Cholesterol Efflux Capacity
Full Steam Ahead or a Bump in the Road?

Amit V. Khera, Daniel J. Rader

High-density lipoprotein cholesterol (HDL-C) levels represent a robust and well-integrated biomarker of cardiovascular risk. The prevalence of low HDL-C is high and likely to increase in coming years in parallel with the epidemic of obesity and type 2 diabetes mellitus. However, the clinical efficacy of raising plasma HDL-C levels to achieve cardiovascular risk reduction has been difficult to prove. Recently published outcomes trials involving the addition of niacin or dalcetrapib to standard low-density lipoprotein cholesterol reduction therapy failed to demonstrate clinical benefit despite increases in HDL-C.1,2 Furthermore, genetic variants associated with increased HDL-C, thus conferring lifelong exposure to higher circulating levels, are not consistently associated with improved vascular outcomes.3 These findings have reinforced the idea that changes in HDL-C levels are an inadequate surrogate for therapeutic use.

The notion of HDL functionality has circulated in the field for years based on known heterogeneity in lipid content, protein cargo, and size across HDL particles.4 For example, assays that quantify apoptotic pathway activation, endothelial vaso-motor effects, and antioxidant properties have suggested HDL functional impairment in cardiometabolic disease states.5–7 Although the relative mechanistic contributions of HDL-mediated atheroprotection remain unclear, the role of HDL in macrophage reverse cholesterol transport is thought to play a key role. This pathway involves efflux of cholesterol from macrophages (such as those within atherosclerotic plaque) to HDL acceptor particles for ultimate return to the liver and biliary excretion.8 Animal studies have suggested that flux through this pathway is a better predictor of the atherosclerotic impact of various genetic and pharmacological perturbations than static, mass-based quantification of circulating HDL-C.9

Rothblat et al10 pioneered methodologies allowing assessment of the cholesterol efflux capacity of human serum or HDL, the basic premise of which is that HDL from different persons varies in its ability to promote the efflux of cholesterol from macrophages ex vivo in a manner that is not simply a function of circulating HDL-C concentration. In this approach, murine J774 macrophages are incubated with radiolabeled cholesterol and then exposed to diluted apolipoprotein-B (apoB)–depleted serum from individual subjects, allowing resultant cholesterol efflux from the cells to be quantified. After extensive testing to optimize assay reproducibility and enhance throughput, this assay was applied to 996 patients in 2 distinct cohorts.11 The assay was designed to integrate the efflux pathways thought to be operative in vivo, with a pathway analysis demonstrating that 34% of the efflux was attributable to ATP-binding cassette transporter A1 (ABCA1), 20% to scavenger receptor class B member 1, and 46% to ABCG1, diffusion, or other pathways. The removal of the HDL-containing fraction using ultracentrifugation led to an 87% decrease in cholesterol efflux capacity, demonstrating that HDL is a key mediator of the efflux.11 More than a 4-fold range was noted across individual values, with HDL-C or apoA-I concentrations explaining a modest proportion of the variability. Finally, standard regression analysis demonstrated that cholesterol efflux capacity was a stronger predictor of prevalent atherosclerotic burden than HDL-C or apoA-I levels in 2 distinct subject cohorts. This observation fueled the concept that HDL function (as assessed by cholesterol efflux capacity) may be more important than HDL-C concentration in influencing risk of coronary artery disease (CAD).

In this issue of Arteriosclerosis, Thrombosis, and Vascular Biology, Li et al12 provide an important addition to our rapidly growing understanding of cholesterol efflux capacity and its relationship to cardiovascular disease. They used apoB-depleted serum with the murine RAW 264.7 macrophage cell line and showed that efflux capacity was highly correlated with that from J774 cells via rigorous validation studies (Figure). The authors observed that the majority (≈75%) of the radiolabeled cholesterol that effluxed from the cells was present in a fraction containing apoA-I; the fact that only ≈40% was present on buoyant HDL suggests that a substantial amount of the efflux was to lipid-poor apoA-I. Although a formal analysis of efflux pathways from the macrophages was not reported, this finding is consistent with previous studies that ABCA1-mediated efflux accounts for approximately one third of the efflux in this assay system.10,11 Additional experimentation suggested that albumin (or an albumin-associated protein) is capable of promoting efflux in this assay, although with dramatically lower efficiency, accounting for <10% of observed efflux. Efflux capacity was significantly correlated with plasma levels of both HDL-C (r=0.45–0.52) and apoA-I (r=0.56–0.61) but not correlated with plasma albumin concentration. Upregulation of ABCA1 did not seem to substantially increase the proportion of radiolabeled cholesterol in the albumin fraction, suggesting that the transporter may not be mechanistically involved in efflux to this protein. These results are thus in keeping with a model

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in which apoA-I–containing particles are the key drivers of cholesterol efflux capacity in apoB-depleted serum.

The most important aspect of the article involves an assessment of the relationship of efflux capacity with prevalent CAD in 2 distinct cohorts and with incident cardiovascular events in 1 cohort. Cholesterol efflux capacity was assessed in a case–control sample of stable patients who underwent coronary angiography (n=1150) and a case–control group of patients referred to an ambulatory cardiology outpatient clinic (n=577). Importantly, cholesterol efflux capacity was significantly inversely associated with prevalent CAD in both case–control cohorts. In the angiographic cohort, the relationship was attenuated after adjustment, although it is notable that the disease-free controls were older and had significantly higher Framingham risk scores than the angiographically confirmed CAD cases. Remarkably, in the outpatient study, the subjects in the highest tertile of efflux capacity had an odds ratio of 0.11 for CAD compared with those in the lowest tertile, with minimal subsequent attenuation after adjustment for traditional risk factors, including HDL-C levels. Overall, these results are consistent with the previous study (while extending it to the outpatient setting) and reinforce a paradigm in which cholesterol efflux capacity is protective against the development of atherosclerosis.13

Unexpectedly, an analysis of incident cardiovascular events in the angiographic cohort alone suggested that individuals in the top tertile of cholesterol efflux capacity had a moderately increased risk of a composite cardiovascular end point of incident myocardial infarction, stroke, or death during 3 years of follow-up after angiography. It seems that all subjects were included whether or not they had obstructive coronary disease at time of angiography. This observation is based on relatively few events (n=113) in the overall cohort and even fewer in the highest tertile. Furthermore, the middle tertile was no different from the lowest tertile in incident events. Few examples of a biomarker having discordant relationships with disease prevalence versus incidence have been described. Seemingly paradoxical findings in assessing recurrent events in patients with existing disease may relate to index event bias,13 which may, for example, underlie the positive association of obesity with an initial coronary event but a null or even a protective relationship with recurrent events. When applying this concept to the study of Li et al.,12 those individuals who developed angiographic CAD despite having high efflux capacity could have disproportionately harbored high-risk attributes that overcame the putative protection afforded by increased cholesterol efflux capacity. These other risk factors may subsequently drive the increased risk noted in a fashion that cannot be eliminated by standard multivariable regression adjustments. As noted above, baseline characteristics in the angiographic study differed substantially between cases and controls. For example, control patients were substantially older than CAD cases (mean 72 versus 61 years) and had higher Framingham risk scores. These controls were overrepresented in the top tertile of efflux capacity based on the prevalence associations but may have been at increased risk of the composite end point for reasons unrelated to efflux capacity. Variability was also noted about using lipid-lowering therapies and presumably other therapeutic or lifestyle interventions, most of which have unknown impact on efflux capacity and are potential sources of bias. Despite these significant caveats, this surprising finding has potentially important implications for the interpretation of cholesterol efflux capacity measurements and their relationship to cardiovascular risk.

Putting this study12 in context with previous findings,11 cholesterol efflux capacity has been found to be inversely associated with prevalent atherosclerotic vascular disease in 4 independent cohorts (2 angiographic CAD, 1 carotid intima-media thickness, and 1 outpatient clinical CAD). In contrast, there is a finding of a positive association with incident cardiovascular events in a single angiographic cohort. This observation emphasizes the importance of additional research in this area. It is plausible that cholesterol efflux capacity may relate more strongly with total atherosclerotic burden rather than the acute plaque rupture that triggers coronary events. It will be critically important to measure baseline cholesterol efflux capacity in both primary and secondary prevention cohorts, optimally in those with a larger number of events and more baseline uniformity, and relate it to incident cardiovascular events. The relationships between various HDL functional parameters remain unclear. Is some HDL universally good or does quality vary across functional assays? Do genetic factors influence HDL function independent of HDL-C levels? Little is known about the effect of various pharmacological or lifestyle interventions on HDL functionality, such as efflux capacity, particularly as it relates to future clinical outcomes.

Importantly, the cholesterol efflux capacity assay has several limitations. Cell-based assays are labor-intensive, require a great deal of effort to standardize, and rarely enter clinical practice. Accordingly, a cell-free efflux capacity assay that is based on the uptake of radiolabeled cholesterol is a valuable alternative. The most important aspect of the article involves an assessment of the relationship of efflux capacity with prevalent CAD in 2 distinct cohorts and with incident cardiovascular events in 1 cohort. Cholesterol efflux capacity was assessed in a case–control sample of stable patients who underwent coronary angiography (n=1150) and a case–control group of patients referred to an ambulatory cardiology outpatient clinic (n=577). Importantly, cholesterol efflux capacity was significantly inversely associated with prevalent CAD in both case–control cohorts. In the angiographic cohort, the relationship was attenuated after adjustment, although it is notable that the disease-free controls were older and had significantly higher Framingham risk scores than the angiographically confirmed CAD cases. Remarkably, in the outpatient study, the subjects in the highest tertile of efflux capacity had an odds ratio of 0.11 for CAD compared with those in the lowest tertile, with minimal subsequent attenuation after adjustment for traditional risk factors, including HDL-C levels. Overall, these results are consistent with the previous study (while extending it to the outpatient setting) and reinforce a paradigm in which cholesterol efflux capacity is protective against the development of atherosclerosis.13

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MACROPHAGE CELL LINE
Cell Plating / Culture
\[ \text{Radio labeling with } ^{3}\text{H-Cholesterol} \]
\[ \text{ABCA1 Upregulation with cAMP} \]
\[ 2\% \text{ApoB-depleted Serum} \]
\[ \text{Cholesterol Efflux Capacity} \]

Figure. Cholesterol efflux capacity assay. Schematic diagram of the generic assay of cholesterol efflux capacity. Macrophages are plated, followed by incubation with radiolabeled cholesterol. cAMP is used to upregulate macrophage expression of the ATP-binding cassette transporter (ABCA1). Finally, an individual's serum depleted of apolipoprotein-B (ApoB) particles using precipitation is applied to the cells. After a few hours, media are harvested, with cholesterol efflux capacity expressed as a percentage using the calculation: \(^{3}\text{H count in media}/(^{3}\text{H count extracted from cells}) \times 100.\)
practice. Furthermore, the assay quantifies only 1 component of the reverse cholesterol transport pathway without addressing the efficiency of individuals’ macrophages to efflux cholesterol or hepatic uptake of macrophage-derived cholesterol. Additional efforts may allow for quantification of integrated flux through the entire macrophage reverse cholesterol transport pathway in humans as a complement to the ex vivo measurement of cholesterol efflux capacity.

Although still a relatively young field, research on cholesterol efflux capacity and HDL functionality at large has already led to new insights into the complex relationship between HDL and atherosclerotic cardiovascular disease. The study by Li et al\textsuperscript{12} clearly suggests that there is much yet to be learned.

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**Disclosures**

D.J. Rader consults for Bristol Myers Squibb, CSL, Eli Lilly, Merck, Novartis, Pfizer, and Regulus and is a founder of Vascular Strategies. A.V. Khera reports no conflicts.

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