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HDL and microRNA Therapeutics in Cardiovascular Disease

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Abstract

microRNAs (miRNA) are small non-coding RNAs (sRNA) that post-transcriptionally regulate gene (mRNA) expression and are implicated in many biological processes and diseases. Many miRNAs have been reported to be altered in cardiovascular disease (CVD); both cellular and extracellular miRNA levels are affected by hypercholesterolemia and atherosclerosis. We and other groups have reported that lipoproteins transport miRNAs in circulation and these lipoprotein signatures are significantly altered in hypercholesterolemia and coronary artery disease (CAD). Extracellular miRNAs are a new class of potential biomarkers for CVD; however, they may also be new drug targets as high-density lipoproteins (HDL) transfer functional miRNAs to recipient cells in an endocrine-like form of intercellular communication that likely suppresses vascular inflammation. Recently, RNA-based drugs have emerged as the next frontier in drug therapy, and there are many miRNA inhibitors and mimics in clinical development. Here, we discuss specific miRNA drug targets and how their manipulation may impact CVD. We also address the potential for manipulating HDL-miRNA levels to treat CVD and the use of HDL as a delivery vehicle for RNA and chemical drugs. Finally, we outline the current and future challenges for HDL and miRNA-based therapeutics for the prevention and treatment of CVD.

Keywords

microRNAs; lipoproteins; HDL; locked-nucleic acids; antisense oligonucleotides

1. Introduction

Over 85% of the human genome harbors actively transcribed non-coding RNAs (Hangauer, Vaughn, & McManus, 2013). microRNAs (miRNA) are one class of non-coding sRNAs (approximately 22 nts in length) which post-transcriptionally regulate gene expression through binding to partial complementary target sites enriched in the 3' untranslated region, and to a lesser degree coding regions and the 5' untranslated region of mRNA targets (Bartel, 2004). Altered miRNA activities likely contribute to the development and

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Conflict of interest statement:

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progression of CVD, as dysregulation of miRNAs have been observed in CAD, peripheral vascular disease, stroke, and other vascular pathologies (Fichtlscherer, et al., 2010; Gao, et al., 2012; van Empel, De Windt, & da Costa Martins, 2012; Y. Wang, Wang, & Yang, 2013). miRNA-mediated gene regulation has been implicated in inflammation, cholesterol homeostasis, oxidative stress, and hypertension; all key biological processes underlying CVD (Cengiz, et al., 2015; Heggermont & Heymans, 2012; Lai, et al., 2013; G. Liu & Abraham, 2013; Sen, et al., 2011; Vickers, et al., 2014; Y. Zhang, et al., 2014). Many types of sRNAs, including miRNAs, are present in biological fluids and are protected from nucleases through their association with lipid and protein carriers (Boon & Vickers, 2013; Vickers & Remaley, 2012; Vickers, Roteta, Hucheson-Dilks, Han, & Guo, 2015). Most interestingly, lipoproteins, namely high-density lipoproteins (HDL), were demonstrated to transport functional miRNAs in plasma, and HDL-miRNAs have been reported to be significantly altered in hypercholesterolemia, CAD, and myocardial infarction (Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2011). As such, HDL-miRNAs are a potential new class of markers for CVD. Furthermore, we reported that HDL transfer miRNAs to recipient cells where they regulate target gene expression, including adhesion molecule expression in recipient coronary artery endothelial cells (Tabet, et al., 2014). Therefore, HDL-miRNAs are bioactive mediators of cell-to-cell communication and represent novel drug targets and therapeutic strategies to prevent and treat CVD. RNA-based therapeutics has many advantages over the limited druggable proteome, most importantly the substantially greater number of possible targets. Currently, multiple miRNA targeted drugs for a compendium of diseases are in clinical development, including a few that would impact features of CVD (Frangogiannis, 2014; Hydbring & Badalian-Very, 2013; Vidigal & Ventura, 2015). The mostly widely-used strategies to inhibit targeted miRNAs are the use of modified antisense oligonucleotides (ASO). Chemical modifications to ASO have been used to increase binding affinities and nuclease protection which include the use of locked-nucleic acids (LNA), 2'-O-methyl or 2'-O-methoxyethyl additions, and phosphorothioate backbone modifications. Inhibition of miRNAs by modified ASOs have been extensively reviewed (Stenvang, Petri, Lindow, Obad, & Kauppinen, 2012).

The functional impact of miRNAs in CVD and their potential as drug targets have gained considerable interest and excitement; however, they do have some limitations. One of which, is that target miRNAs must be highly expressed and more than modestly regulate gene expression and function so that targeting miRNAs will have a meaningful effect on gene expression and disease prevention or resolution. Improvements in ASO bioavailability, stability, and target affinity with chemical modifications have dramatically increased the efficacy of ASO therapies; however, there remain critical limitations to their use. For example, complications in delivery, target miRNA accessibility, dose optimization, and poor pharmacokinetics and pharmacodynamics have reduced enthusiasm. Other impediments to the clinical development of ASO drugs are the costs of synthesis, storage, and lack of oral delivery methods. Here, we discuss the current state-of-the-art in miRNA therapeutics, the potential of HDL-miRNAs as drug targets, and current challenges to miRNA therapeutics in CVD.

2. Current development of miRNA therapeutics that may impact CVD

a. miRNA biomarkers of CVD

Many miRNA profiling studies in plasma or extracellular carriers have found significantly altered miRNAs in a wide-variety of diseases, many of which are now being considered as potential biomarkers for different aspects of CVD, see reviews (Joladarashi, Thandavarayan, Babu, & Krishnamurthy, 2014; Samanta, et al., 2016). At the time of this review, there are currently 159 clinical trials investigating miRNA biomarkers in diseases, including breast cancer (ClinicalTrials.gov identifier: NCT01612871), acute myeloid leukemia (NCT01057199), and periodontal disease (NCT02069574). Currently, there are 37 miRNA biomarker studies for CVD (ClinicalTrials.gov). One benefit of extracellular miRNAs as CVD biomarkers is that they can be sampled non-invasively and are present in all biological fluids (Boon & Vickers, 2013). miRNA biomarker studies also benefit from the high stability and relatively long half-life of miRNAs in samples, the required low input for detection, the high specificity of currently available probes, and the increasing available detection strategies and technologies (Balzano, et al., 2015; Creemers, Tijssen, & Pinto, 2012). Aside from diagnostic potential and monitoring response to drug treatments, miRNA-based therapy is a rapidly emerging market that is proving to be very lucrative to the pharmaceutical industry -- expected to reach 1 billion in 2019 (www.bccresearch.com). In contrast to siRNA-based therapeutics, which target and block a single mRNA transcript, inhibiting the activity of a single miRNA has the potential to affect gene expression of multiple mRNA targets, as miRNAs are known to target many mRNAs in many different tissues (Farh, et al., 2005; Grimson, et al., 2007; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). Therapeutically altering miRNA activity can be achieved through inhibiting miRNAs or replacing miRNAs that are increased and decreased in disease, respectively. miRNA replacement therapy can be achieved with miRNA mimics or precursor forms. Currently, there are several leading pharmaceutical companies that have miRNA research and development programs (Fig. 1). Although many miRNA-based drugs in clinical development are aimed at cancer, a few miRNA targets in development are relevant to CVD (Fig. 2).

b. miRNA inhibitors and loss-of-function therapeutics

Santaris Pharma A/S (recently acquired by Roche) was the first company (in 2011) to reach Phase II trials for its miRNA inhibitor (Miravirsen) against miR-122-5p in hepatitis C virus (HCV) infection. Miravirsen (SPC3649), a 15 nt LNA, was found to suppress HCV infection in primates, and was safe and well-tolerated without dose toxicities in healthy controls in two Phase I trials (NCT00688012 & NCT00979927) (Hildebrandt-Eriksen, et al., 2012; Lanford, et al., 2010). In addition, the Phase IIa trial demonstrated a 2-fold reduction in HCV RNA following 5 weekly doses, as well as a high barrier to resistance (Janssen, Reesink, et al., 2013; Lindow & Kauppinen, 2012; Ottosen, et al., 2015). Liver specific miR-122-5p is also a key regulator of lipid metabolism and miR-122 targeted drugs may also have potential for CVD and dyslipidemias (Esau, et al., 2006). For example, miR-122-5p inhibition was found to reduce plasma cholesterol levels in mice (Esau, et al., 2006; Lynn, 2009). Most interestingly multiple cholesterol synthesis genes (HMGCS1, HMGCR, SQLE, and DHCR7) were found to be suppressed with miR-122 inhibition;

however, these gene are likely indirect targets, and thus the exact mechanism by which miR-122 regulates lipid metabolism is still unclear. Regulus Therapeutics is currently developing a miR-21 targeted drug (RG-012) for kidney fibrosis in Alport syndrome. In a Phase I trial, a LNA against miR-21 is being tested to reduce renal fibrosis in these subjects (NCT02136862); this has great potential as circulating miR-21 is associated with kidney fibrosis in mice (Glowacki, et al., 2013) and anti-miR-21 is shown to reduce fibrosis and improve kidney function *in vivo* (Chau, et al., 2012). We have reported that miR-21 suppresses cholesterol synthesis in hepatoma cells (Selitsky, et al., 2015). As such, miR-21 inhibition in humans may raise hepatic and systemic cholesterol levels and increase risk for CVD. Multiple studies have found that miR-21 levels in atherosclerotic lesions and/or in circulation are associated with atherosclerosis and cerebrovascular disease (Kim, et al., 2015; Urbich, Kuehnbacher, & Dimmeler, 2008; F. Wang, et al., 2014). Nonetheless, miR-21 expression is sensitive to flow dynamics (mechano-sensitive) in endothelial cells and has been reported to have both pro- and anti-atherogenic properties (Kumar, Kim, Simmons, & Jo, 2014). Yet, repression of miR-21 was reported to inhibit neointimal lesion formation in mice and prevent stent restenosis (Ji, et al., 2007; D. Wang, et al., 2015). Based on these reports, it is unclear if anti-miR-21 therapy promotes or represses CVD, and the assessment of lipid levels and indicators of CVD in subjects receiving anti-miR-21 therapy during clinical development is warranted.

In collaboration with Regulus Therapeutics, AstraZeneca have initiated Phase I trials (NCT02612662) for an inhibitor of miR-103 and miR-107 (miR-103/107, RG-125/ AZD4076) to regulate insulin sensitivity in Non-Alcoholic Steatohepatitis (NASH) in Type 2 Diabetes (T2D) subjects; miR-103 and miR-107 differ by a single nt on the 3' end. This work stems from Markus Stoffel's group which demonstrated that suppression of miR-103/107 increased caveolin 1 (CAV1) expression and improved glucose homeostasis and insulin sensitivity in mouse models of obesity and T2D (Trajkovski, et al., 2011). Initial results suggest that RG-125 (AZD4076) was well-tolerated and effective in non-human primates (unpublished data). These pre-clinical and clinical studies demonstrate the outstanding potential for miRNA-based therapy in the future. Based on progress in long-term Phase 2 trials (Janssen, Kauppinen, & Hodges, 2013), Miravirsen (anti-miR-122) will likely to be the first FDA approved miRNA-based drug for clinical use. Conversely, pre-clinical trials for other potential miRNA targets have not been as successful, e.g. miR-33. Initial pre-clinical miR-33 inhibition studies using lentivirus (Rayner, et al., 2010), adenovirus (Allen, et al., 2012), and ASOs all reported an increase in plasma HDL-Cholesterol (HDL-C) levels in rodents (Allen, et al., 2012; Rayner, et al., 2010) and in one study regression of atherosclerosis (Rayner, Sheedy, et al., 2011). Furthermore, anti-miR-33 treatments in non-human primates were also found to increase circulating HDL-C levels (Rayner, Esau, et al., 2011). Moreover, Allen *et al.* demonstrated that inhibition of miR-33 can reduce hepatotoxicity associated with simvastatin therapy, and therefore, may have benefits as a concomitant drug with statins (Allen, et al., 2012). Marquart *et al.* reported that anti-miR-33 treatments in athero-prone mice (*Ldlr*^{-/-}) showed an initial elevation of circulating HDL-C levels with treatment after 2 wks, but HDL-C levels returned to control levels by 14 wks and no significant changes in atherosclerotic plaques were found (Marquart, Wu, Lusis, & Baldan, 2013). Goedeke *et al.* reported that long-term silencing of

miR-33 resulted in dyslipidemia and hepatosteatosis (Goedeke, et al., 2014). The potential side-effect of hepatosteatosis from anti-miR-33 therapy in humans raises a critical obstacle in miRNA-based therapy development. Current miRNA-based drug designs for the most part have yet to overcome the issue of the lack of biodistribution of miRNA inhibitors *in vivo*, as most of the inhibitors are taken up by a few tissues, most notably the liver. Using miR-33 as an example, it would be very beneficial to inhibit miR-33 in macrophages to increase ATP-binding cassette transporter A1 (ABCA1)-mediated cholesterol efflux to increase HDL-C levels and reduce atherogenesis; however, a significant fraction of anti-miR-33 drug would likely be in the liver contributing to hepatosteatosis. Based on the example of miR-33 and the generality of miRNA-mediated regulation of different gene networks in different tissues, systemic safety and targeted tissue delivery of miRNA inhibitors need to be carefully considered in pre-clinical studies prior to clinical trials. As noted above, inhibition of miRNAs *in vivo* can be achieved using chemically-modified and/or conjugated single-strand ASOs; however, increasing miRNA levels *in vivo* present greater challenges as they are largely double-stranded oligonucleotides which can activate immune responses (Kariko, Bhuyan, Capodici, & Weissman, 2004). Many of the strategies used to protect miRNA inhibitor drugs are now being functionalized for targeted tissue delivery of miRNA mimics.

c. miRNA mimics and gain-of-function therapeutics

In contrast to miRNA inhibition approaches that usually involve stable single-stranded oligonucleotides, miRNA replacement therapy is more challenging due to the importance of loading the mimic onto the RNA-Induced Silencing Complex (RISC) complex for mRNA degradation or repression. As such, mimics are designed as double-stranded duplexes, consisting of the guide strand identical to the miRNA and a complementary passenger strand that is often modified to increase or shift utilization to the guide strand (van Rooij, Purcell, & Levin, 2012); however, this renders the mimics difficult to transport through cell membranes and they can be degraded in circulation. To circumvent this barrier, several delivery vehicles have been put forward, including DNA vectors and adeno-associated virus (Kota, et al., 2009), lentivirus (Kortylewski & Nechaev, 2014), nanoparticles (Diaz & Vivas-Mejia, 2013; Kheirrolomoom, et al., 2015), liposomes (Akinc, et al., 2010; Akinc, et al., 2008; Morrissey, et al., 2005), and stents (D. Wang, et al., 2015). Recently, a few novel delivery concepts have merged, including ultrasound-targeted microbubbles destruction (UTMD) (Cao, et al., 2015), polyelectrolyte complex micelles (Kuo, et al., 2014), and self-assembled RNA-triple-helix hydrogel scaffolds (Conde, Oliva, Atilano, Song, & Artzi, 2015). These carriers not only protect against degradation and aid in cellular uptake, they are often functionalized to increased targeted cell delivery and reduce accumulation in the liver. Mirna Therapeutics are using liposomal nanoparticles (approximately 120 nm in diameter) loaded with miR-Rx34 to restore miR-34a levels in primary liver cancer and solid tumors (MRX34, NOV340 technology SMARTICLES). This is the first mimic-based therapy to enter into Phase I trials (NCT01829971) and interim results have shown promising outcomes. Nonetheless, miR-34a has been implicated in apoptosis in hepatosteatosis and miR-34a mimics in humans may result in increased risk of NASH (Shan, et al., 2015). Moreover, miR-34a was reported to suppress endothelial senescence and miR-34a levels are increased in atherosclerotic lesions suggesting that miR-34a mimic therapy may confer some level of risk for atherosclerosis and CVD (Ito, Yagi, & Yamakuchi, 2010; Raitoharju, et al.,

2011). Another miRNA mimic-based study aimed at raising miR-29 levels is set for Phase I trials. Montgomery *et al.* demonstrated that intravenously injected miR-29b synthetic duplexes reduced collagen levels and both blocked and reversed pulmonary fibrosis (Montgomery, et al., 2014). Most interestingly, this miRNA mimic study did not use carrier proteins or liposomes. In agreement with the Montgomery study, Zhang *et al.* reported that inhibition of miR-29 induces cardiac fibrosis further solidifying its potential as a therapeutic target (Y. Zhang, et al., 2014). Nevertheless, increasing miR-29b levels in humans may increase the risk for atherosclerosis and CVD, as miR-29b over-expression has been reported to drive macrophages towards pro-inflammatory phenotypes (Graff, Dickson, Clay, McCaffrey, & Wilson, 2012).

d. Other miRNA targets with CVD potential

In addition to the miRNA targets outlined above, miRagen Therapeutics has other miRNA-based drugs with CVD potential in development, including inhibitors for miR-92a. Reduction of miR-92 has been found to aid in neovascularization and prevent neointima formation following vascular injury (Daniel, et al., 2014). Strikingly, miR-92a is a pro-atherogenic miRNA in endothelial cells and miR-92a inhibition has also been reported to prevent endothelial dysfunction and atherosclerosis in mice (Loyer, et al., 2014). Mirage Therapeutics is currently developing a miR-208 inhibitor (MGN-9103) which has been reported to improve cardiac function in Dahl hypertensive rats and also play a role in regulating heart metabolism (Grueter, et al., 2012; Montgomery, et al., 2011). Lastly, a miR-15/miR-195 inhibitor (MGN-1374) is currently being developed to protect against ischemic injury and post myocardial infarction (Hullinger, et al., 2012; Porrello, et al., 2013).

3. Potential HDL-miRNA targets in CVD

Our lab and others have found that lipoproteins, HDL and LDL, transport miRNAs in circulation (Niculescu, et al., 2015; Vickers, et al., 2011; Wagner, et al., 2013). We have also reported that HDL transfers functional miRNAs, namely miR-223, to recipient cells – Huh7 hepatoma cells and human coronary artery endothelial cells (HCAEC) – where they regulate target gene expression (Tabet, et al., 2014; Vickers, et al., 2011). Moreover, HDL and LDL-miRNAs have been reported to be significantly altered in hypercholesterolemia, atherosclerosis, stable angina (SA), unstable angina (UA), and myocardial infarction (MI) (Niculescu, et al., 2015; Vickers, et al., 2011). These studies support a lipoprotein-mediated intercellular communication network that is altered in CVD. Please see a recent review on the topic (Michell & Vickers, 2016). Although the lipoprotein miRNA field is still in its infancy, manipulation of extracellular miRNAs, particularly miRNAs on lipoproteins are attractive drug targets for a few reasons. First, lipoprotein miRNAs are readily accessible circulating in blood and miRNA inhibitors and mimics are not required to cross cell membranes. Secondly, the impact of targeting lipoprotein miRNAs ultimately is mediated by changing the biological activity of candidate miRNAs upon their entry into recipient cells; and lipoproteins may serve as Trojan horses and provide delivery of inhibitors to the biologically relevant cells. Lastly, lipoprotein miRNA intercellular communication provides an opportunity to understand the flow of information between cells in health; so that in

disease the HDL-miRNA signature can be modulated towards health using inhibitors for multiple miRNAs that went up on HDL in disease and miRNA mimics to restore the levels of multiple miRNAs lost in disease. The most likely candidate miRNAs to target for the prevention or treatment of CVD are miRNAs that have been reported to be significantly altered on HDL and/or LDL in CVD. For example, as discussed above, miR-92a is a pro-atherogenic miRNA in endothelial cells (Loyer, et al., 2014). HDL and LDL-miR-92a levels have been reported to be increased in subjects with CAD – HDL (SA, UA, and MI) and LDL (SA and MI) (Niculescu, et al., 2015). Based on these studies, anti-miR-92a drugs, as currently being developed by miRagen Therapeutics, would be strong candidates to prevent endothelial dysfunction and atherosclerosis and treat subjects with CAD. HDL-miR-24 levels have also been reported to be increased in hypercholesterolemia and atherosclerosis (Vickers, et al., 2011). Inhibition of miR-24 has been demonstrated to prevent hepatosteatosis and hyperlipidemia in mice (Ng, et al., 2014). These studies suggest that miR-24 inhibitors would be a strong candidate to repress diet-induced hyperlipidemia and reduce CVD risk. We have previously reported that HDL-miR-223 is transferred to recipient endothelial cells where miR-223 inhibits adhesion molecule expression (intercellular adhesion molecule-1, ICAM-1) and cytokine expression (colony stimulating factor 2, CSF2) (Tabet, et al., 2014). Furthermore, we also reported that miR-223 coordinates hepatic cholesterol homeostasis through suppression of cholesterol biosynthesis (Vickers, et al., 2014). We found that HDL-miR-223 levels are increased in hypercholesterolemia and atherosclerosis in both mice and humans (Vickers, et al., 2011). Therefore, HDL-miR-223 mimic approaches could be used to prevent or treat hypercholesterolemia (hepatic cholesterol biosynthesis) and endothelial activation (monocyte and neutrophil adhesion) and reduce the burden of atherosclerosis and CVD. Nonetheless, miR-223 has been implicated in many other pathophysiologies, including innate immunity, sepsis, cancer, tuberculosis, infection, obesity, influenza, and T2D. The ability of miRNAs to regulate completely different gene networks in a cell-specific and disease-specific manner preclude the likelihood of their clinical use due to many factors, most notably, systemic safety. Although lipoprotein miRNAs may serve as potential drug targets and represent a new approach to miRNA-based therapy, the use of lipoproteins, namely HDL, as miRNA-based drug delivery vehicles also warrant further investigation.

4. HDL-based drug delivery strategies

Although widely-known for its role in reverse cholesterol transport, HDL carry and deliver a diverse set of cargo, including sRNAs, hormones, steroids, vitamins and bile acids (Vickers & Remaley, 2013). While targeting HDL-C levels for therapy (in CVD) has been tempestuous, the unique composition and properties of HDL particles, including nanoparticle size, relatively long half-life, a hydrophobic core with a hydrophilic shell, have generated much interest for HDL as a drug delivery vehicle, particularly in cancer (Lacko, Nair, Paranjape, Johnso, & McConathy, 2002; Lacko, Nair, Paranjape, Mooberry, & McConathy, 2006; McConathy, Nair, Paranjape, Mooberry, & Lacko, 2008; Sabnis & Lacko, 2012; Shahzad, et al., 2011). Furthermore, recent work has validated the exciting potential of reconstituted HDL (rHDL) for targeted drug delivery in CVD (Ding, et al., 2014; Duivenvoorden, et al., 2014; L. Liu, et al., 2014; W. Zhang, et al., 2015).

In 2007, Wolfrum *et al.* first demonstrated that native HDL (nHDL) and LDL can bind to lipophilic siRNAs, and nHDL was shown to aid in the delivery of siRNAs to the liver, gut, kidney, and ovaries through HDL's receptor, scavenger receptor BI (SR-BI); LDL primarily delivered siRNAs to the liver through the LDL-receptor (LDLR) suggesting that endogenous nHDL can act as a therapeutic delivery vehicle to SR-BI expressing cells (Wolfrum, et al., 2007). During this time, another group also found that nHDL efficiently and effectively delivered cholesterol-conjugated siRNAs to brain capillary endothelial cells (Kuwahara, et al., 2011). Here, they demonstrate that nHDL delivered the siRNAs via apolipoprotein E and LDLR on the recipient cells. The use of rHDL was first described in the 1990s stemming from the discovery of apoA-I Milano a decade earlier; HDL containing apoA-I harboring a gene mutation that was described in an Italian family that exhibited low levels of HDL-cholesterol, but were protected from atherosclerosis (Ameli, et al., 1994; Franceschini, Sirtori, Capurso, Weisgraber, & Mahley, 1980). Since then, many formulations and experimental uses of rHDL have been described, including the alterations of apolipoprotein and lipid content to investigate the structural and functional properties of HDL (Patel, et al., 2009). In addition, several commercial formulations are currently in clinical trials to enhance reverse cholesterol transport and reduce atherosclerosis -- CSL-112 (CSL), CER-001 (Cerenis Therapeutics) and MDCO-216 (Medicine Company).

Historically, rHDL has been very effective as a drug delivery vehicle; mostly due to its hydrophobic core. rHDL has been used to deliver hydrophobic chemotherapy drugs, e.g. Taxol, which was reconstituted with egg yolk phosphatidylcholine, unesterified cholesterol and cholesteryl oleate (Lacko, et al., 2002). In this study, the core components (cholesteryl ester and Taxol) were found to have strong uptake affinity in several cancer cell lines expressing SR-BI compared to control cell lines (Lacko, et al., 2002). Subsequently, this group improved tolerability and achieved greater delivery capacity to cancer cell lines and mice using an improved rHDL/drug complex containing paclitaxel (McConathy, et al., 2008). Nevertheless, paclitaxel has been reported to leak/release from rHDL likely during HDL maturation in the circulation (Jia, et al., 2012). Another group demonstrated that modification of paclitaxel-loaded rHDL improved targeting and limited tumor growth (J. Wang, et al., 2013). In the setting of CVD, rHDL loaded with statins were able to sufficiently reduced inflammation after one-week treatment and inhibit plaque progression after 3-months in athero-prone (apolipoprotein E^{-/-}, *ApoE*^{-/-}) mice (Duivenvoorden, et al., 2014). Moreover, *Zhang et al.* reported that rHDL loaded with tanshinone IIA, a vasoactive cardioprotective drug, showed a greater capacity to target and reduce atherosclerosis and inflammation in New Zealand White rabbits compared to liposomes or nanostructured lipid-based carriers (W. Zhang, et al., 2013). These are a few of the many studies that have functionalized rHDL to deliver drugs to cells and animal models. Currently, there is tremendous interest in the applicability of nanoparticles, and HDL, in a way, could be characterized as the body's own endogenous nanocarrier. As such, harnessing HDL's nanocarrier potential for the delivery of a wide-variety of components is exciting for many diseases. In addition to chemotherapy, statins and other drugs, rHDL can also be loaded with nucleic acids. For example, rHDL complexed with a vascular endothelial growth factor (VEGF) plasmid condensed with stearic-PEI improved outcomes in an ischemia/reperfusion rat model (Sun, et al., 2015). We reported that HDL naturally carries sRNAs, namely

miRNAs, but HDL can also be loaded with synthetic miRNAs and used to treat cells to modify gene expression (Vickers, et al., 2011). Similar to miRNAs, siRNAs are powerful tools to control (inhibit) gene expression and rHDL have great potential as delivery vehicles for siRNAs to cells and animal models of CVD, and potentially human therapy. Yang *et al.* demonstrated that nanocarriers, based on the function and structure of nHDL (HDL-mimicking peptide-phospholipid scaffold, HPPS), were able to have direct cytosolic delivery of cholesterol-modified siRNAs in cells to inhibit the bcl-2 oncogene (Yang, et al., 2011). In addition, rHDL conjugated to two different siRNAs were able to systemically deliver these siRNAs *in vivo*, based on fluorescent labeling of the siRNAs (Shahzad, et al., 2011). Moreover, this approach was also demonstrated to be effective at inhibiting cancer growth and progression (Shahzad, et al., 2011). In a similar fashion, lipopeptide nanoparticles were able to efficiently and effectively deliver siRNAs to hepatocytes in rodents and non-human primates to silence Factor VII, further demonstrating the outstanding potential for rHDL as a therapeutic carrier (Dong, et al., 2014). The unique benefit of rHDL as delivery vehicles for drugs and siRNAs is that this system takes advantage of nHDL's endogenous network of cellular interactions and selective core uptake. Nonetheless, it is likely that this system can be improved and optimized. For example, gold-labeling of HDL (HDL-AuNP) allows nHDL and/or rHDL delivery to be trackable, which will be particularly advantageous for future HDL delivery therapeutics (McMahon, et al., 2011). Due to the significant interest in miRNAs and siRNAs as clinical targets or RNA-based drugs, as discussed above, rHDL delivery will likely be a critical delivery vehicle for future therapies.

5. Current challenges to HDL and miRNA therapeutics

Although miRNA-based drugs and HDL delivery vehicles have tremendous potential for CVD and many other metabolic diseases, there are challenges to these approaches and careful consideration of potential problems is warranted (Table 1). One of the greatest obstacles in miRNA-based therapy is delivery of sufficient concentrations to targeted tissues to suppress or enhance miRNA activity. As discussed above, miRNA inhibitors and mimics are readily taken up by only a few organs, e.g. liver and spleen, and targeting miRNAs in these tissues can be achieved without carrier proteins or liposomes (Geary, 2009). Delivery to most other tissues requires delivery vehicles that can be functionalized to bind to the inhibitor or mimic and target designated tissues. There have been major advances in nanoparticle designs and bilayered liposomes have proven to be effective delivery vehicles in many diseases, including cancer. Nevertheless, each carrier type potentially can activate the adaptive immune system or alter gene expression in different tissues through a variety of processes, including cholesterol and lipid flux. Currently, there are a number of vehicles and strategies for delivery, including liposomes and polymer-based nanoparticles, as mentioned above. Likewise, the delivery and uptake of miRNA inhibitors and mimics was demonstrated to be enhanced by the conjugation of peptides, α -tocopherol, cholesterol, antibodies and CpG oligos (Li & Rana, 2014). It should also be noted that delivery of functional inhibitors or mimics to tissues requires the inhibitors to escape the endosomal compartments and reach the cytoplasm where they can be loaded into RISC and bind to target miRNAs. It has been reported that less than 5% of the payload reaches the cytoplasm (Gillieron, et al., 2013). As such, large doses of miRNA inhibitors and mimics are often required for effective silencing

or restoration of miRNA activity and this greatly increases the likelihood for off-target effects. Although there is tremendous potential for the use of miRNA-based therapy to treat disease, miRNA delivery and the carrier vehicles require careful design and different strategies to overcome these potential barriers.

Many of the concerns for miRNA-based drugs are based on off-target effects due to immunostimulatory responses. For example, single-stranded miRNAs inhibitors have the potential to activate the innate immune response through toll-like receptors (TLR), namely TLR7 and TLR8 (Diebold, Kaisho, Hemmi, Akira, & Reis e Sousa, 2004; Heil, et al., 2004). Some miRNAs (in a family) share a common seed region (bases 2–7 of the 5' terminal end) which is a critical region for mRNA target recognition (Bartel, 2004). As such, miRNA family members can target and repress the same genes, and ASO miRNA inhibitors will only inhibit one of the family members and may not be effective due to compensatory repression of the target gene by other family members. LNA inhibitors have proven to be more effective than other ASO modifications, displaying increased, nuclease protection and target affinity (Elmen, Lindow, Schutz, et al., 2008; Elmen, Lindow, Silahatoglu, et al., 2008; Petersen, Bondensgaard, Wengel, & Jacobsen, 2002). The chemistry underlying LNAs effectiveness include a 2' oxygen bridged (methylene) to the 4' carbon, thus locking the conformation into a 3' endo structure (Petersen, et al., 2002). Full-length LNAs (approximately 22 nts in length) have been demonstrated to be effective in animal models, including primates (Elmen, Lindow, Schutz, et al., 2008). Truncated LNAs, termed tiny LNAs, have proven successful in targeting just the seed region, so that all family members are silenced to release gene repression (Obad, et al., 2011). Moreover, it was reported that ssRNAs less than 12 nts in length fail to activate the innate immune system (Hornung, et al., 2005). As such, tiny LNAs overcome two barriers to miRNA loss-of-function – activation of the innate immune system and silencing entire miRNA families. Double-stranded miRNA mimics can also activate innate immunity, e.g. TLR3 activation, likely through a motif on the 3' end of the guide strand of the duplex (Hornung, et al., 2005; Kariko, et al., 2004). Whether the approach includes ssRNA miRNA inhibitors or dsRNA miRNA mimics, a large fraction of the drug ends up in the liver and hepatotoxicity is always a major concern, as previously mentioned. For example, full length LNAs have been reported to cause some level of liver inflammation and increased plasma transaminase levels (Swayze, et al., 2007). Liver toxicity associated with LNAs and other ASOs is likely dependent upon the position and type of chemical modification (Stanton, et al., 2012). The full impact of the LNA and ASO sequences on liver toxicity still remains to be determined, as conflicting data have been reported (Kakiuchi-Kiyota, et al., 2014; Swayze, et al., 2007). Another issue related to off-target effects is the issue of deleterious effects in normal tissues during the targeting of miRNAs in diseased tissues. miRNAs regulate many different genes in different tissues and the gain-of-function or loss-of-function for a miRNA in a diseased tissue could have a completely different reaction in other tissues that take up the miRNA-based drug. miRNA inhibitors and mimics have also been reported to affect biological processes in blood. For example, some miRNA inhibitors, including LNAs and ASOs containing a phosphorothioate backbone, have been reported to prolong blood clotting time by inhibiting coagulation in monkeys (Bennett & Swayze, 2010; Henry, Novotny, Leeds, Auletta, & Kornbrust, 1997). miRNA inhibitors have also been reported to activate complement cascades and reduce

leukocyte counts (Bennett & Swayze, 2010; Galbraith, Hobson, Giclas, Schechter, & Agrawal, 1994; Henry, et al., 1997). Another issue with off-target gene expression changes is non-specific inhibition. LNAs and ASOs have proven to be effective silencers of target miRNAs; however, they could also bind and inhibit other miRNAs with similar sequences. Moreover, it is possible that LNAs and ASOs can bind to mRNAs similar to miRNAs at sites within the 3' untranslated regions or function similar to siRNAs and target mRNAs at coding region sites. Based on these limitations, off-target effects for potential miRNA-based drugs should be critically and extensively investigated prior to application in animal models or human use.

Other challenges for miRNA-based therapy include miRNA accessibility and biological robustness. For example in health, miRNAs are generally fine-tuners of gene expression for many targets so that the suppression of one miRNA target may not result in robust changes to the gene of interest. This is often observed in cell culture (*in vitro*) where LNA inhibition of a miRNA only results in a modest increase in target gene (mRNA) expression. In metabolic disease, the biological impact of miRNAs may become more robust and silencing miRNAs in this context may be more meaningful. The challenge is delivering enough payload so that the target miRNA activity is effectively lost and the target gene of interest is increased to a degree that achieves functional impact in the tissue and/or the system. Similarly, compensation by other miRNAs on the target gene need to be examined, as silencing the target miRNA may not have an effect on the gene of interest due to other miRNAs suppressing the mRNA target in a compensatory manner. Moreover, some miRNA targets may not be accessible to LNAs and ASOs either through their binding to ribonucleoproteins, RISC, or extracellular lipid and protein carriers. In addition to these obstacles, there are also future challenges to miRNA-based therapies. Currently, miRNA-based drugs have been administered through intravenous and sub-cutaneous injections and skin-penetrating peptides (Hsu & Mitragotri, 2011). To maximize the full potential of miRNA-based drugs, oral delivery will need to be explored and developed. Future challenges may also include the potential for drug resistance to miRNA inhibitors and mimics. Although there are serious concerns for the limitations in pharmacodynamics and pharmacokinetics for miRNA-based drugs, the ability to manipulate the kinetics and dynamics of the inhibitors/mimics and delivery vehicles makes miRNA-based therapies attractive for clinical development and utilization.

In addition to LNA and ASO-based miRNA inhibitors, recent advances in alternative approaches to miRNA inhibition may have potential in future miRNA-based therapies. Recently, small molecule inhibitors of miRNAs have emerged (Jayaraj, Nahar, & Maiti, 2015; Velagapudi, Vummidi, & Disney, 2015). Using a novel identification strategy termed Informa, Velagapudi *et al.* identified small molecules that bind to specific RNA sequences and structures (Velagapudi, Gallo, & Disney, 2014). This study demonstrated that bioactive compounds can bind to miRNA precursor forms and inhibit nuclease miRNA processing; specifically, benzimidazole was found to inhibit miR-96 maturation, which resulted in increased expression of Forkhead Box O1 (FOXO1), a miR-96 target gene (Velagapudi, et al., 2014). Other studies have reported that miR-10b, miR-122, miR-372, miR-373 and miR-21 (azobenzene) can also be inhibited by small molecules (Bose, et al., 2012; Gumireddy, et al., 2008; Velagapudi & Disney, 2014; Vo, et al., 2014; Young, Connelly,

Grohmann, & Deiters, 2010). Small molecules have several advantages over traditional miRNA inhibitors as drugs, including better delivery and increased bioavailability, reduced costs of synthesis, and established history of druggability (Castanotto & Rossi, 2009; Rayburn & Zhang, 2008; Velagapudi, et al., 2015). One defining feature of small molecule miRNA inhibitors is the inhibition of miRNA processing, either primary transcripts or precursor forms, instead of targeting the mature miRNA. As discussed above, sequenced based inhibitors of mature miRNAs can be problematic due to non-specificity for similar sequences or lack of functional impact due to identical seed sequences amongst miRNA family members. As such, an alternative approach to ASO therapy for miRNA inhibition is to design LNAs and ASOs to target pri-miRNAs and pre-miRNAs, thus overcoming the obstacles of targeting mature forms (Kloosterman, Lagendijk, Ketting, Moulton, & Plasterk, 2007). Future approaches at manipulating miRNA activity may include small molecules of ASO technologies that alter miRNA transcription or block mature miRNAs from loading into RISC. Other strategies may include the use of miRNA sponges which contain multiple miRNA binding sites and out-compete endogenous mRNA targets (Ebert, Neilson, & Sharp, 2007; Ebert & Sharp, 2010). Another potential strategy for miRNA inhibition is catalytic oligonucleotide enzymes which can recognize and cleave targeted miRNAs (Jadhav, Scaria, & Maiti, 2009; Santoro & Joyce, 1997). These antagamiRzymes have been coupled with LNA technology to create LNAzymes which have been demonstrated to be effective *in vivo* (Jadhav, et al., 2009; Kaur, Scaria, & Maiti, 2010; Suryawanshi, et al., 2012). Furthermore, self-assembled RNA triple helix hydrogen scaffolds have been reported to inhibit miRNAs in mice (Conde, et al., 2015). Collectively, there are many present and future challenges to miRNA-based therapy; however, there is also great promise and the ability to optimize pharmacokinetics and pharmacodynamics will facilitate the development of this new class of drugs to prevent and treat CVD.

6. Conclusions

RNA-based strategies are the next frontier in drug therapy. Mipomersen, a siRNA against apoB, was the first drug in class to be approved by the F.D.A. for the treated of homozygous familial hypercholesterolemia (Geary, Baker, & Crooke, 2015). Currently, no miRNA-based drugs have gained F.D.A. approval; however, many miRNA inhibitors and mimics are in clinical trials and under development. Multiple groups have found that lipoproteins, namely HDL and LDL, transfer miRNAs between cells in a new form of intercellular communication. Most interestingly, lipoprotein miRNAs are significantly altered in CVD and these miRNAs may hold value as disease biomarkers and/or drug targets. Here, we discuss the current state-of-the-art in miRNA drug development and the possible impact on CVD and candidate lipoprotein miRNA targets. We also discuss the many uses of HDL-based delivery vehicles for RNA and chemical drugs. Finally, we detail the current challenges to HDL and miRNA therapeutics and highlight potential alternative strategies for miRNA inhibition in future approaches (Table 2). In summary, the utilization of HDL as drug delivery vehicles, the development of miRNA-based inhibitors and mimics, and the potential for HDL-miRNA therapy support the great enthusiasm for RNA drugs in CVD

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Abbreviations

ASO	antisense oligonucleotides
CAD	coronary artery disease
CVD	cardiovascular disease
DHCR7	7-dehydrocholesterol reductase
HDL	high-density lipoproteins
HDL-C	HDL-cholesterol
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
LNA	locked-nucleic acid
LDL	low-density lipoproteins
nHDL	native HDL
NASH	non-alcoholic hepatosteatosis
nts	nucleotides
rHDL	reconstituted HDL
RNA	ribonucleic acid
RISC	RNA-Induced Silencing Complex
SQLE	squalene monooxygenase
wks	weeks

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COMPANY	COMPOUND	TARGET	ACTION	PRE-CLINICAL	PHASE I	PHASE II	PHASE III
Santaris Pharma	Miravirsen	miR-122	anti-miR	Hepatitis C virus	▶▶▶▶▶▶▶▶		
EnGeneIC Ltd	MesomiR-1	miR-16	mimic	Mesothelioma	▶▶		
miRagen Therapeutics	MRG-106	miR-155	anti-miR	Hematological Malignancies	▶▶		
	MRG-107	miR-155	anti-miR	Amyotrophic Lateral Sclerosis	▶		
	MRG-201	miR-29b	mimic	Cutaneous and Pulmonary Fibrosis	▶▶		
	MGN-1374	miR-15/195	anti-miR	Post-MI Remodelling	▶		
	MGN-4893	miR-451	anti-miR	RBC Disorders	▶		
	MGN-9103	miR-208	anti-miR	Chronic Heart Failure	▶		
	Unknown	miR-92a	anti-miR	Wound Healing and PAD	▶		
MiRNA Therapeutics	MRX34	miR-34	mimic	Solid Tumors	▶▶		
	MRX34	miR-34	mimic	Hematological Malignancies	▶▶		
	MiR-Rxlet-7	Let-7	mimic	Lung Cancer	▶		
	MiR-Rx-06	miR-16	mimic	Prostate Cancer	▶		
	Unknown	miR-101	mimic	Cancer	▶		
	Unknown	miR-215	mimic	Cancer	▶		
Regulus Therapeutics	RG-012	miR-21	anti-miR	Alport Syndrome	▶▶		
	RG-101	miR-122	anti-miR	Hepatitis C Virus	▶▶▶▶▶▶▶▶		
	RG-125 (AZD4076)	miR-103/107	anti-miR	NASH	▶▶		
	Unknown	miR-10b	anti-miR	Glioblastoma	▶		
	Unknown	miR-221	anti-miR	Hepatocellular Carcinoma	▶		
	Unknown	miR-350	anti-miR	Neuroblastoma	▶		

Figure 1.
Schematic of current miRNA targets in clinical development.

Figure 2.
Schematic of the potential impact of HDL and miRNA therapeutics on features of CVD.

Table 1

Current challenges for HDL-miRNA therapeutic delivery

•	Sufficient payload
•	Targeted tissue delivery
•	Activation of immune/inflammatory responses
•	Off-target effects
•	miRNAs target multiple genes
•	Hepatotoxicity
•	Non-specific inhibition

Table 2

Highlights & Future Directions

•	RNA-based strategies are an exciting new drug class
•	Lipoproteins are an advantageous drug delivery vehicle
•	In addition to the well-established miRNA mimics, LNAs and ASOs, new strategies are being developed including targeting precursor and primary miRNAs by small molecules, miRNA sponges, and catalytic oligonucleotide enzymes
•	Determining drug resistance
•	Strategies are needed to reduce costs and improve largescale production of miRNA mimics and LNAs
•	Development of oral delivery for clinical application