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National Cholesterol Education Program Recommendations for Triglyceride Measurement: Executive Summary

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Triglyceride is found in all plasma lipoproteins but is the major lipid component of those lipoproteins with a density <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.[4] 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% 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 concentrations rising fairly rapidly, reaching peak concentrations ~ 4 h after ingestion of a fat-containing meal. They remain above fasting concentrations for ~ 8 h or more as chylomicrons are removed from the circulation.

Increased triglyceride concentrations in the fasting state are of clinical importance in a number of conditions. With severely increased triglyceride concentrations [>1000 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 increases 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 concentrations necessary and important.

The relation 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 relation with HDL. In most studies, HDL-cholesterol is reduced when triglycerides are increased and increases when the triglyceride increase 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

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[4] Nonstandard abbreviations: NCEP, National Cholesterol Education Program; ATP, Adult Treatment Panel; CDC, Centers for Disease Control and Prevention; and INT, iodonitrotetrazolium.
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Table 1. NIH Consensus Conference "working" classification for fasting triglycerides.[a]

Category	Fasting plasma triglyceride concentration, mg/dL (mmol/L)
Normal	<250 (<2.8)
Borderline hypertriglyceridemia	250–500 (2.8–5.6)
Definite hypertriglyceridemia	>500 (>5.6)

[a] Endorsed by Consensus Development Conference, NIH, 1992 (NIH Consensus Conference 1993).
Source: NIH Consensus Conference 1984.

(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.

A working definition of hypertriglyceridemia has been adopted by the National Institutes of Health Consensus Conference on Hypertriglyceridemia (Table 1). Diet and drug therapy is recommended for patients with fasting triglycerides >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 concentrations <500 mg/dL depends on the presence of other lipid abnormalities such as familial combined hyperlipidemia or dysbetalipoproteinemia. ATP II modified the definitions of hypertriglyceridemia somewhat (Table 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 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. 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_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.

Table 2. ATP II classification for triglyceride concentration.

Category	Serum triglyceride concentration, mg/dL
Normal triglycerides	<200
Borderline-high triglycerides	200 to 400
High triglycerides	400 to 1000
Very high triglycerides	>1000

Source: NCEP 1994.

With a CV_a of ~ 3%, within-month data [5] reveal that the biological variance for triglycerides approximates >90% of the total intraindividual variance. Even in the fasting state, considerable biological variation occurs within individuals. In subjects maintaining a carefully monitored NCEP Step I or better diet, and in whom triglycerides were measured twice over a 2-week period, the percent difference between triglyceride concentrations of the two specimens was approximately five times greater than that for cholesterol; >75% of subjects showed deviations >10% during a 2-week period. The Lipid Research Clinics found the average total intraindividual variation for paired fasting analyses performed an average of 2.5 months apart on samples from 7055 fasting persons was ~25% for triglyceride. Unlike total or HDL-cholesterol, no detectable seasonal differences in triglyceride concentrations have been seen. In the nonfasting state, the CV_T of triglyceride is considerable among individuals, whether for a diurnal (6.3–65%), within-month (12.9–34.8%), or within-year (12.9–39.9%) 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 Wadström (*2*) and the techniques of Van Handel and Zilversmit (*3*) and Lofland (*4*). 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

[5] Unpublished data supplied by one of the authors (E.A.S.).

chromogen whose absorbance is measured at 570 nm. The CDC reference method has been modified to replace chloroform with methylene chloride 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 CVs attainable with this method range from 2% to 6%. The CDC reference method is much too cumbersome for use in the routine clinical setting.

Routine Method

Since the mid-1970s, enzymatic methods have virtually replaced the earlier chemical methods in all routine clinical laboratories. In the current enzymatic methods, triglycerides are first hydrolyzed with a bacterial lipase (triacylglycerol acylhydrolase). 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 iodonitrotetrazolium 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+ by using glycerol phosphate dehydrogenase. The appearance of NADH is then measured at 340 nm.

In another variation, glycerol 3-phosphate is oxidized with glycerol phosphate oxidase to generate hydrogen peroxide. The peroxide is then reacted with 3,5-dichloro-2-hydroxybenzenesulfonic acid 4-aminophenazone in the presence of horseradish peroxidase as the chromogenic system. Ferrocyanide minimizes interference from increased 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 patients' specimens when assayed by enzymatic methods, since they measure triglyceride as the quantity of glycerol in the specimen. Most of the older chemical methods involved 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 with glycerol as the lubricant for stopper insertion.

Quantification of free glycerol to obtain true triglyceride concentrations is of limited importance in most patients' situations, since various studies indicate that the potential "error" due to free glycerol is <9 mg/dL in 99% 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 patients' 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 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' Chemistry Survey, only ~6% 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 2). The

advantage of this approach is 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 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% of reference values *and* precision consistent with a CV ≤3%. These guidelines lead to a total error of 8.9% for a laboratory operating at the limits for accuracy and precision. Thus, a laboratory with a 3.5% bias and a CV of 2.0% would not be within the guidelines because the bias exceeds 3%. However, the total error for the laboratory would be 7.4%, well within a total error criterion of 8.9%. 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: total error ≤15%. This is consistent with limits for accuracy and precision (CV) of ≤ ±5% and ≤5%, respectively. 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 analytical goals as indicated in the accompanying paper (*1*).

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 Healthcare 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 after a 9- to 12-h 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 <9 h. It is likely that, on average, fasting triglyceride will be overestimated [~20 mg/dL (0.23 mmol/L)] in patients who have fasted 9 h. 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 1–4% 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 min 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 min of application.

Stored samples.

- Serum or plasma should be removed from cells within 3 h 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 patient's 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% and a CV_a of 5%, the observed CV for the mean triglyceride value is 14.0%, and the difference between the means of sequential series of three samples should not exceed 39% 95% of the time. The difference between the sequential individual values in each series should not exceed 67%. 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 the proximity of that value 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% and a CV_a of 5%, the observed CV for the mean triglyceride value is 17.1%, and the difference between the means of each sequential series should not exceed 48% 95% of the time. The difference between the sequential individual values within each series should not exceed 67%. 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)].

On the basis of prevailing triglyceride concentrations,[6] 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% of the general population as being above or below the 200 mg/dL cutpoint and 96% 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%. One set of conditions that satisfies this recommendation is that triglycerides be measured with an accuracy of ±5% and a CV_a of ≤5%.

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 who handle 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 healthcare 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:

1) 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 by using a "reflex" ordering system.

2) 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.

3) 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.

4) 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 CDC should take the following steps:

1) The CDC should validate and publish the reference method for triglycerides.

2) The CDC should collaborate with the National Institute of Standards and Technology (NIST) on the development of a definitive method for triglycerides.

3) 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:

[6] Unpublished data from the National Health and Nutrition Examination Survey III, kindly provided to the Working Group on Lipoprotein Measurement by the National Center for Health Statistics.

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

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

1) Continue the present policy of requiring standardized triglyceride measurements for government-supported clinical and epidemiological studies.

2) 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:

1) A definitive method for triglycerides is needed. The relation of the CDC reference method to such a definitive method needs to be established.

2) 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.

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

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