Are advanced lipoprotein testing and subfractionation clinically useful?

**Advanced Lipoprotein Testing and Subfractionation Are Clinically Useful**

H. Robert Superko, MD

Advanced lipoprotein testing and subfractionation have been used for scientific medical investigations in humans for >50 years. Original work from the Framingham Heart Study and the Lawrence Livermore Study, which was conducted at the University of California, first suggested the clinical utility of such advanced testing and provided the groundwork for future research. In the subsequent >50 years, clinical trials have documented that aspects of these tests contribute insight into the atherogenic process that is independent of standard lipid test results. Multiple lifestyle and pharmacological treatment studies have been conducted that reveal significant differences in response to treatment based on lipoprotein subclass classification. These changes in lipoprotein subclass distribution have been linked to differences in arteriographic outcome. Standard tests of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) misidentify coronary heart disease (CHD) risk status in a substantial portion of the population. Tests of apolipoprotein concentrations are superior to standard LDL-C tests, and it can be argued that they should replace standard lipoprotein cholesterol testing. Advanced lipoprotein tests that were previously available only from university research laboratories are now provided by several commercial laboratories. Although clinical utility has been demonstrated, on the basis of research laboratory quality tests, caution is advised relative to quality control issues and commercial laboratory testing.

**Response by Mora on p 2395**

History and Recent Changes

Over the past 2 decades, evidence has revealed that standard lipoprotein measurements of triglycerides, total cholesterol, LDL-C, and HDL-C fail to identify many lipoprotein abnormalities that contribute to CHD and peripheral vascular disease risk. Advanced lipoprotein tests (ALTs) lend insight into subtle yet important aspects of lipoproteins and atherosclerosis that help to explain the relative failure of the LDL-C–lowering strategy to stem the epidemic of atherosclerosis. ALTs can be utilized in 4 basic ways: (1) to enhance the accuracy of atherosclerosis risk prediction, (2) to enhance the accuracy of outcome prediction, (3) to assist in treatment selection and dose adjustment, and (4) to counsel first-degree relatives of patients with atherosclerosis. Historically, ALTs have been relegated to research laboratories and clinical trials and thus unavailable to clinicians. Subfractionation of lipoproteins was accomplished in university research laboratories by methods such as analytic ultracentrifugation, density gradient ultracentrifugation, gradient gel electrophoresis, immunoaffinity chromatography, and 2-dimensional gel electrophoresis. This has changed in the past 10 years, and now several commercial laboratories offer ALTs, some of which...
can be performed in standard hospital laboratories. Some companies are developing point of care systems that in the near future may allow physicians to conduct many of these tests in laboratories associated with relatively small clinical groups. Current Procedural Terminology codes exist for some tests. Thus, “advanced” lipoprotein testing and the interpretation measurements can now be incorporated into routine clinical care. The purpose of this article is to review the clinical utility of ALTs that include apolipoprotein and lipoprotein subclass distribution measurements.

Lipoprotein Metabolism Review

A brief review of lipoprotein metabolism helps to set the stage for ALT utilization. Lipoproteins are a diverse group of spherical or discoid particles composed basically of cholesterol, triglycerides, phospholipids, and apoproteins. They are traditionally separated into categories on the basis of density (Table 1). The traditional categories include the triglyceride-rich very-low-density lipoproteins (VLDLs), the intermediate-density lipoproteins (IDLs), the relatively cholesterol-rich LDLs, and HDLs. IDL is defined as the lipoprotein mass in the Svedberg flotation intervals Sf 12 to 20 and has been linked to coronary artery disease risk and arteriographic progression. This is of clinical relevance because the most common laboratory method of determining LDL-C involves precipitation of apolipoprotein (apo) B–containing lipoprotein particles, measurement of the cholesterol content of the remaining plasma (HDL-C), and then calculation of LDL-C with the Friedewald equation. When this method is used, IDL cholesterol is included in the LDL-C number. When IDL and LDL are individually determined, it has been shown that the natural history of coronary artery disease progression is related to IDL and inversely to HDL, but less to LDL. IDL has also been reported to be significantly associated with atherosclerosis progression as determined by carotid wall intima-media thickness. HDL particles play a role in the process that has been termed “reverse cholesterol transport.”

### Table 1. Definition of Lipoproteins Based on Density Ranges of Lipoprotein Particles

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Hydrated Density, g/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&lt;0.94</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.94–1.006</td>
</tr>
<tr>
<td>LDL</td>
<td>1.006–1.063</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006–1.02</td>
</tr>
<tr>
<td>LDL (LDL2)</td>
<td>1.02–1.063</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063–1.20</td>
</tr>
<tr>
<td>HDL2</td>
<td>1.063–1.125</td>
</tr>
<tr>
<td>HDL3</td>
<td>1.125–1.20</td>
</tr>
<tr>
<td>(HDL1)</td>
<td>Close to 1.06</td>
</tr>
</tbody>
</table>

Enzymes and Transfer Proteins

Five major enzymes play a role in basic lipid metabolism: lipoprotein lipase (LPL), hepatic lipase, endothelial lipase, lecithin-cholesterol acyltransferase, and acyl-CoA:cholesterol acyltransferase. LPL is a lipolytic enzyme located on the surface of vascular endothelial cells and macrophages. It is responsible for triglyceride hydrolysis. Deficiency in LPL activity is often associated with substantial increases in plasma triglycerides and low HDL-C. Normal LPL function is essential for normal triglyceride hydrolysis, and apoC-II is a cofactor for LPL action. ApoC-II deficiency results in elevated triglycerides due to reduced LPL activity. Hepatic lipase is an enzyme synthesized by hepatocytes and binds to endothelial cells, allowing it to interact with lipoproteins as they traverse the liver. In conjunction with cholesteryl ester transfer protein activity, hepatic lipase is believed to reduce the core of large HDL2 particles and play a role in the reconversion of HLD2 to HDL3. Hepatic lipase may play a pivotal role in the production of small, dense LDL. Endothelial lipase is a lipolytic enzyme that uses phospholipids as the substrate. Lecithin-cholesterol acyltransferase is responsible for the esterification of cholesterol molecules in HDL. Acyl-CoA:cholesterol acyltransferase serves to convert free cholesterol to esterified cholesterol intracellularly. Cholesteryl ester transfer protein mediates the exchange of triglycerides from VLDL/LDL particles for cholesterol ester in HDL particles.

Membrane Modulators

Membrane modulators are factors that affect the ability of cholesterol to enter or leave the cell. Lipid-free apoA-I, apoA-II, apoA-IV, apoC, and apoE can cause an efflux of phospholipids and cholesterol. The clinical importance of this knowledge involves the recent reports that use of apoA products may provide a clinical treatment option in the future. The ATP binding cassette transporter 1 is a protein that plays an important role in reverse cholesterol transport through transmembrane lipid transport. This process may serve to “flop” cholesterol and phospholipids from the inner to the outer side of the plasma membrane, where it can be picked up by lipid-poor lipoproteins.

Apolipoproteins

Apolipoproteins are proteins attached to a lipoprotein particle and are given alphabetical names such as apoprotein A, B, C, D, and E. By protruding from the surface of the lipoprotein, they can be recognized by a receptor and assist in uptake or activation of cellular mechanisms. They can also serve as cofactors for specific enzymatic reactions. Inherited defects in the amino acid sequence of these proteins can affect normal lipoprotein metabolism by interfering with receptor binding or their actions as cofactors.

The apoprotein content of lipoproteins varies. The LDL particle is somewhat unique in that it is in part defined by having only 1 apoprotein attached, apoB-100. Because of this
unique characteristic, apoB-100 concentration reflects the number of LDL particles.

ApoA can be identified as several forms including apoA-I and apoA-II and accounts for ≈70% of the apoproteins on the HDL particle. A-I is also a constituent of chylomicrons, and its synthesis in the intestine is increased after a fatty meal.\(^2\) Although apoA-I measurement reflects HDL particle concentration, unlike the close relationship between apoB-100 reflecting LDL particle number, elevated apoA-I values may also reflect triglyceride-rich particles that have apoA-I attached and thus may not accurately reflect HDL number.

Apo(a) is an important apoprotein in regard to cardiovascular risk. When attached to apoB and LDL, it is termed lipoprotein(a), or Lp(a) for short. The importance of this lipoprotein lies in its strong association with CHD and carotid atherosclerosis. Elevated levels may be present in as many as 20% to 40% of individuals with CHD. The gene is located on chromosome number 6 and inherited in a dominant fashion, as well.\(^2\) Apo(a) serves as an identification protein for specific receptors located on hepatic and peripheral cells involved with lipoprotein metabolism.\(^2\) ApoB has been identified as primarily 2 apoproteins that are immunologically distinct. ApoB-100 is produced in the liver and attached to LDL particles. ApoB-48 is derived from the intestines and is approximately half the molecular weight of apoB-100. It is attached to triglyceride-rich particles and not to LDL particles. Most commercial apoB antibody tests are not specific for B-100 but reflect both B-48 and B-100. Although whole plasma determination of apoB concentration tends to reflect LDL particle number, it may be confused by B-48 combined with B-100, particularly in a nonfasting state. Nevertheless, whole plasma apoB has been shown to reflect CHD risk.\(^2\)

ApoC, along with apoA-I, is an activator of lecithin-cholesterol acyltransferase. The hydrolysis of triglycerides by LPL is dependent on apoC-II. This is reflected by the substantial elevation in chylomicrons and VLDL seen in persons lacking this apoprotein.\(^2\) ApoE plays an important role in hepatic clearance of VLDL remnants and HDL recognition. ApoE can be identified as a number of different isoforms or genotypes that are distinguished on the basis of amino acid or DNA differences.\(^2\)

**Lipoprotein Subclasses**

LDL is not a homogeneous category of lipoproteins but consists of a set of discrete subspecies with distinct molecular properties, including size and density.\(^2\) In normal subjects, 7 major LDL subspecies can be identified (I, IIa, IIb, IIIa, IIIb, IVa, IVb). LDL-I is the largest and least dense, and the smallest, LDL-IVb, is the most dense. Accurate and reproducible determination of LDL subspecies historically has been made possible by 2 well-established research laboratory methods: gradient gel electrophoresis, which separates LDL particles on the basis of their differing size, and analytic ultracentrifugation, which separates the particles into 12 regions on the basis of their differing density.\(^2\) In most healthy people, the major LDL subspecies are large or buoyant.

HDL is derived from both intestinal and hepatic sources. Hepatic HDL, in a nascent form, appears as a disk-shaped structure. Intestinally derived HDL is more spherical and varies in its protein composition. Both of these HDL particles are relatively small and cholesterol poor and can be classified as HDL3. After interaction with lecithin-cholesterol acyltransferase and LPL, cholesterol ester content is increased, and the particle becomes less dense and larger and is classified as HDL2. On the basis of the relative density obtained in the analytic ultracentrifugation, the more dense, relatively cholesterol-poor form is termed HDL3 (1.25 to 1.21 g/mL), and the less dense, relatively cholesterol-rich form is termed HDL2 (1.062 to 1.21 g/mL).\(^2\)

**Detailed Lipoprotein Tests Have Clinical Utility**

**LDL Subclass Distribution**

The clinical importance of LDL subclass distribution has been reviewed previously.\(^1\) Determination of the small, dense LDL subclass pattern B identifies a group of individuals that carry a 3-fold increased CHD risk.\(^2\) In the Quebec Cardiovascular Study, statistical adjustment for LDL-C, triglycerides, HDL-C, and apoB had virtually no impact on the relationship of small LDL and CHD risk.\(^3\) However, the presence of other risk factors, such as elevated apoB, magnified the risk associated with small LDL. A second clinical use is the identification of CHD patients with rapid arteriographic disease progression. A nearly 2-fold greater rate of arteriographic progression is reported in LDL pattern B compared with pattern A CHD patients. After treatment, arteriographic benefit appears to be greater in patients classified as LDL pattern B compared with pattern A.\(^3\) A third potential clinical use is based on the observation that reduction in the predominance of small LDL is associated with arteriographic benefit, often independent of other cardiovascular risk factors such as age, smoking, weight, and blood pressure and often stronger than the association of disease change with change in LDL-C or apoB.\(^1,3,3,3,4\)

Another clinically relevant aspect of LDL subclass determination is the differential LDL subclass distribution response to a variety of treatments. Individuals with a stable LDL pattern B tend to have significantly greater reduction in LDL-C, apoB, and small LDL in response to a reduced-fat diet compared with LDL pattern A subjects.\(^5\) Nicotinic acid has a differential effect on LDL subclass distribution depending on whether the patient is classified as LDL pattern A or B. LDL pattern B subjects respond with significantly greater reductions in small LDL mass and increase in LDL size.
compared with LDL pattern A subjects.36 Similarly, treatment with medications that tend to increase triglycerides, such as the selective and nonselective β-blockers, can increase the predominance of small LDL counterbalanced by a reduction in large LDL for no net change in total LDL-C. Drugs that tend to reduce triglycerides, such as prazosin, have the opposite effect.37

HDL Subclass Distribution

Similar to LDL subclasses, determination of HDL subclass distribution provides insight into CHD risk prediction, arteriographic progression, arteriographic outcome, and treatment response. The observation that different “types” of HDL exist was first observed by John Gofman, MD, in 1951.38 The possible role of different HDL subtypes in the atherogenic process was discussed in view of the results of the Lawrence Livermore study in 1966.39 In the intervening >40 years, a body of knowledge has accumulated that indicates that differences in HDL subclass distribution help to predict CHD risk and arteriographic progression. Furthermore, treatment-induced change in HDL subclass distribution has been associated with arteriographic outcome.

A number of investigations have reported significantly lower HDL2 in CHD patients compared with control patients, and this inverse relationship between HDL-C, HDL2, and CHD is particularly strong in men with type 2 diabetes mellitus.39–43 In type 2 diabetes patients, the difference between HDL2-C in the myocardial infarction (MI) and non-MI group persists after adjustment for physical activity, alcohol intake, obesity, duration of diabetes, and glycemic control. Franceschini and colleagues,44 in 1987, reported that compared with “normal” individuals, MI patients had significantly lower HDL-C, and the most striking characteristic was a 30% decrease in HDL2; they suggested that HDL2 deficiency may be a primary alteration in MI patients. Not surprisingly, differences in HDL subclass distribution have been found to differ significantly between patients with peripheral vascular disease and healthy controls.45

The cardioprotection aspects of high HDL-C appear to be related to differences in HDL subclass distribution as well. Hyperalphalipoproteinemia is an elevated HDL-C condition often associated with low CHD risk. When the HDL in these subjects is primarily HDL2 or LpAI, cardioprotection is observed, whereas those with high HDL-C values, but primarily composed of LpA-I: A-II, exhibit less CHD protection.46 The prevalence of disorders of HDL subclass distribution may also differ by ethnic group, and individuals of Asian Indian descent have been found to have abnormally low HDL2 compared with matched white subjects despite “normal” HDL-C values.47 Finally, the inheritance of HDL subclasses has revealed correlations among family members for specific HDL subclasses that are independent of HDL-C and apoA-I.48 This suggests that determining HDL2 levels in first-degree relatives of CHD patients with low HDL2 may be informative.

Not all studies have revealed an association of HDL subclass with CHD. The Caerphilly and Speedwell Collaborative Heart Disease Studies investigated 4860 middle-aged men followed for 3 to 5 years.49 Both HDL2 and HDL3 were inversely associated with CHD, and the relationship appeared to be stronger for HDL3 than HDL2. They concluded that the risk for CHD based on HDL-C could not be improved on by the determination of HDL2 or HDL3. The Atherosclerosis Risk in Communities study has also reported no large differences between HDL2-C in subjects with and without known CHD.50

The severity of atherosclerosis and progression over time have been linked to differences in HDL subclass distribution. In 104 men who underwent coronary arteriography, a negative correlation was noted for HDL2-C and disease severity that was independent of triglycerides.51 Johansson and colleagues52 utilized coronary arteriography in 60 male MI survivors to reveal significant associations between HDL subclass distribution and the extent of CHD (r =−0.53, P<0.001) as well as progression over time (r =−0.38, P<0.01). Of clinical interest was the analysis that in normotriglyceridemic patients (triglycerides <≈200 mg/dL), the correlations were even more striking with severity of lesion (r =−0.72, P<0.001) and rate of lesion progression (r =−0.58, P<0.01).

Evidence suggests that treatment-induced change in HDL subclass distribution can be associated with significant arteriographic benefit and a reduction in cardiovascular events.53 A quantitative 2-dimensional gel electrophoresis, immunoblot, image analysis method separates HDL particles into α1, α2, and α3 with sizes of 11.2, 9.51, and 7.12 nm, respectively. A significant inverse relationship was reported between change in the α1 HDL subclass population and coronary artery stenosis ( r =−0.24, P<0.01).54 The treatment combination of simvastatin plus niacin increased the standard HDL-C measurement 20% but increased α1 HDL 115%. When analyzed by tertile change in α1 HDL, most of the subjects in the third tertile, who received the optimal arteriographic benefit, were in the simvastatin plus niacin group, but 20% of subjects in this treatment group had a poor α1HDL response and poor arteriographic outcome. In addition, a group (15%) in the third and best α1HDL tertile was in the placebo group but received diet and exercise advice. Thus, individual variability makes the measurement of HDL subclass change in the individual patient of clinical utility versus the “1 treatment fits all” approach.

Therapeutic treatments can have a differential effect on HDL subclass distribution. Prospective randomized studies at the University of California have revealed that shifting diet calories from 20% of total calories from fat to 10% results in a 15% reduction in HDL-C but an even greater 56% reduction in HDL2 in subjects carrying the atherosclerosis susceptibility trait.55 A prospective alcohol drinking study revealed that increases in HDL-C level appear to be primarily in HDL3.56 Loss of excess body fat (4 to 6 kg), either through exercise or...
reduced dietary calories, can increase HDL-C 10% but increase HDL2 40%. Thus, lifestyle maneuvers can have a significantly greater effect on HDL subclass distribution than that reflected by standard HDL-C measurements.

Treatment with nicotinic acid has been reported to result in a significant reduction in small, dense LDL, often accompanied by an increase in HDL2. The effect of 3 lipid treatments on standard lipoprotein measurements as well as HDL subclass distribution has been reported. At the dose levels of 20 mg/d atorvastatin, 20 mg/d simvastatin, and 40 mg/d lovastatin plus 1000 mg/d nicotinic acid, the HDL-C was increased 4%, 8%, and 19%, respectively, but the HDL2 was increased 28% in the atorvastatin group, 40% in the simvastatin group, and 119% in the nicotinic acid plus lovastatin group. A similar differential HDL subclass distribution response was seen after treatment with gemfibrozil.

**Should Apolipoprotein Measurements Replace Standard LDL-C and HDL-C Measurements?**

Variability in the accuracy and reproducibility of standard measurements, such as HDL-C, may complicate patient management. It has been suggested that the apolipoprotein measurements replace the standard LDL-C and HDL-C because they are more accurate, more reproducible, and reflect CHD risk better than standard lipoprotein cholesterol measurements.

**Apolipoprotein B**

A substantial body of evidence strongly suggests that apoB should replace LDL-C as the primary measure of atherogenic lipoproteins. The methodology is now standardized, and a national apoprotein standardization program exist. Clinical trials such as the Framingham Heart Study provide ranges of risk that are applicable to the patient population. In the setting of normolipidemia, plasma apoB values are consistently lower than the LDL-C value. However, the condition described as hyperapobetalipoproteinemia is characterized by apoB values higher than predicted on the basis of the LDL-C value. A remarkably high incidence of hyperapobetalipoproteinemia (81%) has been reported in the post-MI population that exhibit relatively “normal” LDL-C values yet have disproportionately elevated LDL apoB.

The clinical importance of determining apoB values lies not only in the identification of hyperapobetalipoproteinemia but in the association of elevated apoB with other cardiovascular risk predictors that results in identification of a group of patients at extremely high risk for cardiovascular events. The Quebec Cardiovascular Study investigated the relationship of small LDL, elevated fasting insulin values, and elevated apoB in relation to CHD risk. Each abnormality individually contributed to CHD risk. However, the combination of all 3 abnormalities identified a group of individuals who were at a 20-fold increased risk for cardiovascular events.

**ApoB/A Ratio**

The apoB/A-I ratio reflects the relative number of apoB-containing atherogenic particles compared with the relatively protective apoA-I. This ratio is superior to the standard LDL-C/HDL-C ratio in predicting MI risk. The Apolipoprotein-related Mortality Risk Study (AMORIS) investigated these relationships in 94,667 men and 75,675 women. The standard total cholesterol/HDL-C ratio underestimated risk in 69% of men and 85% of women while overestimating the risk in 26% or men and 12% of women. Use of the conventional cholesterol ratios rather than the apoB/apoA-I ratio results in frequent and substantial error in estimation of the lipoprotein-related risk of vascular disease. An apoB/A-I ratio $\geq 0.90$ independently predicts MI risk in men and carotid artery intima-media thickness change over 3 years.

**Particle Number**

The concept that the number of atherogenic particles relates to CHD risk and CHD progression is established. The historic method of determining LDL particle number is the apoB-100 measurement. Access to apoB-100 antibodies is not standard in the clinical laboratory community, and whole plasma apoB measurements represent both apoB-48 plus apoB-100. Another method of determining LDL apoB, without the need for an apoB-100 antibody, is to separate atherogenic particles with the ultracentrifuge and determine the apoB concentration in the fraction containing lipoprotein particles of a specific density. However, the concentration of whole plasma apoB provides a direct measure of the number of circulating atherogenic lipoproteins and provides clinical insight beyond the LDL-C measurement. Recent multicountry and Canadian guidelines recommend a target of apoB $<90$ mg/dL in high-risk patients, and others recommend $<80$ mg/dL. Any alternative LDL particle number method must demonstrate adequate correlation with true apoB-100 or LDL apoB measurements.

**ApoE Genotype**

The most common gene affecting LDL-C levels is apoE, which has 3 major genotypes, or isoforms, designated E2, E3, and E4. The most common allele, E3, has a frequency of approximately 0.78, whereas E4 has a frequency of 0.15, and E2 has a frequency of 0.07.

ApoE genotypes can be of use in CHD risk prediction. The Etude Cas-Temoins sur l’Infarctus du Myocarde (ECTIM) study reported a relative risk for MI of 1.33 ($P=0.02$) for subjects carrying the E4 allele, which explained $\approx 12\%$ of MI cases. This finding is consistent with the European Atherosclerosis Research Study (EARS), in which the population-ordered odds ratios for E4/3 and E4/4 were 1.16 and 1.33, respectively. It was concluded that the apoE polymorphism is a major factor responsible for the familial predisposition to CHD.

ApoE genotypes explain part of individual differences in LDL-C response to a reduced-fat diet. Men with the apoE3/4
pattern respond to a reduced-fat diet with significantly greater LDL-C reduction than men with the apoE3/3 pattern. A differential effect of reduced-fat diet–induced reduction in LDL appears to affect large LDL particles more in individuals with the apoE4 allele and least in E3/2 subjects. Because of this, diet-induced LDL-C reduction may have a variable benefit in individuals with different E genotypes and LDL subclass patterns. Postprandial lipid metabolism differences also exist between E3/4 and E3/3 subjects. Subjects with the E4 allele have enhanced postprandial lipemia, which may contribute to increased CHD risk.

Fish oil supplementation has a differential effect on lipids and small LDL based on apoE genotype. Individuals with the E2 allele exhibit significantly (P=0.02) greater small, dense LDL-III and postprandial triglyceride reduction than carriers of the E3 allele.

The apoE4 genotype is also an example of information gained from a blood test that has implications not associated with the primary reason the test was conducted. In families with a history of Alzheimer disease, the presence of the E4 allele increases the risk for developing Alzheimer disease, but the interaction is probably complex and may involve interaction with the amyloid B protein precursor gene on chromosome 21q. It has been recommended that the apoE4 test not be used for the prediction of Alzheimer disease risk.

Remnant Lipoprotein Particles
Remnant lipoprotein particles are another type of atherogenic particle that are the result of triglyceride-rich lipoprotein metabolism. An association of elevated remnant particles with CHD risk has been established, and laboratory assays are currently available. Elevated remnant lipoprotein particle cholesterol levels have been reported to be a significant and independent risk factor for impaired flow-mediated, endothelium-dependent dilatation and angiographically proven coronary artery disease in patients with the metabolic syndrome. The issue of the independent role that remnant lipoprotein particles may play in CHD risk is complicated by the association of other triglyceride-rich particles, small LDL, and low HDL2 with elevated remnant lipoprotein particles.

Analysis by Apolipoproteins
A variety of methods are available for the determination of apolipoproteins and include radial immunodiffusion, immunoturbidimetric method, monoclonal antibody–based radial immunodiffusion, infrared spectroscopy, and nephelometry. All rely on the ability of an antibody to recognize a specific amino acid sequence (epitope) on the apoprotein. This method not only requires a consistently high degree of quality in the antibody production but also recognition that other factors may affect the ability of the antibody to recognize the epitope.

Analysis by Density
The definition of lipoproteins is historically based on density. The gold standard of lipoprotein subclass determination is analytic ultracentrifugation, employed by Dr John Gofman and colleagues to elucidate lipoprotein heterogeneity in the 1960s. This method relies on the ability of lipoproteins to float when exposed to high gravitational forces. Although this method is very precise and reproducible, it requires complicated technology that is expensive, time consuming, and no longer readily available. Several preparative ultracentrifugation methods are available to identify lipoprotein subfractions, including density gradient ultracentrifugation. With this method, 4 distinct LDL subclasses can be identified as LDL-I (largest and most buoyant), LDL-II, LDL-III, and LDL-IV (the smallest and most dense). Although accurate and precise, the technical laboratory demands make this method impractical for common clinical use. However, ultracentrifuge techniques can be used to create standards and controls that may be utilized to ensure quality control with other methodologies.

A rapid ultracentrifugation method, termed vertical auto-profile, has been used to determine relative flotation index as a determination of change in LDL buoyancy. This method determines the cholesterol concentration of multiple lipoprotein fractions based on density. During profile decomposition, peak heights for predefined subcurves for all classes are simultaneously varied until the sum of the squared deviations between the sum of the subcurves and the parent profile is minimized by linear regression.

Analysis by Double Precipitation
A method utilizing double dextran precipitation has been used to separate HDL-C into HDL2-C and HDL3-C. Although relatively inexpensive and rapid, this precipitation method has a degree of laboratory variability that hinders its ability to provide consistently accurate results adequate for individual patient management.

Analysis by Size
Polyacrylamide gradient gel electrophoresis, in nondenaturing conditions, has been widely used to characterize individuals on the basis of LDL and HDL size distribution. With this method, the size of major peaks and percent distribution of 7 LDL subclasses (LDL-I, LDL IIa, LDL IIb, LDL IIIa, LDL IIIb, LDL IVa, LDL IVb) can be determined. With this method, both lipid and protein stains may be utilized. Char-
The characterization of HDL particle size (diameter) by gradient gel electrophoresis has identified 5 subclasses: HDL3c (7.2 to 7.8 nm), HDL3b (7.8 to 8.2 nm), HDL3a (8.2 to 8.8 nm), HDL2a (8.8 to 9.7 nm), and HDL2b (9.7 to 12 nm). It often requires custom-made gels and strict attention to laboratory quality control because small variations in gel quality and laboratory conditions may affect the accuracy. A novel microfluidic gel method has been developed that uses microchip technology with rapid and high throughput capabilities. This method has been standardized against an ultracentrifugation-derived HDL subclass standard.89

Analysis by Immunoaffinity Chromatography
Sequential immunoaffinity chromatography can isolate 2 HDL subclasses defined by their apoA-I and apoA-II content as those with A-I only (LpAI) and those with AI and A-II (LpAI:AII) and their relationship to CHD risk.4 The LpAI only particle is the HDL subclass most associated with cardiovascular protection and is similar to HDL2.90

Analysis by Nuclear Magnetic Resonance
A recent method used to estimate lipoprotein subclass distribution is nuclear magnetic resonance.91 Signals are derived from methyl groups on phospholipids, cholesterol, cholesterol ester, and triglycerides. Nuclear magnetic resonance assumes a constancy of lipid mass contained within a particle of given diameter and phospholipid composition and thus methyl lipid nuclear magnetic resonance signal. This system uses a reference library of lipoprotein subclasses incorporated into a linear least-square fitting computer program and provides a quantitative measurement of lipoprotein subclass distribution.

### Table 2. Laboratory Methods for LDL and HDL Subclass Distribution

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Comment</th>
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<tbody>
<tr>
<td>Analytic ultracentrifugation</td>
<td>Very accurate</td>
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<td>Multiple NIH trials</td>
<td>Time consuming</td>
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<td>Density gradient ultracentrifugation</td>
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<td>Double dextran precipitation</td>
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<td>Accuracy for individual patients is questionable</td>
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<td>Requires no new tests</td>
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<td>Gradient gel electrophoresis</td>
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<td>Multiple NIH trials</td>
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<td>Worldwide research use</td>
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<td>High-performance liquid chromatography</td>
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<td>Difficult to run large numbers of samples</td>
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<td>Immunofinity chromatography</td>
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<td>Inexpensive</td>
<td>Variable results</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Accuracy for individual patients is questionable</td>
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Laboratory methods for LDL and HDL subclass distribution include analytic ultracentrifugation, density gradient ultracentrifugation, gradient gel electrophoresis, immunoaffinity chromatography, double dextran precipitation, microfluidics, vertical autoprofile, nuclear magnetic resonance, high-performance liquid chromatography, light scattering, ion mobility, and estimates based on standard blood lipid measurements. NIH indicates National Institutes of Health.
Figure 1. Correlation of fasting triglycerides (TG) and LDL diameter in angstroms (Dia V11) in 180 subjects participating in a nicotinic acid investigation (r = 0.66, P = 0.0001). Subjects with LDL particle diameter < 257 A may be classified as LDL pattern B, and those with LDL particle diameter > 262 A may be classified as LDL pattern A. Although most subjects with fasting triglycerides > 200 mg/dL are likely to exhibit small LDL pattern B, and those with fasting triglycerides < 70 mg/dL are likely to exhibit LDL pattern A, the overlap in LDL subclass pattern when fasting triglycerides are between 70 and 200 mg/dL makes the use of this relationship hazardous in regard to accuracy of LDL subclass pattern determination.

Figure 2. Correlation of LDL diameter and the triglycerides (TG)/HDL-C ratio in 180 subjects (r = 0.70, P = 0.0001). In this data set, a triglycerides/HDL-C ratio > 4.0 tended to identify LDL pattern B subjects, and a triglycerides/HDL-C ratio < 4.0 could not accurately predict LDL subclass pattern with the reliability necessary for clinical use.

Analysis by Other Methods
High-performance liquid chromatography has been utilized to determine LDL and HDL subclass distribution. Ion mobility is a novel method to determine lipoprotein subclasses and utilizes an electrospray method.

Analysis by Estimates Based on Standard Laboratory Tests
Standard laboratory measures of triglycerides, HDL-C, and apoB have a correlation with LDL and HDL subclass distribution. Within specified ranges, these standard measurements can predict subclass distribution with enough accuracy to allow limited clinical utility. The measurements of most clinical utility include fasting triglycerides, HDL-C, triglyceride/HDL-C, and the LDL-C/apoB ratio.

Classification of LDL subclass as either LDL pattern A or B does not necessarily require advanced blood testing. A strong inverse correlation exists between fasting triglyceride values and LDL size. Although this relationship has strong statistical utility, it has clinical utility only in a triglyceride range that has a low probability of misclassification. When fasting triglycerides are < 70 mg/dL, a high probability exists that individuals have predominantly large LDL particles (90.9% are LDL pattern A), and when the fasting triglyceride values are > 200 mg/dL, a high probability exists that individuals predominantly express small LDL pattern B (84.8% are LDL pattern B) (Figure 1). Thus, to accurately predict LDL size in the triglyceride range of > 200 mg/dL or < 70 mg/dL, simple inexpensive fasting triglycerides may be adequate. However, to accurately determine LDL size and LDL A versus B phenotype in patients with fasting triglycerides between 70 and 200 mg/dL, a detailed LDL subclass determination test may be necessary.

It has been suggested that the triglyceride/HDL-C ratio is of use in assessing the presence of small LDL and that 75% of individuals with predominantly small LDL have a triglyceride/HDL-C ratio > 2.0. Data from a nicotinic acid investigation conducted in the United States (Figure 2) suggest that a value > 4.0 may be more reliable. Because only 1 apoB is attached to the LDL particle, the LDL-C/apoB ratio should provide a sense of LDL size. Although a statistically significant relationship exists (r = 0.24, P < 0.001), the relationship is too weak and imprecise to be of clinical value (Figure 3). Likewise, non-HDL reflects atherogenic particles, and a relationship exists between non-HDL and LDL size (r = 0.34, P < 0.001); however, the relationship is also weak and imprecise (Figure 4).

HDL-C has a strong relationship with HDL2 (r = 0.65, P < 0.0001) that has some clinical utility. Utilizing a definition of ≥ 20% HDL2 as low, HDL-C in men < 40 mg/dL is frequently associated with low HDL2 (97.7%), and HDL-C in men ≥ 65 mg/dL is frequently associated with HDL2 > 20% (82.2%). For the HDL-C range > 40 and < 65 mg/dL, too much overlap exists to be of reliable clinical use as a marker of HDL2 level in an individual person (Figure 5).
Laboratory Quality Control and Standardization

One of the most important concepts of ALT is the need for standards and controls and laboratory quality control issues. Quality control is a laboratory issue that clinicians should be familiar with because quality control issues may account for variability in patient results, and clinicians may make clinical decisions based on laboratory values that may not accurately reflect the patient’s true physiological status.96

Quality assurance involves all aspects of the system that ensure consistently accurate and precise test results. Accuracy refers to the measure of how close the reported values are to the “true” value. Precision is a measure of reproducibility. Specificity refers to the accuracy of a test in quantifying what it is intended to measure. To accurately determine the amount of a substance, standards are used to calibrate the test method. Standard deviation is a description of the dispersion of the test results. The coefficient of variation is a measure of the standard deviation divided by the mean value (coefficient of variation = standard deviation/mean). Quality control issues of importance to clinicians include standards and controls, coefficient of variation, and multiple measurements. Even with reasonable quality control, a standard measurement such as a single HDL-C may not be clinically useful in individual patients to determine whether relatively small changes have actually occurred.97

The accuracy of lipoprotein subclass determination is not monitored by national standardization programs. A split sample assessment of the traditional enzymatic methods of determining lipoprotein cholesterol and the established gradient gel electrophoresis method of determining LDL subclass distribution, compared with vertical autoprofile and nuclear magnetic resonance, revealed significant differences (P < 0.001) between methods for total cholesterol, triglycerides, LDL-C, HDL-C, Lp(a), and LDL and HDL subclass distribution.98,99 Complete agreement among methods with respect to LDL subclass phenotyping occurred in only 8% of cases. Assessment of lipoprotein subclasses should involve the use of well-established laboratory methods with demonstrated and highly significant correlations with ultracentrifugation. If such precise methods are not used, then surrogate markers may be of use for large studies, but they can also be misleading in individual patients.

Conclusion

ALTs have clinical utility based on multiple clinical trials spanning 5 decades. With the growing prevalence of the metabolic syndrome, these issues gain further importance and clinical relevance.100 One advantage of ALTs is the greater

**Figure 3.** The LDL-C/apoB ratio correlates with LDL diameter in angstroms (Dia V11) (r = 0.24, P = 0.001) but lacks the ability to discriminate between LDL pattern A and B subjects.58

**Figure 4.** A relationship exists between non-HDL and LDL diameter in angstroms (Dia V11), reflecting a significant statistical relationship (r = 0.34, P < 0.0001) but also substantial scatter that prevents the use of non-HDL to accurately predict LDL diameter in individual patients.58

**Figure 5.** A statistically significant relationship exists between HDL-C and HDL2% in men (n = 549) (r = 0.72, P < 0.0001). Within the HDL-C range of 40 to 65 mg/dL, significant overlap occurs that prevents clinical utilization of HDL-C to accurately predict HDL2%.
insight they provide clinicians into individual patient disorders often masked by standard lipid tests considered to be within “normal” ranges. Many of the disorders identified by ALTs have an inherited pattern, which suggests that screening first-degree relatives may be informative. Some tests are more reliable both from the standpoint of reflecting the underlying physiological disorder and from the standpoint of laboratory accuracy and precision. ApoB is of particular interest in this regard. Change in lipoprotein subclass distribution has been demonstrated with multiple lifestyle and pharmacological interventions and provides the clinician with the opportunity to custom fit treatments to individual patient disorders. Finally, although these tests have clinical utility, the evidence is based on research laboratory quality tests, and caution is advised relative to the accuracy, reproducibility, and precision of commercially available tests.

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Response to Superko

Samia Mora, MD, MHS

Dr Superko provides a comprehensive review and important insights into lipoprotein metabolism and atherogenicity. We both agree that measuring the concentration of low-density lipoprotein (LDL) particles is generally superior to measuring the concentration of LDL cholesterol. The concentration of LDL particles can be obtained in several ways: (1) apolipoprotein B; (2) nuclear magnetic resonance–measured LDL particle concentration; and (3) non–high-density lipoprotein (HDL) cholesterol. Both non-HDL cholesterol and the total/HDL cholesterol ratio correlate highly (≈0.7 to 0.85) with LDL particle concentration and can be obtained easily at no additional cost to standard lipids. Two large primary-prevention studies (Framingham Heart Study and Women’s Health Study) found no substantial improvement in classifying subjects into National Cholesterol Education Program risk categories with apolipoprotein B or nuclear magnetic resonance–measured LDL particle concentration compared with the total/HDL cholesterol ratio. I also suggest that smaller LDL size is positively associated with cardiovascular risk because individuals with predominantly small LDL size have more LDL particles and not because small LDL particles are inherently more atherogenic than large ones. Compared with LDL, less is known about HDL and very low–density lipoprotein size and subclasses in relation to clinical end points. Whether advanced lipoprotein testing and subfractionation may be useful and cost-effective for certain subgroups or for tailoring lipid-altering therapies is an active area of research and awaits further results from randomized trials. Caution is prudent when results are extrapolated from coronary angiography (a surrogate measure) to hard clinical end points in light of recent trials of cholesterol ester transfer protein inhibition. Finally, we both agree that quality control and standardization are important to streamline results obtained from various laboratory methods and to minimize errors in clinical decision making.
Advanced Lipoprotein Testing and Subfractionation Are Clinically Useful
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