Laboratory Assessment of HDL Heterogeneity and Function

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BACKGROUND: Plasma concentrations of HDL cholesterol (HDL-C) and its major protein component apolipoprotein (apo) A-I are strongly inversely associated with cardiovascular risk, leading to the concept that therapy to increase HDL-C and apoA-I concentrations would be antiatherosclerotic and protective against cardiovascular events. The recent failure of the drug torcetrapib, a cholesteryl ester transfer protein inhibitor that substantially increased HDL-C concentrations, has brought focus on the issues of HDL heterogeneity and function as distinct from HDL-C concentrations.

CONTENT: This review addresses the current state of knowledge regarding assays of HDL heterogeneity and function and their relationship to cardiovascular disease. HDL is highly heterogeneous, with subfractions that can be identified on the basis of density, size, charge, and protein composition, and the concept that certain subfractions of HDL may be better predictors of cardiovascular risk is attractive. In addition, HDL has been shown to have a variety of functions that may contribute to its cardiovascular protective effects, including promotion of macrophage cholesterol efflux and reverse cholesterol transport and antiinflammatory and nitric oxide–promoting effects.

SUMMARY: Robust laboratory assays of HDL subfractions and functions and validation of the usefulness of these assays for predicting cardiovascular risk and assessing response to therapeutic interventions are critically important and of great interest to cardiovascular clinicians and investigators and clinical chemists.

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An estimated 1.2 million Americans experience myocardial infarction annually (1). There is plentiful evidence for the effectiveness of therapies that decrease LDL cholesterol (LDL-C), particularly statins, in both primary and secondary prevention of atherosclerotic vascular disease. Despite the antiatherogenic benefits of statins, however, substantial residual risk remains. Epidemiological studies and prospective randomized trials have consistently shown a powerful inverse association between the magnitude of HDL cholesterol (HDL-C) and coronary heart disease (CHD) (2). In the Framingham Heart Study, each 10 mg/dL (0.26 mmol/L) increase in HDL-C was associated with a significant decrease in relative risk for CHD mortality, of 19% in men and 28% in women (3). The prevalence of low HDL-C concentrations, defined by National Cholesterol Education Program Adult Treatment Panel III guidelines as ≤40 mg/dL (≤1.04 mmol/L) in men and ≤50 mg/dL (≤1.30 mmol/L) in women (4), was found to be as high as 66% in high-risk populations with CHD who are on statin therapy, irrespective of their LDL-C concentrations (5). In the post hoc analysis of the Treating to New Targets trial, Barter et al. (6) found HDL-C to be a significant predictor of major cardiovascular events, including the cohort with LDL-C concentrations below 70 mg/dL (1.81 mmol/L). Thus low HDL-C is predictive of risk even in statin-treated patients.

Despite the overwhelming observational data indicating an inverse link of HDL-C and apolipoprotein A-I (apoA-I) concentrations with CHD risk, there are inconsistencies in the observational and interventional experience that raise important questions about the value of HDL-C and apoA-I steady-state measurements as causal indicators of atherosclerosis risk. There are several genetic syndromes of very low HDL-C and apoA-I that are not clearly associated with increased risk of premature CHD. For example, deficiency of plasma lecithin:cholesterol acyltransferase (LCAT) does not appear to increase cardiovascular risk despite

1 Nonstandard abbreviations: LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; CHD, coronary heart disease; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; ABCA1, ATP-binding cassette A1; VA-HIT, Veterans Affairs High-Density Lipoprotein Intervention trial; CETP, cholesteryl ester transfer protein; RCT, reverse cholesterol transport; SR-BI, scavenger receptor type BI; VAP, vertical auto profile; GGE, gradient gel electrophoresis; NMR, nuclear magnetic resonance; LpA-I, apolipoprotein containing only apoA-I; LpA-I:A-II, apolipoprotein containing both apoA-I and apoA-II; FERred, fractional esterification rate on HDL; PON1, paraoxonase.
HDL-C concentrations <15 mg/dL (<0.39 mmol/L) (7). It is well known that individuals heterozygous for the Milano mutation of the apolipoprotein A-I (APOAI) gene do not develop early CHD despite HDL-C <20 mg/dL (<0.52 mmol/L) (8), and the situation is similar for the APOAI Paris mutation (9). Even Tangier disease, caused by mutations in the ATP-binding cassette A1 (ABCA1) transporter gene (ABCA1), in which HDL-C and apoA-I concentrations are virtually undetectable, is not associated with the marked increase in CHD that might be expected from such a dramatic phenotype (10). Importantly, patients with LCAT deficiency and Tangier disease often have decreased LDL-C concentrations, which may be one factor offsetting the low HDL-C concentrations (7, 10). These genetic disorders are all associated with significantly increased turnover of HDL, so steady-state measures may be less informative than measures of flux through the HDL pathway (11). In any case, these illnesses illustrate how simple measures of HDL mass do not always correlate with cardiovascular risk.

Interventional data from randomized controlled trials targeted to HDL provide a similarly complex picture. Fibrates, or peroxisome proliferator–activated receptor α agonists, have modest HDL-increasing effects in humans, but data regarding their role in reducing cardiovascular events are mixed. Cardioprotective effects of fibrates were suggested in the Helsinki Heart Study (12) and the Veterans Affairs High-Density Lipoprotein Intervention trial (VA-HIT) (13). Indeed the latter trial was specifically targeted to men with low HDL-C and remains one of the best positive examples of clinical trial of increasing HDL, but the magnitude of the HDL increase was small, and the benefit cannot be directly linked to increasing HDL per se. Other studies of fibrates, such as the Bezafibrate Infarction Prevention trial (14) and the Fenofibrate Intervention and Event-Lowering in Diabetes study (15), although suggestive of possible benefit, were disappointing. Nicotinic acid (niacin) is the most effective HDL-increasing drug currently on the market, but clinical outcome trial data showing an outcomes benefit (16, 17) were obtained before the use of statins, and evidence is limited for outcomes benefit in the era of widespread statin treatment. Some atherosclerosis imaging trials using carotid intima–media thickness (IMT) (18) or coronary angiography (19) have suggested a benefit of adding niacin to a statin. A metaanalysis of clinical trials using intravascular ultrasound suggested that changes in atheroma volume were related to changes in HDL-C (20), consistent with the concept that intervention that increases HDL could be beneficial.

Experience with torcetrapib, an inhibitor of the cholesteryl ester transfer protein (CETP), has raised the most profound questioning regarding the value of increasing HDL-C concentrations to decrease cardiovascular risk. Torcetrapib had been shown to substantially increase HDL-C concentrations by 50%–100% (21, 22) and, based on this effect, was advanced into phase-III development. However, the large clinical outcome trial Investigation of Lipid Level Management To Understand its Impact in Atherosclerotic Events was terminated early because of increased cardiovascular morbidity and total mortality despite an observed 72% increase in HDL-C concentrations in individuals treated with the drug (23). Imaging trials using intravascular ultrasound (24) and carotid ultrasound to measure IMT (25) were negative despite similarly favorable increases in HDL-C. However, the interpretation of the clinical trial results with torcetrapib are complicated by the fact that the drug increases blood pressure and increases aldosterone as an off-target, non–mechanism-based effect (26). Indeed, another CETP inhibitor, anacetrapib, was recently reported to increase HDL by up to 128% without an increase in ambulatory blood pressure (27). Nevertheless, the torcetrapib experience strongly suggests that therapeutic measures based solely on increasing HDL-C may not be adequate for cardiovascular risk reduction.

The analytical chemistry related to measurement of HDL-C and HDL subclasses has undergone substantial evolution over the last few decades (28, 29). In light of recent developments, there is a growing need to identify other HDL-related subclasses and functions, and to find biomarkers that will better predict cardiovascular risk and can be used to assess the clinical benefits of novel HDL-targeted therapies. This need poses an opportunity for clinical chemists to take the lead in the development and validation of such biomarkers. In addition to being a source for better markers of cholesterol flux, HDL has pleiotropic functions that include antiinflammatory, antioxidant, antithrombotic, and nitric-oxide–promoting effects. This review focuses on in vitro methods to assay various HDL subfractions and HDL functions that could be more effective than currently available surrogate markers of cardiovascular risk.

RECENT ADVANCES IN THE UNDERSTANDING OF HDL METABOLISM AND REVERSE CHOLESTEROL TRANSPORT

The molecular regulation of HDL metabolism and reverse cholesterol transport (Fig. 1) is complex. Much has been learned over the last decade, however, and this

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2 Human genes: APOAI, apolipoprotein A-I; ABCA1, ATP-binding cassette, sub-family A (ABC1), member 1; ABCG1, ATP-binding cassette, sub-family G (WHITE), member 1; CETP, cholesteryl ester transfer protein, plasma.
The topic has been covered in several excellent reviews (30–32). ApoA-I is the major HDL protein present on almost all HDL particles and is synthesized by both the liver and the intestine. ApoA-I is secreted as a lipid-poor protein, and then rapidly acquires phospholipids and unesterified cholesterol from the same tissues via the ABCA1 transporter to form discoidal HDL particles (33). This nascent HDL acquires additional free cholesterol from other peripheral tissues, and this cholesterol is then esterified to CE by the HDL-associated enzyme LCAT, generating spherical HDL. The CETP transfers CE from HDL to apoB-containing lipoproteins in exchange for triglyceride. The lipolytic enzymes hepatic lipase and endothelial lipase hydrolyze HDL triglycerides and phospholipids and generate smaller HDL particles. ApoA-I that is part of mature HDL is catabolized by the liver, whereas lipid-poor apoA-I is catabolized by the kidneys.

HDL facilitates a process known as reverse cholesterol transport (RCT) in which unesterified cholesterol in peripheral tissues is effluxed to HDL and ultimately returned to the liver for excretion in bile and feces. Whether RCT is an important mechanism by which HDL protects against atherosclerosis remains to be definitively proven. The macrophage is the most important cell type for HDL-mediated promotion of cholesterol efflux, and the molecular regulation of cholesterol efflux from macrophages has been extensively studied. The ABCA1 transporter is instrumental in the classic pathway for macrophage cholesterol efflux and promotes cholesterol efflux to lipid-poor apoA-I as an acceptor. Another member of this family, ABCG1, encoded by the ATP-binding cassette, sub-family G (WHITE), member 1 (ABCG1) gene, promotes macrophage cholesterol efflux to mature HDL particles. ABCA1 and ABCG1 expression are up-regulated by the nuclear liver X receptor, which is activated by oxysterols, products of macrophage cholesterol metabolism. Once cholesterol is effluxed to HDL, uptake by the liver is required to complete the RCT process. Two major pathways of hepatic uptake of HDL cholesterol exist: HDL cholesterol can be taken up selectively by the liver via the scavenger receptor type BI (SR-BI), a pathway very important in rodents, which naturally lack CETP. A second pathway in humans and other species that express CETP involves the transfer of CE via CETP to apoB-containing lipoproteins, which are taken up by the liver. This pathway may be particularly
important in humans for the hepatic uptake of HDL-derived CE.

Although promotion of cholesterol efflux and RCT is thought to be the major mechanism by which HDL protects against atherosclerosis, the last decade has seen substantial growth in interest in other properties of HDL that may be atheroprotective. These include the antioxidant and antiinflammatory properties of HDL (34), as well as endothelial nitric-oxide-promoting and antithrombotic effects of HDL (35). Thus the concept that HDL particles have a plethora of properties that protect against atherosclerosis, and that these functions cannot necessarily be inferred from the measurement of plasma HDL-C concentrations, has led to the interest in assays of HDL subfractions and HDL function to refine the risk assessment associated with HDL.

HDL HETEROGENEITY AND MEASUREMENT OF HDL SUBFRACIONS

HDL is a complex macromolecule comprised of lipids (phospholipids and unesterified free cholesterol on the surface and cholesteryl ester and triglycerides in the core) and proteins (apolipoproteins and a variety of other proteins in smaller amounts). Multiple subfractions of HDL based on density, size, charge, and composition can be identified in plasma. A topic of considerable interest is whether specific subfractions of HDL confer greater ability to predict cardiovascular risk than HDL-C itself or may be of greater utility in assessing the benefits of a therapeutic intervention targeted to HDL. Patients with coronary disease generally have smaller, denser HDL particles, leading to the concept that larger HDL particles may be associated with greater protection from CHD. However, the data regarding the predictive ability of HDL subclasses for CHD risk are not conclusive.

The classic method for separation of lipoprotein subfractions is by density. In 1951, Lindgren et al. first identified 2 HDL subtypes by analytic ultracentrifugation based on their buoyancy (36). HDL2, which has a density range of 1.063–1.125 g/mL, comprises the larger, cholesterol-rich particles and HDL3, which represents the range 1.125–1.210 g/mL, comprises small, lipid-poor particles. Havel et al. first described separation of lipoproteins by repeated ultracentrifugations after progressively increasing the solvent density (37). These lipoprotein factions were divided into the following density groups: <1.019, 1.019–1.063, and >1.063, which were later named very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL), respectively. Density gradient ultracentrifugation continues to be the gold standard for isolation of lipoproteins, even after 50 years. Ultimately, more convenient methods such as preparative ultracentrifugation (38) or differential precipitation were developed. Preparative ultracentrifugation yielded HDL3 after a single spin at density 1.125, and HDL2 is calculated as the difference between total HDL and HDL3. Selective precipitation of HDL subfractions HDL2 and HDL3 was developed as a less labor-intensive analytical method (39). This method does not require any specialized apparatus, and relatively large numbers of plasma samples can be processed and analyzed at a time (40).

The major question is whether separation of HDL subfractions by density provides more information regarding cardiovascular outcomes than measuring HDL-C itself. In general, controversy still exists in regard to whether the antiatherogenic effect of HDL can be attributed to one or both HDL subfractions and what is the relative importance of HDL2 and HDL3 cholesterol. In the Kupio Study (41) and Quebec City Suburbs Study (42), HDL2 was inversely associated with CHD. HDL3 had a stronger inverse association with CHD in the Physician’s Health Study (43), the 5-year follow-up of the CAE Philly and Speedwell study groups (44), and the 9-year follow-up of the Caerphilly study (45). The results of these studies provide no conclusive evidence for the usefulness of routine measurement of HDL subfractions in risk stratification for CHD. The differences in the results of various studies may be attributable to the different assay methods used, to ethnic variations, or to the probable heterogeneity of the subfractions with different physiological functions.

The vertical auto profile (VAP) method is an inverted rate zonal, single vertical spin, density gradient ultracentrifugation technique that separates all lipoproteins in <1 h (46). VAP analysis can be performed in less time than other methods because it uses a vertical rotor in which lipoproteins separate across the shorter horizontal axis of the centrifuge tube rather than a longer vertical axis, as in sequential ultracentrifugation, which generally uses a swinging-bucket rotor. The VAP method is a sensitive test for comprehensive measurement of the major HDL density subclasses. Few data exist, however, associating HDL subfractions as assessed by the VAP method to cardiovascular outcomes. The VAP, marketed by Atherotech, is available to clinicians through various reference laboratories as a fee-for-service test.

HDL subfractions can also be separated based on size. The original method for size-based separation was nondenaturing polyacrylamide gradient gel electrophoresis (GGE) in conjunction with automated densitometry (47). A detailed and comprehensive review of polyacrylamide GGE for determination of lipoprotein, including HDL, size was published recently (48). Although the methods are well established and reproduc-
ible, there are few data suggesting that HDL subclasses determined by polycrylamide GGE are more predictive of CHD than total HDL-C concentrations. GGE, marketed by Berkeley Heart Labs, is available to clinicians as a fee-for-service test.

Charge is another important property that has been used to separate HDL subclasses. Nondenaturing lipoprotein electrophoresis has been used for decades as a standard laboratory technique to analytically separate lipoproteins, and HDL migrates as an α-band. Separation of HDL by 2-dimensional electrophoresis (incorporating a size-based separation in addition to separation based on charge) has revealed additional heterogeneity, with pre-β-1, pre-β-2, and pre-α HDL in addition to the α-migrating species (49). Two-dimensional electrophoresis has been used to resolve at least 12 distinct apoA-I-containing HDL subpopulations. A strong negative correlation has also been observed between the large, cholesterol-rich α-1 particles and CHD (50). In the Framingham Offspring Study (51), CHD cases had higher pre-β-1 and α-3 particle and lower α-1, pre-α-3, and pre-α-1 particle concentrations. α-1 and pre-α-3 concentrations had an inverse association, whereas α-3 and pre-α-1 particle concentrations had a positive association with CHD prevalence after data were adjusted for established CHD risk factors. α-1 HDL was most significantly associated with CHD, and each milligram per deciliter increase in α-1 particle concentration decreased odds of CHD by 26% (P < 0.0001). Significant negative correlations were also observed in the HDL-Atherosclerosis Treatment study (50) and the VA-HIT study (52). Conversely, the VA-HIT study demonstrated a significant positive correlation between CHD events and the concentrations of α-3 as well as small, lipid-poor pre-β-1 particles (52).

Nuclear magnetic resonance (NMR) spectroscopy is another rapid method of assessing HDL subfractions (53). Each subclass of HDL emits distinctive NMR signals whose individual amplitude can be accurately measured, and these amplitudes are directly proportional to the numbers of subclass particles emitting the signal, irrespective of variation in particle lipid composition. HDL particles quantified by NMR are subclassified into 3 size classes, large, medium, and small. Published data indicate a relationship of HDL subclasses as assessed by NMR with cardiovascular disease risk. In the Pravastatin Limitation of Atherosclerosis in the Coronary Arteries statin intervention trial (54), large and small HDL particle concentrations were inversely associated with progression of angiographically documented coronary artery disease, independent of HDL-C and other lipids. In the VA-HIT trial (55), NMR-measured concentrations of total and small HDL particles were independent predictors of recurrent cardiovascular disease. In the MultiEthnic Study of Atherosclerosis trial, total HDL particle number was more strongly associated with carotid atherosclerosis than was HDL-C (56). A recently reported study addressed the issue of HDL particle size and cardiovascular disease risk by analyzing the relationship of HDL-C and HDL particle size (assessed by NMR) to cardiovascular disease risk after controlling for apoA-I and apoB (57). Intriguingly, although HDL-C and HDL particle size were generally inversely associated with cardiovascular disease risk, after results were controlled for apoA-I and apoB, the highest levels of HDL-C and HDL particle size were found to be positively associated with cardiovascular disease events. Lipoprotein NMR analysis, marketed by Liposcience, is available to clinicians as a fee-for-service test.

**APOLIPOPROTEIN COMPOSITION AND OTHER PROTEINS IN HDL**

The most abundant protein in HDL, apoA-I, can be measured in plasma using widely available immunoassays. Whether plasma apoA-I concentrations are more predictive of CHD than HDL-C concentrations is an important question that can be difficult to address statistically because of the very tight correlation between apoA-I and HDL-C. Nevertheless, some large prospective population-based studies have suggested that apoA-I concentrations may be more predictive of future CHD events than HDL-C concentrations (58). Furthermore, even after data analysis was controlled for HDL-C, apoA-I still had a continuous inverse association with cardiovascular disease risk (57). It is possible that apoA-I concentrations may be superior to HDL-C in predicting cardiovascular disease risk, but this approach has not yet been incorporated into national guidelines. Furthermore, novel therapeutic interventions that increase apoA-I concentrations may be superior to those that primarily increase HDL-C concentrations, but this superiority remains largely theoretical at present.

The second most abundant protein in HDL is apoA-II, and controversy exists regarding the role of apoA-II in cardiovascular risk. A nested case-control study in the large European Prospective Investigation into Cancer and Nutrition in Norfolk trial demonstrated that plasma apoA-II concentrations were strongly inversely correlated with CHD events, even after adjustment for traditional cardiovascular risk factors and HDL-C and apoA-I concentrations (59). Thus the concept, based largely on mouse studies (60), that apoA-II may be proatherogenic is not supported by the epidemiologic evidence in humans.

HDL can be separated into 2 primary subclasses according to their major apolipoprotein composition, those containing only apoA-I (LpA-I) and those containing both apoA-I and apoA-II (LpA-I:A-II).
(61, 62). In most people, LpA-I is approximately one-third and LpA-I:A-II approximately two-thirds of the total HDL (63). LpA-I is found more in HDL₂, whereas LpA-I:A-II is found more in HDL₃ (64). With regard to whether either of these particles is more predictive of CHD, there is considerable variability in reported studies. Some studies report that individuals with CHD have reductions in LpA-I only (43, 65), whereas others report reductions in both LpA-I and LpA-I:A-II (66–68). In the Framingham Offspring Study and the VA-HIT (69), LpA-I and LpA-I:A-II HDL subclass quantification by differential electroimmunoassay in male participants provided no additional information about CHD risk, compared to the traditional lipid measurements.

HDL particles carry a large number of additional proteins at considerably lower abundance than apoA-I and apoA-II. Other apolipoproteins that are well known to be associated with HDL include apoA-IV; apoC-I, C-II, and C-III; and apoE. Most of these apolipoproteins are highly exchangeable and can also be associated with apoB-containing lipoproteins. Thus measurement of total plasma concentrations of these apolipoproteins is not specific for their content within the HDL fraction per se, but this measurement can be obtained by first precipitating the apoB-containing lipoproteins and then measuring the apolipoprotein of interest in the supernatant. Indeed, this approach was used to demonstrate that HDL-apoC-III was an important determinant of atherosclerosis progression (70).

Based on the recognition that HDL contains many proteins, formal proteomic analyses of HDL have been performed. One recent publication determined that a large number of proteins involved in inflammation, complement regulation, and innate immunity are physiologically bound to human HDL (71). These observations reinforce the concept that HDL evolved as a component of the innate immune system. One elegant example of this phenomenon is the demonstration that HDL serves as a platform for the assembly of a complex containing apoL-I and haptoglobin-related protein (both found in the proteomics study) that is highly lytic for a species of trypanosome (72). HDL protein composition varies considerably among individuals, consistent with differences in HDL function among individuals (see below). One hope is that specific measures of HDL protein concentrations may ultimately permit links to functionality and thus association with atherosclerosis risk.

LABORATORY ASSESSMENT OF ChOLESTEROL EFFLUX AND REVERSE ChOLESTEROL TRANSPORT

Several properties of HDL have been described that could contribute to the antiatherogenic, cardioprotective effects of HDL (Fig. 2). The most popular hypothesis, however, is that HDL and apoA-I protect against atherosclerosis, at least in part, by promoting cholesterol efflux from cells, particularly via cholesterol-loaded macrophages in the arterial wall, and facilitating the RCT process, the transport of that cholesterol back to the liver for excretion in bile and ultimately feces. Whether RCT is an important mechanism of atheroprotection by HDL remains to be established conclusively. Nevertheless, there has been intense interest in understanding the molecular regulation of this pathway. Studies from several laboratories have confirmed...
that the rate of RCT is not always correlated with the plasma concentrations of HDL-C and apoA-I, indicating that measurements of RCT or its components may provide important clinical information beyond that obtained from measurement of HDL-C and apoA-I. With regard to atherosclerosis, the most important cell type for HDL-mediated promotion of cholesterol efflux is the macrophage, and thus the molecular regulation of cholesterol efflux from macrophages has been of particular interest. This section focuses on laboratory-based assays that may provide information about the function of HDL with regard to promotion of macrophage cholesterol efflux or RCT.

**CHOLESTEROL EFFLUX CAPACITY**

The best-recognized atheroprotective function of HDL and apoA-I is the promotion of cholesterol efflux from cells, particularly macrophages, because they are the primary cell type to accumulate cholesterol within the atherosclerotic plaque. There has been substantial investigation into the molecular mechanisms by which macrophages efflux cholesterol. The ABCA1 transporter promotes cholesterol efflux to lipid-poor apoA-I, or pre-β-HDL, as an acceptor (73). Macrophages from ABCA1-knockout mice have substantially decreased cholesterol efflux to lipid-poor apoA-I, and transplantation of ABCA1-deficient bone marrow resulted in significantly increased atherosclerosis (74). More recently, another member of the ABC gene family, ABCG1, was identified as a promoter of macrophage cholesterol efflux to mature HDL particles (75, 76), the most abundant form of HDL in plasma. ABCA1 and ABCG1 act cooperatively in vivo to promote macrophage cholesterol efflux and RCT (77), consistent with the recent observation that mice deficient in macrophage ABCA1 and ABCG1 develop markedly accelerated atherosclerosis (78). Although SR-BI has been shown to facilitate in vitro cholesterol efflux from macrophages to mature HDL (79), its role in mediating macrophage RCT in vivo is probably not quantitatively important (77). The expression levels of macrophage ABCA1 and ABCG1 are regulated by the nuclear receptors liver X receptors α and β (80). A synthetic liver X receptor agonist significantly promoted macrophage RCT in vivo despite having little effect on plasma HDL-C concentrations (81); synthetic liver X receptors have also been shown to inhibit or cause regression of atherosclerosis (82–84).

Higher concentrations of circulating HDL-C have long been considered to indicate greater promotion of cellular cholesterol efflux and thus greater atheroprotection. However, inconsistencies in the relationship of genetic conditions of HDL metabolism to atherosclerosis, as well as the experience with the CETP inhibitor torcetrapib, have called this assumption into question. Indeed, there is considerable variation among individuals with similar HDL-C concentrations with regard to the ability of their serum to promote cholesterol efflux ex vivo (85, 86), and some evidence that even after normalization, some HDL particles are more effective at promoting efflux than others (87). Thus the ability to assay human serum or isolated HDL for its cholesterol efflux capacity could be an important method of determining HDL function in assessing differences among individuals or in response to novel therapies. Assays have been developed and applied in the research setting. Cells (often macrophages) are labeled with 3H-cholesterol, and then exposed to diluted whole serum, serum depleted of apolipoproteins, or isolated HDL for a defined period of time, after which counts in the media, as well as the remaining cell-associated counts, are determined and used for quantification of the percentage efflux (88). Another approach is focused on cholesterol mass rather than a tracer, and involves the quantification of increased cholesterol mass in the media after incubation with acceptor (87). A third approach is the ability of serum to deplete cellular cholesterol available for esterification by acyl CoA:cholesterol acyl transferase as an indirect measure of cholesterol efflux (89). There are multiple variables in these assays, including the donor cell type, the nature of the acceptor (i.e., whole serum, apoB-depleted serum, or isolated HDL), the readout (i.e., cholesterol tracer, cholesterol mass, or indirect indicators such as the availability of acyl CoA:cholesterol acyl transferase) and cholesterol efflux pathways that are being assessed (i.e., ABCA1, ABCG1, SR-BI, or passive diffusion).

Measurement of cholesterol efflux capacity is being increasingly used in preclinical studies (85, 90–92) to assess effects of genetic manipulation or pharmacologic treatment on efflux capacity independent of HDL-C concentrations. Application to human studies has been less common but is also increasing. In a small study using fibroblasts as the donor cell and serum from men referred for coronary angiography as the acceptor, depletion of fibroblasts from the available pool of acyl CoA:cholesterol acyl transferase, but not total efflux of radiolabeled cholesterol tracer, was shown to be correlated with cardiovascular outcomes (86). HDL isolated from patients with CETP deficiency was shown to be more effective in promoting cholesterol efflux than HDL from healthy individuals (87), and HDL from individuals treated with a high dose of the CETP inhibitor torcetrapib was also shown to be more effective in promoting efflux (93).

Measurement of cholesterol efflux capacity of serum or HDL is conceptually attractive and has the potential to be developed as a clinical laboratory tool for risk assessment and evaluation of pharmacologic effi-
cacy. However, substantial work is needed to relate measurements of efflux capacity to prevalent cardiovascular disease and prospectively to cardiovascular outcomes, and to determine whether the associations are independent of plasma HDL-C or apoA-I concentrations. Furthermore, the optimal method for performing such studies with regard to donor cells, type of acceptor, type of readout, and preferred pathways for interrogation has yet to be determined. Finally, standardization of the method would be necessary for broader use of this approach.

**CHOLESTEROL ESTERIFICATION BY LCAT**

The classic RCT pathway involves the esterification of effluxed cholesterol before transport to the liver (94). LCAT is a lipoprotein-associated enzyme responsible for esterifying free cholesterol to CE within the plasma compartment. LCAT deficiency is associated with markedly decreased HDL-C concentrations, whereas LCAT overexpression in mice and rabbits markedly increases HDL-C concentrations. The hydrophobic CE moves to the core of the HDL particle, contributing to the progressive enlargement of HDL. This process also removes free cholesterol from the surface of HDL, thus helping to maintain a free-cholesterol gradient from cells to HDL (95). Although increased LCAT activity has long been thought to be atheroprotective, inadequate data exist to support this. LCAT-deficient patients do not develop accelerated atherosclerosis, but it was reported that decreased LCAT function was associated with increased carotid IMT (96). Thus, in theory, measuring LCAT activity in human plasma could be a functional test that might provide additional cardiovascular risk prediction. However, methods to measure LCAT activity are not standardized. One approach involves the incubation of an exogenous substrate of apoA-I/phospholipids containing radiolabeled free cholesterol with plasma, followed by separation of free and esterified cholesterol and determination of the percentage of cholesterol that was esterified. This assay, commonly termed the LCAT activity assay, correlates best with measures of LCAT mass. A second approach involves the direct mixing of radiolabeled free cholesterol with plasma, followed by incubation, separation, and determination of the percentage of esterified cholesterol. This assay, commonly termed the cholesterol esterification rate assay, is much more dependent on the metabolic milieu and distribution of lipoproteins.

Neither of these assays have been applied to large scale human studies to address the relationship of LCAT activity or cholesterol esterification rate to prevalent cardiovascular disease or outcomes. Perhaps the most studied LCAT-related assay in humans is known as the fractional esterification rate on HDL (FER_{HDL}). This is a functional test that measures the rate of LCAT-mediated esterification of free cholesterol specifically on HDL in plasma depleted of apoB-containing lipoproteins. In a multivariate logistic model, FER_{HDL} was a significant predictor of the presence of angiographically defined CAD (97). The smallest HDL particles have the fastest esterification rate, whereas the largest particles have the slowest (98). Thus, FER_{HDL} acts as an indirect measure of the lipoprotein particle size. If a standardized assay of LCAT mass were to be developed, this approach would be the easiest to apply to large population-based studies to determine whether LCAT mass is a predictor of cardiovascular events independent of HDL-C and apoA-I concentrations. It remains possible that measurement of LCAT mass or activity could become another approach to assessing HDL function in predicting cardiovascular risk.

**CHOLESTERYL ESTER TRANSFER BY CETP**

CETP mediates the transfer of CE from HDL to apoB-containing lipoproteins in exchange for triglycerides. Because apoB-containing lipoproteins are mostly catabolized by the liver, the CETP pathway may be an important route by which HDL-derived cholesterol is transported back to the liver in humans (99). On the other hand, CETP activity results in decreased HDL-C concentrations, and genetic variation in the cholesteryl ester transfer protein, plasma (CETP) gene is an important source of variation in HDL-C concentrations in humans. Thus, debate has been ongoing about whether CETP is pro- or antiatherogenic, a topic clearly important to the issue of developing CETP inhibitors. Several published studies have addressed the association between plasma CETP protein mass concentrations and CHD outcomes. Higher CETP protein concentrations were cross-sectionally associated with CHD (100), greater progression of angiographic CAD (101), greater progression of carotid IMT (102), and younger age of first presentation with acute myocardial infarction (103). In the European Prospective Investigation into Cancer and Nutrition in Norfolk trial, higher CETP mass concentrations were associated with a greater prospective risk of CHD events, but only in those individuals with higher triglyceride concentrations (104). Results of other studies, however, have not supported CETP mass as a positive risk factor for CHD (105–107). CETP activity could potentially be more informative than mass, because it is more likely to include other factors that influence CE transfer rates beyond the mass of CETP itself, but the assays are difficult to perform and labor-intensive. In only a small number of studies, higher CETP activity was associated with increased atherosclerosis or cardiovascular risk (103, 108, 109). Additional studies of the correlation of both CETP mass and activity to CHD risk are needed. It remains possible that an assay of CETP might help re-
fine cardiovascular risk assessment independent of HDL-C concentrations.

LABORATORY ASSESSMENT OF ANTIOXIDANT AND ANTIINFLAMMATORY FUNCTIONS OF HDL

Over the past decade, HDL has been discovered to have other properties beyond cholesterol efflux promotion that could contribute to its antiatherogenic effects (Fig. 2). In particular, studies from several laboratories have identified the antioxidant and antiinflammatory effects of HDL as potentially important in overall HDL function.

PARAOXONASE ACTIVITY AND ANTIOXIDANT CAPACITY OF HDL

Paraoxonase (PON1) is an HDL-associated esterase/lactonase, which derived its name from one of its most commonly used in vitro substrates, paraoxon. PON1 hydrolyzes a variety of oxidized and modified lipids. Its activity is believed to account for at least some of the antioxidant activity of HDL. PON1 protects both HDL and LDL against lipid peroxidation (110, 111). PON1 activity can be measured using several different assays, but is most commonly measured by monitoring p-nitrophenol formation from the substrate paraoxon (112). Decreased lesion size was observed in human PON1 transgenic mice, and accelerated atherosclerosis was seen in PON1 knockout mice (113, 114). In humans, PON1 activity is inversely related to the risk of cardiovascular diseases. In the Caerphilly Prospective Study, PON1 activity predicted coronary events independent of all other coronary risk factors, including HDL-C (115). D-4F, an apoA-I mimic peptide, increased paraoxonase activity in LDL-receptor–null mice after a Western diet and after influenza infection (116). Thus PON1 activity might be developed as a biomarker of HDL function and cardiovascular risk independent of HDL-C concentrations.

HDL has the ability to inactivate oxidized phospholipids, in part due to PON1 activity but potentially also due to other enzymes or properties of HDL. Thus an integrated assay of HDL antioxidant capacity would be of research interest and may contribute to its antiatherogenic effects (Fig. 2). Other than the antioxidant activity, PON1 has also been shown to have antithrombotic properties (34). Limited data suggest that there is substantial interindividual variability among humans in the ability of isolated HDL to inhibit endothelial adhesion molecule expression that is independent of HDL-C concentrations. A study in humans showed that a saturated fat diet significantly decreased and a polyunsaturated fat increased the ability of isolated HDL to suppress tumor necrosis factor α–induced endothelial expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (118). This cell-based assay will be difficult to standardize, and clearly needs to be validated with regard to correlation with atherosclerotic disease and prediction of cardiovascular events independent of HDL-C concentrations. Nevertheless, it appears to be a robust measure of HDL antiinflammatory function and as such may provide valuable information.

OTHER POTENTIALLY ANTIATHEROGENIC PROPERTIES OF HDL

HDL has been shown to have additional properties that may contribute to its antiatherogenic effects (Fig. 2). These include the ability to stimulate endothelial nitric oxide production and thus enhance endothelial function (35, 119). Observational data are consistent with an inverse association between HDL-C concentrations and endothelial function measures (120, 121). The mechanism of this effect appears to be dependent on endothelial SR-BI and may involve cholesterol efflux as a triggering mechanism (35). The interindividual variation in HDL promotion of endothelial nitric oxide production is unknown, but the concept of establishing an endothelial-based assay for HDL-stimulated nitric oxide production is attractive. Some data suggest that the nitric oxide–promoting effect of HDL is partially dependent on the endothelial lysophospholipid receptor sphingosine-1-phosphate (3, 122), suggesting that the content in HDL of lysophospholipids sphingosine-1-phosphate may influence this function of HDL. Perhaps measurement of HDL-associated sphingosine-1-phosphate could serve as a quantitative surrogate for the nitric oxide–promoting function of HDL. This is a potential example of one direction this field will likely take: identification and measurement of reliable mass-based surrogates of HDL function. HDL has also been shown to have antithrombotic properties (35) involving effects on the coagulation system as well
as on platelets. Measurement of the anticoagulant properties of HDL would be highly labor-intensive and potentially be subject to substantial assay variation. Ideally this and other HDL functions would have one or more mass-based surrogates that are highly correlated with function and could be measured as a panel to gain information on a range of HDL functional properties.

Plasma concentrations of HDL-C, while epidemiologically predictive of atherosclerotic cardiovascular events in large populations, are insufficient to capture the functional variation in HDL particles and the cardiovascular risk associated with HDL. Furthermore, HDL-C concentrations are clearly inadequate for assessment of the potential therapeutic efficacy of novel HDL-targeted therapies. Substantial progress has been made in the development of robust and reproducible methods for assessment of HDL subclasses, and many of these assays are now commercially available. Compelling data are still lacking, however, to indicate that any specific HDL subfractions are clearly more predictive than HDL-C itself. The only exception may be apoA-I, which some data suggest may be more predictive than HDL-C. In contrast to the robust state of clinical chemistry regarding HDL subfractions, the laboratory assessment of HDL function remains in its infancy. In vitro assays of HDL function have been developed by various research laboratories but are laborious, nonstandardized, and poorly validated with regard to human outcomes. There is a major need for further research in this area, particularly the development of standard methods that can be used to apply these assays to large population-based studies to test whether they predict risk independently of HDL-C concentrations. The hope is that eventually mass-based assays can be developed that faithfully reflect the myriad functionality of the HDL particle. Ultimately, a panel of assays that reflect HDL heterogeneity and function may be used for cardiovascular risk prediction, but such a tool would likely have to be incorporated into national guidelines for CHD risk assessment before they would be widely accepted and used. On the other hand, HDL functional assays are likely to be used sooner for the early assessment of the potential efficacy of novel HDL-targeted therapeutics. Robust laboratory assays of HDL function, and their validation with regard to cardiovascular risk prediction and in response to therapeutic interventions, are thus critically important and of great interest to the cardiovascular, clinical chemistry, and pharmaceutical communities.

References


