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From Evolution to Revolution: miRNAs as Pharmacological Targets for Modulating Cholesterol Efflux and Reverse Cholesterol Transport

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Abstract

There has been strong evolutionary pressure to ensure that an animal cell maintain levels of cholesterol within tight limits for normal function. Imbalances in cellular cholesterol levels are a major player in the development of different pathologies associated to dietary excess. Although epidemiological studies indicate that elevated levels of high-density lipoprotein (HDL)-cholesterol reduce the risk of cardiovascular disease, recent genetic evidence and pharmacological therapies to raise HDL levels do not support their beneficial effects. Cholesterol efflux as the first and probably the most important step in reverse cholesterol transport is an important biological process relevant to HDL function. Small non-coding RNAs (microRNAs), post-transcriptional control different aspects of cellular cholesterol homeostasis including cholesterol efflux. miRNA families miR-33, miR-758, miR-10b, miR-26 and miR-106b directly modulates cholesterol efflux by targeting the ATP-binding cassette transporter A1 (ABCA1). Pre-clinical studies with anti-miR therapies to inhibit some of these miRNAs have increased cellular cholesterol efflux, reverse cholesterol transport and reduce pathologies associated to dyslipidemia. Although miRNAs as therapy have benefits from existing antisense technology, different obstacles need to be solved before we incorporate such research into clinical care. Here we focus on the clinical potential of miRNAs as therapeutic target to increase cholesterol efflux and reverse cholesterol transport as a new alternative to ameliorate cholesterol-related pathologies.

Keywords

ABCA1; Cholesterol efflux; HDL; miRNAs

1. Introduction

Sterols are present in different eukaryotic life forms, particularly in large amounts in plasma membranes, while they are universally absent in prokaryotic membranes. However, some bacteria do require sterols for growth, including *Mycoplasma capricolum* and

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Methyloccoccus capsulatus, but with a broader specificity than that exerted by cholesterol in eukaryotes [1]. While cholesterol is the predominant sterol in vertebrates, phytosterols are present in plants, and ergosterol is the major sterol in yeast and other fungi. From a cellular evolutionary point of view, cholesterol and other sterols could have been synthesized after the advent of aerobic metabolism, as their synthesis requires several molecules of oxygen, and may have served as a primitive cellular defense against oxygen rather than merely a consequence of a response to the rise in atmospheric O₂ [2]. Nature has also probably pressured the synthesis of cholesterol because of the requirement of more complex organisms to localize different multiprotein complexes (channels, transporters, etc.) in focal, non-homogeneous areas of cellular membranes for appropriate function [1,3]. The unique spatial structure of cholesterol-*3*- β -face and methylated-*5*- β -face- makes this molecule (but not other sterols) ideal to interact with the sn-1 saturated fatty acyl groups and sn-2 unsaturated fatty acyl chains of phospholipids, respectively [3,4]. Cholesterol has not only a unique ability to increase lipid order in fluid membranes while maintaining fluidity and diffusion rates, but also to promote the formation of special membrane domains, that cannot be formed by their precursors [5,6] or their plants counterparts, the phytosterols. The stringency of cholesterol in higher organisms has probably induced the evolution of a specific energy-requiring mechanism to excrete phytosterols after intestinal absorption [7].

From a physiological evolutionary point of view, cholesterol has allowed the formation of compact myelin in the central nervous system (CNS) that permitted the development of our complex brain with a numerous relatively small-diameter and low-capacitance axons that manifest very high conduction velocities [8]. As a consequence, cholesterol concentration in the CNS of humans is higher than in any other tissue and these and other unique abilities of cholesterol have probably benefit the modern human to evolve with a significant larger and more sophisticated brain than other primates [8]. Moreover, in adult humans the brain represents ~2.3% of body weight but uses ~23% of body's daily energy requirements, which is even greater in the infant developing brain (~74% of body energy) [8,9]. This energy demand of a larger brain could only be met by a high rate of the *de novo* cholesterol synthesis and/or the need of a high quality diet rich in sterols. Thus, sterols and particularly *de novo* cholesterol biosynthesis have evolved as probably the most intense regulated process in biology [10]. Moreover, consumption of meat and other easily digestible foods acquired by hunting could explain how this energy demand of a larger brain would be met and thus reduce the larger intestinal tract in expense of brain size [8,9].

The other major physiological process that controls cholesterol levels within our organism is their transport and elimination. It is well known that fats are a good source of energy for multicellular organisms but their high risk of cytotoxicity has improved their efficient transport in aqueous biological environments through the evolution of plasma lipoproteins. Thus, both intestinal and hepatic lipid metabolism likely evolved package of all dietary fats into these triglyceride rich lipoproteins [3]. In humans, most plasma cholesterol is associated with low-density lipoprotein (LDL) and surprisingly, as the other organic constituents involved in energy transport between tissues, it occurs in high concentrations [11]. Even when most body tissues express the LDL receptor for LDL-derived cholesterol uptake, under dietary conditions equivalent to those found in Western humans, most extra-hepatic tissues synthesize enough cholesterol *de novo* to satisfy their requirements [12]. The lack of biodegradability of cholesterol precludes its use as fuel and mainly the liver possesses the appropriate enzymatic machinery to degrade cholesterol in large quantities in a process different to that of combustion as compared to the other common plasma organic constituents [11]. However, the liver does not provide cholesterol as LDL, but synthesizes very low-density lipoprotein (VLDL) and thus, LDL might have evolved as a spandrel of VLDL natural selection and become crucial to the evolutionary fitness of vertebrates in relation to cholesterol metabolism [11]. Then, when exposed to increased levels of dietary

cholesterol and triacylglycerol, LDL cholesterol is increased and taken up by peripheral tissues and as a consequence, the amount of cholesterol that must be returned to the liver for elimination is also increased. Consequently, Apolipoprotein A-I (apoA-I) and cholesteryl ester transfer protein (CETP), together with High-density lipoprotein (HDL), are increased in order to mitigate the potential toxicity of LDL-cholesterol. Apart from the liver, convincing evidence indicates that the intestine may also contribute to the elimination of excess cholesterol [13,14]. In summary, there has been strong evolutionary pressure to ensure appropriate levels of cholesterol in our organism. However, if we compare our millions of years of evolution containing our limited amount of cholesterol in the hunter-gatherer diet to that of our modern diet after the revolution of industry, it becomes clear that in the last century our organism was unable to evolve to handle levels of cholesterol and lipids available from modern diet. This inability to handle high cholesterol levels, for example accumulation of apolipoprotein B containing -lipoproteins in the artery wall, has led to the pathogenesis of one of the most devastating pathologies that hit our modern society, atherosclerosis [15].

Evidence showing that the ratio of non-coding to protein-coding DNA rises as a function of developmental complexity, suggests that RNA regulatory systems were essential for the evolution of developmentally sophisticated multicellular organisms and their phenotypic complexity [16]. Indeed, most of the human genome consists of non-protein-coding sequences (~98%) [17] most of which was originally thought to be “junk DNA”. Recent data from the Encyclopedia of DNA Elements “ENCODE” project found that 80% of our human genome contain elements linked to biochemical functions [18], while about 75% of our full genome is transcribed at some point in certain cells [19]. Moreover, studying 147 cell types, the ENCODE consortium has defined ~8800 small RNA molecules and ~9600 long noncoding RNA molecules [20]. Most of human small RNAs correspond to four major classes: small nuclear RNAs, small nucleolar RNAs, microRNAs (miRNAs) and transfer RNAs, while ~28% of annotated small RNAs are expressed in at least one cell line [19]. While in the last decade we have witnessed the importance of miRNAs in different biological processes in both human health and disease [21], increasing evidence suggests that long noncoding RNAs may also control certain biological processes in human health and disease [22]. That being said, if evolution has devoted so many protein-coding genes to regulate cellular cholesterol content [3], then it is expected that the complexity of cholesterol homeostasis ranging from biogenesis to transport and metabolism may also have another complex layer of regulation through noncoding RNA molecules. Indeed, recent evidence shows that different aspects of cholesterol metabolism are regulated by small non-coding RNAs [23,24].

2. Cholesterol homeostasis, cholesterol efflux and reverse cholesterol transport

Mammalian cells must maintain a tight control of cellular cholesterol levels. Perturbation of cholesterol homeostasis is the major cause of a number of diseases including atherosclerosis, metabolic syndrome and type-2 diabetes [15,25]. As one of the most intensively regulated processes in biology, cholesterol homeostasis is tightly regulated by complex molecular mechanisms ranging from complex feedback loops to non-coding RNA regulation. Cellular and systemic cholesterol levels are tightly regulated through the coordinated action of the sterol regulatory element-binding protein (SREBP) transcription factors and the liver X receptor (LXR) [10,26]. The maintenance of systemic cholesterol levels in humans is regulated by: a) intake from the diet; b) endogenous biosynthesis; and c) removal from the body via biliary and intestinal excretion.

2.1 Dietary cholesterol

Plasma cholesterol levels normally reflect that of the dietary intake; therefore it depends on the type of diet we consume and thus, that is one of the major origin of disorders of dietary excess, such as atherosclerosis and metabolic syndrome. Dietary cholesterol intake is variable but is often less than ~300 mg/day. Cholesterol is absorbed in the intestines by the enterocyte mainly via the Niemann-Pick type C1-like1 (NPC1L1) protein, the pharmacological target of Ezetimibe [27]. Liver X receptors (LXRs) modulate the expression of the ATP-binding cassette transporter G5 (ABCG5) and G8 (ABCG8), which prevent the accumulation of other sterols by pumping non-cholesterol sterols back into the gut lumen. They also promote biliary excretion of sterols. Loss-of-function mutations in ABCG5 or ABCG8 are responsible for sitosterolemia, a disorder characterized by increased intestinal absorption and decreased biliary excretion of dietary sterols, hypercholesterolemia, and premature coronary atherosclerosis [7]. Cholesterol is packaged into large triglyceride-rich particles, chylomicrons (CM), and enters the thoracic lymph circulation. Most cholesterol absorbed from the intestine comes from the bile, which contributes more than ~70% of total cholesterol that reaches the intestinal lumen. Bile acids are pharmacological targets of polymeric bile acid-binding resins [28]. While the contribution of cholesterol absorbed by the intestine is ~25%, the other ~75% of plasma cholesterol is contributed by endogenous cholesterol biosynthesis. Even when the contribution of dietary cholesterol is relatively low, their absorption efficiency and absorbed dietary cholesterol significantly regulates cholesterol biosynthesis and excretion [29,30].

2.2 Cholesterol biosynthesis

Among the most intensely regulated process in biology, cholesterol biosynthesis is accomplished in the endoplasmic reticulum (ER) in more than 20 precisely regulated enzymatic reactions that depends on the availability of an external source of cholesterol [10,31]. The membrane-bound transcription factors, SREBPs, regulate the expression of several genes involved in this process, including the rate-limiting enzyme in cholesterol biosynthesis –the HMGCoA Reductase– a pharmacological target of the widely used hypocholesterolemic drug, statin. Under normal conditions our body synthesis ~1 g/day of cholesterol and the liver is one of the major producer of cholesterol; other sites of high synthesis rates include the intestine, adrenal glands and reproductive organs. However, cholesterol biosynthesis is highly dependent on dietary cholesterol bioavailability. Thus, under dietary conditions equivalent to the Western human diet, extrahepatic tissues probably account for >80% of total cholesterol synthesis [12]. Cholesterol synthesized from the liver is secreted to the circulation via the triglyceride-rich lipoproteins containing apolipoprotein B100 (apoB-100), the very low-density lipoprotein (VLDL). ApoB is the pharmacological target of an antisense oligonucleotide against apoB-100 biosynthesis (Mipomersen) [32]. Due to triglyceride loss by lipoprotein and hepatic lipases, VLDL is converted to low-density lipoprotein (LDL) particles and, together with VLDL, is removed from circulation mainly by the LDL receptor (LDLR). As cellular cholesterol biosynthesis is highly dependent on the availability of an external source of cholesterol, the LDLR contributes to maintain cholesterol homeostasis by limiting the uptake of cholesterol from lipoproteins (LDL) [33]. LDLR expression is regulated transcriptionally by SREBPs and post-transcriptionally by other posttranscriptional mechanisms, such as the Inducible degrader of the low-density lipoprotein receptor (IDOL) and the protein convertase subtilisin-like/kexin type 9 (PCSK9) [34,35]. IDOL is an E3 ubiquitin ligase that mediates the ubiquitination and degradation of the LDLR [36]. IDOL expression is regulated by LXRs, which are activated in response to rising cellular sterol levels, thereby limiting further uptake of exogenous cholesterol through the LDLR pathway [34]. PCSK9 is a secreted protease that mediates the degradation of LDLR by interacting with it and targeting it for degradation [37]. PCSK9 expression is regulated by SREBPs [38] and is pharmacological inhibited by using

monoclonal antibodies, which significantly reduce circulating LDL cholesterol in humans [39]. Similar results were previously observed in Pcsk9-deficient mice [40].

2.3 Cholesterol removal

As an excess of free cholesterol is toxic to cells, different molecular mechanisms can be used to control intracellular cholesterol content. Some types of cells can accumulate large amounts of cholesterol by their esterification and accumulation in lipid droplets, while other cells also produce and secrete lipoproteins (hepatocytes and enterocytes). In addition to inhibiting cholesterol biosynthesis and uptake, mammalian cells respond to cholesterol excess by activating LXRs. LXR activation promotes cellular cholesterol efflux by activating transcriptionally the ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1). LXRs are the pharmacological target of the experimental selective agonist LXR-623 [41]. ABCA1 regulates cellular cholesterol efflux to poor-lipidated apoA-1, while ABCG1 controls the cholesterol export to mature HDL particles. ABCA1 also plays a major role in the biogenesis of HDL in the liver and intestine. Indeed, mutations in this transporter cause Tangier Disease, which is characterized by severe HDL deficiency and cholesterol accumulation in peripheral tissues and prevalence of atherosclerosis [42]. Cholesterol efflux is the first step in reverse cholesterol transport (RCT), the process of removal excess cholesterol from the body via biliary or intestinal excretion [43,44]. Induction of cholesterol efflux and RCT has shown to reduce atherosclerosis in several mouse models [45,46].

Final removal of cholesterol from our organism is mainly mediated via the intestinal and biliary lumen for fecal excretion. Cytochrome P450 7A1 (CYP7A1) is the rate-limiting enzyme in cholesterol conversion to bile acids, the major product of cholesterol catabolism. The half transporters ABCG5 and ABCG8 that form obligate heterodimers and promote biliary excretion of sterols [47,48] are regulated by LXRs. Most of the bile acids that go into the intestinal tract are reabsorbed (~95%). Although under normal conditions the contribution of intestinal RCT (~30%) is less important than the biliary route to the total neutral sterol loss [49], trans-intestinal cholesterol efflux (TICE) pathway is highly inducible and can be pharmacologically manipulated [14,49,50]; suggesting that the intestine can substantially contribute to RCT. In contrast to the well-known hepatobiliary route-based cholesterol excretion, HDL seems not to play a significant role in TICE in animal models [13]. While it is not clear which pathways participate in TICE, the LXR-mediated induction of TICE indicates that ABCG5/ABCG8 may contribute partially to this process [49].

Other proteins have been described to contribute to cellular cholesterol efflux in different cell types, including the scavenger receptor B1 (SR-BI), CD36 and Caveolin-1 (Cav-1) [51]. However, their contribution to macrophage RCT *in vivo* is not clear and remains uncertain [52]. Several reports have shown that Cav-1, a major component of caveolae, participates in regulating intracellular cholesterol trafficking [53] and cholesterol efflux in macrophages [54]. However, its contribution to cholesterol efflux may not be directly related to caveolae [55]. Other biological processes also contribute to cholesterol efflux. In macrophage foam cells, lipid droplets are delivered to lysosomes via autophagy and lysosomal acid lipases hydrolyze cholesteryl esters to release free cholesterol via ABCA1 [56].

Overall, even when the contribution of total macrophage cholesterol efflux to whole body RCT is insignificant, the induction of cholesterol efflux from cells localized in the artery wall that have the potential to transform into macrophage-foam cells, is relevant for atherosclerosis prevention, treatment and regression [15,57]

2.4 HDL and reverse cholesterol transport

High circulating LDL cholesterol (LDL-C) and low HDL cholesterol (HDL-C) are important risk factors for coronary artery disease (CAD). Pharmacological therapies to reduce plasma LDL-C, even in healthy volunteers greatly reduce CAD [58]. Plasma levels of HDL-C have been also inversely associated with risk of CAD in multiple epidemiological studies [59]. However, pharmacologic interventions that increase HDL-C levels have not led to a clear reduction in CAD [60,61]. In contrast to what occurs to LDL-C levels [62], genetic variations that modify plasma HDL-C levels do not directly associate with CAD risk [63]. Thus, even though rising HDL-C levels in experimental animals by HDL infusion or by apoA-I overexpression have a clear anti-atherogenic effects, interventions to increase HDL-C in humans fail to reduce risk for CAD [64,65]. It is important to note that in all these clinical trials, only either total HDL-cholesterol and/or apoA-I concentration was measured by common routine clinical methodologies available. However, it is well known that HDL is a heterogeneous collection of lipoprotein particles with a density between 1.063 and 1.21 g/mL and in-depth studies of their lipid composition and their proteomics have revealed their high heterogeneity both, structurally and functionally [66,67]. Apart from RCT, HDL has a variety of functions including, anti-inflammatory, antioxidant, antithrombotic, antiglycation and even a transporter of miRNAs. In this context, increasing evidence suggests that the ability of HDL to promote cholesterol efflux from macrophage foam cells is a key property of HDL, relevant to the pathogenesis of atherosclerosis, and not explained simply by HDL levels *per se* or apoA-I levels [68,69]. Thus, in the context of RCT and atherosclerosis, pharmacological therapies intended to raise HDL should consider not only the specific subfraction or type of HDL that wants to be increased, but also their function or dysfunction [70]. Moreover, as the cholesterol efflux process is considered the first and most critical step in macrophage RCT [57], therapies to raise HDL levels should consider this aspect of RCT. In this context, some recent clinical data indicates that, independently of HDL or apoA-I levels, cholesterol efflux from macrophages may be a measure of HDL function [68,69]. Thus, evaluation of HDL function/dysfunction related to cholesterol efflux capacity, should be considered when formulating pharmacological therapies to increase HDL function and RCT related to CAD.

3. miRNAs: underscoring their role in human disease

miRNAs were first described as regulators of developmental timing in the model organism *Caenorhabditis elegans* in 1993 [71,72], but did not received special attention until their identification in other species, including mammals [73,74], and their role in human disease was uncovered [75]. Sequencing studies have identified ~1000 miRNA loci encoded in our human genome that are predicted to regulate ~ a third of our genes (ref, ref). Detailed biogenesis and function of miRNAs can be found elsewhere [76,77]. Briefly mature miRNAs are ~22-nucleotide single-stranded RNAs that exert their function via perfect Watson-Crick base pairing, with sequences most commonly located within the 3'untranslated region (3'-UTR) of target mRNAs. Even when other regions of the miRNA can bind to the target mRNA, the "seed" sequence (nucleotides 2–8 at the 5' end of the mature miRNA) is critical for target selection [77]. Interaction of a miRNA with its target mRNA results in inhibition of translation and/or degradation of mRNAs [76,78]. However, certain miRNAs can interact with other target mRNA regions including the 5'UTR, coding region or intron-exon junction and even increase rather than decrease target mRNA expression [79–82]. Other factors that influence miRNA activity are their tissue distribution, as certain miRNAs are highly expressed or even restricted [83,84] to certain cell types and can only target their mRNA target if they are co-expressed in the same tissue at the same time. Moreover, pseudo genes [85] and long non-coding RNAs (lncRNAs) [86] that contain miRNA binding sites, are new players in miRNA activity, acting as competing endogenous

RNAs (ceRNAs) and thus sequestering miRNAs and preventing them from binding to their mRNA targets [87].

Mainly based on short sequences ('seed'), different miRNA target prediction algorithms reveal that miRNAs can target hundreds of genes [88], which really challenge the dissection of miRNA-mediated phenotypes [21]. From an evolutionary point of view, RNA-based regulatory network appearance suggests that miRNAs have probably evolved as buffers against deleterious variation in gene-expression programs. Thus, our current model of miRNA biology suggests that the primarily role of miRNAs seems to be the 'fine-tuning' of gene expression [89]. However, even when miRNAs exert modest effects on many target mRNA, the additive effect of coordinated regulation of a large suite of transcripts that govern the same biological process is believed to result in strong phenotypic output [21]. On the other hand, loss or gain of function experiments in different animal models have revealed that several developmental processes are dependent on certain miRNAs, suggesting that some miRNA functions are mediated by a strong regulation of one or very few targets. A striking feature of miRNA function is the high redundancy among related and non-related miRNAs in regulating gene expression. First, numerous miRNAs share the same seed sequence, thereby controlling the expression of overlapping predicted targets. Second, different miRNAs not sharing the same seed sequence can target the same predicted targets. These facts, in general, reduce the importance of a particular miRNA under conditions of normal cellular homeostasis. However, compelling evidence suggests that it is under conditions of stress that the function of miRNAs become especially pronounced [21]. A disease can be considered as an abnormal condition that affects the normal cellular physiology and can be caused by external factors (environment), internal dysfunction or an aberrant response to physiologic and pathophysiologic stress. Several models for miRNA functions under stress conditions have been proposed including the stress signal mediation and/or modulation, negative or positive feedback and buffering [21,84]. In sum, the complex function of miRNAs not only increases the complexity of molecular events that drive our modern human disease by adding another layer of complexity, but also opens up the possibility of miRNA-base therapeutics.

Our current understanding of the pathophysiological process of modern human disease, such as cardiovascular disease, cancer, and inflammatory autoimmune disease, where different biological pathways contribute in different ways, points out the need of novel therapeutic approaches to prevent or treat their occurrence. As miRNAs can control several biological processes by targeting different genes, their potential as pharmacological targets opens up the development of miRNA-based therapeutics.

4. miRNAs as pharmacological targets

Even while certain questions regarding their biological function and regulation still remain to be answered, miRNAs as potential therapeutics have received special attention from the scientific and clinical audience due to the following reasons. First, diseases from our modern 'Western-type' life are generally multifactorial and have been difficult to be treated by our current one-target drug arsenal. Second, miRNA basic biological function offers a unique opportunity to target different genes within one biological process or disease. Third, previous existing antisense technology and gene therapy approaches have catalyzed efforts to develop therapies to modulate miRNA levels *in vivo*. To date, several tools have been developed to target miRNA pathways from a therapeutic point of view [90–92]. In principle, as miRNAs are generally recognized as inhibitors of gene expression, the use of therapies to increase miRNA expression, "mimics", will result in a decreased expression of their mRNA targets. Conversely, the delivery of therapies to reduce miRNA expression, "inhibitors", can block the activity of miRNAs and thus de-repress their targeted mRNAs.

The unique ability of a single miRNA to modulate the expression of different components of a complex disease pathway offers a unique opportunity to treat disease in a manner that is completely different and revolutionary from that of classical one target-directed drugs. Moreover, due to their “promiscuity”, pharmacological modulation of miRNA function may also enable one to bypass mechanisms that develop tissue insensitivity, as observed in certain classical one target-directed drugs.

4.1 Therapeutic miRNA mimics

The use of miRNA mimics for therapy has been really challenging and their development has been slow in expense of anti-miR technology and therapies to inhibit miRNA function. miRNA mimics could potentially be used in situations in which a reduction in miRNA levels produce a pathological state such as those produced in the human rare Mendelian disorders or certain types of cancer, where regions containing miRNAs are deleted [75]. Genetic mutation in either miRNA seed region or other miRNA regions, that results in a reduced functional miRNA with a significant reduction of mRNA targeting required for normal function [93,94] could also benefit from these therapies. Different strategies to deliver miRNA mimics have been developed. In animal models, synthetic miRNA or pre-miRNA duplexes within lipid nanoparticles have been systemically delivered and exerted their biological effects without apparent toxicity [95–97]. Synthetic RNA duplex oligonucleotides are normally modified, for better stability and cellular uptake, and incorporated into different delivery systems. To increase tissue/cell specificity, surface receptor ligands or other components could also be added to the formulation. Adeno-associated viruses (AAV) are another promising alternative to deliver miRNAs mimics [98]. Certain tissue specificity could be achieved due to natural tropism of different AAV serotypes [99]. In animal models, other viral-based vectors, including adenoviruses and lentiviruses have been tested [100,101]. As AAV-based gene therapy for lipoprotein lipase deficiency (“Glybera”) has been recently approved in the European Union, the first of its kind in the Western World, we envision that the next few years of research on miRNA mimic therapies will follow the fate of anti-miR technology. Moreover, miRNAs can circulate in the blood or different biological fluids in microvesicles, exosomes, Ago2-containing complexes or HDL [102–104], and thus, opportunities will probably arise for therapeutically exploiting this physiologic form of miRNA delivery.

It is important to note that the use of miRNA mimicry might have potential challenges for miRNA-based therapeutics compared to those of conventional classic drugs, where specificity is desired. The basic characteristic of miRNAs to target different mRNAs, raise the possibility of off-target effect appearance due to unintended or unidentified target inhibition [90]. The introduction of a specific miRNA in a cell system can have both beneficial and pathogenic effects, which will ultimately depend on the cellular status. Moreover, the delivery of a miRNA mimic could result in their uptake by tissues that normally do not express them and thus, by repressing their targets could ultimately cause side effects. Delivery to the appropriate cell/tissue type is an important aspect for the safety of a miRNA mimic therapy. Even when we can get advantage of either the natural tropism for certain tissues from the AAV or, a novel tissue-selective formulation of synthetic miRNA mimics systems to target a specific cell type, there are still questions regarding their biological function, particularly those related to extracellular miRNAs, intercellular communication by miRNAs and their presence in numerous biological fluids, that need to be addressed. As several aspects of miRNA biology are still poorly understood in these processes, we cannot discard that the presence of a particular miRNA, even in its specific target cell, could modify either their own secretion or the secretion of other miRNAs that could target a different cell/tissue type causing unwanted side effects. As proof of concept, data generated in animal models suggests that pharmacological delivery of miRNA mimics

is feasible. Current promising strategies to deliver miRNA mimics therapeutically (miRNA replacement therapy), as those for miR-34, will probably soon reach clinical trials [105].

4.2 Therapeutic miRNA inhibition

In contrast to what has accounted for miRNA mimicry, microRNA inhibition has really benefited from previous available RNA-based therapy. Fomivirsen, the first RNA-based drug approved by the US Food and Drug Administration (FDA) in 1998, is a synthetic 21-long antisense oligonucleotide modified with phosphorothioate (which gives resistance to degradation by nucleases) used as antivirals for the treatment of cytomegalovirus retinitis. Anti-miR technology has rapidly developed as pharmacological therapy to regulate miRNA levels *in vivo*. miRNA inhibitors could potentially be used in situations in which an induced-expression (overexpression) of a particular miRNA plays a causal role in a disease, as is the case for cardiovascular disease and cancer, where the overexpression of particular miRNAs directly contributed to the disease.

During the last decade therapeutic inhibition of miRNA activity has been achieved through the use of chemically modified single-stranded reverse complement oligonucleotides (Figure-1). Antisense oligonucleotide (ASOs) complementary to the mature miRNA sequence, ‘antagomiRs’, were the first miRNA inhibitors in mammals [106], and were ASOs containing various modifications in order to modify their pharmacological properties: Cholesterol, conjugated via a 2'-O-methyl (2'-O-Me) linkage in the 3' end, to increase cellular uptake and stability; phosphorothioate linkage instead of natural phosphate linkage, to increase stability and reduce clearance by promoting plasma protein binding; 2'-O-Methyl (2'-O-methyl) modified ribose sugar to protect from endonuclease activity [106,107]; 2',4'-constrained 2'-O-ethyl (cET) nucleotides to improve potency and stability [108,109]; and 2'-O-methoxyethyl (2'-MOE) and 2'-fluoro (2'-F) to improve *in vivo* efficacy [110]. Lastly, the 2'-fluoro/methoxyethyl (2'-F/MOE)-modified with phosphorothioate backbone-modified anti-miR technology has been shown to be efficacious in non-human primates [111].

Locked nucleic acid (LNA) is a modified RNA or DNA nucleotide mimic, in which the ribose moiety is ‘locked’ with an extra bridge connecting the C(2') and C(4') by an oxymethylene bridge which conformationally ‘locks’ the ribose due to fitting into A-form duplexes [112]. Several unique properties make LNA a therapeutically promising agent in miRNA therapy, including: high-binding affinity and increased selectivity to complementary RNA and thus the sequence length can be reduced. LNA also increase the duplex's melting temperature and stability in biological systems [113–115]. LNA-modified anti-miR technology has not only been shown to be efficacious in non-human primates [116,117], but it was also the first anti-miR therapy to show efficacy in human trials (clinicaltrials.gov). These properties have also allowed the development of a phosphorothioate backbone tiny 8-mer LNA-modified anti-miRs for *in vivo* use [118], which can be used for reducing the activity of entire miRNA families that share a common seed region.

Concerning their pharmacokinetics and pharmacodynamics, anti-miRs currently used *in vivo* are water soluble, but unable to be absorbed by the intestine due to their size and charge, thus bad candidates for oral therapy. For now, their administration is via parenteral. While their exact mode of action is not well understood and depends on their specific chemistry, most of them have been shown to have long lasting effects [92,106,111,116–118], which suggests their potential use for chronic, rather than acute, disease. As the role of fine-tuning of gene expression, and thus relatively mild changes in protein output [119], is the main function of miRNAs under normal conditions, anti-miR efficacy will depend on stress conditions that produce the elevation of the miRNA intended to be pharmacologically treated [21]. However, depending on the cell/tissue type, the severity and the type of stress, we have to consider that other factors including: other miRNAs modified by the stress

conditions, other mRNAs (independent of miRNA-mediated) modified by the stress conditions and other regulatory mechanism exerted by ceRNA and lncRNAs, will greatly influence the pharmacodynamics of every particular anti-miR.

As other oligonucleotide antisense therapies [120], anti-miR therapy might not be free of potential off-target effects and their evaluation might be really challenging. As shown in one study with animal models, LNA-containing ASOs may have risk of hepatotoxicity [121]. However, to date, specific toxicity associated with the inhibition of a particular miRNA has not been reported. By using anti-miR therapy, we intend to de-repress different target genes involved in one pathway or a pathological process, but the same inhibition can probably derepress other unrelated genes and cause undesired changes in gene expression [91]. Moreover, tissue distribution, accumulation and their long lasting effects could also be one source of off-target effects. However, this needs to be experimentally evaluated for every miRNA and every type of anti-miR chemistry.

Although there are many aspects of miRNA mimics and anti-miR biology that need to be addressed, the first anti-miR therapy has shown efficacy in human trials (SPC3649, Miravirsén, Santaris Pharma A/S) and the biological interest in controlling miRNAs level therapeutically anticipates the further development of this new class of drugs.

5. miRNA targets in cholesterol efflux, reverse cholesterol transport and HDL function

As in other biological processes, several miRNAs have been described to modulate different aspects of cholesterol homeostasis both directly at cellular levels and/or indirectly in the whole organism [24,122]. In the context of this review, we will discuss only miRNAs directly or indirectly related to cellular cholesterol efflux, RCT and HDL function that could potentially be used as pharmacological therapy (Figure-1).

5.1 Regulation of cholesterol homeostasis, fatty acid metabolism and insulin signaling by miR-33a/b

Due to its genomic localization and experimental data, several studies reported the discovery of the miR-33 family and their importance in cholesterol metabolism, particularly cholesterol efflux [23,123–125]. *miR-33a/b* are encoded within introns of the *Srebp* genes, master regulators of cholesterol and lipid metabolism [31]. The miR-33 family is conserved from *Drosophila* to humans, but among mammals, *miR-33b* is only present in the genome of certain large mammals and primates. miR-33a and miR-33b are important modulators of cellular cholesterol efflux due to their role in the posttranscriptional repression of ABCA1. miR-33a/b directly bind to the 3'UTR of *Abca1*, which in humans has 3 different binding sites, thus exerting a strong repression activity [23,123,126,127]. As a consequence, modulation of miR-33a/b levels results in changes in cellular cholesterol efflux in macrophages and other cell lines [23,123,126]. Importantly, manipulating the expression of miR-33a/b levels *in vivo* either by target disruption of the gene, LNA anti-miR or viral delivery of sense and antisense oligonucleotides, significantly alters circulating HDL-C [23,123–125]. miR-33a/b also regulate the expression of other genes involved in regulating cholesterol metabolism including Niemann Pick C1 (*Npc1*) and *Abcg1*, the latter only in rodents [23,124].

miR-33 deficiency was also shown to reduce the progression of atherosclerotic plaque in a mouse model of atherosclerosis (ApoE^{-/-} mice) [128]. Moreover, the antisense inhibition of miR-33a for 4 weeks in mice (LDLR^{-/-} mice) enhanced the RCT and consequently regression of atherosclerosis [129]. Anti-miR treatment reduced the plaque size and decreased inflammatory gene expression, while it increased markers of plaque stability. In

addition to the enhanced increase of *Abca1* and *Abcg1* in the liver, it was shown that anti-miR treatment directly targeted the plaque macrophages, enhancing *Abca1* expression and cholesterol removal [129], which points to the miR-33 family as potential therapeutic targets against atherosclerotic cardiovascular disease.

In the context of the real contribution of miR-33a and miR-33b in repressing *Abca1* and thus RCT, it is important to note that the real *in vivo* contribution of either mature miR-33a or miR-33b under physiological or pathological conditions could be completely different [130]. SREBP2, the host of miR-33a, is most profoundly regulated by protein processing rather than mRNA expression [10]. By contrast, SREBP1c, a spliced transcript of SREBP1 (host of miR-33b), is expressed in the liver and their expression is highly regulated by dietary factors. SREBP1c and thus miR-33b is highly upregulated following insulin stimulation [126,130]. In hyperinsulinemia conditions, such as in insulin resistance state, dramatically increased expression of SREBP1c and miR-33b contribute to both increased levels of plasma triglycerides and low HDL levels, a hallmark of metabolic syndrome [130]. As inhibition of miR-33b can only be experimentally evaluated in primates and humans, recent data have confirmed that anti-miR therapy in African green monkeys for 12 weeks reduces liver expression of *Abca1*, increase the function of HDL evaluated as macrophage cholesterol efflux and raises plasma HDL levels [111]. Altogether, this clearly supports the development of anti-miR-33 pharmacological therapies.

The therapeutic potential of anti-miR-33 is not only based on *Abca1*, cholesterol efflux and RCT. miR-33a/b also controls the expression of important genes involved in fatty acid β -oxidation and insulin signaling [111,126,127]. Fatty acid β -oxidation, the process by which fatty acids are catabolized to generate energy in the form of ATP and acetyl-CoA for the citric acid cycle is an important process for fatty acid degradation. The miR-33 family directly targets proteins involved in fatty acid β -oxidation. Carnitine palmitoyltransferase 1A (*Cpt1a*), the rate limiting transporter of fatty acid into the mitochondria for β -oxidation; carnitine O-octanoyltransferase (*Crot*), involved in β -oxidation of long chain fatty acids in the peroxisomes; and the mitochondrial trifunctional enzyme subunit beta Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (*Hadhb*) involved in mitochondrial fatty acid β -oxidation [126,127]. Sirtuin 6 (*Sirt6*), an NAD⁺-dependent histone deacetylase that participates in multiple molecular pathways related to aging, glucose metabolism, inflammation and cancer, together with the 5'-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1 gene, *Ampk*), a cellular energy sensor in response to stress and involved in different aspects of cellular metabolism including fatty acid metabolism, are also direct targets of the miR-33 family [122,126]. Insulin receptor substrate 2 (*Irs2*), a signaling adaptor molecule that mediates insulin signaling to downstream effectors, is also a direct target of miR-33 [126]. Thus, all these other targets make the miR-33 family an attractive target for miRNA-based therapy. However, even while miR-33 inhibition is a promising pharmacological target to increase cholesterol efflux and RCT in atherosclerosis and other cardio-metabolic disorders, their safety should be carefully evaluated as other targets related to cell proliferation, cell cycle and inflammation including: cyclin-dependent kinase 6 (*Cdk6*), cyclin D1 (*Ccnd1*), the tumor suppressor p53 and the nuclear receptor coregulator receptor interacting protein 140 (*Rip140*) have also been described [131–133].

From a pharmacological point of view, different anti-miR chemistries were tested for inhibiting miR-33 family function including LNA-antisense oligonucleotide [123] and 2'F/MOE-modified phosphorothioate backbone-modified ASO [111,129]. It is important to note that mature miR-33a and miR-33b only differ in two nucleotides but should target overlapping genes. Interestingly, the miR-33a/b seed sequence (UGCAUUG) between nucleotides 2–8 at the 5' end of the mature miRNA also have a repetitive sequence similar

to that of seed, between nucleotides 13–19, a characteristic not common in most mammalian miRNAs. Whether this special sequence of the miR-33 family is relevant to their biological function, and most importantly to their pharmacological inhibition, is not known. However, this special structure of mature miR-33 could benefit the use of phosphorothioate backbone tiny 8-mer LNA-modified anti-miRs chemistry. Which anti-miR chemistry will have a better pharmacologic profile for human use is not known, but we can envision that anti-miR-33 therapy will be a topic for intensive research in the years to come.

5.2 Regulation of cholesterol efflux and neurological function by miR-758 and miR-106b

The blood-brain barrier separates the normal regulation of cholesterol homeostasis exerted by the lipoprotein axis intestine-liver-tissues, and thus cholesterol metabolism in the central nervous system is different from the way it is organized in the rest of the body [8,134]. Briefly, while in developing brain, cells must produce autonomously cholesterol to survive, in adult brains cholesterol can also be obtained from other sources. Different apolipoproteins are found in the brain including, apolipoprotein (apo) E, apoD and apoJ/clusterin. Apo A-I is also found, but mainly synthesized by the endothelium of brain capillaries. Astrocytes produce and secrete apoE-derived sterols containing lipoproteins. The LDLR and LDLR-related protein 1 (LRP1) are likely to mediate lipoprotein uptake in the brain. ABCA1, ABCG1 and ABCG4 are expressed in astrocytes and different other cells and cholesterol efflux is also induced by LXR agonists in astrocytes [134,135], indicating that astrocytes secrete cholesterol via apoE containing lipoproteins that are possibly lipidated by ABC transporters. Cholesterol elimination is mediated by their conversion to 24S-hydroxycholesterol by the cholesterol 24-hydroxylase (*Cyp46a1*). Neurons also express ABCA1, ABCG1 and ABCG4 and LXR activation increases cholesterol excretion from the brain [136,137]. However, even when *in vitro* models indicate so, whether ABC transporters mediate cholesterol efflux *in vivo* is not known. The maintenance of cholesterol homeostasis in the brain, as in other tissues, is fundamental for its appropriate function as disruption of cholesterol levels in the CNS have been associated with certain neurological and neurodegenerative disorders and thus the search for pharmacological therapies to maintain this homeostasis is of interest.

In contrast to the focus on miR-33 by their interesting genomic localization, miR-758 was found in an unbiased screen for miRNAs in cholesterol-loaded macrophages [138]. It was found that miR-758 regulates cellular cholesterol efflux by directly targeting the 3'UTR of *Abca1*. As a consequence, miR-758 also regulates cellular cholesterol efflux to apoA-I, but not to HDL. The two binding sites for miR-758 within the 3'UTR of *Abca1* is highly conserved among mammals, suggesting their importance during the evolutionary control of cellular cholesterol levels. Even while the regulation of the expression of miR-758 locus is not well known, it is known that high plasma cholesterol levels and increased cellular cholesterol levels increases its expression, which will ultimately result in reduced cholesterol efflux and increased cellular cholesterol content. Thus, in certain physiological conditions its therapeutic inhibition is desired. Interestingly, the relative expression of miR-758 is high in the heart but particularly elevated in the brain [138]. It seems that miR-758 might not only regulate the expression of ABCA1 but also other important proteins involved in several neurological functions including: SLC38A1, IGF1, NTM, XTXBP1 and EPHA1. In the context of cardiovascular diseases, therapeutic inhibition of miR-758 would result in an increased cholesterol efflux and RTC which will ultimately benefit the treatment for atherosclerosis. However, this needs to be experimentally validated. Although our understanding of the role of miR-758 under physiological and pathological conditions needs to increase first, the development of appropriate anti-miR chemistries for targeting the brain miR-758 still remain to be dealt with, including bypassing the blood-brain barrier and delivery to specific cell types.

Through a bioinformatic analysis to search for conserved miRNAs with predicted targets in the 3'UTR of *Abca1*, miR-106b was found as a strong candidate to target *Abca1* as having a perfect 8-mer and several supplementary pairing sites in mammals, including human and rodents [77,139]. miR-106b directly targets the 3'UTR of *Abca1* and in neuronal cell lines (Neuro2a cells), miR-106b reduces cholesterol efflux to apoA-I both under basal and LXR-stimulated conditions [139]. Alzheimer's disease is a common cause of dementia in the elderly. The production and/or aggregation of amyloid (A β) peptide is believed to play a central role in the pathogenesis of AD. A β peptides are generated by cleavage of amyloid precursor proteins (APP). Thus, factors that either increase their production and oligomerization or that reduce their elimination increases the risk of AD. Increased cellular cholesterol levels are thought to induce A β production [140,141] and thus neuronal cholesterol excess elimination might be a potential therapeutic target for AD. In this context, miR-106b mediated repression of *Abca1* was shown to increase A β secretion and clearance. This cellular effect was counteracted by *Abca1* expression, indicating that this effect might be directly linked to the effect of miR-106b on *Abca1* rather than other target genes in neuronal cells [139]. However, various other proteins related to cell proliferation and differentiation are also targets of miR-106b [142] that should be carefully considered. However, APP is also a target of miR-106b [143] and thus an eventual inhibition of miR-106b could lead to increased APP production and cholesterol efflux associated with *Abca1*. What would be the final phenotype in a context of inhibition of neuronal miR-106b is not known, but in the context of cholesterol efflux in the CNS, miR-106b could be an interesting target for the control neuronal cholesterol excess.

5.3 Regulation of LXR-dependent cholesterol efflux by miR-26

Together with the SREBPs, LXRs control distinct aspects of cholesterol homeostasis at the transcriptional level including, uptake (IDOL) or efflux (ABCA1, ABCG5, ABCG8). It was recently shown that cellular cholesterol efflux is also controlled by a tight balance between repression and derepression posttranscriptionally through miRNAs [144]. Treatment of macrophages with an LXR agonist resulted, among other miRNAs, in a repression of the miR-26 family. LXR activation also resulted in an increased expression of *Abca1* and ADP-ribosylation factor-like7 (*Arf7*) which contrasted with the decreased expression of miR-26. *ARL7* is an LXR target gene that participates in apoA-I dependent cholesterol efflux [145]. miR-26-a-1, localized to the intronic region of the RNA polymerase II polypeptide A small phosphatase-like (CTDSPL), was found to be directly regulated at the transcriptional level by LXR [144]. As miR-26 has a highly conserved binding site in the 3'UTR of *Abca1* and *Arf7*, this miRNA regulates cellular cholesterol levels through the modulation of these proteins. miR-26, a newly identified LXR responsive miRNA, suppresses cholesterol efflux by targeting *Abca1* and *Arf7* [144]. Upon cellular cholesterol excess, LXR activation does not only induce the expression of key genes involved in cholesterol efflux, but also reduces the expression of a miRNA that represses some of the same genes, which will ultimately translate into increased cellular cholesterol elimination. The possible pharmacological use of LXR agonists [41] can thus benefit from these molecular mechanisms, on one side by increasing the expression of *Abca1*, *Abcg1* and other genes related to cholesterol elimination and on the other side by repressing the expression of miR-26a. LXR activation also induces the expression of other miRNAs, including miR-613, which targets LXR- α and mediates a feedback loop of LXR- α auto regulation [146]. Thus, pharmacological targets to increase miR-26 activity seem like a promising approach to increase cholesterol efflux and RCT. Why the same pathway activation leads to the modulation of miRNAs with opposite effect is not clear. But, as cholesterol homeostasis needs to be tightly regulated, evolution has probably provided redundant and opposed mechanisms to make sure that this essential molecule can be regulated under strict limits.

5.4 Regulation of ABCA1/ABCG1-mediated reverse cholesterol transport by miR-10b: the emerging role of microbiota

Microbial inhabitants of our body are ~10 times more our own cell number or genomic amount, and colonize every mucosal surface. While the diversity of microbes differs widely between subjects and within different parts of our body, the metagenomic carriage of the metabolic pathway is relatively stable among individuals [147]. There is a clear evidence of a functional interaction between the gut microbiota and the host metabolism which ultimately can influence human health [148]. Dietary polyphenols are major secondary metabolites of plants and their consumption has been associated with reduced risk of developing CVD [149]. Anthocyanidins are pigmented polyphenols found in different vegetables, fruits and common beverages including grape and berry juice and red wine. Cyanidin-3-O-glucoside (Cy-3-G) is a major anthocyanidin of grape and other fruits. There is some evidence that anthocyanidin-rich extracts may exert antiatherogenic effects [150]. Due to their chemical structure (ionized), anthocyanidins are poorly bioavailable. But polyphenols may be metabolized in the large intestine by the gut microbiota and thus certain metabolites can achieve the circulation after dietary ingestion [151]. Protocatechuic acid (PCA), a metabolite of anthocyanidin, was found to have antiatherosclerotic effects [152]. However the molecular mechanism was not clear. It was recently confirmed, however, that PCA is an intestinal microbiota metabolite of Cy-3-G and its antiatherosclerotic effect might be through a miR-dependent pathway related to cholesterol efflux and RCT [153].

PCA increase macrophage cholesterol efflux through the repression of miR-10b [153]. miR-10b directly represses *Abca1* and *Abcg1* and negatively regulates cholesterol efflux from lipid-loaded macrophages [153]. Thus, inhibition of miR-10b activity by either anti-miR chemistries or dietary intervention with anthocyanidins may be an interesting pharmacological approach to increase cholesterol efflux and RCT. When developing this therapeutic strategy we should consider other confirmed targets of miR-10b, as several genes involved in cancer progression have been validated as targets of the oncogenic miR-10b [154,155]. It is not the first time that a dietary polyphenol exerts its effect by regulating the expression of miRNAs [156] or that the microbiota modulates the host gene expression through miRNAs [157]. However, whether polyphenols and other dietary components can physiologically modulate the expression of miRNAs and exert their diverse effects via this action is still under intense investigation [158]. The possibility of increasing or reducing miRNA activity by using other pharmacological approaches including, the use of polyphenols or other dietary components or the modulation of the microbiota, is an attractive alternative to the use of ASOs or miRNA mimic technology. Future research in this arena will eventually provide solid ground for their use in disease prevention or therapy.

5.5 Potential regulation of cholesterol efflux by targeting other genes related to cholesterol homeostasis

Many proteins and factors participate in cholesterol efflux, but their real physiological contribution or their stringency is not well understood. Autophagy is a process by which the cell degrades unnecessary or dysfunctional cellular components through the lysosomal machinery. Several miRNAs have been described that regulate different targets in autophagy [159]. As lipid droplet cholesteryl ester hydrolysis is being recognized as an important step in cholesterol efflux [56], miRNAs that target key pathways in lipid-loaded macrophage autophagy and/or cholesterol ester hydrolases might be interesting targets to promote cholesterol efflux. Caveolin has been proposed to contribute to cellular cholesterol efflux [51,160]. Several miRNAs including, miR-103, miR-107, miR-133a, miR-802 and others were validated to target Cav-1 [161,162]. It is not known yet the contribution of miRNAs related to either autophagy or miRNAs that directly target Cav-1 in the context of cellular cholesterol efflux and RCT, but if we want to stop the devastating effects of atherosclerotic

cardiovascular disease from different fronts, this and other biological processes related to miRNAs and cholesterol efflux need to be experimentally evaluated.

6. From cholesterol evolution to miRNA revolution: looking to the future

Millions of years of evolution have selected cholesterol as a unique molecule capable of providing the mammalian cell membrane with special physical properties that allow not only the anchorage of different proteins, channels and transport complexes, but also the organization in non-homogeneous areas of high lipid density for special functions. Thus, to maintain this molecule in tight limits, the cell has developed several molecular mechanisms to control their cellular levels. Most of the mechanisms that control cellular cholesterol input from endogenous synthesis or uptake from plasma lipoproteins have been elucidated in the last decades, while mechanisms that control cholesterol output are less known. The discovery of another layer of regulation, through the non-coding RNAs, has emphasized that even then *Abca1* is one of the few genes directly involved in cholesterol excess elimination, its regulation can be really complex probably to handle levels of cholesterol under tight limits. Evolution has provided, through different miRNAs (and probably other noncoding RNAs), the capacity to control cellular cholesterol levels by controlling *Abca1* posttranscriptionally. If we compare the 3'UTR size of *Abca1* (>3.3kb) with other common genes involved in cholesterol metabolism (see Figure 2), we can see that this is particularly long. This unusual long 3'UTR clearly raises the possibility to be regulated posttranscriptionally by miRNAs. Different prediction algorithms indicate that ABCA1 can potentially be regulated by ~100 miRs. Which of these miRNAs are physiologically and pathologically important to cholesterol efflux, RCT and cardiovascular disease still remain to be elucidated.

Even while the wide use of our pharmacological arsenal to either inhibit cholesterol biosynthesis (statins) or absorption (ezetimibe, resins) have greatly reduce CAD, even in healthy subjects, CVD remains the first cause of mortality and morbidity worldwide [163]. Pharmacological targets to raise HDLs until now, have proven to be inefficient to reduce risk factors [64,65] additionally to that exerted by standard therapy. Measurements of HDL cholesterol levels may not reflect the physiologic functions of HDLs, particularly RCT [68,69]. As cholesterol efflux is the first, and probably the most important, step in RCT [57], then therapies to increase cholesterol efflux (rather than HDL levels *per se*) and RCT would be a promising alternative to threat atherosclerotic cardiovascular disease. This offers a unique opportunity to treat disease in a manner that is completely different and revolutionary from that of classical one target-directed drugs. Moreover, due to their “promiscuity”, pharmacological modulation of miRNA function, may also enable to bypass mechanism that develop tissue insensitivity, as observed in certain classical one target-directed drugs.

The unique features of miRNAs to target different genes of a complex disease pathway, give us a unique opportunity to treat diseases as not previously imagined. The preclinical studies detailed above suggest that targeting miRNAs that control cholesterol efflux using anti-miR technology, as that against miR-33, can dramatically increase cholesterol efflux, RCT and HDL levels, moving the field rapidly toward novel therapeutics against atherosclerotic cardiovascular disease. Although anti-miR therapy has really benefited from previous antisense technologies, there are several aspects of miRNA biology and particularly anti-miR chemistry, from the point of pharmacokinetics and pharmacodynamics, which need to be considered when developing this revolutionary therapy.

Several companies are currently developing miRNA-based therapeutic and diagnostic applications [91] and despite all the potential challenges that these technologies need to solve, the reality of Phase III clinical trials from Santaris Pharma (Miravirsen) will really

catapult the development of miRNA-base therapies to target cholesterol efflux and RCT, as that of developing an anti-miR against miR-33. Indeed it will not be difficult to believe that the complex *evolution* in the regulation of cellular cholesterol homeostasis gives us a unique alternative to handle levels of cholesterol that exceed the limits, with a therapeutic *revolution*, pharmacologically targeting miRNAs.

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Figure 1. Schematic model of anti-miR therapy to increase cellular cholesterol efflux and RCT
 Anti-miR (inhibitors of miRNA activity) chemistries are synthesized as antisense oligonucleotides (complementary to the mature miRNA) containing chemical modifications to enhance binding affinity, confer nuclease resistance, facilitate cellular uptake and reduce clearance. The liver and the intestines synthesize apolipoprotein-AI (A-I), which is secreted in a lipid-poor form and promotes the transfer of excess of cellular-free cholesterol (yellow dots) and phospholipids, via the ATP-binding cassette A1 (ABCA1) pathway, forming the nascent high density lipoprotein (HDL). The plasma lecithin cholesterol acyltransferase (LCAT) esterifies free cholesterol to cholesteryl ester (CE), forming mature HDL. In certain cell types, ABCG1 and probably the scavenger receptor, class B type 1 (SR-BI) promotes

cholesterol efflux to mature HDL. Mature HDL can transport cholesterol back to the liver directly via SR-BI or alternatively, HDL cholesteryl ester (HDL-CE) is exchanged for triglycerides (TG) in apolipoprotein-B (apoB)-containing lipoproteins (very low-density lipoprotein [VLDL]/low density lipoprotein [LDL]) through cholesteryl ester transfer protein (CETP) and then taken up by the liver via the LDL receptor (LDLR). Returned cholesterol in the liver is eliminated as cholesterol and bile acids. The whole process is known as reverse cholesterol transport (RCT). As intracellular excess of free cholesterol is toxic, cells trigger different mechanism to eliminate cholesterol excess. Through the SREBPs, the synthesis and uptake of cholesterol is inhibited. High levels of cholesterol induce the formation of oxysterols, natural ligands of the liver X receptors (LXRs). LXRs induce the expression of proteins involved in cholesterol efflux, ABCA1 and ABCG1. Different physiological process induce the expression of several miRNAs that target either ABCA1, ABCG1 or other proteins involved in cholesterol efflux, thus reducing the elimination of free cholesterol excess. Anti-miR therapies against these miRNAs will results in a derepression of their target genes and increase cholesterol efflux. Through a not well understood mechanism, cells also eliminate cholesterol to the intestine for fecal excretion via a mechanism known as trans-intestinal cholesterol efflux (TICE). Pharmacological therapies to either inhibit (anti-miR) or increase (miRNA mimics) the activity of miRNAs directly or indirectly related to cellular cholesterol efflux are potential candidates to increase RCT and treat different pathologies associated to dyslipidemia.

Figure 2. Micro-targeting *Abca1*

Comparative analysis of predicted miRNAs targeting different genes involved in cholesterol homeostasis. Predicted miRNA families conserved among mammals and vertebrates are shown (www.targetscan.org). Validated miRNAs that target *Abca1* are shown relative to their approximate binding sites within the 3'UTR of *Abca1*. The particular long 3'U of *Abca1* increase the susceptibility to be regulated by miRNAs. Length (bp) of 3'UTR are shown.