Are advanced lipoprotein testing and subfractionation clinically useful?

Advanced Lipoprotein Testing and Subfractionation Are Not (Yet) Ready for Routine Clinical Use
Samia Mora, MD, MHS

Standard lipid tests measure the cholesterol or triglyceride content of lipoproteins, expressed as mg/dL (or mmol/L) of cholesterol or triglyceride. A standard lipid panel includes total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides. These lipids are carried within lipoprotein particles that are heterogeneous in size, density, charge, core lipid composition, specific apolipoproteins, and function. A variety of lipoprotein assays have been developed that subfractionate lipoprotein particles according to some of these properties such as size, density, or charge. These lipoprotein assays have been proposed for improving assessment of risk of cardiovascular disease (CVD) and for guiding lipid-lowering therapies. They are mostly used today in specialized lipid clinics or research studies, and their general utility for clinical practice is not well delineated. In this review, I first provide a summary of current guideline statements that address this topic. Then I give a brief overview of the different laboratory methods available for clinical use and discuss the literature evaluating these methods. Finally, I discuss current limitations to the widespread clinical application of these methods.

Guideline Recommendations
The Third Adult Treatment Panel of the National Cholesterol Education Program and the American Heart Association recommend measuring a standard lipid panel in adults and targeting lipid-lowering therapy on the basis of levels of LDL cholesterol, and non-HDL cholesterol in subjects with hypertriglyceridemia. European and Canadian guidelines recommend measuring standard lipids while also emphasizing the total/HDL cholesterol ratio in risk assessment. Current guidelines do not recommend routine use of advanced lipoprotein tests, mainly because it is unclear how much incremental prognostic information is provided by these tests beyond that available from a standard lipid panel. Recently, an international panel proposed that cardiovascular risk may be more closely related to atherogenic lipoprotein particle number than LDL cholesterol (the atherogenic lipoprotein particle paradigm). This paradigm posits that lipid concentrations in lipoproteins do not equal the concentrations of the lipoproteins themselves and that measuring the concentration of lipoproteins may be superior to measuring their lipid concentrations for estimating CVD risk and determining the adequacy of statin therapy. A subsequent consensus statement endorsed by the American Diabetes Association and the American College of Cardiology advocated measuring atherogenic particle concentration either as apolipoprotein B (apoB) or LDL particle concentration in subjects at high risk for cardiometabolic disorders for assessing CVD risk and guiding therapy, in conjunction with using LDL and non-HDL cholesterol.

Laboratory Methods
A thorough discussion of the various advanced lipoprotein tests is beyond the scope of this review, but a brief summary...
of the common tests is provided below. Several tests use proprietary techniques that have not been published. The number and nomenclature of lipoprotein subfractions are not uniform across the different techniques and have not been standardized, making it difficult to compare results from various tests. The National Academy of Clinical Biochemistry recently called for standardization of the technologies used to determine lipoprotein subfractions. Several of these tests can only be performed at the company that markets the test, limiting the ability to obtain independent information on test performance.

Apolipoproteins
Apolipoproteins are measured in routine clinical laboratories with the use of immunonephelometric or immunoturbidimetric assays. Importantly, international standards have been developed for apolipoprotein B100 (apoB) and A-1. ApoB reflects the number of potentially atherogenic lipoprotein particles because each particle of very-low-density lipoprotein (VLDL), β-VLDL, intermediate-density lipoprotein (IDL), LDL, and lipoprotein(a) particle carries on its surface 1 apoB100 protein. Most of plasma apoB is found in LDL particles. HDL particles do not carry apoB but instead carry apolipoprotein A-1 (apoA-1). However, apoA-1 does not correspond directly to the concentration of HDL particles in the 1-to-1 fashion seen for apoB100 and LDL particles because an HDL particle may carry >1 apoA-1 protein.

Gradient Gel Electrophoresis
The gradient gel electrophoresis (GGE) method (available from Berkeley Heart Lab Inc, Berkeley, Calif) determines the distribution of LDL size phenotype by proprietary segmented polyacrylamide gradient gels, which separate lipoproteins in a gradient gel on the basis of their size and, to a lesser extent, their charge. Pattern A corresponds to large LDL particles; B to small, dense LDL particles; and AB to an intermediate phenotype. This method gives the relative, or predominant, distribution of lipoprotein particles as determined by the predominant peak particle size. ApoB or ultra-apoB (LDL apoB) is measured by immunoassay for additional cost per request.

Density Gradient Ultracentrifugation
This method (available from Atherotec Inc, Birmingham, Ala, as the vertical autoprofile [VAP] II test] measures the relative distribution of cholesterol within various lipoprotein subfractions, quantifying the cholesterol content of VLDL, IDL, LDL, lipoprotein(a), and HDL subclasses. The VAP II also determines the predominant LDL size distribution (eg, A, AB, or B phenotype) but does not provide concentrations of the lipoprotein particles themselves. ApoB is provided at no additional cost, although it is not measured directly.

Nuclear Magnetic Resonance Spectroscopy
This technique (available from LipoScience Inc, Raleigh, NC) is based on the concept that each lipoprotein particle in plasma of a given size has its own characteristic lipid methyl group nuclear magnetic resonance (NMR) signal. Particle concentrations of lipoprotein subfractions of different size are obtained from the measured amplitudes of their lipid methyl group NMR signals. Lipoprotein particle sizes are then derived from the sum of the diameter of each subclass multiplied by its relative mass percentage based on the amplitude of its methyl NMR signal. NMR LipoProfile-II simultaneously quantifies lipoprotein concentrations of VLDL, IDL, LDL, and HDL particles and their subfractions, each expressed as a lipoprotein particle concentration (number of particles per liter) or as an average particle size for each of VLDL, LDL, and HDL.15–18

Ion-Mobility Analysis
This is a newly developed method (available from Quest Diagnostics Inc, Madison, NJ) that measures both the size and concentrations of lipoprotein particle subclasses on the basis of gas-phase differential electric mobility.19

Comparisons
Direct comparisons of these techniques are limited. The correlation for LDL size between NMR and GGE was 0.86 in a small study of men. In another study (n = 324 individuals), LDL size by GGE and NMR was only moderately correlated (Spearman correlation 0.4), and the chance-adjusted κ statistic was moderate (0.3). A more recent study by Ensign et al22 (n = 40 individuals) found the agreement between GGE and NMR to be 70%. However, when results were compared across 4 methods that are used to determine LDL size, complete agreement among the 4 methods examined (GGE, NMR, VAP, and tube gel electrophoresis) for LDL size phenotype was only 8% (Figure 1). This highlights the important need for standardization if these measurements are to be more widely used in clinical practice, especially given the fact that the methods use different principles for subfractionation of lipoproteins.23
What Are Advanced Lipoprotein Tests Often Used for in Clinical Practice?

1. CVD Risk Assessment. Advanced lipoprotein tests are often used to enhance CVD risk assessment, especially in individuals with low or normal LDL cholesterol, by detecting (1) the presence of higher concentrations of atherogenic lipoprotein particle concentrations (eg, apoB or NMR LDL particle concentration) or (2) the presence of small dense LDL particles, which are commonly believed to be more atherogenic than larger LDL particles. Emerging and conflicting data exist about the potential atherogenicity of the various HDL and VLDL particle subfractions, although less is known compared with LDL subfractions.

2. Targets for Lipid-Lowering Therapy. Advanced lipoprotein tests are often used to detect “residual risk” in patients who are already treated with lipid-lowering therapy, often with statins. Advocates of advanced lipoprotein testing point out that ~70% of the patients who otherwise would have had CVD events still experience CVD events, despite LDL cholesterol lowering on statin therapy. This is often referred to as residual risk.

CVD Risk Assessment

Currently, the best validated measure of CVD risk obtained from advanced lipoprotein testing is the concentration of atherogenic particles, such as apoB or NMR LDL particle concentration. First, I discuss how apoB and NMR LDL particle concentration compare with non-HDL cholesterol or the total/HDL cholesterol ratio. Second, I discuss how smaller LDL size may not be an independent risk factor for CVD but instead a surrogate marker for a higher concentration of atherogenic particles.

Is ApoB (or LDL Particle Concentration) Superior to Non-HDL Cholesterol or the Total/HDL Cholesterol Ratio for CVD Risk Assessment?

Outside of standard lipids, apoB is the most widely studied lipoprotein parameter in both primary and secondary prevention populations and statin trials. Most of these studies, but not all, found that apoB was more closely associated with CVD risk than LDL cholesterol, as summarized elsewhere. Similarly, although in fewer studies than apoB, CVD risk is more closely associated with NMR LDL particle concentration than LDL cholesterol.

What remains controversial is whether apoB (or LDL particle concentration) is superior to non-HDL cholesterol or total/HDL cholesterol ratio for CVD risk prediction, and whether potentially small improvements in risk prediction are offset by potentially higher healthcare costs from additional tests.

Two large studies (Apolipoprotein-related Mortality Risk [AMORIS] and INTERHEART) have contributed valuable insight to risk factors worldwide and concluded that apoB was superior to standard lipids. However, these studies have study design limitations. AMORIS used lipids that were not measured by recommended standards and did not have full adjustment for other risk factors. INTERHEART, an international case-control study, measured lipids in the setting of acute hospitalization or myocardial infarction, which may affect the association of lipids and apolipoproteins with disease. For example, INTERHEART found that higher triglycerides were associated with statistically significantly lower risk of myocardial infarction, a rather unusual finding that stands in contrast to most studies that have found increased risk of CVD with higher triglycerides. INTERHEART also did not have full adjustment for other risk factors.

Both apoB and LDL particle concentration correlate with LDL cholesterol, but they correlate even more with non-HDL cholesterol and the total/HDL cholesterol ratio (correlation coefficient ~0.7 to 0.8). Measures that are highly correlated, as are apoB (or LDL particle concentration) and non-HDL cholesterol or total/HDL cholesterol, make the results obtained from standard regression models unstable (unreliable) because of colinearity. A better way to assess the clinical utility of a lipoprotein test for CVD risk prediction, in particular for highly correlated measures, is the degree to which it enhances the accuracy of existing cardiovascular risk assessment tools.

From a clinical and public health standpoint, the key issue is whether the use of a test results in more appropriate choice of therapy and improves patient outcomes, when used either in addition to or instead of standard risk factors. For example, the reclassification of individuals, in particular those with intermediate risk, to a higher or lower risk category could have important implications for preventive pharmacotherapy in these patients.

The net reclassification index is a quantitative measure of model fit that compares the proportion of individuals moving up or down in risk categories with the use of a biomarker; it has been proposed to assess whether a biomarker adds information to traditional risk factors. The net reclassification index compares shifts in reclassified categories by observed outcome and is penalized by incorrect classification. To date, 2 studies have evaluated the predictive performance of apoB or NMR LDL particle concentration for risk reclassification of asymptomatic individuals compared with standard lipids. In the Framingham Study, little additional risk information was obtained from apoB or apoB/A-1 ratio compared with the total/HDL cholesterol ratio. The difference in the net reclassification index for apoB/A-1 compared with total/HDL cholesterol was very small (0.1%) and statistically nonsignificant. In the Women’s Health Study, both apoB and NMR LDL particle concentration were significantly associated with CVD over an 11-year follow-up of 27 000 women (Figure 2), with a magnitude of association comparable to total/HDL cholesterol or non-HDL cholesterol. However, the net reclassification index for adding either apoB or NMR LDL particle concentration to the total/HDL cholesterol ratio was <2%.
These studies suggest that only a small percentage of individuals in a predominantly white North American primary prevention population would be reclassified into a higher or lower Third Adult Treatment Panel risk category if the apoB/A-1 ratio were routinely substituted for or added to the total/HDL cholesterol ratio in clinical practice. It is unclear if similar results would be obtained in more diverse populations than these 2 studies. In addition, whether particular subgroups of individuals exist, such as those with cardiovascular disease, cardiometabolic risk factors (eg, diabetes mellitus, hypertriglyceridemia), or familial clustering of disease, in whom CVD risk assessment and treatment could be improved with the use of measures from advanced lipoprotein testing has not been well defined and should be investigated further.9 A clear need also remains for performing cost-benefit analyses that evaluate a 1- or 2-step process of obtaining a standard lipid profile along with apoB (or NMR LDL particle concentration).

LDL Particle Size: Are Small LDL Particles Inherently More Atherogenic Than Large Particles?

Advocates of advanced lipoprotein testing may point out that small LDL particles (sometimes referred to as “dense” or pattern B) are more atherogenic than large LDL particles (also referred to as “buoyant” or pattern A); hence, identifying individuals with a greater concentration of small LDL particles may improve CVD risk assessment or therapeutic decisions. This section examines the evidence for the relative atherogenicity of LDL particles. Data to support the alternative view, namely, that both small and large LDL particles are similarly atherogenic, will also be presented.

First, pathophysiological data supporting the view that small LDL particles may be more atherogenic than large particles include the greater oxidation potential of small particles, their association with endothelial dysfunction and multiple metabolic abnormalities, and impaired clearance from the circulation.1,41 However, several mechanisms may underlie the atherosclerotic effect of large LDL.1 At both extremes of LDL size, decreased receptor-binding affinity for LDL receptors is present.42 Large LDL particles also have higher core cholesterol ester content, potentially delivering more cholesterol per particle to arterial walls.43 Large LDL particles predominate in patients with familial hypercholesterolemia and those consuming high-saturated-fat diets.44

Second, epidemiological data for small LDL particles being more atherogenic than large particles include the greater oxidation potential of small particles, their association with endothelial dysfunction and multiple metabolic abnormalities, and impaired clearance from the circulation.1,41 However, several mechanisms may underlie the atherosclerotic effect of large LDL.1 At both extremes of LDL size, decreased receptor-binding affinity for LDL receptors is present.42 Large LDL particles also have higher core cholesterol ester content, potentially delivering more cholesterol per particle to arterial walls.43 Large LDL particles predominate in patients with familial hypercholesterolemia and those consuming high-saturated-fat diets.44

**Figure 2.** Association of standard lipids, NMR lipoproteins, and immunoassay apolipoproteins with incident CVD in 27 673 initially healthy women in the Women’s Health Study.34 *LDL\textsubscript{NMR} size adjusted for nonlipid risk factors and total LDL\textsubscript{NMR} particle concentration (number). **LDL\textsubscript{NMR} size adjusted only for nonlipid risk factors. †Large and small LDL\textsubscript{NMR} particles adjusted for nonlipid risk factors and for lipoprotein concentrations.
average LDL size; large versus small) but does not quantify the number of large and small LDL particles. This distinction is important because a decrease in average LDL size does not necessarily translate into a greater number of small LDL particles because it could also result from having fewer large LDL particles (Figure 3). Thus, small LDL size may potentially confound the association of LDL particle concentration (number) with CVD. A confounding variable is both associated with the risk factor and causally associated with the outcome. A potential confounder (eg, smaller LDL size) may mask the relationship between the risk factor (eg, LDL particle concentration) and the outcome (eg, CVD). A study population may have a mix of individuals, some with predominantly large LDL, such as those with familial hypercholesterolemia, and others with predominantly small LDL, such as those with diabetes mellitus or insulin resistance. These individuals would also be expected to differ in their concentration of LDL particles. For example, we may not know if risk in the diabetic patient is due to the diabetes mellitus or the small LDL particles, but if the risk is predicted correctly by knowing that the person has diabetes mellitus, high LDL particle concentration, high triglycerides, and low HDL cholesterol, it may not be useful to also measure LDL size.

This is demonstrated by 2 case examples outlined in Figure 4. For the same serum concentration of LDL cholesterol, individual B has smaller average LDL particle size and concomitantly more LDL particles than individual A, who has larger average LDL size. Small LDL particles contain less cholesterol than large ones. Thus, individual B must have a higher concentration of total LDL particles, as measured either by NMR (LDL particle concentration) or immunonoassay (apoB), for the same LDL cholesterol as individual A. Prior studies that suggested that smaller LDL particles were more “atherogenic” did not adequately control for the inverse correlation between small and large LDL particle concentrations and potential confounding due to their differing associations with other lipoproteins, lipids, and cardiovascular risk factors.16,20,50

A report from ≈5500 asymptomatic individuals in the Multi-Ethnic Study of Atherosclerosis (MESA) compared the per-particle associations of small and large LDL with carotid intima-media thickness, a direct and well-validated measure of subclinical atherosclerosis.28 To unmask the association of large LDL with IMT, participants were classified into categories of small LDL particle concentration (Figure 5). In these stratified analyses, higher concentrations of large LDL were significantly associated with intima-media thickness within any particular category of small LDL. This was also confirmed by regression analysis: After particle correlations were accounted for, both small and large LDL were “atherogenic” to a similar extent.

This was subsequently confirmed in relation to clinical CVD events in both primary prevention (Women’s Health Study; Figure 2: adjusted hazard ratios of 1.44 and 1.63 for large and small LDL, respectively, quintile 5 versus quintile 1)34 and secondary prevention (Veterans Affairs HDL Intervention Trial [VA-HIT]).51 After lipoprotein correlations were accounted for, particularly the inverse correlation be-
Is Advanced Lipoprotein Testing Useful for Detecting or Managing Residual Risk?

The relative risk reduction in cardiovascular events with statin therapy is $\approx 30$%. Thus, $\approx 70$% of the patients who otherwise would have had CVD events still experience CVD events on statin therapy. Whether or not advanced lipoprotein tests may aid in detecting and treating this residual risk is uncertain.

Why do patients treated with statins have CVD events? First, risk factors for CVD often cluster, such that individuals with dyslipidemia may also have a number of cardiovascular risk factors, such as obesity, hypertension, and diabetes. Even when low LDL cholesterol levels are achieved, other risk factors may be present that increase CVD risk. In addition, treated patients remain at risk because they have higher levels of atherosclerosis since their lipids may have been elevated for a long time before initiation of therapy. Although statins lower LDL and total cholesterol by $\approx 20$% to 50%, they have a smaller effect on lowering triglycerides (\(\approx 10$% to 30$\)) and raising HDL cholesterol (\(\approx 5$% to 10$\)). Numerous studies have shown that low HDL cholesterol and high triglycerides are risk factors independent of LDL cholesterol.$^4$

A secondary analysis from the Pravastatin or Atorvastatin Evaluation and Infection Therapy—Thrombolysis in Myocardial Infarction (PROVE-IT TIMI) 22 trial found that on-treatment levels of triglycerides were independently associated with recurrent coronary events, with triglycerides $< 150$ mg/dL associated with 20$\%$ relative risk reduction after adjustment for risk factors.$^{53}$ This may be particularly relevant in patients with diabetes or metabolic syndrome in whom elevated triglycerides are common.

The American Diabetes Association/American College of Cardiology consensus statement advocated the use of non-HDL cholesterol and apoB (or NMR LDL particle concentration) in addition to LDL cholesterol in subjects at increased metabolic risk, although the targets for apoB were somewhat arbitrary (LDL cholesterol $< 70$ mg/dL, non-HDL cholesterol $< 100$ mg/dL, apoB $< 80$ mg/dL for those at highest risk).$^9$ A recent study suggested that in statin-treated patients, lower targets of LDL and non-HDL cholesterol may be needed to achieve the same target apoB level compared with untreated patients.$^{54}$ Lowering of therapeutic targets for LDL cholesterol and non-HDL cholesterol may diminish the residual risk on therapy. More data are clearly needed on the utility of advanced lipoprotein tests as well as non-HDL cholesterol, total/HDL cholesterol ratio, or triglycerides as targets of therapy together with LDL cholesterol lowering.

Summary of Current Limitations to the Clinical Utility of Advanced Lipoprotein Tests

In summary, a lipoprotein measure obtained from an advanced test is clinically useful if the following criteria are met: It adds to clinical knowledge; it provides risk information that is independent of established predictors; it is easy to measure and interpret in a clinical setting; it is accurate, reproducible, and internationally standardized; and it has a favorable cost-benefit ratio.$^{55}$ Importantly, the lipoprotein measures should improve patient management, particularly through more accurate risk classification and guiding choice of therapy.$^{55,56}$ Challenges to the routine clinical use of advanced lipoprotein tests are summarized in the Table. To date, the most useful measures obtained from lipoprotein tests relate to atherogenic particle number (apoB or LDL particle concentration), but for now, non-HDL cholesterol or the total/HDL cholesterol ratio is of comparable predictive strength and results in no additional cost. LDL size is correlated with atherogenic particle number, but it has no clear benefit over measuring particle number in most studies to date. Additional research is needed to determine the utility of following changes in lipoproteins as therapeutic targets and whether certain subgroups of individuals, such as those with cardio-

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**Figure 5.** Higher concentrations of either large or small LDL were both positively associated with carotid intima-media thickness (IMT; y axis) in a multiethnic study of asymptomatic individuals (n=5538), the MESA Study. Reprinted from Mora et al,$^{28}$ copyright © 2007, with permission from Elsevier.
Table. Summary of Current Limitations to the Clinical Utility of Advanced Lipoprotein Tests

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<th>Limitation</th>
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<tr>
<td>Lack of standardization and comparability of information provided by various tests</td>
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<td>Information overload can be minimized by focusing on several key lipoprotein measures</td>
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<td>Lack of accessibility</td>
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<tr>
<td>Lack of demonstration that tests alter clinical management and outcomes of patients, such as by improving risk classification or targeting of therapy</td>
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<tr>
<td>Subgroups of individuals have not been identified who may particularly benefit from testing (eg, those with cardiometabolic risk factors)</td>
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<td>Favorable cost-benefit ratio has not been demonstrated</td>
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Disclosures

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Response to Mora

H. Robert Superko, MD, FAHA

The opposing viewpoint to “Advanced Lipoprotein Testing and Subfractionation Are Clinically Useful” by Dr Mora is welcomed and illustrates the service that “Controversies in Cardiovascular Medicine” supplies the medical community. Indeed, there is more agreement than disagreement in our positions. First, I agree that standardization of laboratory methods and quality control issues plague the field. We pointed this out in an American Heart Association presentation of the Lab Comparison Study in 2002. Over the past 1 to 2 years, we have initiated meetings with representatives of the Centers for Disease Control and Prevention’s lipoprotein standardization program in an attempt to establish national standards. One unique aspect of the gradient gel electrophoresis method is the use of ultracentrifugation standards at the University of California, illustrating that a standardization program is possible. These 2 articles contribute to that effort. Second, I agree that new laboratory tests must ultimately improve risk prediction, treatment selection, and outcomes. With the national move to embrace “personalized medicine” and the demise of the “one size fits all” mentality, advanced lipoprotein tests may play a role. For example, although low-density lipoprotein diameter is not statistically independent of triglycerides, it is independent when triglycerides are below a threshold value. Thus, this measurement should not be used in all patients but only in those in whom the results contribute to a productive medical decision. The advent of single-nucleotide polymorphism testing, such as KIF6 and 9p21, provides the physician with tools independent of standard lipid measurements. Physicians need not wait for “consensus statements” to embrace new findings. In specific patient groups, advanced lipoprotein tests can improve risk prediction, guide appropriate treatment, and contribute to personalized medicine.
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