CHOLESTEROL AND ATHEROGENESIS

Beginning with the studies of Nikolay Anichkov, excess cholesterol has been intimately associated with the pathophysiology of atherosclerotic cardiovascular disease (ASCVD). Atherosclerosis begins with cholesterol accumulation within macrophages in the subendothelial space of the arterial wall. As the cholesterol burden grows, cholesterol-rich lesions form on the arterial wall. High plasma levels of low-density lipoprotein cholesterol (LDL-C) is a positive risk factor for ASCVD. Statins reduce that risk by inhibiting cholesterol biosynthesis, thereby increasing the number of hepatic LDL receptors that mediate plasma LDL-C disposal. Even with the success of statins, residual ASCVD still occurs, albeit at a lower rate, due to other lipid risk factors, especially low plasma levels of high-density lipoprotein cholesterol (HDL-C). Although macrophage-derived cholesterol is rapidly cleared from plasma in mice, most macrophage-derived cholesterol in the arterial wall is taken up by multiple tissue and cell types, especially erythrocytes. Further, some cholesterol is cleared by a nonhepatic transintestinal pathway. Lastly, cholesterol movement among lipid surfaces is reversible, so that a higher-than-normal level of HDL-cholesteryl ester (CE) uptake via the hepatic HDL-receptor, scavenger receptor class B type 1 (SR-B1), reduces ASCVD risk. Recent studies have challenged the validity of this model, most notably the role of LCAT, which appears to be of minor importance. In mice, most macrophage-derived cholesterol is rapidly cleared from plasma (t1/2 < 5 min) without esterification by hepatic uptake; the remainder is taken up by multiple tissue and cell types, especially erythrocytes. Further, some cholesterol is cleared by the nonhepatic transintestinal pathway. Lastly, cholesterol movement among lipid surfaces is reversible, so that a higher-than-normal level of HDL-cholesteryl ester (HDL-C) bioavailability—defined by high plasma HDL levels concurrent with a high mol% HDL-cholesteryl ester (HDL-C)—leads to the transfer of excess cholesterol to cells in vivo. SR-B1−/− mice provide an animal model to study the mechanistic consequences of high HDL-cholesteryl ester (HDL-C) bioavailability that provokes atherosclerosis and other metabolic abnormalities. Future efforts should aim to reduce HDL-cholesteryl ester (HDL-C) bioavailability, thereby reducing FC accretion by tissues and the attendant atherosclerosis.

Despite these early reports, a growing body of evidence has challenged the hypothesis that increasing plasma HDL-C is cardioprotective and questioned the direct mechanistic link between HDL-C and cardioprotection based on the following:

1. Genetically elevated plasma apolipoprotein AI (apoAI) and HDL-C levels do not reduce ASCVD risk.8
2. An HDL-C–raising endothelial lipase variant is not associated with reduced ASCVD.9
3. Many patients with low HDL-C levels do not develop ASCVD, and vice versa.
4. In the AIM-HIGH trial, niacin added to a statin increased HDL-C but did not reduce ASCVD.10
5. Patients with cholesteryl ester transfer protein deficiency, which profoundly increases plasma HDL-C levels, do not experience a commensurate ASCVD reduction predicted by observational data.11
6. CETP inhibitors, which increase plasma HDL-C levels, fail to reduce ASCVD events.12-14

Other studies reveal a more complex, nonlinear relationship between plasma HDL-C levels and ASCVD mortality, with the extremes of high and low HDL-C concentrations being associated with more all-cause and, in some reports, cardiovascular disease mortality.15-18 This association, recently reinvestigated using a pooled analysis of a large population (N = 37,059),19 revealed a U-shaped curve, wherein the highest risk was at the extremes of the plasma HDL-C levels.
concentrations (Figure 1 A). The middle quintile, the “sweet spot,” covers a plasma HDL-C range from 58 to < 77 mg/dL. The distribution of mean plasma HDL-C concentrations for 40- to 44-year-old males and females according to percentile is shown in Figure 1 B. Superimposing the middle quintile in Figure 1 A onto the bottom plot (Figure 1 B) shows that most male subjects with a high hazard ratio have low plasma HDL-C concentrations, while approximately 10% of female subjects have a high hazard ratio due to high HDL-C concentration; the middle quintile denoted by the red bar is the hazard-ratio optimum. The underlying cause of the high hazard ratio at low HDL-C concentrations has been linked to factors that cluster with low plasma HDL-C levels—smokers, physical inactivity, elevated body mass index, high systolic blood pressure—collectively simulating the metabolic syndrome (MetS) phenotype. The underlying cause for the increased hazard ratio in the highest quintile of HDL-C levels is not known. In the world of precision medicine, patients at higher ASCVD risk due to high or low plasma HDL-C levels should be treated differently. However, the treatment plan would depend upon underlying causes, which for high plasma HDL-C levels are not known.

**TRANSHEPATIC REVERSE CHOLESTEROL TRANSPORT**

HDL is thought to elicit a cardioprotective effect through its role in reverse cholesterol transport (RCT), the transfer of cholesterol from macrophages in the arterial wall to the liver for intestinal disposal (Figure 2). The initial RCT step—cholesterol efflux from macrophages to apoAI via the ATP-binding cassette transporter member 1 (ABCA1)—forms nascent (n)HDL. This is

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**Figure 1.**
(A) Hazard ratio by high-density lipoprotein cholesterol (HDL-C) quintile. (B) Correlation of HDL percentile with HDL-C level; red bar denotes the range for the “optimal” HDL-C level from A. MetS: metabolic syndrome; DysHDL: dysfunctional HDL.

**Figure 2.**
Traditional reverse cholesterol transport model comprises three steps: (1) macrophage free cholesterol efflux to apolipoprotein AI via the ATP-binding cassette transporter Al (ABCA1) yields nascent high-density lipoprotein (nHDL); (2) nHDL esterification by lecithin:cholesterol acyltransferase (LCAT) gives spherical HDL; and (3) selective hepatic uptake of HDL cholesterol ester. SR-B1: scavenger receptor class B type 1.
followed by esterification of HDL free cholesterol (HDL-FC) by lecithin:cholesterol acyltransferase (LCAT), which forms mature, spherical HDL with a cholesteryl ester (CE) core. Finally, HDL lipids, including FC and CE, are selectively removed by the hepatic HDL receptor, scavenger receptor class B type 1 (SR-B1), leaving a remnant of mostly phospholipid and protein and lipid-free apoAI.21,22

Given the evidence against the raising-HDL-is-better hypothesis, research focus shifted from HDL quantity to HDL qualities that better support RCT. One of these is the capacity of plasma to accept FC from macrophages, which begs the question, “Do patients with ASCVD have impaired FC efflux compared to healthy, ASCVD-free individuals?” Two large studies addressed this question and found that the magnitude of macrophage cholesterol efflux to HDL negatively correlates with ASCVD, a correlation that persists even after adjustment for plasma HDL-C and apoAI concentrations.23,24 Thus, FC efflux is a metric of HDL function that inversely associates with ASCVD. This metric is not predictively axiomatic due to confounders that have yet to be identified.

**REVERSIBILITY OF CHOLESTEROL EFFLUX**

Free cholesterol is sparingly soluble in water, ∼30 nM.25 For this reason, FC can transfer between membrane and lipoprotein surfaces by rate-limiting desorption from a lipid surface into the surrounding aqueous phase. This step is followed by rapid, diffusion-controlled transfer to another lipid surface or return to the same surface. Transfer of FC between lipid surfaces occurs on a measurable time scale; FC transfer from HDL and LDL occurs with t½ = 5 and 45 min, respectively.26 Thus, if the macrophage FC burden is relieved by efflux to HDL, it can also be increased by influx from HDL, the rate of which would depend on qualities of HDL that control the rate of HDL-FC desorption.

**CELLULAR STUDIES OF REVERSIBLE CHOLESTEROL FLUX**

According to the traditional Glomset/Ross RCT hypothesis as it has evolved, FC efflux to HDL relieves the FC burden of arterial-wall macrophages, thereby preventing or reversing atherogenesis. However, FC moves freely and reversibly between and across lipid surfaces of cell membranes and plasma lipoproteins,27 so that dysfunctional HDL that supports FC influx into cells could be atherogenic. The balance of FC efflux versus influx between macrophages and reassembled HDL (rHDL) has been studied as a function of the rHDL-FC content, expressed as mol% FC.28 Decreased and increased cell-associated FC corresponds to efflux and influx, respectively. In the study by Picardo et al., cellular FC efflux to rHDL containing no FC (0 mol%) increased with increasing rHDL concentration. As the rHDL-FC content increased to 50 mol%, FC flux shifted from efflux to influx. Moreover, the magnitude of the efflux and influx increased with rHDL concentrations, reaching a maximum effect at 50 μg/mL HDL protein. Cellular FC biosynthesis according to HMG-CoA reductase (HMGR) activity decreased with increasing cellular FC content. Thus, during efflux and influx, which decrease and increase cell FC content, respectively, HMGR activity increases and decreases. At high cellular FC content, the FC is detoxified by its conversion to CE via acyl-CoA-cholesterol acyltransferase (ACAT); consequently, as cellular FC content increases, so does CE formation. The effects of rHDL on cell FC, HMGR, and ACAT were most profound at high rHDL concentrations. A plot of cell FC, HMGR, and ACAT activities versus the rHDL mol% FC at 50 μg/mL rHDL-protein reveals that between 5 and 20 mol% rHDL-FC, rHDL switches from FC acceptor to FC donor (Figure 3). Extrapolation of these data to human physiology suggests that high plasma concentrations of HDL with high FC content would support net FC transfer to tissues, an atherogenic process.
A MOUSE MODEL OF DYSFUNCTIONAL HDL DUE TO HIGH HDL-FC BIOAVAILABILITY

Mice deficient in the HDL receptor SR-B1 are robust models of dysfunctional high plasma HDL-C levels. Compared to wild type (WT) mice, HDL in SR-B1−/− mice is larger and richer in FC (∼15 vs ∼60 mol%, respectively); plasma HDL levels of SR-B1−/− mice are two-times higher than those of WT.29 SR-B1−/− mice are characterized by multiple metabolic defects, such as abnormal platelet and erythrocyte structure and function and infertility among female mice.30 Moreover, when put on a high-fat, high-cholesterol diet, the mice develop atherosclerosis despite a doubling of the HDL concentration and without a meaningful change in the plasma concentration of apoB-containing lipoproteins.31 Thus, while it is by far the most abundant plasma lipoprotein among SR-B1−/− mice, some property of HDL is toxic. We reiterate our hypothesis that the pathological quality of SR-B1−/− HDL is a high HDL-FC bioavailability that is supported by a high plasma HDL concentration (i.e., a high particle number, and a high mol% FC). Comparison of the aforementioned mol% values with the data of Figure 3 suggests that the HDL of SR-B1−/− mice would strongly support FC influx into cells. Notably, the lipid-lowering drug probucol restores some function, including fertility.30,32 However, this is a partial effect in that probucol reduces the atherogenicity of HDL by reducing its plasma concentration without profoundly affecting HDL-FC content.

The importance of mol% HDL-FC is illustrated in the kinetic model in Figure 4, which compares the transfer mechanism and final transfer of FC between WT versus SR-B1−/− mice. This model shows that the greater amount of FC transfer from SR-B1−/− HDL is due to its higher mol% FC. Given that the rate-limiting step for HDL-FC transfer to all tissue and lipoprotein sites is the initial desorption, HDL-FC transfer to LDL is a valid metric for transfer to all tissues and lipoproteins.

Genetic studies of human SCARB1 (the SR-B1 gene) variants positively associate with plasma HDL-C levels and peripheral cellular SR-B1-protein levels.33 Carriers of an SR-B1 variant have increased HDL-C levels, impaired cholesterol efflux, altered platelet function, and reduced adrenal steroidogenesis.34 In the context of the HDL–SR-B1–atherogenesis axis, patients heterozygous for the SR-B1 variant (P376L, where leucine replaces proline 376) showed increased plasma HDL-C levels and ASCVD35; in addition, the rs10846744 single nucleotide polymorphism in the SCARB1 enhancer region is also associated with ASCVD.36 Although loss of SR-B1 function appears to be pathological, the HDL–SR-B1–atherogenesis axis is complex; ultra-high SR-B1 expression, which induces low HDL levels, is nearly as atherogenic as SR-B1 deficiency.37 Currently, there are no approved cardioprotective therapies for elevated and/or dysfunctional HDL with underlying HDL-FC hyper-bioavailability.

IN VIVO NHDL KINETICS

Although the traditional Glomset/Ross RCT model (Figure 2) has been implicated in many studies of FC transport—including those showing that the magnitude of FC efflux (the first step) inversely correlates with ASCVD incidence—it was never rigorously tested until recently. FC efflux to apoAI produces nascent (n)HDL, which contains FC, apoAI, and cell-derived phospholipids (PL). Xu et al. tested a triply labeled nHDL via the interaction of [125I]apoAI with BHK cells overexpressing the ABCA1 transporter and labeled with [3H]FC and [14C]PL.38 The labeled nHDL was injected into mice, and the rates of clearance and tissue sites of label accumulation were determined. As proposed in the traditional RCT model, most FC and PL were cleared by the liver. However, the data were inconsistent with the traditional RCT model, which implicates LCAT in RCT. The clearance of the main LCAT substrates, FC and PL, was rapid, t1/2 = ∼5 and 2 min, respectively—times during which only ∼2% of FC was esterified. Thus, LCAT plays a minor role in most RCT. Given the rapid clearance of FC and the minor role of LCAT, one would also conclude that selective CE uptake via SR-B1 plays a minor role in RCT. However, in light of other...
data, SR-B1 likely mediates a major fraction of hepatic FC clearance; overexpression of SR-B1 in mice accelerates hepatic FC uptake. SR-B1 also transfers a greater fraction of HDL-FC versus CE into cells. Thus, a major fraction of nHDL is cleared via SR-B1, although spontaneous SR-B1–independent uptake is also likely important because it, too, occurs in minutes. Similar studies in humans showed a t½ < 10 min for plasma clearance of HDL-FC. Thus, these data support the hypothesis that most RCT is simply reverse free cholesterol transport and involves little LCAT-derived CE. Collectively, these data support a revised model of RCT (Figure 5).

TRANSINTESTINAL CHOLESTEROL EXCRETION AND FC BIOAVAILABILITY

Studies show that there is some FC transfer to the feces independent of bile. For example, Pertsemlidis and colleagues found FC in the feces of dogs whose bile was diverted to the urinary system. In another study by Yu, mice with deleted hepatic FC transporter had high fecal neutral sterol levels, but their biliary cholesterol secretion rates were almost nonexistent. These findings suggest that FC elimination may occur through direct transintestinal cholesterol excretion (TICE), which only involves FC, not CE. This can occur via two mechanisms, the first being spontaneous transfer from plasma lipoproteins to the intestine and other tissue sites. The transfer of FC to lipoprotein and membrane surfaces from HDL is rapid, t½ = –5 min, whereas FC transfer across membranes is < 1 sec. Consequently, TICE may start with spontaneous FC transfer from lipoproteins to membranes in contact with the plasma compartment followed by diffusion to numerous phospholipid-containing loci between plasma and the intestine. The other possible TICE mechanism involves cholesterol transporters on the plasma membrane of various cellular interfaces between the plasma compartment and the intestine. One of these transporters is SR-B1, which facilitates bidirectional FC transit. To have net transfer to excretion, this process must include an irreversible step that sequesters FC. Since intestinal PL content is a positive regulator of TICE, and PL is the cholesterophilic component of bile, a high concentration of intestinal bile-phospholipid would support net RCT via TICE. TICE may be stimulated by dietary changes that include and sustain a high bile-phospholipid content, thereby contributing to whole-body FC disposal.

The relative contributions of spontaneous and SR-B1–mediated transfer to TICE are unknown. However, TICE is probably only one component of FC transfer to multiple tissue sites. Although our data showed rapid hepatic FC uptake in mice over longer time intervals, the FC appeared in nearly every tissue site, especially erythrocytes (13% at max). Thus, FC is highly mobile and goes to any tissue or cell site by diffusion.

IMPORTANCE OF PLASMA HDL-FC BIOAVAILABILITY IN VIVO

Comparison of the in vivo rates of FC and CE flux reveals that FC flux is an order of magnitude faster than that of CE and several orders of magnitude faster than lipoprotein turnover, which occurs in hours to days. Thus, one would expect FC flux among cells and lipoproteins to be an important determinant of its metabolic and pathophysiological effects. Although in vitro studies in cells show that a high mol% FC in HDL and high plasma HDL concentration promotes influx, complementary in vivo data are lacking. Moreover, most large clinical trials focus on total cholesterol and LDL-C, which contain the sum of FC and CE; there is little information on the relationship, if any, between plasma and/or HDL-FC and ASCVD and whether FC bioavailability contributes to ASCVD risk reduction achieved by statin therapy. Nevertheless, it is relevant and supportive of our hypothesis that the HDL:(TC/PL) ratio of patients with very high HDL-C was 20% higher and the plasma efflux capacity
was lower than in those with normal HDL-C. Although they did not report HDL-FC influx to cells nor the HDL-(FC/PL) ratio, which would determine mol% FC, this report supports the hypothesis that FC bioavailability is higher among the high HDL-C patients.

CONCLUSION

Free cholesterol transport among plasma lipoproteins and various tissues occurs by multiple mechanisms. One minor pathway is LCAT-mediated conversion to CE, which is hepatically extracted by SR-B1–mediated selective uptake. Another is spontaneous and SR-B1–mediated FC transfer to multiple tissue sites, especially the liver. Spontaneous transfer, such as diffusion, mediates the appearance of lipoprotein-derived FC in nearly all tissues and red blood cells. A high plasma HDL-FC is possibly atherogenic and toxic to some tissues. New approaches to reduce HDL-FC bioavailability are needed to control FC cytotoxicity that is associated with high plasma HDL and high HDL-FC per particle content.

KEY POINTS

• Recent studies support changes to the widely cited reverse cholesterol transport (RCT) mechanism. Foremost among these is the minimal involvement of lecithin:cholesterol acyltransferase (LCAT)-mediated free cholesterol (FC) esterification, which is interesting given that the discovery of LCAT provoked formulation of the traditional RCT mechanism.
• Mechanistically, FC transfer to the liver occurs via spontaneous transfer and scavenger receptor class B type 1–mediated uptake.
• The FC content of high-density lipoprotein (HDL) and the plasma HDL concentration are both determinants of FC bioavailability, which may be an important determinant of several pathological states, including atherosclerosis.

Conflict of Interest Disclosure:
Dr. Gotto is a consultant for Esperion and KOWA Pharmaceuticals America, Inc. and is on the Data Safety Monitoring Board for Ionis Pharmaceuticals.

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