................... 45
   General Recommendations .......................................................... 45
     Maintaining Linkage With the Existing Epidemiological Database .................................. 45
     Reference Method ................................................................... 45
     Criteria for Analytical Performance ................................................................................. 46
     Routine Method ......................................................................... 46
   Recommendations for Manufacturers .................................................................................. 47
   Recommendations for Health Care Providers .................................................................... 47
     Understand What Is Being Measured .............................................................................. 47
     Measure LDL-Cholesterol in Fasting Samples ................................................................ 48
     Use of Serum and Plasma ............................................................................................... 48
     Stored Samples ......................................................................................... 48
     Need for Serial Measurements in Individuals ................................................................ 48
     Screening ....................................................................................... 49
   Recommendations for Laboratories ..................................................................................... 49
   Recommendations for Government Agencies and Other Professional Groups .............. 50
   Recommendations for Further Research ........................................................................... 50

6. CALCULATIONS ............................................................................. 53

REFERENCES .................................................................................. 55

Table

1  NCEP Adult Treatment Panel II Categories for Total and LDL-Cholesterol Concentrations for Patients With and Without Coronary Heart Disease .......................................................... 17
2  NCEP Laboratory Standardization Panel Recommendations for Accuracy and Precision of Clinical Total Cholesterol Measurements ............................................................. 18
3  Plasma Lipoproteins ........................................................................ 19
4  Contribution of Lp(a)-Cholesterol to Measured LDL-Cholesterol Values ........................ 21
5  Lp(a) Concentrations in Various Populations ..................................................................... 22
6  Estimates of Analytical Variation in LDL-Cholesterol Measurements (Standardized Laboratories) ......................................................................................................................... 26
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Estimates of Total Analytical Variation in LDL-Cholesterol Measurements</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Total Coefficient of Variation of Mean LDL-Cholesterol Concentration as a Function of the Number of Specimens Analyzed</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Range of Uncertainty of an Individual’s Average LDL-Cholesterol Concentration at Selected LDL-Cholesterol Levels</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>Use of the Friedewald Equation in Various Studies</td>
<td>32</td>
</tr>
<tr>
<td>11</td>
<td>Current CDC-NHLBI Criteria for Acceptable Performance for Total Cholesterol, Triglyceride, and HDL-Cholesterol Measurements</td>
<td>37</td>
</tr>
<tr>
<td>12</td>
<td>Number (n) of Serial Samples Required To Establish the Usual LDL-Cholesterol Value of an Average Subject Within Selected Error Limits</td>
<td>38</td>
</tr>
<tr>
<td>13</td>
<td>Analytical Goals for the Total Error of Singular Results for LDL-Cholesterol in Relation to Various Statistical Models and the Goals for Analytic Bias and Imprecision</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>Effect of Bias in Cholesterol, Triglyceride, and HDL-Cholesterol Measurements on LDL-Cholesterol Values Estimated With the Friedewald Equation</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>Serum Triglyceride Concentrations in Men Fasting for Various Periods Before Blood Sampling</td>
<td>42</td>
</tr>
<tr>
<td>16</td>
<td>Serum Triglyceride Concentrations in Women Fasting for Various Periods Before Blood Sampling</td>
<td>43</td>
</tr>
</tbody>
</table>
Elevation of low density lipoprotein (LDL)-cholesterol constitutes a major risk factor for the development of coronary heart disease (CHD). In humans, LDL carries most of the circulating cholesterol, and in many cases, the assessment of total cholesterol concentration provides a useful surrogate indicator of LDL-cholesterol concentration. It is necessary in the diagnosis and treatment of hyperlipidemia, however, to assess the distribution of cholesterol among the major plasma lipoproteins, particularly LDL and high density lipoproteins (HDL). It is important therefore to establish recommendations for the reliable measurement of not only total cholesterol but LDL- and HDL-cholesterol and plasma triglycerides as well. Recommendations for reliable total cholesterol measurements were developed by the National Cholesterol Education Program (NCEP) Laboratory Standardization Panel in 1990. For the past several years, the NCEP Working Group on Lipoprotein Measurement has been developing recommendations for LDL- and HDL-cholesterol measurements and triglyceride measurement. The working group’s recommendations for LDL-cholesterol measurement are summarized in this paper along with some of the issues that the group considered in developing the recommendations.

CONSIDERATIONS

Total cholesterol in humans is distributed primarily among three major lipoprotein classes: very low density lipoproteins (VLDL), LDL, and HDL. Smaller amounts of cholesterol are also contained in two minor lipoprotein classes: intermediate density lipoprotein (IDL) and lipoprotein (a) [Lp(a)]. In normal individuals, the minor lipoprotein classes can be expected to contribute on average about 2-4 mg/dL to the total cholesterol measurement. IDL and Lp(a) are themselves atherogenic, however, and on average, their concentrations can be expected to be higher in patients with CHD and in patients at risk for developing CHD by virtue of a dyslipoproteinemia. LDL-cholesterol has been measured using one of two methods in almost all epidemiological and case-control studies on which estimates of the association of LDL-cholesterol level with coronary heart disease are based. Both of these methods involve the measurement of total cholesterol, triglycerides, and HDL-cholesterol. In the more commonly used method, LDL-cholesterol concentration is estimated from these three measurements using the Friedewald equation:

\[ \text{[LDL-chol]} = \text{[Total chol]} - \text{[HDL-chol]} - \frac{\text{[TG]}}{5} \]

where all concentrations are expressed in mg/dL.\(^1\)

The less commonly used method, which is generally referred to as “beta quantification,” requires the use of the preparative ultracentrifuge to prepare a plasma or serum fraction that contains only LDL and HDL. The cholesterol content of this fraction is measured, and LDL-cholesterol is calculated by subtracting HDL-cholesterol, which is measured separately. The ultracentrifugal method is not readily available in the routine laboratory, and its use is primarily confined to research laboratories and specialized lipoprotein laboratories. In both of these methods, the contributions of IDL and Lp(a) are included in the LDL-cholesterol measurement. For this reason, what is commonly referred to as “LDL-cholesterol”

\(^1\) Divide triglyceride by 2.17 when concentrations are expressed in mmol/L.
actually represents cholesterol contained in LDL plus IDL plus Lp(a), and the measurement might better be considered to represent the cholesterol contained in several potentially atherogenic particles. As mentioned above, the proportional contribution of IDL and/or Lp(a) to the “LDL-cholesterol measurement” would be expected to be greater in at-risk populations.

Another complicating issue is that at present there is no true reference method for LDL-cholesterol measurement. Unlike total cholesterol, LDL is not a unique molecular species but rather consists of a population of similar particles that vary somewhat in their chemical composition and physical-chemical properties. LDL is defined functionally in terms of the methods used to separate it from other lipoproteins.

Risk-related cutpoints for LDL-cholesterol (potentially atherogenic particles) were recently reaffirmed by the Second Report of the National Cholesterol Education Program Adult Treatment Panel (ATP II) (table ES-1). In order for the common cutpoints to be used successfully, however, it is necessary that the measurements reflect the contributions of all three lipoprotein contributors to the LDL-cholesterol levels derived from the current epidemiological database. In the absence of a true reference method for LDL, it is necessary to define a reference method that reflects the lipoprotein cholesterol contributors to the current risk estimates. The Working Group on Lipoprotein Measurement takes this approach.

### SOURCES OF VARIATION IN LDL-CHOLESTEROL MEASUREMENT

Variations in LDL-cholesterol concentrations within an individual result from normal physiological fluctuations that occur during the course of day-to-day living and from the analytical error inherent in the measurements. Normal physiological variation occurs independently of analytical error and would occur even under ideal circumstances in which analytical error was zero. Normal physiological variation is seen when an individual is sampled on more than one occasion. Available data suggest that the coefficient of biological variation for LDL-cholesterol lies within the range of about 6 to 11 percent and averages about 8.2 percent.

Analytical variation refers to the fluctuation (i.e., imprecision) of the measurements when a single sample is analyzed several times. Ideally, the measurement would be the same for all replicates, but in practice, replicate measurements in the same sample vary somewhat because of uncertain-

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**TABLE ES-1. NCEP Adult Treatment Panel II categories for total and LDL-cholesterol concentrations for patients with and without coronary heart disease**

<table>
<thead>
<tr>
<th>Total Cholesterol Category</th>
<th>Total Cholesterol mg/dL (^1) (mmol/L)</th>
<th>LDL-Cholesterol mg/dL (^1) (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt;200 (5.17)</td>
<td>Desirable</td>
</tr>
<tr>
<td>Borderline-high</td>
<td>200-239 (5.17-6.18)</td>
<td>Borderline-high risk</td>
</tr>
<tr>
<td>High</td>
<td>&gt;240 (6.21)</td>
<td>High-risk</td>
</tr>
</tbody>
</table>

**Patients With Coronary Heart Disease**

<table>
<thead>
<tr>
<th>LDL-Cholesterol Category</th>
<th>LDL-Cholesterol mg/dL (^1) (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>≤100 (2.6)</td>
</tr>
<tr>
<td>Higher than optimal</td>
<td>&gt;100 (2.6)</td>
</tr>
</tbody>
</table>

\(^1\) To convert mg/dL to mmol/L, divide by 38.7.
ties involved in volume measurements, variation in instrument function, and lot-to-lot variations in reagent formulations. The challenge to the laboratory is to minimize such fluctuation. Currently available information suggests that the analytical coefficient of variation fell in the range of about 3-7 percent in various studies and averaged about 4 percent. With modern laboratory methods, a coefficient of variation of under 4 percent should be readily attainable in the well-controlled clinical laboratory.

The other source of laboratory error is laboratory bias. Laboratory bias is defined as the average deviation of the measured value from the actual value. This issue is of particular concern to the physician who must interpret LDL-cholesterol measurements made in different laboratories. Ideally, a medically useful measurement should be made with a total analytical error of less than 10 percent including both laboratory bias and imprecision. Since LDL-cholesterol is calculated from several primary measurements, the error in the LDL-cholesterol estimate will be influenced by the error in the primary measurements, and the accuracy of an individual measurement will be limited by the biases and imprecision of the methods used to make the primary measurements. The Working Group on Lipoprotein Measurement’s recommendations for reliable LDL-cholesterol measurement represent a compromise between the ideal and that which can be accomplished with existing technology.

RECOMMENDATIONS

The following recommendations are made for measuring LDL-cholesterol. It is recognized that these recommendations may not provide an accurate measurement of LDL-cholesterol, per se, in all individuals but will provide an estimate of the amount of cholesterol carried in total atherogenic particles of d >1.006 kg/L. This includes VLDL remnants, IDL, Lp(a), and LDL. The recommendations are intended to provide accurate and precise measurements that conform to the present epidemiological database relating LDL-cholesterol concentration to cardiovascular risk and to current estimates of the reduction in risk attending LDL-cholesterol lowering. The recommendations will have to be revised as the relative contributions of individual atherogenic particles to risk and treatment modalities directed at lowering the concentration of specific particles are better understood.

General Recommendations

Maintaining Linkage With the Existing Epidemiological Database. Various approaches have been taken in developing methods to measure LDL-cholesterol, and new methods continue to evolve. There is incomplete information available about exactly what is being measured with some of the methods, and there is not yet enough experience with them for them to be relied on by clinicians. The Working Group on Lipoprotein Measurement recommends that the most prudent course at present is to measure LDL-cholesterol by methods similar to those used to establish the epidemiological database on which the relationships between cardiovascular risk and LDL-cholesterol have been established. The existing epidemiological database relating LDL-cholesterol concentration to coronary heart disease risk includes the contributions of other potentially atherogenic particles in addition to LDL, and the methods used to measure LDL-cholesterol should give results equivalent to those used to establish the database.

Reference Method. The current basis for accuracy of LDL-cholesterol measurement should be combined ultracentrifugation-polyanion precipitation. The reference method should be based on the current Centers for Disease Control and Prevention (CDC) reference methods for total cholesterol and HDL-cholesterol and should satisfy the following major criteria:

- The reference method should provide serum-equivalent values.
- The LDL- plus HDL-containing fraction should be prepared by ultracentrifuging an accurately measured aliquot of the specimen at d 1.006 kg/L for the equivalent of 18 hours at 105,000 x g, quantitatively removing the supernatant VLDL- and chylomicron-containing fractions, and reconstituting the infranatant fraction to the original volume with 0.15 M NaCl. The cholesterol content of the reconstituted infranate is measured.
Recommendations on Lipoprotein Measurement—LDL

- The HDL-containing fraction should be prepared from the ultracentrifugal infranate using a polyanion-divalent cation reagent that provides HDL-cholesterol measurements equivalent to those obtained with the heparin-manganese chloride method.

- Cholesterol in the ultracentrifugal bottom fraction should be measured with methods that satisfy the accuracy and precision criteria of the NCEP Laboratory Standardization Panel. Cholesterol in the HDL-containing fraction should be measured with methods that satisfy the accuracy and precision criteria specified for HDL-cholesterol by the NCEP Working Group on Lipoprotein Measurement.

- LDL-cholesterol should be calculated as follows:

\[ [\text{LDL-chol}] = [d > 1.006 \text{ kg/L chol}] - [\text{HDL-chol}] \]

Criteria for Analytical Performance. The goals for LDL-cholesterol measurement are stated in terms of total analytical error, which takes account of both accuracy and imprecision (reproduced here in table ES-2). This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater imprecision can be accepted if the measurements are more nearly accurate. The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV \( \leq 3 \) percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent. The specification of guidelines for accuracy and precision separately can lead to an ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits are considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance. LDL-cholesterol measurements should be within the following routine limit of performance:

- Total error \( \leq 12 \) percent

This is consistent with the following limits for accuracy and precision:

- Accuracy \( \pm 4 \) percent
- CV \( \pm 4 \) percent

Laboratories and others making LDL-cholesterol measurements can assess their individual conformance to the goals as indicated in table ES-2. These criteria should apply regardless of how, where, or by whom the measurements are made.

Routine Method. For routine patient evaluation and followup, and for monitoring nonlaboratory-based measurements in situations where ultracentrifugation is impractical, LDL-cholesterol should be estimated from direct measurements of total cholesterol, triglycerides (TG), and HDL-cholesterol using the Friedewald equation.

Either EDTA plasma or serum can be used. To convert plasma values to equivalent serum values, multiply the plasma value by 1.03. When EDTA plasma is used, the HDL-containing fraction should be prepared using a polyanion-divalent cation reagent that provides results of the same accuracy as obtainable with the modified heparin-manganese chloride method (0.092 M MnCl\(_2\)) as determined by appropriate statistical methods. When serum is used, the polyanion-divalent cation reagent should provide results of the same accuracy as those obtainable with heparin-manganese chloride (0.046 M).

The total cholesterol measurements should conform to the accuracy and precision criteria of the NCEP Laboratory Standardization Panel, and the triglyceride and HDL-cholesterol measurements should conform to the criteria outlined in
TABLE ES-2. Procedure to measure individual laboratory conformance to analytical goals for lipids and lipoprotein-cholesterol

A. Data production within a laboratory measurement system:
1. For a sufficient period (e.g., 1 year) include two levels of matrix internal quality control materials in each analytical run.
2. Assay the matrix control nearest the medical decision cutpoints in duplicate a sufficient number of times (e.g., once per week).
3. Measure bias (%B).*

B. Calculations†:
1. Calculate the CV of the duplicates (CV_W).
2. Calculate the total CV (CV_T) of the control at the same concentration.
3. Calculate \( CV_T^2 - CV_W^2 \)^{1/2} = CV_B.
4. Calculate total error (TE) = %B + 1.96 (CV_T).

C. Goals†:
1. CV_W < G_{CV}.
2. CV_B + %B < G_B.
3. TE ≤ G_T.

Where the goals for lipids and lipoproteins are as follows:

<table>
<thead>
<tr>
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<th>G_T</th>
<th>G_B</th>
<th>G_{CV}</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>8.9%</td>
<td>≤ ± 3%</td>
<td>≤ 3%</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≤ 15%</td>
<td>≤ ± 5%</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>≤ 22%</td>
<td>≤ ± 10%</td>
<td>≤ 6%</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>≤ 12%</td>
<td>≤ ± 4%</td>
<td>≤ 4%</td>
</tr>
</tbody>
</table>

The primary goal, G_T, is met when both G_{CV} and G_B are met. Although G_T can be met when either G_{CV} or G_B are exceeded somewhat, clinical and analytical performance are optimal when all three goals are met. To calculate the total error for cholesterol, LDL-cholesterol, HDL-cholesterol, or triglycerides, substitute the respective goals in C. above.

* Percent bias is calculated as the mean difference between measured value and reference value, expressed as a percent of the reference value. Bias (%B) can be measured by (1) periodic use of fresh frozen sera, prepared to accurately simulate the normative patient sample, the total cholesterol concentration of which is known with a total error <1 percent or (2) routine or periodic use of a reference material, the total cholesterol concentration of which is known with a total error <1 percent, and the analytical specificity of which is known with the method in use. (One such reference material is College of American Pathologists [CAP] Chemistry Survey serum, the target values of which have been validated by direct NIST-confirmed comparisons with fresh frozen sera. For many common methods, this material will be available from the CAP as the 1995 Crosslink® product and the 1994 Survey Validated Reference Material [SVRM] product.)

† CV, coefficient of variation, calculated as \( \frac{\text{standard deviation}}{\text{mean}} \times 100 \)

CV_W, within-run CV
CV_B, among-run CV
CV_T, total CV, includes within-run and among-run variation
TE, total error
G_{CV}, goal for CV_W
G_B, goal for bias
G_{TE}, goal for total error
the respective reports of the NCEP Working Group on Lipoprotein Measurement. The Friedewald equation should not be used as a reference method for LDL-cholesterol measurement.

Limitations of the Routine Method. The Friedewald equation should not be used under the following circumstances.

- When chylomicrons are present.
- When plasma triglyceride concentration exceeds 400 mg/dL (4.52 mmol/L).
- In patients with dysbetalipoproteinemia (type III hyperlipoproteinemia).

In circumstances in which these conditions apply, LDL-cholesterol should be measured with the combined ultracentrifugation-polyanion precipitation method. The first two conditions can generally be recognized readily. Chylomicrons are visible as a floating “cream” layer when the specimen is allowed to stand undisturbed at 4 °C overnight. Samples with triglyceride concentrations exceeding 400 mg/dL (4.52 mmol/L) are generally turbid. The recognition of dysbetalipoproteinemia (type III hyperlipoproteinemia), however, requires the identification of β-VLDL. Since β-VLDL contains proportionately more cholesterol than normal VLDL, the use of the factor [TG]/5 underestimates the amount of cholesterol in the VLDL fraction, and consequently the Friedewald equation overestimates LDL-cholesterol. Use of the Friedewald equation in this case will result in the misidentification of a dysbetalipoproteinemic (type III) patient as having hyperbetalipoproteinemia (type II hyperlipoproteinemia). It can be anticipated that use of the Friedewald equation will result in such misclassification in approximately 2 in 1,000 individuals in the general population.

New methods designed for use as routine methods should be validated against the reference method rather than the routine method.

Recommendations for Manufacturers

- Manufacturers and others developing new methods and instruments for LDL-cholesterol measurement should be aware of the medical community’s need to refer such measurements to the current epidemiological database as it relates to risk for coronary heart disease.
  - As each new method is developed, it should be validated against the reference method using appropriate statistical methods for comparing measurement methods.
  - For all current and future methods, the nature of the lipoprotein particles that contribute to the LDL-cholesterol measurement should be specified.
  - The assigned LDL-cholesterol values for calibration and quality control materials should be traceable to the reference method for LDL-cholesterol. The method(s) used to establish assigned values should be indicated, and bias with respect to reference values should be stated. This is particularly important for methods that may not measure all the atherogenic apo B-containing particles of d >1.006 kg/L. The CDC laboratory, the Cholesterol Reference Method Laboratory Network, and other CDC standardized lipoprotein research laboratories can be of assistance for these purposes.
- Manufacturers should cooperate with CDC and the Cholesterol Reference Method Laboratory Network in developing suitable reference materials for LDL-cholesterol measurement. To the extent possible, such materials should be free of matrix effects.

Recommendations for Health Care Providers

- Understand What Is Being Measured. Physicians and other health care providers should understand which lipoproteins contribute to the measured LDL-cholesterol value. They should also become familiar with the limitations of the Friedewald equation (see General Recommendations) and avoid using this method when it is inappropriate.
- Measure LDL-Cholesterol in Fasting Samples
  - Blood samples should be obtained by venipuncture following a 12-hour period of fasting. If necessary, the patient can take water and prescribed medications during

...
this period. This procedure should be followed for research purposes and in other circumstances in which error in the LDL-cholesterol measurement must be minimized.

— If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not be less than 9 hours. It is likely that, on average, LDL-cholesterol will be underestimated slightly (about 2 to 4 percent) in patients who have fasted for 9 hours. This should be taken into account when interpreting the values.

— To the extent possible, blood should be drawn in the sitting position and the patient should be allowed to sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should be sampled in the same position on each occasion.

• Use of Serum and Plasma

— EDTA plasma should be used when the LDL-cholesterol is to be measured by ultracentrifugation polyanion precipitation.

— Either serum or EDTA plasma can be used when LDL-cholesterol is to be estimated with the Friedewald equation.

— When EDTA plasma is used, the plasma value is converted to the equivalent serum value by multiplying the plasma value by 1.03.

• Stored Samples

— Serum or plasma should be removed from cells within 3 hours of venipuncture.

— Specimens can be stored for up to 3 days at 4 °C. If analysis is delayed, the specimens can be stored for periods up to several weeks at -20 °C in a non-self-defrosting freezer. Specimens should be stored at -70 °C or lower if longer periods of storage are necessary.

— In all cases, the samples should be stored in clean containers that can be sealed to prevent evaporation. Do not use cork stoppers or plastic film to seal the containers since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

• Need for Serial Measurements in Individuals.

When LDL-cholesterol measurements are made for the purpose of diagnosis, treatment, and followup, it is important initially to establish the patient’s usual LDL-cholesterol to assess the efficacy of treatment. Considering the inherent physiological and analytical variability of LDL-cholesterol measurements, LDL-cholesterol measured on a single occasion will not suffice. Although it would be ideal to establish a patient’s LDL-cholesterol value within a 10 percent limit for total error with 95 percent confidence, at present it is not technically or economically feasible due to the excessive number of serial samples (i.e., four) required. For this reason, the following recommendation is made to improve the reliability of LDL-cholesterol measurements. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart. The individual LDL-cholesterol values should be averaged.

Three serial samples: Using three serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 8.2 percent and a CV_a of 4 percent, the observed CV for the mean LDL-cholesterol value is 5.3 percent, and the difference between the means of sequential series of three samples should not exceed 14.6 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 25 percent. If they are further apart, analytical error or a change in the physiological steady state of the patient should be suspected and another sample may be warranted, depending on the patient’s LDL-cholesterol level and its proximity to the concentrations used for decision making.

Two serial samples: For reasons of convenience and considering economic factors, the ATP II report recommended the use of at least two serial samples. Using two serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 8.2 percent and a
CV<sub>a</sub> of 4 percent, the observed CV for the mean LDL-cholesterol value is 6.5 percent. The difference between the means of each sequential series should not exceed 17.9 percent. The difference between individual values in each series should not exceed 25 percent, 95 percent of the time. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s LDL-cholesterol level and its proximity to the concentrations used for decision making.

Based on the prevailing distributions of LDL-cholesterol,* using two serial measurements and considering a cutpoint of 130 mg/dL, a patient’s LDL-cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is >145 mg/dL or <115 mg/dL, respectively. Using a cutpoint of 160 mg/dL, the patient’s LDL-cholesterol value can be confidently assumed to be above or below the cutpoint when the mean value is >178 mg/dL or <142 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 71 percent of the general population as being above or below the 130 mg/dL cutpoint and 73 percent as being above or below the 160 mg/dL cutpoint.

**Screening.** NCEP guidelines do not require initial testing for LDL-cholesterol; LDL measurement has been recommended as a followup procedure when indicated and may be used for initial testing at the physician’s option. It may ultimately prove desirable to substitute LDL-cholesterol measurements for the currently used procedures that rely on total cholesterol measurement, when LDL-cholesterol measurements become more nearly accurate, precise, and economically feasible. At present, however, there is little information available about the accuracy and precision of LDL- or HDL-cholesterol measurements made in field screening or physicians’ office settings. In one recent study, HDL-cholesterol measurements with a desktop analyzer were judged to be excessively variable. In many instances field screening is conducted using capillary blood samples obtained by fingerstick, and there is currently little information about the suitability of such samples for LDL-cholesterol measurements. Another difficulty is the need for fasting specimens. For these reasons, it would be premature to recommend LDL-cholesterol screening until such concerns have been adequately addressed by appropriately designed studies. Even if ultimately recommended, such measurements should be made in a laboratory setting. Accordingly, mass screening for LDL-cholesterol is not recommended at this time.

**Recommendations for Laboratories**

- Laboratories should use procedures that allow the measurement of LDL-cholesterol with a total error ≤12 percent. One set of conditions that satisfies this recommendation is that LDL-cholesterol be measured with an accuracy of ±4 percent and a CV ≤4 percent. In the absence of a formal standardization program for LDL-cholesterol, the adequacy of LDL-cholesterol measurements made with the Friedewald equation is governed by strict adherence to NCEP criteria for cholesterol, triglyceride, and HDL-cholesterol measurement. Until a reference method for LDL-cholesterol is established and suitable reference materials become available, the accuracy of LDL-cholesterol values calculated with the Friedewald equation should be assessed using the laboratory biases for total cholesterol, triglyceride, and HDL-cholesterol as established using reference materials for those analytes. This assessment should be made at two levels of LDL-cholesterol, for example, 130 mg/dL (3.36 mmol/L) and 160 mg/dL (4.13 mmol/L). Precision should be assessed using the LDL-cholesterol values calculated from the total cholesterol, triglyceride, and HDL-cholesterol measurements in those pools.

- All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel.

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* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
Personnel handling blood samples should use gloves and should avoid leaving samples open to the air longer than necessary. Samples should be handled in accordance with CDC guidelines for the prevention of infection in health care workers.

**Recommendations for Government Agencies and Other Professional Groups**

The Centers for Disease Control and Prevention should take the following steps:

- Establish a reference method for LDL-cholesterol measurement. For the present, the method can probably be readily developed by modifying the current CDC ultracentrifugation polyanion precipitation method for HDL-cholesterol. The reference method should include the contributions of all the atherogenic apo B-containing particles reflected in the current epidemiological database that relates LDL-cholesterol concentration to the risk for coronary heart disease.

- Add an LDL-cholesterol standardization program to the current CDC-NHLBI standardization programs for total cholesterol, triglycerides, and HDL-cholesterol. Because of the important and far-reaching consequences of inaccurate LDL-cholesterol measurements, an LDL-cholesterol standardization program should be developed also.

- In cooperation with appropriate experts in the academic, clinical laboratory, and manufacturing sectors, develop reference materials for LDL-cholesterol measurement in which matrix effects are minimized. These reference materials should be suitable for standardization, surveillance, method calibration as appropriate, and bench-level quality control.

The National Heart, Lung, and Blood Institute should take the following steps:

- It has been the policy of NHLBI to require standardized lipid and lipoprotein measurements for Government-supported clinical and epidemiological studies. This policy should be continued.

- Encourage the Cholesterol Reference Method Laboratory Network system to expand its activities to include the certification of LDL-cholesterol, HDL-cholesterol, and triglyceride measurements.

- Encourage the development and preliminary evaluation of new LDL-cholesterol methods and associated instrumentation.

The Cholesterol Reference Method Laboratory Network currently provides assistance to manufacturers and laboratories wishing to establish the traceability of total cholesterol measurements to the cholesterol reference method. The Network should:

- Expand these activities to include LDL-cholesterol, HDL-cholesterol, and triglyceride measurements.

The College of American Pathologists and other professional organizations that operate clinical chemistry survey programs should take the following steps:

- Include LDL-cholesterol measurements in such surveys.

- Provide CDC-confirming values for LDL-cholesterol concentrations in survey pools.

**Recommendations for Further Research**

Further research is needed in several areas.

- New methods for LDL-cholesterol measurement should be developed. Such methods should be capable of quantitating LDL-cholesterol directly; they should not be based on calculations of the difference between two or more measured values. The nature of the lipoprotein species contributing to the LDL-cholesterol measurement should be defined and the relationship established between values obtained and those used to establish the current epidemiological database. This is particularly important for LDL-specific methods, since such methods would exclude the contributions of one or more potentially atherogenic particles that may be present in higher concentrations in individuals who are at increased risk for coronary heart disease.
• Research is needed to define the relative contributions of cholesterol carried in each of the individual apo B-containing lipoproteins of d > 1.006 kg/L (IDL, LDL, Lp(a)) to coronary heart disease risk as presently defined in terms of LDL-cholesterol measurements that include the contributions of all such lipoproteins.

• Suitable reference materials are needed for LDL-cholesterol measurement. Such materials should be essentially free of matrix effects and should be sufficiently stable to allow long-term monitoring of the accuracy and precision of LDL-cholesterol measurements.
Elevated concentrations of low density lipoprotein (LDL) markedly increase the risk for coronary heart disease (CHD) (Pekkanen et al. 1990; Castelli et al. 1986) and lowering plasma LDL concentrations reduces the risk for CHD, myocardial infarction (MI), and CHD-related death (Castelli et al. 1986; Stamler et al. 1986; Coronary Drug Project Research Group 1975; Lipid Research Clinics Program 1984; Frick et al. 1987; Blankenhorn et al. 1987). Furthermore, reducing plasma LDL concentration also reduces the incidence of a second heart attack in myocardial infarction survivors, slows the progression of CHD (Brensike et al. 1984), and may even lead to regression of coronary atherosclerosis. In 1984, the Consensus Development Conference on Lowering Blood Cholesterol to Prevent Heart Disease defined age-specific (but not sex-specific) levels for total cholesterol at which risk for CHD increases significantly and recommended that all physicians include a blood cholesterol measurement when first evaluating their adult patients (NIH Consensus Conference 1985, 1993). Subsequently, the first report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel [ATP]) (1988a) provided recommendations for the detection and treatment of high blood cholesterol in adults, based on a revised non-age- and non-sex-specific definition of risk levels for total and LDL-cholesterol (Table 1). This publication was pivotal in that it led to the recommendation of a single set of cholesterol value decision points for recognizing hypercholesterolemia and high LDL-cholesterol concentration. These

### TABLE 1. NCEP Adult Treatment Panel II categories for total and LDL-cholesterol concentrations for patients with and without coronary heart disease

<table>
<thead>
<tr>
<th>Patients Without Coronary Heart Disease</th>
<th>Total Cholesterol Category</th>
<th>Total Cholesterol mg/dL (mmol/L)</th>
<th>LDL-Cholesterol Category</th>
<th>LDL-Cholesterol mg/dL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desirable</td>
<td>&lt;200 (5.17)</td>
<td>Desirable</td>
<td>&lt;130 (3.36)</td>
<td></td>
</tr>
<tr>
<td>Borderline-high</td>
<td>200-239 (5.17-6.18)</td>
<td>Borderline-high risk</td>
<td>130-159 (3.64-4.11)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>≥240 (6.21)</td>
<td>High-risk</td>
<td>≥160 (4.13)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients With Coronary Heart Disease</th>
<th>LDL-Cholesterol Category</th>
<th>LDL-Cholesterol mg/dL (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>&lt;100 (2.6)</td>
<td></td>
</tr>
<tr>
<td>Higher than optimal</td>
<td>&gt;100 (2.6)</td>
<td></td>
</tr>
</tbody>
</table>

1 To convert mg/dL to mmol/L, divide by 38.7.
cutpoints have been reaffirmed by the ATP’s second report (ATP II) (NCEP 1994). These decision points are based on studies in which the cholesterol measurements were made with standardized procedures (Myers et al. 1989) that are traceable to the reference and definitive methods for cholesterol quantitation. For the decision points to be useful, however, it is necessary that clinical cholesterol measurements be made with similar reliability. As a first step toward ensuring reliability, the NCEP Laboratory Standardization Panel (1988, 1990) discussed at length the various sources of preanalytical and analytical factors that affect the accuracy and precision of total cholesterol measurements, defined analytical performance criteria (table 2), developed detailed recommendations for the laboratory community and its suppliers to ensure acceptable cholesterol measurements, and described the resources currently available to meet this objective.

The NCEP Working Group on Lipoprotein Measurement was organized to consider and make recommendations concerning the measurement of high density lipoprotein (HDL), triglycerides, and LDL. These measurements are considered in three separate parts in this report. This part concerns the measurement of LDL-cholesterol.

LIPOPROTEIN CONTRIBUTORS TO TOTAL CHOLESTEROL

Virtually all the cholesterol in plasma is transported by the plasma lipoproteins. In the fasting state, plasma cholesterol is normally transported primarily in three major lipoprotein fractions: very low density lipoprotein (VLDL) (d <1.006 kg/L), LDL (d 1.019-1.063 kg/L), and HDL (d 1.063-1.21 kg/L) (equation 1).

\[
[\text{Total chol}] = [\text{VLDL-chol}] + [\text{LDL-chol}] + [\text{HDL-chol}]
\]

EQUATION 1

Lesser amounts of cholesterol are also carried in two minor lipoprotein classes, intermediate density lipoproteins (IDL, d 1.006-1.019 kg/L) and Lp(a) (d 1.045-1.080 kg/L). Some of the properties of the lipoproteins are shown in table 3. LDL is the major contributor to plasma total cholesterol concentration in humans, accounting for one-half to two-thirds of the plasma cholesterol. Because of this, there is a high correlation between total plasma cholesterol concentration and LDL-cholesterol concentration (r >0.9), and total cholesterol is often used as a surrogate measure of LDL-cholesterol. Chylomicrons (d <1.006 kg/L) appear in the blood transiently after a fat-containing meal and are normally removed completely by 12 hours. They are rich in triglycerides and responsible for the postprandial increase in plasma triglycerides but usually have no significant effect on the total cholesterol concentration. The major lipoprotein fractions can be readily separated from

<table>
<thead>
<tr>
<th>Performance Criteria$^{1,2}$ (1992)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy $\leq$3%</td>
</tr>
<tr>
<td>Precision $^3$ (CV) $\leq$3%</td>
</tr>
</tbody>
</table>

1 Compared to the reference method (Duncan et al. 1982; Cooper et al. 1986). The guidelines refer only to analytical error; they do not include the contribution of biological variation.

2 Assuming the maximum allowable bias and imprecision, and a 95 percent confidence limit for the imprecision, the allowable total error for single measurements is $\pm$8.9 percent using the 1992 criteria.

3 Precision is assessed by the coefficient of variation (CV), defined as:

\[
\text{CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100
\]
TABLE 3. Plasma lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Density (kg/L)</th>
<th>Electrophoretic Mobility1</th>
<th>Chemical Composition2</th>
<th>Apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC3 TG PL Protein + CE (percent by wt)</td>
<td></td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.95</td>
<td>Origin</td>
<td>4 90 5 1</td>
<td>Al, C's, AIV, E, B48</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>Prebeta</td>
<td>20 55 19 8</td>
<td>C's, B, E</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>Beta-prebeta</td>
<td>Intermediate between VLDL and LDL</td>
<td>C's, B, E</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>Beta</td>
<td>55 5 20 20</td>
<td>B-100</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>Alpha</td>
<td>22 5 28 50</td>
<td>Al, All, D, C, E</td>
</tr>
<tr>
<td>HDL2</td>
<td>1.063-1.12</td>
<td>Alpha</td>
<td>24 8 25 43</td>
<td></td>
</tr>
<tr>
<td>HDL3</td>
<td>1.12-1.21</td>
<td>Alpha</td>
<td>21 2 23 55</td>
<td></td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.045-1.080</td>
<td>Prebeta</td>
<td>46 5 22 27</td>
<td>B-100, Lp(a)</td>
</tr>
</tbody>
</table>

1 By agarose gel electrophoresis.
2 Data from Segal et al. 1984; Gries et al. 1988; Fress et al. 1986; Albers and Hazzard 1974; Gotto et al. 1986; Gaubatz et al. 1983.
3 FC, unesterified cholesterol; CE, cholesterol esters; TG, triglycerides; PL, phospholipids.

MAJOR ISSUES CONCERNING LDL-CHOLESTEROL MEASUREMENT

It is worthwhile to consider first exactly what is being measured and which factors affect the attainable accuracy and precision of LDL-cholesterol measurements. In almost all of the epidemiological studies from which the quantitative association between LDL-cholesterol and cardiovascular risk is derived, this lipoprotein has been measured in one of two ways. Both methods have been applied to plasma or serum collected after a 12-hour period of fasting.

Beta Quantification

The first method, usually referred to as “beta quantification,” is a combined ultracentrifugation polyanion precipitation procedure in which appropriate lipoprotein-containing fractions are prepared and the cholesterol concentrations of the fractions are measured and used to calculate LDL-cholesterol. In this method, an aliquot of plasma is used to measure the total cholesterol concentration. A separate aliquot is centrifuged (10,000 x g) at plasma density (d 1.006 kg/L) for 18 hours at 10 °C. Under these conditions, VLDL (d <1.006 kg/L) accumulates as a layer at the top of the ultracentrifuge tube.

The remaining lipoproteins (IDL, LDL, Lp(a), HDL) and other plasma proteins sediment at d 1.006 kg/L. The VLDL fraction is removed from the top of the tube, the infranatant is reconstituted accurately to a known volume, and its cholesterol content is measured. A separate aliquot of unfractionated plasma is treated to precipitate VLDL, IDL, LDL and Lp(a) (Bachorik and Albers 1986), and the cholesterol concentration of the clear HDL-containing supernatant is measured. LDL-cholesterol is then calculated as the difference in the cholesterol concentrations of other plasma proteins, and from each other, in the ultracentrifuge and are most commonly quantified in terms of their cholesterol content (Bachorik 1989).
the d >1.006 kg/L (bottom) fraction and the HDL-containing supernate (equation 2).

**EQUATION 2**

\[ \text{[LDL-chol]} = \text{[d >1.006 kg/L chol]} - \text{[HDL-chol]} \]

The VLDL (d <1.006 kg/L) fraction may also contain chylomicrons in samples from nonfasting patients or in those from fasting patients with impaired chylomicron clearance. In addition, in patients with dysbetalipoproteinemia (type III hyperlipoproteinemia), the VLDL fraction may also contain lipoproteins with beta rather than prebeta mobility, so-called \( \beta \)-VLDL or “floating beta” lipoproteins (Mahley and Rall 1989). This is an uncommon disorder (estimated prevalence 2 in 1,000 in the general population [LaRosa et al. 1986]), characterized in part by the presence of an unusual beta-migrating lipoprotein that has an electrophoretic mobility similar to LDL (beta lipoprotein), but the flotation characteristics of VLDL, hence its name. This lipoprotein is enriched in cholesterol compared to VLDL (table 3), and its presence can lead to misdiagnosis of dysbetalipoproteinemia (type III hyperlipoproteinemia) as hyperbetalipoproteinemia (type II hyperlipoproteinemia) (see below). Chylomicrons and \( \beta \)-VLDL, if present, do not interfere with the LDL-cholesterol measurement using the ultracentrifugal method because they are not present in the ultracentrifugal infranatant.

Two points should be noted. First, the d >1.006 kg/L fraction contains IDL and Lp(a), and in some cases might also contain partially metabolized chylomicrons and/or VLDL (i.e., remnants) in addition to LDL. The non-HDL associated cholesterol in this infranatant represents the contributions of all these lipoproteins. In most cases, the contributions of remnants, IDL, and Lp(a) are only a few mg/dL; by far the greatest contributor is LDL, and the presence of the other lipoproteins is usually ignored. Nonetheless, in certain patients such particles can contribute a larger percentage of the LDL-cholesterol (Kurschinski et al. 1989). For example, Lp(a) present in concentrations of 22 to 100 mg/dL would be expected to contribute from 5 to 23 percent to the measurement at an LDL-cholesterol concentration of 130 mg/dL (3.36 mmol/L) (table 4). Lp(a) concentrations have been measured in various studies, a number of which are shown in table 5. The median values reported in different studies averaged about 8 mg/dL corresponding to about 2 mg/dL (0.05 mmol/L) of Lp(a) cholesterol, and the 70th percentile was about 20 mg/dL, which would contribute about 6 mg/dL (0.16 mmol/L) Lp(a) cholesterol to the LDL-cholesterol measurement. This would constitute 5 percent of the LDL-cholesterol in an individual with a measured LDL-cholesterol concentration of 130 mg/dL (3.36 mmol/L).

**The Friedewald Equation**

The ultracentrifugal measurement of LDL is time consuming and expensive and requires equipment not commonly available in the clinical laboratory or physician’s office. For this reason, LDL-cholesterol is most commonly estimated from quantitative measurements of total and HDL-cholesterol and plasma triglycerides (TG) using the empirical relationship of Friedewald et al. (1972).

**EQUATION 3**

\[ \text{[LDL-chol]} = \text{[Total chol]} - \text{[HDL-chol]} - \text{[TG]/5} \]

where all concentrations are given in mg/dL.\(^1\)

The quotient [TG]/5 is used as a measure of VLDL-cholesterol concentration. It assumes, first, that virtually all of the plasma TG is carried on VLDL, and second, that the TG:cholesterol ratio of VLDL is constant at about 5:1 (Friedewald et al. 1972). Neither assumption is strictly true, but the equation generally provides LDL-cholesterol values that agree within a few mg/dL of those measured in the ultracentrifuge (Friedewald et al. 1972). Based on an analysis of data collected in

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\(^1\) When cholesterol and triglycerides are expressed in mmol/L, VLDL-cholesterol is estimated as [TG]/2.17.
TABLE 4. Contribution of Lp(a)-cholesterol to measured LDL-cholesterol values

<table>
<thead>
<tr>
<th>Lp(a) Concentration (mg/dL)</th>
<th>Equivalent Lp(a)-Cholesterol Concentration&lt;sup&gt;1&lt;/sup&gt; (mg/dL (mmol/L))</th>
<th>Contribution to Measured LDL-Cholesterol&lt;sup&gt;2&lt;/sup&gt; (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.5 (0.04)</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>3.0 (0.08)</td>
<td>2.3</td>
</tr>
<tr>
<td>15</td>
<td>4.5 (0.12)</td>
<td>3.5</td>
</tr>
<tr>
<td>20</td>
<td>6.0 (0.16)</td>
<td>4.6</td>
</tr>
<tr>
<td>22</td>
<td>6.6 (0.17)</td>
<td>5.0</td>
</tr>
<tr>
<td>30</td>
<td>9.0 (0.23)</td>
<td>6.9</td>
</tr>
<tr>
<td>50</td>
<td>15.0 (0.39)</td>
<td>11.5</td>
</tr>
<tr>
<td>75</td>
<td>22.5 (0.58)</td>
<td>17.3</td>
</tr>
<tr>
<td>100</td>
<td>30.0 (0.78)</td>
<td>23.1</td>
</tr>
</tbody>
</table>

<sup>1</sup> Lp(a)-cholesterol = 0.3 x Lp(a); assumes that 30 percent of the mass of Lp(a) is accounted for by cholesterol (sterol nucleus) and the concentrations are expressed in mg/dL (Kostner et al. 1981).

<sup>2</sup> Assuming measured LDL-cholesterol value = 130 mg/dL (3.36 mmol/L).

the Lipid Research Clinics (LRC) Program Prevalence Studies (Lipid Research Clinics Program 1980), DeLong et al. (1986) recommended the use of the expression 0.16 x TG (or [TG]/6.25) as a better estimator of VLDL-cholesterol, and this value is used in a few laboratories (College of American Pathologists 1988, 1989). However, others feel that in view of its simplicity, the inherent physiological variability of VLDL composition, and the analytical variability of triglyceride measurements, the original factor should be retained (Warnick et al. 1990; McNamara et al. 1990), and most laboratories use the original factor (College of American Pathologists 1989). In either case, large errors in the LDL estimates can occur in samples with triglycerides exceeding 400 mg/dL (4.52 mmol/L) and in those with chylomicrons, and the error continues to increase with increasing triglyceride concentration. The 400 mg/dL (4.52 mmol/L) limit is an arbitrary point at which the error in the LDL-cholesterol estimate is considered to become unacceptably large.

In practice, aliquots of plasma are taken for total cholesterol and triglyceride measurement. HDL is measured in the supernate following the precipitation of the other lipoproteins with a polyanion in the presence of a divalent cation (Bachorik and Albers 1986). The more common precipitants include heparin-MnCl<sub>2</sub>, dextran sulfate-MgCl<sub>2</sub>, phosphotungstate-MgCl<sub>2</sub>, and polyethylene glycol (PEG). LDL-cholesterol estimated with the Friedewald equation also contains the contributions of lipoproteins in the IDL density range and Lp(a) since the precipitation procedures commonly used to remove LDL from plasma also precipitate these lipoproteins. The methods discussed above were used in most of the epidemiological studies on which estimates of the relationship between LDL-cholesterol concentration and CHD risk are based (Bachorik 1989; Castelli et al. 1986), as well as in studies of the benefits of LDL-cholesterol lowering (Stamler et al. 1986; Lipid Research Clinics Program 1984; Frick et al. 1987; Blankenhorn et al. 1987).

**Potentially Atherogenic Particles**

Lp(a) was first recognized about 30 years ago (Berg 1963). It is becoming more widely recognized that a high Lp(a) concentration constitutes an inde-
### TABLE 5. Lp(a) concentrations in various populations

<table>
<thead>
<tr>
<th>Population</th>
<th>n</th>
<th>Mean (SD)</th>
<th>25</th>
<th>33</th>
<th>50</th>
<th>67</th>
<th>75</th>
<th>85</th>
<th>90</th>
<th>95</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>99</td>
<td>17 (15)</td>
<td>14</td>
<td>17</td>
<td>RID</td>
<td>Murai et al. (1986)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients without atherosclerotic vascular disease</td>
<td>124</td>
<td>16 (13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Randomly selected hospital inpatients and outpatients</td>
<td>37</td>
<td>3</td>
<td>5</td>
<td>10</td>
<td>EIA</td>
<td>Zenker et al. (1986)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical students with immunochemically detectable Lp(a)</td>
<td>27</td>
<td>21</td>
<td>4</td>
<td>RID</td>
<td>Ehnholm et al. (1971)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>84</td>
<td>13 (14)</td>
<td>7</td>
<td>30</td>
<td>35</td>
<td>ELISA</td>
<td>Pless et al. (1989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volunteers of Prospective Cardiovascular Munster Study</td>
<td>1,053</td>
<td>5 (2-16)²</td>
<td>16</td>
<td>~20</td>
<td>EIA</td>
<td>Sandkamp et al. (1990)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northwest Lipid Research Clinic Prevalence Study</td>
<td>340</td>
<td>14</td>
<td>8</td>
<td>~20</td>
<td>~36</td>
<td>~50</td>
<td>RID</td>
<td>Albers and Hazzard (1974)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parents and grandparents of newborns with normal cord blood lipid concentration</td>
<td>1,251</td>
<td>10 (13)</td>
<td>6</td>
<td>~50</td>
<td>RID</td>
<td>Albers et al. (1974)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normolipidemic subjects attending a public health survey</td>
<td>55</td>
<td>~30</td>
<td>~50</td>
<td>EIA</td>
<td>Kostner et al. (1981)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy hospital and university employees (white)</td>
<td>134</td>
<td>~16 (19)</td>
<td>16</td>
<td>EIA</td>
<td>Guyton et al. (1985)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Company employees in Westfalia</td>
<td>1,464</td>
<td>~10 (13)</td>
<td>~5</td>
<td>~11</td>
<td>~25</td>
<td>~37</td>
<td>EIA</td>
<td>Schriewer et al. (1984)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northwest Lipid Research Clinic Prevalence Study normolipidemic subjects with immunochemically detectable Lp(a)</td>
<td>320</td>
<td>13</td>
<td>3</td>
<td>8</td>
<td>17</td>
<td>39</td>
<td>51</td>
<td>RID</td>
<td>Albers et al. (1975)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Honolulu Heart Study subjects and sons of subjects without coronary artery disease</td>
<td>408</td>
<td>14 (13)</td>
<td>3</td>
<td>11</td>
<td>20</td>
<td>EIA</td>
<td>Rhoads et al. (1986)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Normal subjects</td>
<td>276</td>
<td>35</td>
<td>ELISA</td>
<td>Rodriguez et al. (1990)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Normolipidemic subjects</td>
<td>178</td>
<td>6</td>
<td>34</td>
<td>ELISA</td>
<td>Panteghini et al. (1990)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal males</td>
<td>984</td>
<td>14</td>
<td>35</td>
<td>ELISA</td>
<td>Schaefer et al. (1990)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Normal males and females, Age: 20-29</td>
<td>78</td>
<td>19 (20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>30-39</td>
<td>84</td>
<td>22 (23)</td>
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<tr>
<td>40-49</td>
<td>64</td>
<td>25 (24)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>50-60</td>
<td>55</td>
<td>32 (26)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-60</td>
<td>119</td>
<td>28 (25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physicians Health Study subjects (males 40-84 years old, who did not develop fatal or nonfatal MI)</td>
<td>296</td>
<td>4</td>
<td>10</td>
<td>23</td>
<td>40</td>
<td>55</td>
<td>ELISA</td>
<td>Ridker et al. (1993)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CARDIA study subjects</td>
<td>2,007</td>
<td>37 (23)</td>
<td>17</td>
<td>32</td>
<td>51</td>
<td>66</td>
<td>79</td>
<td>ELISA</td>
<td>Marcovina et al. (1993)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blacks</td>
<td>2,158</td>
<td>19 (19)</td>
<td>5</td>
<td>10</td>
<td>27</td>
<td>48</td>
<td>60</td>
<td>ELISA</td>
<td>Marcovina et al. (1993)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. RID, radial immunodiffusion; EIA, electroimmunooassay; ELISA, enzyme linked immunosorbent assay.
2. Mean ±SD of Log Lp(a) values.
3. Data were reported in terms of Lp(a) protein concentration. In this table, the data are expressed in terms of total Lp(a) mass using the authors’ estimate of the relationship between Lp(a) protein and total Lp(a) mass.
ependent risk factor for coronary heart disease (Albers et al. 1977; Berg et al. 1979; Kostner et al. 1981; Schriewer et al. 1984; Rhoads et al. 1986; Dahlen et al. 1986; Durrington et al. 1988; Sandkamp et al. 1990; Seed et al. 1990), and may also predispose to cerebrovascular disease (Murai et al. 1986; Zenker et al. 1986). Evidence is accumulating that lipoproteins in the IDL density range may also be atherogenic (Zilversmit 1979; Krauss 1987a). What has generally been called LDL-cholesterol can be thought of as representing the concentration of cholesterol in the major potentially atherogenic particles. Because the concentration of Lp(a) is independent of total cholesterol, triglycerides, or HDL-cholesterol, it is not possible to estimate Lp(a) levels from these analytes (Kurschinski et al. 1989). Given an independent measure of Lp(a) concentration, however, and considering that the sterol nucleus accounts for about 30 percent of the mass of Lp(a), the following modification of Equation 3 has been used to correct the LDL-cholesterol estimate for the concentration of Lp(a)-cholesterol (Jurgens and Koltringer 1987; Sandkamp et al. 1990).

\[
[\text{LDL-chol}] = [\text{Total chol}] - [\text{HDL-chol}] - \frac{[\text{TG}]}{5} - 0.3 \times [\text{Lp(a)}]
\]

where the concentration of Lp(a) is expressed in terms of Lp(a) mass and all concentrations are given in mg/dL.

The distinction between LDL-cholesterol and cholesterol in other atherogenic particles is important for several reasons. First, although these lipoproteins have been measured together as LDL-cholesterol, it will at some point be useful to identify their separate contributions to the measurement. For example, dietary treatment and most drug treatment regimens that successfully lower LDL-cholesterol are not effective in lowering Lp(a) (Albers et al. 1975; Vessby et al. 1982). Second, methods are currently being developed for directly measuring plasma LDL-cholesterol. Such methods should obviate the use of the ultracentrifuge and reduce the number of analytes that must be measured to calculate LDL-cholesterol concentration. This in turn may also reduce the analytical variability associated with LDL-cholesterol measurement. However, it may also increase the specificity of the measurement for LDL-cholesterol. It is uncertain whether LDL-specific methods would improve the estimation of cardiovascular risk. As discussed above, the epidemiological data for such estimates are based on the combined measurement of several potentially atherogenic particles (IDL, LDL, Lp(a)) whose relative individual contributions to the risk profile and differential responses to treatment are not yet fully understood. Thus, it is conceivable that measuring only LDL-cholesterol could provide a less sensitive risk indicator than measurements of cholesterol in total atherogenic particles. For this reason, it is premature to base risk estimates on measurements that reflect the plasma concentration of LDL-cholesterol only. Nonetheless, direct methods for LDL-cholesterol measurement are being developed and marketed, and the Working Group recommends, first, that manufacturers of such methods clearly indicate the lipoprotein species included in the LDL-cholesterol measurement; second, that those who use and interpret the measurements understand precisely what they represent; and third, that priority be given to research aimed at establishing the relative separate contributions of the various potentially atherogenic particles to CHD risk and the efficacy of current treatment regimens with respect to each of them.

For the remainder of this report, the term LDL-cholesterol should be interpreted to indicate the total amount of cholesterol transported by three lipoproteins, IDL + LDL + Lp(a) (i.e., non-HDL lipoproteins of density >1.006 kg/L).
PHYSIOLOGICAL VARIATION

Measured LDL-cholesterol values vary within an individual when sampled on several occasions. This arises from a number of factors that can be broadly separated into two categories, biological and analytical sources of variation. The Laboratory Standardization Panel has discussed these factors at length with respect to total cholesterol measurement (NCEP Laboratory Standardization Panel 1990), and the discussion will not be repeated here. For total cholesterol, the coefficients of biological variation (CV_b) in individuals ranged from 1 to 11 percent and averaged between 4.5 and 8 percent. CV_b for total cholesterol on average can be assumed to be about 6.5 percent (NCEP Laboratory Standardization Panel 1990). Since most of the plasma total cholesterol is transported in LDL, physiological variation of total cholesterol measurements can be expected to reflect primarily the variation in LDL-cholesterol. There is, however, little information available about the magnitude of CV_b for LDL-cholesterol. Friedlander et al. (1985) reported an observed CV of about 13 percent in LDL-cholesterol, based on measurements made in adolescents and adults on two occasions; this included both physiological and analytical components of variation, and the authors did not report the two components separately. In an earlier study that measured the interlaboratory variability of LDL-cholesterol measurements, however, the analytical coefficient of variation (CV_a) was 6.7 percent (Bachorik et al. 1981). Both of the above studies were performed in laboratories of the LRC program, however, and assuming that the CV_a was no more than this in the study of Friedlander et al. (1985), their finding suggests a CV_b of about 11 percent. Demacker et al. (1982) estimated CV_b values for total cholesterol, triglycerides, and HDL-cholesterol. From their data, it can be calculated that the CV_b for LDL-cholesterol was about 10.0 percent over a 1-month period and 9.6 percent over a 1-year period. In one recent study, a CV_b of 5.9 percent was estimated for LDL-cholesterol from three samples obtained from 51 normal volunteers over a 1-week period (Bookstein et al. 1990). In another (Mogadam et al. 1990), the CV_b was 9.6 percent in 20 normal volunteers sampled weekly for 4 weeks. In still another study based on multiple baseline measurements over a median period of 2 months in patients referred to a lipid clinic, the CV_b's for total and LDL-cholesterol were 4.8 and 6.6 percent, respectively (Kafonek et al. 1992). The mean LDL-cholesterol concentration in this study was 160 mg/dL (4.13 mmol/L). Thus, the available data suggest that the CV_b for LDL-cholesterol lies within the range of about 6 to 11 percent and averages about 8.2 percent.

ANALYTICAL VARIATION

Beta Quantification

The measurement of LDL-cholesterol can be thought of as involving two general steps: (1) separation of the appropriate lipoprotein-containing fraction(s) and (2) estimation of the amount of cholesterol in the fraction(s). For combined ultracentrifugation/polyanion precipitation methods (equation 2), two separation procedures are needed to prepare the ultracentrifugal and the HDL-containing fractions, and cholesterol must be measured in each fraction. In methods based on the Friedewald equation (equation 3), a single separation step is needed to prepare the HDL-containing fraction, but three analyses must be performed: total cholesterol, HDL-cholesterol, and triglycerides. The analytical variation of LDL-cholesterol measurement includes that contributed by each of the separation and measurement steps.
The analytical variation inherent in measuring LDL-cholesterol with the combined ultracentrifugation/polyanion precipitation method is shown in table 6. In one study (Bachorik et al. 1981), aliquots of 53 fresh plasma samples were distributed to each of the participating laboratories of the Lipid Research Clinics Program and each aliquot analyzed once. All laboratories used the same methods for plasma fractionation and cholesterol measurement. The analytical coefficient of variation ($CV_a$) for LDL-cholesterol measurement was about 6.6 percent (table 6), which included both within-laboratory and among-laboratory components of variation. By comparison, the $CV_a$ for total cholesterol measurement was about 2.2 percent, similar to that measured in quality control pools in the LRC laboratories (Lippel et al. 1977, 1978). It can be calculated that the variability of the LDL-cholesterol values due to the initial lipoprotein isolation steps (ultracentrifugation and heparin-Mn$^{2+}$ precipitation) was about 5.3 percent (section 6). Thus, the preparation of the lipoprotein-containing fractions accounted for most of the variability. In another study con-

### TABLE 6. Estimates of analytical variation in LDL-cholesterol measurements (standardized laboratories)

<table>
<thead>
<tr>
<th>Period of Study</th>
<th>No. of Pools</th>
<th>No. of Analyses per Pool</th>
<th>LDL-chol Concentration mg/dL (mmol/L)</th>
<th>$CV_a$ (%)</th>
<th>$CV_a$ (LDL-chol)</th>
<th>Method</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 wk</td>
<td>1</td>
<td>44</td>
<td>154 (3.98)</td>
<td>6.8</td>
<td>1.6</td>
<td>UC/Pcpn</td>
<td>Single lab</td>
<td>Siekmeier et al. (1988)</td>
</tr>
<tr>
<td>4 yr</td>
<td>53</td>
<td>12</td>
<td>112 (2.89)</td>
<td>6.6²</td>
<td>2.9</td>
<td>UC/Pcpn 12 labs</td>
<td></td>
<td>Bachorik et al. (1981)</td>
</tr>
<tr>
<td>6 wk</td>
<td>1</td>
<td>20</td>
<td>135 (3.49)</td>
<td>2.7</td>
<td>1.7</td>
<td>UC/Pcpn Single lab</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 wk</td>
<td>1</td>
<td>20</td>
<td>138 (3.57)</td>
<td>2.9</td>
<td>1.8</td>
<td>Friedewald equation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21 mo</td>
<td>128</td>
<td>1</td>
<td>160 (4.13)</td>
<td>4.2</td>
<td>2.1</td>
<td>Friedewald equation</td>
<td>Single lab</td>
<td>Kafonek et al. (1992)</td>
</tr>
<tr>
<td>1 yr</td>
<td>1</td>
<td>376</td>
<td>99 (2.56)</td>
<td>4.5</td>
<td>2.4</td>
<td>Friedewald equation</td>
<td>Single lab</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>376</td>
<td>171 (4.42)</td>
<td>3.7</td>
<td>1.9</td>
<td>Friedewald equation</td>
<td>Single lab</td>
<td>++</td>
</tr>
<tr>
<td>6 wk</td>
<td>1</td>
<td>148</td>
<td>103 (2.66)</td>
<td>4.7²</td>
<td>3.1</td>
<td>Friedewald equation</td>
<td>4 labs</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31</td>
<td>227 (5.87)</td>
<td>2.8²</td>
<td>1.3</td>
<td>Friedewald equation</td>
<td>Single lab</td>
<td></td>
</tr>
<tr>
<td>1 mo</td>
<td>2</td>
<td>Not indicated</td>
<td>Not indicated</td>
<td>4.4</td>
<td>3.4</td>
<td>Friedewald equation</td>
<td>Single lab</td>
<td>Bookstein et al. (1990)</td>
</tr>
<tr>
<td>1 mo</td>
<td></td>
<td>20</td>
<td>Not indicated</td>
<td>6.4³</td>
<td>1.7</td>
<td>Friedewald equation</td>
<td>2 labs</td>
<td>Mogadam et al. (1990)</td>
</tr>
</tbody>
</table>

1. UC/Pcpn, combined ultracentrifugation-polyanion precipitation method.
2. Interlaboratory plus intralaboratory $CV_a$.
3. $CV_a$ includes within- and among-laboratory components of variation, calculated from data given in the paper.

Unpublished data provided by Northwest Lipid Research Clinic, Seattle.

++ Two quality control pools were analyzed, one for cholesterol and triglycerides and one for HDL-cholesterol. LDL-cholesterol was calculated from these measurements; $CV_a$ represents the variability of the LDL estimates. Unpublished data contributed by the Johns Hopkins Lipid Research Clinic, Baltimore.

** Unpublished data contributed for this report by Pacific Biometrics, Inc., Seattle, WA; Medical Research Laboratory, Cincinnati, OH; Johns Hopkins Lipid Research Clinic, Baltimore, MD; Centers for Disease Control and Prevention, Laboratory Standardization Section, Atlanta, GA.
ducted in a single laboratory, a CVₐ of 6.9 percent (not shown in table 6) was reported for LDL-cholesterol measured with a combined ultracentrifugation/polyanion precipitation method (Friedewald et al. 1972). In a more recent evaluation, the analysis of a pooled plasma sample over a 6-week period in a single laboratory revealed a CVₐ of 2.7 percent for LDL-cholesterol (table 6, third line). The lower CVₐ in this study presumably reflects the improvement in methodology, the shorter duration of the study, and the absence of an among-laboratory component of the variation.

The Friedewald Equation

Information available for the CVₐ of LDL-cholesterol estimated with the Friedewald equation was contributed mainly by single laboratories. Siekmeier et al. (1988) reported CVₐ’s of 6 to 12 percent for LDL-cholesterol. Bookstein et al. (1990) recently reported a CVₐ of 4.4 percent for LDL-cholesterol. Mogadam et al. (1990) found a CVₐ of 6.4 percent (includes within-laboratory and among-laboratory components of variation) in samples collected from 20 volunteers sampled weekly for 4 weeks. In another recent study (Kafonek et al. 1992), the intralaboratory CVₐ for LDL-cholesterol was calculated from the individual CVₐ’s for total cholesterol, triglycerides, and HDL-cholesterol for baseline measurements in 128 subjects referred to a lipid clinic. The average CVₐ for LDL-cholesterol was 4.2 percent at a level of 160 mg/dL (4.13 mmol/L) (table 6). In another evaluation conducted by the Johns Hopkins Lipoprotein Analytical Laboratory (unpublished results), the CVₐ for LDL-cholesterol was determined from the analysis of quality control pools furnished by the Centers for Disease Control and Prevention, over a 21-month period. Cholesterol and triglycerides were measured in two frozen serum pools (Q15 and Q18), and HDL-cholesterol was measured in a third pool (AQ11). Since the three analytes were measured independently, two LDL-cholesterol values were calculated using the cholesterol and triglyceride values for pools Q15 and Q18 and the HDL-cholesterol values for pool AQ11. CVₐ’s of 3.6 and 3.4 percent were obtained at LDL-cholesterol levels of 99 mg/dL (2.57 mmol/L) and 171 mg/dL (4.44 mmol/L), respectively (data not shown). In order to provide further information, several members of the Working Group on Lipoprotein Measurement participated in an experiment in which the CVₐ for LDL-cholesterol was estimated from cholesterol, triglyceride, and HDL-cholesterol measurements in each of two serum pools over a 6-week period. CVₐ’s of 4.7 and 2.8 percent were determined at LDL-cholesterol levels of 103 mg/dL (2.68 mmol/L) and 227 mg/dL (5.90 mmol/L), respectively. These values include both interlaboratory and intralaboratory analytical variation (table 6).

All of the foregoing studies were performed in research laboratories, most of which were standardized for cholesterol, triglycerides, and HDL-cholesterol analysis according to the criteria of the CDC-NHLBI Lipid Standardization Program (Myers et al. 1989). A perusal of table 6 reveals that the CVₐ’s for LDL-cholesterol estimated from the Friedewald equation averaged about 4.0 percent over an LDL-cholesterol concentration range of about 100-225 mg/dL (2.60-5.84 mmol/L). The CVₐ’s for LDL-cholesterol were about twofold greater than those for total cholesterol in the same laboratories (table 6). Both CVₐ’s are the standard of performance achievable in well-controlled laboratories.

Surveys of Clinical Laboratories

The College of American Pathologists Comprehensive Chemistry Survey (1989) indicates CVₐ’s of about 12 percent for LDL-cholesterol in eight survey samples analyzed in 1,150 to 1,400 clinical laboratories in the United States (table 7). It must be stressed, however, that the data in tables 6 and 7 cannot be compared directly because they reflect different aspects of LDL-cholesterol analysis. First, the studies in table 6 were performed in one or only a few laboratories using the same or similar methods. Second, the study periods exceeded 6 weeks in only three of the nine studies cited. They therefore reflect primarily the level of precision that can reasonably be expected in a well-controlled laboratory, and as such are best used as a basis for developing performance criteria for LDL-cholesterol measurement.

In contrast, the data in table 7 reflect analyses over a 1-year period in almost 1,400 laboratories operating independently. These laboratories used
many different methods for cholesterol, triglyceride, and HDL-cholesterol, not all of which give equivalent values. Furthermore, the $CV_a$'s shown in table 7 reflect not only the precision of the LDL-cholesterol measurements in a given laboratory, but also the method-specific biases of the laboratories. Such biases are affected not only by the peculiarities of the methods themselves, but also by how closely the survey pools reflect the behavior of fresh patient specimens (i.e., the results will be influenced to some extent by matrix effects). After considering such factors, the apparent precision of the LDL-cholesterol measurements in this survey is encouraging. Nonetheless, the findings do give some indication of the variability of LDL-cholesterol measurement in clinical laboratories as a whole and suggest that improvement in the accuracy and/or precision of LDL-cholesterol measurements will have to be attained so that LDL-cholesterol measurements made in different laboratories can be considered equivalent. Ideally, such measurements should satisfy the generally accepted criterion for the medical usefulness of a clinical measurement: the variability of a measurement due to imprecision should be no greater than half the biological variation (NCEP Laboratory Standardization Panel 1990). The studies cited in table 6 suggest that such a goal is attainable. Interestingly, as observed in the studies in table 6, the CAP surveys also suggest that the $CV_a$ for LDL-cholesterol was about twice that for total cholesterol (table 7). This observation may be more than coincidental. Two of the three measurements needed to estimate LDL-cholesterol require cholesterol analyses. The third measurement, triglycerides, is used to estimate VLDL-cholesterol as [TG]5, and the error in VLDL-cholesterol values due to error in the triglyceride measurement is only one-fifth of the triglyceride error itself. This, combined with the fact that VLDL generally carries only 10 to 20 percent of the circulating cholesterol, suggests that current efforts to improve the analytical accuracy and precision of cholesterol measurements will be accompanied by a concomitant improvement in the accuracy and precision of LDL-cholesterol measurements as well.

As a final precautionary note, data such as those in table 7 should not be used to estimate the precision to be expected for LDL-cholesterol measurements in the individual clinical laboratory since the term “CV” as used in this table includes among-laboratory inaccuracy (bias) as well as within-laboratory analytical $CV$. At present, most individual clinical laboratories surveyed meet or exceed the NCEP guidelines for total cholesterol measurement, and in many, the $CV_a$’s for LDL-cholesterol

### TABLE 7. Estimates of total analytical variation in LDL-cholesterol measurements

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of Concentration</th>
<th>LDL-chol</th>
<th>CV&lt;sub&gt;a&lt;/sub&gt;</th>
<th>CV&lt;sub&gt;a&lt;/sub&gt; (LDL-chol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Laboratories</td>
<td>mg/dL (mmol/L)</td>
<td>(%)</td>
<td>CV&lt;sub&gt;a&lt;/sub&gt; (Total chol)</td>
</tr>
<tr>
<td>C03</td>
<td>1,149</td>
<td>138 (3.57)</td>
<td>10.8</td>
<td>1.9</td>
</tr>
<tr>
<td>C04</td>
<td>1,138</td>
<td>167 (4.32)</td>
<td>11.3</td>
<td>2.0</td>
</tr>
<tr>
<td>C08</td>
<td>1,282</td>
<td>171 (4.42)</td>
<td>11.4</td>
<td>2.0</td>
</tr>
<tr>
<td>C09</td>
<td>1,271</td>
<td>195 (5.04)</td>
<td>12.6</td>
<td>2.1</td>
</tr>
<tr>
<td>C13</td>
<td>1,405</td>
<td>132 (3.41)</td>
<td>11.8</td>
<td>2.0</td>
</tr>
<tr>
<td>C14</td>
<td>1,393</td>
<td>153 (3.95)</td>
<td>11.7</td>
<td>2.1</td>
</tr>
<tr>
<td>C16</td>
<td>1,349</td>
<td>191 (4.94)</td>
<td>13.3</td>
<td>2.2</td>
</tr>
<tr>
<td>C17</td>
<td>1,340</td>
<td>159 (4.11)</td>
<td>12.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Sources of Variation

measurements are undoubtedly similar to those in standardized laboratories. As an example, the CV\(_a\)'s for total cholesterol (1.2 percent), triglycerides (2.6 percent), and HDL-cholesterol (2.9 percent) were furnished to the Working Group on Lipoprotein Measurement by the University of Wisconsin Hospital and Clinics Laboratory. The data were for routinely used quality control pools. From these data, the CV\(_a\) for LDL-cholesterol was about 3.3 percent, which is in the range shown in table 6 for single laboratories. It is prudent for the physician who must interpret serial measurements in the individual patient to use a single, well-controlled clinical laboratory insofar as possible. This would eliminate the among-laboratories component of analytical variation. The CV\(_a\) for LDL-cholesterol measurements within a particular laboratory can be obtained from the laboratory or calculated from the quality control pool concentrations and CV\(_a\)'s for total cholesterol, triglyceride, and HDL-cholesterol (section 6). This information should be readily provided by the laboratory on request.

**NUMBER OF SPECIMENS REQUIRED TO ESTIMATE LDL-CHOLESTEROL**

In view of the variability of LDL-cholesterol values from biological and analytical sources, several samples obtained at intervals of at least several weeks are required to establish an individual’s usual range for circulating LDL-cholesterol. Table 8 reveals that four separate samples, each analyzed in duplicate, would be necessary to establish an individual’s LDL-cholesterol with a total coefficient of variation of 5 percent. If single analyses are used, eight specimens would be required. Two separate specimens, each analyzed once and the results averaged, would establish the patient’s usual LDL-cholesterol concentration with a total coefficient of variation (CV\(_p\)) of 7.0 percent.

The number of specimens actually collected will depend on the purpose of the measurement. When the purpose of the measurement is to determine initially whether the patient’s baseline LDL-cholesterol is above the desirable range, obtain two specimens at least 1 week apart and average the results. The observed CV for the mean is 7.0 percent (table 8), and the two individual values would be expected to fall within ±14 percent of the patient mean 95 percent of the time.

When the purpose of the measurement is to establish the effectiveness of the treatment, obtain three specimens on separate days at least 1 or 2 weeks apart, analyze each specimen once, and calculate the mean. From the data in table 8, the observed CV for the mean is 6.2 percent, and the three individual values would be expected to be within ±12.4 percent of the patient mean 95 percent of the time. The ranges of uncertainty for the average LDL-cholesterol level under selected conditions are shown in table 9. The underlined values are those for two and three specimens taken from a patient, each analyzed once.

It is clear from this table that it would be unlikely for a patient with a usual LDL-cholesterol concentration of 130 mg/dL (3.36 mmol/L) or lower to have a measured LDL-cholesterol of 160 mg/dL (4.13 mmol/L) or higher. Similarly, it would be equally unlikely that a patient with an LDL-cholesterol of 160 mg/dL (4.13 mmol/L) or higher would have a measured value of 130 mg/dL (3.36 mmol/L) or less. The range of overlap decreases from 24 mg/dL (0.28 mmol/L) (single specimen

### TABLE 8. Total coefficient of variation of mean LDL-cholesterol concentration as a function of the number of specimens analyzed

<table>
<thead>
<tr>
<th>No. of Specimens per Patient</th>
<th>No. of Replicate Analyses per Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
</tr>
<tr>
<td>5</td>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
<td>5.2</td>
</tr>
<tr>
<td>7</td>
<td>5.1</td>
</tr>
<tr>
<td>8</td>
<td>4.9</td>
</tr>
</tbody>
</table>

1 Assuming CV\(_a\) = 8.2 percent, CV\(_p\) = 4.0 percent.
per patient analyzed once) to 11 mg/dL (0.28 mmol/L) (two specimens per patient, each analyzed once). Obtaining three specimens per patient reduces the overlap range to 6 mg/dL (0.16 mmol/L) (table 9). Assuming from table 9 an observed coefficient of variation of 6.2 percent for LDL-cholesterol, the values measured in a patient with a usual LDL-cholesterol level of 130 mg/dL (3.36 mmol/L) sampled on three occasions would be expected to fall within the range 114-146 mg/dL (2.95-3.77 mmol/L) 95 percent of the time, if no physiological change has occurred in the patient’s steady state (table 9). This is a total range of 32 mg/dL (0.83 mmol/L). At the level of 160 mg/dL (4.13 mmol/L), the 95 percent range would be from 140-180 mg/dL (3.62-4.65 mmol/L), a difference of 40 mg/dL (1.03 mmol/L). Thus, values measured on three occasions should agree within these limits 95 percent of the time. Larger discrepancies could be due either to laboratory error or to physiological change in the patient between samplings. It would not be possible to decide which, but in this case a fourth sample should be obtained when it is verified that the patient is in steady state.

As seen in table 9, however, a patient with an average LDL-cholesterol concentration of 145 mg/dL (3.75 mmol/L), who is sampled three times and each sample analyzed once, would be expected to have a measured LDL-cholesterol concentration in the range 127 to 163 mg/dL (3.28-4.21 mmol/L), or from 3 mg/dL (0.08 mmol/L) below the maximum desirable level to 3 mg/dL (0.08 mmol/L) above the minimum high-risk level. Thus, patients with usual LDL-cholesterol concentrations in the range 142 to 148 mg/dL (3.67-3.82 mmol/L) would manifest LDL-cholesterol concentrations that span the desirable to high-risk range, and the mean of eight samples, each analyzed once, would be required to place a patient in the borderline-risk range (130-160 mg/dL) (3.36-4.13 mmol/L) with 95 percent certainty.

### TABLE 9. Range of uncertainty of an individual’s average LDL-cholesterol concentration at selected LDL-cholesterol levels

<table>
<thead>
<tr>
<th>Number of Specimens per Patient</th>
<th>Number of Analyses per Specimen</th>
<th>CV (T) (%)</th>
<th>LDL, 95% Range mg/dL (mmol/L)</th>
<th>LDL, 95% Range mg/dL (mmol/L)</th>
<th>LDL, 95% Range mg/dL (mmol/L)</th>
<th>Overlap¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>9.1</td>
<td>106-154 (3.64)</td>
<td>119-171 (3.75)</td>
<td>130-189 (4.13)</td>
<td>130-154 (3.36-3.98)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>7.0</td>
<td>112-148 (2.89-3.82)</td>
<td>125-165 (3.23-4.26)</td>
<td>137-183 (3.54-4.73)</td>
<td>137-148 (3.54-3.82)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>6.2</td>
<td>114-146 (2.95-3.77)</td>
<td>127-163 (3.28-4.21)</td>
<td>140-180 (3.62-4.65)</td>
<td>140-146 (3.62-3.77)</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>5.5</td>
<td>116-144 (3.00-3.72)</td>
<td>129-161 (3.33-4.16)</td>
<td>142-178 (3.67-4.60)</td>
<td>142-144 (3.67-3.72)</td>
</tr>
</tbody>
</table>

¹ Values shown are for overlap for LDL-cholesterol concentrations of 130 mg/dL (3.36 mmol/L) and 160 mg/dL (4.13 mmol/L).
Section 3.
Accuracy of LDL-Cholesterol Measurements

There is at present no formal standardization program for LDL-cholesterol measurement and no available reference method for LDL-cholesterol. Analytical ultracentrifugation has been used as a “reference method” (Lindgren and Jensen 1972). This method provides values in terms of total LDL mass, and conversion to equivalent LDL-cholesterol values requires certain assumptions about the constancy of the cholesterol composition of LDL (Krauss 1987b). For this reason, the combined ultracentrifugation-polyanion precipitation method described above is usually used as the standard by which other methods are judged, and this method has been used to evaluate the accuracy of LDL-cholesterol estimated with the Friedewald equation (table 10). In the original paper, Friedewald et al. (1972) found that LDL-cholesterol estimated from equation 3 agreed with the combined ultracentrifugation-polyanion precipitation method within <0.5 percent on average and that the values obtained with the two methods were highly correlated (r ~ 0.98). The absolute values of the differences between the two methods was 7 percent or less in subjects with TG <400 mg/dL (4.52 mmol/L). Larger differences are encountered in samples with TG >400 mg/dL (4.52 mmol/L), due to the presence of particles that are enriched in TG compared to VLDL from subjects with lower TG concentrations. For this reason, the Friedewald equation has not been used for samples with triglyceride concentrations exceeding 400 mg/dL (4.52 mmol/L).

In contrast, DeLong et al. (1986), in an analysis of data from over 10,000 men, women, and children who participated in the LRC Prevalence Study, found that the original Friedewald equation underestimated LDL-cholesterol to an extent that depended on the TG concentration of the sample; the underestimate was about 4 percent or less at TG concentrations of 200 mg/dL (2.26 mmol/L) or less and was as great as about 10 percent at a TG concentration of 400 mg/dL (4.52 mmol/L) (table 10) (DeLong et al. 1986). The negative bias was even greater at TG concentrations above 400 mg/dL (4.52 mmol/L). It should be noted that the negative bias was somewhat less (<~4 percent) in subjects with hypercholesterolemia, or hypercholesterolemia plus hypertriglyceridemia (DeLong et al. 1986). On the basis of these findings, these investigators suggested that VLDL-cholesterol was better estimated by multiplying the triglycerides concentration by 0.16, corresponding to the factor [TG]/6.25. The reasons for the somewhat discordant findings in the two studies (DeLong et al. 1986; Friedewald et al. 1972) are not entirely clear because the methods used to measure the plasma lipids and lipoproteins in the two studies were similar. The differences could have resulted from factors such as the bias of the triglyceride methods and whether blanked or unblanked triglyceride values were used.

Friedlander et al. (1982), using similar methods, also found that equation 3 underestimated LDL-cholesterol by about 5 percent. These authors found somewhat less bias when a factor that depended on the triglyceride concentration was substituted for [TG]/5 in equation 3 (table 10). The authors noted, however, that the factors determined from their data might not apply equally well to other populations. Rao et al. (1988) also found that equation 3 underestimated LDL-cholesterol by 1.5 to 3.5 percent in most subjects, but the underestimate was about 10 percent in hypertriglyceridemic subjects (table 10). The use of a TG-related factor in place of [TG]/5 was most useful in samples with high
triglyceride concentrations. Siekmeier et al. (1988) found that equation 3 underestimated LDL-cholesterol by about 5 percent. In contrast, it can be calculated from the work of Wilson et al. (1981) that the factor \([TG]/5\) would underestimate the age and sex-adjusted mean VLDL-cholesterol concentration by 13 to 26 mg/dL (0.15-0.29 mmol/L) in adults in the Framingham study. This, in turn, would lead to an overestimate of LDL-cholesterol calculated from equation 3. The authors indicated a 7 to 10 percent error would be expected in LDL-cholesterol (table 10) (Wilson et al. 1981). Again,

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**TABLE 10. Use of the Friedewald equation in various studies**

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>TG VLDL-chol</th>
<th>Error in LDL-cholesterol Using Friedewald Equation</th>
<th>Correlation With Ultra-centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Friedewald et al. 1972</td>
<td>Normal</td>
<td>5.0</td>
<td>&lt;1%</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td></td>
<td></td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Type IV (TG &lt;400)</td>
<td></td>
<td></td>
<td>0.94</td>
</tr>
<tr>
<td>DeLong et al. 1986</td>
<td>Population based (U.S.)</td>
<td>6.25</td>
<td>Up to -10%</td>
<td>—</td>
</tr>
<tr>
<td>Friedlander et al. 1982</td>
<td>Population based (Israel)</td>
<td>TG-related factor</td>
<td>&lt;1%</td>
<td>0.97-0.98</td>
</tr>
<tr>
<td>Wilson et al. 1981</td>
<td>Population based (Framingham)</td>
<td>TG-related factor</td>
<td>Up to +10%</td>
<td>—</td>
</tr>
<tr>
<td>Castelli et al. 1977</td>
<td>Population based (Cooperative Lipoprotein Phenotyping Study)</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Albany</td>
<td>8.3</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Honolulu</td>
<td>5.6</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Evans County</td>
<td>5 to 6.7</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Rao et al. 1988</td>
<td>Clinical lab samples</td>
<td>-1.5 to -9.9%</td>
<td></td>
<td>0.93-0.94</td>
</tr>
<tr>
<td>Siekmeier et al. 1988</td>
<td>Clinical lab samples</td>
<td>-3 to -6%</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Hata and Nakajima 1986</td>
<td>Volunteers</td>
<td>3.0 to 5.0</td>
<td>+2 to +9%</td>
<td>0.73-0.92</td>
</tr>
<tr>
<td>Warnick et al. 1990</td>
<td>Samples submitted for lipid analysis</td>
<td>TG-related factor</td>
<td>&lt;10% in 90% of samples</td>
<td>—</td>
</tr>
<tr>
<td>McNamara et al. 1990</td>
<td>Dyslipidemics and population based (Framingham)</td>
<td>4.5-5.9</td>
<td>&lt;10% in 90% of samples</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>depending on TG concentration</td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Senti et al. 1991</td>
<td>Healthy subjects, patients with peripheral and cerebral vascular disease, and chronic renal failure</td>
<td>2.5-5.0^2</td>
<td>4% to &gt;60%^3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4% to &gt;60%^3</td>
<td>—</td>
</tr>
</tbody>
</table>

1 Using TG/5 to estimate VLDL-cholesterol.
2 Ratio applies to VLDL fraction.
3 Direction of error not specified.
VLDL-cholesterol was more accurately estimated using a triglyceride-dependent factor, but they also pointed out that their factors might not apply to other populations (Wilson et al. 1981). Hata and Nakajima (1986) also found that equation 3 overestimated LDL-cholesterol by 2 to 9 percent in Japanese subjects with various lipid patterns (table 10). They suggested that the factors [TG]/3 and [TG]/4 were more appropriate to their subjects with triglyceride concentrations of <150 mg/dL (1.69 mmol/L) and 150-299 mg/dL (1.69-3.38 mmol/L), respectively, again suggesting that a single factor may not apply equally to all populations. McNamara et al. (1990) examined 4,800 samples using a combined ultracentrifugation/polyanion precipitation method for comparison. They found that the factor [TG]/4 was best for samples containing 50 mg/dL (0.56 mmol/L) triglyceride or less; [TG]/4.5 and [TG]/5 were better for triglyceride concentrations of 51-200 mg/dL (0.58-2.26 mmol/L) and 201-400 mg/dL (0.58-2.26 mmol/L), respectively. However, the mean errors in LDL using the factor [TG]/5 for samples with triglyceride concentrations of up to 400 mg/dL (4.52 mmol/L) were small, ranging from 0 to 3 percent (table 10). They concluded that in view of the variability of VLDL composition, the factor [TG]/5 should be retained and that it provides fairly accurate estimations of LDL-cholesterol in most cases. Warnick et al. (1990), using a combined ultracentrifugation/polyanion precipitation method as the basis for accuracy, compared LDL-cholesterol values obtained in approximately 5,000 samples in the Northwest Lipid Research Clinic laboratory using three versions of the Friedewald equation for estimating LDL-cholesterol: (1) the original Friedewald formula (equation 3); (2) the modification suggested by DeLong et al. (1986); and (3) a formula derived from a stepwise linear regression analysis in which cholesterol, triglyceride, and HDL-cholesterol were all considered to affect the VLDL-cholesterol estimate. They found that (1) all of the three procedures were more nearly accurate in samples with triglyceride concentrations below 200 mg/dL (2.26 mmol/L); (2) all of the methods became progressively less accurate at higher triglyceride concentrations; (3) the direction of the error (positive or negative) was symmetrically distributed about 0 when the unmodified Friedewald equation was used, whereas the modification suggested by DeLong et al. (1986) tended to overestimate LDL-cholesterol. These authors found that the stepwise linear regression procedure slightly improved the accuracy of the equation, but they also cautioned that the regression parameters determined from their data might not apply to other data sets or populations, or in cases in which other methods for lipid measurement were employed. They, too, concluded that overall, it was probably best to continue to use the unmodified Friedewald equation (equation 3).

Finally, Senti et al. (1991) found that the error in LDL-cholesterol calculated from the Friedewald equation increased as the ratio of cholesterol/triglyceride in the VLDL and IDL fractions increased. This effect was more pronounced in patients with atherosclerosis or chronic renal failure. As the IDL-cholesterol/triglyceride ratio increased from <0.3 to >1.21, the error ranged from 1 to 36 percent in patients with atherosclerosis, and 7 to 34 percent in patients with chronic renal failure. The frequency of LDL-cholesterol errors of 10 percent or more increased from about 5 percent to about 50 percent in control subjects and atherosclerosis patients as the VLDL-cholesterol/triglyceride ratio increased from <0.2 to >0.39. The authors did not indicate the direction of the error, but it is known that the presence of cholesterol-enriched VLDL leads to the overestimation of LDL-cholesterol. Since cholesterol-enriched VLDL and IDL are atherogenic, however, it is not clear that this is actually a drawback with respect to the assessment of risk for coronary heart disease. As discussed above, an assay that measures only LDL-cholesterol would exclude these potentially atherogenic particles. This could lead to the underestimation of risk, particularly in patients with atherosclerosis or chronic renal failure. The results of various studies of this kind are summarized in table 10.

The various studies cited in this table must be interpreted cautiously, however, since they did not all use exactly the same methods for measuring cholesterol, triglycerides, and HDL-cholesterol. Also, some of the studies used combined ultracentrifugation/polyanion precipitation procedures similar to those discussed here as the comparison methods, and others used sequential density
ultracentrifugation for this purpose (table 10). As discussed earlier, the combined ultracentrifugation/polyanion precipitation methods would have included IDL-cholesterol and Lp(a) cholesterol in the LDL-cholesterol estimates, whereas sequential density ultracentrifugation may not include IDL and would include only part of the Lp(a) depending on the densities employed. Some of the studies used heparin-MnCl$_2$ precipitation procedures for HDL measurement, whereas others used dextran sulfate-MgCl$_2$ or phosphotungstate methods, which can introduce method-specific biases in the LDL-cholesterol estimate when compared to sequential density ultracentrifugation. In addition, the VLDL-cholesterol value calculated from the quotient [TG]/5 in equation 3 depends on the accuracy of the triglyceride method and on whether triglyceride blanks are measured. Triglyceride blanks were not measured at all in some of these studies (table 10), whereas in one study an estimated average value was used to correct for the triglyceride blank (table 10). Thus, although differences may exist between populations, it cannot be concluded that population differences actually accounted for the various triglyceride devisors that were observed to be optimal in the studies cited. Systematic methodological differences undoubtedly contributed to the findings.

In view of these uncertainties, it is recommended that the original factor [TG]/5 be used when estimating LDL-cholesterol with the Friedewald equation.$^1$

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$^1$ When cholesterol and triglycerides are expressed in mmol/L, VLDL-cholesterol is estimated as [TG]/2.17.
Section 4.
Direct Methods for LDL Measurement

**PRECIPITATION METHODS**

Recently, several “direct methods” for LDL-cholesterol measurement have been developed. In some of these, LDL is precipitated selectively using polyvinyl sulfate or heparin at low pH. LDL-cholesterol is calculated as the difference between total cholesterol and that in the supernatant following LDL precipitation. In another method, the LDL precipitate is recovered for direct cholesterol measurement. Mulder et al. (1984) evaluated the accuracy of two such difference methods and one method in which precipitated LDL-cholesterol was measured directly compared to an ultracentrifugal method that quantitated LDL \((d \, 1.019-1.063 \, \text{kg/L})\). They found that the three methods agreed well in samples with triglyceride concentrations below about 180 mg/dL (2.03 mmol/L), but overestimated LDL-cholesterol concentration considerably in samples with higher triglyceride concentrations. This study cannot be compared directly with the others cited, however, because the \(d \, 1.019-1.063 \, \text{kg/L}\) would not contain lipoprotein particles in the IDL density range and would contain only a portion of the \(\text{Lp(a)}\) that may have been present (table 3). Furthermore, the identity of the lipoprotein species precipitated with the three direct methods was not specified. Lippi et al. (1986) found that LDL-cholesterol determined with a polyvinyl sulfate precipitation method agreed well with values determined by an electrophoretic method and were highly correlated with values obtained with the Friedewald equation in samples with triglyceride concentrations up to about 350 mg/dL (3.95 mmol/L). Again, the presence of lipoproteins other than LDL in the precipitate was not investigated, and the combined ultracentrifugation polyanion precipitation method was not used for comparison. In a new polyclonal antibody-based method developed by Genzyme, Inc., Boston, MA, antibodies against several apolipoproteins other than apo B are coated on latex particles and used to remove lipoproteins other than LDL. The cholesterol in the soluble LDL-containing fraction is measured directly. The method has not been published, and at present, there is insufficient field experience with the method to know how comparable it may be with existing methods, and which if any atherogenic lipoproteins other than LDL are being measured. From the above, it is evident that the currently available “direct LDL methods” still require one or more separation and cholesterol analysis steps. It is not clear whether such methods will ultimately give more reliable results than current methods.

**ELECTROPHORETIC METHODS**

Electrophoretic methods have also been examined for the determination of plasma lipoproteins, including LDL. For reasons that have been discussed in detail (Bachorik 1980), these methods cannot be recommended as the procedures of choice for measuring LDL-cholesterol. Briefly, the electrophoretic methods are generally performed on unfractionated plasma or serum, and the electrophoretic band corresponding to LDL is visualized by staining for lipid or by immunochemical means using antibodies to apo B. The relative amount of material visualized in the LDL region of the electropherogram is determined by densitometric scanning or by eluting and measuring the amount of cholesterol associated with the band. The resolution between LDL and VLDL is not always complete, however, and the intensity of staining is not the same for all the lipoproteins. These factors can interfere with both direct cholesterol measurement and densitometric scanning. The conversion of densitometric scanning values to LDL-cholesterol values requires the use of assumptions about the constancy of the cholesterol
composition of LDL that are not entirely warranted. In addition, neither Lp(a) nor IDL comigrate with LDL in most samples, and the equivalence of the electrophoretic, Friedewald, and ultracentrifugation methods can be uncertain in many samples. In one recent study (Niedbala et al. 1985), the correlation between LDL-cholesterol values determined with an electrophoretic method and equation 3 was only 0.92 to 0.93 in samples with triglycerides of under 400 mg/dL (4.52 mmol/L) and was only 0.87 in samples with LDL-cholesterol concentrations below 200 mg/dL (5.17 mmol/L), reflecting the variability inherent in electrophoretic methods. The electrophoretic values averaged 7 percent lower than those calculated from equation 3. In another evaluation (Graziani et al. 1986), the correlation between an electrophoretic method and the Friedewald method was higher (0.95), but the electrophoretic method gave values about 6 percent lower.

**DENSITY GRADIENT ULTRACENTRIFUGATION AND NUCLEAR MAGNETIC RESONANCE**

Recently, two different approaches have been used to determine the complete lipoprotein profile in a single step. In the first, plasma is subjected to density gradient ultracentrifugation under controlled conditions using the vertical rotor (Chung et al. 1980, 1981, 1986; Cone et al. 1982; Segrest et al. 1983). The cholesterol concentrations of the separated lipoprotein fractions are measured to provide a lipoprotein cholesterol profile. The profile itself reflects the incompletely separated lipoproteins, but a mathematical curve resolution procedure is used to derive the component curves for each of the lipoproteins and to calculate their cholesterol concentrations. This procedure is capable of deriving the lipoprotein-cholesterol concentrations for VLDL, IDL, LDL, Lp(a), and HDL. LDL-cholesterol concentrations can be expressed either in terms of the individual atherogenic particles, IDL, LDL, and Lp(a), or as the sum of total atherogenic particles. The method actually involves several analytical manipulations including the formation of the density gradient, recovery of the lipoproteins, and continuous cholesterol analyses to determine the cholesterol distribution in the gradient. These steps would be expected to contribute to the analytical component of variation. Nonetheless, the method can be automated and addresses a major issue for LDL-cholesterol measurement; it identifies the measured particles and therefore might in principle be readily related to the existing epidemiological database.

In the second method, the nuclear magnetic resonance spectrum for lipoprotein-associated fatty acyl methyl and methylene groups is measured in unfractionated serum (Hamilton and Morrisett 1986; Otvo et al. 1991). The contributions of the major lipoprotein classes, VLDL, LDL, and HDL, are derived mathematically and converted to equivalent lipoprotein-cholesterol concentrations based on certain assumptions about the composition of the lipoproteins. It is unclear at present whether this approach will provide lipoprotein-cholesterol values equivalent to those determined with current methods, since it actually measures lipoprotein fatty acyl groups. On the other hand, since it detects a lipoprotein component other than cholesterol and apolipoprotein, it may ultimately provide useful diagnostic information in conjunction with traditional measurements.

**RECOMMENDED METHODS**

The results obtained by the various investigators have been somewhat inconsistent. There is incomplete information available about exactly what is being measured with some of the methods, and there is not yet enough experience with them for them to be relied on by clinicians. The Working Group on Lipoprotein Measurement recommends that the most prudent course at present is to measure LDL-cholesterol by methods similar to those used to establish the epidemiological database on which the relationships between cardiovascular risk and LDL-cholesterol concentration have been established. The unmodified Friedewald procedure (equation 3) gives reasonably accurate measurements in samples without chylomicrons or β-VLDL and triglyceride concentrations below 200 mg/dL (5.17 mmol/L) and is useful in samples with triglyceride concentrations up to 400 mg/dL (4.52 mmol/L). Thus, the method is suitable in most cases. For samples with β-VLDL, triglycerides exceeding 400 mg/dL (4.52 mmol/L), or chylomicrons, the method of choice is the combined ultracentrifugation polyanion precipitation method. It might also be pointed out that patients with fasting triglyceride concentrations above 250 mg/dL (2.82 mmol/L)
warrant further evaluation in any case (NIH Consensus Conference 1984, 1993), which at least in some would usually require more extensive analytical procedures than use of the Friedewald equation.

STANDARDIZATION OF LDL-CHOLESTEROL MEASUREMENTS

There is at present no formal standardization program for LDL-cholesterol. The CDC, however, currently operates standardization programs for total cholesterol, triglyceride, and HDL-cholesterol, and these programs can be used to develop reference materials and procedures for LDL-cholesterol standardization. In the CDC method used to establish the concentration of HDL-cholesterol in reference materials, serum is ultracentrifuged at 105,000 x g for 18 hours at d 1.006 kg/L. The floating VLDL layer is removed, and the infranate is treated with heparin and MnCl₂ (0.046 mmol/L) to precipitate the remaining apo B-containing lipoproteins. After removing the precipitate, HDL-cholesterol that remains in the supernate is measured with the CDC cholesterol reference method (Duncan et al. 1982). It should be feasible to measure cholesterol in the untreated ultracentrifugal infranate also and establish the LDL-cholesterol concentrations of the reference pools using equation 2. A standardization protocol similar to those for total cholesterol, triglyceride, and HDL-cholesterol could then be developed for LDL-cholesterol.

At present, the adequacy of LDL-cholesterol measurement is governed primarily by strict adherence to currently established criteria for cholesterol, triglyceride, and HDL-cholesterol measurement (Myers et al. 1989). These criteria are shown in table 11. The analytical coefficient of variation of LDL-cholesterol calculated from measured values of the three analytes in a single laboratory can be derived from the means and standard deviations for those measurements (section 6).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration Range (mg/dL)</th>
<th>Accuracy</th>
<th>Maximum Overall Standard Deviation</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>&gt;150</td>
<td>+3% of RV¹</td>
<td>0.03 RV</td>
<td>3%</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0-88</td>
<td>±9 mg/dL (0.10 mmol/L)</td>
<td>7 mg/dL (0.08 mmol/L)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>89-176</td>
<td>±10 mg/dL (0.11 mmol/L)</td>
<td>8 mg/dL (0.09 mmol/L)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>177-220</td>
<td>±11 mg/dL (0.12 mmol/L)</td>
<td>10 mg/dL (0.11 mmol/L)</td>
<td>—</td>
</tr>
<tr>
<td>HLD-cholesterol</td>
<td>&gt;220</td>
<td>±5% of RV</td>
<td>0.05 RV</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td>&lt;40</td>
<td>±10% of RV</td>
<td>2.5 mg/dL (0.06 mmol/L)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>40-60</td>
<td>±10% of RV</td>
<td>3.0 mg/dL (0.08 mmol/L)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>&gt;60</td>
<td>±10% of RV</td>
<td>3.5 mg/dL (0.09 mmol/L)</td>
<td>—</td>
</tr>
</tbody>
</table>

¹ RV, reference value
Assuming the maximum allowable limits for precision in Table 6, and considering samples with total cholesterol concentrations of 200 to 240 mg/dL (5.17 to 6.20 mmol/L), triglycerides of 150 mg/dL (1.69 mmol/L), and HDL-cholesterol of 50 mg/dL (1.29 mmol/L), the CV\textsubscript{a} for LDL-cholesterol calculated from Equation 3 would be about 5.7 percent (section 6), which is in the range of values observed in CDC or Cholesterol Reference Method Laboratory Network standardized laboratories (Table 11). It should be possible, at least on an interim basis, to develop formal criteria for acceptable LDL-cholesterol measurements based on established criteria for cholesterol, triglyceride, and HDL-cholesterol measurement. However, a reference method and a standardization program should be developed for LDL-cholesterol.

**CRITERIA FOR ACCURACY AND PRECISION**

Precision is the primary analytical performance characteristic since accuracy cannot be judged reliably with an imprecise method. The precision of LDL-cholesterol measurements should conform to the generally accepted criterion for the usefulness of a medical test: The random error of the measurement should be no greater than half the average biological variation. In a recent study (Kagonek et al. 1992), the average CV\textsubscript{b} for LDL-cholesterol was found to be approximately 8 percent. The data in Table 6 indicate that a CV\textsubscript{a} of about 4 percent or less can be achieved in a well-controlled laboratory. Thus, a precision goal of 4 percent for LDL-cholesterol testing is consistent with the widely accepted criterion that CV\textsubscript{a} should be less than one-half CV\textsubscript{b} (Elevitch 1977) and also with actual present achievements in good laboratory practice. Accordingly, the LDL-cholesterol precision goal is set at ≤4 percent.

Given this imprecision goal, the goal for bias was developed by relating the error in estimating LDL-cholesterol values to the number of replicate samples that must be obtained from the patient. The 10 percent error limit avoids misclassification of subjects at the midpoint of the NCEP borderline range for LDL-cholesterol, of subjects in the lower borderline range as falsely positive, and of subjects in the upper borderline range as falsely negative. To use an error limit lower than 10 percent in the face of existing biological variability would require sampling rates that are impractical, even if goals for laboratory error are set at zero. As illustrated in Table 12, in the typical situation, the number of serial specimens necessary to establish the patient’s baseline LDL-cholesterol concentration is not affected until the typical analytical bias in the laboratory population reaches about 4 percent. Accordingly, the LDL-cholesterol bias is set at 4 percent.

The specified criteria for accuracy and precision reflect the minimum acceptable levels of routine (i.e., average) laboratory performance that should be achievable within the limits of current methodology. Expressed in this way, the recommendations would lead to average total errors not exceeding 12 percent for LDL-cholesterol (see Table 12).

<table>
<thead>
<tr>
<th>Intraindividual Biologic Variability, Average CV%</th>
<th>Analytic Error</th>
<th>Observed CV%</th>
<th>Number of Serial Samples for Error Limits of 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>8.2</td>
<td>3 5 11 31</td>
</tr>
<tr>
<td></td>
<td>1.5% bias, 2% CV</td>
<td>8.6</td>
<td>3 6 12 34</td>
</tr>
<tr>
<td></td>
<td>4% bias, 4% CV</td>
<td>10.0</td>
<td>4 7 16 44</td>
</tr>
</tbody>
</table>

1 With 95 percent confidence, the subject’s expected LDL-cholesterol value lies within the range defined by the mean of single analyses of n replicate samples sent to different laboratories, ± the stated percent error limit.
The Working Group on Lipoprotein Measurement recognizes that the average total error limit could be exceeded in individual cases. According to table II-3 in appendix II, however, to consider the criteria for bias (≤4 percent) and precision (CV ≤4 percent) as 95 percent limits (see discussion in appendix II) would require an average laboratory bias of 1 percent and a CV of 2.3 percent in order that a total error of less than 10 percent be achieved for the mean of several serial samples with 95 percent confidence using a feasible number (two to three) of serial specimens from an individual. At present, this degree of accuracy and precision is beyond the capability of the currently used methods for LDL-cholesterol.

Assuming a CV of 4 percent with a confidence interval of 95 percent, the maximum error due to imprecision would be 1.96 x 4 percent, or 7.8 percent. Allowing a maximum bias of 4 percent, the maximum total error for a single LDL-cholesterol measurement would be 11.8 percent.

A clinically derived goal is compared to analytically derived goals in table 13. Goals for single point diagnostic LDL-cholesterol testing are set in terms of total error, since the components of bias and precision are not apparent to the clinical user of the result and it is their sum that affects the clinical classification of the patient. The clinical goal is based on a statistical model that relates clinical diagnostic accuracy to biological and analytical variability (Ross 1988). That the clinical goal is larger than laboratory-derived analytical goals is not surprising since laboratory goals evolve from analytical factors susceptible to control, whereas clinical goals depend primarily on the sizable inherent biological variability of analytes that affect diagnostic classification (Elevitch 1977; Hartmann and Ross 1988; Ross and Lawson 1987; Ross 1988). The establishment of error goals for lipids and lipoprotein-cholesterol measurements, along with the associated calculations, are given in more detail in appendix II.

It is useful to consider the maximum bias that might occur for LDL-cholesterol measurements made in a laboratory that meets the minimal CDC-NHLBI criteria for the accuracy of cholesterol, triglyceride, and HDL-cholesterol measurements (table 11). Assuming a total cholesterol of 240 mg/dL (6.20 mmol/L); triglyceride, 150 mg/dL (1.69 mmol/L); HDL-cholesterol, 50 mg/dL (1.29 mmol/L); and LDL-cholesterol, 160 mg/dL (4.13 mmol/L); and maximum allowable biases of 3 percent for total cholesterol, 6.7 percent for triglyceride, and 10 percent for HDL-cholesterol, the expected biases in the LDL-cholesterol estimates can be calculated by considering the direction of the biases (i.e., whether they are positive or negative) for each of the three primary measurements. The results of these calculations are shown in table 14. There would be no bias in LDL-cholesterol values if the cholesterol, triglyceride, and HDL-cholesterol measurements were all biased in the same direction. The error in LDL-

### TABLE 13. Analytical goals for the total error of singular results for LDL-cholesterol in relation to various statistical models and the goals for analytical bias and imprecision

<table>
<thead>
<tr>
<th>Bias</th>
<th>CV&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Quadratic</th>
<th>Linear, 1 Tailed</th>
<th>Linear, 2 Tailed</th>
<th>Clinical Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>4%</td>
<td>4%</td>
<td>11.1%</td>
<td>10.6%</td>
<td>11.8%</td>
<td>14.5%</td>
</tr>
</tbody>
</table>

1 The values were calculated as shown in appendix II.
TABLE 14. Effect of bias in cholesterol, triglyceride, and HDL-cholesterol measurements on LDL-cholesterol values estimated with the Friedewald equation

<table>
<thead>
<tr>
<th>Assumed True Value →</th>
<th>Chol (mg/dL)</th>
<th>Trig (mg/dL)</th>
<th>HDL-chol (mg/dL)</th>
<th>LDL-chol (mg/dL)</th>
<th>% Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(6.20)</td>
<td>(1.69)</td>
<td>(1.29)</td>
<td>(4.13)</td>
<td></td>
</tr>
<tr>
<td>Example</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Bias</td>
<td>+3%</td>
<td>+6.7%</td>
<td>+10%</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Apparent Value</td>
<td>247</td>
<td>160</td>
<td>55</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.38)</td>
<td>(1.81)</td>
<td>(1.42)</td>
<td>(4.13)</td>
<td></td>
</tr>
<tr>
<td>2. Bias</td>
<td>+3%</td>
<td>+6.7%</td>
<td>-10%</td>
<td></td>
<td>+6.3%</td>
</tr>
<tr>
<td>Apparent Value</td>
<td>247</td>
<td>160</td>
<td>45</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.38)</td>
<td>(1.69)</td>
<td>(1.16)</td>
<td>(4.39)</td>
<td></td>
</tr>
<tr>
<td>3. Bias</td>
<td>+3%</td>
<td>-6.7%</td>
<td>+10%</td>
<td></td>
<td>+2.5%</td>
</tr>
<tr>
<td>Apparent Value</td>
<td>247</td>
<td>140</td>
<td>55</td>
<td>164</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.38)</td>
<td>(1.58)</td>
<td>(1.42)</td>
<td>(4.24)</td>
<td></td>
</tr>
<tr>
<td>4. Bias</td>
<td>+3%</td>
<td>-6.7%</td>
<td>-10%</td>
<td></td>
<td>+8.8%</td>
</tr>
<tr>
<td>Apparent Value</td>
<td>247</td>
<td>140</td>
<td>45</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.38)</td>
<td>(1.58)</td>
<td>(1.16)</td>
<td>(4.50)</td>
<td></td>
</tr>
<tr>
<td>5. Bias</td>
<td>-3%</td>
<td>-6.7%</td>
<td>-10%</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Apparent Value</td>
<td>233</td>
<td>140</td>
<td>45</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.02)</td>
<td>(1.58)</td>
<td>(1.16)</td>
<td>(4.13)</td>
<td></td>
</tr>
</tbody>
</table>

cholesterol would be 2.5 percent if the triglyceride bias was opposite those of cholesterol and HDL-cholesterol. The maximum error (8.8 percent) occurs when both triglyceride and HDL-cholesterol exhibit biases opposite that for total cholesterol. In order to achieve accuracy within 4 percent, the bias in LDL-cholesterol at a level of 160 mg/dL (4.13 mmol/L) could not exceed 6.4 mg/dL (0.17 mmol/L). This level of accuracy would be achieved for the examples shown in table 14 with maximal biases of 1.5 percent for total cholesterol, 4 percent for triglycerides, and 3 percent for HDL-cholesterol. Although the extreme examples considered in table 14 are unlikely to occur in any particular laboratory, they nonetheless emphasize the need for a reference method and standardization program for LDL-cholesterol. Moreover, they also indicate the need for methods that allow the measurement of LDL-cholesterol in a way that does not depend on calculating the difference between two or more primary measurements.

CONTROLLABLE PREANALYTICAL FACTORS THAT CAN AFFECT LDL-CHOLESTEROL MEASUREMENTS

Several aspects of patient preparation and blood sampling can affect lipid and lipoprotein measurements (NCEP Laboratory Standardization Panel 1990). Many of these are not under the control of the laboratory, but four issues—the use of fasting samples, serum-plasma differences, posture-related changes in lipid concentrations, and analysis of frozen samples—are discussed here because the laboratory can control them to a greater or lesser extent.

Fasting Samples

Although total cholesterol remains essentially unaffected postprandially (NCEP Laboratory Standardization Panel 1990), the effects of recent food ingestion on plasma LDL- and HDL-cholesterol concentrations are less certain. Cohn et al.
(1988a) found transient decreases of as much as 20 percent in LDL- and HDL-cholesterol concentrations after feeding subjects a single formula meal containing 1 g fat per kg body weight, or one-half to two-thirds the average American daily fat intake. The changes were related to transient changes in the lipid composition of both lipoproteins during the metabolism of the postprandial lipoproteins. In another study, these authors recorded a mean decrease of 7.5 percent in LDL-cholesterol concentration in human subjects following a formula fat meal when LDL-cholesterol was measured with an ultracentrifugation polyanion precipitation method (Cohn et al. 1988b). However, the apparent decrease was about 22 percent when LDL-cholesterol was estimated from equation 3. Thus, LDL-cholesterol decreased postprandially, but the magnitude of the decrease was overestimated by about threefold using equation 3. On the other hand, van Amelsvoort et al. (1989) reported no significant change in the concentration of LDL- or HDL-cholesterol after their subjects consumed a more conventional lunch that provided 40 percent of the total daily caloric requirement and that varied in fat content from 23 to 55 percent of calories. (In neither study was the Friedewald equation used to estimate LDL-cholesterol [see below]). Wilder (1992) found a 2.5 percent decrease in LDL-cholesterol in a group of volunteers 5 hours after ingestion of a self-selected breakfast. In that study, LDL-cholesterol was calculated using the Friedewald equation. The possibility exists, therefore, that LDL-cholesterol might eventually be measured accurately in many nonfasting subjects, but such a recommendation cannot be made at present.

It is also necessary to consider how long patients should fast before blood is drawn. A standard fasting period of 12 hours has long been used for both clinical and research measurements. In view of the wider appreciation of the need for LDL-cholesterol measurements, the revised NCEP ATP guidelines advising the measurement of HDL-cholesterol when screening some categories of individuals, and the inconvenience to the patient of having to fast for 12 hours, the revised ATP guidelines recommend that lipid and lipoprotein measurements can be made following a fasting period of 9 to 12 hours.

Although limited data are available, there is some evidence that the error in lipid and lipoprotein measurements may be acceptable for clinical purposes after a 9-hour fasting period. Havel (1957) fed formula meals containing 1.5 g fat/kg body weight to several subjects who had been on a high-fat diet for a week. The concentrations of serum glycerides 8 hours postprandially averaged 21 mg/dL higher than fasting values. Using equation 3, this would be expected to produce a 4 mg/dL underestimate of LDL-cholesterol, or about 3 percent at an LDL-cholesterol level of 130 mg/dL. There was little change in the concentration of ultracentrifugally isolated LDL-cholesterol, however. Havel et al. (1973) fed several healthy subjects formula meals containing 1.5 g fat/kg body mass and measured postprandial lipoprotein cholesterol concentration in ultracentrifugally isolated lipoproteins. From their data, the 9-hour postprandial LDL-cholesterol values would be about 3 mg/dL below fasting values. This would be a 2.3 percent underestimate at an LDL-cholesterol level of 130 mg/dL. Cross-sectional data from a study of employees of the Southern Pacific Transportation Co. in California were provided to the Working Group on Lipoprotein Measurement by Dr. Richard Havel. These data suggest that serum triglycerides in men whose levels were less than 400 mg/dL and who had fasted 8 to 10 hours are about 15 mg/dL higher than those who had fasted 12 to 14 hours (table 15). This would lead to a 3 mg/dL, or about 2 percent, underestimate at an LDL-cholesterol level of 130 mg/dL when using equation 3. There was no consistent trend in women (table 16). A preliminary analysis of LDL-cholesterol values in Phase I of the National Health and Nutrition Examination Study III (NHANES III) was provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics. The analysis revealed no significant differences in group mean LDL-cholesterol level in subjects who had fasted 9 to 11 hours compared to those who had fasted 12 hours or more. As indicated above, a decrease in HDL-cholesterol has also been observed postprandially and could add to the error if HDL-cholesterol did not return to fasting levels by 9 hours. Havel (1957) and Havel et al. (1973) observed little change in HDL-cholesterol 9 hours after a formula fat meal.
TABLE 15. Serum triglyceride concentrations in men fasting for various periods before blood sampling

<table>
<thead>
<tr>
<th>Hours Since Last Food</th>
<th>&lt;8 (n=85)</th>
<th>8-10 (n=159)</th>
<th>10-12 (n=946)</th>
<th>12-14 (n=1,271)</th>
<th>&gt;14 (n=1,135)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-adjusted mean log TG</td>
<td>2.083</td>
<td>2.072</td>
<td>2.038</td>
<td>2.015</td>
<td>2.057</td>
</tr>
<tr>
<td>Triglyceride concentration (mg/dL)</td>
<td>121.1</td>
<td>118.0</td>
<td>109.2</td>
<td>103.5</td>
<td>113.9</td>
</tr>
</tbody>
</table>

1 n, number of subjects.
2 Age range, 20-74 years.

Source: Data provided by Drs. Richard J. Havel and Nancy Phillips, University of California, San Francisco.

Cohn et al. (1988b) fed 22 normal subjects a fat load of 1 g/kg body weight. They found that triglycerides remained about 50 percent higher than fasting values after 9 hours. At this point, HDL-cholesterol levels were 2 mg/dL lower than after 12 hours, and LDL-cholesterol calculated from equation 3 was 6 percent lower than after 12 hours. Lichtenstein et al. (1993) measured postprandial total cholesterol, triglyceride, and HDL-cholesterol in 14 subjects following three normal meals and a snack. The average fasting concentrations of total cholesterol, triglyceride, and HDL-cholesterol were 222, 105, and 48 mg/dL, respectively. Ten hours after the last food ingestion, total cholesterol and HDL-cholesterol each averaged 4 mg/dL below fasting values, and triglyceride was 98 mg/dL higher than the fasting level. Calculated from these mean values, the fasting LDL-cholesterol value would be 153 mg/dL and the 10-hour postprandial values would be 133 mg/dL, or about 13 percent lower than the fasting values. In contrast, Schneeman et al. (1993) found that plasma triglycerides returned to baseline by 9 hours after the ingestion of an ordinary meal containing one-third the daily intake of fat. Total cholesterol was unchanged; HDL-cholesterol values were not reported. De Bruin et al. (1991) fed a 98 g fat load to six healthy subjects and found that plasma triglycerides had returned to baseline after 7 hours. HDL-cholesterol decreased postprandially and remained 35 percent below the fasting level after 8 hours, however. Assuming that total cholesterol values at 8 hours were the same as fasting values, it can be calculated that LDL-cholesterol estimated with equation 3 would have been about 12 percent higher than fasting values. Samples were not taken in the 9- to 12-hour postprandial period in that study, but it is unlikely that either LDL- or HDL-cholesterol levels would have returned to fasting levels 1 hour later. On the other hand, NHANES III phase I data revealed no significant difference in group mean HDL-cholesterol in subjects who had fasted 9 to 11 hours compared to those who had fasted 12 hours or longer. Overall, the available data from the various studies suggest that, on average, a 9- to 12-hour fast would produce little error in LDL-cholesterol values.

Further studies are needed to assess the effects of fasting for 9 to 12 hours in a “real life” setting, that is, in patients following their normal dietary routines. Nonetheless, some tentative conclusions can be drawn about the magnitude of the error to be expected when LDL-cholesterol is estimated in patients who have fasted for 9 to 12 hours. First, it is likely that LDL-cholesterol will be underestimated slightly in patients who have fasted for 9 hours. When LDL-cholesterol is calculated from equation 3, the error on average will probably be in the range of about 2 to 4 percent and will probably be about 1 to 2 percent if the measurements are made with the beta quantification method. Second, such errors will probably create
the most uncertainty in patients with LDL-
cholesterol concentrations near the medical
decision cutpoints; patients with clearly normal or
elevated LDL-cholesterol concentrations should
not be misclassified. Based on these considera-
tions, the Working Group on Lipoprotein Measure-
ment recommends that LDL-cholesterol measure-
ments can be made most accurately in samples from patients who
have fasted for 12 hours. A 12-hour fasting period
should be used for most research purposes and in other
cases where the error in LDL-cholesterol measure-
ments must be minimized. For purposes of conve-
nience, a 9- to 12-hour fasting period can be used in
many patients, but LDL-cholesterol will be underesti-
mated slightly. This should be taken into account
when interpreting the values.

Serum-Plasma Differences

Plasma prepared using disodium ethylenediamine-
tetraacetate (EDTA) as the anticoagulant has
generally been used for lipoprotein analysis
because EDTA retards certain oxidative and
enzymatic changes that can occur in lipoproteins
during storage and handling (Bachorik 1983).
Such changes can affect certain lipoprotein
methods, particularly ultracentrifugation and
electrophoretic analysis of apolipoproteins
(Bachorik 1983). Total cholesterol, triglyceride,
and HDL-cholesterol also can be measured
accurately in serum, and serum concentrations of
these analytes have been measured in a number of
large-scale epidemiological studies including the
second National Health and Nutrition Examination
Survey (NHANES II) (Sempos et al. 1989;
Linn et al. 1989), the Hispanic Health and
Nutrition Examination Survey (Carroll et al.
1990), and others (Srinivasan et al. 1976); and
guidelines for desirable, borderline, and high LDL-
cholesterol levels are given in terms of serum
concentration (NCEP 1988, 1994). It is recom-
mended that serum LDL-cholesterol values be re-
ported.

EDTA exerts a slight osmotic effect causing a shift
of water from blood cells to the plasma. This
results in slightly lower lipid concentrations in
EDTA plasma compared to serum. In a multi-
laboratory examination of 500 serum-plasma pairs,
the difference was estimated to be about 3.0
percent in samples containing 1 g/L EDTA
(Laboratory Methods Committee, Lipid Research
Clinics Program 1977). More recently, in a
smaller study in a single laboratory, a difference of
4.7 percent was found in blood samples containing
1.5 g/L EDTA (Cloey et al. 1990). The higher
concentration is commonly used in evacuated
blood-drawing tubes that are currently available
commercially. For the sake of simplicity and
consistency with the NCEP Laboratory Standard-
ization Panel recommendations, it is suggested
that plasma LDL-cholesterol values be multiplied
by 1.03 when converting to serum values.

TABLE 16. Serum triglyceride concentrations in women fasting for various periods before blood
sampling

<table>
<thead>
<tr>
<th>Hours Since Last Food</th>
<th>&lt;8 (n=18)</th>
<th>8-9 (n=27)</th>
<th>10-11 (n=251)</th>
<th>12-13 (n=342)</th>
<th>≥14 (n=330)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-adjusted mean log TG$^2$</td>
<td>1.972</td>
<td>1.886</td>
<td>1.919</td>
<td>1.919</td>
<td>1.914</td>
</tr>
<tr>
<td>Triglyceride (mg/dL) concentration</td>
<td>93.6</td>
<td>76.9</td>
<td>83.0</td>
<td>83.0</td>
<td>87.3</td>
</tr>
</tbody>
</table>

1 n, number of subjects.
2 Age range, 20-74 years.

Source: Data provided by Drs. Richard J. Havel and Nancy Phillips, University of California, San Francisco.
Heparin exerts little osmotic effect in concentrations employed as an anticoagulant, and cholesterol concentrations in heparinized plasma are the same as in serum (Ladenson et al. 1974; Lum and Gambino 1974; Cloey et al. 1990). Heparin, however, does not have the antioxidative and antimicrobial properties of EDTA. A further caution: Heparin might possibly interfere with HDL-cholesterol analysis depending on the polyanion divalent cation reagents used to prepare the HDL-containing fraction; at present, however, there are no available data to indicate whether this may actually be a problem.

**Posture**

Plasma volume increases when a standing person lies down, causing a decrease in the plasma concentration of nondiffusible components including lipoproteins (Thompson et al. 1928; Waterfield 1931; Fawcett and Wynn 1960; Stoker et al. 1966; Tan et al. 1973; Humphrey et al. 1977; Dixon and Paterson 1978; Renoe et al. 1979; Miller et al. 1992). This is caused by the redistribution of water between the vascular and extravascular compartments. A significant reduction in total cholesterol occurs within 5 minutes (Tan et al. 1973), and decreases of up to 10 to 15 percent have been observed after 20 minutes (Tan et al. 1973; Dixon et al. 1978). The change in standing individuals who sit is a little smaller, about 6 percent after 10 to 15 minutes (Tan et al. 1973), and the changes are reflected in alteration in individual lipoprotein concentrations (Hagen et al. 1986). Such changes can affect the interpretation of lipid and lipoprotein measurements, and the NCEP Laboratory Standardization Panel (1990) has recommended that blood sampling be standardized to the sitting position if possible and that the patient sit quietly for 5 minutes before blood is drawn. It is recommended that this procedure be used when collecting specimens for LDL-cholesterol measurement also.

**Frozen Specimens**

The safest procedure is to analyze freshly drawn specimens as soon as possible after collection. Specimens can be safely stored for up to 3 days at 4 °C, after which storage-related changes can produce errors in HDL-cholesterol measurements (Bachorik et al. 1980) and thus lead to inaccurate LDL-cholesterol values. Cholesterol, triglycerides, and HDL-cholesterol are stable for periods of at least 1 year when the samples are frozen at temperatures of -70 °C and lower. Frozen sera were used in a number of large-scale epidemiological surveys (Sempos et al. 1989; Linn et al. 1989; Carroll et al. 1990) including the ongoing NHANES III. Specimens can also be stored for periods of a few weeks at -20 °C in a non-self-defrosting freezer. LDL-cholesterol can therefore be measured in suitably stored frozen samples using the Friedewald equation.

Frozen specimens, however, should not be used for ultracentrifugal analysis, since freezing can alter the structure of the lipoproteins, primarily the triglyceride-rich lipoproteins, and alter their distributions in the ultracentrifugal fractions. This can lead to inaccurate LDL-cholesterol measurements.

It should be mentioned that frozen sera may ultimately be found suitable when used as LDL-cholesterol reference materials or for quality control purposes, since such sera might be selected to contain low concentrations of triglyceride-rich lipoproteins or otherwise treated to ensure stability. The LDL-cholesterol concentrations of such pools, although stable during the period of use, might change during their preparation and might not reflect those in fresh samples. The NCEP Working Group on Lipoprotein Measurement recommends that for clinical and research purposes only samples that have not been frozen be used to measure LDL-cholesterol with the ultracentrifugation-polyanion precipitation (beta quantification) method.
In view of the foregoing considerations, the Working Group on Lipoprotein Measurement makes the following recommendations for measuring LDL-cholesterol. It is recognized that these recommendations may not provide an accurate measurement of LDL-cholesterol, per se, in all individuals but will provide an estimate of the amount of cholesterol carried in total atherogenic particles of d >1.006 kg/L. They are intended to provide accurate and reproducible measurements that conform to the present epidemiological database relating LDL-cholesterol concentration to cardiovascular risk and to current estimates of the reduction in risk attending LDL-cholesterol lowering. The recommendations will have to be revised as the relative contributions of individual atherogenic particles to risk and treatment modalities directed at lowering the concentration of specific particles are better understood.

GENERAL RECOMMENDATIONS
Maintaining Linkage With the Existing Epidemiological Database

New methods for LDL-cholesterol continue to evolve, but there is incomplete information available about what is being measured and there is not yet enough experience with them for clinicians to rely on them. The existing epidemiological database relating LDL-cholesterol concentration to coronary heart disease risk includes the contributions of other potentially atherogenic particles in addition to LDL, and the methods used to measure LDL-cholesterol should give results equivalent to those used to establish the database.

Reference Method

The current basis for accuracy of LDL-cholesterol measurement should be combined ultracentrifugation-polyanion precipitation. The reference method should be based on the current CDC reference methods for total cholesterol and HDL-cholesterol and should satisfy the following major criteria:

- The reference method should provide serum-equivalent values.
- The LDL- plus HDL-containing fraction should be prepared by ultracentrifuging an accurately measured aliquot of the specimen at d 1.006 kg/L for the equivalent of 18 hours at 105,000 x g, quantitatively removing the supernatant VLDL- and chylomicron-containing fractions, and reconstituting the infranatant fraction to the original volume with 0.15 M NaCl. The cholesterol content of the reconstituted infranate is measured.
- The HDL-containing fraction should be prepared from the ultracentrifugal infranate using a polyanion divalent cation reagent that provides HDL-cholesterol measurements equivalent to those obtained with the heparin-manganese chloride method (Burstein and Samaille 1960).
- Cholesterol in the ultracentrifugal bottom fraction should be measured with methods that satisfy the accuracy and precision criteria of the NCEP Laboratory Standardization Panel (1988, 1990). Cholesterol in the HDL-containing fraction should be measured with methods that...

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1 Includes VLDL remnants, IDL, Lp(a), and LDL.
Recommendations on Lipoprotein Measurement—LDL

satisfy the accuracy and precision criteria specified for HDL-cholesterol (see part two, HDL recommendations).

- LDL-cholesterol should be calculated from equation 2:

\[ [\text{LDL-chol}] = [d > 1.006 \, \text{kg/L chol}] - [\text{HDL-chol}] \]

- The reference method should be the accuracy base against which new methods are evaluated.

Criteria for Analytical Performance

The goals for LDL-cholesterol measurement are stated in terms of total analytical error, which takes account of both accuracy and imprecision. This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater impression can be accepted if the measurements are more nearly accurate. The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV ≤ 3 percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent. The specification of guidelines for accuracy and precision separately can lead to an ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits are considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance. LDL-cholesterol measurements should be within the following routine limit of performance:

\[ \text{Total error} \leq 12 \text{ percent} \]

This is consistent with the following limits for accuracy and precision:

\[ \text{Accuracy} \leq +4 \text{ percent} \]
\[ \text{CV} \leq 4 \text{ percent} \]

Laboratories and others making LDL-cholesterol measurements can assess their individual conformance to the analytical goals as indicated in appendix II. These criteria should apply regardless of how, where, or by whom the measurements are made.

Routine Method

For routine patient evaluation and followup, and for monitoring non-laboratory-based measurements and in situations where ultracentrifugation is impractical, LDL-cholesterol should be estimated from direct measurements of total cholesterol, triglycerides, and HDL-cholesterol using the original Friedewald equation:

\[ [\text{LDL-chol}] = [\text{Total chol}] - [\text{HDL-chol}] - [\text{TG}] / 5, \]

where all concentrations are expressed in mg/dL.²

Either EDTA plasma or serum can be used. To convert plasma values to equivalent serum values, multiply the plasma value by 1.03.

When EDTA plasma is used, the HDL-containing fraction should be prepared using a polyanion-divalent cation reagent that provides results of the same accuracy as obtainable with the modified heparin-manganese chloride method (0.092 M MnCl₂) (Warnick and Albers 1978) as determined by appropriate statistical methods (Bland and Altman 1986; Brown and Beck 1989; Westgard and Hunt 1973). When serum is used, the polyanion-divalent cation reagent should provide results of the same accuracy as those obtainable with the method of Bursten and Samaille (1960), which uses a manganese chloride concentration of 0.046 M.

² Divide triglyceride by 2.17 when concentrations are expressed in mmol/L.
The total cholesterol measurements should conform to the accuracy and precision criteria of the NCEP Laboratory Standardization Panel (1988, 1990), and the triglyceride and HDL-cholesterol measurements should conform to the criteria outlined in the respective reports of the NCEP Working Group on Lipoprotein Measurement (see part two, HDL recommendations; and part three, triglyceride recommendations). The Friedewald equation should not be used as a reference method for LDL-cholesterol measurement.

**Recommendations for Manufacturers**

Manufacturers and others developing new methods and instruments for LDL-cholesterol measurement should be aware of the medical community’s need to refer such measurements to the current epidemiological database as it relates to risk for coronary heart disease.

- As each new method is developed, it should be validated against the reference method using appropriate statistical methods for comparing measurement methods (Bland and Altman 1986; Brown and Beck 1989; Westgard and Hunt 1973).

- For all current and future methods, the nature of the lipoprotein particles that contribute to the LDL-cholesterol measurement should be specified.

- The assigned LDL-cholesterol values for calibration and quality control materials should be traceable to the reference method for LDL-cholesterol. The method(s) used to establish assigned values should be indicated, and bias with respect to reference values should be stated. This is particularly important for methods that may not measure all the atherogenic apo B-containing particles of d>1.006 kg/L. The CDC laboratory, the Cholesterol Reference Method Laboratory Network, and other CDC standardized lipoprotein research laboratories can be of assistance for these purposes.

Manufacturers should cooperate with CDC and the Cholesterol Reference Method Laboratory Network in developing suitable reference materials for LDL-cholesterol measurement. To the extent possible, such materials should be free of matrix effects.

**Recommendations for Health Care Providers**

**Understand What Is Being Measured**

Physicians and other health care providers should understand which lipoproteins contribute to the measured LDL-cholesterol value. They should also become familiar with the limitations of the Friedewald equation (see General Recommendations).
Measure LDL-Cholesterol in Fasting Samples

- Blood samples should be obtained by venipuncture following a 12-hour period of fasting. If necessary, the patient can take water and prescribed medications during this period. This procedure should be followed for research purposes and in other circumstances in which error in the LDL-cholesterol measurement must be minimized.

- If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not be less than 9 hours. It is likely that, on average, LDL-cholesterol will be underestimated slightly (about 2 to 4 percent) in patients who have fasted for 9 hours. This should be taken into account when interpreting the values.

- To the extent possible, blood should be drawn in the sitting position and the patient should be allowed to sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should be sampled in the same position on each occasion.

Use of Serum and Plasma

- EDTA plasma should be used when the LDL-cholesterol is to be measured by ultracentrifugation-polyanion precipitation.

- Either serum or EDTA plasma can be used when LDL-cholesterol is to be estimated with the Friedewald equation.

- When EDTA plasma is used, the plasma value is converted to the equivalent serum value by multiplying the plasma value by 1.03.

Stored Samples

- Serum or plasma should be removed from cells within 3 hours of venipuncture.

- Specimens can be stored for up to 3 days at 4 °C. If analysis is delayed, the specimens can be stored for periods up to several weeks at -20 °C in a non-self-defrosting freezer. Specimens should be stored at -70 °C or lower if longer periods of storage are necessary.

- In all cases, the samples should be stored in clean containers that can be sealed to prevent evaporation. Do not use cork stoppers or plastic film to seal the containers since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

Need for Serial Measurements in Individuals

When LDL-cholesterol measurements are made for the purpose of diagnosis, treatment, and followup, it is important initially to establish the patient’s usual LDL-cholesterol to assess the efficacy of treatment. Considering the inherent physiological and analytical variability of LDL-cholesterol measurements, LDL-cholesterol measured on a single occasion will not suffice. Although it would be ideal to establish a patient’s LDL-cholesterol value within a 10 percent limit for total error with 95 percent confidence, at present it is not technically or economically feasible due to the excessive number (i.e., four) of serial samples required. For this reason, the following recommendation is made to improve the reliability of LDL-cholesterol measurements. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart. The individual LDL-cholesterol values should be averaged.

Three serial samples: Using three serial samples, each referred to the same laboratory and assayed once, and assuming a CV of 8.2 percent and a CV of 4 percent, the observed CV for the mean LDL-cholesterol value is 5.3 percent, and the difference between the means of sequential series of three samples should not exceed 14.6 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 25 percent. If they are further apart, analytical error or a change in the physiological steady state of the patient should be suspected and another sample may be warranted, depending on the patient’s LDL-cholesterol level and its proximity to the concentrations used for decision making.
Two serial samples: For reasons of convenience and considering economic factors, the ATP II report recommended the use of at least two serial samples. Using two serial samples, each referred to the same laboratory and assayed once, and assuming a CV of 8.2 percent and a CV of 4 percent, the observed CV for the mean LDL-cholesterol value is 6.5 percent. The difference between the means of each sequential series should not exceed 17.9 percent. The difference between individual values in each series should not exceed 25 percent, 95 percent of the time. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s LDL-cholesterol level and its proximity to the concentrations used for decision making.

Based on the prevailing distributions of LDL-cholesterol,* using two serial measurements and considering a cutpoint of 130 mg/dL, a patient’s LDL cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is >145 mg/dL or <115 mg/dL, respectively. Using a cutpoint of 160 mg/dL, the patient’s LDL-cholesterol value can be confidently assumed to be above or below the cutpoint when the mean value is >178 mg/dL or <142 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 71 percent of the general population as being above or below the 130 mg/dL cutpoint and 73 percent as being above or below the 160 mg/dL cutpoint.

Screening

NCEP guidelines do not require initial testing for LDL-cholesterol; LDL measurement has been recommended as a followup procedure when indicated (NCEP 1988, 1994) and may be used for initial testing at the physician’s option. It may ultimately prove desirable to substitute LDL-cholesterol measurements for the currently used procedures that rely on total cholesterol measurement, when LDL-cholesterol measurements become more nearly accurate, precise, and economically feasible. At present, however, there is little information available about the accuracy and precision of LDL- or HDL-cholesterol measurements made in field screening or physicians’ office settings. In one recent study, HDL-cholesterol measurements with a desktop analyzer were judged to be excessively variable (Bachorik et al. 1991). In many instances field screening is conducted using capillary blood samples obtained by fingerstick, and there is currently little information about the suitability of such samples for LDL-cholesterol measurements. Another difficulty is the need for fasting specimens. For these reasons, it would be premature to recommend LDL-cholesterol screening until such concerns have been adequately addressed by appropriately designed studies. Even if ultimately recommended, such measurements should be made in a laboratory setting. Accordingly, mass screening for LDL-cholesterol is not recommended at this time.

RECOMMENDATIONS FOR LABORATORIES

Laboratories should use procedures that allow the measurement of LDL-cholesterol with a total error \(\leq 12\) percent. One set of conditions that satisfies this recommendation is that LDL-cholesterol be measured with an accuracy within \(\pm 4\) percent and a CV \(\leq 4\) percent. In the absence of a formal standardization program for LDL-cholesterol, the adequacy of LDL-cholesterol measurements made with the Friedewald equation is governed by strict adherence to NCEP criteria for cholesterol, triglyceride, and HDL-cholesterol measurement (NCEP Laboratory Standardization Panel 1990; also see part two, HDL, and part three, triglycerides). Until a reference method for LDL-cholesterol is established and suitable reference materials become available, the accuracy of LDL-cholesterol values calculated with the Friedewald equation (equation 3) should be assessed using the laboratory biases for total cholesterol, triglyceride, and HDL-cholesterol as established using reference materials for those analytes. This assessment should be made at two levels of LDL-cholesterol, for example, 130 mg/dL (3.36 mmol/L) and 160 mg/dL (4.13 mmol/L). Precision should be assessed using the LDL-cholesterol values calculated from the total cholesterol, triglyceride, and HDL-cholesterol measurements in those pools.

* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel. Personnel handling blood samples should use gloves and should avoid leaving samples open to the air longer than necessary. Samples should be handled in accordance with CDC guidelines for the prevention of infection in health care workers.

**RECOMMENDATIONS FOR GOVERNMENT AGENCIES AND OTHER PROFESSIONAL GROUPS**

The Centers for Disease Control and Prevention should take the following steps:

- Establish a reference method for LDL-cholesterol measurement. For the present, the method can probably be readily developed by modifying the current CDC ultracentrifugation polyanion precipitation method for HDL-cholesterol. The reference method should include the contributions of all the atherogenic apo B-containing particles reflected in the current epidemiological database that relates LDL-cholesterol concentration to the risk for coronary heart disease.

- Add an LDL-cholesterol standardization program to the current CDC-NHLBI standardization programs for total cholesterol, triglycerides, and HDL-cholesterol. Because of the important and far-reaching consequences of inaccurate LDL-cholesterol measurements, an LDL-cholesterol standardization program should be developed also.

- In cooperation with appropriate experts in the academic, clinical laboratory, and manufacturing sectors, develop reference materials for LDL-cholesterol measurement in which matrix effects are minimized. These reference materials should be suitable for standardization, surveillance, method calibration as appropriate, and bench-level quality control.

The National Heart, Lung, and Blood Institute should take the following steps:

- It has been the policy of NHLBI to require standardized lipid and lipoprotein measure-ments for Government-supported clinical and epidemiological studies. This policy should be continued.

- Encourage the Cholesterol Reference Method Laboratory Network system to expand its activities to include the certification of LDL-cholesterol, HDL-cholesterol, and triglyceride measurements.

- Encourage the development and preliminary evaluation of new LDL-cholesterol methods and associated instrumentation.

The Cholesterol Reference Method Laboratory Network currently provides assistance to manufacturers and laboratories wishing to establish the traceability of total cholesterol measurements to the cholesterol reference method. The Network should:

- Expand these activities to include LDL-cholesterol, HDL-cholesterol, and triglyceride measurements.

The College of American Pathologists and other professional organizations that operate clinical chemistry survey programs should take the following steps:

- Include LDL-cholesterol measurements in such surveys.

- Provide CDC-confirming values for LDL-cholesterol concentrations in survey pools.

**RECOMMENDATIONS FOR FURTHER RESEARCH**

Further research is needed in several areas.

- New methods for LDL-cholesterol measurement should be developed. Such methods should be capable of quantitating LDL-cholesterol directly; they should not be based on calculations of the difference between two or more measured values. The nature of the lipoprotein species contributing to the LDL-cholesterol measurement should be defined and the relationship established between values obtained and those used to establish the current epidemiological database. This is particularly important for LDL-specific methods, since such
methods would exclude the contributions of one or more potentially atherogenic particles that may be present in higher concentrations in individuals who are at increased risk for coronary heart disease.

- Research is needed to define the relative contributions of cholesterol carried in each of the individual apo B-containing lipoproteins of \( d > 1.006 \) kg/L (IDL, LDL, Lp(a)) to coronary heart disease risk as presently defined in terms of LDL-cholesterol measurements that include the contributions of all such lipoproteins.

- Suitable reference materials are needed for LDL-cholesterol measurement. Such materials should be essentially free of matrix effects and should be sufficiently stable to allow long-term monitoring of the accuracy and precision of LDL-cholesterol measurements.
Section 6. Calculations

1. Calculation of component of $CV_a(LDL)$ attributable to preparation of the lipoprotein fractions.

The component of analytical variation attributable to the preparatory steps was calculated as follows:

\[ var_{prep} = (var_{a(LDL)} - var_{a(TC)}) \]

Tot Chol = 193  \quad SD_{TC} = 4.5  \quad var_{TC} = 20.25

LDL = 113  \quad SD_{LDL} = 7.5  \quad var_{LDL} = 55.50

\[ var_{prep} = 35.25 \]

\[ SD_{prep} = 5.9 \quad CV_{prep} = SD_{prep}/LDL\text{ conc} \]

\[ = 5.3\% \]

2. Calculation of $CV_a(LDL)$ from measured values of total cholesterol, triglycerides, and HDL-cholesterol.

The $CV_a$ for LDL-cholesterol measurement was calculated from the variances of the total cholesterol, triglyceride, and HDL-cholesterol measurements as follows:

\[ var_{LDL} = var_{TC} + var_{HDL} + \frac{1}{5} var_{TG/5} \]

3. Calculation of $CV_a(LDL)$ from maximum allowable CDC standardization limits for total cholesterol, triglycerides, and HDL-cholesterol.

Assume a total cholesterol concentration of 200 mg/dL (5.17 mmol/L); triglyceride, 150 mg/dL (1.69 mmol/L); and HDL-cholesterol, 50 mg/dL (1.29 mmol/L). From table 11, the maximum $CV_a$’s allowed for CDC standardization at these levels are: total cholesterol, 3 percent; triglyceride, 5.3 percent; and HDL-cholesterol, 6 percent.

\[ tot\ chol = 200 \quad CV_{a(TC)} = 3.0\% \quad SD_{TC} = 6 \quad var_{TC} = 36 \]

\[ triglyceride = 150 \quad CV_{a(TG)} = 5.3\% \quad SD_{TG} = 8 \quad var_{TG} = 64 \]

\[ 1/5 var_{TG/5} = 2.5 \]

\[ HDL-chol = 50 \quad CV_{a(HDL)} = 6.0\% \quad SD_{HDL} = 3 \quad var_{HDL} = 9 \]

\[ calculated\ LDL = 120 \quad var_{LDL} = 47.5 \quad SD_{LDL} = 6.9 \quad CV_{a(LDL)} = 5.7\% \]
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Part Two: Recommendations for Measurement of High Density Lipoprotein Cholesterol

National Cholesterol Education Program
Working Group on Lipoprotein Measurement
# Table of Contents

EXECUTIVE SUMMARY ................................................................................................................... 67  
Considerations ................................................................................................................................. 67  
Recommendations .............................................................................................................................. 70  
  General Recommendations .......................................................................................................... 70  
  Recommendations for Manufacturers .......................................................................................... 71  
  Recommendations for Health Care Providers ............................................................................ 72  
  Recommendations for Laboratories ............................................................................................ 74  
  Recommendations for Analyses in Nonlaboratory Settings ....................................................... 75  
  Recommendations for Government Agencies and Other Professional Groups ......................... 75  
  Recommendations for Further Research ..................................................................................... 75

1. BACKGROUND ............................................................................................................................ 77

2. CONSIDERATIONS REGARDING ACCURACY IN HDL-CHOLESTEROL MEASUREMENT ........................................................................................................................... 81

3. SOURCES OF VARIATION IN HDL-CHOLESTEROL CONCENTRATION ................................................................. 83  
  Physiological Sources of Variation .............................................................................................. 83  
  Behavioral Sources of Variation ................................................................................................ 84  
  Pathological Sources of Variation ............................................................................................. 85  
  Sampling Sources of Variation Related to Biological Factors ..................................................... 86  
  Analytical Variation .................................................................................................................. 90  
  Matrix Interactions ..................................................................................................................... 90

4. CRITICAL REVIEW OF HDL-CHOLESTEROL METHODS ................................................................................. 93  
  CDC Reference Method .............................................................................................................. 93  
  Field Methods ............................................................................................................................ 94  
    Separation of the HDL Fraction ............................................................................................... 94  
    Cholesterol Analysis ............................................................................................................... 95  
    Precipitation Reagents .......................................................................................................... 96  
    Electrophoretic Methods ......................................................................................................... 99
Recommendations on Lipoprotein Measurement—HDL

Compact Analyzers .................................................................................................................... 99
Quality Control Procedure ........................................................................................................ 100

5. CURRENT LABORATORY PERFORMANCE IN HDL-CHOLESTEROL ANALYSIS ........ 101

6. PERFORMANCE STANDARDS ................................................................................................. 105

7. RECOMMENDATIONS ............................................................................................................... 109
   General Recommendations ............................................................................................................. 109
   Recommendations for Manufacturers ............................................................................................. 110
   Recommendations for Health Care Providers ................................................................................ 111
   Recommendations for Laboratories ................................................................................................ 113
   Recommendations for Analyses in Nonlaboratory Settings .......................................................... 114
   Recommendations for Government Agencies and Other Professional Groups ............................. 114
   Recommendations for Further Research ......................................................................................... 114

REFERENCES ...................................................................................................................................... 115

Table

1. Plasma Lipoproteins ..................................................................................................................... 78
2. NCEP Laboratory Standardization Panel Recommendations for Accuracy and Precision of Clinical Total Cholesterol Measurements .................................................................................. 81
3. Association of HDL-Cholesterol Concentration With Lifestyle and Other Variables .......... 83
4. Recommended Technique for Obtaining Fingerstick Specimens ............................................. 89
5. Trends in Use of HDL-Cholesterol Precipitation Methods by Laboratories Participating in the CAP Comprehensive Chemistry Survey ................................................................................. 97
7. Number (N) of Replicate Samples Required To Establish the Usual HDL-Cholesterol Value of an Average Subject Within Selected Error Limits in Relation to Cholesterol Biological Variability and Analytical Error ......................................................................................... 105

Figure

1. HDL-Cholesterol Method Group Biases in CAP Surveys .......................................................... 98
2. Laboratory Performance Trends in HDL-Cholesterol Analysis on CAP Comprehensive Chemistry Proficiency Survey ................................................................................................................. 102
3. HDL Method Biases on Fresh-Frozen Specimens ...................................................................... 103
Executive Summary

Low levels of high density lipoprotein (HDL)-cholesterol are a strong, independent inverse predictor of coronary heart disease (CHD) risk. There is some epidemiological evidence that low HDL-cholesterol is as important a risk indicator as elevated low density lipoprotein (LDL)-cholesterol. Each 1 percent increase in HDL-cholesterol has been associated with a 2 to 4 percent decrease in CHD risk. Clinical trials of LDL-lowering therapies have observed that concomitant increases in HDL-cholesterol confer an additional independent reduction in CHD risk.

The first report of the Adult Treatment Panel of the National Cholesterol Education Program (NCEP) identified an HDL-cholesterol of <35 mg/dL as a major risk factor for CHD. A National Institutes of Health (NIH)-sponsored consensus conference for HDL and triglycerides held in February of 1992 concluded that evidence is sufficient to accept a causal role for HDL-cholesterol in regard to CHD. The panel recommended that measurements of HDL-cholesterol be made whenever total cholesterol is measured. Treatment of patients with low HDL-cholesterol was considered appropriate, preferably by hygienic measures: smoking cessation, improved diet, weight loss, and increased exercise. The second report of the NCEP Adult Treatment Panel (ATP II), released in June 1993, reaffirmed that a low HDL-cholesterol (<35 mg/dL) is a major risk factor for CHD; identified a high HDL-cholesterol (≥60 mg/dL) as a “negative” risk factor, one that reduces CHD risk; and recommended that testing for HDL-cholesterol be added to total cholesterol in the clinical setting if an accurate result can be obtained.

The enhanced role of HDL-cholesterol in medical practice increases the need for reliable laboratory measurements. The NCEP Working Group on Lipoprotein Measurement was convened in 1989 to develop recommendations for HDL-cholesterol as well as for LDL-cholesterol and triglyceride measurement. Analogous to guidelines published for total cholesterol in 1986 and 1988, comprehensive recommendations in this report for HDL-cholesterol are now available to the medical community to facilitate improvements in measurement performance. This document provides a concise overview of the considerations and recommendations for HDL-cholesterol.

CONSIDERATIONS

Accuracy in the measurement of HDL-cholesterol is especially important, first, because the inverse association with risk for CHD is expressed over a narrow concentration range. Second, unlike other lipid analytes, the cutpoint for increased CHD risk is at the low end of the HDL concentration range where small errors are relatively more serious. Finally, with most current methods, LDL-cholesterol is a calculated value determined in part by subtracting the contributions of HDL-cholesterol and very low density lipoprotein (VLDL)-cholesterol from total cholesterol, or HDL-cholesterol from cholesterol in an HDL- plus LDL-containing fraction. Thus, an error in HDL-cholesterol concentration produces an equal and opposite error in LDL-cholesterol concentration.

For example, a 5 mg/dL negative error in HDL-cholesterol produces a 5 mg/dL positive error in LDL-cholesterol. Although this would produce a relatively small error in LDL-cholesterol (e.g., less than 4 percent at a level of 130 mg/dL), the error could introduce a somewhat disproportionate error in the assessment of CHD risk when the assessment is based on both LDL- and HDL-cholesterol measurements.
Several proficiency surveys have suggested that about two-thirds of HDL measurements are acceptable in routine laboratories and that improvements in laboratory performance have been modest over the past decade. To some extent, these findings undoubtedly reflect the inadequacy of surveillance materials currently used for HDL-cholesterol measurements, since “matrix effects” can severely affect HDL-cholesterol measurements in surveillance pools. Nonetheless, the findings suggest a need for improvement, and providing recommendations for improving the reliability of HDL-cholesterol measurements in routine laboratories is considered an important element in the NCEP effort to improve the detection and treatment of those at increased risk for CHD.

Like the other lipoproteins, HDL is not amenable to easy definition, a prerequisite for a true reference system. HDL comprises a complex, heterogeneous population of particles containing cholesterol along with other lipids and apolipoproteins. The major protein component is apolipoprotein A-I, responsible for about one-third of the particle mass. Even though cholesterol constitutes only about one-sixth of the total mass, HDL is usually quantitated in terms of its cholesterol content because of the long-established association of cholesterol with CHD risk.

Particles in the HDL class have a wide range of composition and include various combinations of apolipoproteins. The particles can be fractionated and separated from other lipoproteins by a variety of methods including chemical precipitation, ultracentrifugation, and electrophoresis. These various methods depend on different properties of the particles and produce method-related differences in results.

Most routine laboratories for reasons of convenience use a separation method based on the chemical precipitation of apo B-containing lipoproteins (VLDL, intermediate density lipoprotein [IDL], LDL, and Lp(a)), sedimentation of the precipitant by low-speed centrifugation, and subsequent quantitation of HDL as cholesterol remaining in the supernate. The most common reagents are sodium phosphotungstate with or without magnesium ion and dextran sulfate with magnesium. These reagents aggregate the positively charged apo B-containing lipoproteins leaving HDL in solution. Commercial and even published research versions of these reagents are quite heterogeneous and give differing results. An alternative method sometimes used in research laboratories employs separation based on buoyant density at extremely high forces in the ultracentrifuge. This approach requires expensive specialized equipment and considerable technical expertise and is quite tedious, making it unsuitable for routine use. A few laboratories have used electrophoresis with lipoprotein separation on a gelatin-like support, but these methods have not been widely accepted.

The basis for accuracy for HDL-cholesterol among the specialty lipid laboratories has been a complicated three-step method used by the Centers for Disease Control and Prevention (CDC). VLDL is first removed by ultracentrifugation. Chemical precipitation is then performed to remove the other apo B-containing lipoproteins (IDL, LDL, and Lp(a)), and quantification of cholesterol in the supernatant is performed using the Abell-Kendall reference method for cholesterol. Even though the CDC method is complicated and expensive, there is justification for continuing its use as the accuracy target for routine laboratories following the precedent established for total cholesterol. Since the CDC method was the accuracy base for national population studies, which in turn are the basis for the current HDL-cholesterol cutpoints, standardizing the routine measurements to the CDC method will achieve appropriate classification of patients. The CDC method has not been validated and approved as an HDL reference method through the Cholesterol Reference Method Laboratory Network as has been done for the Abell-Kendall method for total cholesterol. However, the CDC Cholesterol Reference Method Laboratory Network has a program available for industry to evaluate performance for HDL-cholesterol measurement. For practical reasons the CDC reference method for HDL does not lend itself to large sample comparisons. Therefore, a designated comparison method (precipitation with dextran sulfate followed by quantification with the Abell-Kendall cholesterol method) was evaluated and selected to transfer the CDC accuracy base for HDL. This designated comparison method has been carefully evaluated.
and successfully standardized to the CDC reference method in all of the Cholesterol Reference Method Laboratory Network laboratories. Development of simpler equivalent reference methods with results comparable to those of the present CDC reference method for HDL-cholesterol will also facilitate standardization of HDL-cholesterol in the routine laboratory.

HDL-cholesterol concentrations measured in an individual on several occasions can fluctuate considerably due to normal physiological variations and variations in the analytical methods themselves. HDL-cholesterol concentrations in the blood are strongly affected by lifestyle factors such as recent diet, alcohol consumption, weight changes, physical activity, and smoking. Hormones and other medications also influence the HDL-cholesterol concentrations.

The coefficient of biological variation is generally considered to be about 7.5 percent; thus, in a series of replicate samplings, two-thirds of the values will fall within 7.5 percent of the mean value. Biological variation is the major contributor to the overall variability of HDL-cholesterol values. The effects of biological variation can be controlled to some extent by standardizing the conditions for patient preparation and blood collection, but HDL-cholesterol cannot be estimated reliably from measurements in a single sample. Several samples should be taken, and the overall mean used to determine the patient’s usual HDL-cholesterol concentration or, more accurately, the patient’s usual range of HDL-cholesterol concentrations.

Analytical variation has two components, imprecision and bias. Imprecision is specified in absolute terms as standard deviation (SD) or in relative terms as the coefficient of analytical variation (CVa) and reflects the noise in the measurement process. Differences in technical personnel, reagent batches, and volume measurements and variations in instrument performance contribute to variation in the measured values. Imprecision can be minimized with careful technique and reliable instruments and by adhering to accepted principles of good laboratory practice. Bias refers to consistent or systematic differences between the measured values and the reference value, which in the case of HDL are primarily due to inadequate separation of the HDL fraction and/or improper calibration of the cholesterol method used.

In the past there has been no universally accepted accuracy target for HDL-cholesterol. Methods have been developed and marketed with no formal mechanism to obtain agreement with an accepted reference method. In addition, there have been no generally accepted guidelines for precision. The CDC method has been widely used as the accuracy target in recent national population studies, however, and a CDC-National Heart, Lung, and Blood Institute (NHLBI) standardization program has been in place for a number of years. The major objective of these recommendations is to provide guidelines for minimum accept-

<table>
<thead>
<tr>
<th>TABLE ES-1.</th>
<th>Current CDC-NHLBI criteria for acceptable performance in high density lipoprotein cholesterol</th>
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<tbody>
<tr>
<td>Concentration Range (mg/dL)</td>
<td>Accuracy</td>
</tr>
<tr>
<td>&lt;40</td>
<td>±10% of RV(^1)</td>
</tr>
<tr>
<td>40-60</td>
<td>±10% of RV</td>
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<tr>
<td>&gt;60</td>
<td>±10% of RV</td>
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\(^1\) RV, CDC reference value determined with the combination ultracentrifugation-heparin-MnCl\(_2\) method (see text).
able performance based on what is achievable with current methodology. The recommendations for minimizing analytical error build on the CDC-NHLBI standardization criteria (table ES-1), which call for accuracy within 10 percent of a reference value determined with the CDC reference method for HDL-cholesterol and a concentration-dependent limit on the standard deviations for measurements in pooled sera.

RECOMMENDATIONS

The primary measured values required for the diagnosis and treatment of hyperlipidemia are total cholesterol, triglycerides, and HDL-cholesterol. These measurements are used in current routine practice to calculate the LDL-cholesterol value. The methodology needed for reliable measurements of total cholesterol and triglyceride is currently available. A CDC-NHLBI standardization program for HDL-cholesterol is available. This program has served as the basis for satisfactory laboratory performance in a number of clinical and epidemiological studies for almost 20 years. The criteria for standardization of HDL-cholesterol measurement are given in table ES-1. HDL-cholesterol measurement, however, presents a unique problem; although HDL-cholesterol methods are sufficiently accurate and precise to satisfy the current CDC-NHLBI standardization criteria, performance at the limits of the standardization criteria for accuracy and imprecision would not satisfy the generally accepted criterion for the usefulness of a medical test: The random error of the measurement (CVa) should be no greater than half the average coefficient of biological variation (CVb). Short of using an impractical number of serial specimens, this would lead to insufficiently accurate results in a proportion of patients. Thus it will be necessary to improve performance in measuring HDL-cholesterol particularly in order to classify accurately those patients whose values are near the recommended cutpoints, as well as to facilitate patient treatment and followup. As a first step, the adoption of the current CDC-NHLBI standardization criteria for routine measurements is expected to improve the reliability of these measurements.

However, these considerations complicate the development of recommendations for HDL-cholesterol measurement, and the NCEP Working Group on Lipoprotein Measurement adopted a two-tiered set of performance goals, the first reflecting the current state of the art of HDL-cholesterol measurement methodology. The second is based on stricter criteria and should increase the proportion of acceptable clinical HDL-cholesterol measurements. The following recommendations are made with full appreciation of the need to develop better HDL methods over the next few years and acknowledge the challenge this presents and the uncertainty of when this will be accomplished.

General Recommendations

- Maintaining Linkage With the Existing Epidemiological Database. The existing epidemiological and clinical database relating HDL-cholesterol concentration to coronary risk is largely based on CDC-NHLBI standardized HDL-cholesterol measurements, which are in turn based on use of the heparin-MnCl2 method. It is recommended that this linkage be maintained regardless of which HDL-cholesterol method is used.

- Reference Method. It is recommended that the CDC reference method be used as the accuracy target for HDL-cholesterol measurements. Inasmuch as HDL is defined to some extent by the methods used to isolate it, continuation of the CDC method as a point of reference will maintain linkage between HDL-cholesterol measurements and the existing epidemiological and clinical database, regardless of the method used. The CDC reference method is a three-step method:
  1. Ultracentrifugation at d 1.006 g/mL to remove triglyceride-rich lipoproteins.
  2. Precipitation of apo B-containing lipoproteins from the ultracentrifugal infranatant with heparin and MnCl2.

- Routine Methods. The basis for assessing the accuracy of routine methods should be the CDC reference method. Inasmuch as ultracentrifugation is not practical in most routine laboratories, routine methods can be two-step methods:
1. Precipitation of apo B-containing lipoproteins from serum or ethylenediaminetetraacetic acid (EDTA) plasma.


**Criteria for Analytical Performance.** The following goals are recommended. These goals are stated in terms of total analytical error, which takes account of both accuracy and imprecision. This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater imprecision can be accepted if the measurements are accurate (see appendix II for calculation of total error). The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV ≤3 percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent.

The specification of guidelines for accuracy and precision separately can lead to an ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits is considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance.

**Interim Goals**

Total error, ≤22 percent

One set of conditions that is consistent with this total error limit is:

**Precision:** CV ≤6 percent at 42 mg/dL (1.09 mmol/L) or higher. An SD ≤2.5 mg/dL (0.065 mmol/L) is recommended at levels below 42 mg/dL (1.09 mmol/L).

**Accuracy:** bias ≤±10 percent (compared to CDC reference method)

**Goals for 1998**

Total error, ≤13 percent

One set of conditions that is consistent with this total error limit is:

**Precision:** CV ≤4 percent at 42 mg/dL (1.09 mmol/L) or higher. An SD ≤1.7 mg/dL (0.044 mmol/L) is recommended at levels below 42 mg/dL (1.09 mmol/L).

**Accuracy:** bias ≤±5 percent (compared to CDC reference method)

These criteria should apply regardless of how, where, or by whom the measurements are made.

**Serum and Plasma.** Either serum or plasma can be used for measurements. NCEP guidelines are based on serum values, and when classifying patients, serum or serum-equivalent values should be used. To convert EDTA plasma values to serum values, multiply the plasma values by 1.03.

**Recommendations for Manufacturers**

- Manufacturers and others developing new methods, reagents, and instruments for HDL-cholesterol measurement should be aware of the medical community’s need to refer such measurements to the existing epidemiological database as it relates to risk for coronary heart disease.

  — New methods, reagents, and instruments should be validated against the reference method through split sample comparisons with the Cholesterol Reference Method Laboratory Network, using appropriate statistical methods for comparing measurement methods.

  — The assigned HDL-cholesterol values for calibration and reference materials should be traceable to the reference method for HDL-cholesterol. The CDC laboratory, the Cholesterol Reference Method Laboratory Network, and other CDC standardized lipoprotein research laboratories can be of assistance for these purposes.
• Manufacturers should cooperate with CDC and the Cholesterol Reference Method Laboratory Network in developing reference materials for HDL-cholesterol measurement. Such materials should be commutable (i.e., closely emulate patient specimens).

Recommendations for Health Care Providers

• Minimize preanalytical sources of variation.
  — Patients should maintain their usual diet and a reasonably stable weight for at least 2 weeks prior to blood collection.
  — If measurements are made sooner than 12 weeks after the occurrence of myocardial infarction, values may be lower than typical for the patient. A preliminary measurement made within this period can give a sense of the patient’s HDL-cholesterol value, which if not below the ATP II recommended cutpoints (35 mg/dL [0.90 mmol/L] or 60 mg/dL [1.55 mmol/L]) can assist in initial management decisions. Measurements should not be made sooner than 8 weeks after acute trauma including major surgery, acute bacterial or viral infection or illness, or pregnancy.

• HDL-cholesterol measurement should be made in the fasting and postprandial state.
  — Blood samples should be obtained by venipuncture following a 12-hour period of fasting. If necessary, the patient can take water and prescribed medications during this period. This procedure should be followed for research purposes and in clinical circumstances in which error in the HDL-cholesterol must be minimized.
  — If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not normally be less than 9 hours. It is likely that, on average, HDL-cholesterol will be underestimated slightly (about 1 to 4 percent) in patients who have fasted 9 hours. This should be taken into account when interpreting the values.

• Prolonged venous occlusion should be avoided. ATP II states that HDL-cholesterol can be measured in the nonfasting state. It is likely that, on average, HDL-cholesterol will be underestimated somewhat (5 to 10 percent) in nonfasting patients. This should be taken into account when interpreting the values. The error introduced when measuring HDL-cholesterol in the nonfasting state, however, would be conservative in that it would lead to an overestimation of risk (false positive) in patients with fasting HDL-cholesterol levels at or somewhat above the cutpoints. This would likely be detected with followup measurements in fasting samples. The measurement of HDL-cholesterol in the nonfasting state would not be expected to interfere with the detection of patients with truly low HDL-cholesterol unless the plasma triglyceride level is so high that it interferes with the measurement itself. Based on the HDL-cholesterol distributions in the Lipid Research Clinics Population Studies, a 10 percent negative error in HDL-cholesterol would tend to misclassify about 15 percent of the population with respect to the two ATP II cutpoints: the 10 percent with HDL-cholesterol levels of 35-40 mg/dL (0.90-1.04 mmol/L) and about 5 percent with HDL-cholesterol levels of 60-65 mg/dL (1.55-1.69 mmol/L). Thus, the use of nonfasting HDL-cholesterol measurements would not interfere with the classification of about 85 percent of the population.
  — To the extent possible, blood should be drawn in the sitting position and the patient should sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should be sampled in the same position on each occasion.

• Prolonged venous occlusion should be avoided. The tourniquet should be released within 1 minute of application. If difficulties are encountered, use the other arm, or release the tourniquet for a few minutes before attempting a second venipuncture.
• Use of serum or plasma
  — HDL-cholesterol can be measured in either serum or EDTA plasma.

• Processing
  — Serum or plasma should be removed from cells within 3 hours of venipuncture.

• Need for serial measurements in individuals
  — Considering the physiological variability of HDL-cholesterol, HDL-cholesterol measured on a single occasion is not sufficient to determine the patient’s “usual” HDL-cholesterol concentration. Although it would be ideal to establish a patient’s HDL-cholesterol with a 10 percent limit for total error with 95 percent confidence, at present it is not technically or economically feasible because of the excessive number of serial samples that would be required. For this reason, the following recommendations are made to improve the reliability of HDL-cholesterol measurements. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart.

  — Three serial samples: Using three serial samples, each referred to the same laboratory and analyzed once, and assuming a CV_b of 7.5 percent in an interim CV_a of 6 percent, the observed CV for the mean HDL-cholesterol value is 5.5 percent, and the difference between the means of sequential series of three samples should not exceed 15.4 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 27 percent. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s HDL-cholesterol concentration and its proximity to the concentrations used for decision making (35 mg/dL [0.90 mmol/L] or 60 mg/dL [1.55 mmol/L]). Using the 1998 CV_a goal of 4 percent, the observed CV for the mean HDL-cholesterol value for three serial samples is 4.9 percent, and the difference between the means of sequential series should not exceed 13.6 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 24 percent. If they are further apart, analytical error or a change in the physiological state of the patient should be suspected and another sample may be warranted.

  — Two serial samples: For reasons of convenience and considering economic factors, the ATP II report recommended the use of at least two serial samples. Using two serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 7.5 percent and an interim CV_a of 6 percent, the observed CV for the mean HDL-cholesterol value is 6.8 percent. The difference between the means of each series should not exceed 18.8 percent, and the difference between sequential individual values within each series should not exceed 27 percent, 95 percent of the time. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s HDL-cholesterol concentration and its proximity to the concentrations used for decision making (35 mg/dL [0.90 mmol/L] and 60 mg/dL [1.55 mmol/L]). Using the 1998 CV_a goal of 4 percent, the observed CV for the mean of two serial samples is 6.0 percent and the difference between the means for each series should not exceed 16.7 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 24 percent. If they are farther apart, another sample may be warranted.

Based on prevailing HDL-cholesterol levels,* under the interim goals, using two serial measurements and considering a cutpoint of 35 mg/dL (0.90 mmol/L), a

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* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
patient's HDL-cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is greater than 40.7 mg/dL (1.05 mmol/L) or less than 29.3 mg/dL (0.76 mmol/L), respectively. Using a 60 mg/dL (1.55 mmol/L) cutpoint, the patient's HDL-cholesterol value can be confidently assumed to be above or below the cutpoint when the mean value is greater than 69.9 mg/dL (1.81 mmol/L) or less than 50.1 mg/dL (1.29 mmol/L), respectively. Based on prevailing HDL-cholesterol levels in the U.S. population, two serial specimens are sufficient to categorize 83 percent of the general population as above or below the 35 mg/dL cutpoint and 58 percent of the population as above or below the 60 mg/dL cutpoint.

Under the 1998 goals, using two serial measurements and considering a cutpoint of 35 mg/dL, a patient's HDL-cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is >39.0 mg/dL or <31.0 mg/dL, respectively. Using a 60 mg/dL cutpoint, the patient's HDL-cholesterol value can be confidently assumed to be above or below the cutpoint when the mean value is >66.8 mg/dL or <53.2 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 88 percent of the general population as above or below the 35 mg/dL cutpoint and 70 percent of the population as above or below the 60 mg/dL cutpoint.

- Separations
  - The HDL-containing fraction is best prepared on the day of sample collection. If analyses must be delayed for 1 or 2 days, the serum or plasma can be stored at 4 °C. If the analyses are delayed beyond 3 days, the specimens should be transferred to storage vials that have leak- and evaporation-proof seals, and frozen. For periods up to 1 month, samples can be stored at -20 °C in a non-self-defrosting freezer. For storage periods of 1 month to 2 years, samples should be stored at -70 °C. The storage containers should not be sealed with cork stoppers or plastic film since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

- All blood specimens must be considered potentially infectious and handled according to accepted laboratory safety guidelines.

**Recommendations for Laboratories**

- Laboratories should use methods that are optimized using the CDC reference method for HDL-cholesterol as the point of reference for accuracy. Since matrix effects are significant, accuracy should be verified through method comparison studies using fresh specimens. NCCLS Protocol EP9-P is recommended for the conduct of such studies.

- The specificity of the precipitation step should be verified additionally by appropriate immunochromatographic or electrophoretic analysis of HDL supernates.

- Since incomplete sedimentation of lipemic specimens is a common source of error with the precipitation methods, measures should be incorporated to screen for supernatant turbidity and eliminate the unsedimented apo B-containing lipoproteins.

- Quality control materials should be selected that reasonably emulate performance on patient specimens. At least two levels are recommended, one each near the decision levels of 35 mg/dL (0.90 mmol/L) and 60 mg/dL (1.55 mmol/L).

- Laboratories should participate in an external proficiency testing program.

- All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel. Personnel handling blood samples should use gloves and should avoid leaving samples open to the air longer than necessary. Samples should be handled in accordance with CDC guidelines for the prevention of infection in health care workers.
Recommendations for Analyses in Nonlaboratory Settings

In addition to the recommendations above, the following recommendations apply to HDL-cholesterol testing in nonlaboratory settings.

- Desktop analyzers and other analytical systems should be designed and validated to achieve the requisite performance by operators without formal laboratory training. These systems should operate reliably under conditions that prevail in field screening, physicians’ offices, or other settings outside the conventional laboratory.

- The performance criteria established for laboratory-based measurements should apply to measurements made outside the conventional laboratory setting.

- Operators of desktop analyzers or similar nonlaboratory-based systems should receive training in phlebotomy techniques, safety procedures, and quality control. Such operators should work under the supervision of health care professionals with appropriate education, training, and experience in laboratory measurements.

Recommendations for Government Agencies and Other Professional Groups

The Centers for Disease Control and Prevention should:

- In cooperation with appropriate experts in the academic, clinical laboratory, and manufacturing sectors, develop reference materials for HDL-cholesterol measurement in which matrix effects are minimized. Such materials should be suitable for standardization, surveillance, method calibration as appropriate, and bench-level quality control.

The National Heart, Lung, and Blood Institute should take the following steps:

- Continue the policy of NHLBI to require standardized lipid and lipoprotein measurements for Government-supported clinical and epidemiological studies.

- Encourage the Cholesterol Reference Method Laboratory Network to expand its activities to include the certification of HDL-cholesterol, LDL-cholesterol, and triglyceride measurements.

- Encourage the development and preliminary evaluation of new HDL-cholesterol methods and associated reagents and instrumentation.

The Cholesterol Reference Method Laboratory Network currently provides assistance to manufacturers and laboratories wishing to establish traceability of total cholesterol measurements to the cholesterol reference method. The network should:

- Expand activities to include HDL-cholesterol, LDL-cholesterol, and triglyceride measurements.

The College of American Pathologists and other professional organizations that operate clinical chemistry survey programs should take the following steps:

- Include HDL-cholesterol measurements in such surveys.

- Provide CDC-confirmed values for HDL-cholesterol concentrations in survey pools.

Recommendations for Further Research

- New methods for HDL-cholesterol measurement are needed. Such methods should be capable of providing HDL-cholesterol measurements that meet or exceed the 1998 goals specified above. This will be important both for the measurement of HDL-cholesterol, per se, and for the calculation of LDL-cholesterol from measured values of total cholesterol, triglyceride, and HDL-cholesterol.

- Suitable reference materials are urgently needed for HDL-cholesterol measurements. Such materials should be free of matrix effects and should be sufficiently stable to allow the long-term monitoring of the accuracy and precision of HDL-cholesterol measurements and for use in proficiency surveys to allow the accurate assessment of clinical HDL-cholesterol measurements.
Early observations by Gofman and coworkers (1966) and Barr and colleagues (1951), suggesting that the serum concentration of high density lipoprotein (HDL) cholesterol is related to risk for coronary heart disease (CHD), attracted attention in the mid-seventies (Miller and Miller 1975) and have since led to extensive investigations of HDL metabolism and its association with CHD risk. Several large prospective studies conducted in the United States and elsewhere including the Framingham Study (Abbott et al. 1988), the Lipid Research Clinics Follow-up Study (Bush et al. 1987), and the Donolo-Tel Aviv Study (Brunner et al. 1987) have shown HDL-cholesterol level to be a strong, independent inverse predictor of CHD in men and women. HDL-cholesterol has proved to be as powerful a predictor of risk as low-density lipoprotein (LDL) cholesterol concentration and may be particularly important in women (Abbott et al. 1988; Bush et al. 1987; Brunner et al. 1987). Case-control studies have also shown individuals with manifest CHD to have lower HDL-cholesterol levels than matched, normal controls.

Gordon and coworkers (1989) analyzed data from four major prospective studies and concluded that an increase in HDL-cholesterol of 1 mg/dL is associated with a reduction of 2 to 3 percent in CHD risk (or a 1 percent increase in HDL-cholesterol is equivalent to 1.5 to 2 percent decrease in risk). In the Framingham Heart Study (Abbott et al. 1988), a 1 percent increase in LDL-cholesterol level was associated with approximately a 2 percent increased risk of CHD over 6 years, whereas a 1 percent decrease in HDL-cholesterol level was associated with a 3 to 4 percent increase in CHD risk. The Framingham Heart Study data also suggest that the LDL-cholesterol associated risk for CHD in men and women may be attenuated to some extent in individuals with high HDL-cholesterol levels.

As might be expected, ratios reflecting concentrations of both HDL and LDL, in particular total cholesterol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterol, are powerful predictors of CHD risk. However, these ratios have had rather limited acceptance, and the second report of the National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP II) does not advocate their use, in part because of anomalous relationships with risk at the extremes of the concentration range (NCEP 1994). For instance, an individual with very low values for both LDL-cholesterol and HDL-cholesterol might have a ratio indicating moderate risk, although it is clear that this risk must in fact be very low by virtue of his or her very low level of atherogenic LDL.

HDL encompasses a family of lipoprotein particles in which size, density, flotation rate, and composition vary across a spectrum (table 1). Although more complex subfractionation schemes have been proposed using analytical or preparative ultracentrifugation or electrophoresis, the most commonly used method (differential precipitation) quantifies the smaller, denser HDL$_3$ fraction and the larger, lighter HDL$_2$ fraction. Some early reports suggested that HDL$_2$ is the fraction more directly responsible for the association of total HDL-cholesterol with reduced risk of CHD (Miller et al. 1981); but more recently several reports have suggested that both HDL$_2$ and HDL$_3$ cholesterol concentrations are inversely related to CHD risk. Although quantitation of HDL subclasses by differential precipitation has been considered appropriate for epidemiological studies (Patsch et al. 1989), reservations about the specificity
Recommendations on Lipoprotein Measurement—HDL

(Demacker et al. 1986) as well as clinical utility of separate HDL$_2$ and HDL$_3$ cholesterol measurements preclude a recommendation for use in routine practice.

The major protein constituent of HDL, apolipoprotein A-I (apo AI) (table 1), can be measured by various immunoassay techniques (Labeur et al. 1990) in human serum without the need for a preliminary separation step. This measurement has been considered as an alternative or supplement to the measurement of HDL-cholesterol in characterizing coronary heart disease (CHD) risk (Kottke et al. 1986). Before the apo AI measurement can be recommended for routine application, however, further work will be necessary (Stein 1990). First, refinements in the immunoassay technology are needed. Second, a standardization procedure for apo AI must become widely available. There is still a lack of long-term epidemiological evidence demonstrating prospectively the value in predicting CHD risk or evidence of the efficacy of treatment to raise apo AI levels.

HDL has been demonstrated to consist of metabolically distinct classes of particles, differing in their apolipoprotein constituents (Fruchart 1990). One class containing apo AI without apolipoprotein AII (apo AII) is reportedly more important in protecting against CHD. Another class containing apo AI and apo AII may be less protective. Measurements to distinguish apo AI-only particles from apo AI/AII particles are being made in various studies, but considerably more work is needed before their utility for CHD risk assessment can be judged.

Large-scale, formal controlled clinical trials are generally regarded as the ultimate test of hypotheses about the practical value of altering lipoprotein concentrations with respect to CHD incidence in a population. Major trials, in which reduction of LDL-cholesterol by drug treatment was the most notable lipoprotein change, in particular the Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT), have shown convincingly that LDL lowering is a  

**TABLE 1. Plasma lipoproteins**

<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Density (kg/L)</th>
<th>Electrophoretic Mobility$^1$</th>
<th>CE (percent by wt)</th>
<th>Apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.95</td>
<td>Origin</td>
<td>4 90 5 1</td>
<td>Al, C’s, AIV, E, B48</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>Prebeta</td>
<td>20 55 19 8</td>
<td>C’s, B, E</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>Beta-prebeta</td>
<td>Intermediate between VLDL and LDL</td>
<td>C’s, B, E</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>Beta</td>
<td>55 5 20 20</td>
<td>B-100</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>Alpha</td>
<td>22 5 28 50</td>
<td>Al, AII, D, C, E</td>
</tr>
<tr>
<td>HDL$_2$</td>
<td>1.063-1.12</td>
<td>Alpha</td>
<td>24 8 25 43</td>
<td></td>
</tr>
<tr>
<td>HDL$_3$</td>
<td>1.12-1.21</td>
<td>Alpha</td>
<td>21 2 23 55</td>
<td></td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.045-1.080</td>
<td>Prebeta</td>
<td>46 5 22 27</td>
<td>B-100, Lp(a)</td>
</tr>
</tbody>
</table>

$^1$ By agarose gel electrophoresis.

$^2$ Data from Segal et al. 1984; Gries et al. 1988; Fless et al. 1986; Albers and Hazzard 1974; Gotto et al. 1986; Gaubatz et al. 1983.

KEY: FC, unesterified cholesterol; TG, triglycerides; PL, phospholipids; CE, cholesterol esters.
Background

valuable preventive strategy (Lipid Research Clinics Program 1984). A large trial designed to look specifically at the value of increasing HDL levels has not been conducted but is currently in progress. Indeed, the close metabolic interrelationships of the major lipoprotein families make it difficult to devise a trial in which LDL-cholesterol is lowered substantially without any change in concentration of HDL-cholesterol or its subfractions. In the LRC-CPPT, cholestyramine treatment led to substantial reduction of LDL-cholesterol and a small increase in HDL-cholesterol. Statistical analysis has indicated that both lipoprotein changes contributed independently to the reduction in CHD observed (Gordon et al. 1986). There was a twofold difference in the later occurrence of CHD between the highest and lowest tertiles of HDL-cholesterol at baseline despite LDL-cholesterol levels above 175 mg/dL at entry into the study.

The Helsinki Heart Study (Manninen et al. 1988), in which the drug gemfibrozil lowered LDL-cholesterol and triglycerides and elevated HDL-cholesterol substantially and simultaneously in subjects with elevated cholesterol and triglycerides, showed a correlation between the increase in HDL-cholesterol and the reduction in CHD risk. In this study the predictive value of HDL-cholesterol for CHD risk was very high even at high levels of LDL-cholesterol: There was a fivefold difference in CHD incidence between the highest and lowest tertiles of baseline HDL-cholesterol (Manninen et al. 1990).

The first report of the Adult Treatment Panel of the National Cholesterol Education Program identified a serum HDL-cholesterol of <35 mg/dL as a major risk factor for CHD in the treatment algorithms for both sexes (NCEP 1988a). However, measurement of HDL-cholesterol was not recommended for individuals with total cholesterol <200 mg/dL and for some individuals with total cholesterol of 200-239 mg/dL; and monitoring of HDL and intervention to increase HDL-cholesterol levels were not recommended. In view of the large and increasing body of evidence that high levels of HDL-cholesterol reduce CHD risk, it is not surprising that modification of the ATP guidelines to increase emphasis on HDL- as well as LDL-cholesterol has been urged by some (e.g., Manninen et al. 1990). Hygienic measures such as smoking cessation, weight loss, and exercise increase HDL-cholesterol in at least some proportion of the population with low HDL. Certain drugs used to treat other forms of dyslipidemia (niacin, gemfibrozil, lovastatin) can also raise HDL in patients who are being treated for dyslipidemias that respond to such medications.

On the other hand, there are areas of uncertainty relating to HDL as a risk factor, and the means available for elevation of HDL-cholesterol (Rifkind 1990). A review by members of the first ATP of the role of HDL-cholesterol in management of individuals found to have elevated cholesterol essentially reaffirmed the appropriateness of the 1988 Adult Treatment Panel guidelines (Grundy et al. 1989). However, an NHLBI Consensus Conference panel considering HDL, triglyceride, and coronary heart disease, held February 1992 (NIH Consensus Development Panel 1993), recommended that HDL-cholesterol be measured whenever serum total cholesterol is determined for assessment of CHD risk; that efforts to increase HDL level when it is undesirably low should be made; and that hygienic measures (weight loss, exercise, smoking cessation) should be the primary emphasis for such efforts (NIH Consensus Development Panel 1993). This panel also recommended that HDL-cholesterol-elevating drugs (niacin, gemfibrozil) should be used only as a last resort in certain patients, when hygienic interventions have been exhausted. In its second report (NCEP 1994), the NCEP Adult Treatment Panel reaffirmed that a low HDL-cholesterol (<35 mg/dL [0.90 mmol/L]) is a major risk factor; identified a high HDL-cholesterol (≥60 mg/dL [1.55 mmol/L]) as a negative risk factor, that is, one that reduces CHD risk; and also recommended that HDL-cholesterol be measured along with total cholesterol during routine risk assessment if accurate HDL-cholesterol measurements are available. Like the Consensus Development Panel, the second report of the NCEP Adult Treatment Panel also recommends hygienic measures to raise HDL-cholesterol; when drug therapy is necessary to lower LDL-cholesterol, the panel suggested that agents that concomitantly raise HDL be considered.
Recommendations on Lipoprotein Measurement—HDL

Whatever the outcome of the many ongoing investigations in this area, it seems clear that accurate, precise, and reasonably inexpensive analytical procedures for determination of serum and plasma concentrations of HDL-cholesterol will continue to be important to the progress of national programs to reduce CHD risk via modification of serum lipoprotein concentrations.

The NCEP Working Group on Lipoprotein Measurement was organized to consider and make recommendations concerning the measurement of HDL, triglycerides, and LDL. These measurements are considered in three separate parts in this report. This part concerns the measurement of HDL-cholesterol.
Section 2.
Considerations Regarding Accuracy in HDL-Cholesterol Measurement

Recommendations for laboratory performance for total cholesterol measurement (table 2) were provided by the NCEP Laboratory Standardization Panel (1988b). The necessity for accuracy was emphasized. Accuracy in the measurement of HDL-cholesterol is also important, particularly because the inverse association of HDL-cholesterol with risk of CHD is expressed over a relatively narrow concentration range. Errors in HDL-cholesterol measurement also lead to errors in estimation of the LDL-cholesterol. With most current methods, LDL-cholesterol is a calculated value determined by subtracting the contribution of HDL-cholesterol and VLDL-cholesterol from total cholesterol (Friedewald et al. 1972), or by subtracting HDL-cholesterol from an HDL- and LDL-containing fraction (Lipid Research Clinics Program 1982). Thus an error in HDL-cholesterol concentration produces an equal and opposite error in LDL-cholesterol concentration. For example, a 5 mg/dL negative error in HDL-cholesterol would produce a 5 mg/dL positive error in LDL-cholesterol. Although this would be a relatively small error in LDL-cholesterol per se (e.g., less than 4 percent at a level of 130 mg/dL), this could introduce a somewhat larger error in the assessment of CHD risk when the assessment is based on both LDL- and HDL-cholesterol measurements.

Recognizing this problem and the importance of accuracy in LDL-cholesterol measurements, the NCEP Working Group on Lipoprotein Measurement has recommended development of direct LDL methods that do not depend on measuring the HDL-cholesterol value (see part two, LDL recommendations). Although accuracy in HDL-cholesterol measurement is important, the complex nature of HDL

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**TABLE 2. NCEP Laboratory Standardization Panel recommendations for accuracy and precision of clinical total cholesterol measurements**

| Performance Criteria<sup>1,2</sup> (1992) |  
|---|---|
| Accuracy | ≤3% |
| Precision<sup>3</sup> (CV) | ≤3% |

---

<sup>1</sup> Compared to the reference method (Duncan et al. 1982; Cooper et al. 1986). The guidelines refer only to analytical error; they do not include the contribution of biological variation.

<sup>2</sup> Assuming the maximum allowable bias and imprecision, and a 95 percent confidence limit for the imprecision, the allowable total error for single measurements is ±8.9 percent using the 1992 criteria.

<sup>3</sup> Precision is assessed by the coefficient of variation (CV), defined as:

\[
CV = \frac{\text{standard deviation}}{\text{mean}} \times 100
\]
Recommendations on Lipoprotein Measurement—HDL

presents a substantial analytical challenge. Chol-
esterol is a molecule of unique structure and can
be precisely defined. This provides the basis for
specific definitive (Cohen et al. 1980) and refer-
ence (Duncan et al. 1982; Abell et al. 1952)
methods by which the total cholesterol concen-
tration can be accurately measured and for standard-
ization of cholesterol measurements. In contrast,
the various classes of lipoprotein particles that
contain cholesterol do not lend themselves to
precise definition. They are complex, polydisperse
populations of particles, composed of cholesterol
(unesterified and esterified), triglycerides, and
phospholipids, combined with various proteins
(table 1). The major lipoprotein classes are
heterogeneous and can each be separated into
several subclasses, the exact nature of which
depends on the method of separation.

The common nomenclature for the lipoproteins is
based on separation by ultracentrifugation. HDL
has been considered to correspond to the fraction
of density 1.063 to 1.21 kg/L (Havel et al. 1955)
or, in common practice, the fraction with density
greater than 1.063 kg/L. Other analytical methods
can result in separation of slightly different
populations of particles. For example, ultracen-
trifugal and precipitation techniques do not
separate entirely equivalent fractions; there are
even slight differences between fractions isolated
with different precipitation methods. The HDL
fraction separated by virtue of its density can also
contain lipoprotein particles that are atherogenic,
such as Lp(a), whereas the common precipitation
methods will likely remove these particles. For the
sake of simplicity and to follow common usage, the
term HDL will be used here to designate that
fraction obtained by any of the common labora-
tory procedures, recognizing that the various
methods may give somewhat different results.

The HDL class might better be defined either in
terms of composition or, with better characteriza-
tion of HDL subspecies, in terms of its functional
properties. A more specific definition based on
composition might be “those lipoprotein particles
containing apolipoprotein A1 without apolipo-
protein B.” A definition based on function might
comprise the population of lipoprotein particles
that mitigate the atherogenic process. The
analytical technology will certainly progress to
the point where individual functional subclasses
of particles can be defined, characterized, and
quantified, perhaps improving their usefulness
in predicting risk of CHD and other diseases.
Section 3.
Sources of Variation in HDL-Cholesterol Concentration

The value reported for HDL-cholesterol is subject to variation from a variety of preanalytical and analytical factors (Table 3).

Preanalytical sources of variation are considered in previous reports on total cholesterol measurement of the NCEP Laboratory Standardization Panel (1988b, 1990). This overview will emphasize primarily the factors influencing HDL-cholesterol values, including those from biological sources, sampling variation, and specimen collection.

### PHYSIOLOGICAL SOURCES OF VARIATION

Intraindividual biological variation in serum HDL-cholesterol concentration levels arises from the inherent characteristics of the individual, behavioral factors, disease, environmental factors, and seasonal changes. Biological sources also contribute to sampling variation. Selected representative biological sources of variation are discussed here.

The variation in HDL-cholesterol concentration is somewhat independent of the other lipoproteins, but there is a trend to vary inversely with changes in serum triglyceride levels, primarily associated with VLDL. Representative publications indicate that HDL-cholesterol concentration in men varies during the day consistent with a biological coefficient of variation (CV$_b$) of about 4.5 percent, within a month a CV$_b$ of 7.7 percent, and for a year a CV$_b$ of 8.4 percent (Demacker et al. 1982a). The range in HDL-cholesterol values reported within an individual over 1 year (CV$_b$ of 3.6 to 12.4 percent) is similar to that observed over 1 month (Demacker et al. 1982a). A study of longitudinal variability in HDL-cholesterol levels of healthy Japanese women yielded a CV$_b$ of 10.4 percent, following yearly monitoring for 3 years, with more variability in hyperlipemics (Chiba et al. 1984). In a study over a 3- to 5-month period where specimens were taken at intervals of 1 to 2 weeks, the intraindividual biological CV$_b$ was determined to be 5.5 percent for HDL-cholesterol (Ford 1989). The percent critical difference, which is the largest observed difference of a second result from the first result, was calculated to be 17

<table>
<thead>
<tr>
<th>Variable</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-fat diet</td>
<td>↑</td>
</tr>
<tr>
<td>High activity level</td>
<td>↑</td>
</tr>
<tr>
<td>Obesity</td>
<td>↓</td>
</tr>
<tr>
<td>Smoking</td>
<td>↓</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>↑</td>
</tr>
<tr>
<td>Hospitalization</td>
<td>↓</td>
</tr>
<tr>
<td>Diabetes (type II)</td>
<td>↓</td>
</tr>
<tr>
<td>Antihypertensive drugs:</td>
<td></td>
</tr>
<tr>
<td>thiazides</td>
<td>↓</td>
</tr>
<tr>
<td>some beta blockers</td>
<td>↓</td>
</tr>
<tr>
<td>Estrogen replacement therapy</td>
<td>↑</td>
</tr>
<tr>
<td>Acute infections</td>
<td>↓</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>↓</td>
</tr>
<tr>
<td>Season: winter</td>
<td>↑</td>
</tr>
<tr>
<td>summer</td>
<td>↓</td>
</tr>
<tr>
<td>Recumbent posture</td>
<td>↓</td>
</tr>
<tr>
<td>Venous occlusion</td>
<td>↑</td>
</tr>
</tbody>
</table>

TABLE 3. Association of HDL-cholesterol concentration with lifestyle and other variables
Recommendations on Lipoprotein Measurement—HDL

percent, and from this the investigators concluded that measurement of seven specimens is necessary to reliably establish the underlying HDL-cholesterol value. A study of 20 subjects tested weekly for 4 weeks found variations of more than \( \pm 20 \) percent in the mean HDL-cholesterol in 65 percent of the subjects (Mogadam et al. 1990). In an investigation of reliability of a single measurement of HDL-cholesterol, a CV_{b} of 7.5 percent was observed, and the biologic CV_{b} was 85 percent of total CV (Bookstein et al. 1990). Another study of variation over 18 days reached a maximum with CV_{b}'s of 5 to 6 percent in about a week (Rotterdam et al. 1987); the authors of this study recommended that, approximately 1 week after the initial measurement, a subsequent measurement to determine a patient’s average value should be made in order to include the subject’s usual range of biological variability. For the present purposes, the average CV_{b} for HDL-cholesterol is taken as 7.5 percent.

Genetic factors are associated with both very low and very high concentrations of HDL-cholesterol (Glueck et al. 1977). Age and gender also influence levels; males after puberty have lower values than females (Lipid Research Clinics Program 1980).

BEHAVIORAL SOURCES OF VARIATION

Some behavioral sources of variation in HDL-cholesterol are dietary intake, physical activity, smoking, and alcohol consumption. These lifestyle factors appear to be predominant determinants of variation in HDL-cholesterol concentrations.

Diet

HDL-cholesterol concentrations in serum vary with the fat content of the diet as percent of total calories and in some circumstances according to the fatty acid composition of the fat (Grundy and Denke 1990). Saturated fatty acids appear not to reduce HDL-cholesterol levels; in fact, serum HDL-cholesterol concentrations typically are highest when the diet is high in both fat and saturated fatty acids. Monounsaturated fatty acids do not lower HDL-cholesterol levels when they are substituted for saturated fatty acids, and since exchange of monounsaturated for saturated fatty acids lowers LDL-cholesterol, the result of this exchange is a decrease in the proportion of LDL-to HDL-cholesterol.

High intakes of polyunsaturated fatty acid reduce HDL-cholesterol concentrations, about 1 percent for every 2 percent of total calories in which polyunsaturated fatty acids replace saturated or monounsaturated fatty acids (Grundy and Denke 1990). However, exchange of monounsaturated for polyunsaturated fat at levels commonly consumed in the United States appears not to reduce HDL-cholesterol (Dreon et al. 1990).

Enrichment of the NCEP Step I Diet with monounsaturated fat to change the diet to 10 percent saturated, 18 percent monounsaturated, and 10 percent polyunsaturated fat with 250 mg of cholesterol intake per day did not change the HDL-cholesterol concentration from the slightly lower values observed with the Step I Diet when compared with an average American diet (Ginsberg et al. 1990). A study of free-living men and women demonstrated that low-fat polyunsaturated-enriched diets increased HDL_{2} cholesterol and decreased HDL_{3} cholesterol (Dreon et al. 1990). Strict vegetarians have 12 percent lower HDL-cholesterol levels than control nonvegetarians and 7 percent lower values than lactovegetarians (Sacks et al. 1985).

Obesity

HDL-cholesterol concentrations in serum are frequently reduced in obese patients (Grundy and Denke 1990). Although obesity arising from excess intake of dietary energy lowers HDL-cholesterol, this can be counteracted by the tendency of the increased proportion of energy from total fat in the diet to raise HDL-cholesterol (Knuiiman et al. 1987). During weight loss in obese individuals, HDL-cholesterol concentrations may rise rather slowly. Repeated weight gain and loss in obese individuals is a source of variation of cholesterol in HDL as well as in the other lipoproteins. HDL-cholesterol was shown to increase in moderately overweight men who lose weight, whether the fat is lost by caloric restriction or increased exercise (Wood et al. 1988).
Physical Activity

Long-term, relatively strenuous exercise increases the HDL-cholesterol in serum (Wood and Haskell 1979). Adults who walked for exercise 2 1/2 to 4 hours or more each week averaged 3.5 percent increase in HDL-cholesterol in serum (Tucker and Friedman 1990). Loss of body fat accompanying increased exercise seems to account for at least part of the HDL-elevating effect of exercise (Williams et al. 1990). Addition of a program of regular exercise in overweight men and women losing weight on the NCEP diet counteracts the tendency of such low-fat regimens to lower HDL-cholesterol (Wood et al. 1991).

Smoking

HDL-cholesterol is depressed in cigarette smokers of randomly selected families (Brischetto et al. 1983). Examination of the Framingham cohort of women indicated that HDL-cholesterol in current smokers averaged 13 percent lower than those of nonsmokers (Willett et al. 1983).

Alcohol Intake

HDL-cholesterol in serum is increased by a sustained alcohol intake (Castelli et al. 1977). Alcohol intake in moderate drinkers apparently primarily affects the HDL3 fraction, as determined by total mass (Haskell et al. 1984). Alcohol consumption has been reported to cause about 5 percent of the variance of HDL-cholesterol in the LRC Prevalence Study population (Gordon et al. 1981).

PATHOLOGICAL SOURCES OF VARIATION

Variations in plasma HDL-cholesterol concentration result from diseases such as myocardial infarction, stroke, and diabetes; pharmacological therapy as in treatment of hyperlipidemia and hypertension; and the effects of other changes in physiological state such as pregnancy.

Myocardial Infarction

In a study of 58 patients with myocardial infarction, HDL-cholesterol changed minimally during the first 2 days but showed a significant fall at 9 days and did not fully return to the original values even after 3 months (Ryder et al. 1984).

Stroke

The effect of stroke on serum HDL-cholesterol varied with the ages of the subjects (Mendez et al. 1987). Very little change after stroke occurred in a group 50 to 69 years of age. Stroke victims in the 50- to 59-year-old age group had lower mean HDL-cholesterol levels than those in the 60- to 69-year-old age group (Mendez et al. 1987).

Hospitalization

Hospitalized patients were reported to demonstrate reduced HDL-cholesterol levels (Genest et al. 1988).

Diabetes

HDL-cholesterol levels are lower in individuals with type II (non-insulin-dependent) diabetes due to a decreased rate of HDL synthesis, and this is reflected mainly in decreases in the HDL2 fraction (Howard 1987). In type I (insulin-dependent) subjects, untreated persons have low serum HDL-cholesterol levels, but treated individuals with glycemic control show increases of HDL-cholesterol, even to levels higher than those of controls (Howard 1987).

Subjects with a high degree of insulin resistance have lower HDL-cholesterol than do subjects with low insulin resistance (Laakso et al. 1990). Insulin therapy of non-insulin-dependent diabetes generally improves HDL-cholesterol levels, but major effects on lipid and lipoprotein levels vary with the different phenotypes of hyperlipoproteinemia (Hughes et al. 1987).

Treatment for Hypertension

Subjects undergoing treatment with diuretics experience a small decrease in HDL-cholesterol (Shekelle et al. 1984). Diuretics combined with propranolol therapy cause a substantial decrease in HDL-cholesterol (Lasser et al. 1984).

A study of antihypertensive medications indicated that thiazide-type diuretics and some beta-blockers tend to produce small decreases in HDL-choles-
terol levels, but calcium antagonists and angiotensin-converting enzyme inhibitors increase HDL-cholesterol levels in serum (Krone and Najele 1988).

**Other Medications**

Progestins, anabolic steroids, and cholesterol-lowering drugs such as probucol and neomycin can contribute to reduced levels, and some of the cholesterol-lowering drugs such as niacin, fibrates, HMG CoA reductase inhibitors, and bile acid sequestrants tend to increase HDL-cholesterol (Grundy et al. 1987). Estrogen replacement therapy in postmenopausal women increases HDL-cholesterol, but this effect is modified by some progestational agents (Knopp 1991).

**Other Diseases**

Acute infections and renal disease have been reported to decrease HDL-cholesterol (Vergani and Dioguardi 1978; Baillie and Orr 1979).

**Pregnancy**

Although other lipoproteins increase appreciably during pregnancy, HDL-cholesterol is minimally decreased (Reichel and Widhalm 1988).

**Environmental Seasonal Change**

Plasma levels of HDL-cholesterol are reportedly higher in the January-February period compared to the June-July period of the year (Gordon et al. 1987).

**SAMPLING SOURCES OF VARIATION RELATED TO BIOLOGICAL FACTORS**

Major biologic factors that may affect HDL-cholesterol measurements at the time the specimen is collected are fasting status, posture, and venous occlusion.

**Fasting vs. Nonfasting**

A standard fasting period of 12 hours has long been used for both clinical and research measurements. In view of (1) the wider appreciation of the need for LDL-cholesterol measurements, (2) the revised NCEP ATP II guidelines advising the measurement of HDL-cholesterol when total cholesterol is measured, and (3) the inconvenience to the patient of having to fast for 12 hours, the ATP II guidelines recommended that lipid and lipoprotein measurements can be made following a fasting period of 9 to 12 hours and that the HDL-cholesterol measurements can be made in the nonfasting state. Fasting was not previously considered essential before specimen collection for HDL-cholesterol measurement. Folsom et al. (1983) reported no average difference in HDL-cholesterol values in plasma specimens from fasting compared to nonfasting subjects and concluded that fasting is unnecessary. However, there is evidence that fat feeding can lower HDL-cholesterol in the short term. Cohn et al. (1988a, 1988b) fed 22 normal subjects a fat load of 1 g/kg body weight. They found an 8 to 9 percent decrease in HDL-cholesterol levels at 3 to 6 hours and that HDL-cholesterol levels were 4 to 5 percent lower after 9 hours than after 12 hours. Lichtenstein et al. (1993) measured postprandial total cholesterol, triglyceride, and HDL-cholesterol in 14 subjects following 3 normal meals and a snack. The average fasting concentration of HDL-cholesterol was 48 mg/dL. HDL-cholesterol decreased by 8.3 percent after 5 hours and did not recover for at least 10 hours. Wilder (1992) found a 4 percent decrease in HDL-cholesterol 3 hours after subjects were fed a self-selected breakfast; after 5 hours HDL-cholesterol was 1.5 percent below fasting levels. The magnitude of the decrease was determined primarily by the amount of fat in the meal. De Bruin et al. (1991) fed a 98 g fat load to six healthy subjects and found that HDL-cholesterol decreased postprandially. HDL-cholesterol levels 2 to 4 hours after the meal were over 20 percent lower than fasting levels and remained 35 percent below the fasting level after 8 hours. Havel (1957) and Havel et al. (1973), however, observed little change in HDL-cholesterol between 3 and 24 hours after a formula fat meal. In addition, a preliminary analysis of HDL-cholesterol values in Phase I of the Third National Health and Nutrition Examination Survey was provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics. The analysis revealed no significant difference in the group mean serum HDL-cholesterol in subjects who had fasted 9 to 11 hours compared with those who had fasted 12 hours or
more. Subjects undergoing prolonged fasting for 6 days showed a 22 percent decrease in serum HDL-cholesterol, but no changes were observed if fruit juices or carbohydrates were included in fluid intake (Wallentin and Skoldstam 1980).

Further studies are needed to assess the effects of fasting for 9 to 12 hours in a “real life” setting, that is, in patients following their normal dietary routines. Nonetheless, some tentative conclusions can be drawn about the magnitude of the error to be expected when HDL-cholesterol is measured in nonfasting patients or in patients who have fasted for 9 to 12 hours. First, it is likely that HDL-cholesterol will be underestimated somewhat in patients who have fasted for 9 hours. The error, on average, will probably be in the range of about 1 to 4 percent. The error would be somewhat larger (5 to 10 percent) in nonfasting patients. Second, such errors will probably create the most uncertainty in patients with HDL-cholesterol concentrations near the medical decision cutpoints; patients with clearly normal or elevated HDL-cholesterol concentrations should not be misclassified. Third, the error introduced when measuring HDL-cholesterol in nonfasting or 9- to 12-hour fasting patients would be conservative in that it would lead to somewhat of an overestimation of risk (i.e., false positive) in patients with fasting HDL-cholesterol levels at or somewhat above the cutpoints; this would likely be detected on followup measurements in fasting samples. The measurements of HDL-cholesterol in nonfasting or 9-hour fasting patients would not be expected to interfere with the detection of patients with truly low HDL-cholesterol unless the triglyceride level is so high that it interferes with the measurement itself.

Finally, based on HDL-cholesterol distributions in the Lipid Research Clinics Population Studies (Lipid Research Clinics Program 1980), a 10 percent negative error in HDL-cholesterol would tend to misclassify about 15 percent of the population with respect to the two ATP cutpoints (35 mg/dL [0.90 mmol/L] and 60 mg/dL [1.55 mmol/L]): 10 percent with HDL-cholesterol levels of 35-40 mg/dL (0.90-1.04 mmol/L) and about 5 percent with HDL-cholesterol levels of 60-65 mg/dL (1.55-1.69 mmol/L). Thus the use of nonfasting HDL-cholesterol measurement would not interfere with the classification of about 85 percent of the population.

**Posture and Venous Occlusion**

HDL-cholesterol serum values are increased about 10 percent after a person stands up for 30 minutes compared with those taken after the person has been lying down for 30 minutes (Hagan et al. 1986; Tan et al. 1973; Kjeldsen et al. 1983). The sympathetic nervous system apparently plays a role in postural effects (Howes et al. 1987, 1988). Prolonged venous occlusion also contributes to higher values (Page and Moinuddin 1962).

**Specimen Collection**

The standardization of blood collection is important in minimizing biological variability. The NCEP cutpoints for HDL-cholesterol are based on analysis of serum (NCEP 1993). EDTA has generally been used as the anticoagulant when lipoproteins are to be measured because it retards certain oxidative and enzymatic changes that can occur in lipoproteins during storage and handling (Bachorik 1983). EDTA, however, produces an osmotic dilution estimated to be 3 percent (Laboratory Methods Committee 1977). The NCEP guidelines recommend that total cholesterol values in EDTA plasma be multiplied by 1.03 to convert them to serum equivalents (NCEP 1988a, 1994). A more recent study (Cloey et al. 1990) suggested a factor of 1.045 is more appropriate, because the EDTA concentration in currently available blood-collecting tubes is 50 percent higher than when the earlier study was performed (Laboratory Methods Committee 1977). On the other hand, another study found no significant difference between paired serum and EDTA plasma in
Recommendations on Lipoprotein Measurement—HDL

cholesterol measured by either enzymic or Abell-Kendall methods, when tubes were filled completely and well mixed (Warnick, unpublished observations). When HDL-cholesterol is quantified by precipitation methods, the difference between EDTA plasma and serum is reportedly smaller (Folsom et al. 1983); this might be explained by partial EDTA chelation of the divalent cation, possibly decreasing lipoprotein precipitation and partially compensating for the osmotic dilution. Citrate and oxalate anticoagulants, which also chelate metal ions and substantially interfere with the precipitation methods, should be avoided. For the sake of simplicity and consistency with previous NCEP guidelines, it is recommended that plasma HDL-cholesterol be multiplied by 1.03 when converting them to serum values.

Since posture affects HDL-cholesterol concentration, and the NCEP cutpoints are based on measurements in seated subjects, this position is recommended for blood collection. A standing subject would be expected to have higher lipid values, whereas a reclining subject would have lower values compared to sitting. For best agreement with NCEP values, subjects should be seated quietly for at least 5 minutes prior to collection.

Venous occlusion results in a higher concentration of lipoprotein particles behind the tourniquet. When a tourniquet is used for blood collection, it should be removed within 1 minute to avoid artificially elevated values (NCEP 1990).

Differences in total cholesterol have been observed between venous and capillary specimens (Koch et al. 1987), but the results are contradictory. Some studies (Bachorik et al. 1990; Miller et al. 1990) have suggested higher results in capillary specimens, but others (Kupke et al. 1979; Bachorik et al. 1989; Greenland et al. 1990) have found the opposite. The observed discrepancies may be due to differences in blood collection technique (Bradford et al. 1990) and may be a function of the nature of a particular analytical system. A recent study comparing serum cholesterol values in paired venous and fingerstick capillary specimens using an accurate enzymic assay found no significant difference with proper blood collection technique (Warnick, 1994). Recommendations for obtaining fingerstick specimens are given in table 4 (Dörnor and Dorn-Zachertz 1991). These issues will become increasingly important for HDL-cholesterol measurement with the development of micro-HDL methods for compact analyzers suitable for capillary specimens collected by fingerstick. The effect of the type of specimen and the conditions of fingerstick specimen collection and analysis should be evaluated for each analytical system.

Specimen Storage

HDL-cholesterol values can be unstable with apparent values changing substantially in serum or plasma stored at 4 °C for a few days (Warnick and Albers 1979; Bachorik et al. 1980; Matthew and Duggan 1988). Changes in pH, bacterial action, enzyme and transfer protein activity, and other factors may affect HDL composition, its separation from the apo B-containing lipoproteins, or the cholesterol analysis. Addition of bacterial or protein inhibitors seems not to improve specimen stability (Warnick, unpublished observations).

HDL-cholesterol analyses should preferably be completed on the day of collection or at least within 1 or 2 days. When analysis must be delayed, freezing is the usual method of storage (Bachorik et al. 1980, 1982; Nanjee and Miller, 1990). However, the process of freezing and thawing has been observed to affect results by some methods but not others (Warnick, unpublished observations). In general, the lower the temperature, the better the stability. Serum does not completely freeze above approximately -40 °C; as ice crystals begin to form, solutes concentrate in the remaining liquid, lowering the freezing point. Storage at temperatures below -50 °C is generally considered acceptable for 1 to 2 years. Storage in a conventional non-self-defrosting laboratory freezer, nominally at -15 °C to -20 °C, is acceptable for up to 1 month. Specimens are less stable in a self-defrosting freezer or one that is opened frequently. Self-defrosting freezers cycle between about -20 °C and -2 °C, leading to specimen instability. Frozen specimens must be thoroughly but gently mixed after thawing to eliminate stratification and ensure homogeneity; mixing on a laboratory blood wheel or similar device for 30 minutes after thawing is generally sufficient. In studies using frozen specimens, the effect of storage
TABLE 4. Recommended technique for obtaining fingerstick specimens

Preparation
Assemble the necessary supplies. Ask patients to sit quietly for at least 5 minutes and preferably 15 minutes before blood collection. If patients must move from a waiting area, have them walk quietly to the phlebotomy area.

Prepare hands
If the hands are cold, have the patient rub them together briskly or shake for several minutes. Massaging of the hands by the phlebotomist can relax the hands, straighten the fingers, and promote good blood flow.

Select finger
The nondominant hand is recommended. The ring finger is usually preferred because of less callus, but the middle finger may be better on women or children with small hands. Squeeze and release the fingertip a few times. The “flushing of color” into the area is an indicator of good blood flow.

Cleansing
Cleanse end of finger with alcohol or antiseptic pad. Use a sterile gauze pad or cottonball to thoroughly dry the site.

Fingerstick
Pinch end of finger from the side opposite the puncture site to distract the patient and keep skin taut at the puncture site. Use a spring-loaded device with a chisel or blade type lancet and enough force to give a good puncture or incision. Holding the hand palm up, puncture or cut on the upper side corner of the chosen finger up away from the nail bed. Orient the lancet blade to cut across the fingerprint. Hold the lancet tightly against the skin while activating and do not release pressure during the puncture.

Blood collection
Remove lancet and use a sterile gauze to wipe away the first drop of blood, which can be contaminated with tissue fluid or alcohol. Keeping the hand low and oriented either palm up or palm down, whichever is more convenient; allow drops to form and touch into capillary tube or collection device. Hold the capillary horizontally or tilted slightly upward to avoid air bubbles. Do not touch pipet directly to skin but rather allow droplets to form before touching the capillary to the droplet. When collection is complete, place a sterile pad over the puncture site and have the subject maintain pressure. When blood flow has stopped, a bandaid, preferably the spot type, can be placed over the site.

Stimulating blood flow
If blood flow is slow, the following may help:
   A) Lower hand.
   B) Express blood down from the hand toward the finger by progressively squeezing gently and releasing downward across the hand and finger in a “milking” motion. Avoid squeezing the puncture site directly, which can cause dilution with tissue fluid.
   C) Wipe the puncture site with gauze to clear a developing clot and promote flow.

Precautions
Avoid leaving blood in a capillary tube more than 2 or 3 minutes before analysis. The heparin anticoagulant may be unevenly distributed in the tube, allowing localized clotting to occur.

Source: Dörmor and Dorn-Zachertz 1991
Recommendations on Lipoprotein Measurement—HDL

conditions on the HDL method should be evaluated.

Specimens shipped to another site for analysis should be kept refrigerated or frozen. Shipment in insulated containers (e.g., cardboard outer boxes with styrofoam liners) with water ice or “blue” ice packs is adequate for overnight shipments. Alternatively, dry ice can be used to keep specimens frozen for overnight or longer periods. Securely sealed, break-proof vials should be enclosed in sealed containers with absorbent material. Glass serum bottles of appropriate volume with securely sealed rubber stoppers and aluminum seals are recommended for long-term frozen storage. Guidelines for safe shipment of potentially hazardous biological materials should be followed (U.S. Department of Health and Human Services 1980).

ANALYTICAL VARIATION

Quantitation of HDL-cholesterol requires attention to both the separation and analysis steps to ensure reliable results. Acceptable precision is a prerequisite for accuracy; an analytical system that has no systematic error but that is imprecise will not provide accurate values in many specimens. Thus, achieving the requisite precision is the first consideration in attaining acceptable overall performance. Achieving accuracy in the HDL-cholesterol determination requires both specificity for HDL-cholesterol in the separation step and good precision as well as proper calibration of the cholesterol analysis. A bias in the precipitation step could be offset by a compensating error or calibration adjustment in the cholesterol analysis, but such a system relying on compensating errors is less desirable than a system accurate for both steps.

Chemical precipitation methods, which will be reviewed in detail later, have been most widely used for routine HDL-cholesterol quantification. The accuracy or specificity of a particular precipitation method may best be verified by comparison with a validated precipitation procedure coupled with a reliable cholesterol method (Duncan et al. 1982) that is free of interferences from the precipitating reagents and other substances in the specimen. Once the specificity of the precipitation method has been demonstrated, the accuracy of the combined procedure (precipitation step and cholesterol analysis step) can be verified by comparison with an accepted reference procedure. The accuracy of the reference or comparison method must, of course, also be known in order to make a reliable assessment of another method (Bennett et al. 1991).

It is important to optimize the cholesterol assay for the particular precipitation method. A cholesterol method that is accurate for total serum cholesterol may not necessarily be accurate for HDL-cholesterol. Since about 75 percent of the serum cholesterol is removed with the apo B-containing lipoproteins, HDL-cholesterol concentrations are in the low range and the contributions of endogenous interfering substances are more likely to affect the measurements adversely.

MATRIX INTERACTIONS

Changes in the analyte or in the specimen matrix that interact with one or another component of the analytical method are commonly referred to as matrix interactions. Such matrix interactions can pose a substantial problem in the reliable transfer of accuracy from a reference method to field methods. Manufactured or processed reference materials (i.e., quality control pools, calibrators, or other reference materials) can be altered in ways that affect their assay characteristics such that they no longer behave in the same manner as fresh patient specimens. Changes in the proportion of free and esterified cholesterol, in the nature or distribution of the lipoproteins, or in background interfering substances—any of which might be induced by addition of a concentrate to increase values, freeze drying, or other manufacturing processes—may alter the separation and measurement characteristics of the specimen. As a result, a method calibrated to give accurate values with fresh specimens may appear to be inaccurate with processed material. Recent studies (Kroll et al. 1989; Kroll and Chesler 1990) have described some sources of matrix interactions in total cholesterol analysis. Matrix interactions seem to be more prevalent with the newer dry chemistry analytical systems offering integrated HDL separations such as the Reflotron (Hiller et al. 1987) and the Cholestech LDX.

Improvements in method and reference material technology will help to ameliorate such method-
Sources of Variation

dependent analyte/matrix interactions. Enhancing the specificity of a method through decreased susceptibility to endogenous interfering substances will reduce the potential for differences in reference materials compared to fresh sera. In addition, improvements in manufacturing processes for reference materials that make the assay characteristics of calibrators, bench controls, and proficiency-testing materials more consistent with those of fresh specimens will also help to reduce the matrix effect.

Until commutable reference and quality control materials (those performing exactly like fresh specimens) are available, the use of fresh specimens will be necessary for evaluating HDL-cholesterol method accuracy, especially for those methods that are matrix sensitive. Such studies are best performed by simultaneously measuring HDL-cholesterol with the tested and comparison methods using specimens that have been handled under similar conditions.
Section 4.
Critical Review of HDL-Cholesterol Methods

CDC REFERENCE METHOD

There is at present no well-validated and accepted reference method for HDL-cholesterol; however, a CDC method has been widely used as the accuracy target in recent national population studies. This as yet unpublished method is a complicated, multi-step procedure involving ultracentrifugation, precipitation, and cholesterol analysis. First, VLDL and chylomicrons are removed from serum by ultracentrifugation for 18 hours, at 105,000 x g, at density 1.006 kg/L (the background density of serum), followed by separation using a tube slicing technique (Lipid Research Clinics Program 1982). Ultracentrifugation is included to eliminate the interference from triglyceride-rich lipoproteins.

In the second step of the CDC method, the d >1.006 kg/L fraction containing LDL and HDL is treated with heparin and Mn²⁺ (final concentration 46 mmol/L) to precipitate the remaining apo B-associated lipoproteins (IDL, LDL, and Lp(a)). The precipitate is removed by low-speed centrifugation (Lipid Research Clinics Program 1982). In the third step, the HDL-cholesterol that remains in the supernate is measured by the CDC modification of the reference Abell-Kendall method (Duncan et al. 1982).

Because the CDC reference method was the accuracy target for the population studies from which CHD risk estimates and HDL-cholesterol cutpoints were derived, there is considerable justification for continuing this procedure as the national reference method. However, the equipment requirements, technical demands, large specimen volume, and expense of this three-step procedure preclude its widespread use for the routine laboratory as well as for most research laboratories. Since matrix interactions necessitate method comparisons using fresh specimens for reliable accuracy transfer, the large number of reference analyses required would be logistically impractical and prohibitively expensive using the CDC method. An alternative approach to accuracy transfer through the general distribution of reference materials with target values established by the reference method would decrease the number of reference analyses but will not be reliable because of matrix interactions. Given these considerations, there is a need for simpler equivalent reference methods that have been shown to agree with the CDC reference method. The best candidates for equivalent reference methods will be precipitation methods applied directly to whole serum and coupled with the Abell-Kendall cholesterol assay (Duncan et al. 1982).

HDL separation by sequential density ultracentrifugation has also been used as a reference or comparison method for HDL quantification. Although HDL by definition comprises lipoprotein particles with densities between 1.063 and 1.21 kg/L (Havel et al. 1955), in usual practice a single ultracentrifugation has been used and HDL considered to be particles with density >1.063 kg/L. Ultracentrifugation alone does have major disadvantages as a reference method. In addition to the general complexity and expense involved, the d >1.063 kg/L fraction often contains Lp(a), because its density overlaps that of HDL (table 1). Other apo B-associated lipoproteins may also be suspect due to cross-contamination. Because such apo B-containing lipoproteins are atherogenic, including them with HDL is inappropriate (Warnick and Albers 1978a). Quantitative recovery of the ultracentrifugation fractions is also difficult. For these reasons ultracentrifugal fractionation alone is not recommended as a reference method for HDL quantification.
FIELD METHODS

Most routine laboratories use one of several chemical precipitation methods for separation of HDL (Gibson and Brown 1981; Levin 1989). The first step requires addition of appropriate reagents to precipitate VLDL and LDL and the other apo B-containing lipoproteins from serum (table 1). The precipitate is then sedimented by low-speed centrifugation. In the second step, HDL-cholesterol is measured in the supernate. Such precipitation methods have been used for decades, but many of the current methods, which use different combinations and concentrations of precipitating reagents coupled with enzymic assays, were adopted for routine quantification of HDL-cholesterol in the mid-1970’s. The various precipitation methods, although similar in technique, are not equivalent in terms of their separation characteristics and freedom from interference (Burstein and Legmann 1982). The tendency of the lipoprotein particles to precipitate is a function of their size and lipid composition; the larger and more lipid-rich particles are precipitated more readily. The reagents used for precipitation differ somewhat in their abilities to precipitate lipoproteins. Factors such as reagent concentration, solution pH, ionic strength, and temperature also affect the separations.

Polyanions, including heparin, dextran sulfate, and sodium phosphotungstate, interact with positively charged amino acids (e.g., arginine) on the lipoproteins. Divalent cations, usually Mn$^{2+}$, Mg$^{2+}$, or Ca$^{2+}$, used together with the polyanions, enhance the insolubility of the complexes by interacting with negatively charged groups such as those on phospholipids. Within a class of polyanions, the larger the size and the higher the negative charge, the greater is the tendency for formation of insoluble complexes. Although it is not charged, polyethylene glycol at very high concentrations (approximately 100-fold higher than the polyanions) can also precipitate the lipoproteins. Thus, the major lipoprotein classes can be separated with reasonable specificity using the appropriate reagents and conditions. In general, the more dissimilar the lipoproteins, the better they can be separated with precipitation methods. Precipitation methods have been most successful for separating apo B-containing lipoproteins from HDL.

Separation of the HDL Fraction

To separate HDL, a precipitation reagent ideally should precipitate all the apo B-containing lipoproteins (VLDL, IDL, LDL, and Lp(a)) but should not precipitate any HDL. In practice, however, the methods are not completely specific for HDL in every specimen. For example, VLDL and/or LDL present at very high concentrations may not be completely precipitated at reagent concentrations that are suitable in most specimens. In addition, some HDL may precipitate when VLDL/LDL levels are unusually low. The specimen matrix is also a factor. For example, EDTA used as anticoagulant will chelate part of the divalent metal ion, reducing its effective concentration and inhibiting the complex precipitation of apo B-containing lipoproteins in EDTA plasma. For this reason a higher metal ion concentration is required when EDTA plasma is used compared to serum (Warnick and Albers 1978a). Thus, reagent concentrations and separation conditions must be optimized to maximize precipitation of LDL/VLDL while minimizing precipitation of HDL in most specimens. Other characteristics of the precipitating reagents, such as their stability, freedom from interference, compatibility with cholesterol reagents and assay methods, and cost must be considered when formulating a practical reagent.

Specificity of the separations can be verified approximately by performing lipoprotein electrophoresis of the fractions to detect cross-contaminating material. Immunochemical quantitation of the major apolipoproteins in fractions is also useful. The presence of apo B in the HDL-containing fraction signals incomplete precipitation or sedimentation of apo B-containing lipoproteins. The presence of apo AI in the precipitated lipoproteins indicates the precipitation of some HDL (Warnick and Albers 1978a).

Precipitation methods are subject to interference from triglyceride-rich lipoproteins (Warnick and Albers 1978b). Lipemic specimens contain high levels of VLDL and/or chylomicrons; the triglyceride content of these lipoproteins reduces the density of the insoluble lipoprotein aggregates and may prevent their complete sedimentation. At very high triglyceride levels, the insoluble lipopro-
proteins may actually float to the surface. The triglyceride level at which interference occurs varies considerably among specimens and also depends to some extent on which precipitation method is used.

After centrifugation, the HDL-containing supernates should be screened visually for turbidity, preferably by viewing against a dark background under a light shining perpendicularly to the line of sight. Any turbidity at all can produce a significant error in the HDL-cholesterol value since unsedimented precipitate is rich in cholesterol. Turbid supernates can be cleared in most cases by high-speed centrifugation (Warnick and Albers 1978a). Alternatively, the specimens can be diluted with an equal volume of saline solution (Burstein and Samaille 1960), which decreases the solution density and facilitates sedimentation of the precipitate. The original specimen can be diluted before precipitation, or the dilution can be done on supernates that are turbid after the initial centrifugation. When diluting a turbid supernate, add a volume of saline equal to the original specimen volume and an additional proportionate volume of the precipitating reagents, then remix and recentrifuge the sample. The triglyceride-rich lipoproteins can also be removed by ultracentrifugation (as in the CDC reference method) before the specimen is treated with the precipitating reagent (Lipid Research Clinics Program 1982). Early precipitation methods usually used low-speed centrifugation (1,500 x g) for up to 30 minutes; more recent studies, however, indicate that centrifugation at 10,000-12,000 x g sediments the precipitates in about 5 minutes and better clears lipemic specimens (Nguyen and Warnick 1989).

Methods employing electrophoretic separation with densitometric scanning have also been adapted for quantitation of HDL-cholesterol but are much less common than the precipitation methods. These methods will be reviewed in greater detail later.

**Cholesterol Analysis**

The second step in quantifying HDL-cholesterol is the analysis of cholesterol in the supernate. In recent years enzymic methods have largely replaced the earlier strong-acid methods for routine cholesterol measurements (Zak and Artiss 1990). Enzymic assays, employing cholesterol ester hydrolase, cholesterol oxidase, and horseradish peroxidase enzymes, are reasonably specific for cholesterol and are usually applied directly to serum without extraction or other pretreatment, an advantage over the older strong-acid methods. However, certain substances can interfere with enzymic methods as described below. A factor is applied to correct the measured cholesterol concentration in the supernate for the dilution incurred by adding the precipitation reagents.

Special considerations apply when assaying cholesterol in HDL supernates, since the cholesterol levels of the supernate are lower than in serum; the effects of interfering substances are thus magnified. Supernates obtained by any of the precipitation methods contain on the average only about 25 percent of the serum cholesterol but essentially all of the interfering substances. As an example, interference from ascorbic acid, which decreases color formation with the common enzymic reagents, may be negligible in serum but significant in the HDL supernate. Similarly, bilirubin, which is a reducing agent and has an absorbance overlapping that of the cholesterol chromophor (Perlstein et al. 1978), is more likely to cause noticeable interference in the HDL supernate. Furthermore, the precipitating reagents themselves may interfere with one or more of the enzymatic cholesterol measurement reactions. For example, manganese ion reacts with the phosphate buffer used in some enzymic reagents to produce an insoluble complex and can cause some photometric interference (Steele et al. 1976). Each precipitation reagent may have unique characteristics that can interact with a particular formulation of the cholesterol reagent. Thus, the cholesterol assay must be optimized and validated specifically for the HDL separation method used. Accuracy for total cholesterol measurement does not necessarily imply accuracy for HDL-cholesterol measurement. Both the separation and analytical steps must be validated to ensure acceptable performance.

Since the cholesterol concentration is lower in the HDL supernate than in serum, performance can be improved in most assay systems by increasing the specimen-to-reagent volume ratio, bringing the
Recommendations on Lipoprotein Measurement—HDL

color response into the optimum range. With many common enzymic methods, the appropriate specimen-to-reagent ratio for HDL is usually about 1:25, compared to 1:100 for total serum cholesterol. However, the concentrations of potentially interfering substances such as ascorbic acid and bilirubin are simultaneously increased. The use of a chromophor with a higher extinction coefficient can increase method sensitivity, offsetting the need to increase specimen volume; this can reduce photometric interference (Artiss et al. 1981) but not necessarily chemical interference.

There are many approaches to the calibration of HDL-cholesterol assays, depending on the nature of the analytical system. Calibration with a secondary serum-based standard, closely approximating patient specimens, has proven to be reliable for most systems (Demacker et al. 1983; Boerma et al. 1986). Accuracy is best determined with fresh specimens and when based on comparison analyses using an accurate reference method. Set points on calibrators are then assigned or adjusted to achieve agreement on the patient specimens.

Precipitation Reagents

Of the various available reagents used for HDL separation, the combination of heparin and Mn\textsuperscript{2+} has been most extensively studied and has been used in most major population and clinical studies. This reagent is used for the CDC HDL-cholesterol reference method. This method was developed by Burstein and Samaille (1960) for the fractionation of serum and was subsequently used without modification by Fredrickson and coworkers (1969) and by the Lipid Research Clinics Program (1982) for separating HDL-cholesterol EDTA plasma specimens. Reasonable accuracy, as compared to ultracentrifugation, was demonstrated (Bachorik et al. 1976; Warnick and Albers 1978a) but with indications that the Mn\textsuperscript{2+} concentration (46 mmol/L) might not completely remove the apo B-containing lipoproteins from EDTA plasma (Warnick and Albers 1978a; Ishikawa et al. 1976; Albers et al. 1978; Mao and Kottke 1980). The removal of LDL and VLDL from EDTA plasma was enhanced by increasing the Mn\textsuperscript{2+} concentration to 92 mmol/L. The heparin concentration, when it exceeds about 1-2 mg/mL does not seem to be critical, but the source of heparin can be important. Heparin obtained from lung was reported to give lower results than that obtained from intestine. Intestinal heparin is more common and has been recommended for use (Mayfield et al. 1979). Heparin and Mn\textsuperscript{2+} can be added separately or as a combined solution without affecting results.

Of the common precipitation methods, the heparin-Mn\textsuperscript{2+} reagent seems to be most sensitive to interference by triglyceride-rich lipoproteins (Warnick et al. 1985). An Mn\textsuperscript{2+} concentration of 92 mmol/L facilitates sedimentation compared to the original concentration of 46 mmol/L (Warnick and Albers 1978a). The higher concentration has been recommended for EDTA plasma, whereas the original concentration (46 mmol/L) is used for serum.

Specimens with incomplete sedimentation, indicated by turbidity in the supernate, must be treated further to remove the apo B-containing lipoproteins completely (Warnick and Albers 1978b). The additional manipulations required to clear turbid supernates are inconvenient and time consuming; one report even suggested that the incomplete separations with heparin and Mn\textsuperscript{2+} are not always visually obvious (Kiss et al. 1986). Turbidity from unsedimented lipoproteins in supernates can be removed by dilution, filtration, or high-speed centrifugation, as described previously. In some cases, turbidity that develops in stored heparin-Mn\textsuperscript{2+} supernates may not result from lipemia but from reaction of Mn\textsuperscript{2+} with carbon dioxide in the air to form a slowly developing precipitate that will eventually settle. This type of turbidity develops more readily and quickly in serum than in EDTA plasma.

Mn\textsuperscript{2+} also reportedly interferes with the enzymic color reaction (Steele et al. 1976). The Mn\textsuperscript{2+} interference may be at least partially eliminated by addition of EDTA to the cholesterol reagent (Steele et al. 1976). Mn\textsuperscript{2+} remaining in the supernate after the initial separation can also be precipitated with sodium bicarbonate (Bachorik et al. 1984). These remedies are inconvenient, and the use of heparin-Mn\textsuperscript{2+} reagent by clinical laboratories has been steadily declining in recent years (table 5). Currently less than 2 percent of routine laboratories report the use of this reagent.
TABLE 5. Trends in use of HDL-cholesterol precipitation methods by laboratories participating in the CAP Comprehensive Chemistry Survey

<table>
<thead>
<tr>
<th>Precipitation Method</th>
<th>Comprehensive Chemistry</th>
<th>Number of Laboratories Reporting Use</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1983</td>
<td>1985</td>
</tr>
<tr>
<td>Dextran sulfate, all methods</td>
<td>C-06</td>
<td>C-04</td>
</tr>
<tr>
<td>50 kDa</td>
<td>285</td>
<td>462</td>
</tr>
<tr>
<td>500 kDa</td>
<td>—</td>
<td>42</td>
</tr>
<tr>
<td>Other</td>
<td>—</td>
<td>240</td>
</tr>
<tr>
<td>Heparin, Mn²⁺/Ca²⁺</td>
<td>133</td>
<td>123</td>
</tr>
<tr>
<td>Phosphotungstate, all methods</td>
<td>726</td>
<td>1,197</td>
</tr>
<tr>
<td>with Mg²⁺</td>
<td>1,173</td>
<td>1,220</td>
</tr>
<tr>
<td>without Mg²⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>—</td>
<td>24</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>97</td>
<td>105</td>
</tr>
</tbody>
</table>

The most common methods used in routine clinical laboratories involve precipitation with sodium phosphotungstate, usually in combination with Mg²⁺. Less often phosphotungstate is used at pH 5.2, the isoelectric point of LDL; this does not require the metal ion. In recent CAP surveys about 50 to 60 percent of laboratories report using some version of this reagent (table 5). The reagent was originally described by Burstein and Scholnick (1973) in a preparative-scale technique for isolation of lipoproteins. More recently, it has been adapted to routine quantification (Lopes-Virella et al. 1977; Maddison et al. 1979; Seigler and Wu 1981). The advantages cited for phosphotungstate reagents are their stability and reduced interference from triglyceride-rich lipoproteins compared to heparin and Mn²⁺ (Gibson and Brown 1981; Warnick et al. 1985). Since special treatment of turbid supernates is inconvenient, especially in the high-volume routine laboratory, the triglyceride insensitivity has been considered a substantial advantage. The method seems to be more sensitive to reaction conditions when compared to other precipitation methods. Its specificity is affected by such variables as temperature and separation time (Warnick et al. 1979; Grove 1979). The reagent concentrations originally recommended were found to produce a significant underestimation of HDL-cholesterol levels compared with ultracentrifugation because some of the HDL was also precipitated (Warnick et al. 1979). Recent modifications of the method use lower phosphotungstate and Mg²⁺ concentrations, which may improve its specificity (Kostner et al. 1979; Warnick et al. 1985). The interference of phosphotungstate with an enzymic cholesterol method has been reported (McGowan et al. 1982a, 1982b).

The next most common precipitation reagent is dextran sulfate combined with Mg²⁺ (Burstein and Legmann 1982; Burstein and Scholnick 1973). This reagent is becoming increasingly popular in routine laboratories (table 5). The earlier versions that used dextran sulfate of molecular weight 500 kDa were precise but had a negative bias consistent with the precipitation of some HDL (figure 1) (Warnick et al. 1979). A modified reagent, reported as a Selected Method of Clinical Chemistry, uses a dextran sulfate of intermediate (50 kDa)
Mean biases for HDL methods different from the CDC reference method on various survey materials of the College of American Pathologists' proficiency testing program. The special Lipid Survey used a material that was lyophilized by a low temperature process reputed to preserve the lipoproteins, whereas the Chemistry surveys used conventional lyophilization. (PT, phosphotungstate; DS, dextran sulfate; PEG, polyethylene glycol.) CDC reference values are shown on the right of each panel.

Molecular weight with Mg\(^{2+}\), gave separations in better agreement with those of the heparin-Mn\(^{2+}\) procedures, and avoided the interference of Mn\(^{2+}\) (Warnick et al. 1982). Lipoprotein precipitation has been shown to be a function of the dextran sulfate molecular weight; 2,000 kDa preparations readily precipitate all lipoproteins, and 15 kDa remove only the largest lipoproteins (Burstein and Legmann, 1982). The 50 kDa material seems to achieve specific separation of apo B-containing lipoproteins and is not as sensitive to reaction conditions such as temperature (Warnick et al. 1982a). Mg\(^{2+}\) phosphate or carbonate remains soluble, which is an advantage with enzymic reagents. There have been reports, however (Henderson et al. 1980; Demacker et al. 1980b), suggesting that some dextran sulfate Mg\(^{2+}\) reagents might interfere with certain enzymic assays. One source of interference described recently was turbidity associated with reaction between dextran sulfate and potassium but not sodium in the enzyme reagent (Olmos et al. 1992).

Another reagent described for HDL separation but little used in routine laboratories (table 5) is polyethylene glycol. Although polyethylene glycol (molecular weight 6 kDa) can at high concentrations precipitate all serum proteins, LDL and VLDL are reportedly precipitated with reasonable specificity under appropriate conditions. Polyethylene glycol concentrations used are nearly 100-fold higher than those for the previously described polyanions, making reagents highly viscous and difficult to pipet accurately. An early method recommended a final polyethylene glycol concentration of 12 percent (Viikari 1976). This method was later reported to precipitate some HDL (Warnick et al. 1979), but subsequent decreases in polyethylene glycol concentration or an increase in reagent pH appear to improve its specificity (Allen et al. 1979; Demacker et al. 1980a, 1980b; Izzo et al. 1981; Briggs et al. 1981; Wiebe and Smith 1985). A final polyethylene glycol concentration of approximately 7.5 percent may give the best accuracy (Demacker et al. 1980a, 1980b; Warnick et al. 1985). This method...
seems to be effective in achieving sedimentation in the presence of elevated triglycerides (Warnick et al. 1985) and has been considered attractive as a readily available single-component reagent. Polyethylene glycol can vary from different sources, and the reagent may interfere with some enzymic cholesterol methods (McGowan et al. 1982a).

**Electrophoretic Methods**

The major lipoprotein classes can be separated by electrophoresis on various media including cellulose acetate (Cobb and Sanders 1978), agarose (Lindgren et al. 1977; Conlon et al. 1979), and polyacrylamide (Muniz 1977). Such separations have been used for years in qualitative analysis of the lipoproteins, generally with visual assessment. More recently, methods were adapted for quantitative analysis with densitometric scanning of the colored bands after staining with lipophilic dyes or reaction with enzymic cholesterol reagent. Lipoprotein precipitating reagents can also be used to make the lipoproteins appear as turbid bands on gels (Wieland and Seidel 1978). Total cholesterol is measured in the specimen, and lipoprotein cholesterol is calculated as a fraction of the total cholesterol based on the relative densitometric quantitation of the corresponding bands. Many electrophoretic quantitation methods have unacceptably large analytical variation (Warnick et al. 1982b) and tend to overestimate HDL-cholesterol compared to precipitation methods. For example, in a recent CAP Comprehensive Chemistry Proficiency Survey (figure 1), the mean HDL-cholesterol for all electrophoretic methods was about 20 mg/dL higher than that for the CDC reference method and for the precipitation methods in the same survey pool.

Differences observed by the electrophoretic methods can have several causes. The population of particles constituting “HDL” when separated by electrophoresis does not necessarily correspond exactly to that separated by ultracentrifugation or precipitation. The separations can vary with the porosity of the gel, and polyacrylamide fractionations differ from those on agarose gel. A support medium such as cellulose acetate may be somewhat opaque, limiting the reliability of quantitation by densitometry (Conlon et al. 1979). The lipophilic dyes or enzymic reagents may not fully react with the lipoproteins or may not produce a linear response. Furthermore, the agreement between methods may vary considerably in individual samples. Although some newer electrophoretic methods may be acceptably accurate and precise, these limitations have restricted routine use of electrophoresis for the measurement of HDL-cholesterol. Less than 2 percent of laboratories currently report using electrophoretic methods (table 5).

**Compact Analyzers**

HDL quantification methods are available for compact analyzers. These include the following:

- **VISION**—Abbott Laboratories, North Chicago, IL (Meloy et al. 1990)
- **Ektachem DT-60**—Eastman Kodak, Rochester, NY (Warnick et al. 1986)
- **Reflotron**—Boehringer Mannheim Diagnostics, Indianapolis, IN (Hiller et al. 1987; Ng et al. 1991).
- **LDX**—Cholestech Corporation, Hayward, CA (unpublished)

Most of the current methods require a preliminary HDL separation step. The Reflotron method can be used with fingerstick specimens but requires prior separation of the red cells; EDTA plasma is actually loaded onto the test strip. HDL separation and cholesterol analysis occur in the instrument. A whole blood procedure with EDTA as anticoagulant has been developed for the VISION; the specimen is added to a pretreatment reagent tube with an internal standard, and the mixture is then loaded into the test pack for separation and analysis. Another manufacturer, Cholestech, offers a direct whole blood method suitable for fingerstick specimens. This method employs a telephone-sized analyzer instrument. The DT-60 requires prior removal of apo B-containing lipoproteins by conventional precipitation. Other manufacturers are developing a variety of systems. Since the reliability of some compact analyzer applications for total cholesterol measurement has been questioned (Rastam et al. 1988; Naughton et al. 1990), the newer HDL methods should be thoroughly evaluated before use.
Quality Control Procedure

Analytical performance is generally monitored through the use of internal and external quality control programs. Monitoring the measurement of HDL-cholesterol is complicated by the instability of the lipoproteins and the sensitivity of the methods to the matrix characteristics of the specimen. Many of the commercially available quality control materials are not suitable for HDL-cholesterol analyses (Bullock et al. 1980; Warnick et al. 1981). For example, the use of ethylene glycol as stabilizer compromises the HDL separation step. The requirements for practical commercial quality control materials include stability, clarity, ease of shipping, and competitive cost and are not always compatible with the nature of the HDL analyte. For example, lyophilized materials are suitable for many applications but are not appropriate for HDL-cholesterol. Most specialty laboratories and many routine laboratories prepare fresh frozen serum materials from donors (Warnick et al. 1981; Demacker et al. 1982b). Such pools stored at 70 °C are usually stable for 1 to 2 years. When practical considerations preclude in-house preparation, some of the better commercial materials may be acceptable for monitoring the reproducibility of a method but should be supplemented with fresh specimen comparisons to evaluate accuracy. Two levels are recommended, at or near the decision points of 35 mg/dL (0.90 mmol/L) and about 60 mg/dL (1.55 mmol/L).

Participation in an external proficiency survey is recommended. However, because of marked matrix interactions with the usual lyophilized survey materials, judgments about method performance may not be entirely accurate. Efforts continue on several fronts to develop better quality control and survey materials. Regional specialty surveys are also available that use fresh or fresh frozen specimens (Myers et al. 1991), and such materials more nearly reflect the properties of fresh specimens.
Section 5.
Current Laboratory Performance in HDL-Cholesterol Analysis

The laboratories of the Lipid Research Clinics Program followed a common protocol (Lipid Research Clinics Program 1982) involving heparin-Mn$^{2+}$ (46 mmol/L) precipitation with cholesterol analysis by a Liebermann-Burchard reagent on the Auto Analyzer-II (Technicon Instruments, Tarrytown, NY). The laboratories all participated in the CDC-NHLBI Standardization Program and used common standards and quality control pools prepared by CDC. An examination of analytical performance in 10 laboratories revealed long-term, within-laboratory SD's ranging from 1.68 mg/dL (0.04 mmol/L) to 4.18 mg/dL (0.11 mmol/L) ($CV_a$ 3.40 to 8.41 percent). The overall variance across all laboratories was consistent with an SD of 3.27 mg/dL (0.08 mmol/L); 66 percent of the total variance was within run, 13 percent between run, and 21 percent among laboratories (Albers et al. 1980). Thus, in those studies there was less variation between laboratories than within laboratories. This performance level demonstrates the feasibility of standardizing HDL-cholesterol measurements across laboratories.

A performance survey of HDL-cholesterol analysis in 138 routine and specialized laboratories conducted in 1979 by CDC (Hainline et al. 1980) found that about 40 to 50 percent of the results met the CDC-NHLBI criteria (shown in Table 6) for acceptable precision and accuracy. In three smaller surveys of clinical laboratories conducted in 1979, 1982, and 1986 using fresh-frozen patient specimens, 61 to 63 percent of the results were within ±5 mg/dL of target values (Warnick et al. 1980, 1983; McMillan and Warnick 1988).

The largest U.S. proficiency surveys have been conducted by the College of American Pathologists. Since the CAP surveys prescribe only a single analysis of each specimen by each participating laboratory, the observed variability of the measurements includes both imprecision and bias. The survey SD's over all participating laboratories have been in the 5-7 mg/dL range, suggesting that about two-thirds of the results were within 5 mg/dL of the consensus mean. For the most part, the interlaboratory SD's for HDL-cholesterol

### TABLE 6. Current CDC-NHLBI criteria for acceptable performance in high density lipoprotein cholesterol

<table>
<thead>
<tr>
<th>Concentration Range (mg/dL)</th>
<th>Accuracy</th>
<th>Maximum Imprecision Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;40</td>
<td>±10% of RV$^2$</td>
<td>2.5 mg/dL (0.06 mmol/L)</td>
</tr>
<tr>
<td>40-60</td>
<td>±10% of RV</td>
<td>3.0 mg/dL (0.08 mmol/L)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>±10% of RV</td>
<td>3.5 mg/dL (0.09 mmol/L)</td>
</tr>
</tbody>
</table>

$^1$ See Myers et al. 1989.
$^2$ RV, CDC reference value determined with the combination ultracentrifugation-heparin-MnCl$_2$ method (see text).
measurements has been about 5 mg/dL over the past 10 years (figure 2).

Several points should be noted in drawing conclusions about the reliability of HDL-cholesterol measurements using such survey data. First, the findings reflect the use of a variety of methods in a large number of laboratories; the variability of HDL-cholesterol measurements in individual laboratories using modern analytical methods is undoubtedly lower. Second, part of the variation observed in the surveys can be attributed to the interlaboratory biases that can be expected to decrease with the adoption of a common point of reference by which bias can be judged. Finally, as discussed earlier in this report, HDL-cholesterol measurements in survey pools are subject to matrix effects that do not affect measurements in fresh samples. Indeed there are indications of this in the survey data. The overall performance for the common reagent combinations on several recent CAP proficiency surveys is illustrated in figure 1. Data for all laboratories using each precipitation reagent are grouped so that several cholesterol methods and commercial reagent sources are included in each group. The upper two panels illustrate the apparent biases for these using the conventional cake-type lyophilized survey materials. The lowest panel of the figure illustrates much lower intermethod biases on a newer type of survey material prepared by low-temperature lyophilization, a process that reduces degradation of the lipoproteins. Thus, matrix interactions on lyophilized survey materials may overestimate the actual biases for the most commonly used methods (see also below).

Overall, the electrophoretic methods as a group had a large positive bias with considerable variability among methods. Of the precipitation

**FIGURE 2.** Laboratory performance trends in HDL-cholesterol analysis on CAP Comprehensive Chemistry Proficiency Survey.

Overall interlaboratory variability, coefficient of variation (CV), and standard deviation (SD) for all HDL-cholesterol methods.
methods, the widely used phosphotungstate-magnesium methods were in reasonably close agreement with the CDC reference method. Dextran sulfate (500 kDa) with magnesium gave somewhat lower results, whereas dextran sulfate of 50 kDa gave results nearer those of the CDC method. Phosphotungstic acid without a cation had a variable bias, whereas heparin with manganese gave moderately low results.

More reliable information about the accuracy of precipitation methods can be gained by comparison studies on fresh or fresh-frozen specimens. Intermethod biases are relatively small on fresh specimens with low HDL levels but can be larger on specimens with high HDL-cholesterol levels (figure 3) (Wiebe and Smith 1985). Since the first NCEP HDL-cholesterol cutpoint (35 mg/dL [0.90 mmol/L]) is in the range where current precipitation methods demonstrate less bias, patient classification based on this cutpoint may not be compromised. As discussed previously, errors in HDL-cholesterol contribute reciprocally to errors in LDL-cholesterol, and although such errors may be modest, accuracy at all levels of HDL-cholesterol is important when LDL-cholesterol is to be measured. The apparent bias of HDL-cholesterol measurement, particularly with the newer survey pools (figure 1), suggests that, on average, the positive bias of LDL-cholesterol measurements may be in the range of about 5 mg/dL (0.13 mmol/L) or less.

The intermethod bias can be reduced by adjusting the precipitation reagent concentrations and precipitation conditions (Warnick et al. 1985). Considering that current methods were developed prior to agreement on a reference method, it is not surprising that intermethod differences exist. With the use of the CDC method as the accepted accuracy base, it should be possible for manufacturers and laboratories to optimize their separation methods and/or cholesterol methods to achieve accuracy in HDL-cholesterol measurement.

### FIGURE 3. HDL method biases on fresh-frozen specimens

![Image of bar chart showing HDL method biases on fresh-frozen specimens.](image)

The mean and range of HDL-cholesterol values for 90 serum samples tested by six HDL isolation procedures. HM1M, heparine-manganese chloride (final concentration, 0.046M); HM2M, heparin-manganese chloride (final concentration, 0.09M); DS, dextran sulfate; NaPT, sodium phosphotungstate; PEG, polyethylene glycol.

**Source:** Wiebe and Smith 1985
Recommendations on Lipoprotein Measurement—HDL
The NCEP Adult Treatment Panel has recommended that HDL-cholesterol concentrations <35 mg/dL (0.90 mmol/L) and ≥60 mg/dL (1.55 mmol/L) be considered positive and negative risk factors, respectively, for CHD when selecting patients for treatment. Appropriate patient classification based on these cutpoints requires reasonable agreement of HDL-cholesterol methods with the methods used in earlier studies to establish the relationship between HDL-cholesterol concentration and risk for CHD. These methods were generally standardized to the CDC HDL reference method described above.

Precision is the primary analytical performance characteristic since accuracy cannot be judged reliably with an imprecise method. The precision of HDL-cholesterol measurements should conform to the generally accepted criterion for the usefulness of a medical test: The random error of the measurement should be no greater than half the average biological variation. Assuming the average CVₜ for HDL-cholesterol is 7.5 percent, a precision goal of about 4 percent would satisfy this criterion.

Given this precision goal, a goal for bias was developed by relating the error in HDL-cholesterol values to the number of replicate samples that must be obtained from the patient. Using an error limit lower than 10 percent with 95 percent confidence in the face of existing biological variability would require sampling rates that are impractical, even if the goals for laboratory error are set at zero (table 7). A ±10 percent error limit would require the analysis of three serial samples if bias is 2 percent and the measurements are made with a CVₐ of 2 percent. This level of performance is beyond the capability of present methodology. Allowing a bias of ±5 percent and a CVₐ of

<table>
<thead>
<tr>
<th>Intraindividual Biological Variability, Average CV%</th>
<th>Analytical Error</th>
<th>Observed CV%</th>
<th>N Replicate Samples for Error Limits²</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>None</td>
<td>7.5</td>
<td>10%  7.5%  5%  3%</td>
</tr>
<tr>
<td></td>
<td>2% bias, 2% CV</td>
<td>8.0</td>
<td>3   5   10   28</td>
</tr>
<tr>
<td></td>
<td>5% bias, 4% CV</td>
<td>9.9</td>
<td>4   7   15   42</td>
</tr>
<tr>
<td></td>
<td>10% bias, 6% CV</td>
<td>13.9</td>
<td>8   14  30   83</td>
</tr>
</tbody>
</table>

1 See appendix II for calculations.
2 With 95 percent confidence, the subject’s expected HDL-cholesterol value lies within the range defined by the mean of singlet analysis of n replicate samples sent to different laboratories above or below the stated percent error limit.
4 percent, four specimens would be necessary to satisfy an error limit of 10 percent with 95 percent confidence.

Total error limits for single analyses (table 8) were derived from three statistical models depending on whether bias is assumed to be variable (quadratic model) or fixed (linear model) and on the definition of 95 percent confidence (one or two tailed) with the linear models. Using these models, a bias of 5 percent and a CVₐ of 4 percent leads to a total error of up to 12.8 percent.

The clinically derived goal for HDL-cholesterol is compared to analytically derived goals in table 8. The goals for single point diagnostic HDL-cholesterol testing are set in terms of the total error since the components of bias and precision are not apparent to the clinical user of the result and both parameters affect the clinical classification of the patient. The clinical goal is based on a statistical model that relates clinical diagnostic accuracy to biological variability and to analytical variability (Ross 1988). It is not surprising that the clinical goal is larger than laboratory-derived analytical goals, since laboratory goals evolve from closely controlled analytical factors, whereas clinical goals depend primarily on the sizable inherent biological variability of analytes that affect diagnostic classification (Elevitch 1977; Hartman and Ross 1988; Ross and Lawson 1987; Ross 1988).

The CDC-NHLBI Lipid Standardization Program (Myers et al. 1989), however, allows a precision consistent with an SD of 2.5 mg/dL (0.065 mmol/L) to 3.5 mg/dL (0.078 mmol/L), the allowable SD increasing with HDL-cholesterol concentration (table 6). The precision allowance is 2.5 mg/dL (0.065 mmol/L) up to an HDL-cholesterol level of 40 mg/dL (1.03 mmol/L), 3 mg/dL (0.078 mmol/L) from 40 to 60 mg/dL (1.03-1.55 mmol/L), and 3.5 mg/dL (0.078 mmol/L) above 60 mg/dL (1.55 mmol/L). The precision goal was expressed in terms of SD rather than CV, a relative term, because experience has suggested that the SD for HDL-cholesterol measurement is nearly constant throughout the HDL-cholesterol concentration measurement range. Analytical goals are generally expressed in terms of CV, however, which facilitates communicating the targets and expressing analytical goals when using different units of measurement. In the case of the CDC criteria, a maximum CV of 6.3 percent is allowed at a level of 40 mg/dL (1.03 mmol/L), 6 percent at 50 mg/dL (1.29 mmol/L), and 5.8 percent at 60 mg/dL (1.55 mmol/L). Another unique characteristic of HDL-cholesterol compared to the other lipid/lipoprotein analytes is that one of the decision points, 35 mg/dL (0.90 mmol/L), is at the low rather than the high side of the population distribution. Thus, a precision goal expressed in relative terms allows a narrow tolerance at this decision point. These two characteristics of the HDL-cholesterol measurement necessitate particular care in specifying analytical performance goals for random error.

### TABLE 8. Analytical goals for single point total error for HDL-cholesterol measurement in relation to various statistical models

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Analytical Limits</th>
<th>Analytical Models</th>
<th>Clinical Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bias</td>
<td>CV</td>
<td>Quadratic</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>5%</td>
<td>4%</td>
<td>12.6%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>6%</td>
<td>22.9%</td>
</tr>
</tbody>
</table>

1See appendix II for calculations.
The CDC-NHLBI performance requirements for HDL-cholesterol were based on actual laboratory performance. Experienced lipid laboratories were generally able to achieve or exceed a level of performance consistent with the goals. Using a 10 percent bias and 6 percent CV, however, would require measurements on eight serial specimens among the laboratories to determine the HDL-cholesterol value within 10 percent error at a 95 percent confidence level (table 7) and would increase the analytically derived error goal to about 22 percent (table 8). Using a 5 percent bias and 4 percent CV would reduce the required number of specimens to four, or one more than the number of specimens required due to biological variability alone. The establishment of analytical goals for lipids and lipoprotein-cholesterol measurement and the associated calculations are given in more detail in appendix II.

In view of the physiological variability of HDL-cholesterol and the extreme accuracy and precision that would be necessary to establish the patient’s HDL-cholesterol within 10 percent at a 95 percent confidence level without using an unrealistically large number of serial samples, the following course of action is recommended. The goals are stated in terms of total error, which accounts for both bias and imprecision; bias and imprecision can vary somewhat so long as the criteria for maximum total error are satisfied.

• An interim analytical goal based on the current CDC-NHLBI standardization criteria is recommended. The interim analytical goal is that the total error of the measurement should be \( \leq 22 \) percent. This is consistent with an analytical bias of 10 percent and a precision of \( CV \leq 6 \) percent at a level of 42 mg/dL (1.09 mmol/L) or higher. An SD \( \leq 1.7 \) mg/dL (0.04 mmol/L) is recommended at levels below 42 mg/dL (1.09 mmol/L). The ultimate goals are recommended for adoption by 1998, provided methodological developments make these goals feasible.

It is recognized that there will need to be considerable improvement both in HDL-cholesterol methods themselves and in the reference, quality control, and survey materials used to judge the accuracy and precision of the measurements before the 1998 goals can be met. It is also recognized that these goals may have to be reevaluated depending on the rate of progress in these areas. Nonetheless, it is useful to establish such goals, since they serve to define the accuracy and precision necessary for reliable HDL-cholesterol measurements.

The specified criteria for accuracy and precision reflect the minimum acceptable levels of routine (i.e., average) laboratory performance that should be achievable within the limits of current methodology. Expressed in this way, the recommendations would lead to average total errors not exceeding 22 percent (interim) or 13 percent (by 1998) for HDL-cholesterol (see table II-1 of appendix II) for laboratories operating at the minimum acceptable levels for analytical bias and CV. Accordingly, these goals are recommended for routine total error of HDL-cholesterol measurements, and the goals for accuracy and precision listed above are considered to represent one set of conditions under which the primary goal for total error can be achieved.

The Working Group on Lipoprotein Measurement recognizes that the average total error limit could be exceeded in individual cases. However, to consider the interim criteria for bias and precision as 95 percent limits (see appendix II) would require an average laboratory bias \( \leq 3.7 \) percent and a CV \( \leq 3.3 \) percent in order to achieve a total error of less than 10 percent for the mean of several serial samples with 95 percent confidence using a feasible number (i.e., 4) of serial specimens from an individual. At present, this degree of accuracy and precision is beyond the capability of the currently used methods for HDL-cholesterol.
The primary measured values required for the diagnosis and treatment of hyperlipidemia are total cholesterol, triglycerides, and HDL-cholesterol. These measurements are used in current routine practice to calculate the LDL-cholesterol value. The methodology needed for reliable measurements of total cholesterol and triglyceride are currently available. There is an available CDC-NHLBI standardization program for HDL-cholesterol. This program has served as the basis for satisfactory laboratory performance in a number of clinical and epidemiological studies for almost 20 years. The criteria for standardization of HDL-cholesterol measurement are given in table 6. HDL-cholesterol measurement, however, presents a unique problem. Although HDL-cholesterol methods are sufficiently accurate and precise to satisfy the current CDC-NHLBI standardization criteria, performance at the limits of the standardization criteria for accuracy and imprecision would not satisfy the generally accepted criterion for the usefulness of a medical test. That is, the random error of the measurement \( CV_a \) should be no greater than half the average coefficient of biological variation \( CV_b \). Short of using an impractical number of serial specimens, this would lead to insufficiently accurate results in a proportion of patients. Thus it will be necessary to improve performance in measuring HDL-cholesterol, particularly in order to classify accurately those patients whose values are near the recommended cutpoints as well as to facilitate patient treatment and followup. As a first step, the adoption of the current CDC-NHLBI standardization criteria for routine measurements is expected to improve the reliability of these measurements.

These considerations, however, complicate the development of recommendations for HDL-cholesterol measurement, and the Working Group adopted a two-tiered set of performance goals, the first reflecting the current state of the art of HDL-cholesterol measurement methodology. The second is based on stricter criteria and should increase the proportion of acceptable clinical HDL-cholesterol measurements. The following recommendations are made with full appreciation of the need to develop better HDL-cholesterol methods over the next few years and acknowledges the challenge this presents and the uncertainty of when this will be accomplished.

**GENERAL RECOMMENDATIONS**

- **Maintaining Linkage With the Existing Epidemiological Database.** The existing epidemiological and clinical database relating HDL-cholesterol concentration to coronary risk is largely based on CDC-NHLBI standardized HDL-cholesterol measurements, which are in turn based on use of the heparin-MnCl\(_2\) method. It is recommended that this linkage be maintained regardless of which HDL-cholesterol method is used.

- **Reference Method.** It is recommended that the CDC reference method be used as the accuracy target for HDL-cholesterol measurements. Inasmuch as HDL is defined to some extent by the methods used to isolate it, continuation of the CDC method as a point of reference will maintain linkage between HDL-cholesterol measurements and the existing epidemiological and clinical database regardless of the method used. The CDC reference method is a three-step method:

  1. Ultracentrifugation at \( d 1.006 \) g/mL to remove triglyceride-rich lipoproteins.

  2. Precipitation of apo B-containing lipoproteins from the ultracentrifugal infranatant with heparin and MnCl\(_2\).
Recommendations on Lipoprotein Measurement—HDL


- **Routine Methods.** The basis for assessing the accuracy of routine methods should be the CDC reference method. Inasmuch as ultracentrifugation is not practical in most routine laboratories, routine methods can be two-step methods:
  1. Precipitation of apo B-containing lipoproteins from serum or EDTA plasma.

- **Criteria for Analytical Performance.** The following goals are recommended. These goals are stated in terms of total analytical error, which takes account of both accuracy and imprecision. This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater imprecision can be accepted if the measurements are accurate (see appendix II). The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV ≤3 percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent. The specification of guidelines for accuracy and precision separately can lead to an ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits are considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance.

**Interim Goals**

Total error, ≤22 percent

One set of conditions that is consistent with this total error limit is:

- **Serum and Plasma.** Either serum or plasma can be used for measurements. NCEP guidelines are based on serum values, and when classifying patients, serum or serum-equivalent values should be used. To convert EDTA plasma values to serum values, multiply the plasma values by 1.03.

**RECOMMENDATIONS FOR MANUFACTURERS**

- Manufacturers and others developing new methods, reagents, and instruments for HDL-cholesterol measurement should be aware of the medical community’s need to refer such measurements to the existing epidemiological database as it relates to risk for coronary heart disease.
  
  — New methods, reagents, and instruments should be validated against the reference method through split sample comparisons with the Cholesterol Reference Method Laboratory Network, using appropriate statistical methods for comparing measurement methods (Bland and Altman Precision: CV ≤6 percent at 42 mg/dL (1.09 mmol/L) or higher.
  An SD ≤2.5 mg/dL (0.065 mmol/L) is recommended at levels below 42 mg/dL (1.09 mmol/L).
  Accuracy: bias ≤ ±10 percent (compared to CDC reference method)

**Goals for 1998**

Total error, ≤13 percent

One set of conditions that is consistent with this total error limit is:

- **Serum and Plasma.** Either serum or plasma can be used for measurements. NCEP guidelines are based on serum values, and when classifying patients, serum or serum-equivalent values should be used. To convert EDTA plasma values to serum values, multiply the plasma values by 1.03.

- **Serum and Plasma.** Either serum or plasma can be used for measurements. NCEP guidelines are based on serum values, and when classifying patients, serum or serum-equivalent values should be used. To convert EDTA plasma values to serum values, multiply the plasma values by 1.03.

— The assigned HDL-cholesterol values for calibration and reference materials should be traceable to the reference method for HDL-cholesterol. The CDC laboratory, the Cholesterol Reference Method Laboratory Network, and other CDC standardized lipoprotein research laboratories can be of assistance for these purposes.

• Manufacturers should cooperate with CDC and the Cholesterol Reference Method Laboratory Network in developing reference materials for HDL-cholesterol measurement. Such materials should be commutable (i.e., closely emulate patient specimens).

RECOMMENDATIONS FOR HEALTH CARE PROVIDERS

• Minimize preanalytical sources of variation.

— Patients should maintain their usual diet and a reasonably stable weight for at least 2 weeks prior to blood collection.

— If measurements are made sooner than 12 weeks after the occurrence of myocardial infarction, values may be lower than typical for the patient. A preliminary measurement made within this period can give a sense of the patient’s HDL-cholesterol value, which if not below the ATP II recommended cutpoints (35 mg/dL [0.90 mmol/L] or 60 mg/dL [1.55 mmol/L]) can assist in initial management decisions. Measurements should not be made sooner than 8 weeks after acute trauma including major surgery, acute bacterial or viral infection or illness, or pregnancy.

• HDL-cholesterol measurement in the fasting and postprandial state.

— Blood samples should be obtained by venipuncture following a 12-hour period of fasting. If necessary, the patient can take water and prescribed medications during this period. This procedure should be followed for research purposes and in clinical circumstances in which error in the HDL-cholesterol must be minimized.

— If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not normally be less than 9 hours. It is likely that, on average, HDL-cholesterol will be underestimated slightly (about 1 to 4 percent) in patients who have fasted 9 hours. This should be taken into account when interpreting the values.

— ATP II states that HDL-cholesterol can be measured in the nonfasting state. It is likely that, on average, HDL-cholesterol will be underestimated somewhat (5 to 10 percent) in nonfasting patients. This should be taken into account when interpreting the values. The error introduced when measuring HDL-cholesterol in the nonfasting state, however, would be conservative in that it would lead to an overestimation of risk (false positive) in patients with fasting HDL-cholesterol levels at or somewhat above the cutpoints. This would likely be detected with followup measurements in fasting samples. The measurement of HDL-cholesterol in the nonfasting state would not be expected to interfere with the detection of patients with truly low HDL-cholesterol unless the plasma triglyceride level is so high that it interferes with the measurement itself. Based on the HDL-cholesterol distributions in the Lipid Research Clinics Population studies (Lipid Research Clinic Program 1980), a 10 percent negative error in HDL-cholesterol would tend to misclassify about 15 percent of the population with respect to the two ATP II cutpoints: the 10 percent with HDL-cholesterol levels of 35-40 mg/dL (0.90-1.04 mmol/L) and about 5 percent with HDL-cholesterol levels of 60-65 mg/dL (1.55-1.69 mmol/L). Thus, the use of nonfasting HDL-cholesterol measurements would not interfere with the classification of about 85 percent of the population.

— To the extent possible, blood should be drawn in the sitting position and the patient should sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should
be sampled in the same position on each occasion.

- Prolonged venous occlusion should be avoided. The tourniquet should be released within 1 minute of application. If difficulties are encountered, use the other arm, or release the tourniquet for a few minutes before attempting a second venipuncture.

- Use of serum or plasma
  - HDL-cholesterol can be measured in either serum or EDTA plasma.

- Processing
  - Serum or plasma should be removed from cells within 3 hours of venipuncture.

- Need for serial measurements in individuals
  - Considering the physiological variability of HDL-cholesterol, HDL-cholesterol measured on a single occasion is not sufficient to determine the patient’s “usual” HDL-cholesterol concentration. Although it would be ideal to establish a patient’s HDL-cholesterol with a 10 percent limit for total error with 95 percent confidence, at present it is not technically or economically feasible because of the excessive number of serial samples that would be required. For this reason, the following recommendations are made to improve reliability of HDL-cholesterol measurements. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart.

  - Three serial samples: Using three serial samples, each referred to the same laboratory and analyzed once, and assuming a CV_b of 7.5 percent in an interim CV_a of 6 percent, the observed CV for the mean HDL-cholesterol value is 5.5 percent, and the difference between the means of sequential series of three samples should not exceed 15.4 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 27 percent. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s HDL-cholesterol level and its proximity to the concentrations used for decision making (35 mg/dL [0.90 mmol/L] or 60 mg/dL [1.55 mmol/L]). Using the 1998 CV_a goal of 4 percent, the observed CV for the mean HDL-cholesterol value for three serial samples is 4.9 percent, and the difference between the means of sequential series should not exceed 13.6 percent, 95 percent of the time. The difference between sequential individual values in each series should not exceed 24 percent. If they are further apart, analytical error or a change in the physiological state of the patient should be suspected and another sample may be warranted.

  - Two serial samples: For reasons of convenience and considering economic factors, the ATP II report recommended the use of at least two serial samples. Using two serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 7.5 percent and an interim CV_a of 6 percent, the observed CV for the mean HDL-cholesterol value is 6.8 percent. The difference between the means of each series should not exceed 18.8 percent, and the difference between sequential individual values within each series should not exceed 27 percent, 95 percent of the time. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s HDL-cholesterol level and its proximity to the concentrations used for decision making (35 mg/dL [0.90 mmol/L] and 60 mg/dL [1.55 mmol/L]). Using the 1998 CV_a goal of 4 percent, the observed CV for the mean of two serial samples is 6.0 percent and the difference between the means for each series should not exceed 16.7 percent, 95 percent of the time. The difference between sequential individual values in

* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
each series should not exceed 24 percent. If they are farther apart, another sample may be warranted.

Based on prevailing HDL-cholesterol levels,* under the interim goals, using two serial measurements and considering a cutpoint of 35 mg/dL (0.90 mmol/L), a patient’s HDL-cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is greater than 40.7 mg/dL (1.05 mmol/L) or less than 29.3 mg/dL (0.76 mmol/L), respectively. Using a 60 mg/dL (1.55 mmol/L) cutpoint, the patient’s HDL-cholesterol value can be confidently assumed to above or below the cutpoint when the mean value is greater than 69.9 mg/dL (1.81 mmol/L) or less than 50.1 mg/dL (1.29 mmol/L), respectively. Based on prevailing HDL-cholesterol levels in the U.S. population (Johnson et al. 1993), two serial specimens are sufficient to categorize 83 percent of the general population as above or below the 35 mg/dL cutpoint and 58 percent of the population as above or below the 60 mg/dL cutpoint.

Under the 1998 goals, using two serial measurements and considering a cutpoint of 35 mg/dL, a patient’s HDL-cholesterol can be confidently assumed to be above or below the cutpoint when the mean value is >39.0 mg/dL or <31.0 mg/dL, respectively. Using a 60 mg/dL cutpoint, the patient’s HDL-cholesterol value can be confidently assumed to be above or below the cutpoint when the mean value is >66.8 mg/dL or <53.2 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 88 percent of the general population as above or below the 35 mg/dL cutpoint and 70 percent of the population as above or below the 60 mg/dL cutpoint.

• Separations

— The HDL-containing fraction is best prepared on the day of sample collection. If analyses must be delayed for 1 or 2 days, the serum or plasma can be stored at 4 °C. If the analyses are delayed beyond 3 days, the specimens should be transferred to storage vials that have leak- and evaporation-proof seals, and frozen. For periods up to 1 month, samples can be stored at -20 °C in a non-self-defrosting freezer. For storage periods of 1 month to 2 years, samples should be stored at -70 °C. The storage containers should not be sealed with cork stoppers or plastic film since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

• All blood specimens must be considered potentially infectious and handled according to accepted laboratory safety guidelines.

RECOMMENDATIONS FOR LABORATORIES

• Laboratories should use methods that are optimized using the CDC reference method for HDL-cholesterol as the point of reference for accuracy. Since matrix effects are significant, accuracy should be verified through method comparison studies using fresh specimens. NCCLS Protocol EP9-P is recommended for the conduct of such studies.

• The specificity of the precipitation step should be verified additionally by appropriate immunoochemical or electrophoretic analysis of HDL supernates.

• Since incomplete sedimentation of lipemic specimens is a common source of error with the precipitation methods, measures should be incorporated to screen for supernatant turbidity and eliminate the unsedimented apo B-containing lipoproteins.

• Quality control materials should be selected that reasonably emulate performance on patient specimens. At least two levels are recommended, one near each the decision levels of 35 mg/dL (0.90 mmol/L) and 60 mg/dL (1.55 mmol/L).

• Laboratories should participate in an external proficiency testing program.

• All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel. Personnel handling blood samples should use gloves and should avoid leaving samples open
to the air longer than necessary. Samples
should be handled in accordance with CDC
guidelines for the prevention of infection in
health care workers.

RECOMMENDATIONS FOR ANALYSES
IN NONLABORATORY SETTINGS
In addition to the recommendations above, the
following recommendations apply to HDL-
cholesterol testing in nonlaboratory settings.

• Desktop analyzers and other analytical systems
should be designed and validated to achieve the
requisite performance by operators without
formal laboratory training. These systems
should operate reliably under conditions that
prevail in field screening, physicians’ offices, or
other settings outside the conventional
laboratory.

• The performance criteria established for
laboratory-based measurements should apply to
measurements made outside the conventional
laboratory setting.

• Operators of desktop analyzers or similar
nonlaboratory-based systems should receive
training in phlebotomy techniques, safety
procedures, and quality control. Such operators
should work under the supervision of health
care professionals with appropriate education,
training, and experience in laboratory
measurements.

RECOMMENDATIONS FOR
GOVERNMENT AGENCIES AND OTHER
PROFESSIONAL GROUPS
The Centers for Disease Control and Prevention
should:

• In cooperation with appropriate experts in the
academic, clinical laboratory, and
manufacturing sectors, develop reference
materials for HDL-cholesterol measurement in
which matrix effects are minimized. Such
materials should be suitable for standardization,
surveillance, method calibration as appropriate,
and bench-level quality control.

The National Heart, Lung, and Blood Institute
should take the following steps:

• Continue the policy of NHLBI to require
standardized lipid and lipoprotein measure-
ments for Government-supported clinical and
epidemiological studies.

• Encourage the Cholesterol Reference Method
Laboratory Network to expand its activities to
include the certification of HDL-cholesterol,
LDL-cholesterol, and triglyceride measure-
ments.

• Encourage the development and preliminary
evaluation of new HDL-cholesterol methods and
associated reagents and instrumentation.

The Cholesterol Reference Method Laboratory
Network currently provides assistance to manufac-
turers and laboratories wishing to establish trace-
ability of total cholesterol measurements to the
cholesterol reference method. The Network
should:

• Expand activities to include HDL-cholesterol,
LDL-cholesterol, and triglyceride measurements.

The College of American Pathologists and other
professional organizations that operate clinical
chemistry survey programs should take the follow-
ing steps:

• Include HDL-cholesterol measurements in such
surveys.

• Provide CDC-confirmed values for HDL-
cholesterol concentrations in survey pools.

RECOMMENDATIONS FOR FURTHER
RESEARCH

• New methods for HDL-cholesterol measurement
are needed. Such methods should be capable of
providing HDL-cholesterol measurements that
meet or exceed the 1998 goals specified above.
This will be important both for the measurement
of HDL-cholesterol, per se, and for the calcu-
ation of LDL-cholesterol from measured values
of total cholesterol, triglyceride, and HDL-
cholesterol.

• Suitable reference materials are urgently needed
for HDL-cholesterol measurements. Such
materials should be free of matrix effects and
should be sufficiently stable to allow the long-
term monitoring of the accuracy and precision of
HDL-cholesterol measurements and for use in
proficiency surveys to allow the accurate assess-
ment of clinical HDL-cholesterol measurements.


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Recommendations on Lipoprotein Measurement—HDL


Part Three:
Recommendations for Triglyceride Measurement

National Cholesterol Education Program
Working Group on Lipoprotein Measurement
# Table of Contents

EXECUTIVE SUMMARY .................................................................................................................. 129
   Considerations .......................................................................................................................... 129
   Biological and Preanalytical Variation .................................................................................. 130
   Triglyceride Measurement ....................................................................................................... 131
      Reference Method .................................................................................................................. 131
      Routine Method ...................................................................................................................... 131
   Free Glycerol (Triglyceride Blank) Measurements ................................................................. 132
   Recommendations .................................................................................................................. 132
      General Recommendations .................................................................................................... 132
      Recommendations for Manufacturers .................................................................................. 133
      Recommendations for Health Care Providers ..................................................................... 133
      Recommendations for Laboratories ..................................................................................... 135
      Recommendations for Government Agencies and Other Professional Groups ................. 135
      Recommendations for Further Research ............................................................................. 136

1. BACKGROUND ....................................................................................................................... 137
   Triglyceride Transport ............................................................................................................. 137
   Clinical Importance ............................................................................................................... 138

2. METHODS FOR TRIGLYCERIDE MEASUREMENT ............................................................. 143
   Reference Method .................................................................................................................. 143
   Routine Methods .................................................................................................................... 143

3. MAJOR ISSUES CONCERNING TRIGLYCERIDE MEASUREMENT .................................... 147
   Biological Variation ................................................................................................................. 147
   Preanalytical Factors ............................................................................................................... 148
      Fasting Samples ................................................................................................................... 148
      Posture ................................................................................................................................ 149
      Venous/Capillary Differences and Venous Occlusion ........................................................... 150
      Serum-Plasma Differences ................................................................................................... 150
      Frozen Specimens ............................................................................................................... 150
      Sample Shipment ................................................................................................................ 150
Executive Summary

Triglyceride is found in all plasma lipoproteins but is the major lipid component of those lipoproteins with a density less than 1.019 kg/L. These triglyceride-rich lipoproteins encompass a spectrum of lipoproteins in terms of size, density, and lipid and apolipoprotein composition and include chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), and intermediate density lipoprotein (IDL). Because the catabolic processes involved in VLDL and IDL metabolism are similar to those for chylomicrons, defects in their catabolism result in prolongation of residence time and, therefore, increased concentrations in the circulation. It is necessary in the diagnosis and treatment of hyperlipidemia to assess the plasma concentrations of triglycerides, and it is important to establish recommendations for reliable triglyceride measurement. For the past several years, the National Cholesterol Education Program (NCEP) Working Group on Lipoprotein Measurement has been developing recommendations for triglyceride and low density lipoprotein (LDL)- and high density lipoprotein (HDL)-cholesterol measurement. The working group’s recommendations for triglyceride measurement are summarized in this paper.

CONSIDERATIONS

In the fasting state, chylomicrons are absent in subjects with normal states of triglyceride-rich lipoprotein synthesis and catabolism. In view of the relative paucity of triglyceride in LDL and HDL (<5 percent by weight), most triglyceride is associated with VLDL. When the VLDL concentration is very low, however, a large percentage of the plasma triglyceride may be found in LDL and HDL. In the nonfasting state, plasma triglyceride concentrations vary considerably with levels rising fairly rapidly, reaching peak concentrations about 4 hours after ingestion of a fat-containing meal. They remain above fasting concentrations for about 8 hours or more as chylomicrons are removed from the circulation.

Elevated triglyceride concentrations in the fasting state are of clinical importance in a number of conditions. With severe elevated triglyceride concentrations (>1,000 mg/dL [11-30 mmol/L]), there is a significant association with the development of abdominal pain and pancreatitis. This can occur in subjects with a marked increase in VLDL but is more often encountered in patients with impaired chylomicrons (type I) or both chylomicron and VLDL (type V) catabolism.

Combined elevations of chylomicrons and VLDL are more commonly found secondary to poorly controlled type I or type II diabetes mellitus or excessive alcohol intake, especially if associated with underlying familial hyperlipidemia such as familial combined hyperlipidemia or familial hypertriglyceridemia. In these subjects, clinical manifestations such as eruptive xanthoma, corneal arcus, xanthelasma, and lipemia retinalis make the measurement of plasma triglyceride levels necessary and important.

The relationship of plasma triglycerides, or triglyceride-rich lipoproteins, and atherosclerotic disease is still unclear. For this reason, neither the NCEP’s 1988 Report of the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel or ATP) nor the second report (ATP II) from the same group (1994) identified hypertriglyceridemia as a target for intervention.

One of the most confounding variables in assessing the role of hypertriglyceridemia in atherosclerosis is its close and inverse relationship with HDL. In
most studies, HDL-cholesterol is reduced when triglycerides are elevated and increases when the triglyceride elevation is treated, whether by diet or drug. There is also a growing body of evidence that the catabolic products of chylomicrons, chylomicron remnants, and VLDL (VLDL rem-nants and IDL) may be atherogenic. The lack of adequate techniques for assessing chylomicron remnants or IDL has prevented their evaluation in large-scale, epidemiological, cross-sectional, or interventional studies.

A working definition of hypertriglyceridemia has been adopted by the National Institutes of Health Consensus Conference on Hypertriglyceridemia (table ES-1). Diet and drug therapy is recom-mended for patients with fasting triglycerides exceeding 500 mg/dL, as there is substantial fluctuation in triglycerides in these individuals, which may expose them to pancreatitis. The decision to treat patients with triglyceride con-centrations below 500 mg/dL depends on the presence of other lipid abnormalities such as familial combined hyperlipidemia or dysbetalipoproteinemias. ATP II modified the definitions of hypertriglyceridemia somewhat (table ES-2) and recommended nonpharmacologic therapy in patients with increased triglycerides. In addition, the panel indicated that drug therapy may also be required when increased triglycerides are accompa-nied by forms of hyperlipidemia known to be atherogenic, such as familial combined hyperlipidemia, and that drug therapy is generally required in patients with triglycerides high enough to put them at risk for developing pancreatitis. Triglyceride measurement is also very important in that it provides a simple and inexpensive way to estimate the VLDL-cholesterol content, a factor used in the calculation of LDL-cholesterol.

**BIOLOGICAL AND PREANALYTICAL VARIATION**

When a single measurement for triglyceride is made, it is affected by a number of sources of biological variation present at the moment the sample is drawn. The total variation (CV), as measured by the analytical process, is a combina-tion of the intraindividual biological variation (CV_b) and the analytical variation (CV_a). When interpreting a single result or a series of results from a single person, the total intraindividual variation (CV_T) is of primary interest.

With an analytical coefficient of variation (CV_a) of about 3 percent, within-month data* reveal that the biological variance for triglycerides approxi-mates more than 90 percent of the total intraindi-vidual variance. Even in the fasting state, consider-able biological variation occurs within individuals. In subjects maintaining a carefully monitored NCEP Step I or better diet, and in whom triglycer-ides were measured twice over a 2-week period, the percent differences between triglyceride concentra-tions of the two specimens was approximately five times greater than that for cholesterol; over 75 percent of subjects showed deviations greater than 10 percent during a 2-week period. The Lipid Research Clinics found the total intraindividual variation for paired fasting analyses performed an average of 2.5 months apart on samples from 7,055 fasting persons was about 25 percent for triglyceride. Unlike total or HDL-cholesterol, no detect-able seasonal differences in triglyceride concentra-tions have been seen. In the nonfasting state, the

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**TABLE ES-1. NIH Consensus Conference “working” classification for fasting triglycerides**

<table>
<thead>
<tr>
<th>Category</th>
<th>Fasting Plasma Triglyceride Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;250 mg/dL (&lt;2.8 mmol/L)</td>
</tr>
<tr>
<td>Borderline hypertriglyceridemia</td>
<td>250-500 mg/dL (2.8-5.6 mmol/L)</td>
</tr>
<tr>
<td>Definite hypertriglyceridemia</td>
<td>&gt;500 mg/dL (&gt;5.6 mmol/L)</td>
</tr>
</tbody>
</table>

1 Endorsed by Consensus Development Conference, NIH, 1992 (NIH Consensus Conference 1993)

Source: NIH Consensus Conference 1984

* Unpublished data supplied by one of the authors (EAS).
total CV (CVₜ) of triglyceride is considerable among individuals, whether for a diurnal (6.3 to 65 percent), within-month (12.9 to 34.8 percent), or within-year (12.9 to 39.9 percent) period. Although the fluctuations described above reflect healthy individuals on stable diets, far greater fluctuations are seen in certain physiological and disease states.

Triglyceride measurements are also influenced by other, more definable preanalytical sources of variation that operate before or during blood sampling, storage, and shipment of samples to the laboratory. Sources of variation associated with sample collection and handling include fasting status, posture during collection, venous-capillary differences, venous occlusion, the use of anticoagulants, and the conditions of storage and shipment. Many of these factors can be controlled to some extent.

TRIGLYCERIDE MEASUREMENT

Reference Method

The reference system to standardize triglyceride measurement and evaluate laboratory performance is not as well developed as the one for total cholesterol. Since 1966, an in-house triglyceride method established at the Centers for Disease Control and Prevention (CDC) has been used as a point of reference to standardize triglyceride measurements in laboratories involved in epidemiologic and clinical studies. The CDC triglyceride method is based on the chemical method of Carlson and the techniques of Van Handel and Zilversmit and Lofland. Serum lipids are extracted with chloroform, then treated with silicic acid to remove phospholipids and free glycerol. An aliquot of the extract is saponified to release glycerol, and the glycerol is oxidized with sodium periodate to produce formaldehyde. Formaldehyde is reacted with chromotropic acid to produce a chromogen whose absorbance is measured at 570 nm. The CDC reference method has been modified to replace chloroform with methylene chloride in order to eliminate the need for filtration to remove the silicic acid particles. This semiautomated procedure owes much of its specificity to the extraction procedure that removes phospholipids and free glycerol and retains minimally some monoglycerides and diglycerides. The coefficients of variation attainable with this method range from 2 to 6 percent. The CDC reference method is much too cumbersome for use in the routine clinical setting.

Routine Method

Since the mid-1970’s, enzymatic methods have virtually replaced the earlier chemical methods in all routine clinical laboratories. In the current enzymatic methods, triglycerides are first hydrolyzed using a bacterial lipase (triacylglycerol acylylhydrolase). A surfactant or detergent is often used to facilitate hydrolysis. The glycerol released is quantified by one of a variety of coupled enzyme systems. The initial step in each of these methods is conversion of glycerol to glycerol 3-phosphate plus adenosine 5-diphosphate (ADP).

In one approach, the ADP that is produced is used to generate pyruvate, which is then converted to lactate. The disappearance of reduced nicotinamide adenine dinucleotide (NADH) is measured spectrophotometrically at 340 nm. A different reaction is used to allow measurement in the visible range of the spectrum. The NADH reacts with iodonitro-tetrazolium violet (INT) to produce
a colored product, INT-formazan, which is then measured spectrophotometrically at 505 nm. Other methods use the glycerol 3-phosphate and react it with NAD+ using glycerol phosphate dehydrogenase. The appearance of NADH is then measured at 340 nm.

In another variation, glycerol 3-phosphate is oxidized using glycerol phosphate oxidase to generate hydrogen peroxide. The peroxide is then reacted with 3,5-dichloro-2-hydroxybenzene sulfonic acid 4-aminophenazone in the presence of horseradish peroxidase as the chromogenic system. Ferrocyanide minimizes interference from elevated bilirubin. The red chromophore is measured at 510 nm. This procedure offers greater specificity because it avoids the oxidative/reductive side reactions of NAD, the extreme sensitivity to pH, and unfavorable equilibria that can occur with the other enzymatic systems.

**FREE GLYCEROL (TRIGLYCERIDE BLANK) MEASUREMENTS**

Free glycerol in the plasma/serum, resulting from endogenous and/or exogenous glycerol, may cause an overestimation of triglyceride in patient specimens when assayed by enzymatic methods, since they measure triglyceride as the quantity of glycerol in the specimen. Most of the older chemical methods employed an extraction step that removed or partially removed free glycerol. This free glycerol is termed the triglyceride blank. Most routine clinical laboratories do not correct for free glycerol. Increased concentrations of interfering endogenous glycerol in plasma may arise from a variety of sources: recent exercise, liver disease, diabetes mellitus, hemodialysis, stress, use of glycerol-containing intravenous medications, and parenteral nutrition.

Increased triglyceride blanks can also result from external sources, such as the use of blood-collection tubes with glycerol-coated stoppers, contamination of quality control materials from filters used for sterilization, contamination caused by hand lotion used by laboratory personnel, and from laboratory detergents. The tubes used for collecting specimens can cause an overestimation of plasma triglyceride if they are manufactured using glycerol as the lubricant for stopper insertion.

Quantitation of free glycerol in order to obtain true triglyceride concentrations is of limited importance in most patient situations, since various studies indicate that the potential “error” due to free glycerol is less than 9 mg/dL in 99 percent of subjects.

Glycerol blanking does take on importance, however, when attempting to standardize triglyceride measurements or to establish traceability to a reference method. The accuracy standard is based on blanked triglyceride measurements because in many cases the contribution of free glycerol to unblanked triglyceride value would exceed the recommended error goal. The amount of free glycerol in processed materials used as survey samples, reference materials, and calibrators can vary considerably and is usually greater than in patient specimens. This situation makes standardizing triglyceride measurements and comparing results across the Nation’s clinical laboratories very difficult.

The most common approach to correct for the glycerol in the enzymatic assays involves omitting the lipase reagent. The contribution of free glycerol is subtracted from the value obtained in the presence of lipase. However, at present this approach may require a separate analysis for free glycerol. Preliminary studies at CDC indicate that it is important to use free glycerol reagent and triglyceride reagent from the same manufacturer in order to get consistent “net” triglyceride results.

In another approach, the free glycerol is eliminated by using a preincubation step, in which the sample is pretreated with glycerol kinase, glycerol phosphate oxidase, and peroxidase followed by addition of lipase and chromogen. From data collected in the College of American Pathologists’ (CAP) Chemistry Survey, only about 6 percent of all participants correct for free glycerol.

**RECOMMENDATIONS**

**General Recommendations**

- **Reference Method.** The current basis for accuracy of triglyceride measurement should be the CDC reference method.

- **Criteria for Analytical Performance.** The goals for triglyceride measurement are stated in terms of total analytical error, which takes
account of both accuracy and imprecision (reproduced here in table ES-2). This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater imprecision can be accepted if the measurements are more nearly accurate. The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV ≤ 3 percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent. The specification of guidelines for accuracy and precision separately can lead to an ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits are considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance. Triglyceride measurements should be within the following minimum limits of performance.

\[ \text{Total Error} \leq 15\% \]

This is consistent with the following limits for accuracy and precision:

\[ \text{Accuracy} \leq 5\% \]
\[ \text{CV} \leq 5\% \]

Because of the large biological variation of triglycerides, the recommendations are more stringent than would be necessary for the measurement of triglycerides, per se. This is necessary because triglyceride measurements are also required for the estimation of LDL-cholesterol. These criteria should apply regardless of how, where, or by whom the measurements are made.

Laboratories and others making triglyceride measurements can assess their individual conformance to the analytic goals as indicated in appendix II.

• Triglycerides can be measured in either serum or plasma. Serum or serum-equivalent values should be reported. To convert measurements made in EDTA plasma to serum-equivalent values, multiply the plasma value by 1.03. Triglyceride measurements made in heparin plasma are equivalent to serum values.

Recommendations for Manufacturers

• The assigned values for the triglyceride concentrations of calibration and quality control materials should be traceable to the reference method for triglycerides, and the bias with respect to reference values should be stated.

• Instrument and reagent suppliers should provide systems in which glycerol blanking can be easily and economically incorporated into all triglyceride assays.

• Manufacturers should help develop and provide reference materials that are free of matrix effects.

Recommendations for Health Care Providers

• Triglycerides should be measured when the patient is in a metabolic steady state.

• Triglycerides should be measured in fasting samples.

— Blood samples should be obtained by venipuncture following a 9- to 12-hour period of fasting. If necessary the patient can take water and prescribed medications during this period. This procedure should be followed for research purposes and in other circumstances in which the error in the fasting triglyceride must be minimized.

— If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not be less than 9 hours. It is likely that, on average, fasting triglyceride will be overestimated (about 20 mg/dL [0.23 mmol/L]) in patients who have fasted 9 hours. This would contribute to a 4 mg/dL (0.10 mmol/L) underestimate of LDL-cholesterol calculated from the Friedewald equation but would be partially compensated by an accompanying 1 to 4 percent underestimate of HDL-cholesterol.
under these conditions. This should be taken into account when interpreting the values.

— To the extent possible, blood should be drawn in the sitting position and the patient should be allowed to sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should be sampled in the same position on each occasion.

— Prolonged venous occlusion should be avoided. The tourniquet should be removed within 1 minute of application.

• Stored samples

— Serum or plasma should be removed from cells within 3 hours of venipuncture.

— Specimens can be stored for up to 3 days at 4 °C. If the analysis is delayed, the specimens can be stored for up to several weeks at -20 °C in a non-self-defrosting freezer. Specimens should be stored at -70 °C or lower if longer periods of storage are necessary.

— In all cases, the samples should be stored in clean containers that can be sealed to prevent evaporation. Do not use cork stoppers or plastic film to seal the containers since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

• Need for serial measurements in individuals.

Considering the physiological variability of triglycerides, triglyceride measured on a single occasion is not sufficient to determine the patients’ usual triglyceride concentration. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart. The individual values should be averaged.

Three serial samples: Using three serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 23.7 percent and a CV_a of 5 percent, the observed CV for the mean triglyceride value is 14.0 percent, and the difference between the means of sequential series of three samples should not exceed 39 percent, 95 percent of the time. The difference between the sequential individual values in each series should not exceed 67 percent. If they are further apart, analytical error or a change in the physiological status of the patient should be suspected and another sample may be warranted depending on the patient’s triglyceride concentration and its proximity to the concentrations used for decision making.

Two serial samples: For reasons of convenience and considering economic factors, the ATP II report recommended the use of at least two serial samples. Using two serial samples, each referred to the same laboratory and analyzed once, and assuming a CV_b of 23.7 percent and a CV_a of 5 percent, the observed CV for the mean triglyceride value is 17.1 percent, and the difference between the means of each sequential series should not exceed 48 percent, 95 percent of the time. The difference between the sequential individual values within each series should not exceed 67 percent. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient’s triglyceride concentration and its proximity to the concentrations used for decision making (200 mg/dL [2.26 mmol/L] and 400 mg/dL [4.52 mmol/L]).

Based on prevailing triglyceride concentrations,* using two serial measurements and considering a cutpoint of 200 mg/dL, a patient’s triglyceride can be confidently assumed to be above or below the cutpoint when the mean value is >257 mg/dL or <143 mg/dL, respectively. Using a 400 mg/dL cutpoint, the patient’s triglyceride value can be confidently assumed to be above or below the cutpoint when the mean value is >514 mg/dL or <286 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 74 percent of the general population as being above or below the 200 mg/dL cutpoint and 96 percent as being above or below the 400 mg/dL cutpoint.

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* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
Recommendations for Laboratories

- Laboratories should use procedures that allow the measurement of triglycerides with a total error ≤15 percent. One set of conditions that satisfies this recommendation is that triglycerides be measured with an accuracy of ±5 percent and a CV of ≤5 percent.

- Given the marked intraindividual fluctuation in plasma or serum triglyceride concentrations and the controversy regarding the exact clinical significance of mild increases in plasma triglycerides, stringent accuracy and precision goals are not as crucial for triglyceride measurements when the goal is to establish the patient’s mean triglyceride concentration per se. The recommendations are primarily influenced by the current use of triglyceride measurements in the estimation of LDL-cholesterol.

- All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel. Personnel handling blood samples should use gloves and should avoid leaving samples open to the atmosphere longer than necessary. Samples should be handled in accordance with current CDC guidelines for the prevention of infection in health care workers.

- Glycerol Blanking. Several professional organizations are currently reviewing the problems associated with the measurement of triglyceride in routine laboratories. Specifically, the Ad Hoc Triglyceride Review Committee of the Lipids and Lipoproteins Division, American Association for Clinical Chemistry (AACC) has made interim recommendations regarding glycerol blanking. The NCEP Working Group on Lipoprotein Measurement endorses the following recommendations, adapted from the AACC Lipids and Lipoproteins Division:
  - All laboratories should offer a glycerol-blanked triglyceride analysis, even though it may be performed only when requested. Any specimen with triglyceride concentration >200 mg/dL (2.26 mmol/L) should be glycerol blanked using a “reflex” ordering system.
  - Reports from the laboratory should clearly state whether the triglyceride analysis was glycerol blanked (e.g., designated as “Blanked Triglyceride” or “Unblanked Triglyceride”). Physicians need to be educated as to how the inclusion of a glycerol blank may alter the meaning of the results.
  - Glycerol blanking of triglyceride measurements should be mandatory in laboratories that specialize in assessment of lipid status, have large populations of hyperlipidemic subjects, or participate in clinical or basic research.
  - Glycerol blanking of triglyceride analyses need not be routinely conducted on outpatients’ samples, unless economically feasible. However, because of the potential for higher glycerol concentrations in hospital inpatient specimens, all inpatient specimens should be routinely glycerol blanked.

Recommendations for Government Agencies and Other Professional Groups

The Centers for Disease Control and Prevention should take the following steps:

- The CDC should validate and publish the reference method for triglycerides.
- The CDC should collaborate with the National Institute of Standards and Technology (NIST) on the development of a definitive method for triglycerides.
- In cooperation with appropriate experts in the academic, clinical laboratory, and manufacturing sections, the CDC should develop reference materials for triglycerides that manifest the minimum matrix effects and have low blanks.

The Cholesterol Reference Method Laboratory Network currently provides assistance to manufacturers and laboratories wishing to establish the traceability of total cholesterol measurements to the cholesterol reference method. The network should:
• Expand these activities to include triglyceride, HDL-cholesterol, and LDL-cholesterol measurements.

The National Heart, Lung, and Blood Institute should take the following steps:

• Continue the present policy of requiring standardized triglyceride measurements for Government-supported clinical and epidemiological studies.

• Encourage the Cholesterol Reference Method Laboratory Network system to expand its activities to include the certification of triglyceride, HDL-cholesterol, and LDL-cholesterol measurements.

Recommendations for Further Research

Further research is needed in several areas:

• A definitive method for triglycerides is needed. The relationship of the CDC reference method to such a definitive method needs to be established.

• Reference materials should be developed that are free of matrix effects and have blank values that are similar to those in fresh specimens. Such materials should be sufficiently stable to allow long-term monitoring of the accuracy and precision of triglyceride measurements.

• Triglyceride methods are needed that incorporate a correction for the triglyceride blank. Such methods should be easy to use, economical, and preferably not require that the blanked triglyceride be calculated from two primary measurements.
Section 1. Background

TRIGLYCERIDE TRANSPORT

Triglyceride is an ester consisting of a glycerol molecule coupled to three fatty acid residues (R) of varying carbon chain lengths and degrees of saturation:

\[
\begin{align*}
&\text{O} \\
&\text{R}_1 \quad \text{C} \quad \text{O} \quad \text{C} \quad \text{O} \quad \text{R}_2 \\
&\text{O} \\
&\text{R}_3 \quad \text{C} \quad \text{O} \quad \text{C} \quad \text{O} \quad \text{R}_4 \\
\end{align*}
\]

The two terminal carbons (Cambien et al. 1986; Stein 1987) in glycerol are chemically equivalent. Triglycerides are the most prevalent form of fat in the human diet. Triglycerides of plant origin, other than those from equatorial regions, contain large amounts of C18:2 residues, are liquid at room temperature, and are termed “polyunsaturates.” Animal triglycerides are generally saturated, are solid at room temperature, and contain C12:0 through C18:0 fatty acids (Stein 1987).

During digestion, triglycerides are hydrolyzed to form glycerol, monoglycerides, and unesterified fatty acids that are subsequently absorbed into the intestinal epithelium and resynthesized into triglyceride.

Triglyceride is found in all plasma lipoproteins but is the major lipid component of those lipoproteins with a density less than 1.019 kg/L. These triglyceride-rich lipoproteins encompass a spectrum of lipoproteins in terms of size, density, lipid, and apolipoprotein composition (table 1) and include chylomicrons, chylomicron remnants, very low density lipoprotein (VLDL), and intermediate density lipoprotein (IDL). The chylomicrons are synthesized by the intestine and transport dietary triglyceride and cholesterol from their site of absorption in the intestinal tract to peripheral tissues to be used as an energy supply, to adipose tissue for storage, and to the liver for further utilization and/or storage.

Chylomicrons are cleared within 6 to 8 hours of a meal and, in the fasting state, are absent from the plasma unless a metabolic defect in their clearance occurs. VLDL transports triglycerides of hepatic origin, and its metabolic route is similar to that of chylomicrons. Because the catabolic processes involved in VLDL and IDL metabolism are similar to those for chylomicrons, defects in their catabolism result in prolongation of residence time and, therefore, increased levels in the circulation.

In the fasting state, chylomicrons are absent in subjects with normal states of triglyceride-rich lipoprotein synthesis and catabolism. In view of the relative paucity of triglyceride in low density lipoprotein (LDL) and high density lipoprotein (HDL) (<5 percent by weight), most triglyceride is associated with VLDL (table 1). When the VLDL concentration is very low, however, a large percentage of the plasma triglyceride may be found in LDL and HDL. In the nonfasting state, plasma triglyceride levels vary considerably with levels rising fairly rapidly, reaching peak levels about 4 hours after ingestion of a fat-containing meal. They remain above fasting levels for about 8 hours as chylomicrons are removed from the circulation.

Triglyceride-rich lipoproteins are also distinct from LDL and HDL in that, when present in sufficiently high concentrations, their large size creates turbidity or opalescence in the plasma, and visual inspection of plasma is often useful to detect
TABLE 1. Plasma lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein Class</th>
<th>Density (kg/L)</th>
<th>Electrophoretic Mobility</th>
<th>Chemical Composition&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>FC</td>
<td>TG</td>
</tr>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.95</td>
<td>Origin</td>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95-1.006</td>
<td>Prebeta</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006-1.019</td>
<td>Beta-prebeta</td>
<td>Intermediate between VLDL and LDL</td>
<td>C's, B, E</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019-1.063</td>
<td>Beta</td>
<td>55</td>
<td>5</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.121</td>
<td>Alpha</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.063-1.121</td>
<td>Alpha</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>HDL&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.12-1.21</td>
<td>Alpha</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>1.045-1.080</td>
<td>Prebeta</td>
<td>46</td>
<td>5</td>
</tr>
</tbody>
</table>

1 By agarose gel electrophoresis.
2 Data from Segal et al. 1984; Gries et al. 1988; Fless et al. 1986; Albers and Hazzard 1974; Gotto et al. 1986; Gaubatz et al. 1983.

KEY: FC, unesterified cholesterol; TG, triglycerides; PL, phospholipids; CE, cholesterol esters.

their presence. Plasma is generally turbid when triglyceride concentration exceeds 400 mg/dL (4.52 mmol/L). Because of their large lipid content, they are readily separated from LDL and HDL by ultracentrifugation at plasma density (d <1.006 kg/L). They can also be separated from each other and from LDL and HDL by electrophoresis. The apolipoproteins in the triglyceride-rich lipoproteins play crucial metabolic roles in their catabolism.

CLINICAL IMPORTANCE

Hypertriglyceridemia is of interest and importance in a number of clinical conditions. With severe elevations (>1,000 mg/dL), there is a significant association with the development of abdominal pain and pancreatitis. This can occur in subjects with a marked increase in VLDL but is more often encountered in patients with impaired chylomicron (type I) or both chylomicron and VLDL (type V) catabolism. Pure hyperchylomicronemia in a fasting patient is rare, being found only in subjects with defects in lipoprotein lipase (LPL) or in apolipoprotein CII deficiency. Apo CII is a required cofactor for LPL activation. Combined elevations of chylomicrons and VLDL are more commonly found secondary to poorly controlled type I or type II diabetes mellitus or excessive alcohol intake, especially if associated with an underlying familial hyperlipidemia such as familial combined hyperlipidemia or familial hypertriglyceridemia. In these subjects, clinical manifestations such as eruptive xanthoma, corneal arcus, xanthelasma, and lipemia retinalis make the measurement of plasma triglyceride levels necessary and important.

The relationship of plasma triglycerides, or triglyceride-rich lipoproteins, and atherosclerotic disease is still unclear. For this reason, neither the National Cholesterol Education Program’s (NCEP) Adult Treatment Panel (ATP) 1988 report nor the second report from the same group (ATP II) (NCEP 1994) identified hypertriglyceridemia as a target for intervention. The Consensus Development Conference Statement (February 26-28, 1992) following the NIH Consensus
Conference on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease concluded that “current evidence is inadequate to conclude that high plasma triglyceride levels are associated with an increased risk for CHD or that lowering triglyceride levels will decrease CHD risk.” They did, however, conclude that “patients with high triglyceride and low HDL even in the presence of a desirable total cholesterol should be treated with hygienic means or with drugs if the former proves ineffective” (NIH Consensus Conference 1993).

More than 30 years have elapsed since an association between coronary artery disease and hypertriglyceridemia was first noted (Albrink and Man 1959). Although this association has been confirmed both in prospective studies (Cambien et al. 1986; Carlson and Bottiger 1985; Carlson et al. 1979; Fager et al. 1981; Kaukola et al. 1980) and cross-sectional epidemiological studies (Albrink and Man 1959; Antonis and Bersohn 1960; Davignon et al. 1977; Kukita et al. 1982; Tan et al. 1980), much of the association may be due to other closely associated factors such as obesity, hyperglycemia, hyperinsulinemia, hypertension, cigarette smoking, elevated total cholesterol, and low HDL-cholesterol that often accompany hypertriglyceridemia. Even with adjustment for other factors, there remain specific population subgroups in which triglycerides remain a strong and statistically significant risk factor for coronary artery disease (Castelli 1986). These groups include postmenopausal women and patients in whom total and HDL-cholesterol are low (ratio <3.5:1) but triglycerides are elevated (Castelli 1986). Triglycerides may also play a significant and independent role in the development of coronary atherosclerosis in men over age 50. In any event, fasting triglyceride levels of 250-500 mg/dL are probably a marker for other atherosclerotic risk factors. Austin (1991) reviewed the relationship of plasma triglycerides and coronary heart disease and provided excellent commentary.

One of the most confounding variables in assessing the role of hypertriglyceridemia in atherosclerosis is its close and inverse relationship with HDL. In most studies, HDL-cholesterol is reduced when triglycerides are elevated and increases when triglyceride elevation is treated, whether by diet or drug. Furthermore, there is a growing body of evidence that the catabolic products of chylomicrons, chylomicron remnants, and VLDL (VLDL remnants and IDL) may be atherogenic. The lack of adequate techniques for assessing chylomicron remnants or IDL has prevented their evaluation in large-scale, epidemiological, cross-sectional, or interventional studies. There is a need to develop such methods.

The association between hypertriglyceridemia and peripheral vascular disease is stronger and more consistent than that for coronary atherosclerosis (Beyrer et al. 1968; Shepherd et al. 1985). This relationship remains strong even after adjusting for other lipoprotein components and nonlipoprotein risk factors such as hypertension and cigarette smoking.

A working definition of hypertriglyceridemia was adopted by the National Institutes of Health Consensus Conference on Hypertriglyceridemia (NIH Consensus Conference 1984) and endorsed by the recent NIH Consensus Development Conference (NIH Consensus Conference 1993) (table 2). Therapy is recommended for patients with triglycerides exceeding 500 mg/dL in the fasting state because there is substantial fluctuation in triglycerides in these individuals. The decision to treat triglyceride levels below 500 mg/dL depends on the presence of other lipid abnormalities such as familial combined hyperlipidemia or dysbetalipoproteinemia. The NCEP Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, in its second report (NCEP 1994), modified the definitions of hypertriglyceridemia somewhat (table 3) and recommended nonpharmacologic therapy in patients with elevated triglycerides. In addition, the panel indicated that drug therapy may also be required when elevated triglycerides are accompanied by forms of hyperlipidemia known to be atherogenic, such as familial combined hyperlipidemia, and that drug therapy is generally required in patients with triglycerides high enough to put them at risk for developing pancreatitis. Reference intervals for triglycerides (Lipid Research Clinics [LRC] Program Epidemiology Committee 1979) are age- and sex-related (table 4), and triglycerides of over 150 mg/dL in the first two decades of life should be considered abnormal. Triglyceride measurement has a third
TABLE 2. NIH Consensus Conference “working” classification for fasting triglycerides

<table>
<thead>
<tr>
<th>Category</th>
<th>Fasting Plasma Triglyceride Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;250 mg/dL (&lt;2.8 mmol/L)</td>
</tr>
<tr>
<td>Borderline hypertriglyceremia</td>
<td>250-500 mg/dL (2.8-5.6 mmol/L)</td>
</tr>
<tr>
<td>Definite hypertriglyceremia</td>
<td>&gt;500 mg/dL (&gt;5.6 mmol/L)</td>
</tr>
</tbody>
</table>

1 Endorsed by Consensus Development Conference, NIH, 1992 (NIH Consensus Conference 1993)
Source: NIH Consensus Conference 1984

TABLE 3. ATP II classification for triglyceride levels

<table>
<thead>
<tr>
<th>Category</th>
<th>Serum Triglyceride Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal triglycerides</td>
<td>&lt;200 mg/dL</td>
</tr>
<tr>
<td>Borderline-high triglycerides</td>
<td>200 to 400 mg/dL</td>
</tr>
<tr>
<td>High triglycerides</td>
<td>400 to 1,000 mg/dL</td>
</tr>
<tr>
<td>Very high triglycerides</td>
<td>&gt;1,000 mg/dL</td>
</tr>
</tbody>
</table>
Source: NCEP 1994

Important, although indirect, role in clinical medicine. This is to provide a simple and inexpensive way to estimate the VLDL-cholesterol content, a factor used in the calculation of LDL-cholesterol.

In summary, triglyceride measurement is of clinical importance for three reasons: (1) its relationship to pancreatitis; (2) its association with disturbances in other lipoproteins and possible relationship to atherosclerosis; and (3) calculation of LDL-cholesterol by the Friedewald formula (Friedewald et al. 1972):

\[
[\text{LDL-chol}] = [\text{Total chol}] - [\text{HDL-chol}] - \frac{[\text{TG}]}{5}
\]

The NCEP Working Group on Lipoprotein Measurement was organized to consider and make recommendations concerning the measurement of HDL, triglycerides, and LDL. These measurements are considered in three separate parts of this report. This part concerns the measurement of triglycerides.
### TABLE 4. Reference values for triglycerides (mg/dL)\(^1\)

<table>
<thead>
<tr>
<th>Age in Years</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentiles</td>
<td>Percentiles</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5-9</td>
<td>28</td>
<td>34</td>
</tr>
<tr>
<td>10-14</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>15-19</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>20-24</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>25-29</td>
<td>45</td>
<td>51</td>
</tr>
<tr>
<td>30-34</td>
<td>46</td>
<td>57</td>
</tr>
<tr>
<td>35-39</td>
<td>52</td>
<td>58</td>
</tr>
<tr>
<td>40-44</td>
<td>56</td>
<td>69</td>
</tr>
<tr>
<td>45-49</td>
<td>56</td>
<td>65</td>
</tr>
<tr>
<td>50-54</td>
<td>63</td>
<td>75</td>
</tr>
<tr>
<td>55-59</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>60-64</td>
<td>56</td>
<td>65</td>
</tr>
<tr>
<td>65-69</td>
<td>54</td>
<td>61</td>
</tr>
<tr>
<td>70+</td>
<td>63</td>
<td>71</td>
</tr>
</tbody>
</table>

\(^1\) To convert to mmol/L, multiply by 0.0113

Source: LRC Program Epidemiology Committee 1979
Section 2.
Methods for Triglyceride Measurement

REFERENCE METHOD
The reference system to standardize triglyceride measurement and evaluate laboratory performance is not as well developed as it is for total cholesterol. Since 1966, an in-house triglyceride method established at the Centers for Disease Control and Prevention (CDC) has been used as a point of reference to standardize triglyceride measurements in laboratories involved in epidemiologic and clinical studies (Centers for Disease Control 1973). The CDC triglyceride method is based on the method of Carlson (1963) and Carlson and Wadström (1959) and the techniques of Van Handel and Zilversmit (1957) and Lofland (1964). The procedure can be summarized as follows:

1. Serum lipids are extracted with chloroform, then treated with silicic acid to remove phospholipids and free glycerol.

2. An aliquot of the extract is saponified to release glycerol:

\[ \text{Triglyceride} + \text{KOH} \rightarrow \text{glycerol} + \text{fatty acids} \]

70 °C; 30 minutes

3. Glycerol is oxidized with sodium periodate to produce formaldehyde.

\[ \text{Glycerol} + 2\text{NaIO}_4 \rightarrow 2\text{HCHO} + \text{HCOOH} + 2\text{NaIO}_3 + \text{H}_2\text{O} \]

4. The formaldehyde produced reacts with chromotropic acid to produce a chromogen whose absorbance is measured at 570 nm.

\[ \text{Formaldehyde} + \text{chromotropic acid} \rightarrow \text{chromogen} \]

This semiautomated procedure owes much of its specificity to the chloroform-silicic acid extraction procedure that removes phospholipids, free glycerol, and other interfering substances and retains minimally some monoglycerides and diglycerides (Jover 1963). The precision attainable with this method ranges from coefficients of variation of 2 to 6 percent. Table 5 illustrates the precision achieved with this method by the CDC Lipid Reference Laboratory analyzing in-house control pools. The CDC reference method, however, is much too cumbersome for use in the routine clinical setting.

ROUTINE METHODS
The early chemical methods used in the clinical analysis of triglyceride required solvent extraction of the specimen and treatment of the solvent extract with an adsorbent to remove phospholipids and other interfering substances. Since the mid-1970's, however, enzymatic methods have virtually replaced the earlier chemical methods in all routine clinical laboratories. In the current enzymatic methods, triglycerides are first hydrolyzed using a bacterial lipase (triacylglycerol acylhydrolase). A surfactant or detergent is often used to facilitate hydrolysis. The glycerol released is quantitated by a variety of coupled enzyme systems. The reactions are summarized below. The initial step in each of these methods is conversion of glycerol to glycerol-3-phosphate.

1. Triglyceride + H\(_2\)O \(\xrightarrow{\text{lipase}}\) glycerol + FFA

2. Glycerol + ATP \(\xrightarrow{\text{glycerol kinase}}\) glycerol-3-phosphate + ADP

Several different reaction sequences have been used to quantitate triglycerides. In one approach, the adenosine 5-diphosphate (ADP) produced in reaction 2 is used to generate pyruvate, which is then converted to lactate (Bucolo and David...
TABLE 5. Analytical performance of CDC reference method for triglyceride

<table>
<thead>
<tr>
<th>Pool ID</th>
<th>Dates of Analysis</th>
<th>Number of Measurements</th>
<th>Triglyceride Concentration (mg/dL [mmol/L])</th>
<th>Coefficient of Variation (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPN-4</td>
<td>11/80 - 1/88</td>
<td>1,290</td>
<td>78.4 (0.886)</td>
<td>4.27</td>
</tr>
<tr>
<td>LPN-5</td>
<td>6/87 - 1/88</td>
<td>106</td>
<td>80.1 (0.905)</td>
<td>5.43</td>
</tr>
<tr>
<td>LPH-5</td>
<td>10/84 - 1/88</td>
<td>570</td>
<td>209.0 (2.362)</td>
<td>2.24</td>
</tr>
</tbody>
</table>

1973). The disappearance of nicotinamide adenine dinucleotide phosphate (NADH) is measured spectrophotometrically at 340 nm.

3. ADP + phosphoenolpyruvate $\rightarrow$ ATP + pyruvate
4. Pyruvate + NADH + H$^+$ $\rightarrow$ lactate + NAD$^+$

An additional reaction is used to allow measurement in the visible range of the spectrum. The NADH reacts with iodonitrotetrazolium violet (INT) to produce a colored product, INT-formazan, which is then measured spectrophotometrically at 505 nm.

5. NADH + INT$^+$ $\rightarrow$ NAD + INT-formazan

Other methods use the glycerol-3-phosphate produced in reaction 2.

6. Glycerol-3-phosphate + NAD$^+$ $\rightarrow$ dihydroxyacetone phosphate + NADH + H$^+$

The appearance of NADH is measured at 340 nm.

In another variation, glycerol-3-phosphate is oxidized using glycerol phosphate oxidase to generate hydrogen peroxide (Fossati and Prencipe 1982).

The peroxide is then reacted with 3,5 dichloro-2-hydroxybenzene sulfonic acid 4-aminophenazone in the presence of horseradish peroxidase as the chromogenic system. The red chromophore is measured at 510 nm.

7. Glycerol-3-phosphate + O$_2$ $\rightarrow$ H$_2$O$_2$ + dihydroxyacetone phosphate

8. H$_2$O$_2$ + 4-aminophenazone $\rightarrow$ HCl + H$_2$O + quinonemonoimine dye + 3,5 dichloro-2-hydroxybenzene sulfonic acid

Ferrocyanide is included to minimize interference from elevated bilirubin (Spain and Wu 1986).

The last procedure offers greater specificity because it avoids the oxidative/reductive side reactions of NAD, the extreme sensitivity to pH, and unfavorable equilibria that can occur with the other enzymatic systems. The reconstituted reagent is also more stable and gives a more nearly linear response to triglyceride concentration. Information from the College of American Pathologists (CAP) Chemistry Survey (table 6) illustrates the widespread use of enzymatic methods in clinical laboratories.
**TABLE 6. Enzymatic triglyceride methods in routine laboratories from the CAP Comprehensive Chemistry Survey**

1990 Set C-A, Specimen C-04
(All enzymatic methods include lipase and glycerol kinase with coupled reaction as indicated)

<table>
<thead>
<tr>
<th>Methods</th>
<th>Glycerol Blank</th>
<th>Serum Blank</th>
<th>Proportion of Labs&lt;sup&gt;1&lt;/sup&gt; (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Colorimetric</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol phosphate oxidase</td>
<td>NO</td>
<td>NO</td>
<td>54</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>YES</td>
<td>NO</td>
<td>3</td>
</tr>
<tr>
<td>UV GPDH (up reaction)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol phosphate dehydrogenase</td>
<td>NO</td>
<td>NO</td>
<td>15</td>
</tr>
<tr>
<td>Produces NADH</td>
<td>NO</td>
<td>YES</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YES</td>
<td>YES</td>
<td>1</td>
</tr>
<tr>
<td>UV PK (down reaction)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>NO</td>
<td>YES</td>
<td>13</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>NO</td>
<td>NO</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>YES</td>
<td>YES</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YES</td>
<td>NO</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>1</sup> n=3,672
Section 3.
Major Issues Concerning Triglyceride Measurement

**BIOLOGICAL VARIATION**

When a single measurement for triglyceride is made, it is affected by all the specific sources of biological variation present at the time the specimen is obtained. The total variation ($CV_T$), as measured by the analytical process, is a combination of the intraindividual biological variation ($CV_b$) and the analytical variation ($CV_a$). When interpreting a single result or a series of results from a single person, the total intraindividual variation ($CV_T$) is of primary interest. For triglyceride, the average total intraindividual variation ($CV_T$), measured during nonfasting, has been found to remain essentially the same for diurnal (35.7 percent), within-month (24.2 percent), and within-year (25.9 percent) periods (Demacker et al. 1982). Variation of triglyceride was considerable among the individuals in the study group, whether for a diurnal (6.3-65.0 percent), within-month (12.9-34.8 percent), or within-year (12.9-39.9 percent) period (Demacker et al. 1982). With an analytical coefficient of variation ($CV_a$) of about 3 percent, the within-month data reveal that the biological variation for triglycerides accounts for more than 90 percent of the total intraindividual variation. Even in the fasting state, considerable biological variation occurs within individuals. In subjects in the placebo phase of a clinical trial in which the subjects were maintained on a carefully monitored NCEP Step I or stricter diet, the variation for triglycerides over a 2-week period was approximately five times greater than that for cholesterol (table 7). The data were derived from 631 male and female subjects with moderate hypercholesterolemia (LDL >160 mg/dL and triglycerides <350 mg/dL). All samples were collected after a 12- to 14-hour fasting period and analyzed in a CDC standardized laboratory. The data indicate that triglycerides fluctuate widely; over 75 percent of subjects showed variations >10 percent during a 2-week period. The Lipid Research Clinics found the total intraindividual variation for paired fasting analyses performed an average of 2.5 months apart on samples from 7,055 fasting persons was about 25 percent for triglyceride (Jacobs and Barrett-Connor 1982). Unlike the total cholesterol, no detectable seasonal differences in triglyceride concentrations have been seen (Gordon et al. 1987). For the present purposes, $CV_b$ for triglycerides is assumed to be 23.7 percent.

<table>
<thead>
<tr>
<th>TABLE 7. Variation of plasma lipid levels over 2 weeks in 631 subjects following NCEP Step I Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation (percent) = [(Visit 1 – Visit 2/Visit 1) x 100]</td>
</tr>
<tr>
<td>Population Percentile</td>
</tr>
<tr>
<td>5th              10th              25th              50th              75th              90th              95th</td>
</tr>
<tr>
<td>Cholesterol      0.6%             0.9%             2.2%             4.9%             9.0%             14.6%           18.5%</td>
</tr>
<tr>
<td>Triglyceride     2.3%             3.9%             9.4%             23.5%            42.6%            70.9%           93.3%</td>
</tr>
<tr>
<td>HDL-cholesterol  0.0%             1.6%             3.0%             6.9%             12.1%            20.0%           25.0%</td>
</tr>
<tr>
<td>Calculated LDL-cholesterol       0.5%             1.0%             3.0%             6.5%             12.5%            18.7%           23.3%</td>
</tr>
</tbody>
</table>
Although the fluctuations described above reflect healthy individuals on stable diets, far greater fluctuations are seen in certain physiological and disease states. With dietary alteration, for example, switching from a high-fat to a high-carbohydrate diet or vice versa will result in rapid and often dramatic changes in plasma triglycerides. In patients with chronic diseases, especially metabolic conditions such as diabetes, hypothyroidism, glycogen storage disorders, and alcoholism, triglyceride levels are often severely elevated. Even in acute illness such as viral infections, post myocardial infarction or post surgery, triglyceride levels can be markedly increased. A number of drugs such as thiazide, diuretics, and estrogens in postmenopausal women can cause triglycerides to rise into the borderline range.

PREANALYTICAL FACTORS

Triglyceride measurements are influenced by both preanalytical and analytical sources of variation. As efforts to reduce analytical inaccuracy and imprecision progress, the effect of preanalytical factors that operate before or during blood sampling, during storage, and during shipment of samples to the laboratory must be considered in the measurement of triglyceride and the interpretation of results.

Representative behavioral sources of variation including diet, obesity, exercise, alcohol intake, and smoking for the most part reflect an individual's usual state of health. These are lifestyle sources of variation that will not be under the control of the laboratory.

Sources of variation associated with sample collection and handling include fasting status, posture during collection, venous-capillary differences, venous occlusion, the use of anticoagulants, and the conditions of storage and shipment (Lipid Research Clinics Program 1982). Many of these factors can be controlled to some extent (Bachorik 1982).

Fasting Samples

A fasting specimen is required for the determination of triglycerides because triglycerides may increase markedly postprandially as a result of triglyceride-rich chylomicrons entering the circulation (Terpstra et al. 1978). The increase is due to the appearance of chylomicrons in the circulation after a fat-containing meal. It is also necessary to consider how long patients should fast before blood is drawn. A standard fasting period of 12 hours has long been used for both clinical and research measurements. In view of the wider appreciation of the need for LDL-cholesterol measurements, the NCEP ATP II guidelines advising the measurement of HDL-cholesterol when screening some categories of individuals, and the inconvenience to the patient of having to fast for 12 hours, the ATP II guidelines recommend that lipid and lipoprotein measurements can be made following a fasting period of 9 to 12 hours.

Although limited data are available, there is some evidence that the error in lipid and lipoprotein measurements may be acceptable for clinical purposes after a 9-hour fasting period. Havel (1957) fed formula meals containing 1.5 g fat/kg body weight to several subjects who had been on a high-fat diet for a week. The concentrations of serum glycerides 8 hours postprandially averaged 21 mg/dL higher than fasting values. Cross-sectional data were provided to the Lipoprotein Measurement Working Group by Dr. Richard Havel. These data suggest that serum triglyceride levels in men who had fasted 8 to 10 hours are about 15 mg/dL higher than those who had fasted 12 to 14 hours (table 8). There was no consistent trend in women. A preliminary analysis of serum triglyceride levels in Phase I of the Third National Health and Nutrition Examination Survey (NHANES III) was provided to the lipoprotein measurement working group by the National Center for Health Statistics. The analysis revealed no significant differences in triglyceride levels of subjects who had fasted 9 to 11 hours compared to those who had fasted 12 hours or more. Cohn et al. (1988) fed 22 normal subjects a fat load of 1 g/kg body weight. They found that triglycerides remained about 50 percent higher than fasting values after 9 hours. Lichtenstein et al. (1993) measured postprandial triglyceride in 14 subjects following three normal meals and a snack. The average fasting concentration of triglyceride was 105 mg/dL. Ten hours after the last food ingestion, triglyceride remained 98 mg/dL higher than the fasting level. In contrast, Schneeman et al. (1993) found that plasma triglycerides returned to baseline by 9 hours after the ingestion of an ordinary meal containing one-third the daily
intake of fat. De Bruin et al. (1991) fed a 98 g fat load to six healthy subjects and found that plasma triglycerides had returned to baseline after 7 hours. Further studies are needed to assess the effects of fasting for 9 to 12 hours in a “real life” setting, that is, in patients following their normal dietary routines. Nonetheless, some tentative conclusions can be drawn about the magnitude of the error to be expected when triglyceride is estimated in patients who have fasted for 9 to 12 hours. First, overall, it is likely that triglycerides will be overestimated somewhat (about 20 mg/dL) in patients who have fasted for 9 hours. This would contribute to a 4 mg/dL (0.10 mmol/L) underestimate of LDL-cholesterol but would be partially compensated by an accompanying 1 to 4 percent underestimate of HDL-cholesterol under these conditions. Second, such errors will probably create the most uncertainty in patients with triglyceride concentrations near the medical decision cutpoints; patients with clearly normal or elevated triglyceride concentrations should not be misclassified. Based on these considerations, the Working Group on Lipoprotein Measurement recommends that triglyceride measurements can be made most accurately in samples from patients who have fasted for 12 hours. A 12-hour fasting period should be used for most research purposes and in other cases where the error in triglyceride measurements must be minimized. For purposes of convenience, a 9- to 12-hour fasting period can be used in many patients, but triglyceride will be overestimated somewhat. This should be taken into account when interpreting the values.

**Posture**

Changes in posture have been shown to be associated with rapid and progressive hemodilution and hemoconcentration (Hagan et al. 1986). Posture-induced increases in serum triglyceride concentration after 30 minutes in the supine position followed by 30 minutes of standing averaged about 12 percent for serum specimens from fasting subjects (Hagan et al. 1986). Triglyceride concentration was decreased about 6 percent after a standing patient assumed and remained in a seated position for 15 or 20 minutes (Tan et al. 1973). Miller et al. (1992) reported a 10 percent decrease in triglyceride concentration when a subject sits and an 18 percent decrease when subjects recline. These fluctuations reflect changes in individual triglyceride concentrations and may complicate the interpretation of results; therefore, it is recommended that blood sampling conditions be standardized to the seated position and that patients be allowed to sit quietly for 5 minutes before drawing the sample. This is the position most lipid investigators use for the collection of blood samples. If it is not possible to use the sitting position, the same position should be used each time that particular patient is sampled.

### TABLE 8. Serum triglyceride concentrations in men fasting for various periods before blood sampling

<table>
<thead>
<tr>
<th>Hours Since Last Food</th>
<th>&lt;8 (n=85)</th>
<th>8-10 (n=159)</th>
<th>10-12 (n=946)</th>
<th>12-14 (n=1,271)</th>
<th>&gt;14 (n=1,135)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age-adjusted mean log TG²</td>
<td>2.083</td>
<td>2.072</td>
<td>2.038</td>
<td>2.015</td>
<td>2.057</td>
</tr>
<tr>
<td>Triglyceride concentration (mg/dL)</td>
<td>121.1</td>
<td>118.0</td>
<td>109.2</td>
<td>103.5</td>
<td>113.9</td>
</tr>
</tbody>
</table>

1 Number of subjects.
2 Age range, 20-74 years.

Source: Data provided by Dr. Richard J. Havel and Dr. Nancy Phillips, University of California, San Francisco.
Venous/Capillary Differences and Venous Occlusion

Differences in triglyceride concentrations measured in the serum of capillary blood have been found to average about 8.5 percent lower than in the serum of venous blood (Kupke et al. 1979) and lipid measurements in capillary samples are more variable than in venous samples (Bachorik et al. 1991). For this reason, it is recommended that measurements used for diagnosis and followup be made in venous samples. Prolonged venous occlusion prior to venipuncture has been associated with increases in serum lipid concentrations of as much as 10 to 15 percent (Koerselman et al. 1961; Page and Moinuddin 1962). Venous occlusion for up to 1 minute had no detectable effect on serum lipids and hematocrit (Tan et al. 1973). It is recommended that the tourniquet be applied for not more than 1 minute, and it should be released as soon as the blood begins to flow.

Serum-Plasma Differences

Triglycerides can be measured on either serum or plasma. If plasma is used for triglyceride determinations, ethylenediaminetetraacetic (EDTA) is the preferred anticoagulant because it retards auto-oxidation of unsaturated fatty acids and cholesterol and helps maintain lipoproteins in natural physical states (Lipid Research Clinics Program 1982). EDTA, however, can cause shifts of water from red cells to plasma, diluting nondiffusible plasma constituents and resulting in concentration differences between serum and plasma samples. In a study of 500 serum-plasma pairs, the use of EDTA (1 g/L) as anticoagulant decreased cholesterol and triglyceride in plasma by about 3 percent (Laboratory Methods Committee, Lipid Research Clinics Program 1977). Heparin has little osmotic effect in the concentration used for anticoagulation and decreases the cholesterol and triglyceride concentrations by less than 1 percent (Lum and Gambino 1974).

Frozen Specimens

The safest procedure is to analyze freshly drawn specimens as soon as possible after collection. Effects of storage of serum on triglyceride are minimal within 4 days when the samples are stored at 2 °C to 4 °C. When HDL-cholesterol and LDL-cholesterol are to be measured also, the specimens should be stored no longer than 3 days at 4 °C (see part two, LDL). Samples can be stored for at least several weeks at -20 °C, and for several years if stored at -50 °C to -80 °C (Kuchmak et al. 1982; Stokes et al. 1986; Tiedink and Katan 1988). When storing samples at -20 °C, use a non-self-defrosting freezer.

Sample Shipment

Because there is an increasing tendency for patient samples to be sent to central, well-standardized laboratories for lipid and other analyses, especially for clinical and epidemiological research, it is important that such shipments be carried out under optimal conditions. Once samples have been collected and serum or plasma separated, they should be stored either at 4 °C or frozen (-20 °C or -80 °C) depending on the circumstances. Be sure that the labels used to identify the individual samples will not become unfastened or illegible at shipment temperatures or if they should get wet. If stored at 4 °C, shipment should occur within 24 to 48 hours and temperatures should be maintained at or close to 4 °C. This can be achieved by utilizing a tight-sealing styrofoam container within a protective cardboard box and containing an “artificial” freezer pack (frozen at -20 °C for 24 hours). Direct contact of sample and freezer pack should be avoided, otherwise freezing of the serum/plasma could occur. This can be achieved by placing patient samples in separate plastic containers within the styrofoam box. Crushed ice, well sealed in a plastic bag, also can be used in place of the freezer pack but may present logistical problems and may not last as long. Samples are generally stable in this transport system for at least 24 hours. Samples previously frozen at -20 °C or -80 °C should be shipped in similar styrofoam boxes containing solid CO₂ (“dry ice”) in sufficient quantity to maintain constant freezing temperatures for at least 24 hours. Samples shipped frozen should be unpacked at the laboratory in a “cold room” (4 °C) if the samples will continue to be stored frozen. If analysis is to commence immediately, samples should be unpacked and kept at room temperature until they reach room temperature. The samples must be well mixed prior to analysis. This is a crucial step because a representative aliquot cannot be obtained from an incompletely
mixed specimen. The thawed samples should be mixed for 30 minutes on a blood rotator or similar device before analysis.
Section 4.
Triglyceride Blank Measurements

Triglyceride blanking is a laboratory procedure that determines the amount of glycerol not bound with fatty acids, known as “free glycerol.” This is then taken into account in the final expression of the triglyceride concentration, known as “true,” “net,” or “blanked” triglyceride. Triglyceride blanks resulting from endogenous and/or exogenous glycerol may cause an overestimation of triglyceride in patient specimens, since most current methods measure the total amount of glycerol in the specimen (unesterified as well as esterified, see section 2). Most of the older chemical methods employed an extraction step that partially removed free glycerol. In the enzymatic methods, however, all the free glycerol present contributes to total triglyceride concentration if it is not taken into consideration, and triglyceride blanks are higher with the enzymatic methods than with the chemical methods used formerly. Most routine clinical laboratories do not correct for free glycerol. Increased levels of endogenous glycerol in plasma may arise from a variety of sources: recent exercise, liver disease, diabetes mellitus, hemodialysis, stress, use of glycerol-containing intravenous medications, and parenteral nutrition (McPherson et al. 1985; Ng et al. 1986; ter Welle et al. 1984). Increased triglyceride blanks can also result from external sources, such as the use of blood-collection tubes with glycerol-coated stoppers, contamination of quality control materials from filters used for sterilization, contamination caused by hand lotion used by laboratory personnel, and from laboratory detergents (Cheung and Swaminathan 1987; Ryder et al. 1986). The collection tubes used for specimens can cause an overestimation of plasma triglyceride if they are manufactured using glycerol as the lubricant for the stopper (Stein 1987). Small volume tubes used for pediatric specimens are more likely to use glycerol-lubricated stoppers.

The controversy over whether to blank triglyceride analyses for free glycerol continues (Cole 1990; Jessen et al. 1990; Rautela et al. 1973). In a recent study the need for free glycerol blanking was evaluated (Jessen et al. 1990). Triglyceride and free glycerol concentrations were measured in 419 inpatient samples and in 339 outpatient samples at two clinics. It was found that all outpatient samples had glycerol values (expressed as equivalent triglyceride concentration) <25 mg/dL (0.28 mmol/L), with 99 percent being <8.8 mg/dL (0.10 mmol/L) (Jessen et al. 1990). For the inpatients, 97 percent had glycerol values <25 mg/dL (0.28 mmol/L); the remaining 3 percent were quite variable (Jessen et al. 1990). Thus, omission of the triglyceride blank introduced an error exceeding about 10 mg/dL (0.11 mmol/L) in fewer than 1 percent of the outpatients. A major justification for accurate triglyceride measurements is its use for estimating LDL-cholesterol in the Friedewald equation (Friedewald et al. 1972).

\[
[\text{LDL-chol}] = [\text{Total chol}] - [\text{HDL-chol}] - \frac{[\text{TG}]}{5}
\]

where all concentrations are expressed in mg/dL.

\[\frac{[\text{TG}]}{2.17}\] is used when concentration is expressed in mmol/L.

In this equation, \([\text{TG}] / 5\) is an estimate of VLDL-cholesterol concentration. From the study cited
above, if the glycerol blank is 25 mg/dL (0.28 mmol/L), LDL-cholesterol would be underestimated by 5 mg/dL (0.06 mmol/L), or 3.8 percent, at a level of 130 mg/dL. LDL-cholesterol would be underestimated by 2 mg/dL (0.05 mmol/L) or less in most individuals if the glycerol blank is omitted.

Glycerol blanking does take on more importance, however, when attempting to standardize triglyceride measurements or to establish traceability to the reference method. Since the glycerol blank is removed from the specimen when analyzed with the reference method, the accuracy of triglyceride measurements must be based on blanked values. The triglyceride blank in processed materials used as survey samples, reference materials, and calibrators can vary considerably and can be greater than in patient specimens (table 9). This situation makes standardizing triglyceride measurements and comparing results among clinical laboratories very difficult. For example, a clinical laboratory might employ a lyophilized calibration serum that has a true triglyceride value of 150 mg/dL (1.69 mmol/L) and a triglyceride blank of 50 mg/dL (0.56 mmol/L). Using such a calibration pool without correcting for the contribution of the blank could produce as much as a 25 percent underestimate of the values in fresh specimens, which generally have much lower blanks. Using such a calibrator, a patient sample with a true triglyceride value of 275 mg/dL would have an apparent value of 206 mg/dL. The 69 mg/dL underestimation of triglyceride would result in the overestimation of LDL-cholesterol by 14 mg/dL (0.36 mmol/L) or about 11 percent in a patient with a true LDL-cholesterol value of 130 mg/dL (3.36 mmol/L). Such an error in LDL-cholesterol could have a significant impact, particularly for LDL-cholesterol concentrations near or at the NCEP LDL-cholesterol cutpoints for decision making.

Several methods have been used to compensate for the presence of free glycerol in the sample. With the older chemical methods, a constant correction factor (0.11 mmol/L [about 10 mg/dL]) was advocated (Carlstrom and Christensson 1971; Stinshoff et al. 1977) to compensate for the absence of an extraction step. The more common approach in the enzymatic assays involves omitting the lipase reagent from the reaction mixture. The contribution of free glycerol is subtracted from the value obtained in the presence of lipase.

The CAP Chemistry Survey, only about 6 percent of participants correct for free glycerol (table 6). Studies at CDC comparing commercial enzymatic kit results for free glycerol with an isotope-dilution gas chromatography-mass spectrometry method sometimes gave substantially different results with reference serum pools (Bernert et al. 1992). Thus, consideration must also be given to the potential for matrix effects with enzymatic free glycerol assays.

<table>
<thead>
<tr>
<th>TABLE 9. Glycerol blanks in some processed materials</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAP Reference Materials</strong></td>
</tr>
<tr>
<td>CAP Survey Materials</td>
</tr>
<tr>
<td>1989 Lipid Survey, Sets LS-A, LS-B</td>
</tr>
<tr>
<td>1990 Lipid Survey, Set LS-A</td>
</tr>
<tr>
<td><strong>CDC Reference Materials</strong></td>
</tr>
<tr>
<td><strong>National Institute of Standards and Technology Reference Materials</strong></td>
</tr>
</tbody>
</table>

Source: Unpublished data from CDC Lipid Reference Laboratory.
There is some difficulty in selecting a triglyceride standard when results are to be expressed in mg/dL, because the molecular weight of a particular triglyceride depends on its fatty acid composition. Furthermore, triglycerides and the fatty acid composition of triglycerides vary in individuals and are influenced by diet. This is not an issue when triglycerides are expressed in mmol/L.

A tripalmitin triglyceride standard is available from the National Institute of Standards and Technology (certified 99 percent). Tripalmitin, however, is insoluble in aqueous media and is not useful for enzymatic methods. Triolein standards and glycerol standards are available from various manufacturers for use on enzymatic systems. Glycerol standards have the advantage that their concentrations are accurately known because glycerol has a unique molecular structure. They have the disadvantage of not being subject to the hydrolysis by lipase, however, and the use of glycerol does not monitor the hydrolysis step in the enzymatic procedures. Triolein requires the use of surfactants for solubility in saline- or serum-based standards. Triolein is not suitable for kinetic procedures and may require a longer incubation time when enzymatic procedures are used. It has the further disadvantage of being less stable during storage in some solvent systems than tripalmitin or glycerol. CDC uses triolein/tripalmitin (2:1) as a standard in order to reflect the unsaturated/saturated composition of human serum.

A plasma- or serum-based secondary standard can be prepared by freezing aliquots of a pool of plasma with a triglyceride level of about 200 mg/dL (Bonderman et al. 1976). Pooled plasma used as a calibrator must be tested and found to be negative for hepatitis B surface antigen and human immunodeficiency virus (HIV) antibodies. The triglyceride concentration of the calibration pool should be assigned based on the reference triglyceride method. Primary standards should be included in each run to confirm the linearity of the response as a function of concentration.
Section 6.
Performance and Standardization
of Triglyceride Measurements

The use of the enzymatic methods for triglyceride analysis has contributed to an improvement in clinical laboratory performance in recent years. Assessment of the reliability of triglyceride measurements can be obtained from a review of the College of American Pathologists Comprehensive Chemistry Survey. Reviews of previous surveys for triglyceride indicate that both inaccuracy and imprecision are problems in triglyceride measurements. Table 10 summarizes the performance of over 3,000 laboratories during 1990. Comparing the overall means for each survey set to CDC-confirming values indicates positive biases ranging from 3.1 to 8.5 percent. The range of means for each survey set is quite large, from 40 mg/dL (0.45 mmol/L) to 56 mg/dL (0.63 mmol/L), indicating considerable variation in triglyceride measurement. The overall coefficient of variation for each of the survey sets, which includes both within-laboratory and among-laboratory variation, averages about 7.6 percent. The range of CV’s for individual peer groups is extremely large, from 2.2 to 21 percent, indicating significant imprecision in the measurement of triglyceride. The large deviations in results reported by CAP participants clearly show the need to improve the inter-laboratory comparability of this assay. It should be stated, however, that such data should not be used to judge the precision of triglyceride measurements in individual clinical laboratories, since within-laboratory imprecision is generally much smaller than indicated in table 10. The findings indicate, however, that triglyceride measurements will have to be improved in order for measurements in different laboratories to be considered equivalent.

TABLE 10. CAP 1990 Comprehensive Survey (triglyceride results)

<table>
<thead>
<tr>
<th>Survey Sets</th>
<th>Number Labs</th>
<th>RVⁱ mg/dL</th>
<th>Overall Means mg/dL</th>
<th>Percent Bias</th>
<th>Range of Means mg/dL</th>
<th>CV² Range (percent)</th>
<th>CV (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-A C-03</td>
<td>3,688</td>
<td>159.8</td>
<td>173.4</td>
<td>+8.5</td>
<td>142.5-186.4</td>
<td>2.2-15.5</td>
<td>7.9</td>
</tr>
<tr>
<td>C-04</td>
<td>3,672</td>
<td>190.5</td>
<td>201.9</td>
<td>+6.0</td>
<td>169.3-218.6</td>
<td>2.5-11.9</td>
<td>7.5</td>
</tr>
<tr>
<td>C-B C-09</td>
<td>4,388</td>
<td>159.8</td>
<td>172.5</td>
<td>+7.9</td>
<td>144.4-184.9</td>
<td>3.0-10.5</td>
<td>7.8</td>
</tr>
<tr>
<td>C-10</td>
<td>4,424</td>
<td>219.2</td>
<td>233.1</td>
<td>+6.3</td>
<td>199.5-254.6</td>
<td>6.1-21.6</td>
<td>7.8</td>
</tr>
<tr>
<td>C-C C-15</td>
<td>4,574</td>
<td>190.5</td>
<td>201.3</td>
<td>+5.7</td>
<td>173.6-220.3</td>
<td>3.1-16.3</td>
<td>7.4</td>
</tr>
<tr>
<td>C-16</td>
<td>4,599</td>
<td>219.2</td>
<td>233.1</td>
<td>+6.3</td>
<td>201.0-257.2</td>
<td>6.7-10.5</td>
<td>7.6</td>
</tr>
<tr>
<td>C-D C-21</td>
<td>3,968</td>
<td>189.3</td>
<td>195.1</td>
<td>+3.1</td>
<td>164.8-212.0</td>
<td>2.9-10.6</td>
<td>7.4</td>
</tr>
<tr>
<td>C-22</td>
<td>3,972</td>
<td>189.3</td>
<td>195.4</td>
<td>+3.2</td>
<td>165.8-212.0</td>
<td>3.2-10.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>

¹ RV = Reference value determined by CDC chromotropic acid method.
² CV = Coefficient of variation (includes both within- and between-laboratory variation).
Source: From the College of American Pathologists 1990 Comprehensive Chemistry Survey Reports.
Since 1966, CDC has been offering standardization assistance for the measurement of triglyceride to epidemiologic and lipid research laboratories (Myers et al. 1989). The CDC-NHLBI Lipid Standardization Program criteria for acceptable performance for triglyceride measurement is summarized in table 11. For a sample with triglyceride concentration of 177 mg/dL (2.00 mmol/L), the allowable CV is 5.5 percent. The CDC uses serum-based reference materials that are intended to approximate actual patient specimens (Kuchmak et al. 1982; Williams et al. 1970). Elevated levels of triglyceride are prepared by adding egg yolk extract (Williams et al. 1970). These reference materials tend to have higher blanks than normal patient specimens; however, since the CDC triglyceride reference method removes the blank before the samples are analyzed (see above), participants in the CDC standardization program must correct for the triglyceride blank in order to meet CDC performance criteria. The LRC, which were standardized by CDC, achieved CVs of about 4 percent for pools with concentration ranges from 0.41 to 3.09 mmol/L (36-273 mg/dL) (Ahmed et al. 1979).

The measurement of triglyceride is made either for the purpose of establishing its concentration, per se, or as part of the battery of analyses necessary to estimate LDL-cholesterol with the Friedewald equation. The requirements for accuracy and precision, therefore, depend to some extent on the intended purpose for the measurement. When measured to establish the triglyceride concentration, the need for accurate and precise results is less stringent than for cholesterol; the CV for triglyceride is about 24 percent compared to 6 percent for cholesterol. For example, an evaluation of the LRC Prevalence Study population found that a 95 percent confidence interval corresponding to a single measurement of 250 mg/dL would range from 155 to 404 mg/dL; the CV for the LRC laboratories was about 4.0 percent (Brenner and Heiss 1990).

**PERFORMANCE GOALS FOR TRIGLYCERIDE MEASUREMENT**

The performance goals for triglyceride analysis are based on the degree of accuracy and precision necessary to give reliable LDL-cholesterol measurements using the Friedewald equation. Table 12 presents analytical goals for allowable error. The single point total error goals in the table were derived from three statistical models depending on whether bias is assumed to be variable (quadratic model) or fixed (linear model) and on the definition of 95 percent confidence (one- or two-tailed) with the linear models. The slightly larger single point goal for the linear two-tailed model is conservative analytically and more specific for assessment of unsatisfactory accuracy (NCEP Laboratory Standardization Panel 1990).

Because of the large biological variation of triglyceride, clinical goals for triglyceride testing are unsatisfactory, particularly when the triglyceride

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**TABLE 11. Criteria for acceptable performance for triglyceride measurements. CDC-NHLBI Lipid Standardization Program**

<table>
<thead>
<tr>
<th>Triglyceride Concentration</th>
<th>Maximum Bias of the Mean from RV(^1)</th>
<th>Overall Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range/CDC Pool (mmol/L)</td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
</tr>
<tr>
<td>0.00 - 0.99</td>
<td>±0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>1.00 - 1.99</td>
<td>±0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>2.00 - 2.49</td>
<td>±0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>≥2.50</td>
<td>±0.05(^2)</td>
<td>0.05(^2)</td>
</tr>
</tbody>
</table>

\(^1\) RV = CDC reference value

\(^2\) The limits of the acceptable values for each CDC reference pool may be obtained by multiplying the CDC reference values by the factor listed.
measurements are used to calculate LDL-cholesterol concentration. Instead, the goals for triglyceride measurement were evaluated for consistency with clinical and analytical goals for total cholesterol, HDL-cholesterol, and LDL-Cholesterol testing. The goal for total analytical error of a single result is that triglyceride should be measured with a total error of 15 percent or less. (See table II-1 of appendix II.) Performance goals of <5 percent bias and <5 percent CV for triglyceride testing are consistent with the overall goal of no more than 10 percent error in the mean of serial samples, with 95 percent confidence at borderline LDL-cholesterol concentrations (145 mg/dL), using a practical number of serial samples in individuals.

The specified criteria for accuracy and precision reflect the minimum acceptable levels of routine (i.e., average) laboratory performance that should be achievable within the limits of current methodology. Expressed in this way, the recommendations would lead to average total errors not exceeding 15 percent for triglyceride (see table II-1 of appendix II) for laboratories operating at the minimum acceptable levels for analytic bias and CV. Accordingly, it is recommended that the goal for routine total error of triglyceride measurements not exceed 15 percent, and the goals for accuracy and precision listed above are considered to represent one set of conditions under which the primary goal for total error can be achieved.

RECOMMENDATIONS

General Recommendations

- **Reference Method.** The current basis for accuracy of triglyceride measurement should be the CDC reference method.

- **Criteria for Analytical Performance.** The goals for triglyceride measurement are stated in terms of total analytical error, which takes account of both accuracy and imprecision. This approach has the advantage that a slightly greater inaccuracy can be tolerated if the measurements are very precise; conversely, a slightly greater imprecision can be accepted if the measurements are more nearly accurate. The advantage of using total error is best understood by an example. In the case of total cholesterol, the NCEP Laboratory Standardization Panel specified criteria for acceptable performance as accuracy within 3 percent of reference values and precision consistent with a CV ≤3 percent. These guidelines lead to a total error of 8.9 percent for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5 percent bias and a CV of 2.0 percent would not be within the guidelines because the bias exceeds 3 percent. However, the total error for the laboratory would be 7.4 percent, well within a total error criterion of 8.9 percent. The specification of guidelines for accuracy and precision separately can lead to an

<table>
<thead>
<tr>
<th>Analytical Limits</th>
<th>Analytical Models</th>
<th>Clinical Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bias 5%</td>
<td>CV&lt;sub&gt;a&lt;/sub&gt; 5%</td>
<td>13.9% Quadratic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13.3% Linear, 1 Tailed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.8% Linear, 2 Tailed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.9%</td>
</tr>
</tbody>
</table>

<sup>1</sup> The values were calculated as shown in appendix II.

<sup>2</sup> Assuming CV<sub>T</sub> = 25 percent for triglycerides and that 90 percent of the variance is physiological. CV<sub>b</sub> taken as 23.7 percent.
ambiguous situation in which the performance of laboratories that are actually within acceptable total error limits are considered unacceptable. A total error criterion obviates this situation and is the primary criterion used to determine acceptable performance. Triglyceride measurements should be within the following minimum limits of performance.

Total Error \leq 15\%

This is consistent with the following limits for accuracy and precision:

Accuracy \leq \pm 5\%

\text{CV} \leq 5\%

Because of the large biological variation of triglycerides, the recommendations are more stringent than would be necessary for the measurement of triglycerides, per se. This is necessary because triglyceride measurements are also required for the estimation of LDL-cholesterol. Laboratories and others making triglyceride measurements can assess their individual conformance to the analytic goals as indicated in appendix II.

These criteria should apply regardless of how, where, or by whom the measurements are made.

- Triglycerides can be measured in either serum or plasma. Serum or serum-equivalent values should be reported. To convert measurements made in EDTA plasma to serum-equivalent values, multiply the plasma value by 1.03. Triglyceride measurements made in heparin plasma are equivalent to serum values.

Recommendations for Manufacturers

- The assigned values for the triglyceride concentrations of calibration and quality control materials should be traceable to the reference method for triglycerides, and the bias with respect to reference values should be stated.

- Instrument and reagent suppliers should provide systems in which glycerol blanking can be easily and economically incorporated into all triglyceride assays.

- Manufacturers should help develop and provide reference materials that are free of matrix effects.

Recommendations for Health Care Providers

- Triglycerides should be measured when the patient is in a metabolic steady state.

- Triglycerides should be measured in fasting samples.

- Blood samples should be obtained by venipuncture following a 9- to 12-hour period of fasting. If necessary the patient can take water and prescribed medications during this period. This procedure should be followed for research purposes and in other circumstances in which the error in the fasting triglyceride must be minimized.

- If, as a matter of convenience to the patient, a shorter fasting period must be used for routine clinical purposes, the fasting period should not be less than 9 hours. It is likely that, on average, fasting triglyceride will be overestimated (about 20 mg/dL [0.23 mmol/L]) in patients who have fasted 9 hours. This would contribute to a 4 mg/dL (0.10 mmol/L) underestimate of LDL-cholesterol calculated from the Friedewald equation but would be partially compensated by an accompanying 1 to 4 percent underestimate of HDL-cholesterol under these conditions. This should be taken into account when interpreting the values.

- To the extent possible, blood should be drawn in the sitting position and the patient should be allowed to sit quietly for at least 5 minutes before sampling. If the sitting position is not feasible, the patient should be sampled in the same position on each occasion.

- Prolonged venous occlusion should be avoided. The tourniquet should be removed within 1 minute of application.

- Stored samples

- Serum or plasma should be removed from cells within 3 hours of venipuncture.
— Specimens can be stored for up to 3 days at 4 °C. If the analysis is delayed, the specimens can be stored for up to several weeks at -20 °C in a non-self-defrosting freezer. Specimens should be stored at -70 °C or lower if longer periods of storage are necessary.

— In all cases, the samples should be stored in clean containers that can be sealed to prevent evaporation. Do not use cork stoppers or plastic film to seal the containers since such materials may not completely prevent evaporation. In addition, screw-type caps can loosen at freezer temperatures.

Need for serial measurements in individuals. Considering the physiological variability of triglycerides, triglyceride measured on a single occasion is not sufficient to determine the patients' usual triglyceride concentration. The patient should be sampled on several occasions within an 8-week period, and the samples should be obtained at least 1 week apart. The individual values should be averaged.

Three serial samples: Using three serial samples, each referred to the same laboratory and assayed once, and assuming a CV_b of 23.7 percent and a CV_a of 5 percent, the observed CV for the mean triglyceride value is 14.0 percent, and the difference between the means of sequential series of three samples should not exceed 39 percent, 95 percent of the time. The difference between the sequential individual values within each series should not exceed 67 percent. If they are farther apart, analytical error or a change in the physiological status of the patient should be suspected. Another sample may be warranted depending on the patient's triglyceride concentration and its proximity to the concentrations used for decision making (200 mg/dL [2.26 mmol/L] and 400 mg/dL [4.52 mmol/L]).

Based on prevailing triglyceride concentrations,* using two serial measurements and considering a cutpoint of 200 mg/dL, a patient's triglyceride can be confidently assumed to be above or below the cutpoint when the mean value is >257 mg/dL or <143 mg/dL, respectively. Using a 400 mg/dL cutpoint, the patient's triglyceride value can be confidently assumed to be above or below the cutpoint when the mean value is >514 mg/dL or <286 mg/dL, respectively. Thus, two serial specimens are sufficient to categorize 74 percent of the general population as being above or below the 200 mg/dL cutpoint and 96 percent as being above or below the 400 mg/dL cutpoint.

Recommendations for Laboratories

- Laboratories should use procedures that allow the measurement of triglycerides with a total error ≤15 percent. One set of conditions that satisfies this recommendation is that triglycerides be measured with an accuracy of ±5 percent and a CV_a of ≤5 percent.

- Given the marked intraindividual fluctuation in plasma or serum triglyceride concentrations and the controversy regarding exact clinical significance of mild increases in plasma triglycerides, stringent accuracy and precision goals are not as crucial for mean triglyceride measurements when the goal is to establish the patient's mean triglyceride concentration per se. The recommendations are primarily influenced by the current use of triglyceride measurements in the estimation of LDL-cholesterol.

---

* Unpublished data from the 1988-91 National Health and Nutrition Examination Survey, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.
• All blood samples should be considered potentially infectious and should be handled appropriately. Care should be taken that the sample is not ingested, inhaled, or otherwise brought into contact with laboratory personnel. Personnel handling blood samples should use gloves and should avoid leaving samples open to the atmosphere longer than necessary. Samples should be handled in accordance with current CDC guidelines for the prevention of infection in health care workers.

• Glycerol Blanking. Several professional organizations are currently reviewing the problems associated with the measurement of triglyceride in routine laboratories. Specifically, the Ad Hoc Triglyceride Review Committee of the Lipids and Lipoproteins Division, American Association for Clinical Chemistry (AACC) has made interim recommendations regarding glycerol blanking (Cole 1990). The NCEP Working Group on Lipoprotein Measurement endorses the following recommendations adapted from the AACC Lipids and Lipoproteins Division:

— All laboratories should offer a glycerol-blanked triglyceride analysis, even though it may be performed only when requested. Any specimen with triglyceride concentration >200 mg/dL (2.26 mmol/L) should be glycerol blanked using a “reflex” ordering system.

— Reports from the laboratory should clearly state whether the triglyceride analysis was glycerol-blanked (e.g., designated as “Blanked Triglyceride” or “Unblanked Triglyceride”). Physicians need to be educated as to how the inclusion of a glycerol blank may alter the meaning of the results.

— Glycerol blanking of triglyceride measurements should be mandatory in laboratories that specialize in assessment of lipid status, have large populations of hyperlipidemic subjects, or participate in clinical or basic research.

— Glycerol blanking of triglyceride analyses need not be routinely conducted on outpatients’ samples, unless economically feasible. However, because of the potential for higher glycerol concentrations in hospital inpatient specimens, all inpatient specimens should be routinely glycerol blanked.

Recommendations for Government Agencies and Other Professional Groups

The Centers for Disease Control and Prevention should take the following steps.

• CDC should validate and publish the reference method for triglycerides.

• CDC should collaborate with NIST on the development of a definitive method for triglycerides.

• In cooperation with appropriate experts in the academic, clinical laboratory, and manufacturing sections, CDC should develop reference materials for triglycerides that manifest the minimum matrix effects and have low blanks.

The Cholesterol Reference Method Laboratory Network currently provides assistance to manufacturers and laboratories wishing to establish the traceability of total cholesterol measurements to the cholesterol reference method. The network should:

• Expand these activities to include triglyceride, HDL-cholesterol, and LDL-cholesterol measurements.

The National Heart, Lung, and Blood Institute should take the following steps.

• Continue the present policy of requiring standardized triglyceride measurements for Government-supported clinical and epidemiological studies.

• Encourage the Cholesterol Reference Method Laboratory Network system to expand its activities to include the certification of triglyceride, HDL-cholesterol, and LDL-cholesterol measurements.
Recommendations for Further Research

Further research is needed in several areas.

- A definitive method for triglycerides is needed. The relationship of the CDC reference method to such a definitive method needs to be established.

- Reference materials should be developed that are free of matrix effects and have blank values that are similar to those in fresh specimens.

Such materials should be sufficiently stable to allow long-term monitoring of the accuracy and precision of triglyceride measurements.

- Triglyceride methods are needed that incorporate a correction for the triglyceride blank. Such methods should be easy to use, economical, and preferably not require that the blanked triglyceride be calculated from two primary measurements.
References


Carlson LA, Bottiger LE. Risk factors for ischaemic heart disease in men and women. Results of the 19-year follow-up of the Stockholm
Recommendations on Lipoprotein Measurement—Triglyceride


Centers for Disease Control. Semiautomated procedure for the determination of triglycerides in serum as performed in the Lipid Standardization Laboratory. Atlanta (GA): Centers for Disease Control; 1973.


Lofland HB Jr. A semiautomated procedure for the determination of triglycerides in serum. Anal Biochem 1964 Dec;9(4):393-400


Myers GL, Cooper GR, Winn CL, Smith SJ. The Centers for Disease Control-National Heart, Lung, and Blood Institute Lipid Standardization Program: an approach to accurate and precise lipid
Recommendations on Lipoprotein Measurement—Triglyceride


References
### Appendix I.
**Glossary of Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AACC</td>
<td>American Association for Clinical Chemistry</td>
</tr>
<tr>
<td>[analyte]</td>
<td>Square brackets indicate the concentration of the analyte in question</td>
</tr>
<tr>
<td>ATP</td>
<td>Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults</td>
</tr>
<tr>
<td>CAP</td>
<td>College of American Pathologists</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation, defined as SD/mean</td>
</tr>
<tr>
<td>CV&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Coefficient of analytical variation</td>
</tr>
<tr>
<td>CV&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Coefficient of normal physiological variation</td>
</tr>
<tr>
<td>CV&lt;sub&gt;T&lt;/sub&gt;</td>
<td>Total coefficient of variation, also referred to as observed variation; includes the contributions of CV&lt;sub&gt;a&lt;/sub&gt; and CV&lt;sub&gt;b&lt;/sub&gt; and calculated as: ( CV_T = (CV_a^2 + CV_b^2)^{1/2} )</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
</tr>
<tr>
<td>INT</td>
<td>Iodonitrotetrazolium</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRC</td>
<td>Lipid Research Clinics</td>
</tr>
<tr>
<td>NCEP</td>
<td>National Cholesterol Education Program</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>R</td>
<td>Residue</td>
</tr>
<tr>
<td>TGRL</td>
<td>Triglyceride-rich lipoproteins</td>
</tr>
<tr>
<td>β-VLDL</td>
<td>beta-VLDL, also referred to as &quot;floating beta&quot; lipoprotein</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
</tbody>
</table>
Recommendations on Lipoprotein Measurement
Appendix II.
Analytical Goals for Lipoprotein Cholesterol
and Procedure To Measure
Individual Laboratory Conformance to Goals

Analytical goals for the performance characteristics of a measurement process may be specifications either for the expected performance under stable conditions or for the limits of acceptable performance. To quantify typical routine performance, analytical goals can be expressed as the expectation for the 50th percentile or the standard normal deviation of process performance. To quantify performance limits, the analytical goals can be expressed as the expectation for a high percentile of the population, say the 95th or the 99th, or a multiple of the standard normal deviation. In this presentation, analytical goals for bias and coefficient of variation (CV) are set for the limits of acceptable performance. They are expressed as specifications for the value of the 95th percentile of the population distribution of these performance parameters.

This interpretation is based on three lines of reasoning. Measurement processes for total cholesterol whose routine performance for bias and CV approximate one-half of the analytical goals advanced by the NCEP Lipid Standardization Panel can be controlled with customary internal process control procedures and yet ensure process output meeting the analytical goals (Westgard 1992; Westgard and Burnett 1990; Westgard et al. 1991; Westgard and Wiebe 1991). Second, results calculated by use of statistical models of measurement processes are usually expressed at the traditional 95 percent confidence level. Third, the available data for total cholesterol suggest that existing population distributions of bias and CV can be improved to meet these goals (Ross et al. 1992, 1993).

The skewed population distribution of the CV of measurement processes complicates theoretical goal setting (Ross and Fraser 1982). However, available data suggest that the standard deviation of the logarithm CV of total cholesterol, HDL-cholesterol, and triglycerides is a relatively stable parameter that is independent of analyte, analyte concentration, and CV in the range of 2 to 10 percent CV (Ross et al. 1992). The stability of this parameter enables calculation of the standard deviation and various percentiles of the population distribution of CV’s under the provision that 95 percent of the CV values meet the analytical goal.

The CV goal must be carefully defined because it may include a contribution due to random analytical bias that is systematic within analytical runs and that artificially inflates estimates of pure random error (Ross 1982; Ross and Fraser 1982). For this presentation, the CV goal is applied only to the pure random error (within-run random variation) of the measurement process.

The bias goal must account for the primary performance characteristics of the calibration process: accuracy and stability. The inaccuracy of the calibration process is defined as bias measured over a large number of analytical runs (“fixed” bias). The instability of the calibration process is a random effect that gives rise to the variation observed among the individual means of analytical runs (between-run variation).

Among laboratories, fixed- and within-run bias are considered to be normally distributed random variables with means equal to zero. When these random variables are defined as percent biases of a true mean (in a medically useful range), their variances are equal to their squared CV’s. The standard deviations of these distributions are (numerically) equal to the CV’s of percent biases relative to means in the medically useful ranges. Thus, the bias goals can be denoted in terms of
percent CV or the numerically equal percent bias in calculation formulas.

Only one analytical goal for bias is specified by the NCEP Laboratory Standardization Panel for each lipoprotein measurement. This goal is the maximum allowable bias of analytic runs. This bias, when added to the within-run imprecision, gives at the specified confidence level the total analytical error that is just acceptable. Larger total analytical error should be detected by the process control system.

For this presentation, the bias goal is decomposed into fixed and random components whose linear sum is the goal. This requirement is stringent but is imposed to ensure that laboratories meet both the fixed and random bias components. The requirement does not allow laboratories to meet the overall bias goal by combinations of extreme fixed- and within-run bias with opposite signs whose sum is acceptable (Ross and Fraser 1993).

Instability of calibration is a universal characteristic of current measurement processes. In this presentation, a limit equal to one-half of the within-run CV is imposed on between-run variation so that the long-term CV observed in the measurement process is only minimally increased.

A limit on the clinical accuracy of medical tests is imposed by the biological variability of the analyte (Ross 1988; Ross and Fraser 1993; Ross and Lawson 1987). Biological variation can be controlled by proper preanalytical preparation of the patient. However, at some point, biological variation becomes irreducible. At this point, replicate sampling of the patient is the only means to further improve test accuracy (Bookstein et al. 1990; Cooper et al. 1992; Dujovne and Harris 1990; Mogadam et al. 1990).

The pattern by which multiple samples from one patient are referred to laboratories greatly changes the effect of analytical goals on observed test accuracy (Ross and Fraser 1993). Possible statistical sampling patterns include referral of repeated samples among all measurement processes, repeated referral to a single measurement process, replications within a sample over measurement processes, and others. For clarity, this presentation considers only the first two possibilities. Further, we assume that each sample generates only one specimen and that each specimen is analyzed in singlet in different analytical runs.

We consider each measurement process as a single measurement process within one laboratory. Although laboratories may have multiple measurement processes for one analyte, referral of replicate samples from a patient to one laboratory is considered equivalent to referrals to the same measurement process. Thus, the fixed analytical bias term includes a laboratory-specific component and a method-specific component.

The goals for each component of bias are applied as follows: If replicates are referred among laboratories, the fixed- and between-run bias specifications are the standard deviations of normally distributed populations whose linear sum is the goal for the limit of acceptable performance, as previously discussed. However, if replicates obtained from a patient are repeatedly referred to the same laboratory, an amount equal to the standard deviation of the between-run bias variable is first subtracted linearly from the total bias goal. The remainder is then the fixed bias of the measurement process within the laboratory and is numerically equal to the standard deviation of the fixed-bias variable (Ross and Fraser 1993). For each of the two sampling patterns (among and within laboratories), the effect of analytical error on test accuracy is calculated for measurement processes whose routine performance characteristics are representative of those of a population 95 percent of which meet the goal. The bias and CV of these measurement processes are approximately one-half of the analytical goals. Such measurement processes have little apparent impact on test accuracy. If instability of these error conditions occur, customary internal process control systems are likely to detect an increase in bias or CV before the analytical goal is exceeded.

For each sampling pattern, the effect of analytical accuracy on test accuracy is also calculated for measurement processes whose performance characteristics are equal to the analytical goals. Such measurement processes have an apparent impact on test accuracy, and the impact of analyti-
Analytical errors are unacceptable in the case of replicate measurements of HDL-cholesterol within laboratories whose biases are near the interim goal for analytic bias. Measurement processes should not routinely operate at the interim or final analytical goals, because an increase in bias or CV exceeding the analytical goal will not be detected by available systems for internal process control (Westgard 1992; Westgard and Burnett 1990; Westgard et al. 1991; Westgard and Wiebe 1991).

An overview of lipoprotein analytical goals for bias, CV, and total error is presented in table II-1. Total error limits for singlet results calculated by various analytical statistical models using the goals for bias and CV are in close agreement with one another. With the exception of triglyceride, they are in reasonable agreement with the limits required by the clinical statistical model. The clinical model (Ross 1988; Ross and Fraser 1993) is based on the effect of biological variation (inherent test error) on test accuracy. Because the biological variation of triglyceride is large, the clinical total error goal is large. However, the triglycerides are also measured as a component of the LDL-cholesterol determination. The 5 percent goals for the bias and CV of triglyceride measurement are adopted to maintain analytical consistency with the requirements of LDL-cholesterol measurements.

The impact of biological variation and analytical error on the test accuracy of total cholesterol, LDL-cholesterol, and HDL-cholesterol is presented in tables II-2 through II-4. In the average patient, two or three replicate samples are required to narrow the total error of the test, including biological variation, to less than 10 percent, when testing is conducted in laboratories whose measurement processes are typical of laboratory populations whose members meet the final goals in 95 percent of the cases. However, two to five replicates are required by those measurement processes operating at bias and CV equal to their final respective goals.

HDL-cholesterol cannot be measured with 10 percent accuracy and 95 percent confidence by use of a measurement process whose bias is equal to the interim goals. Even typical performance expected under the interim goals requires averaging the singlet results from four to six samples from a patient if 10 percent test accuracy is desired with 95 percent confidence.

### TABLE II-1. Analytical goals for the total analytical error of singlet results in relation to various statistical models and the goals for analytical bias imprecision (percent)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Analytical Limits</th>
<th>Total Error Goal for Singlet Points</th>
<th>Clinical Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bias</td>
<td>CV</td>
<td>Quadratic</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>3</td>
<td>3</td>
<td>8.3</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>4</td>
<td>4</td>
<td>11.1</td>
</tr>
<tr>
<td>HDL-cholesterol (1994)*</td>
<td>10</td>
<td>6</td>
<td>22.9</td>
</tr>
<tr>
<td>HDL-cholesterol (1998)*</td>
<td>5</td>
<td>4</td>
<td>12.6</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>5</td>
<td>5</td>
<td>13.9</td>
</tr>
</tbody>
</table>

*Dates refer to interim goal and 1998 goal. See main report.
**TABLE II-2. Total cholesterol**

<table>
<thead>
<tr>
<th>Source of Test Error</th>
<th>Biological Variation, % CV_b</th>
<th>Performance Specification, %CV</th>
<th>No. of Patient Samples With &lt;10% Total Error in Mean, p ≥ 0.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological only</td>
<td>5.4</td>
<td>0</td>
<td>2 2</td>
</tr>
<tr>
<td>Biological plus routine analytical</td>
<td>5.4</td>
<td>0.76</td>
<td>2 2</td>
</tr>
<tr>
<td>Biological plus limit for analytical</td>
<td>5.4</td>
<td>1.50</td>
<td>2 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CV_b *, CV_f, CV_e</td>
<td></td>
</tr>
</tbody>
</table>

* CV = β within lab

CV_b - Average intraindividual CV = 5.4%
- 95% range of intraindividual CV’s = 14.0%

CV_a - Polynomial coefficients for regression of mean lnCV_a upon analyte concentration = 1.421; - 0.00435370; 0.0000100144.
- s lnCV_a = 0.405914.

Average long-term intralaboratory CV_a in 1989/90 at medical decision levels:
- 200 mg/dL - 2.59% CV
- 240 mg/dL - 2.56% CV

Average bias of singlet results among laboratories in 1989/90: 0%

Average bias among methods in 1989/90: 0.1%
### TABLE II-3. LDL-cholesterol

<table>
<thead>
<tr>
<th>Source of Test Error</th>
<th>Biological Variation, % CV&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Performance Specification, %CV</th>
<th>No. of Patient Samples With ≤10% Total Error in Mean, p ≥ .95</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CV&lt;sub&gt;β&lt;/sub&gt;</td>
<td>CV&lt;sub&gt;γ&lt;/sub&gt;</td>
<td>CV&lt;sub&gt;ε&lt;/sub&gt;</td>
</tr>
<tr>
<td>Biological only</td>
<td>8.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Biological plus routine analytical</td>
<td>8.2</td>
<td>1.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Biological plus limit for analytical</td>
<td>8.2</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* CV = β within lab

- CV<sub>b</sub>- Average intraindividual CV = 8.2%
- 95% range of intraindividual CV’s = 20.0%

- CV<sub>a</sub>- s lnCV<sub>a</sub> unknown. Assumed to be the same as that for cholesterol.

The mean CV and average interlaboratory bias are unknown.
### Table II-4. **HDL-cholesterol**

**Analytical Goals - Interim**: 10% Bias; 6% CV; **Proposed 1998**: 5% Bias, 4% CV

<table>
<thead>
<tr>
<th>Source of Test Error</th>
<th>Biological Variation, % CVₐ</th>
<th>Performance Specification, %CV</th>
<th>No. of Patient Samples With  ≤10% Total Error in Mean, p ≥.95</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological only</td>
<td>7.5</td>
<td>0</td>
<td>3 Among Lab 3 Within Lab</td>
</tr>
<tr>
<td>Biological plus routine analytical</td>
<td>7.5</td>
<td>Interim: 3.65</td>
<td>4 Among Lab 6 Within Lab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1998: 1.58</td>
<td></td>
</tr>
<tr>
<td>Biological plus limit for analytical</td>
<td>7.5</td>
<td>Interim: 7.00</td>
<td>6 Among Lab 5 ▲</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1998: 3.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**CVₐ** - Average intraindividual CV = 7.5%
- 95% range of intraindividual CV's = 24.5%

**CVₐ** - Polynomial coefficients for regression of mean lnCVₐ upon analyte concentration = 2.455; -0.0118116; 0.0000521829.
- s lnCVₐ = 0.4400118.

Average long-term intralaboratory CVₐ in 1989/90 at medical decision levels.
- 35 mg/dL - 8.21% CV
- 45 mg/dL - 7.61% CV

Average bias of singlet results and of methods is unknown.

*CVₐ = β within lab.

▲10% error in the test mean cannot be attained with 95% confidence under these goals and allocation of components of error.
Appendix II

Formulas Used for Calculations for Tables II-2 through II-4

For referral of samples from one patient among all laboratories, each sample analyzed in singlet.

\[
\left(\frac{G_T}{1.96}\right)^2 = \frac{CV_b^2 + CV_\beta^2 + CV_\gamma^2 + CV_\varepsilon^2}{n}
\] (1)

For referral of all samples from one patient to a single laboratory, each sample analyzed in singlet in separate analytical runs.

\[
\left(\frac{G_T}{1.96}\right)^2 - \beta^2 = \frac{CV_b^2 + CV_\gamma^2 + CV_\varepsilon^2}{n}
\] (2)

\[
N = \text{int} (n)
\] (3)

\[
CV_\gamma = \frac{1}{2} CV_\varepsilon
\] (4)

\[
CV_\beta = |\beta|
\] (5)

Analytical performance of a measurement process at the limits of acceptable analytical performance:

\[
CV_\varepsilon = e^{(\ln Gcv)} = Gcv
\] (6)

\[
G_B = CV_\beta + CV_\gamma \text{ (among labs)}
\] (7)

\[
G_B = |\beta| + CV_\gamma \text{ (within lab)}
\] (8)

Analytical performance of a stable measurement process representative of population 95 percent of which conform to the analytical goals:

\[
CV_\varepsilon = e^{(\ln Gcv - 1.645 (\ln CV_a))}
\] (9)

\[
\frac{G_B}{1.96} = CV_\beta + CV_\gamma \text{ (among labs)}
\] (10)

\[
\frac{G_B}{1.96} = |\beta| + CV_\gamma \text{ (within lab)}
\] (11)
Recommendations on Lipoprotein Measurement

Procedure to measure individual laboratory conformance to analytical goals for lipids and lipoprotein-cholesterol

A. Data production within a laboratory measurement system:
   1. For a sufficient period (e.g., 1 year) include two levels of matrix internal quality control materials in each analytical run.
   2. Assay the matrix control nearest the medical decision cutpoints in duplicate a sufficient number of times (e.g., once per week).
   3. Measure bias (%B).*

B. Calculations†:
   1. Calculate the CV of the duplicates (CV_w).
   2. Calculate the total CV (CV_t) of the control at the same concentration.
   3. Calculate (CV_t^2 – CV_w^2)^(1/2) = CV_b.
   4. Calculate total error (TE) = %B + 1.96 (CV_t).

C. Goals†:
   1. CV_w < G_cv.
   2. CV_b + %B < G_b.
   3. TE ≤ G_te.

Where the goals for lipids and lipoproteins are as follows:

<table>
<thead>
<tr>
<th></th>
<th>G_T</th>
<th>G_B</th>
<th>G_cv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>8.9%</td>
<td>≤ ± 3%</td>
<td>≤ 3%</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≤ 15%</td>
<td>≤ ± 5%</td>
<td>≤ 5%</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>≤ 22%</td>
<td>≤ ± 10%</td>
<td>≤ 6%</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>≤ 12%</td>
<td>≤ ± 4%</td>
<td>≤ 4%</td>
</tr>
</tbody>
</table>

The primary goal, G_te, is met when both G_cv and G_b are met. Although G_te can be met when either G_cv or G_b are exceeded somewhat, clinical and analytical performance are optimal when all three goals are met.

To calculate the total error for cholesterol, LDL-cholesterol, HDL-cholesterol, or triglycerides, substitute the respective goals in C. above.

* Percent bias is calculated as the mean difference between measured value and reference value, expressed as a percent of the reference value. Bias (%B) can be measured by (1) periodic use of fresh frozen sera, prepared to accurately simulate the normative patient sample, the total cholesterol concentration of which is known with a total error <1 percent or (2) routine or periodic use of a reference material, the total cholesterol concentration of which is known with a total error <1 percent, and the analytical specificity of which is known with the method in use. (One such reference material is College of American Pathologists [CAP] Chemistry Survey serum, the target values of which have been validated by direct NIST-confirmed comparisons with fresh frozen sera. For many common methods, this material will be available from the CAP as the 1995 Crosslink© product and the 1994 Survey Validated Reference Material [SVRM] product.)

† CV, coefficient of variation, calculated as standard deviation / mean x 100

W, within-run CV
B, among-run CV
T, total CV, includes within-run and among-run variation
TE, total error
G_cv, goal for CV_w
G_b, goal for bias
G_te, goal for total error
The Symbols and Abbreviations Are Denoted as Follows:

- **CV<sub>b</sub>**: Intraindividual biological variation of the analyte as % CV. The average CV<sub>b</sub> is used for calculations.
- **CV<sub>β</sub>**: Variation of the fixed analytical bias of measurement processes among all laboratories as % CV. β is a random normally distributed variable with mean equal to zero.
- **β**: The fixed analytical bias of a specific measurement process within a laboratory as a percent of the true value of a sample that is numerically equal to CV<sub>β</sub>.
- **CV<sub>γ</sub>**: Variation of the means of analytical runs as % CV. γ is a random, normally distributed variable with mean equal to zero.
- **CV<sub>ε</sub>**: Variation of results within an analytical run about their mean as % CV. ε is a random, normally distributed variable with mean equal to zero.
- **CV<sub>a</sub>**: Analytical CV (empirical).
- **G<sub>T</sub>**: The goal for allowable total error of the test as percent of the expected value of a patient.
- **G<sub>CV</sub>**: The goal for allowable analytical imprecision (CV<sub>ε</sub>) as % CV.
- **G<sub>B</sub>**: The goal for allowable analytical bias as the percent difference between the mean of an analytical run and the true value of the specific sample.
- **N**: The number of samples obtained from a single patient at different times and each sample analyzed in singlet.
- **1.96**: Two-sided standard normal deviate for 95% of a normally distributed population.
- **1.645**: One-sided standard normal deviate for 95% of a normally distributed population.
- **s**: Standard deviation of ln of CV<sub>a</sub>.
Recommendations on Lipoprotein Measurement
References for Appendix II


Recommendations on Lipoprotein Measurement
Appendix III.
National Reference Method Laboratory Network
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