More than 60 years ago, an observation was made that would change the way we understood how cholesterol contributes to cardiovascular disease (CVD) development. In 1951, Barr et al.1 noted that individuals who have atherosclerosis tended to have low levels of plasma \(\alpha\)-lipoproteins, now widely known as high-density lipoprotein (HDL). He then put forth the notion that measurement of the levels of these lipoproteins would be a valuable tool for assessing an individual’s risk of developing atherosclerosis and even perhaps aid in its early detection. These observations, combined with the findings of the Framingham Study2 set in motion decades of research that would eventually contribute to the HDL-cholesterol (HDL-C) hypothesis that states that low levels of circulating HDL are a causative factor in the development of CVD. Since then, a wealth of epidemiological studies have demonstrated an inverse correlation between plasma levels of HDL-C and the risk of CVD and its thrombotic complications.3 This correlation is thought to reflect the ability of HDL particles to remove excess cholesterol from peripheral cells, particularly macrophages in atherosclerotic plaques, for return to the liver. Supporting this, several studies in animal models have demonstrated that raising the number of HDL particles by either direct infusion of HDL or by overexpressing apolipoprotein A-I (a major protein component of HDL) can reduce atherosclerosis progression or promote its regression.4,5 As a result of these collective observations, HDL has earned the moniker of the 'good cholesterol'.

MicroRNA Control of High-Density Lipoprotein Metabolism and Function
Katey J. Rayner, Kathryn J. Moore

Abstract: Recent discoveries of microRNAs (miRNAs) that control high-density lipoprotein abundance and function have expanded our knowledge of the mechanisms regulating this important lipoprotein subclass. miRNAs have been shown to regulate gene networks that control high-density lipoprotein biogenesis and uptake, as well as discrete steps in the reverse cholesterol transport pathway. Furthermore, high-density lipoprotein itself has been shown to transport miRNAs selectively in health and disease, offering new possibilities of how this lipoprotein may alter gene expression in distal target cells and tissues. Collectively, these discoveries offer new insights into the mechanisms governing high-density lipoprotein metabolism and function and open new avenues for the development of therapeutics for the treatment of cardiovascular disease. (Circ Res. 2014;114:183-192.)

Key Words: cholesterol, HDL ■ lipid metabolism ■ microRNAs
good cholesterol. Yet, recent Mendelian randomization studies have shown that certain single-nucleotide polymorphisms that raise plasma HDL-C levels do not lower the risk of myocardial infarction, challenging the concept that HDL is atheroprotective. Compounding these findings, several clinical trials of HDL-C–raising therapeutics, including niacin and inhibitors of cholesterol ester transfer protein (CETP), have failed to show benefit. These studies have begun to cast doubt on HDL's atheroprotective functions and spurred further investigations of the molecular mechanisms regulating levels of plasma HDL and its function. One area of rapid growth in this regard has been the discovery of microRNAs (miRNA) as potent regulators of the gene pathways controlling HDL genesis, cholesterol efflux, and reverse cholesterol transport (RCT).

For the past decade, the importance of noncoding RNA as regulators of pre-, co-, and post-transcriptional gene expression has emerged. Of the various classes of noncoding RNAs, miRNAs are currently the most widely studied and have established roles in regulating a myriad of biological processes. The human genome contains >2500 unique mature miRNAs, and these are encoded in both intergenic and intronic regions of the genome. A number of reviews describing the transcription and processing of miRNAs have recently been published. In their mature form, these small, noncoding RNAs of ≈22 nucleotides bind to partially complementary sites primarily found in the 3′ untranslated region (UTR) region of target mRNAs and inhibit gene expression via induction of mRNA degradation or translational repression. Although the founding work on miRNAs was performed in Caenorhabditis elegans, this mechanism of post-transcriptional gene regulation has been highly conserved throughout evolution. It is now estimated that >60% of all human genes are regulated by miRNAs. Studies of miRNA expression and function have demonstrated that miRNAs allow for context-dependent fine-tuning of gene expression, for example during embryonic development or environmental stresses (eg, starvation). In this regard, a powerful aspect of miRNAs is their ability to repress numerous genes simultaneously to influence the output of biological gene networks. Although the magnitude of a miRNA's effect on any 1 gene may be modest (often on the order of 10%–30%), the ability of miRNAs to repress multiple genes or steps in a pathway can lead to robust inhibition of the output.

Recent studies in the field of lipid metabolism have identified microRNAs as regulators of plasma levels of lipoproteins, novel intercellular signaling molecules, and plasma biomarkers of physiological status. As described below, multiple genes affecting HDL and the RCT pathway have been shown to be under control of these small noncoding RNAs, including those affecting HDL biogenesis, cellular cholesterol efflux, selective cholesterol uptake from HDL, and bile transport (Figure). These studies have revealed how single miRNAs can target multiple components of this pathway and also identified key genes that are under control of multiple miRNAs. These findings have garneried considerable interest not only for their insight into how cells and tissues regulate HDL function, but also for their potential as targets for miRNA-based therapies.

**MicroRNAs Controlling HDL Biogenesis**

Nascent HDL is generated in the liver through the efflux of cholesterol and phospholipid across the hepatocyte cell membrane onto newly synthesized lipid-poor apolipoprotein A-I. The ATP-binding cassette transporter A1 (ABCA1) plays a critical role in this process as evidenced by the near-absence of plasma HDL-C in patients with Tangier disease, which results from mutations in the ABCA1 gene. The levels of ABCA1 at the plasma membrane controls the rate of cholesterol efflux to apolipoprotein A-I, and hepatic-specific deletion of ABCA1 results in an ≈85% loss of total HDL-C, with ABCA1 in the adipose and intestine contributing to the residual balance. Several miRNAs have recently been identified that target ABCA1 and thus regulate plasma levels of HDL-C. Among these, miR-33a and miR-33b were the first to be reported and were intriguing because of their genomic context: miR-33a and miR-33b are embedded in intronic regions of the sterol regulatory element–binding protein 2 (SREBP2) and SREBP1 genes that code for the SREBP2 and SREBP1 transcription factors that control the expression of genes involved in cholesterol and fatty acid synthesis. MiR-33a/miR-33b are coregulated with their host genes and act to repress gene programs that oppose SREBP functions (eg, cholesterol efflux and fatty acid oxidation). For example, under low-cholesterol conditions that trigger transcription of SREBP2 and the regulation of genes involved in cholesterol synthesis and uptake, cotranscription of miR-33a acts to inhibit cellular cholesterol export by targeting ABCA1. The 3′UTRs of mouse and human ABCA1 mRNA harbor 4 miR-33a binding sites, resulting in strong repression of ABCA1 mRNA and protein. Furthermore, consistent with the ability of most miRNAs to mediate pathway regulation, miR-33a/miR-33b also target other genes that contribute to cholesterol mobilization and efflux from the cell, including NPC1 and ABCG1. The physiological relevance of miR-33 targeting of ABCA1 was initially demonstrated using inhibitors of miR-33, which increased cholesterol efflux from hepatocytes to apolipoprotein A-I in vitro and raised levels of plasma HDL-C in mice by 25% to 30%. These findings were subsequently confirmed by targeted deletion of miR-33, which resulted in 25% and 40% increases in plasma HDL-C in male and female miR-33 null mice, respectively.

The 2 members of the miR-33 family, miR-33a and miR-33b, differ by only 2 nucleotides in their mature form; however, these nucleotides lie outside the seed region (5′ bases 2–8 of the mature miRNA) that dictates target recognition. Thus, miR-33a and miR-33b are predicted to target a similar subset...
of genes, and experiments to date have shown comparable repression of ABCA1 and other known targets by these 2 isoforms. Nonetheless, differences in their genomic context and evolutionary conservation may influence biological outcomes. For example, the abundance of miR-33a and miR-33b is controlled by factors that regulate their host genes and the amplitude of the induction of SREBF2/1 (eg, levels of SREBP2 mRNA are increased 2- to 3-fold by sterol depletion, whereas levels of SREBP1 can be induced >10× that amount by insulin). Furthermore, the presence of miR-33a within the SREBF2 locus is highly conserved across species, whereas miR-33b is present in primates, but lacking in rodents and lower organisms. Such differences would lead to high levels of miR-33b in insulin-resistant states in humans, and thus repression of ABCA1 and plasma HDL, that would not be observed in mice.

To determine whether the findings of miR-33 inhibition in mice were translatable to primates, a study was undertaken in African green monkeys using 2′F/MOE anti–miR-33 oligonucleotides designed to inhibit both miR-33a and miR-33b. Anti–miR-33 treatment increased hepatic expression of ABCA1 and plasma HDL-C in monkeys fed both a chow diet and a high-carbohydrate diet designed to increase levels of SREBP1 and thus miR-33b. Notably, these effects of miR-33 inhibition were accompanied by increases in the number of large HDL particles and apolipoprotein A-I in the circulation, attributes that have been shown to be atheroprotective in studies of other HDL-raising therapies. These studies solidified the notion that miR-33 represses hepatic ABCA1 expression and thus dampens plasma HDL-C levels in a model highly relevant to humans and highlighted its potential as a therapeutic target to raise HDL.

Although the majority of miR-33 studies have focused on the 5p strand, a recent report indicates that the miR-33 passenger strand or * strand may also be active in certain cell types or tissues. An increasing number of miRNA* sequences with abundant expression have been reported to act as guide miRNA*s, prompting renewed interest in their function. Goedeke et al showed that miR-33a* and miR-33b* accumulate under steady state conditions in various mouse, monkey, and human tissues, and other groups have noted the specific regulation of miR-33a* in endothelial cells subjected to hypoxia and in M2-polarized macrophages. The miR-33a* and miR-33b* strands are highly conserved across species, suggesting a conserved function. Interestingly, miR-33a* and miR-33b* were shown to target a similar subset of lipid metabolism genes (NPC1, CROT, IRS2, SREC3, NFYC, RIP140, ABCA1 indirectly) as their sister strands, implying that both strands of the miR-33 locus may work in concert to regulate cellular cholesterol metabolism. miR-33a* and miR-33b* have different seed sequences from miR-33a and miR-33b and thus are predicted to bind different sites in the 3′UTR of their target genes. This dual targeting of lipid metabolism genes by both strands of the miR-33 duplex would result in strong repression of their targets. Future studies examining the regulation of miR-33 and miR-33* abundance will be important to understand the factors that support their activities.
accumulation in the cell. The mechanisms of miRNA strand selection and loading into the RNA-induced silencing complex remain obscure but are thought to be related to the thermodynamic stability of each strand of the duplex. Interestingly a recent study reported stabilization of strands by their target miRNAs, suggesting a scenario in which miR-33 may be stabilized to help miR-33 regulate genes involved in cholesterol efflux and fatty acid oxidation. The functional effects of miR-33 on HDL and triglyceride levels in vivo have yet to be examined but are likely to be relevant to the design of current therapeutic strategies to inhibit miR-33 for the treatment of atherosclerosis, as targeting of 1 arm of the duplex would not necessarily inhibit the functionality of the other and may in fact lead to stabilization of the nontargeted strand.

ABCA1 has an uncommonly long 3′UTR of >3.3 kb, rendering it particularly susceptible to post-transcriptional regulation by miRNAs. Indeed, shortly after the discovery of miR-33, other miRNAs were found to repress ABCA1 and cholesterol efflux in vitro, including miR-75854, miR-26a, and miR-106b. Recently, 2 groups reported that miR-144, an intergenic miRNA present in a bicistronic cluster with miR-451, also targets ABCA1 in the liver and modulates plasma HDL-C levels. Interestingly, miR-144 expression is regulated by 2 members of the nuclear hormone receptor family: the farnesoid X receptor and the liver X receptors (LXR), providing fine-tuning of ABCA1 expression under specific biological contexts. These ligand-activated transcription factors contribute to the regulation of cholesterol homeostasis through transcriptional regulation of lipid-associated genes: farnesoid X receptor controls hepatic steroid and bile acid levels, and LXR controls components of the cellular cholesterol efflux pathway in the liver and macrophages, including ABCA1. Farnesoid X receptor induction of miR-144 transcription may channel cholesterol to the bile for excretion by repressing hepatic ABCA1 and thus HDL biogenesis. Although this serves to reduce plasma HDL-C levels, this effect of miR-144 may be favorable overall by promoting RCT, as has been observed with probucol treatment. However, LXR upregulation of miR-144 during cholesterol excess may function as a feedback mechanism to prevent uncontrolled LXR-induced cholesterol efflux through ABCA1. In both studies, anti-miR inhibition of miR-144 in mice resulted in increased hepatic ABCA1 expression and plasma HDL-C levels. However, further studies of the effect of miR-144 inhibition on RCT will be required to determine its effect on biliary cholesterol excretion.

Collectively, the studies of miR-33 and miR-144 have begun to illuminate the intricate network that fine-tunes ABCA1-dependent cholesterol efflux from the liver to regulate plasma HDL-C. It is likely that numerous other microRNAs will be identified to act in concert to regulate ABCA1 expression in the liver, with their individual and combined contributions determined by factors that regulate miRNA expression and abundance. The identification of such metabolic rheostats will no doubt provide new opportunities for therapeutic manipulation of plasma HDL-C levels and RCT.

**MicroRNAs Controlling Cellular Cholesterol Mobilization**

The efflux of excess cholesterol from peripheral tissues, particularly macrophages in the artery wall, is essential for maintaining cholesterol homeostasis. At the cellular level, this requires that cholesterol first be mobilized from internal stores via cooperation of the lysosome, lipid droplets, neutral cholesterol ester hydrolase, and the autophagy machinery. The final step of cholesterol efflux from the plasma membrane is mediated by ABCA1 to lipid-poor apolipoprotein A-I (ApoE in the brain) and through the related transporter, ABCG1, to mature HDL particles. This ability of HDL and apolipoprotein A-I to act as acceptors of excess cholesterol from cells is thought to be central to their protective functions and this constitutes the first step in the RCT pathway through which HDL ferries cholesterol back to the liver for excretion. Through the coordination of these cholesterol mobilization pathways, net cholesterol balance in the arterial wall is maintained and pro-inflammatory responses by arterial cholesterol-loaded macrophages are reduced. Because each of these steps represents potential points of microRNA control, the complexity of microRNA regulation of cholesterol efflux is likely to be much greater than originally anticipated.

As an example, autophagy, which regulates the availability of free cholesterol for efflux and contributes prominently to macrophage RCT in vivo, is a complex process that requires multiple sequential membrane remodeling and trafficking events, orchestrated by a small army of autophagy-related gene (ATG) proteins. This pathway has recently been shown to be regulated by several miRNAs, including miR-18a, miR-20a, miR-30a, miR-30d, miR-101, miR-106b, miR-132, miR-181a, miR-196, miR-212, miR-221, miR-222, miR-376b, miR-502, which act by targeting ATG proteins (ATG2, ATG4, ATG5, and ATG12) or their upstream effectors (BECN1, mTOR, and ULK1). To date, the majority of these miRNAs regulating autophagy have been characterized in different types of cancer (breast cancer, hepatocellular carcinoma, chronic myelogenous leukemia, colon cancer, and melanomas), as well as in cardiac hypertrophy, Parkinson disease, and Crohn disease. Although the role of these miRNAs in regulating lysosomal trafficking of cholesterol has yet to be investigated, they are likely to have a major effect on cholesterol efflux, RCT, and HDL function. Because the activation of autophagy in macrophages has been shown to suppress foam cell formation and atherosclerosis in mice, therapeutic targeting of microRNAs that limit this pathway may provide new therapeutic targets for enhancing cholesterol flux.

Although macrophage RCT does not significantly alter total plasma HDL-C levels, its contribution is critical to atheroprotection. Indeed, Khra et al showed that the efflux capacity of HDL is an independent and robust predictor of atherosclerosis in humans, which is not simply explained by levels of HDL-C in the circulation. Because ABCA1 and ABCG1 control the terminal steps of cholesterol efflux to nascent and mature HDL from extrahepatic cells, microRNAs that target these genes would be predicted to inhibit RCT. In mice, miR-33 targets both ABCA1 and ABCG1 (ABCG1 is not a target in humans), and miR-33 inhibitors enhance macrophage cholesterol efflux to apolipoprotein A-I and HDL in vivo. Furthermore, parenteral delivery of anti-miR-33 oligonucleotides in mice increased RCT from labeled macrophages in vivo and directly upregulated ABCA1 in...
atherosclerotic plaque macrophages to reduce plaque cholesterol content.38 Although miR-33 has been the most extensively studied in vivo, several other microRNAs have been shown to regulate ABCA1 in macrophages and other cell types, including miR-758, miR-26, miR-106 and miR-144.40-48 Like miR-33, miR-26 also downregulates other genes involved in cholesterol mobilization in addition to ABCA1, such as ADP-ribosylation factor-like 7, an intracellular transport protein that moves cholesterol to the membrane for removal by ABCA1.47 Expression of miR-26 is suppressed by LXR and thus would be predicted to be downregulated under conditions of cholesterol excess during which increased levels of ABCA1 would be needed. However, miR-144 would be induced by LXR to target ABCA1 under similar conditions,49 and thus further studies of the temporal expression and abundance of these 2 microRNAs will be needed to resolve their relative contribution to cholesterol efflux control. Finally, miR-758 and miR-106b have been found to be highly enriched in the brain where ABCA1 plays a key role in effluxing excess cholesterol to apoE, the predominant apolipoprotein in the brain.50,51 Notably, ABCA1-dependent cholesterol efflux seems to reduce the accrual of amyloid-β in the brain, and in accordance with this, overexpression of miR-106b in neuronal cells increased the accumulation of amyloid-β in these cells.46 Thus, although multiple miRNAs can mediate post-transcriptional regulation of ABCA1, their individual effect on cholesterol efflux and RCT will be influenced by factors, such as their relative tissue enrichment, transcriptional regulation, as well as miRNA cooperation and competition.

Recent studies have highlighted the complex interplay of dietary nutrients and intestinal microbiota composition in influencing cardiometabolic diseases.61,62 Dietary anthocyanins, such as the cyanidin-3-O-B-glucoside (Cy-3-G) polyphenol commonly found in fruits, berries, and red wine, have been associated with reduced risk of CVD. This has now been linked in a series of studies to the actions of a Cy-3-G metabolite, protocatechuic acid (PCA), which reduces levels of miR-10b, a newly identified repressor of ABCA1 and ABCG1.63,64 Studies in antibiotic-treated and germ-free mice established that dietary Cy-3-G conversion to PCA, and its downstream enhancement of RCT, was dependent on the gut microbiota.64 Using physiological concentrations of PCA achieved with Cy-3-G dietary supplementation, the authors showed that PCA treatment of macrophages reduced miR-10b, causing derepression of its target genes ABCA1 and ABCG1, and increasing cholesterol efflux capacity. In Apoe−/− mice treated for 4 weeks with dietary Cy-3-G or PCA, these observed changes in miR-10b, macrophage cholesterol efflux and RCT were associated with a reduction in atherosclerotic plaque size. This study underscores the complex interaction of the gut microbiome with risk factors for CVD and highlights a new mechanism through which microRNAs that regulate cholesterol homeostasis could be modulated. Future studies investigating how other gut-microbiota–derived compounds, such as the recently identified plasma metabolite trimethylamine N-oxide that promotes macrophage cholesterol accumulation and atherosclerosis,62 might also alter cholesterol-associated microRNAs will no doubt reveal new mechanistic links between metabolism and host–microbial interactions.

**MicroRNAs Targeting Hepatic HDL Uptake and Excretion**

Transport of HDL-C to the liver for bile acid synthesis and excretion is the final step of RCT and can occur either directly via the scavenger receptor B-1 (SR-B1) or after transfer to apolipoprotein B–containing lipoproteins by the CETP present in humans.3 MicroRNAs targeting these pathways are just beginning to be explored and represent exciting new therapeutic targets to influence the route of delivery of HDL’s cargo. SR-B1 is a plasma membrane glycoprotein structurally similar to CD36 that is most highly expressed in liver and steroidogenic tissues, where it delivers HDL-derived cholesterol for excretion and steroid hormone synthesis. SR-B1–mediated selective uptake of HDL-C is considered a beneficial pathway because it both increases the rate of delivery of cholesterol to the liver and results in the release of cholesterol-depleted HDL particles that are recycled to promote cholesterol efflux further. The level of SR-B1 expression is controlled at the transcriptional level by nuclear hormone receptor transcription factors, such as PPARg and LXR, and at the post-transcriptional level by alternative splicing of the mRNA. The additional posttranscriptional control of this pathway by microRNAs was recently demonstrated using small interfering RNA silencing of the miRNA-processing enzymes Drosha and Dicer, which resulted in a marked increase in SR-B1 mRNA and protein in HEPG2 cells.65 Bioinformatic prediction algorithms, such as Targetscan and MiRanda, indicate that ≤50 microRNAs may target the 3′UTR of human SR-B1. Among these, miR-185, miR-96, and miR-223 were validated as strong repressors of SR-B1 mRNA and cell surface expression, and their inhibition in HEPG2 cells increased SR-B1 expression and selective HDL-C uptake.66 Notably, when 2 or 3 of these miRNAs were combined, there was greater repression of SR-B1 than that conferred by any single miRNA, suggesting that these microRNAs may coordinately repress SR-B1 mRNA by simultaneously binding to different regions of the SR-B1 3′UTR. Interestingly, miR-185, miR-96, and miR-223 have all been reported to regulate genes involved in the proliferation of various tumor cell lines although changes in proliferation were not observed in the HEPG2 cells used for the study of SR-B1. Although the factors regulating expression of these microRNAs have not yet been explored, miR-185 is located within the first intron of a gene of unknown function, C22orf25, whereas miR-96 and miR-223 are intergenic microRNAs encoded on chromosomes 7 and X, respectively. Of these microRNAs, only miR-223 does not have conserved target sites in the rodent SR-B1 3′UTR. An analogous screen of microRNAs targeting the 3′UTR of mouse SR-B1 identified miR-125a and miR-455 as potent regulators of SR-B1 expression in murine steroidogenic and hepatic cell lines.67 Overexpression of these microRNAs reduced both SR-B1–mediated selective HDL uptake and HDL-stimulated progestosterone production. The miR-125a–binding site is conserved in the human SR-B1 3′UTR; however, studies of its function in human cells have yet to be performed. Although in vivo studies demonstrating that inhibition of mouse and primate SR-B1 targeting microRNAs increase HDL-C uptake are still lacking, these hold promise as a therapeutic approach to hold to increase RCT through this pathway.
During its journey to the liver, HDL undergoes numerous remodeling events that affect its size and composition. CETP mediates the exchange of HDL-cholesteryl esters for triglycerides from apoB-containing lipoproteins, thus shifting HDL’s cholesteryl cargo for uptake by the LDL receptor in the liver. Inhibitors of CETP have been actively pursued by the pharmaceutical industry as a means to raise plasma HDL-C and RCT.\(^7\)\(^8\) Although no microRNAs have yet been described to target the CETP gene, and its relatively small UTR (<200 nucleotides) is only predicted to contain few miRNA-binding sites, it is likely that the expression of this and other enzymes that modulate HDL composition and function, such as lecithin-cholesterol acyltransferase, hepatic lipase, and endothelial lipase, will be found to be under microRNA control.

Hepatic cholesterol delivered via either SR-BI or the LDL receptor can be oxygenated, converted into bile acids, and secreted into the intestine via canalicular transporters. Although the majority of bile acids are reabsorbed in the intestines, a proportion is eliminated in the feces, thereby ridding the body of excess cholesteryl. In addition to its roles in regulating HDL biogenesis and macrophage cholesteryl efflux, miR-33 has also been shown to regulate hepatic bile metabolism.\(^7\)\(^9\) miR-33 targets the 3′UTRs of ABCB11 and ATP8B1, transporters that reside in hepatic canalicular membranes and play essential roles in regulating biliary output (Figure). Using locked nucleic acid–mediated silencing of miR-33, Allen et al.\(^9\) showed that miR-33 inhibition increased sterols in the bile and enhanced RCT in vivo. Similar studies in Ldlr\(^−/−\) mice by Rayner et al\(^9\) using 2′F/2′MOE oligonucleotide inhibitors of miR-33 noted a step-wise increase in RCT to the serum, liver, and feces—30%, 50%, and 85%, respectively—reinforcing the notion that miR-33 coordinates RCT at multiple levels.

**HDL Transport of MicroRNAs: A Predictor of Functionality?**

Although microRNAs act intracellularly, microRNAs have been shown to be exported from both healthy and diseased tissues and cells. Extracellular microRNAs can be transported in membrane-derived vesicles (exosomes and microparticles), on lipoproteins, or bound to proteins, such as Argonaute2, and these circulating microRNAs are remarkably stable in plasma. As a result, extracellular microRNAs are being studied as novel biomarkers of disease states, including CVD, and distinct circulating microRNA signatures are beginning to be identified in health and disease.\(^9\)\(^0\) For example, recent studies have identified miRNA-208b and miR-499 as promising biomarkers of acute myocardial infarction; however, these remain to be validated in larger populations.\(^9\)\(^0\)\(^9\) Perhaps more exciting is the recognition that extracellular microRNAs represent a novel class of signaling molecules that may mediate cell-to-cell communication. A key role for such extracellular miRNA signaling was demonstrated in the artery wall between endothelial and smooth muscle cells and was shown to mediate atheroprotection.\(^9\)\(^2\) Hergenreider et al.\(^9\) showed that atheroprotective shear stress regulates the expression of multiple microRNAs in endothelial cells via the transcription factor KLF2, which are exported in extracellular vesicles. Most prominent among these were miR-143 and miR-145, which can regulate smooth muscle cell phenotype and prevent dedifferentiation. Indeed, extracellular vesicles derived from KLF2-overexpressing endothelial cells, injected intravenously in Apoe\(^−/−\) mice for 6 weeks, protected from atherosclerotic lesion formation and this could be reversed by inhibiting miR-143/145. Endothelial functions, such as migration, have also been shown to be regulated by such cell-to-cell microRNA-based communication from monocyte-derived (miR-150)\(^9\)\(^3\) and apoptotic cell–derived (miR-126)\(^9\) \(^4\) miRNAs.

The discovery that HDL can transport microRNAs in the plasma, and stably deliver these to cells for uptake, suggests that HDL may participate in extracellular microRNA signaling.\(^9\)\(^4\)\(^9\)\(^5\) Vickers et al.\(^9\) demonstrated that several species of RNAs, including microRNAs and tRNA-derived and RNase P–derived RNA fragments, are carried on plasma HDL. Although microRNAs can also be isolated from LDL, for reasons that are unclear they seem to be more highly enriched on HDL. For example, miR-223 is one of the most abundant microRNAs on both HDL and LDL, yet it is \(7\)-fold higher on HDL (10000 copies/\(\mu\)g of HDL).\(^9\)\(^5\) Other microRNAs with defined roles in vascular biology and inflammation are also present on HDL, albeit at lower levels than miR-223: the endothelial-enriched microRNAs, miR-126 and miR-92a, are present at 3000 copies/\(\mu\)g of HDL, whereas the inflammation-associated microRNAs, miR-146a and miR-155, and the metabolically controlled miR-378 are present at <120 copies/\(\mu\)g of HDL.\(^9\)\(^5\) Notably, of these microRNAs, only the proinflammatory miR-155 was found to be greater quantities on LDL than HDL, a distinction that bears further investigation given the finding that miR-155 is proatherosclerotic. Similar to what has been reported for exosomes and microvesicles, HDL-derived miR-223 was shown to be delivered to cells, including hepatocytes and a kidney cell line overexpressing the HDL receptor SR-BI, which was shown to be required for HDL-mediated delivery of microRNAs to recipient cells.\(^9\)\(^4\) Notably, HDL-transferred miR-223 reduced target gene expression in the recipient cells, including the miR-223 target SR-BI.\(^9\)\(^5\)\(^4\) However, a second study of HDL–transported microRNAs in which the C.\(\text{elegans}\) miRNA, cel-miR-39, was used to track delivery of HDL–derived microRNAs reported that only a small number of cel-miR-39 copies could be detected in recipient cells, such as endothelial cells, monocytes, and smooth muscle cells.\(^9\)\(^5\) Thus, further investigation will be needed to understand the functional relevance and physiological effect of HDL–derived microRNAs, as well as such basic questions as how such microRNAs are selected for export and associate with HDL.

In addition to its critical role in RCT, HDL can exert anti-inflammatory, antioxidant, and antithrombotic effects—and these functions seem to vary among individuals. It is thus possible that these functions of HDL may be mediated in part by, or altered by, the subset of microRNAs that it carries. Indeed, the microRNA cargo of HDL has been shown to be altered in both mice and humans by hypercholesterolemia and atherosclerosis.\(^9\)\(^4\) An analysis of HDL from subjects with familial hypercholesterolemia revealed \(22\) miRNAs that were significa ntly altered when compared with HDL from normal subjects. Moreover, HDL from familial hypercholesterolemia subjects was found to affect target gene expression in recipient hepatoma cells when compared with HDL from normal...
subjects. These findings offer insight into new potential mechanisms by which HDL may mediate pleiotropic effects; however, whether microRNAs are responsible for some of the observed variations in HDL’s anti-inflammatory, antioxidant, and antithrombotic effects awaits further testing.

**Therapeutic Targeting of MiRNAs to Increase HDL Abundance and Function**

MicroRNA-based therapeutics represent a new class of drugs that hold promise for the treatment of cardiovascular and other diseases. The recent Food and Drug Administration approval of Kynamro (previously known as Mipomersen), a first-in-class antisense oligonucleotide inhibitor that targets apolipoprotein B-100 to reduce LDL cholesterol for the treatment of homozygous familial hypercholesterolemia,96 represents a giant leap forward for oligonucleotide-based therapies, including miRNA therapeutics. The first anti-microRNA therapy has yet to reach the clinic, yet antisense oligonucleotides against miR-122 (known as Miraversen) have shown efficacy against miRNA therapeutics. The first anti-microRNA therapy has yet to reach the clinic, yet antisense oligonucleotides against miR-122 (known as Miraversen) have shown efficacy.

The beneficial effects of miR-33 targeting on atherosclerosis were confirmed in miR33+/− mice crossed onto the Apoe−/− background, which showed 20% to 25% reductions in plaque size and lipid content when compared with control Apoe−/− mice after 14 weeks on a 0.15% cholesterol-containing Western diet.100 Peritoneal macrophages isolated from miR33+/− Apoe−/− mice showed enhanced cholesterol efflux to apolipoprotein A-I and HDL-C when compared with macrophages from Apoe−/− mice, reinforcing the concept that miR-33 targeting enhances RCT at the level of both HDL biogenesis and macrophage cholesterol efflux. However, studies in mice treated with miR-33 inhibitors for 12 weeks during the progression of atherosclerosis have been less clear. One study by Rotllan et al101 in Ldlr−/− mice fed a Western diet (0.3% cholesterol) together with treatment with 2′F/MOE–modified anti–miR-33 oligonucleotides showed 20% reductions in both plaque size and macrophage content and a decrease in miR-33 target genes in the aorta, whereas a second study by Marquart et al102 in Ldlr−/− mice fed a Western diet (1.25% cholesterol) together with treatment with an anti–miR-33 locked nucleic acid failed to show any benefit. Notably, both groups reported that although miR-33 inhibitors increased plasma HDL-C in mice fed a chow diet, this effect was lost when the mice were switched to the atherogenic Western diets. This absence of effect of miR-33 inhibition on HDL-C in mice fed a diet enriched in cholesterol may be because of low levels of hepatic miR-33 under these conditions: miR-33 is coregulated with its host gene SREBF2, whose transcription in the liver is decreased by dietary cholesterol.103 However, the reasons for the divergent outcomes of miR-33 inhibition on atherosclerosis are less clear but may relate to differences in the cholesterol contents of the different Western diets (0.3% and 1.25%) or the bioavailability of the miR-33 inhibitors used (ie, locked nucleic acid versus 2′F/MOE). Although the ability of the anti–miR-33 locked nucleic acid used by Marquart et al103 to reach macrophages in the plaque has not been tested, this could potentially account for the efficacy of the 2′F/MOE–modified anti–miR-33 in reducing atherosclerosis in the absence of an increase in plasma HDL. The study by Rotllan et al101 used the same 2′F/MOE–modified anti–miR-33 oligonucleotides that had proven to be effective at increasing ABCA1 in plaque macrophages and regressing atherosclerosis in the previous study by Rayner et al;104 however, miR-33 target gene expression in these cells was not evaluated. Future studies will be needed to determine whether anti–miR-33 targeting of plaque macrophages is responsible for its atheroprotective effects in the absence of increased plasma HDL. These studies further underscore the importance of studying how miRNAs modulate cholesterol flux through the HDL pathway and not HDL cholesterol alone (Table).

Inhibition of miR-33, as well as other ABCA1-targeting microRNAs, may also prove advantageous in a number of other conditions in which increased cholesterol efflux is thought to be beneficial. For example, to reduce islet cholesterol levels, which impair β-cell function and glucose tolerance,105 and to reduce the secretion of β-amyloid in the brain, which is inversely correlated with ABCA1-mediated cholesterol efflux to apoE.106 Studies of these approaches are eagerly awaited. Furthermore, although the preclinical studies of miR-33 inhibitors in mice and monkeys seem promising, many questions remain to be addressed, such as the effects of long-term suppression of miR-33, and whether compensatory mechanisms may become activated during miR-33 inhibition, such as the upregulation of other ABCA1-targeting miRNAs. Finally, as the miRNA networks that regulate hepatic and systemic lipid homeostasis are unraveled, this will no doubt be paralleled by the identification of additional targets for therapeutic intervention.
### Summary and Future Directions

HDL’s halo has become somewhat tarnished as a series of HDL-C–raising therapies have failed to confer protection from CVD. This has prompted a reconsideration of the HDL-C hypothesis and its evolution into the HDL flux hypothesis because of the renewed interest in understanding the mechanisms controlling HDL flux and functionality. The discoveries of microRNAs that control HDL biosynthesis, function, and uptake have greatly expanded our understanding of the molecular mechanisms regulating plasma levels of HDL-C and components of the RCT pathway and have identified new therapeutic targets to regulate HDL flux (Table). The list of microRNAs targeting lipoprotein metabolism pathways continues to grow at a rapid pace and will no doubt expand to include microRNAs targeting other genes involved in HDL biogenesis (eg, apolipoprotein A-I), remodeling (eg, CETP and lecithin-cholesterol acyltransferase), and functionality. There remains much to understand about how microRNAs contribute to HDL functionality in health and disease. For example, whether dysregulation of miRNA activity contributes to the pathogenesis of CVD or HDL dysfunction remains to be ascertained. The prospect that HDL-carried microRNAs contribute to the heterogeneous effects of HDL on endothelial cells, macrophages, and other cell types that influence vascular health is intriguing and may provide insight into how the protective effects of HDL may be altered in disease or enhanced for therapeutic purposes. As our knowledge of these points of post-transcriptional control of HDL increases, so too will the potential for translating these discoveries from animal models to humans and, eventually, new therapies to treat and prevent CVD burden.

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### Disclosures

K.J. Moore is a member of the miR-33 Clinical Advisory Board of Regulus Therapeutics. K.J. Moore and K.J. Rayner have a patent application on the use of miR-33 inhibitors for the treatment of atherosclerosis.

### References


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