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miRNA regulation of LDL-cholesterol metabolism

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Abstract

In the past decade, microRNAs (miRNAs) have emerged as key regulators of circulating levels of lipoproteins. Specifically, recent work has uncovered the role of miRNAs in controlling the levels of atherogenic low-density lipoprotein LDL (LDL)-cholesterol by post-transcriptionally regulating genes involved in very low-density lipoprotein (VLDL) secretion, cholesterol biosynthesis, and hepatic LDL receptor (LDLR) expression. Interestingly, several of these miRNAs are located in genomic loci associated with abnormal levels of circulating lipids in humans. These findings reinforce the interest of targeting this subset of non-coding RNAs as potential therapeutic avenues for regulating plasma cholesterol and triglyceride (TAG) levels. In this review, we will discuss how these new miRNAs represent potential pre-disposition factors for cardiovascular disease (CVD), and putative therapeutic targets in patients with cardiometabolic disorders. This article is part of a Special Issue, entitled: MicroRNAs and lipid/energy metabolism and related diseases, edited by Carlos Fernández-Hernando and Yajaira Suárez.

INTRODUCTION

Cellular and plasma cholesterol levels are maintained through tightly controlled mechanisms, which regulate the expression and activity of key metabolic genes at both the

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CONFLICTS OF INTEREST

AMN has patents issued and pending on the use of miR-128-1 inhibitors, AMN and AW have patents pending on the use of miR-148a inhibitors, AMN and CF-H have patents issued and pending on the use of miR-33 inhibitors, and CF-H and LG have patents pending on the use of miR-27b and miR-148a inhibitors.

transcriptional and post-transcriptional level. Alterations in the control of cholesterol and lipid homeostasis can lead to cardiometabolic diseases, including atherosclerosis, a prominent cause of human morbidity and mortality in Western societies [1, 2]. Among the numerous environmental and genetic factors identified to contribute to atherogenesis, increased levels of low-density lipoprotein (LDL)-cholesterol are sufficient to drive the progression of this disease. For this reason, the pathways governing plasma LDL-cholesterol (LDL-C) levels and their translation into effective therapies [e.g. statins and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors] have been extensively studied. At the forefront of these discoveries are genome-wide association studies (GWAS), which, over the years, have allowed major advances in the identification of novel genetic variants that affect plasma lipoprotein metabolism and increase the risk of developing cardiovascular disease (CVD).

The majority of hypercholesterolemia patients are prescribed statin drugs, which competitively inhibit 3-hydroxy-3methylgutaryl-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol biosynthesis [3, 4]. The therapeutic benefit of statins is due, at least in part, to the sterol regulatory element-binding protein (SREBP)-mediated increase in the expression of the LDL receptor (LDLR), which promotes the clearance of pro-atherogenic lipoproteins from circulation [5]. Although effective at lowering cholesterol levels and reducing cardiovascular-related deaths, statin therapies are not well tolerated by all individuals and two-thirds of statin patients still experience adverse coronary events [3, 4]. As a result, there has been a significant effort in developing new classes of cholesterol-lowering drugs that can be used alone or in combination with statins, including novel approaches that exploit miRNA-dependent gene silencing [6, 7]. In this review, we summarize the therapeutic potential of manipulating miRNA expression to control plasma LDL-C levels, as well as highlight the most recent findings that uncover the importance of miRNAs in directly regulating LDLR activity *in vitro* and *in vivo*. In addition, we focus on several genome-wide association studies (GWAS), which have uncovered a critical role for miRNAs in controlling circulating levels of plasma LDL-C in humans.

miRNAS and LDL-C METABOLISM

It is well-established that miRNAs can have profound effects on cholesterol and lipid homeostasis. Specifically, a great deal of work has identified miRNAs as important regulators of high-density lipoprotein (HDL), increased levels of which are associated with reduced risk for developing CVD [8]. The most well-studied of these miRNAs, miR-33, has been demonstrated to target ATP-binding cassette transporter A1 (ABCA1). ABCA1 is a critical factor for HDL biogenesis and reverse cholesterol transport (RCT), the process through which cells efflux cellular cholesterol for transport to and removal by the liver [9–13]. In addition, miR-33 has been shown to negatively regulate numerous other genes involved in RCT and metabolic function, such as ATP-binding cassette transporter G1 (ABCG1), Niemann-Pick disease, type C1 (NPC1), cholesterol 7α-hydroxylase (CYP7A1), ATP- binding cassette, sub-family B, member 11 (ABCB11), and ATPase, aminophospholipid transporter, class I, type 8B, member 1 (ATP8B1) [9, 14, 15]. Consistent with this, therapeutic inhibition of miR-33 was found to significantly increase circulating levels of HDL-C in mice and non-human primates [9, 10, 13, 16, 17] Moreover, numerous

studies have also demonstrated the beneficial effects of miR-33 inhibition on reducing atherosclerotic plaque burden, thus highlighting the therapeutic potential of manipulating miRNA expression to treat CVD [18–22].

While the role of miRNAs in regulating HDL-C metabolism has been extensively studied, the importance of miRNAs in controlling LDL-C have lagged behind, perhaps due to differences in lipoprotein metabolism between mice and humans. Nevertheless, a number of studies have reported the importance of miRNAs in controlling plasma LDL-C levels by regulating very-low density lipoprotein (VLDL) secretion, cholesterol biosynthesis and LDLR activity (Figure 1).

The liver-restricted miR-122 and miR-30c were the first miRNAs shown to play a role in altering plasma LDL-C *in vivo* by controlling VLDL secretion and cholesterol biosynthesis. While miR-30c controls plasma cholesterol levels by decreasing lipid synthesis and the secretion of ApoB-containing lipoproteins through direct targeting of the microsomal triglyceride transfer protein (MTP) [23], antisense targeting of miR-122 in mice and non-human primates significantly lowers total plasma cholesterol and triglyceride (TAG) levels [24, 25]. While this highlights the use of miR-122 inhibitors to treat dyslipidemias, germ-line and liver-specific deletion of *miR-122* results in increased hepatic steatosis, liver fibrosis and hepatocellular carcinoma [26, 27], thus raising questions about the potential therapeutic value of this treatment. In addition to miR-122 and miR-30c, inhibitors of miR-33 were also shown to modulate VLDL-TAGs in several mouse and non-human primate models, however these results have been controversial. While studies by Goedeke *et al* and Allen *et al* showed that long term inhibition of miR-33 resulted in increased levels of VLDL-TAGs in mice fed a high-fat and chow diet, respectively, no adverse effects were observed with long-term inhibition of miR-33 in non-human primates [16, 17, 28, 29]. Indeed, studies by Moore and Temel showed that anti-miR-33 treatment significantly reduced circulating levels of VLDL-TAGs in rhesus monkeys [17]. Additionally, several other miRNAs (e.g. the miR-96/182/183 locus [30]) have been shown to control LDL-C metabolism by regulating the SREBP2-mediated transcription of LDLR. For a comprehensive review of miRNAs that control cholesterol metabolism by affecting VLDL-secretion and/or cholesterol biosynthesis see review by Osborne and Hussain's groups in this Special Issue of BBA.

A role for miRNAs in contributing to the post-transcriptional regulation of LDLR expression has recently emerged. Notably, miR-27a/b, miR-185, miR-199a, miR-148a, miR-128-1, miR-130b, and miR-301, were shown to directly target the 3′UTR of *LDLR* and modulate LDLR expression in human and mouse hepatic cells [31–34]. Of these miRNAs, only miR-128-1, miR-148a and miR-185 significantly altered plasma LDL-C *in vivo.* Specifically, $ApoE^{-/-}$ and *APOB*Tg;$Ldlr^{-/+}$ mice had markedly less circulating levels of plasma LDL-C when treated with inhibitors of miR-128-1 and miR-148a, respectively (see below for more details [34, 35]). Inhibition of miR-185 expression was also shown to significantly reduce circulating levels of plasma LDL-C in $ApoE^{-/-}$ mice, as well as slow the progression of atherosclerosis [33]. Interestingly, while modulation of miR-27b markedly altered hepatic LDLR expression in wild-type mice, no significant differences in circulating LDL-C levels were observed [32]. As miR-27b plays an extensive role in the

processes governing atherosclerosis [36], additional long-term *in vivo* studies, as well as tissue-specific knockout mice, are needed to further elucidate the therapeutic potential of miR-27b inhibitors.

miRNAS AND GWAS

Since the advent of high-density single nucleotide polymorphism (SNP) genotyping arrays, GWAS have largely supplanted linkage studies to identify genes involved in various disease etiologies [37]. Numerous GWAS have been completed evaluating genomic linkage to various traits, including anthropometric measures such as weight, height, body mass index, as well as diseases such as metabolic disorders, cancers, and neurodegenerative diseases [38]. Importantly, essentially all GWAS to date have focused on the association of SNPs with nearby protein-coding genes, largely ignoring the presence of regulatory non-coding RNAs such as miRNAs in those genomic regions. Typically, DNA variants are located in intergenic or intronic portions of the genome, not within the coding sequences of genes or non-coding RNAs, requiring further post-GWAS functional characterization in order to determine (i) the causal DNA variant (ii) the gene(s) or non-coding RNAs involved the etiology of the disease, and (iii) the mechanism by which the DNA variant affects the expression of the causative agent.

Given the importance of circulating lipids in the development of cardiometabolic diseases (e.g., atherosclerosis, metabolic syndrome, type 2 diabetes (T2D)), a large number of GWAS have been carried out to characterize new genomic loci associated with circulating levels of lipids and lipoproteins [39]. Recently, a meta-analysis of the previously published GWAS of 188,577 European-ancestry individuals and 7,898 non-European-ancestry individuals performed by The Global Lipids Genetics Consortium led to the identification of 62 new loci within 100 kb of SNPs associated with abnormal circulating lipid [total cholesterol (TC), LDL-C, HDL-C, and TAGs] levels (at $p<5\times10^{-8}$) [40]. In addition to the well-characterized lipid-regulatory genes such as LDLR, ABCA1, and LIPA, new genes not previously known to play a role in lipid homeostasis were found. In particular, SNPs near Sortilin 1 (SORT1), on chromosome 1p13, are strongly associated with LDL-C levels ($P=1\times10^{-170}$) and are also linked to elevated risk of myocardial infarction, the leading cause of death in the Western world [41–44]. SORT1 alters serum levels of LDL-C and very low-density lipoprotein (VLDL) particles by modulating hepatic VLDL secretion [45]. Although the physiological function of SORT1 in regulating circulating lipoprotein homeostasis is still strongly debated, its identification by GWAS highlights the utility of this method to uncover new factors important for the regulation of circulating lipid levels.

To determine whether miRNAs might also be linked to abnormal blood lipids, Wagschal *et al* re-analyzed the Global Lipids Genetics Consortium GWAS data, identifying 69 miRNAs within 100 kb of signature lipid SNPs (Figure 2) [34]. Several unbiased bioinformatic approaches were then used to further narrow down potential miRNA regulators of circulating blood lipids. Specifically, predicted target genes of each miRNA were extracted using the software prediction program TargetScan [46], and combined with enrichment analyses [functional Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation] using the Database for Annotation, Visualization

and Integrated Discovery tool (DAVID: http://david.abcc.ncifcrf.gov). From this analysis miR-130b, miR-301b, miR-148a, and miR-128-1 emerged as potential regulators of circulating blood lipids and metabolic homeostasis.

miR-130b and miR-301b

miR-130b and miR-301b are located close to one another on human chromosome 22 in a locus associated with abnormal TC and HDL-C levels [34]. These miRNAs share the same seed sequence and are predicted to target the same metabolic factors. For example, both miRNAs directly target the 3′UTR of *LDLR* and *ABCA1*, thereby significantly reducing LDL-C uptake and binding and cholesterol efflux to ApoA1 in human hepatoma cells and mouse macrophages, respectively [34]. miR-130b and miR-301b also control the expression of salt-inducible kinase 1 (SIK1), a regulator of hepatic gluconeogenesis and SREBP1-dependent lipogenesis [47], as well as the expression of AMP-activated protein kinase alpha 2 (AMPKα-PRKAA2), a component of the trimeric stress- and energy-sensing kinase AMPK, which monitors and regulates cellular energy status. In addition, miR-130b modulates the expression of PPAR gamma coactivator 1 alpha (PGC-1α, PPARGC1A), a transcriptional coactivator, and insulin-induced gene 1 (INSIG1), a negative regulator of SREBP and HMGCR activities. Thus, these miRNAs may orchestrate tight physiological regulation of lipogenesis through the coordinated control of several upstream regulators of SREBP.

miR-148a

miR-148a is encoded within a gene-poor intergenic region of human chromosome 7 and highly expressed in the adult liver. Interestingly, several labs independently correlated SNPs (rs4722551, rs4719841 and rs6951827) in the promoter region of miR-148a with altered blood levels of TC, LDL-C and TAGs [40, 48, 49]. In particular, a miR-eQTL analysis performed in human livers revealed a strong correlation between SNP status and miR-148a expression [34]. Dietary lipids have also been shown to regulate the expression of miR-148a in the livers of mice and non-human primates, suggesting a conserved link to its metabolic function [35]. Specifically, Goedeke *et al* identified the presence of several SREBP1 binding sites in the promoter region of miR-148a and demonstrated through a series of *in vitro* and *in vivo* experiments that SREBP1c transcriptionally controls the expression of miR-148a in mouse and human hepatocytes [35]. As such, it is likely that these DNA variants in the miR-148a promoter might impact miR-148a transcription levels through altering SREBP1 binding sites and therefore, be considered as predisposition factors for CVD.

Although the precise mechanism by which these SNPs contribute to altered plasma lipids remains unknown, recent studies provide mechanistic insight into how miR-148a variants may modify circulating levels of LDL-C in humans. In particular, miR-148a was demonstrated to directly target the 3′UTR of *LDLR*, as well as several other genes involved in lipid metabolism, including *ABCA1*, *SIK1*, *PGC1a*, *AMPK* and *INSIG1* [34, 35]. Importantly, overexpression and inhibition of miR-148a significantly decreases and increases, respectively, hepatic LDLR expression in mice, suggesting a physiological role for miR-148a in controlling LDLR activity *in vivo*. Indeed, inhibition of miR-148a markedly lowered circulating levels of LDL-C in two different mouse models of hypercholesterolemia

[34, 35]. While the effects of long-term inhibition of miR-148a on serum LDL kinetics, hepatic and serum lipids, and the distribution of ApoB and other lipoproteins should be addressed, collectively these studies underscore the therapeutic potential of modulating miR-148a expression to treat dyslipidemias. Moreover, given that miR-148a regulates ABCA1 expression in hepatocytes and macrophages and in the liver *in vivo* [34, 35], as well as modulating HDL-C levels in both normal mice and $ApoE^{-/-}$ mice fed a high fat diet [34, 35], therapies directed against miR-148a represent viable options to simultaneously increase circulating levels of HDL-C and lower LDL-C. Future assessment of atherosclerotic CVD in *miR-148a* knockout mice, as well as in mice treated with miR-148a antisense inhibitors, are needed to determine the role of miR-148a during the progression and regression of atherosclerosis.

In addition to altered LDL-C, SNPs in the miR-148a promoter region (rs4719841 and rs6951827) have also been linked to altered levels of circulating TAGs [34, 40, 49]. Interestingly, the rs472251 variant was also found to be associated with altered TAGs, however this association did not reach statistical significance [48]. It is thus plausible that in addition to regulating cholesterol homeostasis, miR-148a may also contribute to the regulation of fatty acid and TAG metabolism. Indeed, miR-148a is predicted to target numerous SREBP1-responsive genes. Furthermore, miR-148a is also highly expressed in adipose tissue. Taken together, these observations necessitate further investigation into the role of miR-148a in fine-tuning SREBP-mediated processes, such as fatty acid/TAG synthesis. While no appreciable differences in circulating TAGs were observed in *APOB*Tg;$Ldlr^{-/+}$ and $ApoE^{-/-}$ mice treated with inhibitors of miR-148a, future studies using additional mouse and non-human primate models may uncover new roles of miR-148a in regulation of whole-body lipid metabolism.

miR-128-1

Wagschal *et al* uncovered miR-128-1 as strongly associated with altered levels of circulating TC and LDL-C [34]. A miR-eQTL analysis of 424 liver samples from gastric bypass surgery patients showed a correlation between the SNPs associated with the circulating levels of TC and LDL-C and the SNPs associated with miR-128-1 expression [34]. While this suggests that certain SNPs may affect DNA regulatory sequences linked to miR-128-1 expression, more work is needed to determine how these SNPs regulate miR-128-1 expression and whether they represent predisposition factors for cardiovascular risk.

miR-128-1 is located within an intron of *R3HDM1*, a gene with unknown function. Biochemical and physiological studies showed that miR-128-1 plays an important role in lipid and carbohydrate metabolism, suggesting that the association of SNPs in this genomic locus with abnormal blood lipids is indeed due to altered miR-128-1 expression [34]. In particular, long-term inhibition of miR-128-1 in hyperlipidemic ApoE-deficient mice leads to (i) a ~35% decrease in circulating TC, (ii) a ~25% decrease in TAGs associated with VLDL (triglyceridemia), (iii) a decrease in hepatic TAGs (steatosis) and (iv) improved glucose tolerance and insulin sensitivity [34]. Although the precise mechanisms responsible for these complex phenotypes are not yet fully understood, the involvement of miR-128-1 in

the regulation of lipoprotein transport and insulin signaling highlight the crucial role that miR-128-1 plays in these observed phenotypes.

miR-128-1 controls circulating lipoprotein metabolism by directly targeting the 3′UTR of *LDLR* and *ABCA1*. In accordance with the function of ABCA1, miR-128-1 regulates cellular cholesterol efflux to ApoA1 in human hepatoma cells and mouse macrophages [34]. Furthermore, overexpression of miR-128-1 (using viral particles encoding the miR-128-1 precursor) decreased hepatic ABCA1 expression and circulating levels of HDL-C in C57BL/6J mice. While other miRNAs have been shown to regulate ABCA1 expression and HDL-C levels (e.g. miR-33), miR-128-1 also regulates hepatic LDLR expression, similar to miR-148a. Specifically, modulation of miR-128-1 was shown to strongly affect LDL binding in human hepatoma cells and LDL-C clearance in $ApoE^{-/-}$ mice. Indeed, acute (5 days) inhibition of miR-128-1 was sufficient to significantly alter lipoprotein metabolism, resulting in decreased plasma VLDL-C and LDL-C levels. Based on the ability of miR-128-1 inhibitors to affect both HDL-C and LDL-C, miR-128-1 antisense treatment may decrease atherosclerosis by improving the LDL-C/HDL-C ratio.

In addition to controlling cholesterol metabolism, miR-128-1 also regulates hepatic insulin signaling. In particular, miR-128-1 directly controls the expression of the insulin receptor (INSR), insulin receptor substrate 1 (IRS-1), and the downstream phosphorylation levels of Akt [34, 50]. Importantly, antisense inhibition of miR-128-1 improved both glucose tolerance and insulin sensitivity in $ApoE^{-/-}$ mice fed a Western-type diet [34], indicating that miR-128-1 plays a critical physiological role in regulating insulin signaling and glucose homeostasis *in vivo*. Given the key role of insulin in controlling carbohydrate and lipid metabolism in skeletal muscle, further studies on miR-128-1 are needed to shed light on its insulin-dependent functions in other metabolic tissues.

In the liver of patients with T2D, abnormal activation of the insulin-signaling pathway leads to a deregulation of lipid metabolism [51]. Indeed, insulin suppresses hepatic ApoB secretion and promotes the clearance of ApoB-containing circulating lipoproteins via several receptors, including the LDLR, LDLR-related protein 1 (LRP1), and heparan sulfate proteoglycans [52]. Among the most common lipoprotein abnormalities, patients with T2D frequently present low circulating HDL-C and high TAGs, consequently leading to elevated TAG-rich remnant lipoproteins, in both fasting and postprandial states [52]. As such, the role of miR-128-1 in suppressing hepatic insulin signaling could contribute to the decreased lipoprotein levels observed in mice treated with anti-miR-128-1, specifically with respect to altered TAGs and VLDL.

In addition to the effect of miR-128-1 on hepatic insulin signaling, miR-128-1 was also found to control synthesis of free fatty acids through the regulation of fatty acid synthase (*FASN*) expression [34]. Interestingly, miR-128-1 was shown to negatively regulate the expression of SIRT1, an NAD^+-dependent energy sensor and lysine deacetylase that can directly deacetylate and inactivate SREBP1 and thus modulate SREBP1-dependent lipogenesis [53]. It is thus tempting to speculate that the effects of anti-miR-128-1 on *FASN* and hepatic steatosis may at least partially be due to the miR-128-1-mediated repression of

SIRT1, however, additional studies are needed to reveal the precise function of miR-128-1 in regulating lipogenesis and the potential role of SIRT1 in this process.

CONCLUSIONS AND FUTURE MIRNA-BASED THERAPEUTICS

The recent discoveries of miRNAs that control LDLR expression and activity have greatly expanded our understanding of the molecular mechanisms that govern plasma LDL-C. This miRNA-centered regulatory network continues to grow at a rapid pace, and will no doubt expand to include other miRNAs that regulate LDL-C levels, both directly, by regulating VLDL secretion and LDL catabolism, and indirectly, by controlling hepatic cholesterol biosynthesis. While the regulatory function of these miRNAs in health and disease states, as well as how these networks fit into the already well-established transcriptional and post-transcriptional pathways that control hepatic LDLR expression, remain to be fully elucidated, future studies in humanized mouse models and non-human primates will likely help determine the relative contribution of each of these miRNAs in controlling LDLR activity and LDL-C trafficking and cholesterol homeostasis.

The recent findings that manipulating miRNA expression may reduce circulating LDL-C opens new avenues for the treatment of dyslipidemias, especially for those patients who are resistant to statin treatment. Indeed, clinical trials using antisense inhibitors targeting miR-122 are currently underway [54]. Furthermore, multiple groups have demonstrated that inhibition of miR-33 results in a significant increase in plasma HDL-C in mouse and non-human primates and protection against atherosclerosis in hyperlipidemic mouse models [9, 10, 13, 16–22]. In addition, a report from Hussain's group showed that prolonged delivery of miR-30c mimics markedly reduced plasma LDL-C levels and atherogenesis in mice [23].

The recent GWAS discussed in this review also highlights the therapeutic potential of inhibiting miR-128-1 and miR-148a expression to simultaneously lower and increase circulating levels of LDL-C and HDL-C, respectively. As inhibitors of these miRNAs promote cholesterol efflux in macrophages, as well as HDL biogenesis in the liver, antisense treatments would be expected to not only increase HDL-C levels, but increase HDL functionality as well, and thus be of greater clinical relevance. Moreover, given the role of miR-128-1 in regulating insulin signaling and lipoprotein metabolism, inhibition of this miRNA also represents an attractive therapeutic strategy to combat metabolic syndrome and T2D-associated dyslipidemia, such as increased VLDL-TAGs and decreased HDL-C.

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Highlights

- Discussion of the role of miRNAs in regulation of LDL-cholesterol homeostasis
- Unbiased high-throughput and GWAS approaches identify lipid regulatory miRNAs
- Highlights miRNAs as potential therapeutic targets for cardiometabolic diseases

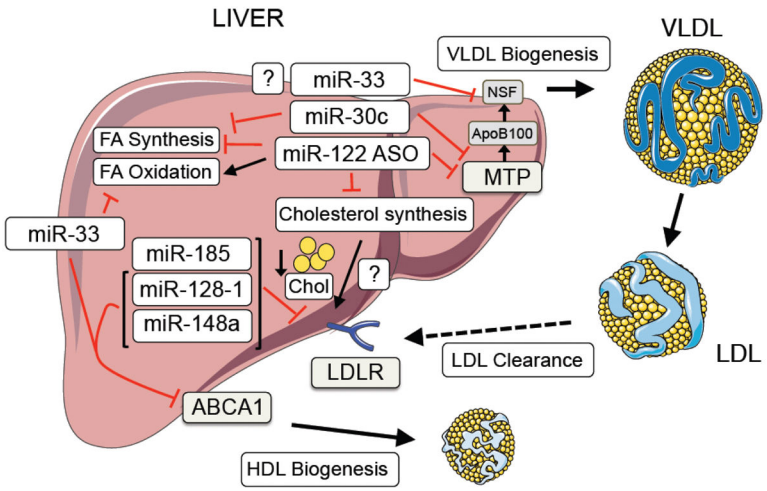

Figure 1. miRNA regulation of LDL-cholesterol metabolism

Schematic representation of miRNAS that control circulating levels of LDL-C *in vivo*.

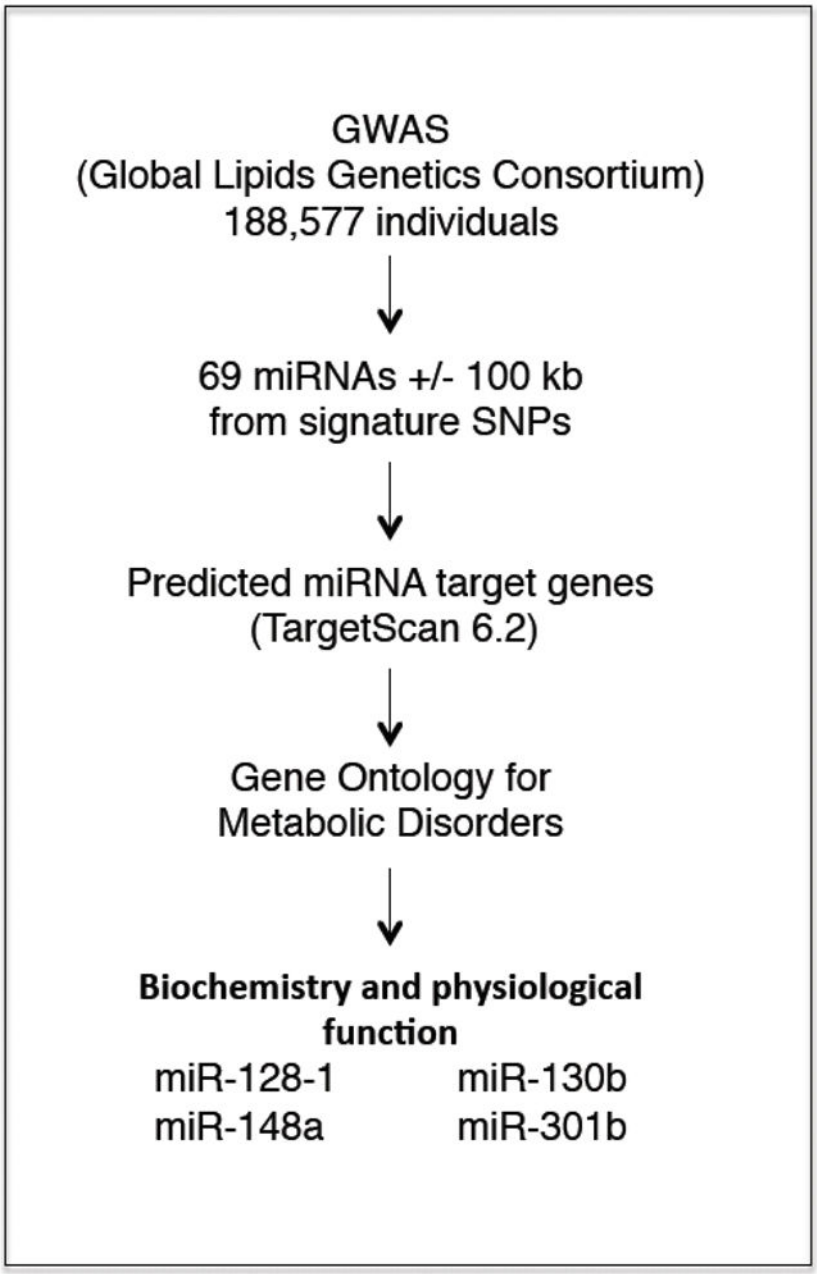

Figure 2. Identification of miRNAs located in genomic loci enriched for SNPs associated with abnormal circulating total cholesterol, triglycerides, LDL-C and HDL-C levels

Description of the GWAS meta-analysis method used to identify cholesterol/lipid-regulating miRNAs located in genomic regions associated with abnormal circulating cholesterol/lipids.