Identification of MicroRNAs Regulating Apolipoprotein Secretion

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Abbreviation

3'-UTR:	3´-untranslated region
ABCA1:	ATP-binding cassette transporter, sub-family A, member 1
ABCG1:	ATP-binding cassette transporter, sub-family G, member 1
Ac-LDL:	Acetylated-low density lipoprotein
apoAI:	Apolipoprotein A
apoB:	Apolipoprotein B
apoE:	Apolipoprotein E
B-lps:	ApoB-containing lipoproteins
BSA	Bovine serum albumin
CE:	Cholesteryl ester
CETP:	Cholesteryl ester transfer protein
CVD:	Cardiovascular diseases
DEPC	Diethylpyrocarbonate
ER:	Endoplasmic reticulum
FBS	Fetal bovine serum
FC:	Free cholesterol
FDA:	Food and Drug Administration
FH:	Familial hypercholesterolemia
FPLC:	Fast protein liquid chromatography
HCV:	Hepatitis C virus
HDL:	High density lipoprotein
HoFH	Homozygous familial hypercholesterolemia
IDL:	Intermediate density lipoprotein
LCAT:	Lecithin-Cholesterol Acyltransferase
LDL:	Low density lipoprotein
LDLR:	Low density lipoprotein receptor
LPL:	Lipoprotein lipase
miR:	MicroRNA
MTP:	Microsomal triglyceride transfer protein
oxLDL:	Oxidized low-density lipoprotein
PCSK9:	Proprotein convertase subtilisin/kexin type 9
Pre-miR	Precursor microRNA
Pri-miR	Primary microRNA
RCT:	Reverse cholesterol transport
RISC:	RNA-induced silencing complex
ROS:	Reactive oxygen species
SR-BI:	Scavenger receptor class B member 1
TG:	Triglyceride
VLDL:	Very low density lipoprotein
WT:	Wild type

Abstract

A major risk factor for cardiovascular disease (CVD) is plasma cholesterol that is carried in plasma by two lipoproteins LDL and HDL. Currently available therapies lower LDL and attempts are undergoing to enhance HDL levels for therapeutic purposes. With advances in microRNAs (miRs), small non-coding RNAs that affect multiple pathways, we hypothesize that there might be miRs that regulate plasma lipoproteins. The major proteins in LDL and HDL are apoB and apoA1. To identify miRs that regulate both LDL and HDL, I took two different approaches. In the first approach, I searched for miRs that could potentially modulate apoB and apoA1 expression. From TargetScan, I found that human apoB mRNA contained two predicted targeting sites for miR-548p. In both Huh-7 and HepG2 cells, overexpression of miR-548p significantly decreased intracellular and secreted medium apoB without affecting apoAI. Mechanistic studies revealed that miR-548p decreased apoB expression levels by destabilizing its mRNA. Luciferase reporter assay indicated that miR-548p interacts with apoB 3'-UTR. Site-directed mutagenesis revealed that both seed and supplementary sequences on Site II, not Site I, were required for the targeting of miR-548p on apoB 3'-UTR. Additionally, the cholesterol and fatty acid syntheses rates were decreased in miR-548p overexpressing Huh-7 cells. We also found that Hmgcr and ACSL4, two important enzymes in cholesterol and fatty acid synthesis process, were predicted targets of miR-548p. Indeed, the mRNA and protein levels of Hmgcr and ACSL4 were significantly lowered by miR-548p. The direct targeting of miR-548p on the 3'-UTR of Hmgcr and ACSL4 was studied by luciferase reporter assay. In summary, a novel miR-548p decreased apoB secretion from Huh-7 cells by targeting apoB 3'-UTR and promoting its posttranscriptional degradation. MiR-548p also reduces cholesterol and fatty acid synthesis by down-regulating Hmgcr and ACSL4 expression levels.

In the second approach, I took an unbiased global approach to identify miRs that individually and simultaneously affect apoB and apoA1. I transfected human hepatoma Huh7 cells in duplicate with a library of 1237 miRs and measured apoB and apoA1 in the media. This resulted in the identification of 13 and 28 miRs that increased and decreased apoB secretion. In addition, I identified 11 and 23 miRs that increased and decreased apoA1 secretion. Most interestingly, I identified 3 miRs that simultaneously increased apoB and reduced apoA1 levels. I further characterized miR-1200 and explained mechanisms how it regulates apoB and apoA1 secretion.

Mechanistic studies showed that miR-1200 reduced apoB secretion by lowering the mRNA levels of apoB. In addition, post-transcriptional degradation of apoB was promoted in miR-1200 transfected cells. Bioinformatic analysis revealed apoB as a predicted target of miR-1200. Luciferase reporter assay and site-directed mutagenesis were used to confirm the direct miR-mRNA interaction. In contrast, miR-1200 increased apoAI protein and mRNA levels by increasing its *de novo* transcription. The mechanistic studies resulted in the identification of a novel transcription repressor of apoAI, BCL11B. Therefore, miR-1200 increases the transcription of apoAI by reducing the expression levels of BCL11B, and promotes the secretion of apoAI. I also observed that the overexpression of miR-1200 in Huh-7 cells decreased lipid synthesis, which might avoid the lipid accumulation in the liver. In summary, these studies identified a novel human microRNA-1200 that could potentially reduce apoB-containing lipoproteins and increase HDL production from the liver, as well as decreasing lipid synthesis in human hepatoma cell line. These three different roles of miR-1200 in lipid metabolism regulation suggest that it may have protective function against atherosclerosis.

In short, I have identified two novel miRs-548p and 1200 as potent regulators of apolipoprotein and lipid metabolism in hepatoma cells. Both have the potential to be developed as therapeutic agents for the treatment of dyslipidemia and atherosclerosis while avoiding hepatic steatosis. As these are primate specific miRs, the physiological effect of overexpressing or inhibiting endogenous miR-548p or miR-1200 could be tested in humanized mice or non-human primate models.

Chapter 1

Introduction

I. Dyslipidemia, Lipid and Lipoprotein Metabolism

Cardiovascular diseases (CVD) remain the top killer in western world. In 2010, CVD accounted for 36.7% and 34.0% of deaths in males and females. And the total cost of CVD and stroke for 2010 was about $315.4 billion (1). According to the Centers for Disease Control and Prevention, 31 and 32 percent of males and females in the USA have high LDL-cholesterol levels, the “bad cholesterol”. Clinical studies show that low LDL and elevated HDL-cholesterol levels are inversely correlated with the risk of CVD. Thus, elevated LDL and low HDL are two well-established risk factors for atherosclerosis and CVD. In the guidelines released by American Association of Clinical Endocrinologists for the management of dyslipidemia and prevention of atherosclerosis, the recommended optimal concentrations of plasma lipids are: total cholesterol<200 mg/dL; HDL-cholesterol≥60 mg/dL; LDL-cholesterol< 100 mg/dL; triglyceride <150 mg/dL (2). People with high plasma ApoB/ApoAI or LDL/HDL ratio are associated with high CVD risk (3-5). The apoB/apoAI ratio, the particle number ratio of LDL and HDL, higher than 0.9 is accepted as a high risk indicator of CVD (4).

Lipids are insoluble in water. A majority of lipids are transported in blood circulation as lipid/protein complexes called lipoproteins. Lipoproteins have a hydrophobic lipid core containing triglyceride (TG) and cholesteryl esters. This core is wrapped around by a monolayer of phospholipids. The surface monolayer also harbors free cholesterol and apolipoproteins. Proteins and lipids have different densities. The density of lipoprotein is determined by the lipid/ protein ratio. Lipoproteins can be classified into five classes, according to different buoyant densities: Chylomicron (and Chylomicron remnant)(<0.95g/ml), Very Low-Density Lipoprotein (VLDL)(0.95-1.006g/ml), Intermediate Low-Density Lipoprotein (IDL) (1.006-1.019g/ml), Low-Density Lipoprotein (LDL) (1.019-1.063g/ml) and High-Density Lipoprotein (HDL) (>1.063g/ml). Chylomicron, VLDL, IDL and LDL share the same structural protein, apolipoproteinB (apoB). So they are also called apoB-containing lipoproteins (B-lps). These lipoproteins deliver lipids from the liver and intestine to peripheral tissues, including blood vessels. When plasma concentrations and decay times of these lipoproteins increase, they are modified and taken up by macrophages. The accumulation of lipids in macrophages converts macrophages to foam cells. Hence, these modified lipoproteins are also referred to as "atherogenic lipoproteins" or "bad cholesterol". On the other hand, apoAI is the major protein on HDL. HDL does not contain apoB. HDL is involved in several functions such as reverse cholesterol transport, antioxidant, anti-inflammation, and anti-thrombotic. As several of these functions confer vascular protection, HDL is also called "good cholesterol".

II. Synthesis, Secretion, and Catabolism of ApoB-containing Lipoproteins

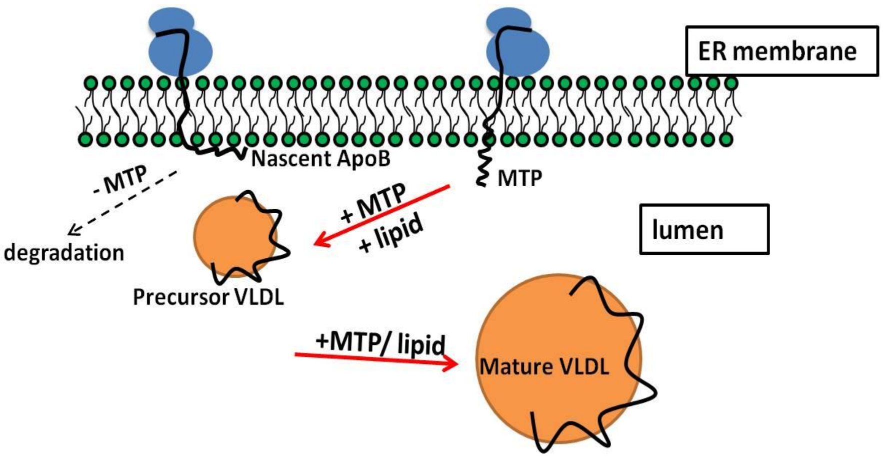

Fig 1. Roles of apoB and MTP in B-lps (VLDL as example) assembly. ApoB and MTP are translated by ribosomes on the rough ER membrane. MTP transfers lipids to nascent apoB peptide to form precursor VLDL. Without MTP, apoB is going to be degraded. Further, MTP transfers more lipids to precursor VLDL, makes it mature VLDL.

ApoB protein has two isoforms in the plasma: apoB48 and apoB100. In humans, apoB48 is exclusively expressed in the small intestine for the assembly for chylomicron; and apoB100 is produced in the liver incorporated in VLDL. ApoB48 and apoB100 are encoded by one apoB gene. ApoB48 is expressed from a posttranscriptional editing of apoB mRNA by apobec-1. Apobec-1 converts the C at the residue 6666 to a U (CAA to UAA), resulting in an early terminating stop codon. In mouse, both apoB48 and apoB100 are secreted from the liver. Mutations in apoB usually result in truncations of apoB peptide, cause hypobetalipoproteinemia, exhibiting low plasma cholesterol, TG, and apoB levels (6, 7).

In humans, a majority of plasma cholesterol is carried in the blood by B-lps. B-lps are mainly synthesized and secreted by the liver and small intestine. Newly translated apoB is lipidated in the endoplasmic reticulum with the help of Microsomal Triglyceride Transfer Protein (MTP) (Fig 1) (8, 9). MTP transfers TG, cholesteryl ester, phospholipids, ceramide, and sphingomyelin to lipid poor nascent apoB protein (8, 10-13). MTP physically interacts with apoB, and acts as a chaperone in B-lps assembly. In the absence of MTP, the apoB peptide is degraded by proteasomes. Loss-of-function mutations of MTP are found in abetalipoproteinemia subjects that have no apoB lipoprotein in plasma (14-16). Chylomicron is formed in the small

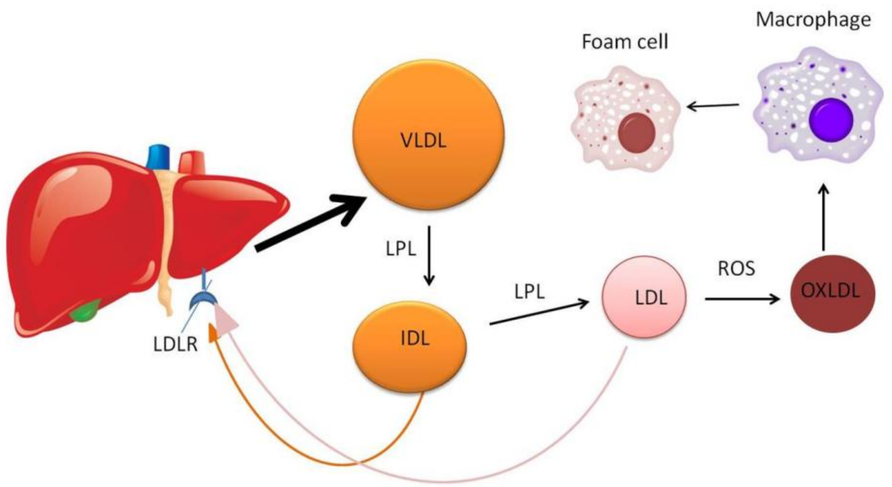

Fig 2. Metabolism of Hepatic B-lps. VLDL is produced by the liver. Lipoprotein lipase hydrolyzes triglyceride in VLDL, converts VLDL into IDL, and IDL is further converted to LDL. Both LDL and IDL can be taken up by the liver via LDLR. Once LDL is oxidized by reactive oxygen species in plasma, ox-LDL is taken up by macrophages via scavenger receptor. Accumulation of lipids in macrophage induces foam cell formation.

intestine to transport dietary fat. VLDL is produced from the liver to deliver endogenous lipids to the peripheral tissues. Lipoprotein Lipase (LPL) in the circulation hydrolyzes TG in TG-rich VLDL and converts these lipoproteins to denser particles IDL and further to LDL (Fig 2). Free fatty acid generated from lipolysis of TG-rich B-lps is mainly carried by albumin in plasma (17). LDL Receptor (LDLR) localized on cell surface recognizes apoB and apoE on B-lps and takes up these lipoproteins. Internalized apoB is directed to lysosomes for degradation and LDLR is recycled to cell surface. Some of the LDL particles get oxidized if not removed faster by hepatocytes. The modified LDL is taken up by macrophages via SR-BI.

III. Regulation of VLDL Production

Several transcription factors of apoB have been identified including: HNF4α, BRF-2, ARP-1, EAR-3, C/EBPA, and P53 (18-20). As one of the well-studied transcription factors in lipid metabolism regulation, HNF4α is a member of nuclear receptor family, highly expressed in the liver. HNF4α binds to promoter regions of both apoB and MTP to activate their transcription and regulates B-lps production (21). Secretion of B-lps can also be modulated by lipid availability (22-26). A majority of synthesized apoB peptides are degraded intracellularly before secretion from hepatocytes. Lipids availability regulates assembly and secretion of B-lps via inhibiting intracellular degradation of nascent apoB. The rate of apoB secretion from human hepatoma cells can be stimulated by incubating cells with oleic acid and inhibiting the proteasomal degradation of apoB (23-27). Omega-3 and omega-6 polyunsaturated fatty acids lower plasma lipid levels by enhancing post-ER presecretory proteolysis to degrade apoB peptides (28, 29). ER-stress induced autophagy may also promote posttranslational degradation of apoB (30). Insulin is another potent regulator of VLDL production (31). Insulin does not change apoB mRNA levels. Insulin inhibits apoB translation by inducing intracellular trafficking of apoB mRNA to P-bodies (32, 33). Meanwhile, insulin also activates Akt to inhibit FoxO1, a transcription activator of MTP. Therefore, insulin induces apoB protein degradation by reducing expression of MTP (34).

IV. Synthesis, and Catabolism of HDL

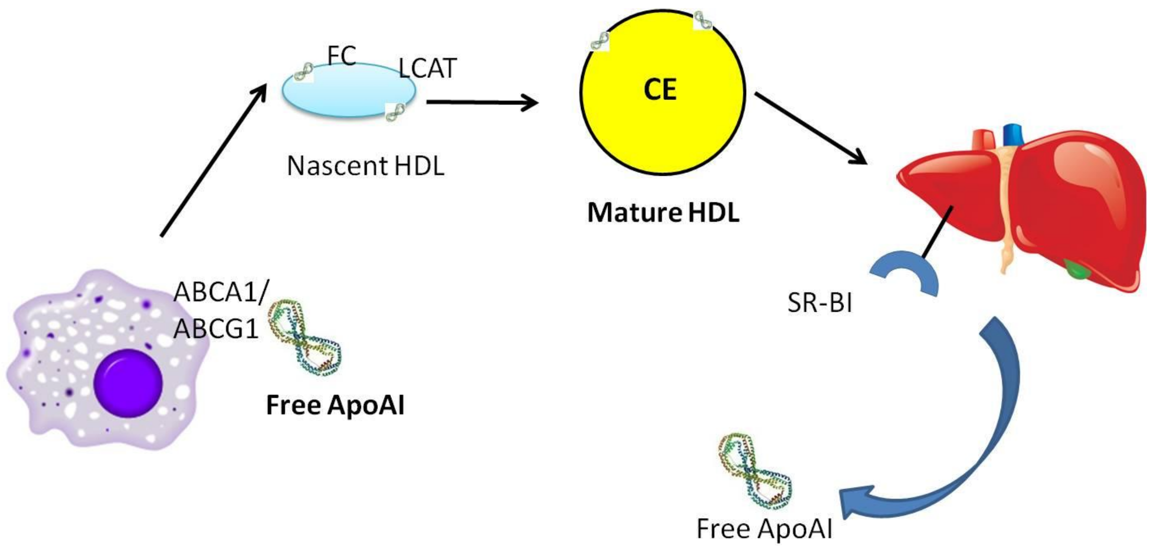

Fig 3. HDL biogenesis and Reverse Cholesterol Transport. Lipid free ApoAI extracts cholesterol from peripheral tissues and becomes nascent HDL. LCAT in HDL esterifies cholesterol to cholesteryl esters and converts nascent HDL to mature HDL. Mature HDL is recognized by SR-BI in the liver, cholesteryl ester in HDL is selectively taken up by the liver and HDL particles are returned to plasma

Similar to B-lps, HDL is produced by the liver and small intestine, however via a different pathway. ApoAI is synthesized by hepatocytes and enterocytes, and secreted in a lipid poor form. It recruits lipids such as phospholipids and free cholesterol via interaction with ATP-binding cassette transporter, sub-family A member 1 (ABCA1) on the plasma membrane of different cells, forming a nascent HDL particle (Fig 3). Free cholesterol in nascent HDL is esterified by an enzyme, Lecithin-Cholesterol Acyltransferase (LCAT) to cholesteryl ester. Mature HDL contains phospholipids, cholesterol, cholesteryl ester and two to four apoAI proteins. Mature HDL interacts with ABCG1 to gain more lipids (35). After this, HDL delivers cholesterol to the liver by delivering cholesteryl ester via interacting with Scavenger receptor class B member 1 (SR-BI) on the surface of hepatocytes. In this selective uptake process, cholesterol ester is taken up by cells and apoAI is recycled for further rounds of HDL biosynthesis. Small lipid-poor apoAI is excreted by the kidney.

Besides this synthetic pathway, HDL is also formed as a by-product of the lipolysis of B-lps. As described before, Lipoprotein Lipase (LPL) hydrolyzes TG in apoB-lipoproteins. LPL deficiency has been identified in human (36). Impaired LPL activity leads to extremely high chylomicron and low HDL concentrations in plasma (36, 37). During TG hydrolysis in B-lps, some of the surface cholesterol and phospholipids can peel off from the surface with apolipoproteins to form HDL. In the absence of LPL, the synthesis of HDL via this pathway might be impaired.

V. Reverse Cholesterol Transport

ApoAI and HDL can efflux lipids especially cholesterol from the peripheral cells such as macrophages after interacting with ABCA1 and ABCG1 (Fig 3). The cholesterol is then delivered to the liver. Via this process, cholesterol is transported from peripheral tissues back to the liver and eventually excreted in the feces (38, 39). This process is referred to as reverse cholesterol transport (RCT), and is believed to be the major anti-atherosclerotic function of HDL (39). The main purpose of this RCT is to bring cholesterol from peripheral tissues back to the liver for excretion from the body. Loss-of-function mutations in ABCA1 gene induce HDL deficiency, Tangier disease (40). Patients with Tangier disease usually have premature CVD, due to the absence of HDL biosynthesis and low RCT.

VI. Modified Lipoproteins

From the observation that incubating macrophages with ox-LDL, but not native LDL, can cause cellular cholesteryl ester accumulation, oxidative modified lipoproteins have caught peoples’ attention (41, 42). Oxidative modification on LDL creates a negative net charge, which can be recognized by SR-BI for uptake by macrophages. Both lipids and apoB amino acids in LDL undergo oxidative modification during oxidation of LDL. It is well accepted that the uptake of ox-LDL by macrophages contributes to foam cell formation, which is the initiation of atherosclerosis. Although researchers have reported that minimally oxidized LDL produced from short time exposure to oxidants may have protective function to cholesteryl ester uptake and foam cell formation (43). Therefore the use of antioxidant, such as vitamin D and E has been tested in different animal models and patient cohorts showing beneficial effect in preventing cardiovascular diseases (44). Oxidative modification is not limited to LDL. Modified HDL is also appreciated for its impaired anti-atherogenic function (45-47). Nitrated ApoAI is enriched in human atherosclerotic lesions, even though its circulating levels are very low. In addition, apoAI recovered from plaques shows impaired cholesterol efflux potency. ApoAI and Myeloperoxidase (MPO), as well as paraoxonase 1 (PON1), form a ternary complex on HDL particle. MPO can site-specifically oxidize apoAI to lower its anti-atherosclerotic function in cholesterol efflux, anti-inflammation, or anti-oxidant. Meanwhile, PON1 inhibits the activity of MPO and protects apoAI from oxidative stress (48). Therefore, not only the levels of LDL and HDL levels in plasma should be considered as factors of CVD, the modified forms of lipoproteins are also very important and informatics parameters for the functionality of lipoprotein in the circulation.

VII. Atherosclerosis

Atherosclerosis is the build-up of lipids in the inner wall of blood vessels. There are several known risk factors of atherosclerosis such as high plasma cholesterol, hypertension, diabetes mellitus, cigarette smoking, and obesity. Excess accumulation of oxidized B-lps in the plasma, such as oxidized LDL (ox-LDL), contribute to atherosclerosis as these modified lipoproteins are taken up by macrophages (47, 49, 50). It is believed that atherosclerosis initiates after the trapping of LDL in the sub-endothelial matrix (51, 52). LDL can bind to the proteoglycans present in the intima, where it gets trapped, oxidized by reactive oxygen species (ROS) or aggregated. Oxidatively modified and aggregated LDL can induce native and adaptive immune response to recruit monocyte, increase the production and secretion of growth factors and proinflammatory cytokines from vascular smooth muscle and endothelial cells, which further causes the proliferation and differentiation of macrophages. Activated macrophages recognize and take up more modified lipoproteins by scavenger receptors expressed on their surfaces. Uptake of ox-LDL leads to the formation of foam cells and is a critical step in the initiation of atherosclerosis. Over time, fats, cholesterol, and calcium accumulate in the lesion leading to increases in plaque burden, narrowing of the vessels, thickening of the artery walls, reductions in blood flow and reduced oxygen supply to the heart muscle. A sudden rapture of plaques causes heart attack or stroke.

VIII. Therapies for Hyperlipidemia and Atherosclerosis

Most therapies focus on lowering plasma cholesterol levels because elevated plasma cholesterol is the greatest risk factor for atherosclerosis. Statins, the most widely prescribed therapeutic drugs, lower plasma cholesterol by reducing hepatic cholesterol synthesis and increasing the expression levels of LDLr to promote LDL clearance; however these drugs only decrease CVD events by 30-40% (53), and almost 20% of the populations do not respond or cannot tolerate statins (54, 55). High doses of statins in some cases cause unmanageable side effects, such as muscle pain, elevated levels of liver function enzymes, and new onset of diabetes mellitus (55, 56). Homozygous familial hypercholesterolemia (HoFH) patients have very high plasma cholesterol levels due to the absence of LDLr expression. In these patients, the expression levels of LDLr cannot be upregulated, therefore, statins are not efficacious in these patients (57, 58).

PCSK9 (proprotein convertase subtilisin/kexin type 9) antibodies have also emerged as potential drugs for hyperlipidemia. PCSK9 is a critical regulator of LDLr expression. It binds to LDLr and targets LDLr for intracellular degradation in lysosomes. Therefore, inhibition of PCSK9 leads to reduced hepatic LDLr degradation, increased LDLr expression, increased clearance of B-lps, and decreased plasma LDL concentrations. Several clinical trials have shown that PCSK9 antibodies effectively reduce LDL cholesterol and cardiovascular events in hypercholesterolemia, heterozygous familial hypercholesterolemia, and statin intolerant patients (59-61). Evolocumab, a PCSK9 antibody that has been recently Food and Drug Administration (FDA) approved, reduces LDL-cholesterol by greater than 50% in statin intolerant patients (59). Other studies have shown that evolocumab can decrease plasma cholesterol by as much as 75%. The long term efficacy and tolerability of PCSK9 still needs to be evaluated. Further, PCSK9 inhibitors are not very effective in homozygous familial hypercholesterolemic patients because they do not have functional LDLr. In addition, neurocognitive adverse events, such as memory lost and confusion, may be potentially induced by PCSK9 inhibition (60).

MTP has been a favored target for treatment of high plasma cholesterol for decades. Since MTP is critical for the biosynthesis of B-lps, MTP inhibitors lower plasma cholesterol by blocking B-lps synthesis and secretion (62). One of the MTP inhibitors, lomitapide, has recently been approved by the FDA for the treatment of HoFH patients. However, because MTP inhibitors induce lipid accumulation in the liver, hepatic steatosis, it cannot be used as a common treatment modality for hyperlipidemia (62).

As apoB is required for B-lps assembly, downregulation of apoB is also being used as a strategy in lowering plasma lipids. Mipomersen (an antisense oligonucleotide of apoB) has also been recently approved by FDA for treating HoFH (63, 64). In recent phase II and phase III clinical trial studies, mipomersen reduced LDL cholesterol levels by 25 to 47% (65). However, similar to the drawbacks of MTP inhibitors, long term treatment of mipomersen causes hepatic steatosis. Therefore, the administration of mipomerson is limited to homozygous FH or heterozygous FH patients who don't respond to statins.

Many attempts have been made to identify a therapy that could increase plasma HDL and reduce atherosclerotic burden. However, so far, no effective therapeutic agents are available to increase functional HDL to prevent CVD events (66). CETP has been studied as a potential drug target. Inhibition of CETP increases HDL and decreases LDL-cholesterol in rodents, nonhuman primates, and humans (67-71). The strategy of inhibiting CETP to differentially regulate HDL and LDL fractions was considered after identification and characterization of CETP deficiency in a population of Japanese people with extremely high HDL levels and low CVD risk (72, 73). However, in a phase III clinical trial with over 15,000 people with high risk of CVD, no significant differences were observed between placebo and the torcetrapid, a CETP inhibitor, treatment groups in terms of atherosclerosis plaque burden, even though the torcetrapid significantly reduced LDL and increased HDL cholesterol levels (74). Failure in the clinical trial of CETP inhibitors raised the question about whether higher plasma concentration of HDL is sufficient to prevent CVD events (75, 76). A recent study reported a loss-of-function variant in SCARB1, the gene encoding SR-BI, identified from a human cohort with extremely high HDL cholesterol levels (77). This variant impairs cholesteryl ester uptake of HDL to hepatocytes. People carrying this variant have significantly high HDL and increased CVD risk. Also, depletion of SR-BI in apoE null mice results in early onsite of atherosclerosis compared to the control apoE null mice, even though these double knockout mice show higher apoAI levels in plasma (78). Therefore, it seems like the HDL concentration in plasma is not indicating the function of HDL in protecting atherosclerosis burden. If the HDL particles are not able to carry cholesterol to flux out of the body, then the high HDL is not sufficient to decrease CVD risk.

Thus, there is a need to find novel therapeutic agents to decrease LDL and increase HDL-cholesterol and reduce atherosclerosis without causing adverse effects.

IX. MicroRNAs Biosynthesis and Mode of Action

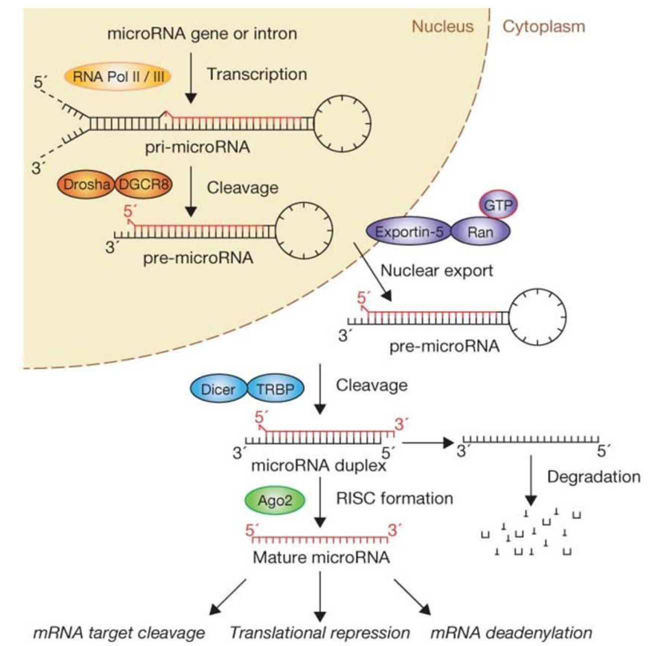

Fig 4. Biosynthesis of microRNAs and the mechanisms of microRNA regulation of gene expression. Pri-miR is transcribed in the nucleas from independent genes or the introns of host mRNA. Drosha cleave the long RNA into shorter pre-miRs into mature miRs. RISK complex helps miRs loading onto 3'-UTR of target mRNAs, which either cause mRNA degradation or stop translation. Graph adapted from "Winter, J., et al., *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nat Cell Biol, 2009. **11**(3): p. 228-34."

MicroRNAs (miRs) are a class of small (~22 nucleotides) non-coding RNAs, which are widely accepted as negative regulators of gene expression. Primary miRs (pri-miRs) are transcribed by RNA polymerase II or III from introns of host genes or their own genes in the nucleus. Primary miRs are the earliest and longest transcripts (Fig 4). Drosha, an enzyme with nuclease activity can co-transcriptionally cleave the hair-pin structure in pri-miRs to produce precursor pre-miRs (79). An interesting recent study about pri-miRs in plants showed that pri-miR contains short open reading frame and encodes for short peptide, which can regulate the expression of miR (80, 81). The pre-miRs are transported by Exportin-5 into the cytoplasm. Cytosolic enzyme Dicer cuts pre-miRs producing the double stranded mature miRs. The RNA-induced silencing complex (RISC) in the cytoplasm helps mature single stranded miRs to interact with target mRNAs, and one of the proteins in the complex Ago-2 will cleave the mRNA (82). Usually miRs regulate gene expression by binding to 3′-UTR of target mRNA, and then inducing posttranscriptional degradation or blocking translation machinery (83). There are also some reports about miR recognizing gene regions outside of 3′-UTR, such as promoter and 5′-UTR (84-86). In most of the mammalian cases (higher than 70%), miR mediated regulation is via mRNA degradation (87). MiRs regulate multiple target genes in diverse biological pathways, as they bind to the target mRNA via seed and supplementary sequences. Seed sequence, present at 2-7 nucleotides from the 5´-end of the miR, forms perfect complementary base pairs with the target mRNA (88). The supplementary site in the 3´-region may or may not form perfect base pairs with target mRNA (89, 90). MiRs with same seed sequence belong to the same family (91).

After more than a decade of research, two miR-based drugs, anti-miR-122 (treatment of HCV) and miR-34 mimic (anti-cancer) are in phase II clinical trials (92, 93). It is likely that discovery of new miRs and their roles in the regulation of different biological pathways may lead to new therapeutic treatments.

X. MiRs Involved in Lipid Metabolism

Several miRs have been identified as potent lipid metabolism regulators. MiR-122 was first identified as the most abundant liver-specific miR, which accounts for more than 70% of miRs in the liver (94). MiR-122 has been shown to play a role in cholesterol and TG synthesis in the liver by several independent groups (95-98). *In vivo* treatment of mice with anti-miR-122 significantly reduced plasma cholesterol in both HDL and LDL fractions (97, 98). The hepatic cholesterol and fatty acid synthesis was also decreased by inhibition of miR-122. In non-human primate animal model, similar effects were observed that anti-miR-122 reduced cholesterol levels in both HDL and LDL (99). Liver specific and global miR-122 knockout mice reproduced the same phenotype with anti-miR, which confirmed the role of miR-122 in regulating plasma cholesterol and hepatic lipid synthesis (95, 96). The function of miR-30c in lipid metabolism has been identified previously in our lab (90, 100). In Western diet fed hyperlipidemic mice, overexpression of miR-30c reduces hepatic MTP activity and TG synthesis to reduce VLDL production resulting in lower plasma LDL without causing lipid accumulation in the liver. In *Apoe*$^{-/-}$ mice, overexpression of miR-30c reduces plasma cholesterol levels and atherosclerosis plaque burden (100). In this case, overexpression of miR-30c achieves the plasma cholesterol lowering goal, and overcomes the adverse affects of MTP inhibitors by suppressing lipid synthesis in the liver (100, 101). Another well-studied HDL regulating miR is miR-33a. MiR-33a negatively regulates of ABCA1 and ABCG1 expression. Inhibition of miR-33 can increase plasma HDL-cholesterol by 20% and promote reverse cholesterol transport to reduce atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice (102-104). Moreover, miR-33a has also been shown to modulate fatty acid oxidation and insulin signaling by targeting genes involved in fatty acid β-oxidation and insulin signaling mediator (105, 106). MiR can also target important transcription factors to alter lipoprotein assembly and plasma lipid levels. MiR-34 reduces HNF4α, a transcription activator of both MTP and apoB, to diminish the production of B-lps (107). However, the inhibition of VLDL secretion causes hepatic steatosis. More detailed information was summarized in a recently published review about miRs that regulate apoB-containing lipoprotein assembly and secretion (108). Very recently, miR-148a was identified from a LDL receptor activity screening. In addition, it can also reduce the expression levels of ABCA1. More importantly, inhibition of endogenous expressed miR-148a resulted in decreased LDL and increased HDL in mouse model (109). Therefore, identification and characterization of novel miRs may result in the development of new therapeutic agents for CVD.

XI. Present Study and Hypothesis

Hyperlipidemia is one of the major risk factors of atherosclerosis. High plasma LDL and low plasma HDL cholesterol levels are two risk factors for CVD. A preferred strategy of managing dyslipidemia and atherosclerosis is to lower LDL and increase HDL in plasma. Statins, lomitapide and mipomersen are available to reduce plasma LDL. However, a significant subpopulation does not respond or is intolerant to statins (55). Lomitapide and mipomersen induce hepatic steatosis and increase plasma transaminases (110). So far, there is no effective therapeutic agent to increase functional HDL to reduce cardiovascular events. Further, no effective agent that concomitantly modulates plasma LDL and HDL is available at this time. MiR-30c and miR-33 decrease LDL and HDL, respectively. However, miRs that concurrently decrease LDL and increase HDL are not known.

Previously, our lab identified miR-30c, which could potently reduce LDL cholesterol levels in hyperlipidemic mice and atherosclerosis in apoE deficient mice by targeting MTP mRNA. In addition, apoB ASO has been used in treating HoFH patients by enhancing posttranscriptional degradation of apoB mRNA. In Chapter 2, I first hypothesized that miRs could potentially interact with the 3'-UTR of apoB mRNA might have the capacity to reduce apoB-containing lipoproteins secretion from liver cells. ApoB is predicted as a well conserved target of miR-548p in TargetScan. I hypothesized that miR-548p reduced apoB secretion by promoting its mRNA degradation, and the predicted interacting sites were contributing the targeting of miR-548p on apoB 3'-UTR.

In Chapter 3, I hypothesized that miRs modulating apoB or apoAI secretion could be identified from a genome-wide functional screening in human hepatoma cell line. MiR-1200 is identified from the screening for its role of differentially regulating apoB and apoAI secretion simultaneously. I hypothesized that miR-1200 modulates apoB and apoAI secretion by two independent mechanisms. It reduces apoB secretion by targeting apoB mRNA and increased apoAI by reducing a repressor.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation.* 2014;129(3):e28-e292.
2. Jellinger PS, Smith DA, Mehta AE, Ganda O, Handelsman Y, Rodbard HW, Shepherd MD, Seibel JA, Dyslipidemia ATFfMo, and Prevention of A. American Association of Clinical Endocrinologists' Guidelines for Management of Dyslipidemia and Prevention of Atherosclerosis. *Endocr Pract.* 2012;18 Suppl 1(1-78.
3. Panayiotou A, Griffin M, Georgiou N, Bond D, Tyllis T, Tziakouri-Shiakalli C, Fessas C, and Nicolaides A. ApoB/ApoA1 ratio and subclinical atherosclerosis. *Int Angiol.* 2008;27(1):74-80.
4. Kaneva AM, Potolitsyna NN, Bojko ER, and Odland JO. The apolipoprotein B/apolipoprotein A-I ratio as a potential marker of plasma atherogenicity. *Dis Markers.* 2015;2015(591454.
5. Lemieux I, Lamarche B, Couillard C, Pascot A, Cantin B, Bergeron J, Dagenais GR, and Despres JP. Total cholesterol/HDL cholesterol ratio vs LDL cholesterol/HDL cholesterol

ratio as indices of ischemic heart disease risk in men: the Quebec Cardiovascular Study. *Arch Intern Med.* 2001;161(22):2685-92.

6. Miller SA, Hooper AJ, Mantiri GA, Marais D, Tanyanyiwa DM, McKnight J, and Burnett JR. Novel APOB missense variants, A224T and V925L, in a black South African woman with marked hypocholesterolemia. *J Clin Lipidol.* 2016;10(3):604-9.
7. Hooper AJ, Robertson K, Champain D, Hua J, Song S, Parhofer KG, Barrett PH, van Bockxmeer FM, and Burnett JR. Lipoprotein metabolism in an apoB-80 familial hypobetalipoproteinemia heterozygote. *Clin Biochem.* 2016;49(9):720-2.
8. Hussain MM, Rava P, Walsh M, Rana M, and Iqbal J. Multiple functions of microsomal triglyceride transfer protein. *Nutr Metab (Lond).* 2012;9(14.
9. Hussain MM, Shi J, and Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *Journal of lipid research.* 2003;44(1):22-32.
10. Walsh MT, and Hussain MM. Targeting microsomal triglyceride transfer protein and lipoprotein assembly to treat homozygous familial hypercholesterolemia. *Crit Rev Clin Lab Sci.* 2016:1-23.
11. Walsh MT, Di Leo E, Okur I, Tarugi P, and Hussain MM. Structure-function analyses of microsomal triglyceride transfer protein missense mutations in abetalipoproteinemia and hypobetalipoproteinemia subjects. *Biochim Biophys Acta.* 2016;1861(11):1623-33.
12. Khatun I, Zeissig S, Iqbal J, Wang M, Curiel D, Shelness GS, Blumberg RS, and Hussain MM. Phospholipid transfer activity of microsomal triglyceride transfer protein produces apolipoprotein B and reduces hepatosteatosis while maintaining low plasma lipids in mice. *Hepatology.* 2012;55(5):1356-68.
13. Iqbal J, Walsh MT, Hammad SM, Cuchel M, Tarugi P, Hegele RA, Davidson NO, Rader DJ, Klein RL, and Hussain MM. Microsomal Triglyceride Transfer Protein Transfers and Determines Plasma Concentrations of Ceramide and Sphingomyelin but Not Glycosylceramide. *J Biol Chem.* 2015;290(43):25863-75.
14. Khatun I, Walsh MT, and Hussain MM. Loss of both phospholipid and triglyceride transfer activities of microsomal triglyceride transfer protein in abetalipoproteinemia. *J Lipid Res.* 2013;54(6):1541-9.
15. Welty FK. Hypobetalipoproteinemia and abetalipoproteinemia. *Curr Opin Lipidol.* 2014;25(3):161-8.
16. Walsh MT, Iqbal J, Josekutty J, Soh J, Di Leo E, Ozaydin E, Gunduz M, Tarugi P, and Hussain MM. Novel Abetalipoproteinemia Missense Mutation Highlights the Importance

of the N-Terminal beta-Barrel in Microsomal Triglyceride Transfer Protein Function. *Circ Cardiovasc Genet.* 2015;8(5):677-87.

17. Chung BH, Tallis GA, Cho BH, Segrest JP, and Henkin Y. Lipolysis-induced partitioning of free fatty acids to lipoproteins: effect on the biological properties of free fatty acids. *J Lipid Res.* 1995;36(9):1956-70.
18. Ladias JA, Hadzopoulou-Cladaras M, Kardassis D, Cardot P, Cheng J, Zannis V, and Cladaras C. Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3. *J Biol Chem.* 1992;267(22):15849-60.
19. Zhuang H, Chuang SS, and Das HK. Transcriptional regulation of the apolipoprotein B100 gene: purification and characterization of trans-acting factor BRF-2. *Mol Cell Biol.* 1992;12(7):3183-91.
20. Novak EM, Dantas KC, Charbel CE, and Bydlowski SP. Association of hepatic nuclear factor-4 in the apolipoprotein B promoter: a preliminary report. *Braz J Med Biol Res.* 1998;31(11):1405-8.
21. Hayhurst GP, Lee YH, Lambert G, Ward JM, and Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol.* 2001;21(4):1393-403.
22. Fisher EA, and Ginsberg HN. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J Biol Chem.* 2002;277(20):17377-80.
23. Dixon JL, Furukawa S, and Ginsberg HN. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *The Journal of biological chemistry.* 1991;266(8):5080-6.
24. Davis RA, and Boogaerts JR. Intrahepatic assembly of very low density lipoproteins. Effect of fatty acids on triacylglycerol and apolipoprotein synthesis. *The Journal of biological chemistry.* 1982;257(18):10908-13.
25. Bostrom K, Wettesten M, Boren J, Bondjers G, Wiklund O, and Olofsson SO. Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. *The Journal of biological chemistry.* 1986;261(29):13800-6.
26. Borchardt RA, and Davis RA. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *The Journal of biological chemistry.* 1987;262(34):16394-402.

27. Moberly JB, Cole TG, Alpers DH, and Schonfeld G. Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. *Biochimica et biophysica acta.* 1990;1042(1):70-80.
28. Pan M, Cederbaum AI, Zhang YL, Ginsberg HN, Williams KJ, and Fisher EA. Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL production. *J Clin Invest.* 2004;113(9):1277-87.
29. Fisher EA, Pan M, Chen X, Wu X, Wang H, Jamil H, Sparks JD, and Williams KJ. The triple threat to nascent apolipoprotein B. Evidence for multiple, distinct degradative pathways. *J Biol Chem.* 2001;276(30):27855-63.
30. Ron D, and Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol.* 2007;8(7):519-29.
31. Haas ME, Attie AD, and Biddinger SB. The regulation of ApoB metabolism by insulin. *Trends Endocrinol Metab.* 2013;24(8):391-7.
32. Karimian Pour N, and Adeli K. Insulin silences apolipoprotein B mRNA translation by inducing intracellular traffic into cytoplasmic RNA granules. *Biochemistry.* 2011;50(32):6942-50.
33. Parker R, and Sheth U. P bodies and the control of mRNA translation and degradation. *Mol Cell.* 2007;25(5):635-46.
34. Kamagate A, Qu S, Perdomo G, Su D, Kim DH, Slusher S, Meseck M, and Dong HH. FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice. *J Clin Invest.* 2008;118(6):2347-64.
35. Kennedy MA, Barrera GC, Nakamura K, Baldan A, Tarr P, Fishbein MC, Frank J, Francone OL, and Edwards PA. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell metabolism.* 2005;1(2):121-31.
36. Reymer PW, Gagne E, Groenemeyer BE, Zhang H, Forsyth I, Jansen H, Seidell JC, Kromhout D, Lie KE, Kastelein J, et al. A lipoprotein lipase mutation (Asn291Ser) is associated with reduced HDL cholesterol levels in premature atherosclerosis. *Nat Genet.* 1995;10(1):28-34.
37. Clee SM, Zhang H, Bissada N, Miao L, Ehrenborg E, Benlian P, Shen GX, Angel A, LeBoeuf RC, and Hayden MR. Relationship between lipoprotein lipase and high density lipoprotein cholesterol in mice: modulation by cholesteryl ester transfer protein and dietary status. *J Lipid Res.* 1997;38(10):2079-89.

38. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, Neeland IJ, Yuhanna IS, Rader DR, de Lemos JA, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med.* 2014;371(25):2383-93.
39. Kingwell BA, Chapman MJ, Kontush A, and Miller NE. HDL-targeted therapies: progress, failures and future. *Nature reviews Drug discovery.* 2014;13(6):445-64.
40. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, et al. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet.* 1999;22(4):352-5.
41. Quinn MT, Parthasarathy S, Fong LG, and Steinberg D. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci U S A.* 1987;84(9):2995-8.
42. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, and Steinberg D. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci U S A.* 1984;81(12):3883-7.
43. Meyer JM, Ji A, Cai L, and van der Westhuyzen DR. Minimally oxidized LDL inhibits macrophage selective cholesteryl ester uptake and native LDL-induced foam cell formation. *J Lipid Res.* 2014;55(8):1648-56.
44. Cherubini A, Vigna GB, Zuliani G, Ruggiero C, Senin U, and Fellin R. Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Des.* 2005;11(16):2017-32.
45. DiDonato JA, Aulak K, Huang Y, Wagner M, Gerstenecker G, Topbas C, Gogonea V, DiDonato AJ, Tang WH, Mehl RA, et al. Site-specific nitration of apolipoprotein A-I at tyrosine 166 is both abundant within human atherosclerotic plaque and dysfunctional. *J Biol Chem.* 2014;289(15):10276-92.
46. Undurti A, Huang Y, Lupica JA, Smith JD, DiDonato JA, and Hazen SL. Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *J Biol Chem.* 2009;284(45):30825-35.
47. Huang Y, DiDonato JA, Levison BS, Schmitt D, Li L, Wu Y, Buffa J, Kim T, Gerstenecker GS, Gu X, et al. An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nat Med.* 2014;20(2):193-203.

48. Huang Y, Wu Z, Riwanto M, Gao S, Levison BS, Gu X, Fu X, Wagner MA, Besler C, Gerstenecker G, et al. Myeloperoxidase, paraoxonase-1, and HDL form a functional ternary complex. *J Clin Invest.* 2013;123(9):3815-28.
49. Trpkovic A, Resanovic I, Stanimirovic J, Radak D, Mousa SA, Cenic-Milosevic D, Jevremovic D, and Isenovic ER. Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases. *Critical reviews in clinical laboratory sciences.* 2014:1-16.
50. Moore KJ, Sheedy FJ, and Fisher EA. Macrophages in atherosclerosis: a dynamic balance. *Nature reviews Immunology.* 2013;13(10):709-21.
51. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999;340(2):115-26.
52. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;362(6423):801-9.
53. Gu Q, Paulose-Ram R, Burt VL, and Kit BK. Prescription cholesterol-lowering medication use in adults aged 40 and over: United States, 2003-2012. *NCHS data brief.* 2014177):1-8.
54. Finkel JB, and Duffy D. 2013 ACC/AHA cholesterol treatment guideline: Paradigm shifts in managing atherosclerotic cardiovascular disease risk. *Trends in cardiovascular medicine.* 2014.
55. Cornier MA, and Eckel RH. Non-traditional dosing of statins in statin-intolerant patients-is it worth a try? *Current atherosclerosis reports.* 2015;17(2):475.
56. Finkel JB, and Duffy D. 2013 ACC/AHA cholesterol treatment guideline: Paradigm shifts in managing atherosclerotic cardiovascular disease risk. *Trends Cardiovasc Med.* 2015;25(4):340-7.
57. Mohrschladt MF, Westendorp RG, Gevers Leuven JA, and Smelt AH. Cardiovascular disease and mortality in statin-treated patients with familial hypercholesterolemia. *Atherosclerosis.* 2004;172(2):329-35.
58. Raper A, Kolansky DM, and Cuchel M. Treatment of familial hypercholesterolemia: is there a need beyond statin therapy? *Current atherosclerosis reports.* 2012;14(1):11-6.
59. Stroes E, Colquhoun D, Sullivan D, Civeira F, Rosenson RS, Watts GF, Bruckert E, Cho L, Dent R, Knusel B, et al. Anti-PCSK9 antibody effectively lowers cholesterol in patients with statin intolerance: the GAUSS-2 randomized, placebo-controlled phase 3 clinical trial of evolocumab. *J Am Coll Cardiol.* 2014;63(23):2541-8.
60. Raal FJ, Stein EA, Dufour R, Turner T, Civeira F, Burgess L, Langslet G, Scott R, Olsson AG, Sullivan D, et al. PCSK9 inhibition with evolocumab (AMG 145) in heterozygous familial

hypercholesterolaemia (RUTHERFORD-2): a randomised, double-blind, placebo-controlled trial. *Lancet.* 2015;385(9965):331-40.

61. Robinson JG, Farnier M, Krempf M, Bergeron J, Luc G, Averna M, Stroes ES, Langslet G, Raal FJ, El Shahawy M, et al. Efficacy and safety of alirocumab in reducing lipids and cardiovascular events. *The New England journal of medicine.* 2015;372(16):1489-99.
62. Cuchel M, Blom DJ, and Averna MR. Clinical experience of lomitapide therapy in patients with homozygous familial hypercholesterolaemia. *Atherosclerosis Supplements.* 2014;15(2):33-45.
63. Bell DA, Hooper AJ, and Burnett JR. Mipomersen, an antisense apolipoprotein B synthesis inhibitor. *Expert Opin Investig Drugs.* 2011;20(2):265-72.
64. Toth PP. Emerging LDL therapies: Mipomersen-antisense oligonucleotide therapy in the management of hypercholesterolemia. *J Clin Lipidol.* 2013;7(3 Suppl):S6-10.
65. Gebhard C, Huard G, Kritikou EA, and Tardif JC. Apolipoprotein B antisense inhibition--update on mipomersen. *Curr Pharm Des.* 2013;19(17):3132-42.
66. Barter PJ, Caulfield M, Eriksson M, Grundy SM, Kastelein JJ, Komajda M, Lopez-Sendon J, Mosca L, Tardif JC, Waters DD, et al. Effects of torcetrapib in patients at high risk for coronary events. *The New England journal of medicine.* 2007;357(21):2109-22.
67. Okamoto H, Yonemori F, Wakitani K, Minowa T, Maeda K, and Shinkai H. A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature.* 2000;406(6792):203-7.
68. Zak Z, Gautier T, Dumont L, Masson D, Deckert V, Duverneuil L, Pais De Barros JP, Le Guern N, Schneider M, Moulin P, et al. Effect of cholesteryl ester transfer protein (CETP) expression on diet-induced hyperlipidemias in transgenic rats. *Atherosclerosis.* 2005;178(2):279-86.
69. Briand F, Thieblemont Q, Andre A, Ouguerram K, and Sulpice T. CETP inhibitor torcetrapib promotes reverse cholesterol transport in obese insulin-resistant CETP-ApoB100 transgenic mice. *Clin Transl Sci.* 2011;4(6):414-20.
70. Parini P, and Rudel LL. Is there a need for cholesteryl ester transfer protein inhibition? *Arteriosclerosis, thrombosis, and vascular biology.* 2003;23(3):374-5.
71. Mohammadpour AH, and Akhlaghi F. Future of cholesteryl ester transfer protein (CETP) inhibitors: a pharmacological perspective. *Clin Pharmacokinet.* 2013;52(8):615-26.

72. Brown ML, Inazu A, Hesler CB, Agellon LB, Mann C, Whitlock ME, Marcel YL, Milne RW, Koizumi J, Mabuchi H, et al. Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. *Nature.* 1989;342(6248):448-51.
73. Inazu A, Jiang XC, Haraki T, Yagi K, Kamon N, Koizumi J, Mabuchi H, Takeda R, Takata K, Moriyama Y, et al. Genetic cholesteryl ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. *J Clin Invest.* 1994;94(5):1872-82.
74. Tall AR. CETP inhibitors to increase HDL cholesterol levels. *The New England journal of medicine.* 2007;356(13):1364-6.
75. Tall AR, Yvan-Charvet L, and Wang N. The failure of torcetrapib: was it the molecule or the mechanism? *Arterioscler Thromb Vasc Biol.* 2007;27(2):257-60.
76. Sheridan C. CETP inhibitors boost 'good' cholesterol to no avail. *Nat Biotechnol.* 2016;34(1):5-6.
77. Zanoni P, Khetarpal SA, Larach DB, Hancock-Cerutti WF, Millar JS, Cuchel M, DerOhannessian S, Kontush A, Surendran P, Saleheen D, et al. Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease. *Science.* 2016;351(6278):1166-71.
78. Trigatti B, Rayburn H, Vinals M, Braun A, Miettinen H, Penman M, Hertz M, Schrenzel M, Amigo L, Rigotti A, et al. Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc Natl Acad Sci U S A.* 1999;96(16):9322-7.
79. Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, and Proudfoot NJ. Primary microRNA transcripts are processed co-transcriptionally. *Nat Struct Mol Biol.* 2008;15(9):902-9.
80. Lauressergues D, Couzigou JM, Clemente HS, Martinez Y, Dunand C, Becard G, and Combier JP. Primary transcripts of microRNAs encode regulatory peptides. *Nature.* 2015;520(7545):90-3.
81. Waterhouse PM, and Hellens RP. Plant biology: Coding in non-coding RNAs. *Nature.* 2015;520(7545):41-2.
82. Winter J, Jung S, Keller S, Gregory RI, and Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol.* 2009;11(3):228-34.
83. Valencia-Sanchez MA, Liu J, Hannon GJ, and Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev.* 2006;20(5):515-24.

84. Younger ST, Pertsemlidis A, and Corey DR. Predicting potential miRNA target sites within gene promoters. *Bioorg Med Chem Lett.* 2009;19(14):3791-4.
85. Place RF, Li LC, Pookot D, Noonan EJ, and Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A.* 2008;105(5):1608-13.
86. Orom UA, Nielsen FC, and Lund AH. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell.* 2008;30(4):460-71.
87. Izaurralde E. GENE REGULATION. Breakers and blockers-miRNAs at work. *Science.* 2015;349(6246):380-2.
88. Obad S, dos Santos CO, Petri A, Heidenblad M, Broom O, Ruse C, Fu C, Lindow M, Stenvang J, Straarup EM, et al. Silencing of microRNA families by seed-targeting tiny LNAs. *Nature genetics.* 2011;43(4):371-8.
89. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-33.
90. Soh J, and Hussain MM. Supplementary site interactions are critical for the regulation of microsomal triglyceride transfer protein by microRNA-30c. *Nutr Metab (Lond).* 2013;10(1):56.
91. Kamanu TK, Radovanovic A, Archer JA, and Bajic VB. Exploration of miRNA families for hypotheses generation. *Sci Rep.* 2013;3(2940.
92. Bouchie A. First microRNA mimic enters clinic. *Nat Biotechnol.* 2013;31(7):577.
93. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, et al. Treatment of HCV infection by targeting microRNA. *N Engl J Med.* 2013;368(18):1685-94.
94. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, and Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol.* 2002;12(9):735-9.
95. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Invest.* 2012;122(8):2884-97.
96. Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest.* 2012;122(8):2871-83.
97. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, and Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. *Nature.* 2005;438(7068):685-9.

98. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 2006;3(2):87-98.
99. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjarn M, Hansen HF, Berger U, et al. LNA-mediated microRNA silencing in non-human primates. *Nature.* 2008;452(7189):896-9.
100. Soh J, Iqbal J, Queiroz J, Fernandez-Hernando C, and Hussain MM. MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. *Nat Med.* 2013;19(7):892-900.
101. Irani S, Pan X, Peck BC, Iqbal J, Sethupathy P, and Hussain MM. MicroRNA-30c Mimic Mitigates Hypercholesterolemia and Atherosclerosis in Mice. *J Biol Chem.* 2016.
102. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, Fisher EA, Moore KJ, and Fernandez-Hernando C. MiR-33 contributes to the regulation of cholesterol homeostasis. *Science.* 2010;328(5985):1570-3.
103. Rayner KJ, Sheedy FJ, Esau CC, Hussain FN, Temel RE, Parathath S, van Gils JM, Rayner AJ, Chang AN, Suarez Y, et al. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. *The Journal of clinical investigation.* 2011;121(7):2921-31.
104. Rayner KJ, Esau CC, Hussain FN, McDaniel AL, Marshall SM, van Gils JM, Ray TD, Sheedy FJ, Goedeke L, Liu X, et al. Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides. *Nature.* 2011;478(7369):404-7.
105. Davalos A, Goedeke L, Smibert P, Ramirez CM, Warrier NP, Andreo U, Cirera-Salinas D, Rayner K, Suresh U, Pastor-Pareja JC, et al. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. *Proc Natl Acad Sci U S A.* 2011;108(22):9232-7.
106. Chen J, Young ME, Chatham JC, Crossman DK, Dell'Italia LJ, and Shalev A. TXNIP regulates myocardial fatty acid oxidation via miR-33a signaling. *Am J Physiol Heart Circ Physiol.* 2016:ajpheart 00151 2016.
107. Xu Y, Zalzala M, Xu J, Li Y, Yin L, and Zhang Y. A metabolic stress-inducible miR-34a-HNF4alpha pathway regulates lipid and lipoprotein metabolism. *Nat Commun.* 2015;6(7466.
108. Zhou L, Irani S, Sirwi A, and Hussain MM. MicroRNAs regulating apolipoprotein B-containing lipoprotein production. *Biochim Biophys Acta.* 2016.

109. Goedeke L, Rotllan N, Canfran-Duque A, Aranda JF, Ramirez CM, Araldi E, Lin CS, Anderson NN, Wagschal A, de Cabo R, et al. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. *Nat Med.* 2015;21(11):1280-9.

110. Cuchel M, and Rader DJ. Microsomal transfer protein inhibition in humans. *Current opinion in lipidology.* 2013;24(3):246-50.

111. de Aguiar Vallim TQ, Tarling EJ, Kim T, Civelek M, Baldan A, Esau C, and Edwards PA. MicroRNA-144 regulates hepatic ATP binding cassette transporter A1 and plasma high-density lipoprotein after activation of the nuclear receptor farnesoid X receptor. *Circ Res.* 2013;112(12):1602-12.

Chapter 2

The following chapter is an accepted manuscript. Author contributions to the manuscript are defined below:

Liye Zhou – I performed and analyzed all experiments presented within this manuscript.

M. Mahmood Hussain – Dr. Hussain is the primary investigator in this study and provided the financial and idea leadership and direction for the work presented in this manuscript

Human MicroRNA-548p Decreases Hepatic ApoB Secretion and Lipid Synthesis

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Abstract

Objective: MicroRNAs (miRs) play important regulatory roles in lipid metabolism. ApoB, as the only essential scaffolding protein in the assembly of very low density lipoproteins, is a target to treat hyperlipidemia and atherosclerosis. We aimed to find out miRs that reduce apoB expression.

Approach and Results: Bioinformatics analyses predicted that hsa-miR-548p can interact with apoB mRNA. MiR-548p or Control miR was transfected in human and mouse liver cells to test its role in regulating apoB secretion and mRNA expression levels. Site-directed mutagenesis was used to identify the interacting site of miR-548p in human apoB 3´-untranslated region. Fatty acid oxidation and lipid syntheses were examined in miR-548p overexpressing cells to investigate its function in lipid metabolism. We observed that miR-548p significantly reduces apoB secretion from human hepatoma cells and primary hepatocytes. Mechanistic studies showed that miR-548p interacts with the 3´-untranslated region of human apoB mRNA to enhance posttranscriptional degradation. Bioinformatics algorithms suggested two potential binding sites of miR-548p on human apoB mRNA. Site-directed mutagenesis studies revealed that miR-548p targets site I involving both seed and supplementary sequences. MiR-548p had no effect on fatty acid oxidation but significantly decreased lipid synthesis in human hepatoma cells by reducing HMGCR and ACSL4 enzymes involved in cholesterol and fatty acid synthesis. In summary, miR-548p reduces lipoprotein production and lipid synthesis by reducing expression of different genes in human liver cells.

Conclusions: These studies suggest that miR-548p regulates apoB secretion by targeting mRNA. It is likely that it could be useful in treating atherosclerosis, hyperlipidemia and hepatosteatosis.

Abbreviations used:

ApoB	apolipoprotein B
ASO	antisense oligonucleotides
Ctrl	control
FBS	fetal bovine serum
HDL	high density lipoproteins
HoFH	homozygous familial hypercholesterolemia
LDL	low density lipoproteins
miR	microRNA
MTP	microsomal triglyceride transfer protein
UTR	untranslated region
VLDL	very low density lipoproteins

Introduction

MicroRNAs (miRs) are small endogenous non-coding RNAs. Since 1993, when the first miR was identified and cloned from *C. elegans* (1), the regulatory functions of miRs have been studied extensively in different species (2). Further, significant attempts have been made to test potential use of miRs as therapeutic agents. MiR-122, the most abundant miR expressed in the liver, binds to the 5´-UTR of the Hepatitis C virus genome to positively affect RNA stability and replication (3). Therefore, inhibition of endogenous miR-122 shows efficacy in treating chronic Hepatitis C infection. And, a locked nucleic acid-modified anti-miR-122, miraversin, is in Phase II clinical trials (4-6). MiR-34, which is induced by a tumor suppressor p53, reduces the expression of multiple genes in apoptosis and cell cycle arrest to suppress tumor genesis (7,8). MRX34, a double-stranded miR-34 mimic has been shown to enhance cancer cell death and inhibit metastasis in a Phase I clinical trial (9). These promising results from preclinical and clinical studies encourage us to seek for miRs that can potentially treat cardiovascular diseases that remain the major cause of morbidity and mortality in western world (10).

Atherosclerosis, the major cause of cardiovascular diseases, occurs due to excess lipid accumulation on the inner wall of arteries (11). Atherosclerotic lesions in major arteries narrow their diameters and reduce blood circulation (12). Several risk factors of atherosclerosis have been identified such as hyperlipidemia, hypertension, smoking, lack of exercise and family history. Hyperlipidemia refers to increases in plasma lipids and lipoproteins. High plasma concentrations of low-density lipoproteins (LDL) and their modified forms, such as oxidized-LDL, contribute to atherosclerosis. And, lowering their concentrations is associated with reduced incidence of cardiovascular disease (13,14).

Plasma lipid levels can be lowered by increasing their catabolism or decreasing production. Statins and PCSK9 inhibitors reduce plasma LDL by increasing their clearance via upregulating hepatic LDL receptors (15,16). Hence, these drugs are not useful in the treatment of homozygous familial hypercholesterolemia (HoFH) subjects with null LDL receptor mutations (17,18). Hypercholesterolemia in these patients can be lowered using drugs that decrease apolipoproteins B (apoB) or microsomal triglyceride transfer protein (MTP); two critical proteins required for the assembly and secretion of very low-density lipoprotein (VLDL) (17,19,20). During VLDL assembly, newly translated apoB peptides are lipidated in the endoplasmic reticulum by MTP (19-21). ApoB siRNAs and antisense oligonucleotides (ASOs) reduce lipoprotein production and plasma cholesterol in both rodents and non-human primates (22-25). Mipomersen, a specific apoB ASO, has been approved to treat HoFH (17,19). Mipomersen reduces plasma cholesterol but is associated with lipid accumulation in the liver (17,19,26-29). Similarly, small molecule inhibitors and ASO mediated reductions in MTP lower plasma cholesterol but are also associated with increases in hepatic lipids (19,20,30-32). Therefore, novel therapeutic agents that reduce VLDL production while avoiding hepatosteatosis might be useful treatment modalities.

Few miRs have been reported to regulate metabolism of apoB-containing lipoproteins (33,34). Inhibition of miR-122 results in significant reductions in plasma cholesterol, apoB and triglyceride (35-37). Further, miR-122 knockout mice have lower plasma cholesterol and triglyceride due to reductions in both LDL and HDL. These mice have higher hepatic lipids and plasma alanine aminotransferase (ALT) and alkaline phosphatase and develop hepatocellular carcinoma with age (38,39). MiR-34a expression reduces lipoprotein production and causes hepatosteatosis (40). *Mir34a*$^{-/-}$ mice have high plasma triglyceride and cholesterol (40). Recently, we showed that miR-30c reduces MTP expression and lowers plasma cholesterol and atherosclerosis without causing steatosis (41,42). Mechanistic studies indicated that miR-30c reduces lipoprotein production and lipid synthesis. Moreover, miR-33a/b was identified for its role in down regulating ABCA1 in macrophage to inhibit cholesterol efflux and HDL biosynthesis (43-45). Here, we investigated whether there are miRs that target apoB to regulate lipoprotein production and identified a novel human miR-548p, which targets apoB mRNA, decreases apoB secretion and reduces hepatic lipid synthesis in human hepatoma cells.

Results

MiR-548p lowers apoB secretion from human liver cells.

Using TargetScan 5.2, we observed that human apoB mRNA has a target site for hsa-miR-548p (**Supplementary Fig I**) that is conserved in primates, but not in other organisms including dog, mouse, rat, chicken, and frog (**Fig 1A**). To test whether hsa-miR-548p affects human apoB secretion, different indicated concentrations of control (Ctrl) miR, miR-548p, and anti-548p were transfected in Huh-7 cells (**Fig 1B**). MiR-548p dose-dependently reduced media and cellular apoB without changing apoAI levels (**Fig 1B**). Anti-548p, an inhibitor of miR-548p, increased both media and cellular apoB without affecting apoAI secretion indicating the role of endogenous miR-548p in apoB regulation (**Fig 1B**). Further studies showed that miR-548p reduces media and cellular apoB in a time dependent manner (**Fig 1C**). The maximum reduction of 67% was observed after 72 hours. Anti-548p increased media and cellular apoB levels without affecting apoAI (**Fig 1C**). In addition, we observed the same effect of miR-548p on apoB in another human hepatoma cell line, HepG2 (**Fig 1D**). Forced expression of miR-548p reduced media and cellular apoB, but had no effect on apoAI (**Fig 1D**). On the other hand, anti-548p increased media and cellular apoB. We also tested the effect of miR-548p on apoB and apoAI secretion in human primary hepatocytes compared to negative control Ctrl. MiR-30c was used as a positive control of transfection efficiency, as it is known to target MTP and reduce apoB secretion (41,42). MiR-548p significantly reduced apoB secretion from the primary hepatocytes without affecting apoAI (Fig 1E). Thus, miR-548p reduces cellular and media apoB in human liver cells without altering apoAI levels.

MiR-548p reduces apoB mRNA levels in human, but not in mouse, liver cells

To begin understand how miR-548p regulates apoB secretion, we quantified apoB, MTP and apoAI mRNA levels in miR-548p, miR-30c and Ctrl transfected human primary hepatocytes. MiR-548p significantly reduced apoB mRNA levels without affecting MTP or apoAI, while miR-30c only reduced MTP mRNA levels (**Fig 2A**). In Huh-7 cells, miR-548p reduced and anti-548p increased apoB mRNA levels. MiR-548p and anti-548 had no effect on MTP and apoAI mRNA levels (**Fig 2B**). These studies indicate that miR-548p reduces steady state apoB mRNA levels in liver cells.

Since mouse is the most widely used animal model to study physiological effects of genetic manipulations and drug treatments, we transfected miR-548p in mouse primary hepatocytes (**Fig 2C**) and in mouse hepatoma AML12 cells (**Fig 2D**). MiR-548p and anti-548p had no effect on mouse apoB, MTP or apoAI. This is consistent with the absence of miR-548p interacting site in the 3´-UTR of mouse apoB (**Fig 1A**). Thus, miR-548p reduces apoB mRNA levels in human liver cells.

MiR-548p interacts with the 3´-UTR of apoB to induce posttranscriptional degradation

MiRs, in general, reduce mRNA levels by interacting with 3´-untranslated region (3´-UTR) of mRNAs and inducing post-transcriptional degradation (2,46). Therefore, we hypothesize that miR-548p may induce post-transcriptional degradation of apoB mRNA. ApoB mRNA disappeared faster in miR-548p expressing cells than in Ctrl transfected cells treated with actinomycin D to inhibit transcription **(Fig 3A)**. Hence, miR-548p promotes post-transcriptional degradation of apoB mRNA.

To identify interactions between miR-548p and apoB mRNA, we cloned wild type 3´-untranslated region (3´-UTR) of apoB mRNA at the end of luciferase cDNA in psiCHECK plasmid **(Fig 3B)** and expressed in Huh-7 cells. Compared to the Ctrl, miR-548p reduced and anti-548p increased luciferase activity in cells transfected with luciferase containing wild type apoB 3´-UTR **(Fig 3C)**. These studies suggest that miR-548p interacts with 3´-UTR of apoB.

Both seed and supplementary sites in miR-548p interact with the 3´-UTR of apoB mRNA at site II

Attempts were then made to identify miR-548p interacting site in the apoB mRNA. MiRanda predicted that human apoB 3´-UTR contains two potential target sites for miR-548p (**Fig 3D**). Both sites contain perfect complementary binding sites for miR-548p seed sequence and also contain possible interaction sites involving supplementary sequences. To determine if miR-548p interacts with these sequences, we mutated these sites to perturb potential base-paring **(Fig 3D)**. Mutations in either seed or supplementary sequences in Site I resulted in loss of miR-548p effect on luciferase activity (**Fig 3E**). However, mutations of Site II seed and supplementary sequences did not affect luciferase activity (**Fig 3E**). These studies suggest that miR-548p interacts with the 3´-UTR of apoB by interacting with both seed and supplementary sequences at Site I.

MiR-548p decreases hepatic lipid synthesis

Inhibition of apoB secretion is usually associated with cellular lipid accumulation (47). Therefore, we asked whether miR-548p would increase cellular lipids. No significant differences were observed in total cellular triglyceride and cholesterol levels in cells transfected with Ctrl and miR-548p (**Fig 4A-B**). Thus, miR-548p expression does not cause lipid accumulation in Huh-7 cells.

To explain reasons for the absence of lipid accumulation in miR-548p expressing cells, we examined the effects of miR-548p on fatty acid oxidation and lipid synthesis (**Fig 4C-D**). Fatty acid oxidation was unaffected (**Fig 4C**). However, miR-548p significantly decreased cholesterol, fatty acid, and triglyceride synthesis by 20%, 30%, and 33%, respectively, but had no effect on phospholipid synthesis **(Fig 4D)**. These studies indicate that miR-548p decreases lipid synthesis.

To explain how miR-548p decreases cholesterol and fatty acid synthesis, we measured mRNA levels of genes involved in lipid synthesis that are predicted to contain potential target sites of miR-548p (**Fig 4E**). HMGCR and ACSL4 mRNA levels were significantly reduced in miR-548p expressing cells. However, mRNA levels of other enzymes involved in triglyceride and fatty acid biosynthesis, as well as fatty acid oxidation were not affected (**Fig 4E**). Further, cellular proteins levels of these enzymes were significantly reduced (Fig 4F) in miR-548p expressing cells while GAPDH protein levels were unaffected. Further, media apoE and PCSK9 levels were unaffected by miR-548p expression. HMGCR is a rate-limiting enzyme in cholesterol synthesis (48). ACSL4 plays a role in fatty acid and triglyceride synthesis (49,50). Thus, miR-548p may target HMGCR and ACSL4 to reduce lipid synthesis.

To determine whether miR-548p directly regulates ACSL4, we utilized luciferase constructs containing the 3'-UTRs of ACSL4. MiR-548p overexpression significantly decreased the luciferase activity when it was expressed with theACSL4 3'-UTR indicating a direct interaction between ACSL4 3'-UTR and miR-548p (Fig 1G).

Discussion

Here we report identification of a novel miR, miR-548p, that targets apoB mRNA and reduces intracellular and media apoB levels. MiR-548p interacts with the 3´-UTR of apoB mRNA involving both seed and supplementary sequences at site I. These interactions induce post-transcriptional degradation of mRNA and reduce secretion of apoB from human liver cells. In addition, we observed that miR-548p significantly reduces lipid synthesis. This is most likely due to diminished expression of ACSL4 and HMGCR, two enzymes involved in fatty acid and cholesterol biosynthesis. Thus, miR-548p reduces lipid synthesis and lipoprotein production via independent mechanisms targeting different mRNAs.

The importance of interactions between apoB mRNA and miR-548p in the regulation of apoB secretion is supported by several experiments. Bioinformatics analyses predict conserved interacting sites in primates. Cloning of 3´-UTR of apoB mRNA at the end of luciferase renders luciferase susceptible to miR-548p dependent regulation. Mutagenesis of 3´-UTR sequence abolishes the regulation by miR-548p. Mouse apoB is not regulated by miR-548p most likely due to the absence of miR-548p interacting site.

According to miRanda, human apoB 3´-UTR contains two targeting sites for miR-548p involving perfect seed sequence Watson-Crick base pair interactions. In contrast, TargetScan predicts only one site that corresponds to the site II predicted by miRanda. Site-directed mutagenesis studies revealed that only one predicted site, site I, contributes to the regulation of apoB mRNA (**Fig 3**). This is consistent with the general understanding that the first miR interacting site after the stop codon is important for physiological activity. However, this interaction starts with the residue 3 of the miR-548p. Usually, residue 2 of miR is involved in the first H-bonding with mRNAs. In fact, the predicted binding of miR-548p at site II starts with the second residue. It is likely that this site is not available for miR-548p as the binding start site is ~ 12 nucleotides away from the end of the first binding site. Thus, if the site I is occupied by a RISC complex then the site II may not be accessible for additional interactions. At this site I, both seed and supplementary site interactions appear important for miR-548p to regulate apoB mRNA levels as mutagenesis of residues only involved in these supplementary site interactions abolish response to miR-548p. A previous study about the interaction of miR-30c and MTP has highlighted the importance of supplementary site interactions (51). Therefore, it is likely that miR-548p interacts with the first possible interaction site available after the stop codon and these interactions involve both seed and supplementary sequences.

MiR-548p is a member of a large primate specific family (52). Most of the miRs are conserved in different species (42,53,54). The conservation allows an easy way to evaluate physiological effects of overexpression or loss-of-function of specific miRs in model organisms before human studies. Nevertheless, many human or primate specific miRs have been identified that play important roles in physiological and pathological pathways, such as cellular differentiation and cancer development (55-57). It is likely that primate miRs have evolved to regulate species specific phenotypes. Primates carry most of the cholesterol in LDL, whereas mice carry it in HDL. Consistent with this, 3´-UTR of primate apoB mRNA contains miR-548p targets sites that carry cholesterol mainly in LDL (Fig 1A). And, this interacting site is not present in animals that carry cholesterol mainly in HDL, such as mouse. Thus, miR-548p might have evolved to regulate plasma LDL levels.

MiR-548p is predicted to be transcribed from an independent gene (*MIR548P*) that is present on the opposite strand of the ST8 alpha-N-acetyl-neuraminide alpha-2, 8-sialyltransferase 4 (*ST8SIA4*) gene (**Supplementary Fig IIA**) that encodes an enzyme required for the synthesis of polysialic acid (58). MiRs are known to be transcribed as pri-miR, undergo cleavage and transported across the nucleus as pre-miR. Not only the mature miR, but also the pre-miR548p is conserved in primates (**Supplementary Fig IIB**). It has been reported that miR-548p is present in several human tissues including blood, brain, liver, lung etc. (**Supplementary Fig III**).

To our knowledge, miR-548p is the first miR that has been shown to target apoB mRNA and decrease apoB secretion from human hepatoma cells. MTP and apoB are the two critical proteins in apoB-containing lipoprotein assembly and secretion. Inhibition of MTP causes hepatosteatosis and elevates plasma AST and ALT activities. In the absence of MTP, lipids are not transferred from endoplasmic reticulum (ER) membrane to ER lumen. Therefore, the newly synthesized lipids, mainly triglyceride, are stored in cytosolic lipid droplets. However, when apoB expression is reduced, lipids can still be transferred to ER lumen by MTP. Lipid accumulation in the ER lumen has been shown to activate autophagy, which engulfs and hydrolyzes lipids in lysosomes (25). Fatty acids generated from lipolysis are then oxidized in mitochondria. Therefore, in mice treated with apoB ASOs, hepatic triglyceride levels increase soon after injection (first 3 weeks) and later resolve due to higher fatty acid oxidation (25). Hence, targeting apoB might be an appropriate way to reduce VLDL production. In this study, we show that miR-548p reduces apoB mRNA to decrease apoB protein secretion from human hepatocytes, and Huh-7 and HepG2cells (**Fig 1**) and also lowers hepatic cholesterol and fatty acid synthesis by suppressing the expression of HMGCR and ACSL4 (**Fig 4**). Thus, it is possible that early steatosis seen with apoB ASO can be avoided by miR-548p overexpression, and may be evaluated in the future in primates.

With this report, we have now identified two miRs that appear to have similar physiological regulatory effects. MiR-30c reduces lipoprotein production by targeting MTP and lipid synthesis by targeting ELOVL5 and LPGAT3 (41,42). MiR-548p reduces lipoprotein production by targeting apoB and lipid synthesis by lowering ACSL4 and HMGCR. To our knowledge, this is the first report describing two different miRs affecting the same physiological processes by targeting different genes. It is likely that nature has evolved multiple miRs to regulate similar pathways due to their physiological importance. Alternatively, the evolution of different miRs may represent redundant mechanisms for their regulation.

From genetic and therapeutic studies, it is well known that separate inhibition of lipoprotein synthesis and lipid synthesis results in different diseases. For example, defects in lipoprotein production result in hypolipidemia that is associated with accumulation of lipids in tissues (19). On the other hand, defects in lipid synthesis are associated with lipodystrophy (59). In contrast, combined reduction of both these metabolic pathways appears to avoid pathologies associated with defects in individual pathways. Therefore, targeting of multiple related pathways might be more beneficial than the classical approach of targeting individual proteins/pathways.

Although these studies advocate for the identification and use of miRs that affect multiple pathways with beneficial outcomes, these approaches need extensive evaluation. A major concern about miR therapies is unanticipated effects. Hence, a rigorous evaluation of all anticipated and unanticipated effects are warranted before advocating their use in humans.

In short, these studies have identified miR-548p that reduces expression of apoB, HMGCR and ACSL4 to diminish lipoprotein production and lipid synthesis. Due to its ability of controlling these two pathways, similar to miR-30c, it might reduce plasma cholesterol and atherosclerosis without causing steatosis. However, this can only be evaluated in primates. Such studies will provide strong evidence for further clinical investigations in humans.

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Materials: All chemicals and solvents were purchased from Fisher Scientific. ^{14}C-oleate and ^{3}H-acetate were from NEN Life Science Products. The monoclonal capture antibodies used in ELISA for human apoB and apoAI (1D1 and 4H1, respectively) were obtained from University of Ottawa Heart Institute. Secondary goat anti-human apoB and apoAI polyclonal antibodies were from Academy Bio-Medical Company (20S-G2, 11A-G2). Swine Anti-Goat IgG (H+L)-alkaline phosphatase was purchased from Southern Biotech (6300-04). Alkaline phosphatase substrate p-nitrophenyl phosphate (PNPP) used for color development was obtained from ThermoFisher Scientific (34045).

Cell culture transfection: Huh-7 and HepG2 cells were obtained from American Type Culture Collection and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplied with 10% of Fetal Bovine Serum (FBS) in cell culture incubators (37° C and 5% CO_2). Cells were transfected with miR mimic using reverse transfection procedure. Briefly, miR mimic and RNAiMAX were diluted with serum reduced Opti-MEM (Life Technologies) separately. After 5 min, diluted miR mimic and RNAiMAX were combined and incubated at room temperature for 20 to 30 min for complex formation. Meanwhile, cells were trypsinized, centrifuged, counted, and 250,000 cells were added into each well of 6-well plates that already contained miR and anti-miRs complexed with RNAiMAX. After 24 hours, media were changed to complete growth medium containing 10% FBS. Media and cells were usually collected 48 h later for analyses. Human plateable cryopreserved hepatocytes were purchased from BioreclamationIVT (M00995-P). Cells were thawed in prewarmed InVitroGRO CP Medium with antibiotics and counted using Trypan Blue. Cells were plated in 6-well plate 7*10^5 viable cells/well. After overnight incubation in 37 °C, 5% CO_2, cells were transfected with miR-548p, miR-30c, or Ctrl mimic (100 nM) using standard forward transfection procedure. MiR mimic and RNAiMAX were diluted with serum reduced Opti-MEM separately. After 5 min, diluted miR mimic and RNAiMAX were combined and incubated at room temperature for 20-30 min. Meanwhile, media in wells were replaced with serum reduced Opti-MEM. The complexes with different miR mimics were added to wells. 6 hours after transfection, media in wells were replaced with InVitroGRO CP Medium with antibiotics. 48 hours after transfection, media were used for apoB and apoAI measurement. Total RNAs were extracted from cells for mRNA quantification.

Measurements of media apoB and apoAI: The concentrations of apoB and apoAI in media were quantified by ELISA (1-3). For cellular total protein quantification, 100 µl of 1N NaOH was added to each well to lyse cells. ApoB and apoAI levels in 2 hour conditioned media were normalized against the cellular protein concentration. For ELISA, high binding 96-well plates (Thermo Fisher Scientific, 07-200-640) were coated with monoclonal human apoB or apoAI antibodies (1:1000 in PBS) by incubating overnight at 4° C. The next day, wells were blocked with 3% BSA in PBS containing 0.05% of Tween 20 (PBS-T) for 1 hour at room temperature. After three washes with PBS-T, 100 µl of samples were added to wells and incubated for 2 hours at 37° C. Purified human LDL and HDL were used to generate standard curve in parallel. The wells were washed with PBS-T, incubated with goat polyclonal human apoB or apoAI antibodies (1:1000 in PBS) for 1 hour at 37° C, washed with PBS-T, incubated with swine anti-goat IgG labeled with alkaline phosphatase (1:1000 in PBS) for 1 hour and washed. We then added 100 µl of substrate PNPP (1 mg/ml) in the reaction buffer (10 mM ethanolamine and 0.5 mM $MgCl_2$, pH 9.5) and measured absorbance at 405 nm wavelength.

Intracellular apoB measurement: Cells were homogenized in 100 mM Tris buffer containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 0.5% SDS (2, 3). And apoB in these cell lysates was measured using the ELISA assay described above.

ApoB mRNA half-life measurement: Huh-7 cells were transfected with 50 nM miR-548p or Ctrl. After 24 hours, media were changed to complete growth medium containing 1 µg/mL actinomycin D to inhibit *de novo* transcription. Total RNA was extracted at different time points to quantify apoB expression using qRT-PCR.

Cloning and mutagenesis of apoB 3´-UTR in a luciferase reporter plasmid: ApoB 3´-UTR was amplified using primers (**Table 1**) from genomic DNA by PCR and cloned into psiCHECK2 plasmid at the end of the Renilla luciferase cDNA. Seed and supplementary sequences that could intercat with miR-548p were mutated via site-directed mutagenesis using specific primers (**Table 2**).

Luciferase reporter assay:

Plasmid harboring the 3'-UTR of ACSL4 (HmiT005090-MT06) and Hmgcr (HmiT008891-MT06) were purchased from Genecopoeia. Huh-7 cells (60-70% confluent in 10 cm petri dishes) were forward transfected with 1.5 µg of reporter plasmids using Turbofect (Life Technologies). After 24 hours, cells were reverse transfected with miR-548p or Ctrl mimics (100 nM). Dual luciferase activities (Promega) were measured in cell lysates after another 48 hours. Firefly luciferase activity was divided by the internal control Renilla luciferase activity for normalization of transfection efficiency.

Immunoprecipitation and western blot: Media from cells transfected with Ctrl or miR-548p were collected in eppendorf tubes with proteinase inhibitor cocktails. 1ug of antibodies against human apoE (abcam, ab1906) or PCSK9 (LSBio, C170825)were added to 1ml of medium and incubated for 1 hour at 4°C on a rotator. 40 µl of Protein A/G Agarose beads (Santa Cruz, sc-2003) was added and incubated overnight at 4°C on a rotator. Beads were centrifuged at 100g, washed with PBS-T, and resuspended in SDS loading buffer for western blot. The same human apoE and PCSK9 antibodies were used for western blot. For the measurement of cellular ACSL4 and Hmgcr, RIPA buffer was used for homogenization of cells. 20ug of total protein was used for separation on 10% SDS-PAGE gels. Antibodies specific for human ACSL4 (Santa Crus, sc-134507), Hmgcr (abcam, ab174830), and GAPDH (Santa Cruz, sc-48167) were used for recognizing specific proteins. ECL was used for protein detection. Antibodies used in western blot were all in 1:1000 dilution.

Cellular lipid extraction and measurement: Cells were washed with cold PBS, scraped from dishes, and centrifuged at 3,000 rpm for 5 min at 4° C. Cell pellets were resuspended in 200 µl of isopropanol and incubated overnight at 4° C on a rotating shaker. Samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were used for lipid measurements. Total cholesterol and triglyceride were measured using commercial kits (Pointe Scientific). The pellets were resuspended in 0.1N NaOH to determine protein for normalization.

Fatty acid oxidation and lipid synthesis: For fatty acid oxidation, Huh-7 cells were transfected with 100 nM miR-548p or Ctrl during plating in 12-well plates. After 48 hours, cells were incubated with DMEM containing 0.4 µCi/ml of ^{14}C-oleate and covered with phenylethylamine soaked Whatman filter paper for 3 hours at 37° C. At the end of incubation, 200 µl of 1M perchloric acid was added to media and incubated for 1 h at room temperature to precipitate acid-insoluble metabolites, and centrifuged at 4° C (10 min, 12,000g). The radioactivities in the supernatant and the filter paper were counted. For lipid synthesis, Huh-7 cells transfected with 50 nM of miR-548p or Ctrl in 12-well plates were incubated with 1 µCi ^{3}H-acetate for three hours. Total lipids were extracted from cells using isopropanol and separated on silica-60 thin layer chromatography plates (hexane:diethyl ether:glacial acetic acid, 82:17:2, v/v). The plates were exposed to iodine to visualize bands. The bands corresponding to cholesteryl ester, triglyceride, fatty acid, cholesterol and phospholipid were scraped and counted in a scintillation counter.

RNA isolation and qRT-PCR: Total RNA from cells was extracted using TRIzol (Invitrogen), and reverse transcribed into cDNA with the Omniscript RT kit (QIAGEN). Expression levels of different genes were quantified by qRT-PCR using SYBR Green qPCR Core Kit (Eurogentec) and specific primers (**Table 3**). Data were analyzed using ΔΔCT method and normalized to 18S.

Statistics: Data are presented as the mean ± SD. The statistical significance was determined by student's t-test, one-way or two-way ANOVA (Graphpad Prism) and significant differences $p<0.05$, $p<0.01$, and $p<0.001$ were symbolized as *, **, and ***, respectively.

Table 1. Primers used to amplify apoB 3´-UTR from human genomic DNA

Forward	5´ CCGCTCGAGGAGAACTTACTATCATCCTC 3´
Reverse	5´ AGCTTTGTTTAAACACATTCAGTGGTATGATACAC 3´

Table 2. Primers used for site-directed mutagenesis

Site	Sequence
Seed1	Forward: 5´- AGATCCTGATTTTCTTTAACTTGCGGGAA ATGCCATCCTTCTGAGTTCAGAGAC-3´ Reverse: 5´- GTCTCTGAACTCAGAAGGATGGCATTTCC CGCAAGTTAAAGAAAATCAGGATCT-3´
Sup1	Forward: 5´- CCTGATTTTCTTTAACTTGCAAAAAAAAAC ATCCTTCTGAGTTCAGAGACCTTCC-3´ Reverse: 5´- GGAAGGTCTCTGAACTCAGAAGGATGTTTT TTTTTGCAAGTTAAAGAAAATCAGG-3´
Seed II	Forward: 5´- CCTGATTTTCTTTAACTTGCAAAAAAAAAC ATCCTTCTGAGTTCAGAGACCTTCC-3´ Reverse: 5´- GGAAGGTCTCTGAACTCAGAAGGATGTTTT TTTTTGCAAGTTAAAGAAAATCAGG-3'
Sup II	Forward: 5´-CCTCCTCCCCCAAGTTTACGCAAATAACTCAG ATCCTGATTTTCTTTAACTTG-3´ Reverse: 5´-CAAGTTAAAGAAAATCAGGATCTGAGTTATT TGCGTAAACTTGGGGGAGGAGG-3´

Table 3. Primers used for quantitative RT-PCR

Gene	Forward	Reverse
hMTP	5´ ACGGCCATTCCCATTGTG 3´	5´ GCCAGAGCTCCGAGAGAGAA 3´
hApoAI	5´ GCAGAGACTATGTGTCCCAGTTTG 3´	5´ CCAGTTGTCAAGGAGCTTTAG 3´

hApoB	5´ TGACCTTGTCCAGTGAAGTC 3´	5´ GTTCTGAATGTCCAGGGTGA 3´
hMGAT1	5´ CCAAGAAAAGTGTGTCCTACAT 3´	5´ GCACCCGCAAGGACAATG 3´
hMGAT2	5´ CCATCTCGCTGGTCAAGAC 3´	5´ GAAGAGAAGCCTGTGCTCTCA 3´
hMGAT3	5´ACTCTGGCCCTTCTCTGTTTT 3´	5´ AACGCCTTCCACCTTGGT 3´
hDGAT2	5´ CAGTTTGGAGACCGGGAGTTC 3´	5´ GATGTTCCAGTTCTGCCAGAAGT 3´
hHMGCR	5´ GTCATTCCAGCCAAGGTTGT 3´	5´ GGGACCACTTGCTTCCATTA 3´
hACSL3	5´ CCCCTGAAACTGGTCTGGTG 3´	5´ TCCGCCTGGTAATGTGTTTTAA 3´
hACSL4	5´ GGCGTACTTTATTGTCGGCTTC 3´	5´ TACAGCCAAGGCAGTTCAATCTTAG 3´
hELOVL7	5´ GGCCAGCCTACCAGAAGTATTTG 3´	5´ GGCGACAATAACAAACTGGACAAG 3´
hPPARA	5´ CAAATGCCAGTATTGTCGATTTCA 3´	5´– CAAAACGAATCGCGTTGTGT 3´
mMTP	5´ CACACAACTGGCCTCTCATTAAAT 3´	5´ TGCCCCCATCAAGAAACACT 3´
mApoB	5´ CTCGACCATCGGCACTGT 3´	5´ AGTTTCTTCTCTGGAGGGGACT 3´
mApoAI	5´ GGCCGTGGCTCTGGTCTT 3´	5´ GGTTCATCTTGCTGCCATACC 3´

References

1. Hussain MM, Zhao Y, Kancha RK, Blackhart BD, and Yao Z. Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100. *Arterioscler Thromb Vasc Biol.* 1995;15(4):485-94.
2. Bakillah A, Zhou Z, Luchoomun J, and Hussain MM. Measurement of apolipoprotein B in various cell lines: correlation between intracellular levels and rates of secretion. *Lipids.* 1997;32(10):1113-8.
3. Walsh MT, Iqbal J, Josekutty J, Soh J, Di Leo E, Ozaydin E, Gunduz M, Tarugi P, and Hussain MM. Novel Abetalipoproteinemia Missense Mutation Highlights the Importance of the N-Terminal beta-Barrel in Microsomal Triglyceride Transfer Protein Function. *Circ Cardiovasc Genet.* 2015;8(5):677-87.

References

1. Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-854
2. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233
3. Thibault, P. A., Huys, A., Amador-Canizares, Y., Gailius, J. E., Pinel, D. E., and Wilson, J. A. (2015) Regulation of Hepatitis C Virus Genome Replication by Xrn1 and MicroRNA-122 Binding to Individual Sites in the 5' Untranslated Region. *J. Virol.* **89**, 6294-6311
4. Gebert, L. F., Rebhan, M. A., Crivelli, S. E., Denzler, R., Stoffel, M., and Hall, J. (2014) Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res.* **42**, 609-621
5. Janssen, H. L., Reesink, H. W., Lawitz, E. J., Zeuzem, S., Rodriguez-Torres, M., Patel, K., van der Meer, A. J., Patick, A. K., Chen, A., Zhou, Y., Persson, R., King, B. D., Kauppinen, S., Levin, A. A., and Hodges, M. R. (2013) Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* **368**, 1685-1694
6. Qiu, Z., and Dai, Y. (2014) Roadmap of miR-122-related clinical application from bench to bedside. *Expert Opin Investig Drugs* **23**, 347-355
7. Misso, G., Di Martino, M. T., De Rosa, G., Farooqi, A. A., Lombardi, A., Campani, V., Zarone, M. R., Gulla, A., Tagliaferri, P., Tassone, P., and Caraglia, M. (2014) Mir-34: a new weapon against cancer? *Mol Ther Nucleic Acids* **3**, e194
8. Raver-Shapira, N., Marciano, E., Meiri, E., Spector, Y., Rosenfeld, N., Moskovits, N., Bentwich, Z., and Oren, M. (2007) Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol. Cell* **26**, 731-743
9. Agostini, M., and Knight, R. A. (2014) miR-34: from bench to bedside. *Oncotarget* **5**, 872-881
10. Mendis, S., Davis, S., and Norrving, B. (2015) Organizational update: the world health organization global status report on noncommunicable diseases 2014; one more landmark step in the combat against stroke and vascular disease. *Stroke* **46**, e121-122
11. Ross, R. (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809
12. Singh, R. B., Mengi, S. A., Xu, Y. J., Arneja, A. S., and Dhalla, N. S. (2002) Pathogenesis

of atherosclerosis: A multifactorial process. *Exp. Clin. Cardiol.* **7**, 40-53

13. Gotto, A. M., Jr. (2011) Jeremiah Metzger Lecture: cholesterol, inflammation and atherosclerotic cardiovascular disease: is it all LDL? *Trans. Am. Clin. Climatol. Assoc.* **122**, 256-289

14. Holvoet, P., Harris, T. B., Tracy, R. P., Verhamme, P., Newman, A. B., Rubin, S. M., Simonsick, E. M., Colbert, L. H., and Kritchevsky, S. B. (2003) Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1444-1448

15. Choumerianou, D. M., and Dedoussis, G. V. (2005) Familial hypercholesterolemia and response to statin therapy according to LDLR genetic background. *Clin Chem Lab Med* **43**, 793-801

16. Alkindi, M., Siminovitch, K. A., Gupta, M., and Genest, J. (2016) Monoclonal Antibodies for the Treatment of Hypercholesterolemia: Targeting PCSK9. *Can J Cardiol*

17. Rader, D. J., and Kastelein, J. J. (2014) Lomitapide and mipomersen: two first-in-class drugs for reducing low-density lipoprotein cholesterol in patients with homozygous familial hypercholesterolemia. *Circulation* **129**, 1022-1032

18. Raal, F. J., Honarpour, N., Blom, D. J., Hovingh, G. K., Xu, F., Scott, R., Wasserman, S. M., Stein, E. A., and Investigators, T. (2015) Inhibition of PCSK9 with evolocumab in homozygous familial hypercholesterolaemia (TESLA Part B): a randomised, double-blind, placebo-controlled trial. *Lancet* **385**, 341-350

19. Walsh, M. T., and Hussain, M. M. (2016) Targeting microsomal triglyceride transfer protein and lipoprotein assembly to treat homozygous familial hypercholesterolemia. *Crit. Rev. Clin. Lab. Sci.*, 1-23

20. Hussain, M. M., Shi, J., and Dreizen, P. (2003) Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J. Lipid Res.* **44**, 22-32

21. Davidson, N. O., and Shelness, G. S. (2000) APOLIPOPROTEIN B: mRNA editing, lipoprotein assembly, and presecretory degradation. *Annu. Rev. Nutr.* **20**, 169-193

22. Zimmermann, T. S., Lee, A. C., Akinc, A., et al. (2006) RNAi-mediated gene silencing in non-human primates. *Nature* **441**, 111-114

23. Nakayama, T., Butler, J. S., Sehgal, A., et al. (2012) Harnessing a physiologic mechanism

for siRNA delivery with mimetic lipoprotein particles. *Mol. Ther.* **20**, 1582-1589

24. Tadin-Strapps, M., Peterson, L. B., Cumiskey, A. M., et al. (2011) siRNA-induced liver ApoB knockdown lowers serum LDL-cholesterol in a mouse model with human-like serum lipids. *J Lipid Res* **52**, 1084-1097
25. Conlon, D. M., Thomas, T., Fedotova, T., Hernandez-Ono, A., Di Paolo, G., Chan, R. B., Ruggles, K., Gibeley, S., Liu, J., and Ginsberg, H. N. (2016) Inhibition of apolipoprotein B synthesis stimulates endoplasmic reticulum autophagy that prevents steatosis. *J. Clin. Invest.* **126**, 3852-3867
26. Gebhard, C., Huard, G., Kritikou, E. A., and Tardif, J. C. (2013) Apolipoprotein B antisense inhibition--update on mipomersen. *Curr Pharm Des* **19**, 3132-3142
27. Bell, D. A., Hooper, A. J., and Burnett, J. R. (2011) Mipomersen, an antisense apolipoprotein B synthesis inhibitor. *Expert Opin Investig Drugs* **20**, 265-272
28. Crooke, S. T., and Geary, R. S. (2013) Clinical pharmacological properties of mipomersen (Kynamro), a second generation antisense inhibitor of apolipoprotein B. *Br J Clin Pharmacol* **76**, 269-276
29. Hashemi, N., Odze, R. D., McGowan, M. P., Santos, R. D., Stroes, E. S., and Cohen, D. E. (2014) Liver histology during Mipomersen therapy for severe hypercholesterolemia. *J. Clin. Lipidol.* **8**, 606-611
30. Letteron, P., Sutton, A., Mansouri, A., Fromenty, B., and Pessayre, D. (2003) Inhibition of microsomal triglyceride transfer protein: another mechanism for drug-induced steatosis in mice. *Hepatology* **38**, 133-140
31. Joy, T. R., and Hegele, R. A. (2008) Microsomal triglyceride transfer protein inhibition-friend or foe? *Nat. Clin. Pract. Cardiovasc. Med.* **5**, 506-508
32. Hussain, M. M., and Bakillah, A. (2008) New approaches to target microsomal triglyceride transfer protein. *Curr. Opin. Lipidol.* **19**, 572-578
33. Zhou, L., Irani, S., Sirwi, A., and Hussain, M. M. (2016) MicroRNAs regulating apolipoprotein B-containing lipoprotein production. *Biochim. Biophys. Acta*
34. Irani, S., and Hussain, M. M. (2015) Role of microRNA-30c in lipid metabolism, adipogenesis, cardiac remodeling and cancer. *Curr. Opin. Lipidol.* **26**, 139-146
35. Esau, C., Davis, S., Murray, S. F., et al. (2006) miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* **3**, 87-98

36. Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H. F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E. M., and Kauppinen, S. (2008) LNA-mediated microRNA silencing in non-human primates. *Nature* **452**, 896-899
37. Lanford, R. E., Hildebrandt-Eriksen, E. S., Petri, A., Persson, R., Lindow, M., Munk, M. E., Kauppinen, S., and Orum, H. (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**, 198-201
38. Tsai, W. C., Hsu, S. D., Hsu, C. S., et al. (2012) MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J. Clin. Invest.* **122**, 2884-2897
39. Hsu, S. H., Wang, B., Kota, J., et al. (2012) Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J. Clin. Invest.* **122**, 2871-2883
40. Xu, Y., Zalzala, M., Xu, J., Li, Y., Yin, L., and Zhang, Y. (2015) A metabolic stress-inducible miR-34a-HNF4alpha pathway regulates lipid and lipoprotein metabolism. *Nat Commun* **6**, 7466
41. Irani, S., Pan, X., Peck, B. C., Iqbal, J., Sethupathy, P., and Hussain, M. M. (2016) MicroRNA-30c Mimic Mitigates Hypercholesterolemia and Atherosclerosis in Mice. *J. Biol. Chem.*
42. Soh, J., Iqbal, J., Queiroz, J., Fernandez-Hernando, C., and Hussain, M. M. (2013) MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. *Nat. Med.* **19**, 892-900
43. Rayner, K. J., Suarez, Y., Davalos, A., Parathath, S., Fitzgerald, M. L., Tamehiro, N., Fisher, E. A., Moore, K. J., and Fernandez-Hernando, C. (2010) MiR-33 contributes to the regulation of cholesterol homeostasis. *Science* **328**, 1570-1573
44. Fernandez-Hernando, C., Suarez, Y., Rayner, K. J., and Moore, K. J. (2011) MicroRNAs in lipid metabolism. *Curr. Opin. Lipidol.* **22**, 86-92
45. Marquart, T. J., Allen, R. M., Ory, D. S., and Baldan, A. (2010) miR-33 links SREBP-2 induction to repression of sterol transporters. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 12228-12232
46. Izaurralde, E. (2015) GENE REGULATION. Breakers and blockers-miRNAs at work. *Science* **349**, 380-382
47. Koornneef, A., Maczuga, P., van Logtenstein, R., Borel, F., Blits, B., Ritsema, T., van

Deventer, S., Petry, H., and Konstantinova, P. (2011) Apolipoprotein B knockdown by AAV-delivered shRNA lowers plasma cholesterol in mice. *Mol Ther* **19**, 731-740

48. Sharpe, L. J., and Brown, A. J. (2013) Controlling cholesterol synthesis beyond 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). *J. Biol. Chem.* **288**, 18707-18715
49. Cooke, M., Orlando, U., Maloberti, P., Podesta, E. J., and Cornejo Maciel, F. (2011) Tyrosine phosphatase SHP2 regulates the expression of acyl-CoA synthetase ACSL4. *J. Lipid Res.* **52**, 1936-1948
50. Golej, D. L., Askari, B., Kramer, F., Barnhart, S., Vivekanandan-Giri, A., Pennathur, S., and Bornfeldt, K. E. (2011) Long-chain acyl-CoA synthetase 4 modulates prostaglandin E(2) release from human arterial smooth muscle cells. *J. Lipid Res.* **52**, 782-793
51. Soh, J., and Hussain, M. M. (2013) Supplementary site interactions are critical for the regulation of microsomal triglyceride transfer protein by microRNA-30c. *Nutr. Metab. (Lond.)* **10**, 56
52. Piriyapongsa, J., and Jordan, I. K. (2007) A family of human microRNA genes from miniature inverted-repeat transposable elements. *PLoS One* **2**, e203
53. Ouimet, M., Ediriweera, H. N., Gundra, U. M., et al. (2015) MicroRNA-33-dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. *J. Clin. Invest.* **125**, 4334-4348
54. Hsu, S. H., Delgado, E. R., Otero, P. A., Teng, K. Y., Kutay, H., Meehan, K. M., Moroney, J. B., Monga, J. K., Hand, N. J., Friedman, J. R., Ghoshal, K., and Duncan, A. W. (2016) MicroRNA-122 regulates polyploidization in the murine liver. *Hepatology* **64**, 599-615
55. Hu, H. Y., He, L., Fominykh, K., Yan, Z., Guo, S., Zhang, X., Taylor, M. S., Tang, L., Li, J., Liu, J., Wang, W., Yu, H., and Khaitovich, P. (2012) Evolution of the human-specific microRNA miR-941. *Nat Commun* **3**, 1145
56. Hu, H. Y., Guo, S., Xi, J., Yan, Z., Fu, N., Zhang, X., Menzel, C., Liang, H., Yang, H., Zhao, M., Zeng, R., Chen, W., Paabo, S., and Khaitovich, P. (2011) MicroRNA expression and regulation in human, chimpanzee, and macaque brains. *PLoS Genet* **7**, e1002327
57. Hu, B., Ying, X., Wang, J., et al. (2014) Identification of a tumor-suppressive human-specific microRNA within the FHIT tumor-suppressor gene. *Cancer Res.* **74**, 2283-2294
58. Sato, C., and Kitajima, K. (2013) Impact of structural aberrancy of polysialic acid and its

synthetic enzyme ST8SIA2 in schizophrenia. *Front Cell Neurosci* **7**, 61

59. Simha, V., and Garg, A. (2006) Lipodystrophy: lessons in lipid and energy metabolism. *Curr. Opin. Lipidol.* **17**, 162-169

Highlights:

- Hsa-miR-548p reduces apoB-containing lipoprotein secretion in human liver cells.
- MiR-548p interacts with apoB 3´-UTR involving both seed and supplementary sequences at Site I.
- MiR-548p decreases lipid synthesis in human hepatoma cells by suppressing the expression levels of ACSL4 and HMGCR.
- MiR-548p does not alter apoB expression levels in mouse liver cells.

Figure Legends

Figure 1. MiR-548p decreases cellular and secreted apoB from human liver cells without affecting apoAI secretion. A. TargetScan 6.2 was used to predict putative miRs that could target apoB 3´-UTR. The miR-548p seed sequence interacting site in apoB mRNA is well conserved in primates, including human, chimpanzee and rhesus monkeys, but not in mouse and rat. **B**. Different amounts of Ctrl, miR-548p, and anti-548p (100 nM) were reverse transfected in Huh-7 cells. Cellular apoB, as well as media apoB and apoAI were quantified using ELISA 48 hours after transfection. The data are representative of two independent experiments. **C.** Time course experiments in Ctrl, miR-548p, and anti-548p (100 nM) transfected Huh-7 cells. Media and cell lysates were collected at indicate time points to measure secreted and cellular apoB and apoAI. **D**. MiR-548p (100 nM) decreased apoB secretion in HepG2 cells without affecting apoAI. ApoB and apoAI in cells and media were measured 48 hours after transfection. The data are representative of two independent experiments. **E.** MiR-548p and miR-30c (100 nM) decreased apoB secretion in human primary hepatocytes without affecting apoAI. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significance was calculated by two-way ANOVA and student's t-test.

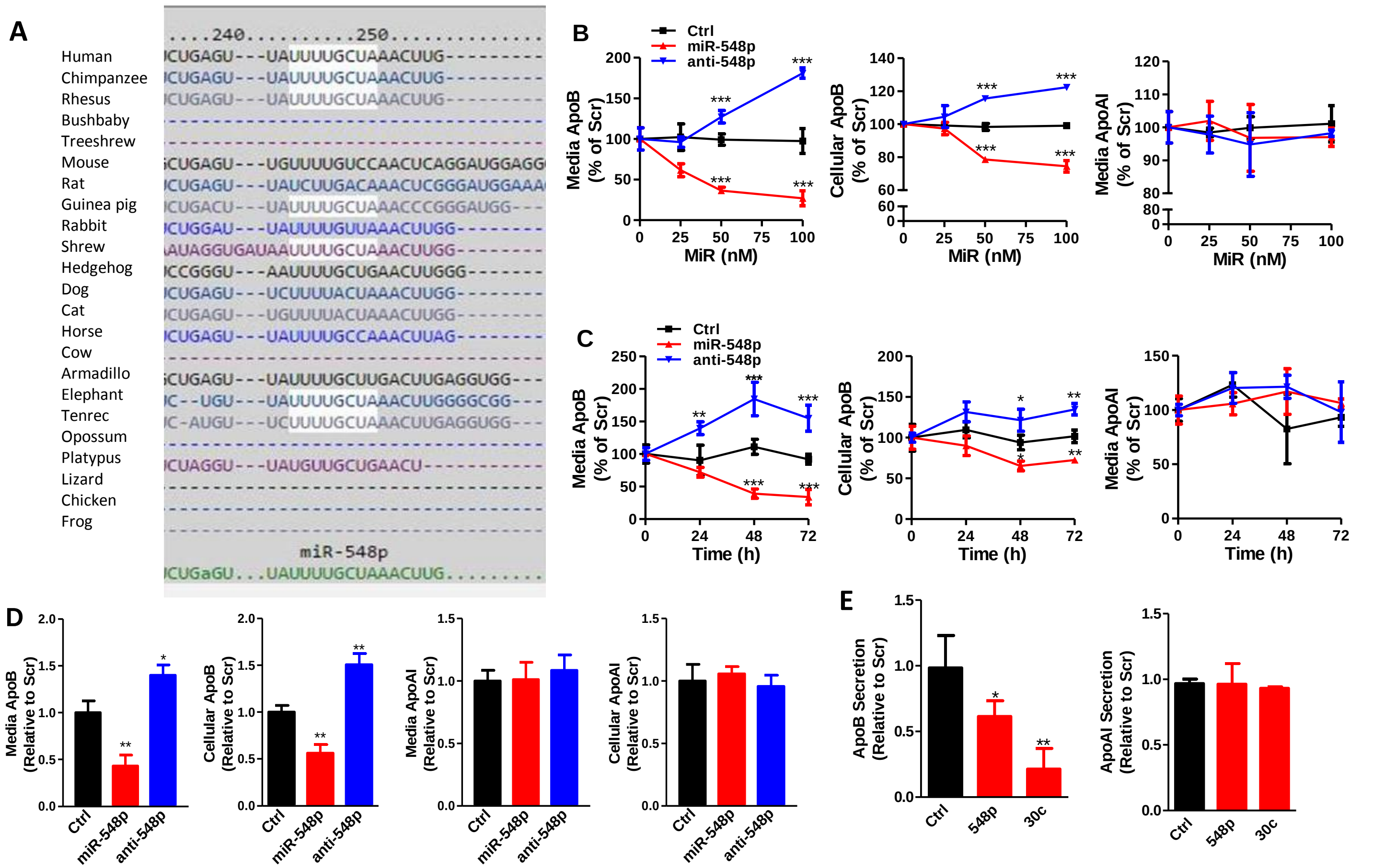
Figure 2. MiR-548p reduces apoB mRNA levels in human primary hepatocytes and Huh7 cells. **A.** In the human primary hepatocytes, miR-548p (100 nM) reduced apoB mRNA levels, miR-30c (100 nM) reduced MTP mRNA levels. **B.** MiR-548p and anti-miR-548p (100 nM) decreased and increased apoB mRNA levels repectively in Huh-7 cells. The data are representative of two independent experiments. **C.** MiR-548p or anti-miR-548p (100 nM) has no effect on mouse apoB in mouse primary hepatocytes. **D.** MiR-548p (100 nM) has no effect on mouse apoB in AML12 cells. The data are representative of two independent experiments. The mRNA expression levels of apoB, MTP or apoAI were quantified after 48 h of transfection. Relative mRNA levels were normalized to 18S. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significance was calculated by student's t-test.

Figure 3. MiR-548p interacts with 3´-UTR of apoB mRNA to promote posttranscriptional degradation. A. Huh-7 cells were transfected with Ctrl or miR-548p (100 nM). After 24 hours, cells were incubated with actinomycin D (1 μg/ml, from 1mg/ml stock). ApoB mRNA levels at different time points were normlaized using 18S as internal control. The apoB mRNA levels relative to time point 0 wereplotted. **B.** ApoB 3´-UTR was amplified from genomic DNA by PCR and cloned into psiCHECK2 vector at the end of Renilla luciferase cDNA. **C**. Plasmids (1.5 μg) containing human apoB 3´-UTR were transfected in Huh-7 cells using transfection reagent Turbofect (Life Technologies). After 24 h, Ctrl, miR-548p and anti-548p (100 nM) were introduced into cells. After 48 h, dual luciferase activities were assayed. The data are representative of two independent experiments. **D.** Two potential targeting sites (Site I and Site II) were predicted by miRanda. ApoB 3´-UTR has complementary bases that could interact with both seed and supplementary sequences. Every seed and supplementary sequence was mutated using site-directed mutagenesis. **E**. Luciferase reporter plasmids (1.5 μg) containing wild type or different mutant human apoB 3´-UTR were transfected in Huh7 cells. Cells were then transfected with Scr or miR-548p (100 nM). Luciferase activity was measured after 24 h. The data are representative of two independent experiments. $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significance was calculated by two-way ANOVA and student's t-test.

Figure 4. MiR-548p decreases lipid synthesis and the expression of genes involved in cholesterol and fatty acid synthesis. (A-B) Overexpression of miR-548p had no effect on cellular lipid levels. Cellular triglyceride and cholesterol were extracted and quantified in Ctrl or miR-548p (100 nM) transfected Huh-7 cells. (**C-D)** MiR-548p (100 nM) reduced cholesterol and triglyceride synthesis without affecting fatty acid oxidation in Huh-7 cells. PL, phospholipid; FA, fatty acid; TG, triglyceride; CE cholesteryl ester. The data are representative of three independent experiments. (**E)**

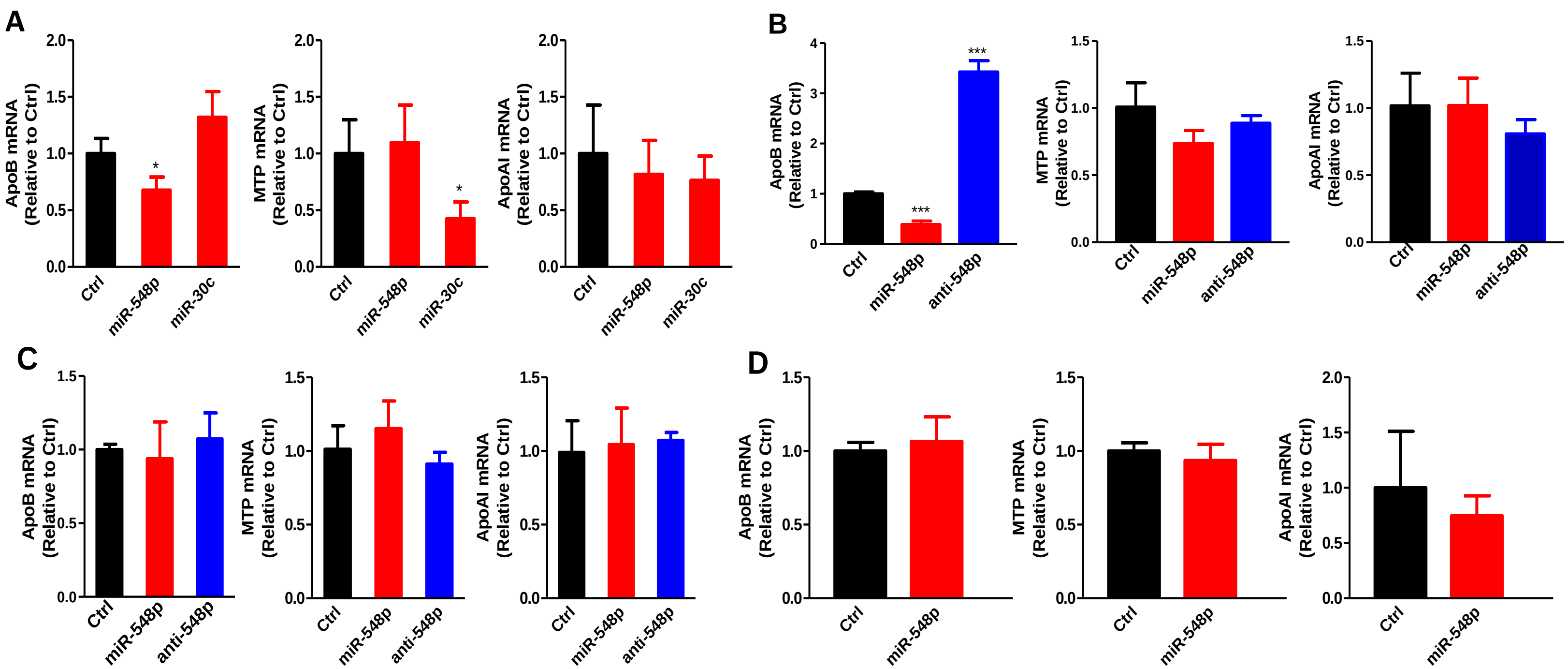
Genes in lipoprotein assembly, triglyceride, cholesterol, and fatty acid biosynthesis were quantified using quantitative RT-PCR. The data are representative of three independent experiments. (**F)** Western blots of GAPDH, ACSL4, and HMGCR in Ctrl or miR-548p (100 nM) transfected Huh-7 cells (left). Medium from transfected cells was used to immunoprecipitate apoE and PCSK9 and then subjected to western blotting (left). Specific protein bands were quantified using ImageJ and plotted as bar graphs (right). **(G)** Luciferase reporter plasmids (1.5 μg) containing human ACSL4 or HMBCR 3´-UTRs were transfected in Huh-7 cells. Cells were then distributed in different wells and transfected with Ctrl or miR-548p (100 nM). Luciferase activity was measured after 24 h. p<0.05, **p<0.01, ***p<0.001. Significance was calculated by student's t-test. The data are representative of two independent experiments.

Fig 1.



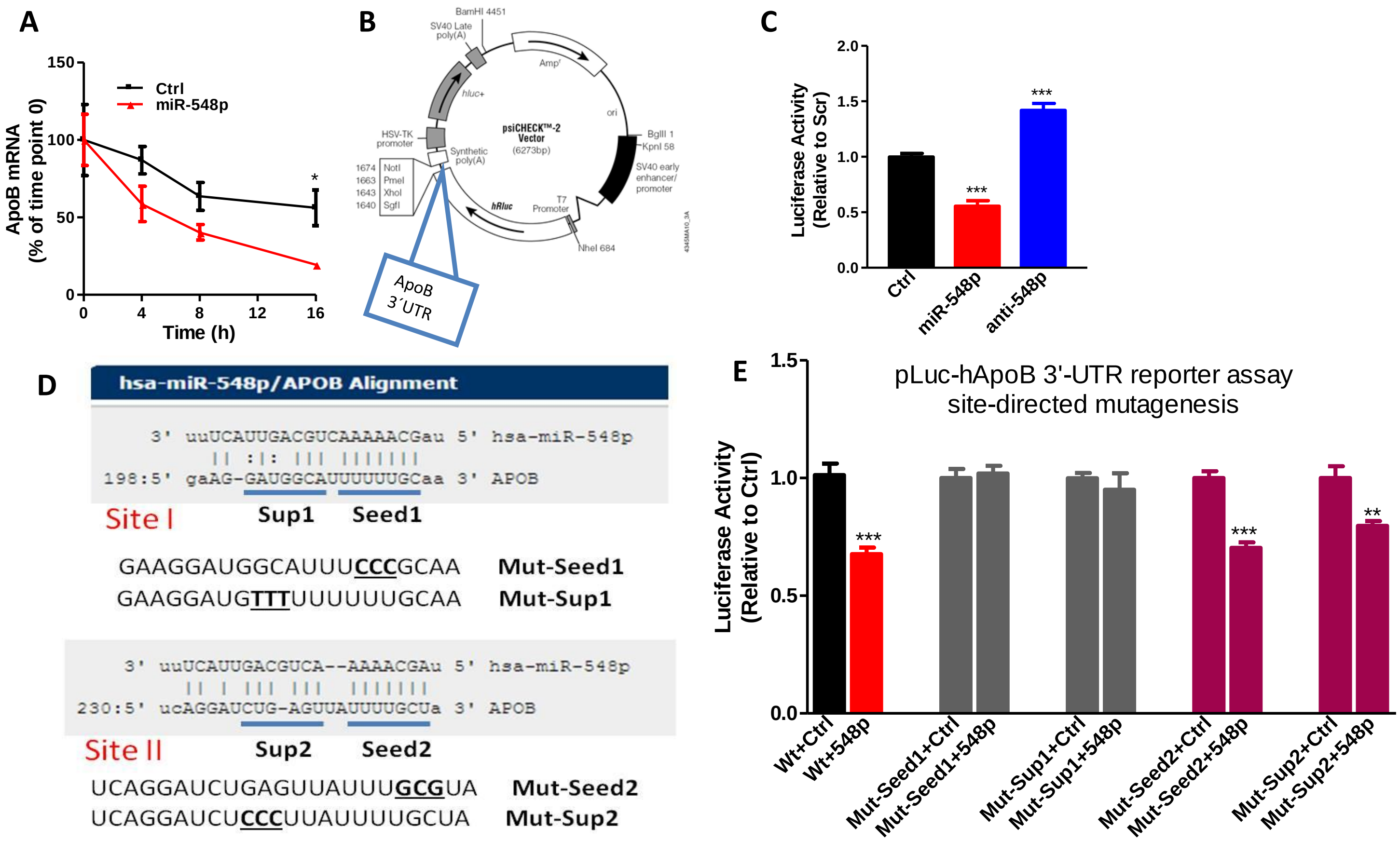
A
Human
Chimpanzee
Rhesus
Bushbaby
Treeshrew
Mouse
Rat
Guinea pig
Rabbit
Shrew
Hedgehog
Dog
Cat
Horse
Cow
Armadillo
Elephant
Tenrec
Opossum
Platypus
Lizard
Chicken
Frog
miR-548p
B
Ctrl
miR-548p
anti-548p
Media ApoB (% of Scr)
Cellular ApoB (% of Scr)
Media ApoAI (% of Scr)
MiR (nM)
C
Time (h)
D
Media ApoB (Relative to Scr)
Cellular ApoB (Relative to Scr)
Media ApoAI (Relative to Scr)
Cellular ApoAI (Relative to Scr)
E
ApoB Secretion (Relative to Scr)
ApoAI Secretion (Relative to Scr)
548p
30c

Fig 2.



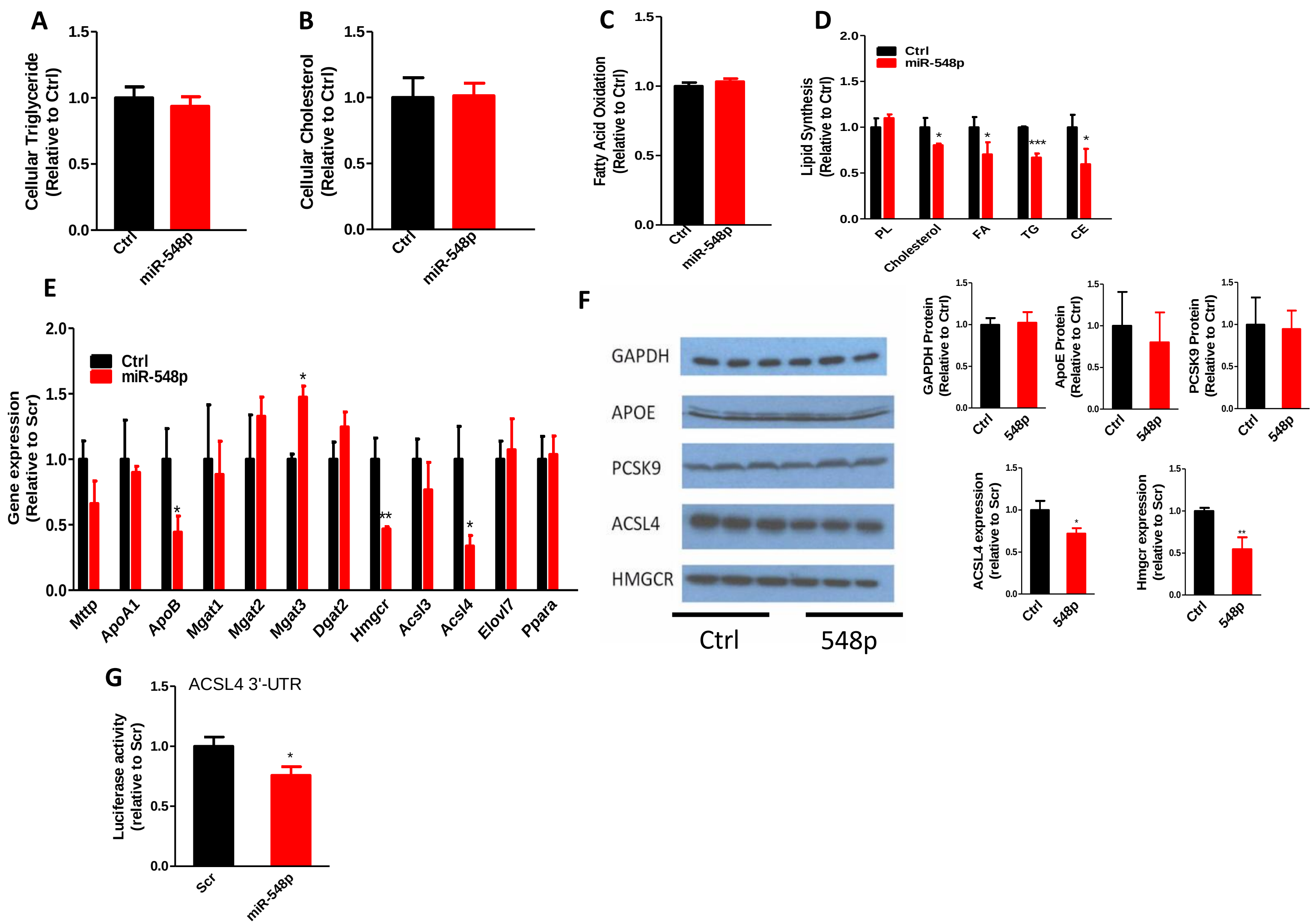
A
ApoB mRNA
(Relative to Ctrl)
MTP mRNA
(Relative to Ctrl)
ApoAI mRNA
(Relative to Ctrl)
Ctrl
miR-548p
miR-30c
*
B
anti-548p
***
C
D

Fig 3.

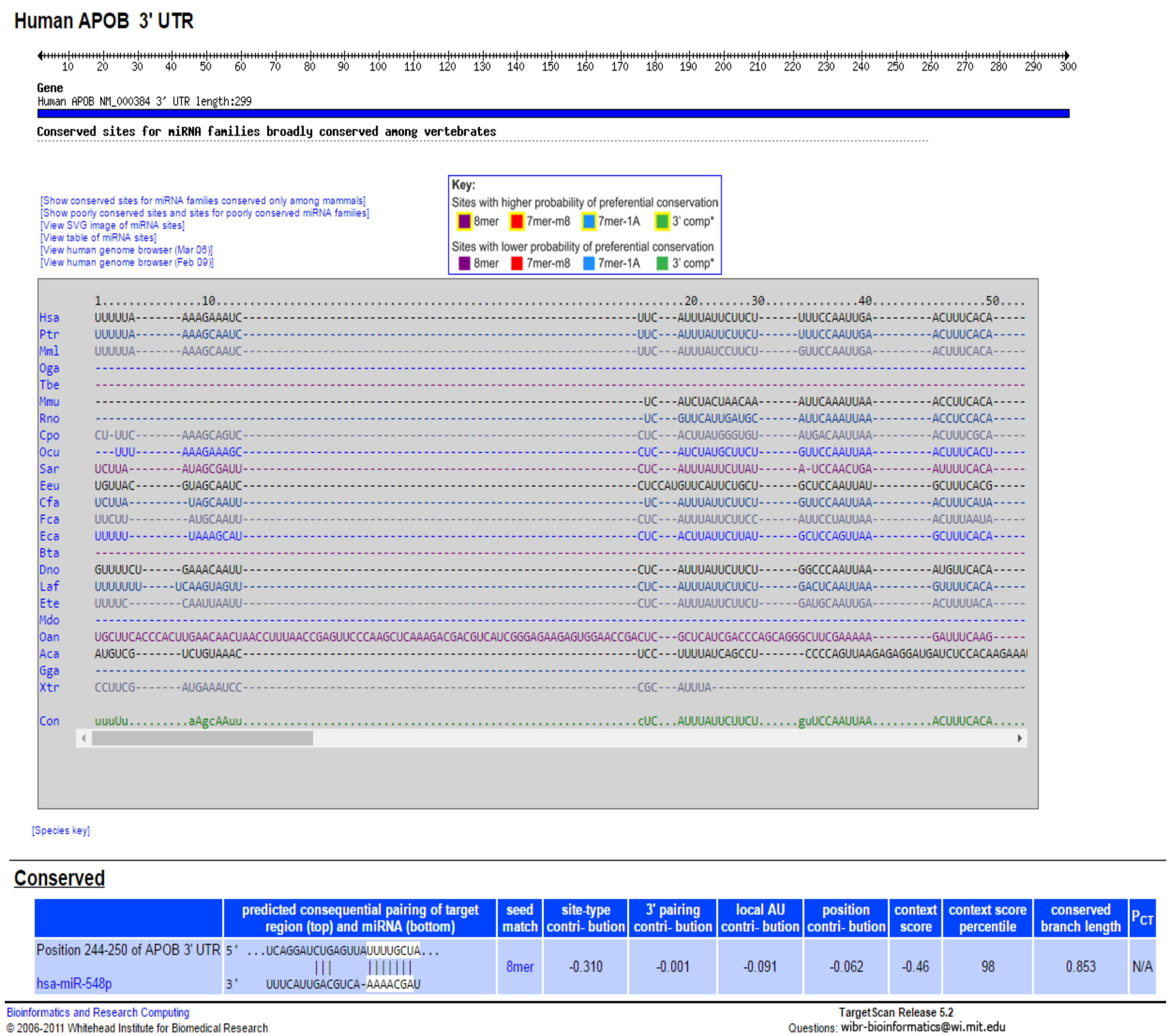


A
ApoB mRNA
(% of time point 0)
150
100
50
0
Ctrl
miR-548p
*
0
4
8
12
16
Time (h)
B
BamHI 4451
SV40 Late poly(A)
Amp
hluc+
psiCHECK™-2 Vector
(6273bp)
ori
HSV-TK promoter
Synthetic poly(A)
BglII 1
KpnI 58
SV40 early enhancer/ promoter
1674 NotI
1663 PmeI
1643 XhoI
1640 SgfI
hRluc
T7 Promoter
NheI 684
ApoB 3´UTR
C
Luciferase Activity
(Relative to Scr)
2.0
1.5
1.0
0.5
0.0
***
***
Ctrl
miR-548p
anti-548p
D
hsa-miR-548p/APOB Alignment
3' uuUCAUUGACGUCAAAAACGau 5' hsa-miR-548p
198:5' gaAG-GAUGGCAUUUUUUGCaa 3' APOB
Site I
Sup1
Seed1
GAAGGAUGGCAUUUCCCGCAA Mut-Seed1
GAAGGAUGTTTUUUUUUGCAA Mut-Sup1
3' uuUCAUUGACGUCA--AAAACGAu 5' hsa-miR-548p
230:5' ucAGGAUCUG-AGUUAUUUUGCUa 3' APOB
Site II
Sup2
Seed2
UCAGGAUCUGAGUUAUUUGCGUA Mut-Seed2
UCAGGAUCUCCCUUAUUUUGCUA Mut-Sup2
E
pLuc-hApoB 3'-UTR reporter assay
site-directed mutagenesis
Luciferase Activity
(Relative to Ctrl)
1.5
1.0
0.5
0.0
***
***
**
Wt+Ctrl
Wt+548p
Mut-Seed1+Ctrl
Mut-Seed1+548p
Mut-Sup1+Ctrl
Mut-Sup1+548p
Mut-Seed2+Ctrl
Mut-Seed2+548p
Mut-Sup2+Ctrl
Mut-Sup2+548p

Fig 4.



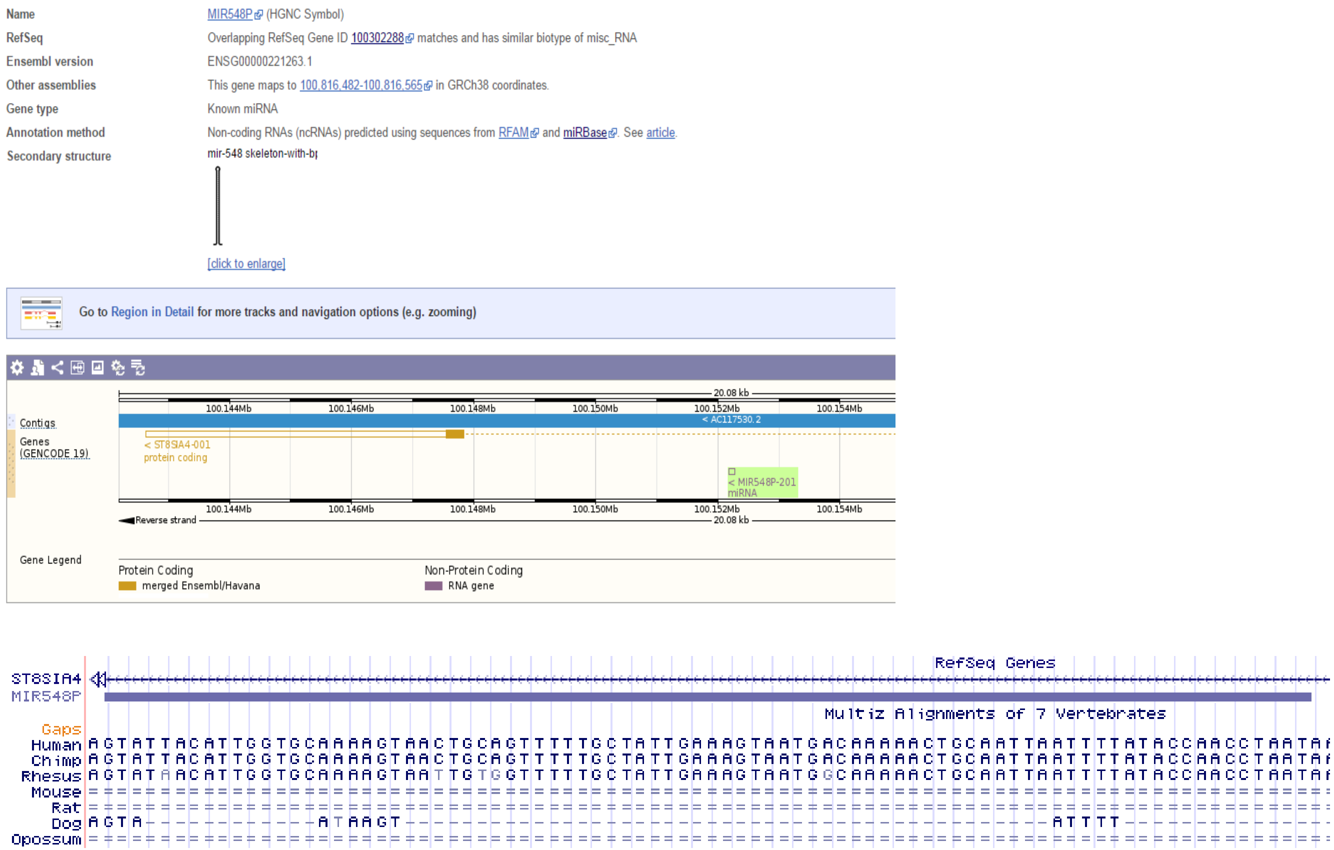
A
Cellular Triglyceride (Relative to Ctrl)
Ctrl
miR-548p
B
Cellular Cholesterol (Relative to Ctrl)
C
Fatty Acid Oxidation (Relative to Ctrl)
D
Lipid Synthesis (Relative to Ctrl)
PL
Cholesterol
FA
TG
CE
E
Gene expression (Relative to Scr)
Mttp
ApoA1
ApoB
Mgat1
Mgat2
Mgat3
Dgat2
Hmgcr
Acsl3
Acsl4
Elovl7
Ppara
F
GAPDH
APOE
PCSK9
ACSL4
HMGCR
Ctrl
548p
GAPDH Protein (Relative to Ctrl)
ApoE Protein (Relative to Ctrl)
PCSK9 Protein (Relative to Ctrl)
ACSL4 expression (relative to Scr)
Hmgcr expression (relative to Scr)
G
ACSL4 3'-UTR
Luciferase activity (relative to Scr)
Scr
miR-548p



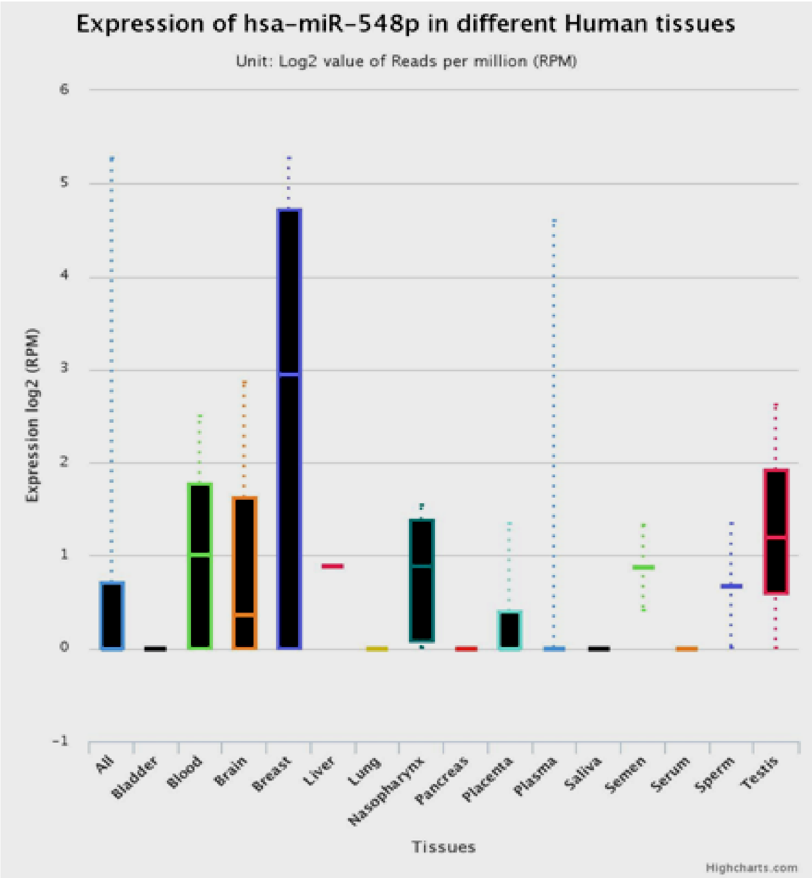
Supplementary Fig I. Interrogation of Target Scan 5.2 for potential miRs that could regulate *Apob* identified hsa-miR-548p as a conserved miR that could potentially target *Apob* mRNA.

A

B



Supplementary Fig II. **A**, miR-548p is located on human chromosome 5 (location; [5: 100,152,186-100,152,269]). It is present on the reverse strand of ST8SIA4-001 protein coding strand and is predicted to be a non-coding RNA with a stem-loop secondary structure. **B**, conservation of pre-miR-548p in primates.



Supplementary Fig III. Tissue distribution of endogenous miR-548p in human, from miRmine.

Chapter 3

The following chapter is a manuscript in preparation. Author contributions to the manuscript are defined below:

Liye Zhou – I performed and analyzed all experiments presented within this manuscript.

Sara Irani – Ms. Irani was helpful in microRNA quantification in tissues and cells.

M. Mahmood Hussain – Dr. Hussain is the primary investigator in this study and provided the financial and idea leadership and direction for the work presented in this manuscript

Identification of miR-1200 differentially regulating apoB and apoAI secretion and lipid synthesis from a High Throughput Functional Screening of Human MicroRNAs

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Abbreviations used: Apo, apolipoprotein; BCL11B, B Cell Lymphoma 11B; miR, microRNA; LDL, low density lipoproteins; HDL, high density lipoproteins; MTP, microsomal triglyceride transfer protein; ABCA1, ATP-binding cassette transporter family A and protein 1; UTR, untranslated region;

Keywords: ApoB, apoAI, cholesterol, fatty acid, microRNA, atherosclerosis, dyslipidemia,

Abstract

High plasma low density (LDL) and low high density lipoprotein (HDL) cholesterol are well-established risk factors for CVD. Therefore lowering LDL and increasing HDL cholesterol levels is one of the major strategies in treating or preventing CVD. MicroRNA, as a class of small non-coding RNAs, have been shown to be important regulators in different biological processes, including lipoprotein metabolism. To identify miRs that could regulate the entrance of apoB-containing lipoproteins and HDL to blood stream from the liver, I used a human microRNA library containing 1237 miRs for a functional screening in human hepatoma Huh-7 cell line. From these miRs, 28 and 13 miRs decreased or increased apoB secretion, respectively. Additionally, 23 and 11 miRs decreased or increased apoAI secretion, respectively. Among these miRs, I found that miRs-1200, 634, and 509 simultaneously decreased apolipoprotein (apo) B (LDL structural protein) and increased apoAI (main HDL protein) secretion.

I concentrated on miR-1200 for its potency on lipoprotein secretion regulation. Mechanistically, miR-1200 reduced apoB expression by targeting the 3'-untranslated region of mRNA and enhancing posttranscriptional degradation. Conversely, miR-1200 increased apoAI transcription by reducing a repressor, BCL11B. In addition, miR-1200 transfected hepatoma cells also showed significantly decreased cholesterol, triglyceride, and phospholipid synthesis rate.

In summary, these studies show that miR-1200 differentially regulates apoB and apoAI secretion, and reduces lipid synthesis in human hepatoma cells. The present study has shown the potential of miR-1200 being developed as a therapeutic agent for the treatment of dyslipidemia and atherosclerosis in humans.

Introduction

Despite significant advances in lowering risk factors, cardiovascular diseases (CVD) accounted for 30.8% of deaths in 2003-2013 in the United States. And the estimated annual cost of CVD and stroke for 2011-2012 was about $316.6 billion (1). Dyslipidemia, a major cause of CVD, is characterized by high LDL cholesterol and total triglyceride, as well as low HDL cholesterol. Human plasma cholesterol is mainly carried in the blood by apolipoprotein B (apoB)-containing low-density lipoproteins (LDL) and non-apoB-containing high-density lipoproteins (HDL). ApoB-containing lipoproteins are mainly synthesized and secreted by the liver and small intestine to transport lipids to other peripheral tissues. Excess accumulation of these lipoproteins and their modifications in the plasma contribute to atherosclerosis as these modified lipoproteins are taken up by macrophages (2, 3). ApoAI interacts with ATP-binding cassette transporter family A and protein 1 (ABCA1) present on the plasma membrane of different cells, especially macrophages, extracts cholesterol and transports to the liver for excretion from the body (4). This reverse cholesterol transport (RCT) is believed to be anti-atherogenic (5). For these reasons, elevated LDL and low HDL cholesterol are two well-established risk factors for atherosclerosis.

Accumulation of LDL in plasma can be caused by either overproduction or impaired removal from the circulation. Many attempts have been made to control LDL-cholesterol. Targeting of MTP, a critical chaperon protein in the assembly of VLDL, efficiently reduces plasma cholesterol and atherosclerosis by inhibiting hepatic VLDL production (6, 7). However, inhibition of MTP causes hepatosteatosis and modest increase of ALT, AST activities in plasma. Statins, the most popular lipid lowering drug, lower plasma LDL-cholesterol by reducing hepatic cholesterol synthesis and increasing LDL clearance; however these drugs only decrease the disease by 30-40% (8), and almost 20% of the population does not respond or cannot tolerate statins (9-12). Further, high doses of statins sometimes cause muscle pain, elevations in plasma levels of liver and muscle enzymes, and new onset of diabetes mellitus (10, 11). Recently, PCSK9 inhibitors have also been shown to lower plasma cholesterol but they could be associated with neurocognitive side effects (12, 13). Both of these drugs work via increasing LDL receptor; therefore, they are not useful in the treatment of homozygous familial hypercholesterolemia subjects that are deficient in this receptor (10, 11, 13, 14). MTP inhibitor and antisense oligonucleotide against apoB, lomitapide and mipomersen, have been approved for the treatment of these patients, even though they cause steatosis (15).

HDL has different vascular protective functions, such as anti-inflammation, anti-oxidation, reverse cholesterol transport. Similar with LDL, higher HDL can be achieved from increasing production rate or decreasing its catabolism. Overexpression of human apoAI using adenovirus significantly reduced atherosclerotic plaque burden in apoE or LDLR knockout mice fed on high-cholesterol diet (16-18). ABCA1 interacts with apoAI and extracts lipids from tissues for HDL biogenesis and reverse cholesterol transport. Patients with loss-of-function mutations of ABCA1 are carriers of Tangier disease. These patients suffer from completely absence of HDL and premature atherosclerosis (19). SR-BI is a major HDL receptor highly expressed on the surface of hepatocytes for the clearance of HDL from circulation. Hepatic overexpression of SR-BI resulted in dramatically decreased HDL and less arthrosclerosis in mice by increasing reverse cholesterol transport (20, 21). However, previous studies have shown that SR-BI deficient mice and human carriers of loss-of-function mutations are pro-atherogenic with extremely high plasma HDL cholesterol levels (22-26). These studies have shown that high HDL cholesterol levels caused by impaired function of SR-BI is not associated with lower risk of cardiovascular disease. The inhibition of CETP increases HDL and decreases LDL by blocking the exchange of TG in VLDL for cholesteryl ester in HDL, however fails to reduce cardiovascular disease events (27). Therefore, it is reasonable to hypothesize that modulating the biosynthesis of HDL is more likely to achieve anti-atherosclerotic effects. So far, no effective therapeutic methods are available to increase functional HDL to prevent CVD (28). Thus, there is still a need to find novel therapeutic agents that modulate plasma LDL and HDL for beneficial outcomes without causing adverse effects.

MicroRNAs (miRs) are small (~22 nucleotides) non-coding RNAs. MiRs target multiple genes and affect multiple pathways by interacting with the 3′-untranslated region (3′-UTR) of mRNA and destabilizing mRNA or blocking translation. In >70% cases, miRs induce mRNA degradation (29). MiRs bind to the target mRNA via seed and supplementary sequences. Seed sequence (2-7 nucleotides from the 5'-end of the miR) forms perfect complementary base pairs while the supplementary site in the 3'-region may or may not form perfect base pairs with the target mRNA(30, 31). MiRs with the same seed sequence belong to the same family. MiR-30c and miR-33 have been identified to decrease LDL and HDL, respectively (32-34). MiR-148a consistently decreased HDL but had variable effects on plasma LDL levels (35, 36). However, miRs that concurrently decrease LDL and increase HDL are unknown; such miRs might be more efficacious in treating atherosclerosis.

Liver is the major organ producing both apoB-containing lipoprotein and HDL. I aimed to identify the miRs that can potentially regulate the secretion of apoB and apoAI from the liver to bloodstream. These miRs may be used as therapeutic agents to treat dyslipidemia and cardiovascular diseases. To achieve this goal, I performed an unbiased screening using a human miR mimic library in human hepatoma cell line. In this chapter, I established the method of *in vitro* large-scale screening using miR mimic transfection coupled with the measurement of apoB and apoAI secretion. The first and second screening results in identification of 75 miRs that potently regulate apoB or apoAI secretion from Huh-7 cells. MiR-1200, one of the dual regulators of apoB and apoAI secretion, was further studied to reveal its mechanism of action.

Results

Establishment and optimization of screen method and condition

To select a suitable cell model for this large-scale screen, I considered a series of factors. Our goal is to discover potential miRs that could regulate plasma lipids levels in human and may be used as therapies of dyslipidemia and cardiovascular disease. Therefore, screening a human miR library in a human cell line will give us relatively reliable information about the function of miRs in human. Liver is a major organ producing lipoproteins to circulation. In addition, for a screen containing more than 2000 transfection reactions, it is important to have cell model, which is easy to maintain and transfect. Therefore, I decided to use human hepatoma Huh-7 cell line to perform the screen. In preliminary studies, a siRNA against MTP and miR-30c were used for the optimization of reverse transfection procedure in a 96-well format, as these two short RNA are proofed to reduce apoB secretion (37). Also in every plate of library screening, miR-30c was included as a positive control for transfection efficiency. Further, each plate had wells transfected with scrambled miR (Scr) as a negative control. The screening was performed in duplicated plates.

High throughput screening in Huh-7 cells

Secreted apoB and apoAI levels were normalized relative to a Scr transfected negative control and plotted as percentages (**Fig 1**). The replicates in two independent plates showed very good reproducibility (Spearman r=0.96 for apoB and 0.92 for apoAI; **Fig 1**). In the first screening, 60 and 57 miRs decreased and increased apoB secretion by over 50%, and 34 and 38 miRs decreased and increased apoAI secretion by over 35% (**Table 1, Supplementary Table 1**). In addition, I observed that miRs in same families induced similar effect on apoB and apoAI secretion (**Fig 2**). 4 and 2 miR families were able to decreased apoB and apoAI secretion, respectively (**Fig 2**). Sharing the same seed sequence for mRNA targeting, a miR family is supposed to regulate a similar set of genes. Hence, this observation provided a good internal control for the reproducibility of the whole screening.

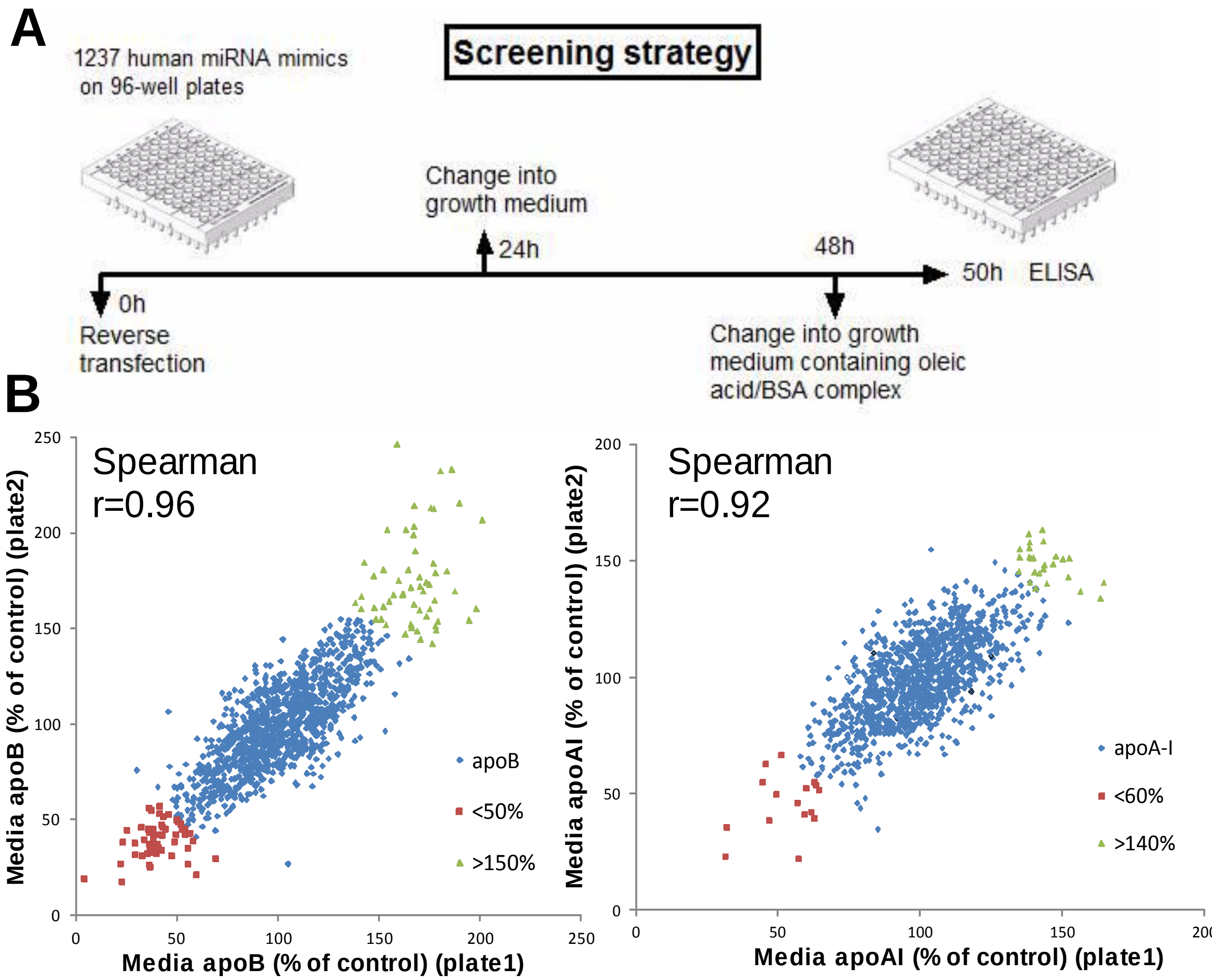


Figure 1: High throughput screening identifies miRNA regulating apoB and apoAI in Huh7 cells. **(A)** Huh-7 cells were reverse transfected in duplicate plates with a human miRDIAN mimic 16.0 library (Dharmacon) of 1237 miRs. After 24 hours, cells received complete media with 10% FBS. After another 24 hours, cells were incubated with complete media containing 10% FBS and OA/BSA complexes (0.4 mM/1.5%) for 2 hours to avoid identification of miRs that affect posttranslational degradation of apoB. Media apoB and apoAI were quantified by ELISA. For control, different wells in each plate were transfected with Scr (negative) or miR-30c (positive, reduces apoB). **(B)** Secretion of apoB and apoAI from Huh7 cells following treatment with 1237 miRsin two different plates.

After first screening, 102 miRs highly regulating apoB and apoAI secretion were repeated in triplicate for secondary screening. 75 miRs gave similar results as in the first screening; the other miRs that did not reproduce the same effect were categorized as false positive (**Fig 3-6**). Among the 75 miRs, 28 miR reduced apoB secretion; 13 miRs increased apoB secretion; 23 miRs reduced apoAI secretion; 11 miRs reduced apoAI secretion. Moreover, I found miRs-1200, 634, and 509 simultaneously decreased apoB and increasin apoAI secretion **(Fig 3, 6)**. I concentrated on miR-1200 because it decreased apoB secretion by 61±2%, and increased apoAI secretion by 54±17%.

ApoB Secretion	# of miRs	ApoAI Secretion	# of miRs
50% ↓	60	25% ↓	92
60% ↓	29	35% ↓	34
70% ↓	3	45% ↓	11
50% ↑	57	25% ↑	82
60% ↑	41	35% ↑	38
70% ↑	16	45% ↑	27

Table 1: Number of miRs that changed media apoB and apoAI to different extents in the second screening were tabulated.

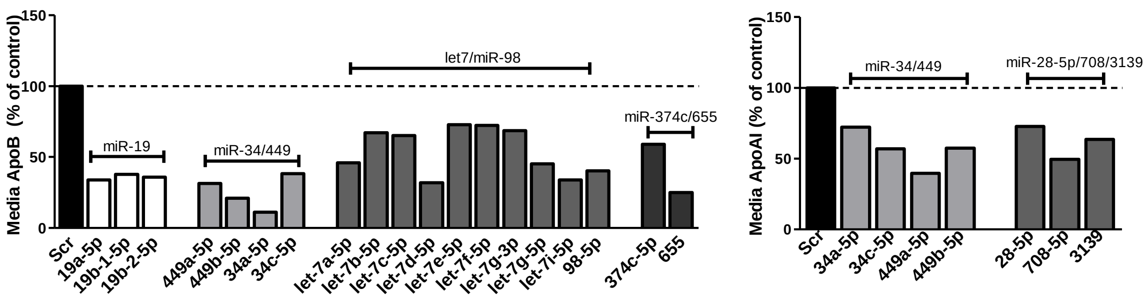


Fig 2: Different miR family members with the same seed sequence showed similar effects on media apoB and apoAI.

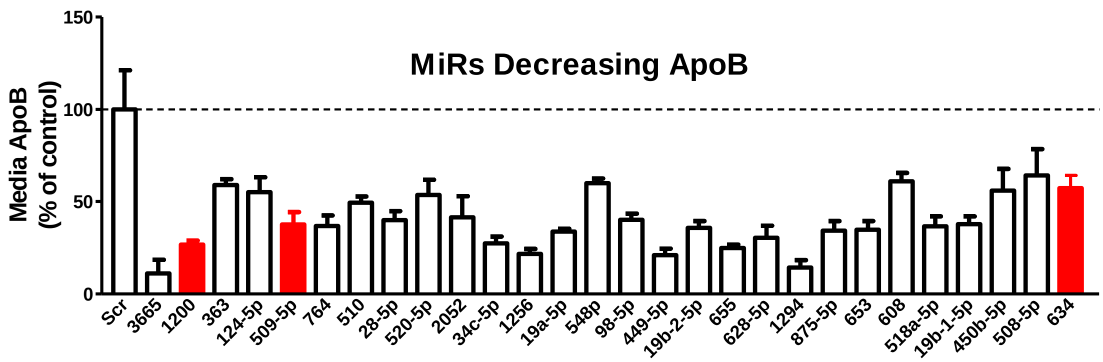


Fig 3: All the miRs that lowered apoB in the first screen, reduced media apoB in the second screen indicating no false positives.

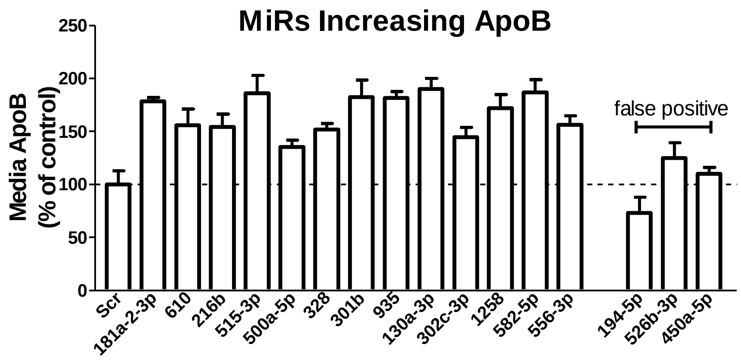


Fig 4: Three out of sixteen miRs did not significantly increase media apoB in the second screening representing false positives.

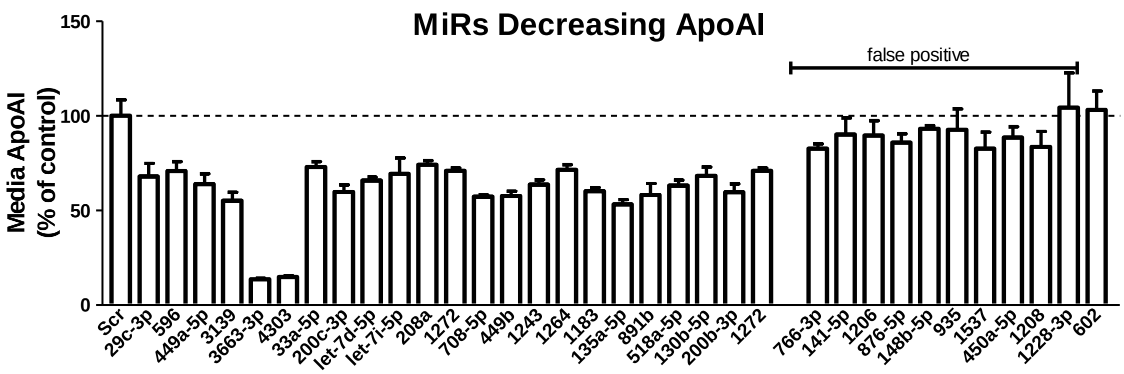


Fig 5: Secondary screening of miRs reducing apoAI revealed several false positives.

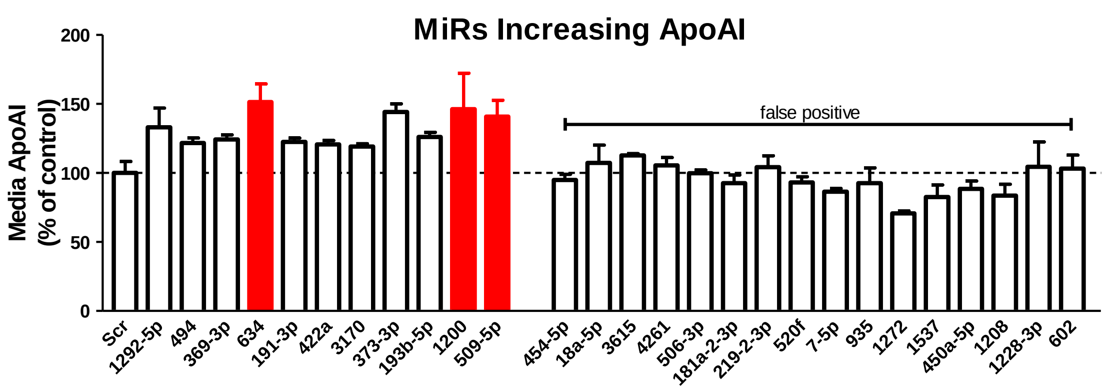


Fig 6: A higher numbers of miRs were false positives when miRs that increased apoAI in the first screen were tested again.

MiR-1200 decreases apoB secretion by enhancing posttranscriptional mRNA degradation

Hsa-miR-1200 is located in the 6^{th} intron of Engulfment and cell motility protein 1 (ELMO1) on human chromosome 7 and the precursor miR-1200 is conserved in primates, not in other species (**Fig 7**). To study its role, Huh-7 cells were transfected with miR-1200 to increase cellular concentrations (**Fig 8A**). MiR-1200 decreased media and cellular apoB in a dose-dependent manner (**Fig 8B, C**). Hairpin inhibitor of miR-1200, anti-1200, dose-dependently increased apoB suggesting that endogenous miR-1200 regulates apoB production (**Fig 8B, C**). The effects of miR-1200 and anti-1200 on cellular and media apoB were maximum at 48 hours post transfection (**Fig 8D, E**). These studies showed that miR-1200 reduces, whereas anti-1200 increases, cellular and media apoB.

Lower cellular apoB protein levels could be due to reductions in mRNA levels or in protein synthesis. Quantifications revealed that apoB mRNA levels were reduced in miR-1200 and increased in anti-1200 over-expressing cells suggesting that miR-1200 modulates mRNA levels (**Fig 8F**). To investigate how miR-1200 reduces apoB mRNA, I determined mRNA degradation after treating cells with actinomycin D to inhibit transcription. ApoB mRNA disappeared faster in miR-1200 expressing cells (**Fig 8G**) indicating that miR-1200 enhances posttranscriptional degradation.

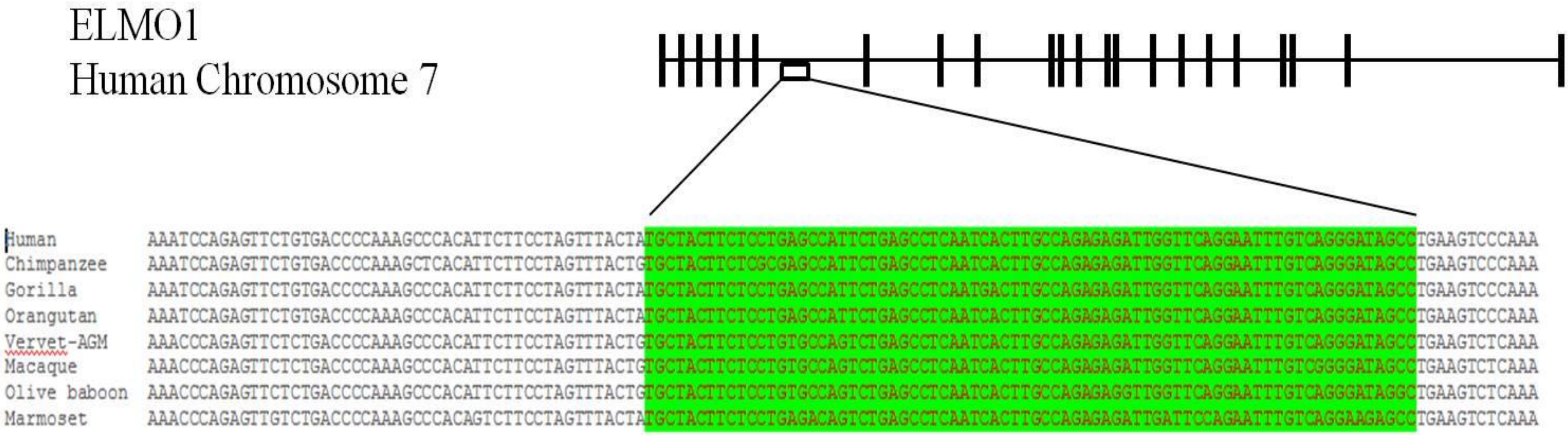
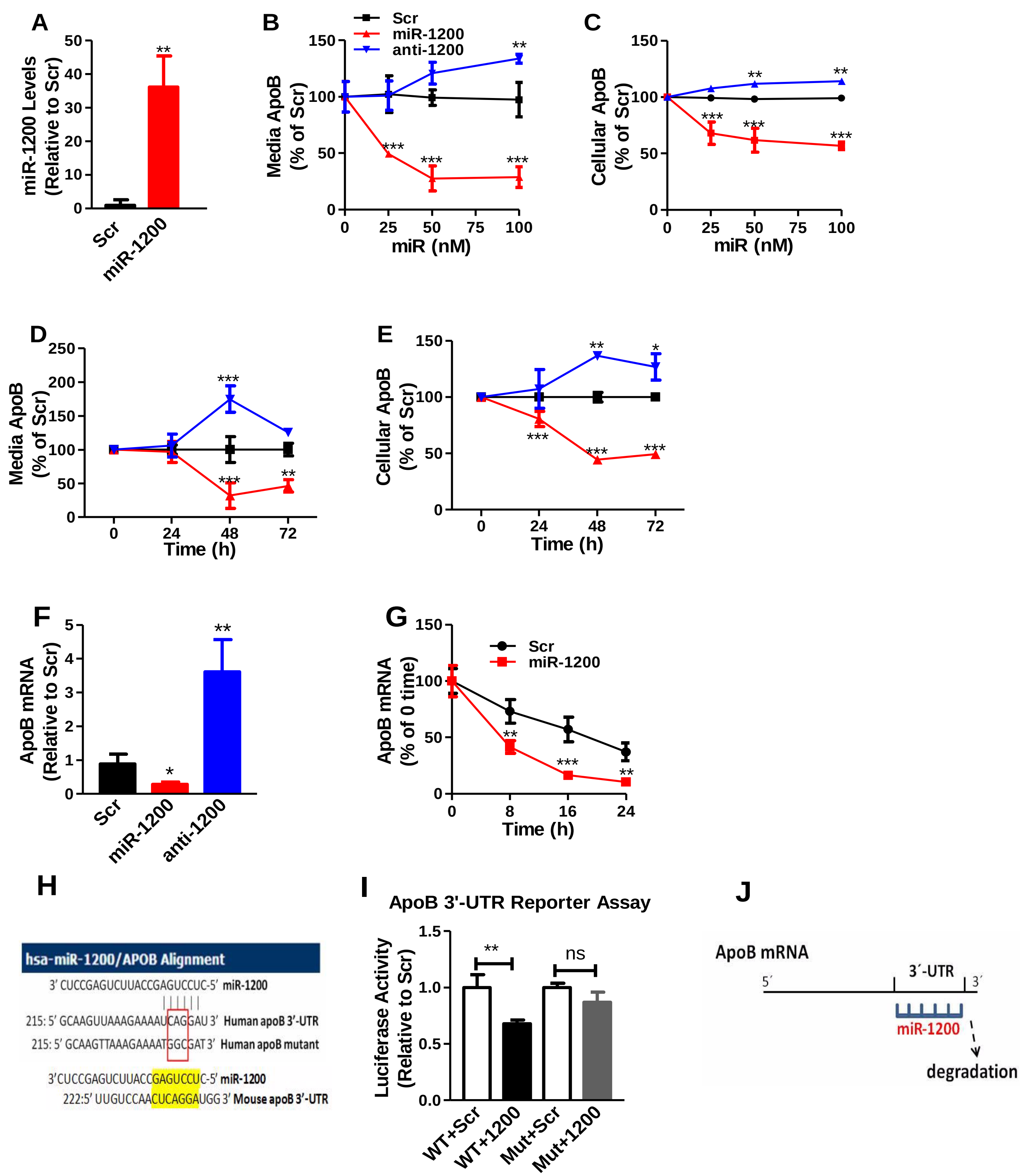


Figure 7. Hsa-miR-1200 is present in the intron of ELMO1 and is conserved in primates. Top line shows schematic representation of different introns and exons in the human ELMO1 gene. MiR-1200 resides in intron 6 of the gene. Pre-miR-1200 sequences are highly conserved in primates and highlighted with green after alignment using Clustral W.



A
miR-1200 Levels (Relative to Scr)
Scr
miR-1200
B
Media ApoB (% of Scr)
Scr
miR-1200
anti-1200
miR (nM)
C
Cellular ApoB (% of Scr)
miR (nM)
D
Media ApoB (% of Scr)
Time (h)
E
Cellular ApoB (% of Scr)
Time (h)
F
ApoB mRNA (Relative to Scr)
Scr
miR-1200
anti-1200
G
ApoB mRNA (% of 0 time)
Scr
miR-1200
Time (h)
H
hsa-miR-1200/APOB Alignment
3′ CUCCGAGUCUUACCGAGUCCUC-5′ miR-1200
215: 5′ GCAAGUUAAAGAAAAUCAGGAU 3′ Human apoB 3′-UTR
215: 5′ GCAAGTTAAAGAAAATGGCGAT 3′ Human apoB mutant
3′CUCCGAGUCUUACCGAGUCCUC-5′ miR-1200
222:5′ UUGUCCAACUCAGGAUGG 3′ Mouse apoB 3′-UTR
I
ApoB 3'-UTR Reporter Assay
Luciferase Activity (Relative to Scr)
ns
WT+Scr
WT+1200
Mut+Scr
Mut+1200
J
ApoB mRNA
5′
3′-UTR
3′
miR-1200
degradation

Figure 8: Regulation of apoB secretion by miR-1200 in human hepatoma cells. (A) Reverse transfection of miR-1200 mimic [50 nM] in Huh-7 cells significantly increased miR-1200 levels after 48 h. **(B, C)** Dose-dependent effects of miR-1200 and anti-1200 on media **(B)** and cellular **(C)** apoB. **(D, E)** Temporal changes in media **(D)** and cellular **(E)** apoB levels after treatment with 50 nM of miR-1200, anti-1200 or Scr control. **(F)** The effect of miR-1200 and anti-1200 on apoB mRNA levels normalized to Scr. **(G)** Time dependent disappearance of apoB mRNA in cells transfected with miR-1200 and treated with actinomycin D (1 µg/mL). The apoB/18S rRNA ratio at time 0 was set to 100%. **(H)** Top: Predicted interactions between miR-1200 and apoB mRNA from miRanda. Bottom: The seed interacting site was mutated as indicated. **(I)** Cells were first transfected with psiCHECK luciferase constructs (1.5 µg/well) containing normal (WT) or mutated (Mut) apoB 3'-UTR sequences. These cells were then reverse transfected with either Scr or miR-1200 [50 nM]. The ratios of firefly and Renilla luciferase activities determined after 48 hours are shown relative to Scr. **(J)** Proposed working model: miR-1200 targets the 3´-UTR of apoB, induces mRNA degradation, and decreases the production of apoB-containing lipoproteins. Data are represented as mean ± SD. *P<0.05, ** P<0.01 and *** P<0.001.

To begin understand how miR-1200 regulates apoB mRNA degradation, I performed *in silico* analysis using miRanda (http://www.microrna.org/microrna/home.do) and found that human apoB mRNA contained miR-1200 interacting sites in their 3´-UTR (**Fig 8H**). Therefore, I hypothesized that miR-1200 interacts with the 3´-UTR of apoB mRNA to increase degradation. To evaluate this hypothesis, 3´-UTR of human apoB mRNA was cloned in psiCheck plasmid after the luciferase cDNA. Luciferase activity of this plasmid was significantly reduced by miR-1200 and this inhibition was avoided after mutagenesis of the complementary site that interacts with the seed sequence (**Fig 8I**). These studies informed that miR-1200 interacts with the 3´-UTR of apoB to increase mRNA degradation (**Fig 8J**).

MiR-1200 increases apoAI secretion by reducing BCL11B, a repressor of apoAI transcription

Next, I concentrated to find out how miR-1200 increases apoAI secretion. MiR-1200 dose-dependently enhanced apoAI secretion by ~41% in Huh-7 cells compared to Scr (**Fig 9A**). Time course studies showed that media apoAI continued to increase until 72 hours after miR-1200 transfection (**Fig 9B**). MiR-1200 increased apoAI mRNA by ~6-fold (**Fig 9C**). Therefore, overexpression of miR-1200 increases media apoAI by elevating mRNA levels. In these studies, anti-1200 had no effect on apoAI expression (**Fig 9A-C**) indicating a complex mode of apoAI regulation by miR-1200 different from that of apoB. I hypothesized that miR-1200 could increase apoAI mRNA by inhibiting post-transcriptional degradation or by enhancing *de novo* transcription. MiR-1200 had no effect on apoAI mRNA degradation (**Fig 9D**). However, it increased the activity of a 1.2 kb apoAI promoter by ~67% (**Fig 9E**) demonstrating that miR-1200 increases apoAI mRNA by enhancing transcription.

Although miRs normally reduce gene expression, they have been shown to activate transcription by interacting with promoter sequences involving complementary base pairing via RNA activation (38, 39). I did not find miR-1200 complementary sequences in the 1.2-kb apoAI promoter and theorized that miR-1200 may increase apoAI transcription by suppressing a transcriptional repressor(s). To begin identify a repressor(s), I selected 3 transcriptional repressors from a list of predicted miR-1200 target genes generated by TargetScan (http://www.targetscan.org/) as they had the potential to bind the apoAI promoter, and the target sites were conserved in human and mouse. Huh-7 cells were then transfected with siRNAs against NRIP1 (Nuclear Receptor Interacting Protein 1), BCL11B (B-Cell Lymphoma 11B), or ZBTB7A (Zinc Finger and BTB Domain Containing 7A) (**Fig 9F**). As expected, miR-1200 reduced apoB; however siNRIP1 increased while siBCL11B and siZBTB7A had no effect on apoB indicating that these repressors do not regulate apoB secretion like miR-1200. However, similar to miR-1200, both siNRIP1 and siBCL11B increased media apoAI by about ~46-53%, but siZBTB7A had no effect. Therefore, NRIP1 and BCL11B may work as apoAI repressors.

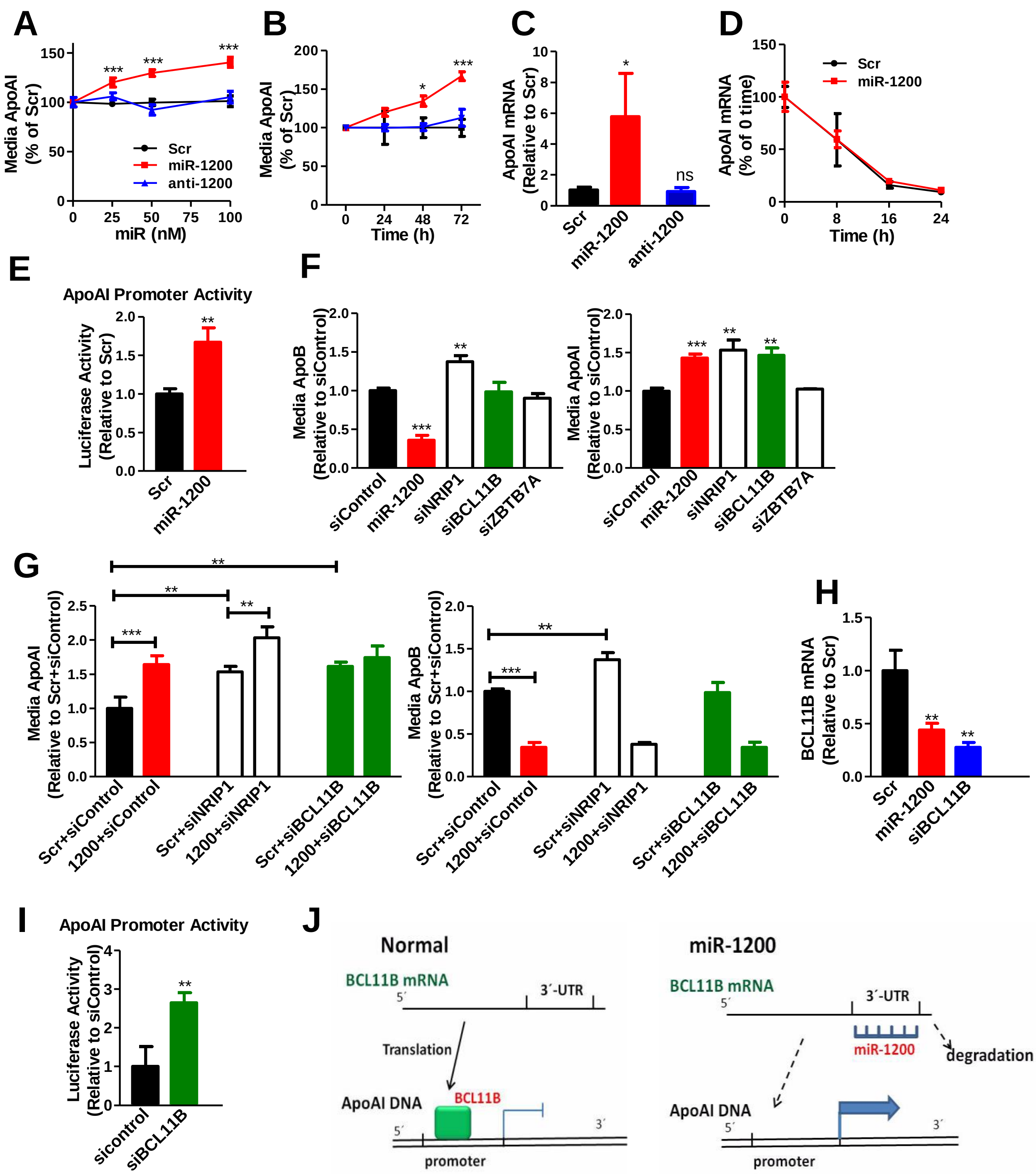
I then asked whether BCL11B is an intermediary in the regulation of apoAI by miR-1200. To test this, I co-transfected siRNAs and miR-1200 in Huh-7 cells (**Fig 9G**). MiR-1200 and siNRIP1 alone increased apoAI secretion by 64 and 50%, respectively; and a combination of both increased it by 104% compared to Scr+siControl suggesting that NRIP1 and miR-1200 additively increase apoAI secretion possibly involving two independent mechanisms (**Fig 9G**). On the other hand, miR-1200, siBCL11B and siBCL11B+miR-1200 increased apoAI to similar extents (**Fig 9G**). In contrast, miR-1200 reduced apoB secretion in cells treated with both siNRIP1 and siBCL1B. These data show that miR-1200 is unable to increase apoAI secretion in siBCL11B treated cells but is able to reduce apoB secretion. Thus, miR-1200 may reduce BCL11B expression to increase apoAI production.

Next, I asked whether miR-1200 modulates BCL11B to regulate apoAI expression. Bioinformatics analyses showed that the 3´-UTR of the human BCL11B mRNA contained 4 miR-1200 binding sites (**Fig 10**). To test whether miR-1200 regulates BCL11B, mRNA levels were quantified in miR-1200 transfected cells. BCL11B mRNA levels were decreased by ~56% in miR-1200 and siBCL11B expressing cells (**Fig 9H**) indicating that miR-1200 regulates BCL11B expression. Further, siBCL11B increased apoAI promoter activity by ~ 2.6-fold (**Fig 9I**) suggesting that BCL11B represses apoAI transcription. These studies indicate that miR-1200 increases apoAI expression by reducing BCL11B (**Fig 9J**).

MiR-1200 reduces apoB and increases apoAI in other human hepatoma cell lines

To ascertain that the regulation of apoB and apoAI by miR-1200 is not specific to Huh-7 cells, I studied its effects in another human hepatoma HepG2 cells. MiR-1200 and anti-1200, respectively, decreased and increased media and cellular apoB (**Fig 12A**). Further, miR-1200 increased media and cellular apoAI levels by ~ 48-54% while anti-1200 had no effect (**Fig 12B**). These studies showed that miR-1200 regulates apoB and apoAI levels in HepG2 cells.

Figure 9: MiR-1200 increases apoAI secretion by reducing expression of BCL11B, a repressor. **(A)** Dose-dependent effect of miR-1200 and anti-1200 on apoAI in Huh-7 cells measured after 48 h. **(B)** Time-dependent changes in media apoAI levels in Huh-7 cells transfected with 50 nM miR-1200, anti-1200 or Scr control. **(C)** Effect of miR-1200 and anti-1200 on mRNA levels of apoAI normalized to Scr. **(D)** Temporal changes in apoAI mRNA levels in cells transfected with Scr or miR-1200 and treated with actinomycin D (1 µg/mL). **(E)** Luciferase activity in Huh-7 cells transfected first with a vector in which human apoAI promoter was cloned upstream of Gaussia luciferase cDNA and then transfected with either miR-1200 or Scr. The luciferase activities were determined after 48 h. Data are presented relative to Scr. **(F)** MiR-1200 or different siRNAs [50 nM] were reverse transfected in Huh-7 cells. ApoAI (left) and apoB (right) were measured in the media after 48 h. **(G)** Huh-7 cells were co-transfected with different miRs and siRNAs [50 nM]. Secreted apoAI and apoB levels were measured after 48 h. **(H)** BCL11B mRNA levels were quantified in Scr control, miR-1200, or siBCL11B transfected Huh-7 cells. **(I)** Cells were first transfected with a plasmid expressing luciferase under the control of apoAI promoter and then transfected with miR-1200 or Scr. The luciferase activities were determined after 48 h. **(J)** Proposed working model: Under normal conditions, BCL11B binds to the apoAI promoter to repress transcription. In miR-1200 overexpressing cells, miR-1200 decreases mRNA levels of BCL11B leading to de-repression of apoAI transcription and increases in mRNA levels. Data are represented as mean ± SD. *$P<0.05$, ** $P<0.01$ and *** $P<0.001$.



A
Media ApoAI (% of Scr)
miR (nM)
Scr
miR-1200
anti-1200
B
Media ApoAI (% of Scr)
Time (h)
C
ApoAI mRNA (Relative to Scr)
ns
D
ApoAI mRNA (% of 0 time)
Time (h)
E
ApoAI Promoter Activity
Luciferase Activity (Relative to Scr)
F
Media ApoB (Relative to siControl)
Media ApoAI (Relative to siControl)
siControl
siNRIP1
siBCL11B
siZBTB7A
G
Media ApoAI (Relative to Scr+siControl)
Media ApoB (Relative to Scr+siControl)
Scr+siControl
1200+siControl
Scr+siNRIP1
1200+siNRIP1
Scr+siBCL11B
1200+siBCL11B
H
BCL11B mRNA (Relative to Scr)
I
ApoAI Promoter Activity
Luciferase Activity (Relative to siControl)
sicontrol
siBCL11B
J
Normal
BCL11B mRNA
3′-UTR
Translation
ApoAI DNA
BCL11B
promoter
miR-1200
degradation

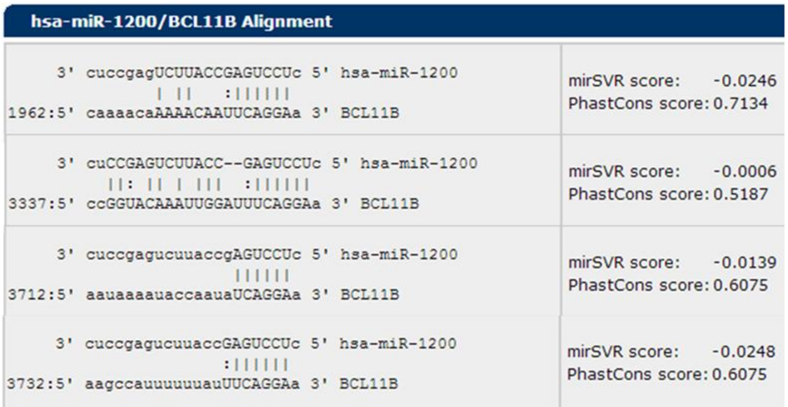


Figure 10. Predicted base-paring at 4 different sites between miR-1200 and the 3'UTR of human BCL11B. MiRanda was used to predict potential targets of miR-1200.

Human Apoa1 promoter sequence

CTTCCAGGAGAAACCTGACAAGATGGTGCTGCAAACACCAACGGACACACAGCACTTT
ACATTCACAGGCTGTCTCAGGGGCCTCCAACAACCCTGACCATTCTTGCCCCATTTTGCA
GATAGAAAACCGAGGCTCAGAGAGATTATATAACTTGCCCACGATCTTCCTCCAGCAAG
ATGGAGGCCAAGTGAAATGAGAAAGCAGGTCTCCTGCCACTTCCTTTGCCCAGAGGTCT
TCTCCCCACACCAGGGCTTCCCAAGGGCTGAGATCCAGTCACACCTGTGCGTGATCAAA
TATAAGTGTGAACAATGCAAAGGGAGACGTCTTCAATCTAAGGGGCTTCAATTCTGTAA
TGTAATTCTGAGATTATGCCCTTTTTTGTTAAAGCCTTTCCTTTTTGAAGTGATGGTCACT
GTAGATGGTGAGGGTTTTTTGGAGGCGGACAATATCTTTACATGACAAAATTAAAAGTT
GGCAGCTCCGAATTGATCTCTGGAGTGTTTTGAAATGCAAGAGGTCTCCGAAACCTCAG
TCTGGGAGCCACGGAGGGCTCTCCCCTCTCCCCAGGTTTACCAGTTTGGGAGGCTTGGA
GAGAGGCCTGGAGGACCTGCTGGGGACTAAAGAAGAGCACTGGTGGGAGGACAGGGC
GGGGGAAGGGGGAGGGGAGTGAAGTAGTCTCCCTGGAATGCTGGTGGTGGGGGAGGC
AGTCTCCTTGGTGGAGGAGTCCCAGCGTCCCTCCCCTCCCCTCCTCTGCCAACACAATG
GACAATGGCAACTGCCCACACACTCCCATGGAGGGGAAGGGGATGAGTGCAGGGAACC
CCGACCCCACCCGGGAGACCTGCAAGCCTGCAGACACTCCCCTCCCGCCCCCACTGAAC
CCTTGACCCCTGCCCTGCAGCCCCCGCAGCTTGCTGTTTGCCCACTCTATTTGCCCAGCC
CCAGGGACAGAGCTGATCCTTGAACTCTTAAGTTCCACATTGCCAGGACCAGTGAGCAG
CAACAGGGCCGGGGCTGGGCTTATCAGCCTCCCAGCCCAGACCCTGGCTGCAGACATA

AATAGGCCCTGCAAGAGCTGGCTGCTTAGAGACTGCGAGAAGGAGGTGCGTCCTGCTG
CCTGCCCCGGTCACTCTGGCTCCCCAGCTCAAGGTTCAGGCCTTGCCCCAGGCCGGGCC
TCTGGGTACCTGAGGTCTTCTCCC

Fig 11: Identification of putative BCL11B binding sites in the human apoAI promoters. The putative binding sites are highlighted in yellow.

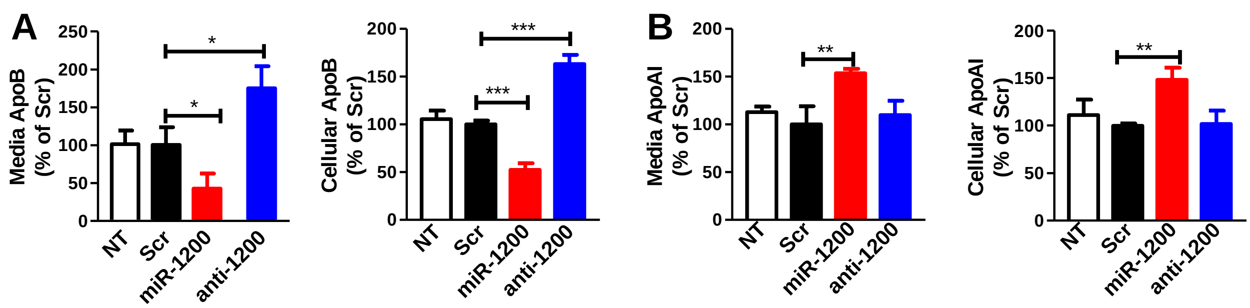


Figure 12. MiR-1200 regulates apoB and apoAI in HepG2 cells. Human hepatoma HepG2 cells were reverse transfected with miRs [50nM]. NT: non-transfected. Media and cellular apolipoproteins were measured after 48 hours.

MiR-1200 reduces lipid synthesis without affecting fatty acid oxidation in Huh-7 cells.

To investigate the effect of miR-1200 on cellular lipid metabolism homeostasis, the synthesis rate of different lipids and fatty acid oxidation were measured in miR-1200 transfected Huh-7 cells. The syntheses of phospholipid, cholesterol, and fatty acid were significantly decreased by miR-1200 (**Fig 13**). Fatty acid oxidation was not affected (**Fig 13**).

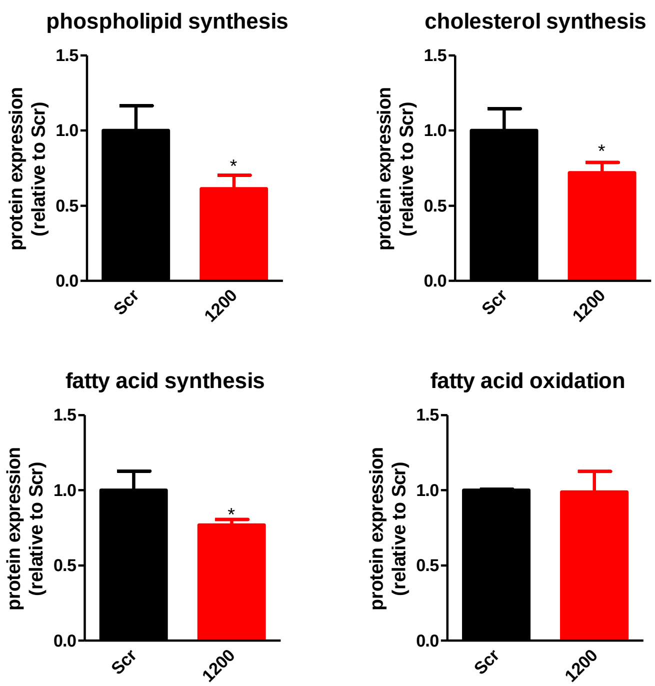


Figure 13. The effects of miR-1200 overexpression of lipid metabolism in Huh-7 cells. MiR-1200 or Scr control (50nM) was transfected in Huh-7 cells. 48 hours after transfection, lipid synthesis and fatty acid oxidation were measured.

Discussion

High LDL and low HDL-cholesterol levels are two well-accepted risk factors for CVD. Each miR targets multiple genes to regulate various biological processes. Our lab previously has shown that miR-30c reduces hepatic apoB-containing lipoprotein secretion by targeting MTP (31, 37). The inhibition of miR-30c on MTP overcomes the lipid accumulation and elevated plasma transaminase activities induced by MTP inhibitors by suppressing hepatic synthesis. Regulation of multiple targets by miR may be able to overcome the side affect of targeting a single gene of interest or achieve more beneficial phenotypes. In present study, I sought to identify miRs that can potentially regulate plasma lipoprotein metabolism using an unbiased *in vitro* high throughput screening. First and secondary screening resulted in the identification of 75 human miRs modulating apoB or apoAI secretion in Huh-7 cell line. Previous studies have used *in silico* predictions to identify miRs that specifically target candidate proteins (37, 40). Instead of the candidate approach, I opted for an unbiased screening to identify miRs that might concurrently regulate apoB and apoAI secretion and identified miR-1200 as a novel modulator of apolipoprotein secretion and lipid synthesis in hepatoma cell line. Thus, miR-1200 could be a novel biologic to mitigate dyslipidemia and atherosclerosis.

In the screening, oleic acid was added to the incubation media to inhibit the proteasomal degradation. Most of the newly translated apoB peptides are degraded before secreted out of cells. MTP transfers lipids to nascent apoB to protect it from degradation. Only well-lipidated apoB peptides can escape from cells for apoB-containing lipoprotein secretion. I am more interested in discovering novel miRs and genes in regulating apoB-containing lipoprotein assembly and secretion, other than proteaosomal degradation pathway. Therefore, I used oleic acid to inhibit the posttranslational degradation of apoB (41, 42). Oleic acid in medium has no effect on apoAI secretion.

The selection of candidate miRs for secondary screening was based on the threshold of reducing and increasing apoB secretion by 60% and 70%, as well as reducing and decreasing apoAI secretion by 35% and 45% to narrow the number of candidate miRs to about 100. From the miRs that replicated the effects of the first screening, I identified 3 miRs-1200, 509, and 634 that were able to decrease apoB and increase apoAI secretion simultaneously. Therefore, it is reasonable to hypothesize that humans may express these miRs to modulate the hepatic production of apoB and apoAI and control plasma lipid levels. From the results of first screening, there are also some miRs that concurrently increased apoB and decreased apoAI secretion, miRs-1231, 1264, and 130b for example, although they were not included in the secondary screening for validation. It is also worthy to test whether inhibition of these miRs can decrease apoB and increase apoAI secretion from hepatocytes to modulate plasma lipoprotein concentrations. Two miRs-4303, and 3663-3p also attracted our attention because they dramatically reduced apoAI secretion by almost 90%. In addition, bioinformatics analysis suggests that the 3′-UTR of human apoAI mRNA may harbor interacting sites of these two miRs. This raises the possibility of targeting the endogenous miRs using antimiR oligonucleotides to increase apoAI production from the liver.

From the results of the first screening, I noticed that more miRs are regulating apoB secretion than that regulating apoAI **(Fig 1)**. 60 miRs decreased apoB secretion by over 50%, whereas only 11 miRs decreased apoAI secretion by over 45%. In addition, 57 miRs increased apoB secretion by more than 50%, whereas only 27 miRs regulating apoAI secretion by more than 50% **(Table 1)**. This difference about miR regulation on hepatic apoB and apoAI secretion could be explained by the different intracellular metabolism complexities. The biosynthesis and assembly of apoB-containing lipoprotein happens intracellularly. Newly synthesized nascent apoB peptides are chaperoned by MTP for getting lipidated and protected from degradation (43). In the process of intracellular trafficking, VLDL is carried in a special VLDL transport vesicle from ER to *cis*-Golgi. In Golgi, these particles are further lipidated and modified to be secreted out of cells for delivery of triglyceride and cholesterol to extra hepatic tissues (44, 45). On the other hand, the majority of secreted apoAI protein is in the form of lipid poor. The discovery of ABCA1 from Tangier diseases patients, who do not have detectable plasma HDL or apoAI and suffer from premature atherosclerosis, revealed the importance of extracellular lipidation of apoAI for the biosynthesis and assembly of HDL (46). Several elegant studies have reported the intracellularly assembled nascent HDL, suggesting that about 20% of secreted apoAI from hepatocyte is intracellularly lapidated (47, 48). Therefore, the extracellular cholesterol efflux is the main contributor of apoAI lipidation (49). Hence, our *in vitro* screening model only concentrated on the hepatic secretion of apoAI, which is the intracellular part of HDL biosynthesis.

Besides the effect of miRs on apolipoprotein secretion, the characterization of miRs might also help us gain new knowledge about unknown functions of genes. When the available biologic knowledge is not sufficient to explain the phenotype induced by miR, it gives us an opportunity to propose for new insights of biological pathways. These studies identified BCL11B as a novel regulator of apoAI secretion. MiR-1200 increased media apoAI by reducing BCL11B, a repressor, and increasing *Apoa1* gene transcription. BCL11B, also known as CTIP2, is a member of the B cell leukemia (BCL) family. It plays a crucial role in the development, proliferation, differentiation and subsequent survival of T cells. It is a zinc finger protein that binds GC-rich elements and interacts with several co-factors and acts both as a repressor and an activator (50). BCL11B has been shown to interact with 3 different sequences in different genes; 5´-TGGGC-3´, 5´-AGGTG-3´ and 5´-TGGCCGG-3´ (51, 52). The human apoAI promoter contains all three sequences and the mouse apoAI promoter contains two of these sequences (**Fig 11**). Moreover, a German study of 20,634 participants reported that genetic variations in the 3´-BCL11B gene desert are associated with elevated risks for cardiovascular diseases (53). Thus, BCL11B may directly interact with the apoAI promoter to act as a repressor and studying the modulation of apoAI repression may be physiologically significant yielding new information about the regulation of plasma HDL levels.

Although several miRs and their inhibitors are being evaluated for different treatment modalities (54-56), there is general skepticism about their utility as it is presumed that their ability to modulate different mRNAs might cause unanticipated side effects. In fact, studies with anti-miR-122 and miR-34a suggest that these miRs besides lowering plasma LDL also cause reductions in plasma HDL and hepatosteatosis (34, 57-59). However, the current study showed that miR-1200 has multiple desirable properties and provides proof of concept that miRs exist to regulate several pathways with predominantly beneficial outcomes. Hence, this study might pave the way for discovering other regulatory non-coding RNAs to treat complex metabolic syndromes with overall beneficial outcomes; such biologics might be better substitutes for various combination therapies. Furthermore, identifications of such miRs and other non-coding RNAs may provide comprehensive novel knowledge about complex physiological regulatory mechanisms.

Materials: A human miRIDIAN Mimic 16.0 library was purchased from Dharmacon. [^{14}C]oleic acid and [^{3}H]acetate were from NEN Life Science Products. Chemicals and solvents were from Fisher Scientific.

Methods:

Cell culture: Human hepatoma Huh-7 and HepG2 cells were obtained from American Type Culture Collection. Huh-7 and HepG2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 1% penicillin-streptomycin and 1% L-glutamine. Cells were incubated in a 37°C, 5% CO_2 cell culture incubator.

Screening: MiRs were suspended in RNase free water to obtain 2 µM stocks and 3 µL of each miR stock were added in duplicate wells to obtain a final concentration of 50 nM. To each well, I then added 7 µl of Opti-MEM and 10 µl of lipofectamine RNAiMAX (Life technologies) diluted 1:20 in Serum Reduced Opti-MEM. After 20 to 30 minutes, 25,000 cells in 100 µl of Opti-MEM were added to each well. After additional 24 hours, culture media were changed with fresh DMEM containing 10% fetal bovine serum. Media were changed 24 hours later and cells were incubated with DMEM containing oleic acid/BSA complex ((oleic acid (0.4mM)/BSA (1.5%)) for 2 hours. ApoB and apoAI concentrations in medium were measured by ELISA (60). Cells were used for protein measurements.

Intracellular apoB measurement: Cells were homogenized in 100 mM Tris buffer (pH7.4) containing 150 mM NaCl, 1 mM EGTA, 1mM EDTA, 1% Triton X-100 and 0.5% SDS. ApoB was measured via ELISA.

mRNA half-life measurement: Huh-7 cells ($1.2*10^5$ / well) in 12-well plates were reverse transfected with miR-1200 or Scr (50 nM). After 24 hours, cells were treated with 1 µg/mL actinomycin D in growth medium. Total RNA was collected at different time points to quantify mRNA levels by qRT-PCR.

Luciferase reporter assay: The 3´-UTR of human apoB mRNA was cloned after the luciferase cDNA in psiCHECK2 plasmid to obtain pLuc-apoB-3´-UTR expression plasmid. This plasmid or control psiCHECK2 plasmid (1.5 µg) was transfected using TurboFect transfection reagent (Dharmacon) in Huh-7 cells ($1.2*10^6$) plated in 10 cm Petri dishes one day before transfection. After 24 hours of transfection, cells were detached and plated in 6-well plates containing miRs+RNAiMAX for reverse transfection (final concentration: 50nM). Luciferase activity was measured after 48 hours with Dual-Luciferase Reporter Assay System (Promega). ApoAI promoter luciferase reporter construct was purchased from GeneCopoeia.

Fatty acid oxidation and synthesis of fatty acids, cholesterol and phospholipids: For fatty acid oxidation, Huh-7 cells in 6-well plates were transfected with 50 nM of miR-1200, or Scr control. 48 hours, after transfection, cells were incubated with DMEM containing 0.4 µCi/ml of ^{14}C-oleate and covered with phenylethylamine soaked Whatman filter paper for 3 hours at 37°C. At the end of incubation, 200 µl of 1M perchloric acid was added to media and incubated for 1 h at room temperature to precipitate acid-insoluble metabolites, and centrifuged (10 min 12,000*g). The radioactivity in the supernatant and the filter paper was counted. For lipid synthesis, cells were incubated with 1 µCi ^{3}H-acetate. After 3 hours, cells were washed with PBS and subjected to total lipid extraction using chloroform/methanol. Dried lipid was dissolved in chloroform and separated on silica-60 Thin Layer Chromatography plates. The bands containing cholesteryl, fatty acid, cholesterol, or phospholipid were scraped off from the plates and counted in a scintillation counter.

RNA isolation and qRT-PCR: Total RNA from tissues and cells were extracted using TRIzol (Invitrogen). RNA was reverse transcribed into cDNA with the Omniscript RT kit (QIAGEN). Expression levels of gene are quantified by qRT-PCR using SYBER Green qPCR Core Kit (Eurogentec), and data was analyzed with ΔΔCT method and normalized to 18S. Primers specific for miR-1200, snoRNA 202 were purchased from Life Technologies.

Quantitative RT-PCR primers:

Gene	**Forward primer**	**Reverse primer**
Human ApoAI	5´ -GCAGAGACTATGTGTCCCAGTTTG-3	5´ -CCAGTTGTCAAGGAGCTTTAG-3´
Human ApoB	5´ -TGACCTTGTCCAGTGAAGTC-3´	5´ -GTTCTGAATGTCCAGGGTGA-3´
Human ABCA1	5´ -TGGTCTCCAAGCAGAGTGTG-3´	5´ -GAGCAGCAGCTCCCAATAC-3´
Human BCL11B	5´ -CACCCCCGACGAAGATGACCAC-3´	5´ -CGGCCCGGGCTCCAGGTAGATG-3´
18s rRNA	5´ -AGTCCCTTGCCCTTTGTACACA-3´	5´ -GATCCGAGGGCCTCACTAAAC-3´

Primers used for site-directed mutagenesis

ApoB $_{C231G\_A232G\_G233C\_}$
Forward: 5'-TAGCAAAATAACTCAGATCGCCATTTTCTTTAACTTGCAAAAAATGCCATCCTTCTG-3'
Reverse: 5'-CAGAAGGATGGCATTTTTTGCAAGTTAAAGAAAATGGCGATCTGAGTTATTTTGCTA-3'

All the siRNAs and miRs used were from Dharmacon.

Catalog Number	Gene Symbol	Gene Accession	Sequence
D-006686-01	NRIP1	NM_003489	GAACAAAGGUCAUGAGUGA
D-005082-01	BCL11B	NM_022898	GAGCAAGUCGUGCGAGUUC
D-020818-01	ZBTB7A	NM_015898	UCACCGCGCUCAUGGACUU
C-301326-00	hsa-miR-1200	MIMAT0005863	CUCCUGAGCCAUUCUGAGCCUC

Statistics: Data were presented as the mean ± SD. The statistical significance was determined by student's t-test, one-way or two-way ANOVA (Graphpad Prism) and significant differences $p<0.05$, $p<0.01$, and $p<0.001$ were symbolized as *, **, and ***, respectively.

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Author contributions: LZ performed all the experiments, analyzed data, made figures and wrote the manuscript. SI measured microRNA levels in cells and tissues. MMH conceived the project, supervised the progress, critically evaluated data and extensively edited the manuscript.

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References

1. Writing Group M, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation.* 2016;133(4):e38-360.
2. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. *Nat Rev Immunol.* 2010;10(1):36-46.
3. Moore KJ, Rayner KJ, Suarez Y, and Fernandez-Hernando C. The role of microRNAs in cholesterol efflux and hepatic lipid metabolism. *Annu Rev Nutr.* 2011;31(49-63.
4. Kingwell BA, Chapman MJ, Kontush A, and Miller NE. HDL-targeted therapies: progress, failures and future. *Nature reviews Drug discovery.* 2014;13(6):445-64.
5. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, Neeland IJ, Yuhanna IS, Rader DR, de Lemos JA, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med.* 2014;371(25):2383-93.
6. Chang G, Ruggeri RB, and Harwood HJ, Jr. Microsomal triglyceride transfer protein (MTP) inhibitors: discovery of clinically active inhibitors using high-throughput screening and parallel synthesis paradigms. *Curr Opin Drug Discov Devel.* 2002;5(4):562-70.
7. Rizzo M. Lomitapide, a microsomal triglyceride transfer protein inhibitor for the treatment of hypercholesterolemia. *IDrugs.* 2010;13(2):103-11.
8. Gu Q, Paulose-Ram R, Burt VL, and Kit BK. Prescription cholesterol-lowering medication use in adults aged 40 and over: United States, 2003-2012. *NCHS data brief.* 2014177):1-8.
9. Ahmad Z. Statin intolerance. *Am J Cardiol.* 2014;113(10):1765-71.
10. Finkel JB, and Duffy D. 2013 ACC/AHA cholesterol treatment guideline: Paradigm shifts in managing atherosclerotic cardiovascular disease risk. *Trends Cardiovasc Med.* 2015;25(4):340-7.
11. Cornier MA, and Eckel RH. Non-traditional dosing of statins in statin-intolerant patients-is it worth a try? *Current atherosclerosis reports.* 2015;17(2):475.
12. Lipinski MJ, Benedetto U, Escarcega RO, Biondi-Zoccai G, Lhermusier T, Baker NC, Torguson R, Brewer HB, Jr., and Waksman R. The impact of proprotein convertase subtilisin-kexin type 9 serine protease inhibitors on lipid levels and outcomes in patients with primary hypercholesterolaemia: a network meta-analysis. *Eur Heart J.* 2016;37(6):536-45.

13. Dadu RT, and Ballantyne CM. Lipid lowering with PCSK9 inhibitors. *Nat Rev Cardiol.* 2014;11(10):563-75.
14. Raal FJ, Stein EA, Dufour R, Turner T, Civeira F, Burgess L, Langslet G, Scott R, Olsson AG, Sullivan D, et al. PCSK9 inhibition with evolocumab (AMG 145) in heterozygous familial hypercholesterolaemia (RUTHERFORD-2): a randomised, double-blind, placebo-controlled trial. *Lancet.* 2015;385(9965):331-40.
15. Rader DJ, and Kastelein JJ. Lomitapide and mipomersen: two first-in-class drugs for reducing low-density lipoprotein cholesterol in patients with homozygous familial hypercholesterolemia. *Circulation.* 2014;129(9):1022-32.
16. Tangirala RK, Tsukamoto K, Chun SH, Usher D, Pure E, and Rader DJ. Regression of atherosclerosis induced by liver-directed gene transfer of apolipoprotein A-I in mice. *Circulation.* 1999;100(17):1816-22.
17. Kawashiri MA, Zhang Y, Pure E, and Rader DJ. Combined effects of cholesterol reduction and apolipoprotein A-I expression on atherosclerosis in LDL receptor deficient mice. *Atherosclerosis.* 2002;165(1):15-22.
18. Benoit P, Emmanuel F, Caillaud JM, Bassinet L, Castro G, Gallix P, Fruchart JC, Branellec D, Denefle P, and Duverger N. Somatic gene transfer of human ApoA-I inhibits atherosclerosis progression in mouse models. *Circulation.* 1999;99(1):105-10.
19. Puntoni M, Sbrana F, Bigazzi F, and Sampietro T. Tangier disease: epidemiology, pathophysiology, and management. *Am J Cardiovasc Drugs.* 2012;12(5):303-11.
20. Kozarsky KF, Donahee MH, Glick JM, Krieger M, and Rader DJ. Gene transfer and hepatic overexpression of the HDL receptor SR-BI reduces atherosclerosis in the cholesterol-fed LDL receptor-deficient mouse. *Arterioscler Thromb Vasc Biol.* 2000;20(3):721-7.
21. Wang N, Arai T, Ji Y, Rinninger F, and Tall AR. Liver-specific overexpression of scavenger receptor BI decreases levels of very low density lipoprotein ApoB, low density lipoprotein ApoB, and high density lipoprotein in transgenic mice. *J Biol Chem.* 1998;273(49):32920-6.
22. Trigatti B, Rayburn H, Vinals M, Braun A, Miettinen H, Penman M, Hertz M, Schrenzel M, Amigo L, Rigotti A, et al. Influence of the high density lipoprotein receptor SR-BI on reproductive and cardiovascular pathophysiology. *Proc Natl Acad Sci U S A.* 1999;96(16):9322-7.

23. Covey SD, Krieger M, Wang W, Penman M, and Trigatti BL. Scavenger receptor class B type I-mediated protection against atherosclerosis in LDL receptor-negative mice involves its expression in bone marrow-derived cells. *Arterioscler Thromb Vasc Biol.* 2003;23(9):1589-94.
24. Chadwick AC, and Sahoo D. Functional characterization of newly-discovered mutations in human SR-BI. *PLoS One.* 2012;7(9):e45660.
25. Khovidhunkit W. A genetic variant of the scavenger receptor BI in humans. *N Engl J Med.* 2011;364(14):1375-6; author reply 6.
26. Zanoni P, Khetarpal SA, Larach DB, Hancock-Cerutti WF, Millar JS, Cuchel M, DerOhannessian S, Kontush A, Surendran P, Saleheen D, et al. Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease. *Science.* 2016;351(6278):1166-71.
27. Filippatos TD, Klouras E, Barkas F, and Elisaf M. Cholesteryl ester transfer protein inhibitors: challenges and perspectives. *Expert Rev Cardiovasc Ther.* 2016:1-10.
28. Rosenson RS, Brewer HB, Jr., Ansell BJ, Barter P, Chapman MJ, Heinecke JW, Kontush A, Tall AR, and Webb NR. Dysfunctional HDL and atherosclerotic cardiovascular disease. *Nat Rev Cardiol.* 2016;13(1):48-60.
29. Izaurralde E. GENE REGULATION. Breakers and blockers-miRNAs at work. *Science.* 2015;349(6246):380-2.
30. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-33.
31. Soh J, and Hussain MM. Supplementary site interactions are critical for the regulation of microsomal triglyceride transfer protein by microRNA-30c. *Nutr Metab (Lond).* 2013;10(1):56.
32. Fernandez-Hernando C, Suarez Y, Rayner KJ, and Moore KJ. MicroRNAs in lipid metabolism. *Curr Opin Lipidol.* 2011;22(2):86-92.
33. Irani S, and Hussain MM. Role of microRNA-30c in lipid metabolism, adipogenesis, cardiac remodeling and cancer. *Curr Opin Lipidol.* 2015;26(2):139-46.
34. Zhou L, Irani S, Sirwi A, and Hussain MM. MicroRNAs regulating apolipoprotein B-containing lipoprotein production. *Biochim Biophys Acta.* 2016.

35. Goedeke L, Rotllan N, Canfran-Duque A, Aranda JF, Ramirez CM, Araldi E, Lin CS, Anderson NN, Wagschal A, de Cabo R, et al. MicroRNA-148a regulates LDL receptor and ABCA1 expression to control circulating lipoprotein levels. *Nat Med.* 2015;21(11):1280-9.
36. Wagschal A, Najafi-Shoushtari SH, Wang L, Goedeke L, Sinha S, deLemos AS, Black JC, Ramirez CM, Li Y, Tewhey R, et al. Genome-wide identification of microRNAs regulating cholesterol and triglyceride homeostasis. *Nat Med.* 2015;21(11):1290-7.
37. Soh J, Iqbal J, Queiroz J, Fernandez-Hernando C, and Hussain MM. MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion. *Nat Med.* 2013;19(7):892-900.
38. Huang V, Place RF, Portnoy V, Wang J, Qi Z, Jia Z, Yu A, Shuman M, Yu J, and Li LC. Upregulation of Cyclin B1 by miRNA and its implications in cancer. *Nucleic acids research.* 2012;40(4):1695-707.
39. Place RF, Li LC, Pookot D, Noonan EJ, and Dahiya R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A.* 2008;105(5):1608-13.
40. de Aguiar Vallim TQ, Tarling EJ, Kim T, Civelek M, Baldan A, Esau C, and Edwards PA. MicroRNA-144 regulates hepatic ATP binding cassette transporter A1 and plasma high-density lipoprotein after activation of the nuclear receptor farnesoid X receptor. *Circ Res.* 2013;112(12):1602-12.
41. Arrol S, Mackness MI, and Durrington PN. The effects of fatty acids on apolipoprotein B secretion by human hepatoma cells (HEP G2). *Atherosclerosis.* 2000;150(2):255-64.
42. Kurokawa M, Hirano T, Furukawa S, Nagano S, and Adachi M. Similar to oleic acid, eicosapentaenoic acid stimulates apolipoprotein B secretion by inhibiting its intracellular degradation in Hep G2 cells. *Atherosclerosis.* 1995;112(1):59-68.
43. Hussain MM, Shi J, and Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res.* 2003;44(1):22-32.
44. Tiwari S, and Siddiqi SA. Intracellular trafficking and secretion of VLDL. *Arterioscler Thromb Vasc Biol.* 2012;32(5):1079-86.
45. Bamberger MJ, and Lane MD. Possible role of the Golgi apparatus in the assembly of very low density lipoprotein. *Proc Natl Acad Sci U S A.* 1990;87(7):2390-4.

46. Oram JF. Molecular basis of cholesterol homeostasis: lessons from Tangier disease and ABCA1. *Trends Mol Med.* 2002;8(4):168-73.
47. Kiss RS, McManus DC, Franklin V, Tan WL, McKenzie A, Chimini G, and Marcel YL. The lipidation by hepatocytes of human apolipoprotein A-I occurs by both ABCA1-dependent and -independent pathways. *J Biol Chem.* 2003;278(12):10119-27.
48. Chisholm JW, Burleson ER, Shelness GS, and Parks JS. ApoA-I secretion from HepG2 cells: evidence for the secretion of both lipid-poor apoA-I and intracellularly assembled nascent HDL. *J Lipid Res.* 2002;43(1):36-44.
49. Rader DJ. Molecular regulation of HDL metabolism and function: implications for novel therapies. *J Clin Invest.* 2006;116(12):3090-100.
50. Avram D, and Califano D. The multifaceted roles of Bcl11b in thymic and peripheral T cells: impact on immune diseases. *J Immunol.* 2014;193(5):2059-65.
51. Cismasiu VB, Ghanta S, Duque J, Albu DI, Chen HM, Kasturi R, and Avram D. BCL11B participates in the activation of IL2 gene expression in CD4+ T lymphocytes. *Blood.* 2006;108(8):2695-702.
52. Tang B, Di Lena P, Schaffer L, Head SR, Baldi P, and Thomas EA. Genome-wide identification of Bcl11b gene targets reveals role in brain-derived neurotrophic factor signaling. *PLoS One.* 2011;6(9):e23691.
53. Mitchell GF, Verwoert GC, Tarasov KV, Isaacs A, Smith AV, Yasmin, Rietzschel ER, Tanaka T, Liu Y, Parsa A, et al. Common genetic variation in the 3'-BCL11B gene desert is associated with carotid-femoral pulse wave velocity and excess cardiovascular disease risk: the AortaGen Consortium. *Circ Cardiovasc Genet.* 2012;5(1):81-90.
54. Bouchie A. First microRNA mimic enters clinic. *Nat Biotechnol.* 2013;31(7):577.
55. Qiu Z, and Dai Y. Roadmap of miR-122-related clinical application from bench to bedside. *Expert Opin Investig Drugs.* 2014;23(3):347-55.
56. van Rooij E, and Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med.* 2014;6(7):851-64.
57. Xu Y, Zalzala M, Xu J, Li Y, Yin L, and Zhang Y. A metabolic stress-inducible miR-34a-HNF4alpha pathway regulates lipid and lipoprotein metabolism. *Nat Commun.* 2015;6(7466.

58. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, Huang Y, Chen HC, Lee CH, Tsai TF, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Invest.* 2012;122(8):2884-97.
59. Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Invest.* 2012;122(8):2871-83.
60. Hussain MM, Zhao Y, Kancha RK, Blackhart BD, and Yao Z. Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100. *Arterioscler Thromb Vasc Biol.* 1995;15(4):485-94.

Supplementary Table I. The effect of 1237 miRs on apoB and apoAI secretion in Huh7 cells.
Medium apoB and apoAI were normalized against protein levels and calculated to percentages of Scr control.

			apoB		apoAI	
Mature Name	Mature Accession	Mature Sequence	plate1	plate2	plate1	plate2
hsa-let-7a-2-3p	MIMAT0010195	CUGUACAGCCUCCUAGCUUUCC	110.79	94.94	114.44	102.87
hsa-let-7a-3p	MIMAT0004481	CUAUACAAUCUACUGUCUUUC	112.31	86.33	73.30	59.51
hsa-let-7a-5p	MIMAT0000062	UGAGGUAGUAGGUUGUAUAGUU	35.73	56.36	72.34	83.45
hsa-let-7b-3p	MIMAT0004482	CUAUACAACCUACUGCCUUCCC	105.54	109.86	90.36	91.48
hsa-let-7b-5p	MIMAT0000063	UGAGGUAGUAGGUUGUGUGGUU	77.29	57.43	107.87	115.59
hsa-let-7c	MIMAT0000064	UGAGGUAGUAGGUUGUAUGGUU	67.69	62.76	78.38	70.12
hsa-let-7c*	MIMAT0004483	UAGAGUUACACCCUGGGAGUUA	89.15	94.94	114.44	107.62
hsa-let-7d-3p	MIMAT0004484	CUAUACGACCUGCUGCCUUUCU	158.43	246.59	91.70	95.20
hsa-let-7d-5p	MIMAT0000065	AGAGGUAGUAGGUUGCAUAGUU	32.34	31.45	62.74	39.59
hsa-let-7e-3p	MIMAT0004485	CUAUACGGCCUCCUAGCUUUCC	125.04	144.66	96.92	102.60
hsa-let-7e-5p	MIMAT0000066	UGAGGUAGGAGGUUGUAUAGUU	78.48	67.11	89.71	77.22
hsa-let-7f-1-3p	MIMAT0004486	CUAUACAAUCUAUUGCCUUCCC	122.09	92.95	100.81	95.83
hsa-let-7f-2-3p	MIMAT0004487	CUAUACAGUCUACUGUCUUUCC	138.71	148.89	93.54	97.85
hsa-let-7f-5p	MIMAT0000067	UGAGGUAGUAGAUUGUAUAGUU	72.94	71.62	60.78	71.53
hsa-let-7g-3p	MIMAT0004584	CUGUACAGGCCACUGCCUUGC	69.96	67.38	110.83	99.48
hsa-let-7g-5p	MIMAT0000414	UGAGGUAGUAGUUUGUACAGUU	55.08	35.56	91.47	83.59
hsa-let-7i-3p	MIMAT0004585	CUGCGCAAGCUACUGCCUUGCU	86.46	92.51	108.30	108.19
hsa-let-7i-5p	MIMAT0000415	UGAGGUAGUAGUUUGUGCUGUU	35.04	32.74	64.23	51.90
hsa-miR-1	MIMAT0000416	UGGAAUGUAAAGAAGUAUGUAU	144.14	142.57	125.78	120.96
hsa-miR-100-3p	MIMAT0004512	CAAGCUUGUAUCUAUAGGUAUG	102.52	82.20	100.81	113.61
hsa-miR-100-5p	MIMAT0000098	AACCCGUAGAUCCGAACUUGUG	107.18	111.45	102.86	80.38
hsa-miR-101-3p	MIMAT0000099	UACAGUACUGUGAUAACUGAA	107.83	106.29	94.22	81.17
hsa-miR-101-5p	MIMAT0004513	CAGUUAUCACAGUGCUGAUGCU	47.51	60.73	67.47	70.52
hsa-miR-103a-2-5p	MIMAT0009196	AGCUUCUUUACAGUGCUGCCUUG	87.04	114.00	94.41	92.61
hsa-miR-103a-3p	MIMAT0000101	AGCAGCAUUGUACAGGGCUAUGA	93.20	105.24	79.17	81.24
hsa-miR-103b	MIMAT0007402	UCAUAGCCCUGUACAAUGCUGCU	91.33	92.95	125.87	122.49
hsa-miR-105-3p	MIMAT0004516	ACGGAUGUUUGAGCAUGUGCUA	162.70	201.90	107.31	122.44
hsa-miR-105-5p	MIMAT0000102	UCAAAUGCUCAGACUCCUGUGGU	89.81	80.09	82.59	115.36
hsa-miR-106a-3p	MIMAT0004517	CUGCAAUGUAAGCACUUCUUAC	100.65	91.42	71.76	71.72
hsa-miR-106a-5p	MIMAT0000103	AAAAGUGCUUACAGUGCAGGUAG	130.76	120.29	91.17	123.07
hsa-miR-106b-3p	MIMAT0004672	CCGCACUGUGGGUACUUGCUGC	42.28	41.93	101.74	71.50
hsa-miR-106b-5p	MIMAT0000680	UAAAGUGCUGACAGUGCAGAU	105.69	78.40	119.00	137.63
hsa-miR-107	MIMAT0000104	AGCAGCAUUGUACAGGGCUAUCA	152.95	152.07	93.21	120.04
hsa-miR-10a-3p	MIMAT0004555	CAAAUUCGUAUCUAGGGGAAUA	112.50	89.12	107.03	85.44
hsa-miR-10a-5p	MIMAT0000253	UACCCUGUAGAUCCGAAUUUGUG	111.97	124.33	90.01	79.79
hsa-miR-10b-3p	MIMAT0004556	ACAGAUUCGAUUCUAGGGGAAU	138.06	125.51	96.13	76.86
hsa-miR-10b-5p	MIMAT0000254	UACCCUGUAGAACCGAAUUUGUG	110.05	109.81	111.83	81.95
hsa-miR-1178-3p	MIMAT0005823	UUGCUCACUGUUCUUCCCUAG	116.70	96.58	101.86	91.31
hsa-miR-1179	MIMAT0005824	AAGCAUUCUUUCAUUGGUUGG	113.16	124.38	102.85	112.60

hsa-miR-1180	MIMAT0005825	UUUCCGGCUCGCGUGGGUGUGU	95.23	86.33	78.32	84.34
hsa-miR-1181	MIMAT0005826	CCGUCGCCGCCACCCGAGCCG	122.09	106.78	105.94	82.51
hsa-miR-1182	MIMAT0005827	GAGGGUCUUGGGAGGGAUGUGAC	138.79	128.61	126.82	128.87
hsa-miR-1183	MIMAT0005828	CACUGUAGGUGAUGGUGAGAGUGGGCA	52.19	45.32	64.36	59.02
hsa-miR-1184	MIMAT0005829	CCUGCAGCGACUUGAUGGCUUCC	144.95	126.62	95.92	122.56
hsa-miR-1184	MIMAT0005829	CCUGCAGCGACUUGAUGGCUUCC	129.95	123.14	91.66	112.83
hsa-miR-1185-5p	MIMAT0005798	AGAGGAUACCCUUUGUAUGUU	94.13	79.12	104.80	114.24
hsa-miR-1193	MIMAT0015049	GGGAUGGUAGACCGGUGACGUGC	118.45	81.94	95.19	81.56
hsa-miR-1197	MIMAT0005955	UAGGACACAUGGUCUACUUCU	113.14	88.51	119.20	99.16
hsa-miR-1200	MIMAT0005863	CUCCUGAGCCAUUCUGAGCCUC	28.99	31.74	145.08	155.96
hsa-miR-1201	MIMAT0005864	AGCCUGAUUAAACACAUGCUCUGA	82.47	111.85	83.60	78.97
hsa-miR-1202	MIMAT0005865	GUGCCAGCUGCAGUGGGGGAG	75.58	65.19	112.88	98.47
hsa-miR-1203	MIMAT0005866	CCCGGAGCCAGGAUGCAGCUC	135.30	129.60	134.57	130.80
hsa-miR-1204	MIMAT0005868	UCGUGGCCUGGUCUCCAUUAU	85.73	109.22	106.35	102.34
hsa-miR-1205	MIMAT0005869	UCUGCAGGGUUUGCUUUGAG	83.53	96.30	108.09	106.54
hsa-miR-1206	MIMAT0005870	UGUUCAUGUAGAUGUUUAAGC	62.56	77.76	63.54	58.24
hsa-miR-1207-3p	MIMAT0005872	UCAGCUGGCCCUCAUUUC	61.40	60.18	113.17	95.95
hsa-miR-1207-5p	MIMAT0005871	UGGCAGGGAGGCUGGGAGGGG	112.87	117.90	151.94	123.51
hsa-miR-1208	MIMAT0005873	UCACUGUUCAGACAGGCGGA	114.87	109.58	151.91	161.83
hsa-miR-122-3p	MIMAT0004590	AACGCCAUUAUCACACUAAAUA	114.53	99.16	87.63	104.83
hsa-miR-1224-3p	MIMAT0005459	CCCCACCUCCUCUCUCCUCAG	84.09	100.83	93.28	100.31
hsa-miR-1224-5p	MIMAT0005458	GUGAGGACUCGGGAGGUGG	86.15	78.45	104.32	102.15
hsa-miR-1225-3p	MIMAT0005573	UGAGCCCCUGUGCCGCCCCCAG	96.94	99.01	112.33	107.03
hsa-miR-1225-5p	MIMAT0005572	GUGGGUACGGCCCAGUGGGGGG	131.99	140.53	102.55	92.98
hsa-miR-122-5p	MIMAT0000421	UGGAGUGUGACAAUGGUGUUUG	123.66	141.89	94.10	115.04
hsa-miR-1226-3p	MIMAT0005577	UCACCAGCCCUGUGUUCCCUAG	96.52	85.57	98.76	104.73
hsa-miR-1226-5p	MIMAT0005576	GUGAGGGCAUGCAGGCCUGGAUGGGG	136.29	122.54	111.73	135.94
hsa-miR-1227-3p	MIMAT0005580	CGUGCCACCCUUUUCCCCAG	102.71	126.71	126.08	149.56
hsa-miR-1228-3p	MIMAT0005583	UCACACCUGCCUCGCCCCCC	166.12	150.46	140.76	137.86
hsa-miR-1228-5p	MIMAT0005582	GUGGGCGGGGGCAGGUGUGUG	101.82	104.19	103.67	155.07
hsa-miR-1229-3p	MIMAT0005584	CUCUCACCACUGCCCUCCCACAG	83.85	99.11	105.37	102.92
hsa-miR-1231	MIMAT0005586	GUGUCUGGGCGGACAGCUGC	140.49	134.25	75.53	78.78
hsa-miR-1233-3p	MIMAT0005588	UGAGCCCUGUCCUCCCGCAG	66.51	70.96	99.71	102.70
hsa-miR-1233-3p	MIMAT0005588	UGAGCCCUGUCCUCCCGCAG	76.74	64.70	109.18	101.08
hsa-miR-1234-3p	MIMAT0005589	UCGGCCUGACCACCCACCCCAC	101.30	96.12	113.06	121.44
hsa-miR-1236-3p	MIMAT0005591	CCUCUUCCCCUUGUCUCUCCAG	134.26	123.35	122.18	115.82
hsa-miR-1237-3p	MIMAT0005592	UCCUUCUGCUCCGUCCCCCAG	63.63	72.23	98.39	128.44
hsa-miR-1238-3p	MIMAT0005593	CUUCCUCGUCUGUCUGCCCC	107.18	97.60	85.01	35.11
hsa-miR-1243	MIMAT0005894	AACUGGAUCAAUUAUAGGAGUG	119.29	108.32	56.96	22.21
hsa-miR-124-3p	MIMAT0000422	UAAGGCACGCGGUGAAUGCC	85.48	81.06	100.65	109.74
hsa-miR-1244	MIMAT0005896	AAGUAGUUGGUUUGUAUGAGAUGGUU	99.83	80.53	85.69	101.62

hsa-miR-1244	MIMAT0005896	AAGUAGUUGGUUUGUAUGAGAUGGUU	73.40	91.43	91.12	106.18
hsa-miR-1245a	MIMAT0005897	AAGUGAUCUAAAGGCCUACAU	141.91	145.35	113.00	129.59
hsa-miR-124-5p	MIMAT0004591	CGUGUUCACAGCGGACCUUGAU	39.80	37.58	93.96	99.34
hsa-miR-1246	MIMAT0005898	AAUGGAUUUUUGGAGCAGG	86.87	106.17	103.77	110.50
hsa-miR-1247-5p	MIMAT0005899	ACCCGUCCCGUUCGUCCCCGGA	95.76	130.95	105.47	87.89
hsa-miR-1248	MIMAT0005900	ACCUUCUUGUAUAAGCACUGUGCUAAA	110.60	90.56	114.00	80.92
hsa-miR-1249	MIMAT0005901	ACGCCCUUCCCCCCCUUCUUCA	80.02	96.24	93.06	108.84
hsa-miR-1250	MIMAT0005902	ACGGUGCUGGAUGUGGCCUUU	71.49	61.09	110.40	94.68
hsa-miR-1251	MIMAT0005903	ACUCUAGCUGCCAAAGGCGCU	121.16	116.00	107.65	106.62
hsa-miR-1252	MIMAT0005944	AGAAGGAAAUUGAAUUCAUUUA	126.57	104.36	127.72	113.14
hsa-miR-1253	MIMAT0005904	AGAGAAGAAGAUCAGCCUGCA	102.13	80.01	72.53	84.02
hsa-miR-1254	MIMAT0005905	AGCCUGGAAGCUGGAGCCUGCAGU	49.17	59.73	83.65	99.30
hsa-miR-1255a	MIMAT0005906	AGGAUGAGCAAAGAAAGUAGAUU	88.54	80.66	107.65	118.68
hsa-miR-1255b-5p	MIMAT0005945	CGGAUGAGCAAAGAAAGUGGUU	74.20	67.39	109.32	98.34
hsa-miR-1256	MIMAT0005907	AGGCAUUGACUUCUCACUAGCU	38.12	42.15	72.95	79.25
hsa-miR-1257	MIMAT0005908	AGUGAAUGAUGGGUUCUGACC	197.57	160.70	135.85	123.64
hsa-miR-1258	MIMAT0005909	AGUUAGGAUUAGGUCGUGGAA	179.87	232.76	108.22	116.78
hsa-miR-1259	MIMAT0005910	AUAUAUGAUGACUUAGCUUUU	76.42	94.49	83.16	92.03
hsa-miR-125a-3p	MIMAT0004602	ACAGGUGAGGUUCUUGGGAGCC	66.15	67.12	104.32	117.84
hsa-miR-125a-5p	MIMAT0000443	UCCCUGAGACCCUUUAACCUGUGA	88.45	101.98	97.01	95.28
hsa-miR-125b-1-3p	MIMAT0004592	ACGGGUUAGGCUCUUGGGAGCU	110.90	129.83	87.71	102.82
hsa-miR-125b-2-3p	MIMAT0004603	UCACAAGUCAGGCUCUUGGGAC	65.00	51.04	62.15	88.87
hsa-miR-125b-5p	MIMAT0000423	UCCCUGAGACCCUAACUUGUGA	62.52	83.48	73.26	115.05
hsa-miR-1260a	MIMAT0005911	AUCCCACCUCUGCCACCA	171.48	169.77	102.52	111.07
hsa-miR-1260b	MIMAT0015041	AUCCCACCACUGCCACCAU	93.11	103.66	115.34	121.75
hsa-miR-1261	MIMAT0005913	AUGGAUAAGGCUUUGGCUU	76.42	92.95	100.24	102.82
hsa-miR-1262	MIMAT0005914	AUGGGUGAAUUUGUAGAAGGAU	66.49	63.99	96.22	79.39
hsa-miR-1263	MIMAT0005915	AUGGUACCCUGGCAUACUGAGU	102.52	103.71	129.86	137.72
hsa-miR-126-3p	MIMAT0000445	UCGUACCGUGAGUAAUAAUGCG	112.30	124.65	118.57	101.17
hsa-miR-1264	MIMAT0005791	CAAGUCUUAUUUGAGCACCUGUU	138.86	129.83	59.80	52.68
hsa-miR-1265	MIMAT0005918	CAGGAUGUGGUCAAGUGUUGUU	134.20	146.73	105.37	107.89
hsa-miR-126-5p	MIMAT0000444	CAUUAUUACUUUUGGUACGCG	113.16	90.56	103.41	78.35
hsa-miR-1266	MIMAT0005920	CCUCAGGGCUGUAGAACAGGGCU	134.15	118.21	95.23	85.17
hsa-miR-1267	MIMAT0005921	CCUGUUGAAGUGUAAUCCCCA	110.76	101.11	107.61	108.03
hsa-miR-1268a	MIMAT0005922	CGGGCGUGGUGGUGGGGG	94.84	100.76	73.66	78.84
hsa-miR-1269a	MIMAT0005923	CUGGACUGAGCCGUGCUACUGG	59.36	21.49	80.55	48.38
hsa-miR-1270	MIMAT0005924	CUGGAGAUAUGGAAGAGCUGUGU	125.32	138.58	88.91	81.83
hsa-miR-1270	MIMAT0005924	CUGGAGAUAUGGAAGAGCUGUGU	118.58	138.58	81.45	81.40
hsa-miR-1271-5p	MIMAT0005796	CUUGGCACCUAGCAAGCACUCA	99.97	110.28	82.76	115.02
hsa-miR-1272	MIMAT0005925	GAUGAUGAUGGCAGCAAAUUCUGAAA	141.70	139.97	163.23	134.14
hsa-miR-1273a	MIMAT0005926	GGGCGACAAAGCAAGACUCUUUCUU	135.71	143.44	83.52	110.63

hsa-miR-1273c	MIMAT0015017	GGCGACAAAACGAGACCCUGUC	130.63	118.27	106.82	117.69
hsa-miR-1273d	MIMAT0015090	GAACCCAUGAGGUUGAGGCUGCAGU	97.89	94.62	97.34	86.90
hsa-miR-1273e	MIMAT0018079	UUGCUUGAACCCAGGAAGUGGA	70.09	81.33	79.73	77.49
hsa-miR-127-3p	MIMAT0000446	UCGGAUCCGUCUGAGCUUGGCU	67.87	73.75	68.92	79.61
hsa-miR-1274a	MIMAT0005927	GUCCCUGUUCAGGCGCCA	89.33	96.24	94.77	111.23
hsa-miR-1274b	MIMAT0005938	UCCCUGUUCGGGCGCCA	109.93	126.62	111.55	104.07
hsa-miR-1275	MIMAT0005929	GUGGGGGAGAGGCUGUC	25.09	44.86	79.58	120.32
hsa-miR-127-5p	MIMAT0004604	CUGAAGCUCAGAGGGCUCUGAU	88.18	130.82	70.19	70.88
hsa-miR-1276	MIMAT0005930	UAAAGAGCCCUGUGGAGACA	159.21	175.34	102.31	120.83
hsa-miR-1277-3p	MIMAT0005933	UACGUAGAUAUAUAUGUAUUUU	112.99	115.43	106.71	100.13
hsa-miR-1278	MIMAT0005936	UAGUACUGUGCAUAUCAUCUAU	98.64	97.59	110.61	119.82
hsa-miR-1279	MIMAT0005937	UCAUAUUGCUUCUUUCU	169.64	159.80	117.45	112.66
hsa-miR-128	MIMAT0000424	UCACAGUGAACCGGUCUCUUU	119.68	102.74	94.52	90.63
hsa-miR-1280	MIMAT0005946	UCCCACCGCUGCCACCC	88.40	80.08	108.55	113.85
hsa-miR-1281	MIMAT0005939	UCGCCUCCUCCUCUCCC	90.29	83.83	107.03	121.36
hsa-miR-1282	MIMAT0005940	UCGUUUGCCUUUUUCUGCUU	118.45	134.23	110.53	99.21
hsa-miR-1283	MIMAT0005799	UCUACAAAGGAAAGCGCUUUCU	140.73	129.83	103.66	114.24
hsa-miR-1284	MIMAT0005941	UCUAUACAGACCCUGGCUUUUC	109.30	90.73	101.22	99.81
hsa-miR-1285-3p	MIMAT0005876	UCUGGGCAACAAAGUGAGACCU	89.47	97.56	80.31	101.55
hsa-miR-1286	MIMAT0005877	UGCAGGACCAAGAUGAGCCCU	88.16	82.27	88.36	106.93
hsa-miR-1287	MIMAT0005878	UGCUGGAUCAGUGGUUCGAGUC	100.35	99.72	100.06	123.73
hsa-miR-1288	MIMAT0005942	UGGACUGCCCUGAUCUGGAGA	125.82	105.24	84.86	78.06
hsa-miR-1289	MIMAT0005879	UGGAGUCCAGGAAUCUGCAUUUU	102.96	79.43	92.91	81.85
hsa-miR-1290	MIMAT0005880	UGGAUUUUUGGAUCAGGGA	129.92	136.08	100.39	110.04
hsa-miR-1291	MIMAT0005881	UGGCCCUGACUGAAGACCAGCAGU	125.63	98.77	126.67	110.42
hsa-miR-129-1-3p	MIMAT0004548	AAGCCCUUACCCCAAAAAGUAU	147.09	133.17	127.98	123.87
hsa-miR-129-2-3p	MIMAT0004605	AAGCCCUUACCCCAAAAAGCAU	130.20	145.87	116.12	118.43
hsa-miR-1292-5p	MIMAT0005943	UGGGAACGGGUUCCGGCAGACGCUG	114.12	114.84	156.17	136.96
hsa-miR-1293	MIMAT0005883	UGGGUGGUCUGGAGAUUUGUGC	42.19	34.36	119.24	138.84
hsa-miR-1294	MIMAT0005884	UGUGAGGUUGGCAUUGUUGUCU	36.74	33.06	62.19	72.23
hsa-miR-1295a	MIMAT0005885	UUAGGCCGCAGAUCUGGGUGA	116.97	105.37	108.17	91.69
hsa-miR-129-5p	MIMAT0000242	CUUUUUGCGGUCUGGGCUUGC	127.86	101.94	107.82	115.57
hsa-miR-1296	MIMAT0005794	UUAGGGCCCUGGCUCCAUCUCC	135.30	130.50	99.05	87.84
hsa-miR-1297	MIMAT0005886	UUCAAGUAAUUCAGGUG	67.92	63.87	80.62	83.49
hsa-miR-1298	MIMAT0005800	UUCAUUCGGCUGUCCAGAUGUA	131.10	120.16	115.11	128.44
hsa-miR-1299	MIMAT0005887	UUCUGGAAUUCUGUGUGAGGGA	177.44	151.54	96.24	102.34
hsa-miR-1300	MIMAT0005888	UUGAGAAGGAGGCUGCUG	130.16	110.81	72.64	78.04
hsa-miR-1301	MIMAT0005797	UUGCAGCUGCCUGGGAGUGACUUC	99.24	97.89	106.40	82.57
hsa-miR-1302	MIMAT0005890	UUGGGACAUACUUAUGCUAAA	98.92	120.50	76.87	108.47
hsa-miR-1302	MIMAT0005890	UUGGGACAUACUUAUGCUAAA	109.87	107.42	94.54	110.95
hsa-miR-1303	MIMAT0005891	UUUAGAGACGGGGUCUUGCUCU	130.73	124.03	112.08	128.49

hsa-miR-1304-5p	MIMAT0005892	UUUGAGGCUACAGUGAGAUGUG	67.90	64.48	93.93	95.04
hsa-miR-1305	MIMAT0005893	UUUUCAACUCUAAUGGGAGAGA	127.16	113.37	84.08	106.46
hsa-miR-1306-3p	MIMAT0005950	ACGUUGGCUCUGGUGGUG	84.02	78.02	110.74	122.94
hsa-miR-1307-3p	MIMAT0005951	ACUCGGCGUGGCGUCGGUCGUG	84.09	98.50	120.45	87.36
hsa-miR-1308	MIMAT0005947	GCAUGGGUGGUUCAGUGG	122.50	119.68	100.34	112.19
hsa-miR-130a-3p	MIMAT0000425	CAGUGCAAUGUUAAAAGGGCAU	175.16	213.42	115.12	111.32
hsa-miR-130a-5p	MIMAT0004593	UUCACAUUGUGCUACUGUCUGC	101.58	74.52	89.42	86.31
hsa-miR-130b-3p	MIMAT0000691	CAGUGCAAUGAUGAAAGGGCAU	121.07	141.58	120.69	86.88
hsa-miR-130b-5p	MIMAT0004680	ACUCUUUCCCUGUUGCACUAC	115.73	115.22	51.01	66.79
hsa-miR-1321	MIMAT0005952	CAGGGAGGUGAAUGUGAU	51.50	68.29	86.22	89.75
hsa-miR-1322	MIMAT0005953	GAUGAUGCUGCUGAUGCUG	81.08	76.05	88.85	76.16
hsa-miR-1323	MIMAT0005795	UCAAAACUGAGGGGCAUUUUCU	79.22	66.83	113.34	125.66
hsa-miR-132-3p	MIMAT0000426	UAACAGUCUACAGCCAUGGUCG	138.93	154.68	84.13	97.21
hsa-miR-1324	MIMAT0005956	CCAGACAGAAUUCUAUGCACUUUC	120.17	134.11	117.88	115.53
hsa-miR-132-5p	MIMAT0004594	ACCGUGGCUUUCGAUUGUUACU	75.49	89.88	105.94	116.14
hsa-miR-133a	MIMAT0000427	UUUGGUCCCCUUCAACCAGCUG	99.46	112.98	79.75	69.91
hsa-miR-133b	MIMAT0000770	UUUGGUCCCCUUCAACCAGCUA	142.29	185.05	118.31	104.55
hsa-miR-134	MIMAT0000447	UGUGACUGGUUGACCAGAGGGG	91.39	97.33	102.40	93.98
hsa-miR-135a-3p	MIMAT0004595	UAUAGGGAUUGGAGCCGUGGCG	124.92	128.04	96.87	84.85
hsa-miR-135a-5p	MIMAT0000428	UAUGGCUUUUUAUUCCUAUGUGA	84.81	71.44	46.70	38.71
hsa-miR-135b-3p	MIMAT0004698	AUGUAGGGCUAAAAGCCAUGGG	102.13	103.45	96.49	112.19
hsa-miR-135b-5p	MIMAT0000758	UAUGGCUUUUCAUUCCUAUGUGA	69.21	80.66	90.85	77.25
hsa-miR-136-3p	MIMAT0004606	CAUCAUCGUCUCAAAUGAGUCU	109.04	97.56	74.61	62.83
hsa-miR-136-5p	MIMAT0000448	ACUCCAUUUGUUUUGAUGAUGGA	109.99	122.90	97.74	97.25
hsa-miR-137	MIMAT0000429	UUAUUGCUUAAGAAUACGCGUAG	116.59	99.52	80.64	64.36
hsa-miR-138-1-3p	MIMAT0004607	GCUACUUCACAACACCAGGGCC	95.41	99.17	111.75	103.93
hsa-miR-138-2-3p	MIMAT0004596	GCUAUUUCACGACACCAGGGUU	100.39	84.97	93.49	108.37
hsa-miR-138-5p	MIMAT0000430	AGCUGGUGUUGUGAAUCAGGCCG	75.53	66.62	76.18	88.27
hsa-miR-139-3p	MIMAT0004552	GGAGACGCGGCCCUGUUGGAGU	78.33	86.48	124.22	117.39
hsa-miR-139-5p	MIMAT0000250	UCUACAGUGCACGUGUCUCCAG	68.75	29.95	77.76	46.24
hsa-miR-140-3p	MIMAT0004597	UACCACAGGGUAGAACCACGG	161.52	168.23	90.07	112.61
hsa-miR-140-5p	MIMAT0000431	CAGUGGUUUUACCCUAUGGUAG	106.14	122.03	93.72	79.92
hsa-miR-141-3p	MIMAT0000432	UAACACUGUCUGGUAAAGAUGG	96.55	97.06	99.61	114.49
hsa-miR-141-5p	MIMAT0004598	CAUCUUCCAGUACAGUGUUGGA	87.00	86.32	60.12	57.76
hsa-miR-142-3p	MIMAT0000434	UGUAGUGUUUCCUACUUUAUGGA	135.31	126.32	88.46	84.83
hsa-miR-142-5p	MIMAT0000433	CAUAAAGUAGAAAGCACUACU	114.35	119.68	93.92	83.06
hsa-miR-143-3p	MIMAT0000435	UGAGAUGAAGCACUGUAGCUC	97.71	121.47	83.17	103.36
hsa-miR-143-5p	MIMAT0004599	GGUGCAGUGCUGCAUCUCUGGU	123.05	106.37	80.10	89.82
hsa-miR-144-3p	MIMAT0000436	UACAGUAUAGAUGAUGUACU	161.18	167.61	82.85	83.90
hsa-miR-144-5p	MIMAT0004600	GGAUAUCAUCAUAUACUGUAAG	112.14	121.62	130.25	112.25
hsa-miR-145-3p	MIMAT0004601	GGAUUCCUGGAAAUACUGUUCU	53.83	42.58	110.06	106.57

hsa-miR-145-5p	MIMAT0000437	GUCCAGUUUUCCCAGGAAUCCCU	112.60	116.38	121.53	135.30
hsa-miR-1468	MIMAT0006789	CUCCGUUUGCCUGUUUCGCUG	73.03	65.59	84.08	100.25
hsa-miR-1469	MIMAT0007347	CUCGGCGCGGGGCGCGGGCUCC	93.26	101.48	104.82	103.18
hsa-miR-146a-3p	MIMAT0004608	CCUCUGAAAUUCAGUUCUUCAG	116.68	102.55	71.67	87.84
hsa-miR-146a-5p	MIMAT0000449	UGAGAACUGAAUUCCAUGGGUU	149.05	142.62	98.29	118.51
hsa-miR-146b-3p	MIMAT0004766	UGCCCUGUGGACUCAGUUCUGG	103.50	113.79	125.67	112.21
hsa-miR-146b-5p	MIMAT0002809	UGAGAACUGAAUUCCAUAGGCU	107.18	92.81	77.26	94.72
hsa-miR-1470	MIMAT0007348	GCCCUCCGCCCGUGCACCCCG	71.76	76.05	117.90	123.76
hsa-miR-1471	MIMAT0007349	GCCCGCGUGUGGAGCCAGGUGU	81.18	89.48	81.94	83.54
hsa-miR-147a	MIMAT0000251	GUGUGUGGAAAUGCUUCUGC	116.76	96.30	102.04	96.89
hsa-miR-147b	MIMAT0004928	GUGUGCGGAAAUGCUUCUGCUA	41.11	57.15	107.15	104.58
hsa-miR-148a-3p	MIMAT0000243	UCAGUGCACUACAGAACUUUGU	109.32	122.35	94.43	103.16
hsa-miR-148a-5p	MIMAT0004549	AAAGUUCUGAGACACUCCGACU	109.66	84.33	75.58	80.63
hsa-miR-148b-3p	MIMAT0000759	UCAGUGCAUCACAGAACUUUGU	123.55	90.26	125.26	126.32
hsa-miR-148b-5p	MIMAT0004699	AAGUUCUGUUAUACACUCAGGC	89.47	96.02	63.22	53.95
hsa-miR-149-3p	MIMAT0004609	AGGGAGGGACGGGGGCUGUGC	88.79	126.88	95.20	79.10
hsa-miR-149-5p	MIMAT0000450	UCUGGCUCCGUGUCUUCACUCCC	97.27	103.45	98.92	105.58
hsa-miR-150-3p	MIMAT0004610	CUGGUACAGGCCUGGGGGACAG	133.80	127.73	126.67	113.99
hsa-miR-150-5p	MIMAT0000451	UCUCCCAACCCUUGUACCAGUG	97.47	106.53	104.04	95.51
hsa-miR-151a-3p	MIMAT0000757	CUAGACUGAAGCUCCUUGAGG	85.74	63.76	113.91	123.13
hsa-miR-151a-5p	MIMAT0004697	UCGAGGAGCUCACAGUCUAGU	153.49	146.50	133.77	120.32
hsa-miR-152	MIMAT0000438	UCAGUGCAUGACAGAACUUGG	177.78	149.43	82.79	108.37
hsa-miR-153	MIMAT0000439	UUGCAUAGUCACAAAAGUGAUC	57.63	39.04	104.80	118.78
hsa-miR-1537	MIMAT0007399	AAAACCGUCUAGUUACAGUUGU	135.14	123.68	140.11	145.34
hsa-miR-1538	MIMAT0007400	CGGCCCGGGCUGCUGCUGUUCCU	90.29	75.90	112.08	111.85
hsa-miR-1539	MIMAT0007401	UCCUGCGCGUCCCAGAUGCCC	194.06	154.64	114.88	115.90
hsa-miR-154-3p	MIMAT0000453	AAUCAUACACGGUUGACCUAUU	113.95	74.32	84.36	87.13
hsa-miR-154-5p	MIMAT0000452	UAGGUUAUCCGUGUUGCCUUCG	84.17	100.90	89.06	87.46
hsa-miR-155-3p	MIMAT0004658	CUCCUACAUAUUAGCAUUAACA	128.61	139.04	104.23	111.07
hsa-miR-155-5p	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU	148.36	130.95	84.38	115.16
hsa-miR-15a-3p	MIMAT0004488	CAGGCCAUAUUGUGCUGCCUCA	110.85	116.18	77.92	63.41
hsa-miR-15a-5p	MIMAT0000068	UAGCAGCACAUAAUGGUUUGUG	104.58	85.91	73.92	87.59
hsa-miR-15b-3p	MIMAT0004586	CGAAUCAUUAUUUGCUGCUCUA	177.07	165.16	129.29	135.82
hsa-miR-15b-5p	MIMAT0000417	UAGCAGCACAUCAUGGUUUACA	69.16	88.37	86.77	82.99
hsa-miR-16-1-3p	MIMAT0004489	CCAGUAUUAACUGUGCUGCUGA	116.50	89.88	101.95	93.30
hsa-miR-16-2-3p	MIMAT0004518	CCAAUAUUACUGUGCUGCUUUA	80.60	67.44	85.79	76.46
hsa-miR-16-5p	MIMAT0000069	UAGCAGCACGUAAAUAUUGGCG	68.47	70.77	75.07	90.15
hsa-miR-17-3p	MIMAT0000071	ACUGCAGUGAAGGCACUUGUAG	73.85	80.73	109.66	103.18
hsa-miR-17-5p	MIMAT0000070	CAAAGUGCUUACAGUGCAGGUAG	95.94	87.30	85.46	102.44
hsa-miR-181a-2-3p	MIMAT0004558	ACCACUGACCGUUGACUGUACC	176.77	212.80	151.87	143.32
hsa-miR-181a-3p	MIMAT0000270	ACCAUCGACCGUUGAUUGUACC	68.79	74.76	98.54	105.59

hsa-miR-181a-5p	MIMAT0000256	AACAUUCAACGCUGUCGGUGAGU	114.26	113.40	82.66	84.47
hsa-miR-181b-5p	MIMAT0000257	AACAUUCAUUGCUGUCGGUGGGU	119.01	94.44	108.04	89.75
hsa-miR-181c-3p	MIMAT0004559	AACCAUCGACCGUUGAGUGGAC	124.58	106.81	99.90	95.48
hsa-miR-181c-5p	MIMAT0000258	AACAUUCAACCUGUCGGUGAGU	95.95	91.75	74.66	85.13
hsa-miR-181d	MIMAT0002821	AACAUUCAUUGUUGUCGGUGGGU	173.03	156.59	83.97	92.51
hsa-miR-182-3p	MIMAT0000260	UGGUUCUAGACUUGCCAACUA	75.49	76.05	82.02	93.93
hsa-miR-1825	MIMAT0006765	UCCAGUGCCCUCCUCUCC	174.60	160.38	94.82	89.07
hsa-miR-182-5p	MIMAT0000259	UUUGGCAAUGGUAGAACUCACACU	92.30	106.34	117.84	108.36
hsa-miR-1826	MIMAT0006766	AUUGAUCAUCGACACUUCGAACGCAAU	93.40	102.55	124.30	111.23
hsa-miR-1827	MIMAT0006767	UGAGGCAGUAGAUUGAAU	76.05	78.02	92.87	102.87
hsa-miR-183-3p	MIMAT0004560	GUGAAUUACCGAAGGGCCAUAA	116.30	86.44	103.67	74.59
hsa-miR-183-5p	MIMAT0000261	UAUGGCACUGGUAGAAUUCACU	106.08	90.86	112.04	93.19
hsa-miR-184	MIMAT0000454	UGGACGGAGAACUGAUAAGGGU	85.71	72.95	110.41	102.36
hsa-miR-185-3p	MIMAT0004611	AGGGGCUGGCUUUCCUCUGGUC	43.80	45.32	87.14	101.55
hsa-miR-185-5p	MIMAT0000455	UGGAGAGAAAGGCAGUUCCUGA	143.83	131.44	94.56	92.57
hsa-miR-186-3p	MIMAT0004612	GCCCAAAGGUGAAUUUUUUGGG	140.49	130.73	103.96	120.30
hsa-miR-186-5p	MIMAT0000456	CAAAGAAUUCUCCUUUUGGGCU	128.58	107.84	115.34	86.50
hsa-miR-187-3p	MIMAT0000262	UCGUGUCUUGUGUUGCAGCCGG	96.93	90.32	79.00	74.12
hsa-miR-187-5p	MIMAT0004561	GGCUACAACACAGGACCCGGGC	91.43	71.14	114.11	118.98
hsa-miR-188-3p	MIMAT0004613	CUCCCACAUGCAGGGUUUGCA	69.78	74.84	72.98	69.06
hsa-miR-188-5p	MIMAT0000457	CAUCCCUUGCAUGGUGGAGGG	130.25	129.52	83.63	79.40
hsa-miR-18a-3p	MIMAT0002891	ACUGCCCUAAGUGCUCCUUCUGG	79.51	94.85	101.41	110.02
hsa-miR-18a-5p	MIMAT0000072	UAAGGUGCAUCUAGUGCAGAUAG	100.26	126.11	142.80	163.49
hsa-miR-18b-3p	MIMAT0004751	UGCCCUAAAUGCCCCUUCUGGC	90.40	116.00	129.86	127.57
hsa-miR-18b-5p	MIMAT0001412	UAAGGUGCAUCUAGUGCAGUUAG	94.45	108.74	122.87	106.26
hsa-miR-1908	MIMAT0007881	CGGCGGGGACGGCGAUUGGUC	78.33	61.62	125.91	124.79
hsa-miR-1909-3p	MIMAT0007883	CGCAGGGGCCGGGUGCUCACCG	82.01	82.20	115.62	116.14
hsa-miR-1909-5p	MIMAT0007882	UGAGUGCCGGUGCCUGCCCUG	143.40	132.64	110.58	92.84
hsa-miR-190a	MIMAT0000458	UGAUAUGUUUGAUAUAUUAGGU	66.13	75.39	85.77	90.24
hsa-miR-190b	MIMAT0004929	UGAUAUGUUUGAUAUUGGGUU	78.15	63.78	97.83	98.47
hsa-miR-1910	MIMAT0007884	CCAGUCCUGUGCCUGCCGCCU	138.86	123.68	109.92	110.43
hsa-miR-1911-3p	MIMAT0007886	CACCAGGCAUUGUGGUCUCC	84.13	92.67	85.01	87.34
hsa-miR-1911-5p	MIMAT0007885	UGAGUACCGCCAUGUCUGUUGGG	111.36	127.21	94.56	97.06
hsa-miR-1912	MIMAT0007887	UACCCAGAGCAUGCAGUGUGAA	71.29	81.37	91.78	99.77
hsa-miR-1913	MIMAT0007888	UCUGCCCCCUCCGCUGCUGCCA	88.32	105.52	120.42	104.02
hsa-miR-191-3p	MIMAT0001618	GCUGCGCUUGGAUUUCGUCCCC	122.77	149.72	152.22	151.35
hsa-miR-1914-3p	MIMAT0007890	GGAGGGGUCCCGCACUGGGAGG	110.90	114.46	120.75	121.22
hsa-miR-1914-5p	MIMAT0007889	CCCUGUGCCCGGCCCACUUCUG	125.63	126.14	106.16	110.82
hsa-miR-1915-3p	MIMAT0007892	CCCCAGGGCGACGCGGCGGG	128.53	108.52	102.97	97.08
hsa-miR-1915-5p	MIMAT0007891	ACCUUGCCUUGCUGCCCGGGCC	81.37	77.55	95.96	67.87
hsa-miR-191-5p	MIMAT0000440	CAACGGAAUCCCAAAAGCAGCUG	94.13	84.13	131.97	96.76

hsa-miR-192-3p	MIMAT0004543	CUGCCAAUUCCAUAGGUCACAG	131.07	142.51	103.92	89.56
hsa-miR-192-5p	MIMAT0000222	CUGACCUAUGAAUUGACAGCC	93.88	102.09	91.85	86.35
hsa-miR-193a-3p	MIMAT0000459	AACUGGCCUACAAAGUCCCAGU	102.91	90.56	112.33	72.78
hsa-miR-193a-5p	MIMAT0004614	UGGGUCUUUGCGGGCGAGAUGA	114.60	115.93	106.51	105.74
hsa-miR-193b-3p	MIMAT0002819	AACUGGCCCUCAAAGUCCCGCU	81.18	77.31	115.74	85.93
hsa-miR-193b-5p	MIMAT0004767	CGGGGUUUUGAGGGCGAGAUGA	166.57	199.38	141.69	145.03
hsa-miR-194-3p	MIMAT0004671	CCAGUGGGGCUGCUGUUAUCUG	97.86	91.42	110.49	97.74
hsa-miR-194-5p	MIMAT0000460	UGUAACAGCAACUCCAUGUGGA	172.95	174.42	60.48	78.78
hsa-miR-195-3p	MIMAT0004615	CCAAUAUUGGCUGUGCUGCUCC	120.57	142.81	102.35	105.16
hsa-miR-195-5p	MIMAT0000461	UAGCAGCACAGAAAUAUUGGC	59.59	92.58	78.07	75.30
hsa-miR-196a-3p	MIMAT0004562	CGGCAACAAGAAACUGCCUGAG	114.75	108.52	122.90	120.98
hsa-miR-196a-5p	MIMAT0000226	UAGGUAGUUUCAUGUUGUUGGG	92.57	92.83	78.04	68.80
hsa-miR-196b-3p	MIMAT0009201	UCGACAGCACGACACUGCCUUC	115.52	104.36	124.30	91.66
hsa-miR-196b-5p	MIMAT0001080	UAGGUAGUUUCCUGUUGUUGGG	89.02	74.44	72.24	54.49
hsa-miR-1972	MIMAT0009447	UCAGGCCAGGCACAGUGGCUCA	134.19	146.58	109.52	117.99
hsa-miR-1972	MIMAT0009447	UCAGGCCAGGCACAGUGGCUCA	117.74	87.84	87.12	102.80
hsa-miR-1973	MIMAT0009448	ACCGUGCAAAGGUAGCAUA	112.50	89.12	99.28	89.66
hsa-miR-197-3p	MIMAT0000227	UUCACCACCUUCUCCACCCAGC	54.84	55.26	92.84	85.51
hsa-miR-1974	MIMAT0009449	UGGUUGUAGUCCGUGCGAGAAUA	119.67	101.48	105.57	112.62
hsa-miR-1975	MIMAT0009450	CCCCCACAACCGCGCUUGACUAGCU	106.24	91.42	69.49	69.18
hsa-miR-1976	MIMAT0009451	CCUCCUGCCCUCCUUGCUGU	64.48	73.64	93.93	79.20
hsa-miR-1977	MIMAT0009452	GAUUAGGGUGCUUAGCUGUUAA	82.93	90.38	85.36	110.28
hsa-miR-1978	MIMAT0009453	GGUUUGGUCCUAGCCUUUCUA	75.49	77.59	121.89	104.09
hsa-miR-1979	MIMAT0009454	CUCCCACUGCUUCACUUGACUA	141.23	137.14	96.26	124.51
hsa-miR-198	MIMAT0000228	GGUCCAGAGGGGAGAUAGGUUC	117.20	115.10	106.39	93.73
hsa-miR-199a-3p	MIMAT0000232	ACAGUAGUCUGCACAUUGGUUA	123.08	121.94	99.48	94.52
hsa-miR-199a-5p	MIMAT0000231	CCCAGUGUUCAGACUACCUGUUC	80.32	72.95	108.55	90.87
hsa-miR-199b-5p	MIMAT0000263	CCCAGUGUUUAGACUAUCUGUUC	90.49	118.78	86.22	90.23
hsa-miR-19a-3p	MIMAT0000073	UGUGCAAAUCUAUGCAAAACUGA	107.81	113.91	117.27	115.72
hsa-miR-19a-5p	MIMAT0004490	AGUUUUGCAUAGUUGCACUACA	50.23	49.19	124.73	109.32
hsa-miR-19b-1-5p	MIMAT0004491	AGUUUUGCAGGUUUGCAUCCAGC	36.55	45.32	90.42	76.21
hsa-miR-19b-2-5p	MIMAT0004492	AGUUUUGCAGGUUUGCAUUUCA	37.00	35.70	103.66	92.57
hsa-miR-19b-3p	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA	154.62	164.41	94.91	122.60
hsa-miR-200a-3p	MIMAT0000682	UAACACUGUCUGGUAACGAUGU	82.95	80.66	110.49	92.03
hsa-miR-200a-5p	MIMAT0001620	CAUCUUACCGGACAGUGCUGGA	82.47	98.23	100.73	91.69
hsa-miR-200b-3p	MIMAT0000318	UAAUACUGCCUGGUAAUGAUGA	50.55	52.33	57.75	66.35
hsa-miR-200b-5p	MIMAT0004571	CAUCUUACUGGGCAGCAUUGGA	75.58	89.15	119.57	113.45
hsa-miR-200c-3p	MIMAT0000617	UAAUACUGCCGGGUAAUGAUGGA	82.47	83.97	61.63	63.38
hsa-miR-200c-5p	MIMAT0004657	CGUCUUACCCAGCAGUGUUUGG	108.11	120.61	102.52	107.89
hsa-miR-202-3p	MIMAT0002811	AGAGGUAUAGGGCAUGGGAA	91.09	85.98	135.72	103.64
hsa-miR-202-5p	MIMAT0002810	UUCCUAUGCAUAUACUUCUUUG	97.50	90.18	106.19	93.41

hsa-miR-203a	MIMAT0000264	GUGAAAUGUUUAGGACCACUAG	106.73	110.55	110.04	101.95
hsa-miR-204-5p	MIMAT0000265	UUCCCUUUGUCAUCCUAUGCCU	107.48	101.94	117.07	120.93
hsa-miR-2052	MIMAT0009977	UGUUUUGAUAACAGUAAUGU	40.43	35.71	100.73	92.51
hsa-miR-2053	MIMAT0009978	GUGUUAAUUAAACCUCUAUUUAC	53.83	45.30	74.24	92.61
hsa-miR-205-3p	MIMAT0009197	GAUUUCAGUGGAGUGAAGUUC	40.99	53.26	83.90	86.81
hsa-miR-2054	MIMAT0009979	CUGUAAUAUAAAUUUAAUUUAUU	102.96	104.72	106.68	98.26
hsa-miR-205-5p	MIMAT0000266	UCCUUCAUUCCACCGGAGUCUG	85.84	97.14	105.47	119.35
hsa-miR-206	MIMAT0000462	UGGAAUGUAAGGAAGUGUGUGG	143.45	121.41	80.81	73.61
hsa-miR-208a	MIMAT0000241	AUAAGACGAGCAAAAAGCUUGU	109.11	120.13	56.69	46.31
hsa-miR-208b	MIMAT0004960	AUAAGACGAACAAAAGGUUUGU	112.15	124.71	79.73	80.71
hsa-miR-20a-3p	MIMAT0004493	ACUGCAUUAUGAGCACUUAAAG	117.03	108.50	107.64	84.78
hsa-miR-20a-5p	MIMAT0000075	UAAAGUGCUUAUAGUGCAGGUAG	122.90	124.82	108.55	124.10
hsa-miR-20b-3p	MIMAT0004752	ACUGUAGUAUGGGCACUUCCAG	111.46	126.50	120.13	124.59
hsa-miR-20b-5p	MIMAT0001413	CAAAGUGCUCAUAGUGCAGGUAG	124.49	105.05	114.87	111.62
hsa-miR-210	MIMAT0000267	CUGUGCGUGUGACAGCGGCUGA	117.72	95.35	115.90	97.41
hsa-miR-2110	MIMAT0010133	UUGGGGAAACGGCCGCUGAGUG	84.81	74.52	106.51	84.41
hsa-miR-2113	MIMAT0009206	AUUUGUGCUUGGCUCUGUCAC	146.94	177.83	120.02	118.87
hsa-miR-2114-3p	MIMAT0011157	CGAGCCUCAAGCAAGGGACUU	78.42	79.72	77.84	82.63
hsa-miR-2114-5p	MIMAT0011156	UAGUCCCUUCCUUGAAGCGGUC	97.35	108.45	86.13	84.46
hsa-miR-2115-3p	MIMAT0011159	CAUCAGAAUUCAUGGAGGCUAG	67.88	70.54	85.66	79.24
hsa-miR-2115-5p	MIMAT0011158	AGCUUCCAUGACUCCUGAUGGA	71.96	66.09	97.82	82.85
hsa-miR-211-5p	MIMAT0000268	UUCCCUUUGUCAUCCUUCGCCU	112.02	92.64	93.49	101.68
hsa-miR-2116-3p	MIMAT0011161	CCUCCCAUGCCAAGAACUCCC	89.02	77.22	87.87	79.21
hsa-miR-2116-5p	MIMAT0011160	GGUUCUUAGCAUAGGAGGUCU	76.57	78.94	85.09	128.63
hsa-miR-2117	MIMAT0011162	UGUUCUCUUUGCCAAGGACAG	85.49	92.42	117.80	112.80
hsa-miR-212-3p	MIMAT0000269	UAACAGUCUCCAGUCACGGCC	105.69	107.21	62.78	81.69
hsa-miR-21-3p	MIMAT0004494	CAACACCAGUCGAUGGGCUGU	86.67	83.73	97.39	89.49
hsa-miR-214-3p	MIMAT0000271	ACAGCAGGCACAGACAGGCAGU	113.07	138.59	86.42	93.32
hsa-miR-214-5p	MIMAT0004564	UGCCUGUCUACACUUGCUGUGC	121.57	78.40	94.27	79.61
hsa-miR-215	MIMAT0000272	AUGACCUAUGAAUUGACAGAC	90.86	96.00	107.37	116.87
hsa-miR-21-5p	MIMAT0000076	UAGCUUAUCAGACUGAUGUUGA	110.59	121.46	112.80	105.38
hsa-miR-216a-5p	MIMAT0000273	UAAUCUCAGCUGGCAACUGUGA	116.50	124.28	96.76	99.23
hsa-miR-216b	MIMAT0004959	AAAUCUCUGCAGGCAAAUGUGA	169.78	172.43	116.53	118.25
hsa-miR-217	MIMAT0000274	UACUGCAUCAGGAACUGAUUGGA	109.75	99.72	64.39	80.92
hsa-miR-218-1-3p	MIMAT0004565	AUGGUUCCGUCAAGCACCAUGG	91.81	110.29	72.19	62.93
hsa-miR-218-2-3p	MIMAT0004566	CAUGGUUCUGUCAAGCACCGCG	112.15	106.28	89.33	107.52
hsa-miR-218-5p	MIMAT0000275	UUGUGCUUGAUCUAACCAUGU	83.07	86.29	99.94	99.21
hsa-miR-219-1-3p	MIMAT0004567	AGAGUUGAGUCUGGACGUCCCG	78.15	99.72	111.21	116.45
hsa-miR-219-2-3p	MIMAT0004675	AGAAUUGUGGCUGGACAUCUGU	49.33	57.21	138.44	141.06
hsa-miR-219-5p	MIMAT0000276	UGAUUGUCCAAACGCAAUUCU	84.15	98.59	73.01	90.63
hsa-miR-220a	MIMAT0000277	CCACACCGUAUCUGACACUUU	80.32	93.05	105.19	91.69

hsa-miR-220b	MIMAT0004908	CCACCACCGUGUCUGACACUU	92.26	82.20	105.94	104.09
hsa-miR-221-3p	MIMAT0000278	AGCUACAUUGUCUGCUGGGUUUC	142.31	139.73	110.78	120.82
hsa-miR-221-5p	MIMAT0004568	ACCUGGCAUACAAUGUAGAUUU	108.04	92.67	97.28	104.46
hsa-miR-222-3p	MIMAT0000279	AGCUACAUCUGGCUACUGGGU	97.75	118.23	79.09	87.81
hsa-miR-222-5p	MIMAT0004569	CUCAGUAGCCAGUGUAGAUCCU	45.34	106.70	89.53	91.03
hsa-miR-223-3p	MIMAT0000280	UGUCAGUUUGUCAAAUACCCCA	113.82	98.12	82.72	76.97
hsa-miR-223-5p	MIMAT0004570	CGUGUAUUUGACAAGCUGAGUU	83.86	74.16	83.23	82.97
hsa-miR-22-3p	MIMAT0000077	AAGCUGCCAGUUGAAGAACUGU	56.06	43.12	78.01	67.08
hsa-miR-224-3p	MIMAT0009198	AAAAUGGUGCCCUAGUGACUACA	65.71	61.75	99.32	110.46
hsa-miR-224-5p	MIMAT0000281	CAAGUCACUAGUGGUUCCGUU	76.04	63.08	109.42	104.22
hsa-miR-22-5p	MIMAT0004495	AGUUCUUCAGUGGCAAGCUUUA	90.29	103.40	85.12	117.66
hsa-miR-2276	MIMAT0011775	UCUGCAAGUGUCAGAGGCGAGG	89.12	89.44	98.34	105.48
hsa-miR-2277-3p	MIMAT0011777	UGACAGCGCCCUGCCUGGCUC	103.84	120.29	120.76	115.88
hsa-miR-2277-5p	MIMAT0017352	AGCGCGGGCUGAGCGCUGCCAGUC	62.31	46.63	95.20	82.85
hsa-miR-2278	MIMAT0011778	GAGAGCAGUGUGUGUUGCCUGG	101.10	126.54	96.74	97.36
hsa-miR-2355-3p	MIMAT0017950	AUUGUCCUUGCUGUUUGGAGAU	89.56	97.60	120.80	114.49
hsa-miR-2355-5p	MIMAT0016895	AUCCCCAGAUACAAUGGACAA	144.95	144.71	103.03	119.72
hsa-miR-23a-3p	MIMAT0000078	AUCACAUUGCCAGGGAUUUCC	123.02	117.53	73.47	88.85
hsa-miR-23a-5p	MIMAT0004496	GGGGUUCCUGGGGAUGGGAUUU	120.64	136.43	88.90	97.80
hsa-miR-23b-3p	MIMAT0000418	AUCACAUUGCCAGGGAUUACC	103.77	112.40	116.79	98.04
hsa-miR-23b-5p	MIMAT0004587	UGGGUUCCUGGCAUGCUGAUUU	106.45	78.96	77.14	73.53
hsa-miR-23c	MIMAT0018000	AUCACAUUGCCAGUGAUUACCC	85.10	101.04	75.80	90.37
hsa-miR-24-1-5p	MIMAT0000079	UGCCUACUGAGCUGAUAUCAGU	97.27	78.96	96.88	84.56
hsa-miR-24-2-5p	MIMAT0004497	UGCCUACUGAGCUGAAACACAG	93.84	96.75	83.13	84.17
hsa-miR-24-3p	MIMAT0000080	UGGCUCAGUUCAGCAGGAACAG	93.28	105.52	106.94	125.15
hsa-miR-25-3p	MIMAT0000081	CAUUGCACUUGUCUCGGUCUGA	63.24	65.68	97.73	78.77
hsa-miR-25-5p	MIMAT0004498	AGGCGGAGACUUGGGCAAUUG	99.22	93.27	91.90	110.65
hsa-miR-26a-1-3p	MIMAT0004499	CCUAUUCUUGGUUACUUGCACG	93.69	103.54	103.25	107.42
hsa-miR-26a-2-3p	MIMAT0004681	CCUAUUCUUGAUUACUUGUUUC	110.79	88.07	100.29	86.49
hsa-miR-26a-5p	MIMAT0000082	UUCAAGUAAUCCAGGAUAGGCU	113.07	120.86	99.95	80.42
hsa-miR-26b-3p	MIMAT0004500	CCUGUUCUCCAUUACUUGGCUC	98.64	91.73	82.79	105.50
hsa-miR-26b-5p	MIMAT0000083	UUCAAGUAAUUCAGGAUAGGU	70.46	91.93	76.84	105.02
hsa-miR-27a-3p	MIMAT0000084	UUCACAGUGGCUAAGUUCCGC	170.19	144.71	111.55	127.42
hsa-miR-27a-5p	MIMAT0004501	AGGGCUUAGCUGCUUGUGAGCA	112.61	89.93	128.15	121.73
hsa-miR-27b-3p	MIMAT0000419	UUCACAGUGGCUAAGUUCUGC	132.98	149.59	104.30	89.81
hsa-miR-27b-5p	MIMAT0004588	AGAGCUUAGCUGAUUGGUGAAC	98.79	112.92	95.12	104.72
hsa-miR-28-3p	MIMAT0004502	CACUAGAUUGUGAGCUCCUGGA	54.99	26.89	111.63	94.57
hsa-miR-28-5p	MIMAT0000085	AAGGAGCUCACAGUCUAUUGAG	40.74	34.42	73.17	72.82
hsa-miR-2861	MIMAT0013802	GGGGCCUGGCGGUGGGCGG	66.92	88.91	100.30	94.96
hsa-miR-2909	MIMAT0013863	GUUAGGGCCAACAUCUCUUGG	78.10	95.31	103.97	79.61
hsa-miR-296-3p	MIMAT0004679	GAGGGUUGGGUGGAGGCUCUCC	100.76	100.24	95.92	88.42

hsa-miR-296-5p	MIMAT0000690	AGGGCCCCCCCUCAAUCCUGU	75.42	93.65	95.40	99.61
hsa-miR-297	MIMAT0004450	AUGUAUGUGUGCAUGUGCAUG	60.75	60.73	84.53	79.10
hsa-miR-298	MIMAT0004901	AGCAGAAGCAGGGAGGUUCUCCCA	84.61	98.50	108.71	97.57
hsa-miR-299-3p	MIMAT0000687	UAUGUGGGAUGGUAAACCGCUU	128.60	104.32	99.51	110.03
hsa-miR-299-5p	MIMAT0002890	UGGUUUACCGUCCCACAUACAU	96.31	85.59	94.84	82.38
hsa-miR-29a-3p	MIMAT0000086	UAGCACCAUCUGAAAUCGGUUA	54.08	79.25	67.99	84.28
hsa-miR-29a-5p	MIMAT0004503	ACUGAUUUCUUUUGGUGUUCAG	112.31	125.79	110.65	129.72
hsa-miR-29b-1-5p	MIMAT0004514	GCUGGUUUCAUAUGGUGGUUUAGA	93.52	101.83	125.15	124.59
hsa-miR-29b-2-5p	MIMAT0004515	CUGGUUUCACAUGGUGGCUUAG	74.65	61.98	108.65	97.69
hsa-miR-29b-3p	MIMAT0000100	UAGCACCAUUUGAAAUCAGUGUU	118.09	132.64	101.97	72.19
hsa-miR-29c-3p	MIMAT0000681	UAGCACCAUUUGAAAUCGGUUA	73.93	91.96	44.61	55.13
hsa-miR-29c-5p	MIMAT0004673	UGACCGAUUUCUCCUGGUGUUC	95.15	90.44	113.92	113.19
hsa-miR-300	MIMAT0004903	UAUACAAGGGCAGACUCUCUCU	126.73	137.37	97.91	116.74
hsa-miR-301a-3p	MIMAT0000688	CAGUGCAAUAGUAUUGUCAAAGC	86.15	103.73	113.09	93.98
hsa-miR-301b	MIMAT0004958	CAGUGCAAUGAUAUUGUCAAAGC	200.53	207.16	116.74	113.85
hsa-miR-302a-3p	MIMAT0000684	UAAGUGCUUCCAUGUUUUGGUGA	123.05	121.62	146.53	148.85
hsa-miR-302a-5p	MIMAT0000683	ACUUAAACGUGGAUGUACUUGCU	120.90	72.56	107.17	98.25
hsa-miR-302b-3p	MIMAT0000715	UAAGUGCUUCCAUGUUUUAGUAG	123.02	126.75	129.86	126.30
hsa-miR-302b-5p	MIMAT0000714	ACUUUAACAUGGAAGUGCUUUC	88.64	86.87	96.20	93.50
hsa-miR-302c-3p	MIMAT0000717	UAAGUGCUUCCAUGUUUCAGUGG	185.42	233.52	130.29	123.47
hsa-miR-302c-5p	MIMAT0000716	UUUAACAUGGGGGUACCUGCUG	116.50	106.78	112.77	109.16
hsa-miR-302d-3p	MIMAT0000718	UAAGUGCUUCCAUGUUUGAGUGU	89.25	89.85	134.07	144.28
hsa-miR-302d-5p	MIMAT0004685	ACUUUAACAUGGAGGCACUUGC	92.67	115.93	71.08	76.21
hsa-miR-302e	MIMAT0005931	UAAGUGCUUCCAUGCUU	146.11	139.37	143.10	133.24
hsa-miR-302f	MIMAT0005932	UAAUUGCUUCCAUGUUU	118.05	99.53	82.11	105.64
hsa-miR-3065-3p	MIMAT0015378	UCAGCACCAGGAUAUUGUUGGAG	90.69	78.54	99.51	91.43
hsa-miR-3065-5p	MIMAT0015066	UCAACAAAAUCACUGAUGCUGGA	128.50	114.95	108.00	122.54
hsa-miR-3074-3p	MIMAT0015027	GAUAUCAGCUCAGUAGGCACCG	42.83	52.05	85.07	65.69
hsa-miR-30a-3p	MIMAT0000088	CUUUCAGUCGGAUGUUUGCAGC	129.95	118.27	69.39	81.23
hsa-miR-30a-5p	MIMAT0000087	UGUAAACAUCCUCGACUGGAAG	94.27	78.71	104.53	98.77
hsa-miR-30b-3p	MIMAT0004589	CUGGGAGGUGGAUGUUUACUUC	118.82	128.78	102.76	80.18
hsa-miR-30b-5p	MIMAT0000420	UGUAAACAUCCUACACUCAGCU	118.05	104.07	107.06	112.21
hsa-miR-30c-1-3p	MIMAT0004674	CUGGGAGAGGGUUGUUUACUCC	102.06	104.65	134.62	121.16
hsa-miR-30c-2-3p	MIMAT0004550	CUGGGAGAAGGCUGUUUACUCU	121.07	107.99	110.42	127.52
hsa-miR-30c-5p	MIMAT0000244	UGUAAACAUCCUACACUCUCAGC	62.44	55.93	81.77	93.80
hsa-miR-30d-3p	MIMAT0004551	CUUUCAGUCAGAUGUUUGCUGC	102.91	120.86	86.13	83.06
hsa-miR-30d-5p	MIMAT0000245	UGUAAACAUCCCCGACUGGAAG	69.99	78.45	119.30	109.34
hsa-miR-30e-3p	MIMAT0000693	CUUUCAGUCGGAUGUUUACAGC	104.62	27.13	78.88	44.10
hsa-miR-30e-5p	MIMAT0000692	UGUAAACAUCCUUGACUGGAAG	101.04	89.22	119.54	109.60
hsa-miR-3115	MIMAT0014977	AUAUGGGUUUACUAGUUGGU	81.25	117.34	68.31	90.93
hsa-miR-3116	MIMAT0014978	UGCCUGGAACAUAGUAGGGACU	84.89	82.42	109.60	137.02

hsa-miR-3117-3p	MIMAT0014979	AUAGGACUCAUAUAGUGCCAG	85.77	97.58	84.64	83.11
hsa-miR-3118	MIMAT0014980	UGUGACUGCAUUAUGAAAAUUCU	108.26	69.40	112.27	101.62
hsa-miR-3119	MIMAT0014981	UGGCUUUUAACUUUGAUGGC	66.25	61.63	86.07	104.30
hsa-miR-3120-3p	MIMAT0014982	CACAGCAAGUGUAGACAGGCA	97.91	109.31	122.03	114.28
hsa-miR-3121-3p	MIMAT0014983	UAAAUAGAGUAGGCAAAGGACA	125.85	115.49	71.29	76.78
hsa-miR-3122	MIMAT0014984	GUUGGGACAAGAGGACGGUCUU	76.87	107.66	97.28	96.58
hsa-miR-3123	MIMAT0014985	CAGAGAAUUGUUUAAUC	175.93	142.40	127.65	101.27
hsa-miR-3124-5p	MIMAT0014986	UUCGCGGGCGAAGGCAAAGUC	92.68	101.94	120.80	108.06
hsa-miR-3125	MIMAT0014988	UAGAGGAAGCUGUGGAGAGA	100.35	103.93	97.84	95.78
hsa-miR-3126-3p	MIMAT0015377	CAUCUGGCAUCCGUCACACAGA	89.56	107.36	100.00	73.74
hsa-miR-3126-5p	MIMAT0014989	UGAGGGACAGAUGCCAGAAGCA	121.87	115.42	101.78	105.09
hsa-miR-3127-5p	MIMAT0014990	AUCAGGGCUUGUGGAAUGGGAAG	109.22	112.44	124.42	104.76
hsa-miR-3128	MIMAT0014991	UCUGGCAAGUAAAAAACUCUCAU	65.20	78.81	107.64	121.30
hsa-miR-3129-5p	MIMAT0014992	GCAGUAGUGUAGAGAUUGGUUU	117.45	127.33	82.32	79.95
hsa-miR-3130-3p	MIMAT0014994	GCUGCACCGGAGACUGGGUAA	79.22	72.37	100.31	107.81
hsa-miR-3130-5p	MIMAT0014995	UACCCAGUCUCCGGUGCAGCC	80.28	89.43	95.97	93.77
hsa-miR-3131	MIMAT0014996	UCGAGGACUGGUGGAAGGGCCUU	83.26	124.16	115.03	102.51
hsa-miR-3132	MIMAT0014997	UGGGUAGAGAAGGAGCUCAGAGGA	93.66	90.84	95.07	101.72
hsa-miR-3133	MIMAT0014998	UAAAGAACUCUUAAAACCCAAU	60.02	68.42	75.39	78.60
hsa-miR-3134	MIMAT0015000	UGAUGGAUAAAAGACUACAUAUU	86.91	99.64	88.12	110.22
hsa-miR-3135a	MIMAT0015001	UGCCUAGGCUGAGACUGCAGUG	84.81	112.63	85.66	101.23
hsa-miR-3136-5p	MIMAT0015003	CUGACUGAAUAGGUAGGGUCAUU	78.12	82.82	97.29	119.72
hsa-miR-3137	MIMAT0015005	UCUGUAGCCUGGGAGCAAUGGGGU	95.35	78.96	140.39	138.44
hsa-miR-3138	MIMAT0015006	UGUGGACAGUGAGGUAGAGGGAGU	70.84	65.69	106.19	114.43
hsa-miR-3139	MIMAT0015007	UAGGAGCUCAACAGAUGCCUGUU	84.11	60.73	65.33	61.94
hsa-miR-31-3p	MIMAT0004504	UGCUAUGCCAACAUAUUGCCAU	110.60	106.77	112.33	139.14
hsa-miR-3140-3p	MIMAT0015008	AGCUUUUGGGAAUUCAGGUAGU	85.77	99.88	93.82	111.38
hsa-miR-3141	MIMAT0015010	GAGGGCGGGUGGAGGAGGA	92.30	88.04	99.57	116.86
hsa-miR-3142	MIMAT0015011	AAGGCCUUUCUGAACCUUCAGA	101.98	77.22	124.81	83.66
hsa-miR-3143	MIMAT0015012	AUAACAUUGUAAAGCGCUUCUUUCG	130.06	146.40	114.93	126.83
hsa-miR-3144-3p	MIMAT0015015	AUAUACCUGUUCGGUCUCUUUA	125.38	120.28	84.98	118.38
hsa-miR-3144-5p	MIMAT0015014	AGGGGACCAAAGAGAUAUAUAG	73.21	65.07	76.00	76.95
hsa-miR-3145-3p	MIMAT0015016	AGAUAUUUUGAGUGUUUGGAAUUG	79.06	105.20	82.01	95.87
hsa-miR-3146	MIMAT0015018	CAUGCUAGGAUAGAAAGAAUGG	98.13	121.46	84.00	87.14
hsa-miR-3147	MIMAT0015019	GGUUGGGCAGUGAGGAGGGUGUGA	77.69	82.76	121.40	115.60
hsa-miR-3148	MIMAT0015021	UGGAAAAAACUGGUGUGUGCUU	108.63	126.76	133.10	128.28
hsa-miR-3149	MIMAT0015022	UUUGUAUGGAUAUGUGUGUGUAU	118.82	107.26	78.23	88.35
hsa-miR-3150a-3p	MIMAT0015023	CUGGGGAGAUCCUCGAGGUUGG	59.00	75.14	111.55	118.10
hsa-miR-3150b-3p	MIMAT0018194	UGAGGAGAUCGUCGAGGUUGG	90.38	112.71	99.24	123.77
hsa-miR-3151	MIMAT0015024	GGUGGGGCAAUGGGAUCAGGU	86.15	75.83	103.59	92.02
hsa-miR-3152-3p	MIMAT0015025	UGUGUUAGAAUAGGGGCAAUAA	121.87	104.72	100.62	112.83

hsa-miR-3153	MIMAT0015026	GGGGAAAGCGAGUAGGGACAUUU	164.33	134.47	135.73	134.87
hsa-miR-3154	MIMAT0015028	CAGAAGGGGAGUUGGGAGCAGA	87.59	88.70	83.14	87.96
hsa-miR-3155a	MIMAT0015029	CCAGGCUCUGCAGUGGGAACU	150.31	129.05	114.93	120.93
hsa-miR-3156-5p	MIMAT0015030	AAAGAUCUGGAAGUGGGAGACA	104.81	109.88	102.28	89.45
hsa-miR-3157-5p	MIMAT0015031	UUCAGCCAGGCUAGUGCAGUCU	82.20	69.57	82.66	85.28
hsa-miR-3158-3p	MIMAT0015032	AAGGGCUUCCUCUCUGCAGGAC	106.21	102.17	98.92	114.52
hsa-miR-3159	MIMAT0015033	UAGGAUUACAAGUGUCGGCCAC	164.05	180.89	114.39	130.66
hsa-miR-31-5p	MIMAT0000089	AGGCAAGAUGCUGGCAUAGCU	84.58	86.58	133.23	137.00
hsa-miR-3160-3p	MIMAT0015034	AGAGCUGAGACUAGAAAGCCCA	132.73	116.71	98.24	93.50
hsa-miR-3161	MIMAT0015035	CUGAUAAGAACAGAGGCCCAGAU	84.74	85.68	90.08	92.22
hsa-miR-3162-5p	MIMAT0015036	UUAGGGAGUAGAAGGGUGGGGAG	86.91	106.34	116.01	87.77
hsa-miR-3163	MIMAT0015037	UAUAAAAUGAGGGCAGUAAGAC	97.53	124.25	125.86	124.01
hsa-miR-3164	MIMAT0015038	UGUGACUUUAAGGGAAAUGGCG	101.11	117.37	108.67	104.62
hsa-miR-3165	MIMAT0015039	AGGUGGAUGCAAUGUGACCUCA	42.63	46.61	61.34	75.15
hsa-miR-3166	MIMAT0015040	CGCAGACAAUGCCUACUGGCCUA	75.38	82.09	93.08	76.78
hsa-miR-3167	MIMAT0015042	AGGAUUUCAGAAAUACUGGUGU	91.78	108.28	84.29	105.46
hsa-miR-3168	MIMAT0015043	GAGUUCUACAGUCAGAC	111.67	84.98	100.56	90.23
hsa-miR-3169	MIMAT0015044	UAGGACUGUGCUUGGCACAUAG	56.96	52.87	119.13	116.48
hsa-miR-3170	MIMAT0015045	CUGGGGUUCUGAGACAGACAGU	133.06	143.82	139.03	151.18
hsa-miR-3171	MIMAT0015046	AGAUGUAUGGAAUCUGUAUAUAUC	85.29	92.31	93.26	101.67
hsa-miR-3173-3p	MIMAT0015048	AAAGGAGGAAAUAGGCAGGCCA	73.28	81.16	101.82	102.79
hsa-miR-3174	MIMAT0015051	UAGUGAGUUAGAGAUGCAGAGCC	60.53	76.59	92.20	98.05
hsa-miR-3175	MIMAT0015052	CGGGGAGAGAACGCAGUGACGU	81.51	95.31	112.50	94.60
hsa-miR-3176	MIMAT0015053	ACUGGCCUGGGACUACCGG	125.63	97.58	126.67	111.61
hsa-miR-3177-3p	MIMAT0015054	UGCACGGCACUGGGGACACGU	28.82	37.96	92.00	82.32
hsa-miR-3178	MIMAT0015055	GGGGCGCGGCCGGAUCG	95.97	97.61	119.44	119.88
hsa-miR-3179	MIMAT0015056	AGAAGGGGUGAAAUUUAAACGU	81.94	88.40	83.28	102.68
hsa-miR-3180	MIMAT0018178	UGGGGCGGAGCUUCCGGAG	73.63	113.65	105.04	116.69
hsa-miR-3180-3p	MIMAT0015058	UGGGGCGGAGCUUCCGGAGGCC	91.12	72.66	121.33	137.02
hsa-miR-3180-5p	MIMAT0015057	CUUCCAGACGCUCCGCCCCACGUCG	88.36	103.54	101.78	105.09
hsa-miR-3181	MIMAT0015061	AUCGGGCCCUCGGCGCCGG	54.91	60.53	118.18	78.40
hsa-miR-3182	MIMAT0015062	GCUUCUGUAGUGUAGUC	134.06	152.53	117.47	102.36
hsa-miR-3183	MIMAT0015063	GCCUCUCUCGGAGUCGCUCGGA	113.73	109.08	122.79	117.15
hsa-miR-3184-5p	MIMAT0015064	UGAGGGGCCUCAGACCGAGCUUUU	65.14	53.57	103.50	95.82
hsa-miR-3185	MIMAT0015065	AGAAGAAGGCGGUCGGUCUGCGG	138.13	153.06	104.45	120.13
hsa-miR-3186-3p	MIMAT0015068	UCACGCGGAGAGAUGGCUUUG	59.19	41.21	85.60	91.97
hsa-miR-3186-5p	MIMAT0015067	CAGGCGUCUGUCUACGUGGCUU	72.11	99.17	85.09	77.57
hsa-miR-3187-3p	MIMAT0015069	UUGGCCAUGGGGCUGCGCGG	93.46	124.71	108.53	131.65
hsa-miR-3188	MIMAT0015070	AGAGGCUUUGUGCGGAUACGGGG	131.77	107.26	106.19	90.30
hsa-miR-3189-3p	MIMAT0015071	CCCUUGGGUCUGAUGGGGUAG	88.45	64.50	95.55	84.50
hsa-miR-3190	MIMAT0015073	UGUGGAAGGUAGACGGCCAGAGA	123.81	131.49	125.29	108.38

hsa-miR-3191-3p	MIMAT0015075	UGGGGACGUAGCUGGCCAGACAG	95.16	88.36	112.97	107.16
hsa-miR-3192	MIMAT0015076	UCUGGGAGGUUGUAGCAGUGGAA	92.30	71.48	70.70	61.29
hsa-miR-3193	MIMAT0015077	UCCUGCGUAGGAUCUGAGGAGU	74.69	98.79	87.39	113.64
hsa-miR-3194-5p	MIMAT0015078	GGCCAGCCACCAGGAGGGCUG	127.73	108.45	81.87	64.62
hsa-miR-3195	MIMAT0015079	CGCGCCGGGCCCGGGUU	111.97	108.00	95.89	82.13
hsa-miR-3196	MIMAT0015080	CGGGGCGGCAGGGGCCUC	95.16	88.36	98.29	114.86
hsa-miR-3197	MIMAT0015082	GGAGGCGCAGGCUCGGAAAGGCG	94.56	108.13	138.78	127.39
hsa-miR-3198	MIMAT0015083	GUGGAGUCCUGGGGAAUGGAGA	151.77	180.89	83.60	71.91
hsa-miR-3199	MIMAT0015084	AGGGACUGCCUUAGGAGAAAGUU	121.87	116.46	94.50	89.87
hsa-miR-3200-3p	MIMAT0015085	CACCUUGCGCUACUCAGGUCUG	89.95	115.43	82.88	93.80
hsa-miR-3200-5p	MIMAT0017392	AAUCUGAGAAGGCGCACAAGGU	83.03	74.48	108.48	102.55
hsa-miR-3201	MIMAT0015086	GGGAUAUGAAGAAAAAU	117.60	123.63	113.33	111.27
hsa-miR-3202	MIMAT0015089	UGGAAGGGAGAAGAGCUUUAAU	125.70	99.88	98.58	97.85
hsa-miR-320a	MIMAT0000510	AAAAGCUGGGUUGAGAGGGCGA	70.62	68.41	98.12	85.54
hsa-miR-320b	MIMAT0005792	AAAAGCUGGGUUGAGAGGGCAA	120.48	111.87	127.59	135.09
hsa-miR-320c	MIMAT0005793	AAAAGCUGGGUUGAGAGGGU	86.83	98.74	97.88	123.20
hsa-miR-320d	MIMAT0006764	AAAAGCUGGGUUGAGAGGA	112.50	122.45	92.20	78.84
hsa-miR-320e	MIMAT0015072	AAAGCUGGGUUGAGAAGG	118.23	114.14	105.12	115.34
hsa-miR-323a-3p	MIMAT0000755	CACAUUACACGGUCGACCUCU	73.44	86.87	69.63	81.51
hsa-miR-323a-5p	MIMAT0004696	AGGUGGUCCGUGGCGCGUUCGC	85.38	91.52	92.99	90.38
hsa-miR-323b-3p	MIMAT0015050	CCCAAUACACGGUCGACCUCUU	69.58	52.18	79.91	74.92
hsa-miR-323b-5p	MIMAT0001630	AGGUUGUCCGUGGUGAGUUCGCA	107.40	90.18	111.59	97.69
hsa-miR-32-3p	MIMAT0004505	CAAUUUAGUGUGUGUGAUAUUU	112.02	128.25	112.75	123.17
hsa-miR-324-3p	MIMAT0000762	ACUGCCCCAGGUGCUGCUGG	96.55	117.16	101.63	109.47
hsa-miR-324-5p	MIMAT0000761	CGCAUCCCCUAGGGCAUUGGUGU	74.34	82.25	103.99	83.06
hsa-miR-325	MIMAT0000771	CCUAGUAGGUGUCCAGUAAGUGU	94.39	84.31	109.45	101.47
hsa-miR-32-5p	MIMAT0000090	UAUUGCACAUUACUAAGUUGCA	86.67	74.03	99.25	88.63
hsa-miR-326	MIMAT0000756	CCUCUGGGCCCUUCCUCCAG	76.41	97.04	78.06	61.68
hsa-miR-328	MIMAT0000752	CUGGCCCUCUCUGCCCUUCCGU	183.06	180.43	84.95	103.13
hsa-miR-329	MIMAT0001629	AACACACCUGGUUAACCUCUUU	76.53	71.45	82.37	98.82
hsa-miR-330-3p	MIMAT0000751	GCAAAGCACACGGCCUGCAGAGA	140.42	154.18	105.68	113.43
hsa-miR-330-5p	MIMAT0004693	UCUCUGGGCCUGUGUCUUAGGC	102.21	102.13	141.60	121.45
hsa-miR-331-3p	MIMAT0000760	GCCCCUGGGCCUAUCCUAGAA	112.24	130.82	125.31	99.03
hsa-miR-331-5p	MIMAT0004700	CUAGGUAUGGUCCCAGGGAUCC	107.42	110.88	94.68	78.28
hsa-miR-335-3p	MIMAT0004703	UUUUUCAUUAUUGCUCCUGACC	81.56	85.62	87.24	78.35
hsa-miR-335-5p	MIMAT0000765	UCAAGAGCAAUAACGAAAAAUGU	84.55	86.47	116.49	114.82
hsa-miR-337-3p	MIMAT0000754	CUCCUAUAUGAUGCCUUUCUUC	91.12	101.94	113.33	110.74
hsa-miR-337-5p	MIMAT0004695	GAACGGCUUCAUACAGGAGUU	127.73	109.53	102.13	111.81
hsa-miR-338-3p	MIMAT0000763	UCCAGCAUCAGUGAUUUUGUUG	110.65	124.17	77.06	105.17
hsa-miR-338-5p	MIMAT0004701	AACAAUAUCCUGGUGCUGAGUG	98.24	83.48	87.19	82.18
hsa-miR-339-3p	MIMAT0004702	UGAGCGCCUCGACGACAGAGCCG	121.51	95.78	73.14	78.33

hsa-miR-339-5p	MIMAT0000764	UCCCUGUCCUCCAGGAGCUCACG	122.90	128.71	79.13	85.54
hsa-miR-33a-3p	MIMAT0004506	CAAUGUUUCCACAGUGCAUCAC	72.94	78.40	80.46	70.81
hsa-miR-33a-5p	MIMAT0000091	GUGCAUUGUAGUUGCAUUGCA	105.87	110.97	65.48	65.39
hsa-miR-33b-3p	MIMAT0004811	CAGUGCCUCGGCAGUGCAGCCC	98.24	111.45	100.67	100.76
hsa-miR-33b-5p	MIMAT0003301	GUGCAUUGCUGUUGCAUUGC	75.23	113.91	76.14	69.34
hsa-miR-340-3p	MIMAT0000750	UCCGUCUCAGUUACUUUAUAGC	111.36	121.39	103.66	113.70
hsa-miR-340-5p	MIMAT0004692	UUAUAAAGCAAUGAGACUGAUU	73.74	88.44	141.77	110.64
hsa-miR-342-3p	MIMAT0000753	UCUCACACAGAAAUCGCACCCGU	118.09	100.43	96.21	91.04
hsa-miR-342-5p	MIMAT0004694	AGGGGUGCUAUCUGUGAUUGA	98.70	79.78	106.67	90.93
hsa-miR-345-5p	MIMAT0000772	GCUGACUCCUAGUCCAGGGCUC	108.92	87.21	81.18	82.90
hsa-miR-346	MIMAT0000773	UGUCUGCCCGCAUGCCUGCCUCU	115.52	110.67	102.48	116.48
hsa-miR-34a-3p	MIMAT0004557	CAAUCAGCAAGUAUACUGCCCU	70.46	98.74	78.84	66.74
hsa-miR-34a-5p	MIMAT0000255	UGGCAGUGUCUUAGCUGGUUGU	37.93	39.04	68.79	75.93
hsa-miR-34b-3p	MIMAT0004676	CAAUCACUAACUCCACUGCCAU	67.80	70.55	98.20	104.07
hsa-miR-34b-5p	MIMAT0000685	UAGGCAGUGUCAUUAGCUGAUUG	84.31	73.10	132.29	128.37
hsa-miR-34c-3p	MIMAT0004677	AAUCACUAACCACACGGCCAGG	147.12	161.18	142.49	124.44
hsa-miR-34c-5p	MIMAT0000686	AGGCAGUGUAGUUAGCUGAUUGC	33.46	39.89	80.23	91.66
hsa-miR-3605-3p	MIMAT0017982	CCUCCGUGUUACCUGUCCUCUAG	97.59	97.07	105.37	102.44
hsa-miR-3605-5p	MIMAT0017981	UGAGGAUGGAUAGCAAGGAAGCC	102.06	100.90	118.63	102.92
hsa-miR-3606-5p	MIMAT0017983	UUAGUGAAGGCUAUUUUAAUU	100.09	88.94	83.16	91.98
hsa-miR-3607-3p	MIMAT0017985	ACUGUAAACGCUUUCUGAUG	67.69	101.66	95.93	127.87
hsa-miR-3607-5p	MIMAT0017984	GCAUGUGAUGAAGCAAAUCAGU	113.58	135.74	111.96	114.43
hsa-miR-3609	MIMAT0017986	CAAAGUGAUGAGUAAUACUGGCUG	106.30	109.48	109.49	105.28
hsa-miR-3610	MIMAT0017987	GAAUCGGAAAGGAGGCGCCG	125.39	151.82	88.27	77.49
hsa-miR-3611	MIMAT0017988	UUGUGAAGAAAGAAAUUCUUA	96.95	90.18	86.00	83.11
hsa-miR-3612	MIMAT0017989	AGGAGGCAUCUUGAGAAAUGGA	48.32	67.43	97.29	91.03
hsa-miR-3613-3p	MIMAT0017991	ACAAAAAAAAAAGCCCAACCCUUC	101.42	92.48	106.73	98.81
hsa-miR-3613-5p	MIMAT0017990	UGUUGUACUUUUUUUUUUGUUC	67.79	73.85	108.15	112.09
hsa-miR-361-3p	MIMAT0004682	UCCCCCAGGUGUGAUUCUGAUUU	131.44	109.64	99.29	93.35
hsa-miR-3614-3p	MIMAT0017993	UAGCCUUCAGAUCUUGGUGUUUU	127.90	118.96	113.37	114.52
hsa-miR-3614-5p	MIMAT0017992	CCACUUGGAUCUGAAGGCUGCCC	120.40	128.01	87.39	110.00
hsa-miR-3615	MIMAT0017994	UCUCUCGGCUCCUCGCGGCUC	166.78	162.80	134.76	151.73
hsa-miR-361-5p	MIMAT0000703	UUAUCAGAAUCUCCAGGGGUAC	91.08	102.49	97.84	97.84
hsa-miR-3616-3p	MIMAT0017996	CGAGGGCAUUUCAUGAUGCAGGC	101.30	94.64	112.08	104.31
hsa-miR-3616-5p	MIMAT0017995	AUGAAGUGCACUCAUGAUAUGU	132.55	145.68	88.55	80.55
hsa-miR-3617-5p	MIMAT0017997	AAAGACAUAGUUGCAAGAUGGG	125.85	144.71	103.50	109.19
hsa-miR-3618	MIMAT0017998	UGUCUACAUUAAUGAAAAGAGC	119.16	137.73	110.67	127.90
hsa-miR-3619-5p	MIMAT0017999	UCAGCAGGCAGGCUGGUGCAGC	98.13	121.46	67.47	76.42
hsa-miR-3620-3p	MIMAT0018001	UCACCCUGCAUCCCGCACCCAG	113.57	126.62	100.18	112.83
hsa-miR-3621	MIMAT0018002	CGCGGGUCGGGGUCUGCAGG	110.44	87.21	96.87	92.24
hsa-miR-3622a-3p	MIMAT0018004	UCACCUGACCUCCCAUGCCUGU	103.98	89.93	100.96	111.13

hsa-miR-3622a-5p	MIMAT0018003	CAGGCACGGGAGCUCAGGUGAG	102.83	99.83	106.68	100.42
hsa-miR-3622b-3p	MIMAT0018006	UCACCUGAGCUCCCGUGCCUG	104.40	88.09	86.08	93.62
hsa-miR-3622b-5p	MIMAT0018005	AGGCAUGGGAGGUCAGGUGA	37.17	55.14	69.57	70.45
hsa-miR-362-3p	MIMAT0004683	AACACACCUAUUCAAGGAUUCA	98.59	86.38	84.42	90.57
hsa-miR-362-5p	MIMAT0000705	AAUCCUUGGAACCUAGGUGUGAGU	70.92	76.43	103.66	79.10
hsa-miR-363-3p	MIMAT0000707	AAUUGCACGGUAUCCAUCUGUA	129.28	84.59	104.27	130.04
hsa-miR-363-5p	MIMAT0003385	CGGGUGGAUCACGAUGCAAUUU	36.49	37.31	100.66	89.33
hsa-miR-3646	MIMAT0018065	AAAAUGAAAUGAGCCCAGCCCA	116.58	67.71	90.67	87.29
hsa-miR-3647-3p	MIMAT0018067	AGAAAAUUUUUGUGUGUCUGAUC	57.70	50.29	72.07	80.51
hsa-miR-3647-5p	MIMAT0018066	CUGAAGUGAUGAUUCACAUUCAU	137.02	129.37	120.90	117.62
hsa-miR-3648	MIMAT0018068	AGCCGCGGGGAUCGCCGAGGG	131.01	93.15	102.76	102.36
hsa-miR-3649	MIMAT0018069	AGGGACCUGAGUGUCUAAG	135.51	134.47	104.27	105.38
hsa-miR-365*	MIMAT0009199	AGGGACUUUCAGGGGCAGCUGU	83.51	75.96	108.04	95.95
hsa-miR-3650	MIMAT0018070	AGGUGUGUCUGUAGAGUCC	61.93	54.72	89.12	79.96
hsa-miR-3651	MIMAT0018071	CAUAGCCCGGUCGCUGGUACAUGA	71.53	68.86	124.78	117.51
hsa-miR-3652	MIMAT0018072	CGGCUGGAGGUGUGAGGA	88.64	81.25	135.29	139.64
hsa-miR-3653	MIMAT0018073	CUAAGAAGUUGACUGAAG	147.98	155.08	95.20	87.14
hsa-miR-3654	MIMAT0018074	GACUGGACAAGCUGAGGAA	112.21	137.75	76.50	101.08
hsa-miR-3655	MIMAT0018075	GCUUGUCGCUGCGGUGUUGCU	117.39	96.81	121.69	93.26
hsa-miR-3656	MIMAT0018076	GGCGGGUGCGGGGGUGG	127.86	102.34	120.02	113.59
hsa-miR-3657	MIMAT0018077	UGUGUCCCAUUAUUGGUGAUU	51.79	47.88	98.84	92.24
hsa-miR-3658	MIMAT0018078	UUUAAGAAAACACCAUGGAGAU	74.77	87.84	86.67	95.19
hsa-miR-3659	MIMAT0018080	UGAGUGUUGUCUACGAGGGCA	110.05	114.67	107.07	92.05
hsa-miR-365a-3p	MIMAT0000710	UAAUGCCCCUAAAAAUCCUUAU	90.34	73.74	95.73	109.13
hsa-miR-3660	MIMAT0018081	ACUGACAGGAGAGCAUUUUGA	86.57	92.08	69.76	81.69
hsa-miR-3661	MIMAT0018082	UGACCUGGGACUCGGACAGCUG	112.92	116.71	100.96	82.14
hsa-miR-3662	MIMAT0018083	GAAAAUGAUGAGUAGUGACUGAUG	87.05	93.24	92.80	96.16
hsa-miR-3663-3p	MIMAT0018085	UGAGCACCACACAGGCCGGGCGC	103.33	84.49	31.32	23.35
hsa-miR-3663-5p	MIMAT0018084	GCUGGUCUGCGUGGUGCUCGG	94.66	112.02	92.89	89.09
hsa-miR-3664-5p	MIMAT0018086	AACUCUGUCUUCACUCAUGAGU	108.69	82.95	108.92	96.92
hsa-miR-3665	MIMAT0018087	AGCAGGUGCGGGGCGGCG	3.89	19.52	98.40	85.53
hsa-miR-3666	MIMAT0018088	CAGUGCAAGUGUAGAUGCCGA	120.99	107.12	91.64	83.06
hsa-miR-3667-3p	MIMAT0018090	ACCUUCCUCUCCAUGGGUCUUU	67.88	62.37	92.46	71.03
hsa-miR-3667-5p	MIMAT0018089	AAAGACCCAUUGAGGAGAAGGU	113.73	119.79	80.66	94.20
hsa-miR-3668	MIMAT0018091	AAUGUAGAGAUUGAUCAAAAU	99.82	107.27	103.68	103.16
hsa-miR-3669	MIMAT0018092	ACGGAAUAUGUAUACGGAAUAUA	100.75	123.17	103.27	99.75
hsa-miR-3670	MIMAT0018093	AGAGCUCACAGCUGUCCUUCUCUA	107.40	105.03	89.03	93.02
hsa-miR-3671	MIMAT0018094	AUCAAAUAAGGACUAGUCUGCA	126.56	108.22	116.88	108.79
hsa-miR-3672	MIMAT0018095	AUGAGACUCAUGUAAAACAUCUU	109.40	132.11	114.06	124.81
hsa-miR-3673	MIMAT0018096	AUGGAAUGUAUAUACGGAAUA	112.48	112.18	81.18	86.62
hsa-miR-367-3p	MIMAT0000719	AAUUGCACUUUAGCAAUGGUGA	51.75	52.20	95.89	102.36

hsa-miR-3674	MIMAT0018097	AUUGUAGAACCUAAGAUUGGCC	57.75	59.08	88.17	80.59
hsa-miR-3675-3p	MIMAT0018099	CAUCUCUAAGGAACUCCCCCAA	95.99	94.26	117.61	112.83
hsa-miR-3675-5p	MIMAT0018098	UAUGGGGCUUCUGUAGAGAUUUC	124.45	136.85	105.37	109.44
hsa-miR-367-5p	MIMAT0004686	ACUGUUGCUAAUAUGCAACUCU	96.08	93.38	85.57	63.36
hsa-miR-3676-3p	MIMAT0018100	CCGUGUUUCCCCCACGCUUU	140.19	93.26	111.20	103.77
hsa-miR-3677-3p	MIMAT0018101	CUCGUGGGCUCUGGCCACGGCC	102.38	102.94	98.92	100.02
hsa-miR-3678-3p	MIMAT0018103	CUGCAGAGUUUGUACGGACCGG	71.47	87.49	87.95	81.58
hsa-miR-3678-5p	MIMAT0018102	UCCGUACAAACUCUGCUGUG	106.21	104.72	95.52	84.56
hsa-miR-3679-3p	MIMAT0018105	CUUCCCCCCAGUAAUCUUCAUC	120.91	118.50	95.52	100.75
hsa-miR-3679-5p	MIMAT0018104	UGAGGAUAUGGCAGGGAAGGGGA	69.92	77.92	94.50	71.10
hsa-miR-3680-3p	MIMAT0018107	UUUUGCAUGACCCUGGGAGUAGG	152.65	96.52	93.60	81.78
hsa-miR-3680-5p	MIMAT0018106	GACUCACUCACAGGAUUGUGCA	94.07	104.21	113.19	100.02
hsa-miR-3681-3p	MIMAT0018109	ACACAGUGCUUCAUCCACUACU	108.77	109.48	94.84	75.69
hsa-miR-3681-5p	MIMAT0018108	UAGUGGAUGAUGCACUCUGUGC	68.54	68.32	114.40	106.98
hsa-miR-3682-3p	MIMAT0018110	UGAUGAUACAGGUGGAGGUAG	93.63	121.93	96.72	118.81
hsa-miR-3683	MIMAT0018111	UGCGACAUUGGAAGUAGUAUCA	88.96	94.26	93.14	87.46
hsa-miR-3684	MIMAT0018112	UUAGACCUAGUACACGUCCUU	118.38	88.93	82.93	79.64
hsa-miR-3685	MIMAT0018113	UUUCCUACCCUACCUGAAGACU	102.06	101.66	90.42	114.52
hsa-miR-3686	MIMAT0018114	AUCUGUAAGAGAAAGUAAAUGA	80.02	85.08	80.56	93.74
hsa-miR-3687	MIMAT0018115	CCCGGACAGGCGUUCGUGCGACGU	112.73	119.13	118.45	110.54
hsa-miR-3688-3p	MIMAT0018116	UAUGGAAAGACUUUGCCACUCU	138.10	121.79	103.49	118.05
hsa-miR-3689a-3p	MIMAT0018118	CUGGGAGGUGUGAUAUCGUGGU	130.83	110.67	119.47	116.76
hsa-miR-3689a-5p	MIMAT0018117	UGUGAUAUCAUGGUUCCUGGGA	93.69	125.81	100.31	119.88
hsa-miR-3689b-3p	MIMAT0018181	CUGGGAGGUGUGAUAUUGUGGU	89.22	94.14	128.80	113.59
hsa-miR-3689b-5p	MIMAT0018180	UGUGAUAUCAUGGUUCCUGGGA	101.30	100.88	97.34	114.05
hsa-miR-3690	MIMAT0018119	ACCUGGACCCAGCGUAGACAAAG	91.75	83.49	104.92	116.48
hsa-miR-3691-5p	MIMAT0018120	AGUGGAUGAUGGAGACUCGGUAC	102.30	91.52	113.82	106.07
hsa-miR-3692-3p	MIMAT0018122	GUUCCACACUGACACUGCAGAAGU	93.57	131.08	112.22	114.07
hsa-miR-3692-5p	MIMAT0018121	CCUGCUGGUCAGGAGUGGAUACUG	137.84	140.69	106.39	111.86
hsa-miR-369-3p	MIMAT0000721	AAUAAUACAUGGUUGAUCUUU	116.11	113.15	138.23	155.59
hsa-miR-369-5p	MIMAT0001621	AGAUCGACCGUGUUAUAUUCGC	105.87	108.74	116.98	115.99
hsa-miR-370	MIMAT0000722	GCCUGCUGGGGUGGAACCUGGU	92.72	101.48	101.47	97.85
hsa-miR-3713	MIMAT0018164	GGUAUCCGUUUGGGGAUGGU	81.53	70.60	106.15	102.48
hsa-miR-3714	MIMAT0018165	GAAGGCAGCAGUGCUCCCCUGU	68.39	74.18	101.17	87.86
hsa-miR-371a-3p	MIMAT0000723	AAGUGCCGCCAUCUUUUGAGUGU	135.14	135.97	129.29	128.84
hsa-miR-371a-5p	MIMAT0004687	ACUCAAACUGUGGGGGCACU	139.61	137.14	101.10	110.15
hsa-miR-372	MIMAT0000724	AAAGUGCUGCGACAUUUGAGCGU	86.91	115.93	121.86	128.30
hsa-miR-373-3p	MIMAT0000726	GAAGUGCUUCGAUUUUGGGGUGU	139.07	135.84	138.34	158.15
hsa-miR-373-5p	MIMAT0000725	ACUCAAAAUGGGGGCGCUUUCC	151.73	161.56	111.33	92.81
hsa-miR-374a-3p	MIMAT0004688	CUUAUCAGAUUGUAUUGUAAUU	80.02	97.14	82.37	97.39
hsa-miR-374a-5p	MIMAT0000727	UUAUAAUACAACCUGAUAAGUG	87.82	96.20	114.49	84.95

hsa-miR-374b-3p	MIMAT0004956	CUUAGCAGGUUGUAUUAUCAUU	49.17	42.60	114.46	90.70
hsa-miR-374b-5p	MIMAT0004955	AUAUAAUACAACCUGCUAAGUG	64.48	70.12	116.79	117.31
hsa-miR-374c-5p	MIMAT0018443	AUAAUACAACCUGCUAAGUGCU	60.53	57.53	96.54	93.74
hsa-miR-375	MIMAT0000728	UUUGUUCGUUCGGCUCGCGUGA	115.48	101.41	97.56	93.74
hsa-miR-376a-3p	MIMAT0000729	AUCAUAGAGGAAAAUCCACGU	77.76	102.14	94.06	84.77
hsa-miR-376a-5p	MIMAT0003386	GUAGAUUCUCCUUCUAUGAGUA	104.10	94.86	84.07	96.59
hsa-miR-376b-3p	MIMAT0002172	AUCAUAGAGGAAAAUCCAUGUU	111.64	104.67	102.79	87.26
hsa-miR-376c-3p	MIMAT0000720	AACAUAGAGGAAAUUCCACGU	86.18	69.76	82.27	67.03
hsa-miR-377-3p	MIMAT0000730	AUCACACAAAGGCAACUUUUGU	98.23	106.76	93.14	109.20
hsa-miR-377-5p	MIMAT0004689	AGAGGUUGCCCUUGGUGAAUUC	57.65	56.03	76.09	69.78
hsa-miR-378a-3p	MIMAT0000732	ACUGGACUUGGAGUCAGAAGG	105.43	116.51	111.95	112.54
hsa-miR-378a-5p	MIMAT0000731	CUCCUGACUCCAGGUCCUGUGU	76.95	105.18	98.12	106.33
hsa-miR-378b	MIMAT0014999	ACUGGACUUGGAGGCAGAA	135.51	142.06	125.60	133.26
hsa-miR-378c	MIMAT0016847	ACUGGACUUGGAGUCAGAAGAGUGG	106.30	91.63	101.17	110.82
hsa-miR-379-3p	MIMAT0004690	UAUGUAACAUGGUCCACUAACU	122.50	136.81	102.48	102.16
hsa-miR-379-5p	MIMAT0000733	UGGUAGACUAUGGAACGUAGG	98.45	88.91	69.61	60.31
hsa-miR-380-3p	MIMAT0000735	UAUGUAAUAUGGUCCACAUCUU	82.26	82.76	97.85	94.58
hsa-miR-380-5p	MIMAT0000734	UGGUUGACCAUAGAACAUGCGC	65.49	58.77	134.01	130.53
hsa-miR-381-3p	MIMAT0000736	UAUACAAGGGCAAGCUCUCUGU	104.62	91.26	126.82	110.46
hsa-miR-382-5p	MIMAT0000737	GAAGUUGUUCGUGGUGGAUUCG	97.14	87.75	102.56	98.51
hsa-miR-383	MIMAT0000738	AGAUCAGAAGGUGAUUGUGGCU	49.96	61.66	125.15	132.29
hsa-miR-384	MIMAT0001075	AUUCCUAGAAAUUGUUCAUA	92.00	117.16	74.67	84.91
hsa-miR-3907	MIMAT0018179	AGGUGCUCCAGGCUGGCUCACA	49.80	49.98	81.21	88.26
hsa-miR-3908	MIMAT0018182	GAGCAAUGUAGGUAGACUGUUU	71.96	89.05	96.39	73.13
hsa-miR-3909	MIMAT0018183	UGUCCUCUAGGGCCUGCAGUCU	126.95	142.06	80.80	85.00
hsa-miR-3910	MIMAT0018184	AAAGGCAUAAAACCAAGACA	81.97	61.29	81.32	99.55
hsa-miR-3911	MIMAT0018185	UGUGUGGAUCCUGGAGGAGGCA	73.21	57.48	97.33	106.98
hsa-miR-3912	MIMAT0018186	UAACGCAUAAUAUGGACAUGU	96.86	118.84	86.55	116.34
hsa-miR-3913-5p	MIMAT0018187	UUUGGGACUGAUCUUGAUGUCU	125.31	115.46	108.72	103.35
hsa-miR-3914	MIMAT0018188	AAGGAACCAGAAAAUGAGAAGU	119.95	112.63	105.04	120.32
hsa-miR-3915	MIMAT0018189	UUGAGGAAAAGAUGGUCUUAUU	97.89	101.57	93.08	90.55
hsa-miR-3916	MIMAT0018190	AAGAGGAAGAAAUGGCUGGUUCUCAG	74.50	90.71	106.23	122.56
hsa-miR-3917	MIMAT0018191	GCUCGGACUGAGCAGGUGGG	107.79	93.61	101.73	123.09
hsa-miR-3918	MIMAT0018192	ACAGGGCCGCAGAUGGAGACU	72.78	87.36	91.69	85.87
hsa-miR-3919	MIMAT0018193	GCAGAGAACAAAGGACUCAGU	78.10	71.66	86.92	107.97
hsa-miR-3920	MIMAT0018195	ACUGAUUAUCUUAACUCUCUGA	123.81	122.45	103.97	83.26
hsa-miR-3921	MIMAT0018196	UCUCUGAGUACCAUAUGCCUUGU	82.51	103.53	98.95	105.67
hsa-miR-3922-3p	MIMAT0018197	UCUGGCCUUGACUUGACUCUUU	75.55	116.04	105.33	80.17
hsa-miR-3923	MIMAT0018198	AACUAGUAAUGUUGGAUUAGGG	86.15	79.32	98.84	102.15
hsa-miR-3924	MIMAT0018199	AUAUGUAUAUGUGACUGCUACU	107.57	88.52	92.16	84.36
hsa-miR-3925-5p	MIMAT0018200	AAGAGAACUGAAAGUGGAGCCU	87.28	90.12	117.40	99.17

hsa-miR-3926	MIMAT0018201	UGGCCAAAAAGCAGGCAGAGA	116.71	119.00	97.29	112.80
hsa-miR-3927-3p	MIMAT0018202	CAGGUAGAUAUUUGAUAGGCAU	96.95	90.95	82.26	92.53
hsa-miR-3928	MIMAT0018205	GGAGGAACCUUGGAGCUUCGGC	83.26	101.94	101.73	94.20
hsa-miR-3929	MIMAT0018206	GAGGCUGAUGUGAGUAGACCACU	96.57	93.26	90.93	103.23
hsa-miR-3934-5p	MIMAT0018349	UCAGGUGUGGAAACUGAGGCAG	95.89	99.17	109.49	105.67
hsa-miR-3935	MIMAT0018350	UGUAGAUACGAGCACCAGCCAC	67.87	59.83	77.45	69.89
hsa-miR-3936	MIMAT0018351	UAAGGGGUGUAUGGCAGAUGCA	83.79	96.86	84.61	106.26
hsa-miR-3937	MIMAT0018352	ACAGGCGGCUGUAGCAAUGGGGG	89.12	117.65	103.25	89.91
hsa-miR-3938	MIMAT0018353	AAUUCCCUUGUAGAUAACCCGG	94.07	116.71	90.08	90.60
hsa-miR-3939	MIMAT0018355	UACGCGCAGACCACAGGAUGUC	91.43	83.30	118.36	113.19
hsa-miR-3940-3p	MIMAT0018356	CAGCCCGGAUCCCAGCCCACUU	131.99	111.31	103.03	123.37
hsa-miR-3941	MIMAT0018357	UUACACACAACUGAGGAUCAUA	79.70	87.38	81.24	81.18
hsa-miR-3942-5p	MIMAT0018358	AAGCAAUACUGUUACCUGAAAU	86.68	94.90	93.44	87.25
hsa-miR-3943	MIMAT0018359	UAGCCCCCAGGCUUCACUUGGCG	115.78	91.67	114.53	113.65
hsa-miR-3944-3p	MIMAT0018360	UUCGGGCUGGCCUGCUGCUCCGG	128.50	147.49	83.47	72.66
hsa-miR-3945	MIMAT0018361	AGGGCAUAGGAGAGGGUUGAUAU	105.11	91.67	99.33	117.54
hsa-miR-409-3p	MIMAT0001639	GAAUGUUGCUCGGUGAACCCCU	89.12	94.64	68.91	79.01
hsa-miR-409-5p	MIMAT0001638	AGGUUACCCGAGCAACUUUGCAU	101.74	98.34	91.44	94.22
hsa-miR-410	MIMAT0002171	AAUAUAACACAGAUGGCCUGU	86.25	78.13	120.83	119.59
hsa-miR-411-3p	MIMAT0004813	UAUGUAACACGGUCCACUAACC	113.07	110.81	118.49	112.90
hsa-miR-411-5p	MIMAT0003329	UAGUAGACCGUAUAGCGUACG	92.18	96.39	99.51	96.18
hsa-miR-412	MIMAT0002170	ACUUCACCUGGUCCACUAGCCGU	117.10	74.16	69.14	75.67
hsa-miR-421	MIMAT0003339	AUCAACAGACAUUAAUUGGGCGC	104.26	84.09	86.65	67.60
hsa-miR-422a	MIMAT0001339	ACUGGACUUAGGGUCAGAAGGC	132.40	119.29	143.20	146.67
hsa-miR-423-3p	MIMAT0001340	AGCUCGGUCUGAGGCCCCUCAGU	104.04	95.64	95.89	82.26
hsa-miR-423-5p	MIMAT0004748	UGAGGGGCAGAGAGCGAGACUUU	89.15	82.78	126.58	115.28
hsa-miR-424-3p	MIMAT0004749	CAAAACGUGAGGCGCUGCUAU	74.39	67.76	121.21	120.82
hsa-miR-424-5p	MIMAT0001341	CAGCAGCAAUUCAUGUUUUGAA	81.40	85.91	86.20	83.08
hsa-miR-4251	MIMAT0016883	CCUGAGAAAAGGGCCAA	121.17	108.29	82.88	100.53
hsa-miR-4252	MIMAT0016886	GGCCACUGAGUCAGCACCA	89.70	83.49	101.60	112.83
hsa-miR-4253	MIMAT0016882	AGGGCAUGUCCAGGGGGU	63.86	50.97	96.80	80.17
hsa-miR-425-3p	MIMAT0001343	AUCGGGAAUGUCGUGUCCGCCC	85.25	77.76	97.34	77.81
hsa-miR-4254	MIMAT0016884	GCCUGGAGCUACUCCACCAUCUC	88.79	68.32	81.87	81.24
hsa-miR-4255	MIMAT0016885	CAGUGUUCAGAGAUGGA	81.51	84.88	110.13	101.49
hsa-miR-425-5p	MIMAT0003393	AAUGACACGAUCACUCCCGUUGA	50.61	74.16	92.31	101.25
hsa-miR-4256	MIMAT0016877	AUCUGACCUGAUGAAGGU	67.19	73.75	90.24	80.42
hsa-miR-4257	MIMAT0016878	CCAGAGGUGGGGACUGAG	114.60	102.85	103.96	87.77
hsa-miR-4258	MIMAT0016879	CCCCGCCACCGCCUUGG	92.18	86.47	87.87	102.51
hsa-miR-4259	MIMAT0016880	CAGUUGGGUCUAGGGGUCAGGA	88.45	67.99	122.59	119.47
hsa-miR-4260	MIMAT0016881	CUUGGGGCAUGGAGUCCCA	115.22	83.30	100.62	100.53
hsa-miR-4261	MIMAT0016890	AGGAAACAGGGACCCA	132.67	140.53	147.55	152.13

hsa-miR-4262	MIMAT0016894	GACAUUCAGACUACCUG	102.38	119.01	90.08	92.77
hsa-miR-4263	MIMAT0016898	AUUCUAAGUGCCUUGGCC	100.80	119.86	94.67	89.41
hsa-miR-4264	MIMAT0016899	ACUCAGUCAUGGUCAUU	78.10	80.70	129.08	103.92
hsa-miR-4265	MIMAT0016891	CUGUGGGCUCAGCUCUGGG	51.79	67.17	116.49	117.54
hsa-miR-4266	MIMAT0016892	CUAGGAGGCCUUGGCC	86.83	71.63	108.65	96.14
hsa-miR-4267	MIMAT0016893	UCCAGCUCGGUGGCAC	93.69	89.44	69.41	76.29
hsa-miR-4268	MIMAT0016896	GGCUCCUCCUCUCAGGAUGUG	109.22	95.01	82.76	85.15
hsa-miR-4269	MIMAT0016897	GCAGGCACAGACAGCCCUGGC	99.78	102.06	84.61	105.09
hsa-miR-4270	MIMAT0016900	UCAGGGAGUCAGGGGAGGGC	89.70	92.53	115.34	103.92
hsa-miR-4271	MIMAT0016901	GGGGGAAGAAAAGGUGGGG	84.61	63.63	70.70	65.87
hsa-miR-4272	MIMAT0016902	CAUUCAACUAGUGAUUGU	108.92	68.53	94.99	75.29
hsa-miR-4273	MIMAT0016903	GUGUUCUCUGAUGGACAG	100.35	95.60	113.92	106.07
hsa-miR-4274	MIMAT0016906	CAGCAGUCCCUCCCCCUG	103.59	104.29	80.69	70.84
hsa-miR-4275	MIMAT0016905	CCAAUUACCACUUCUUU	94.31	100.27	97.76	108.31
hsa-miR-4276	MIMAT0016904	CUCAGUGACUCAUGUGC	63.86	66.15	79.73	75.88
hsa-miR-4277	MIMAT0016908	GCAGUUCUGAGCACAGUACAC	87.95	84.97	88.38	98.17
hsa-miR-4278	MIMAT0016910	CUAGGGGGUUUGCCCUUG	80.76	68.86	89.34	94.96
hsa-miR-4279	MIMAT0016909	CUCUCCUCCCGGCUUC	145.42	133.13	129.58	146.11
hsa-miR-4280	MIMAT0016911	GAGUGUAGUUCUGAGCAGAGC	111.97	119.13	102.27	114.43
hsa-miR-4281	MIMAT0016907	GGGUCCCGGGGAGGGGGG	109.68	108.00	115.02	107.81
hsa-miR-4282	MIMAT0016912	UAAAAUUUGCAUCCAGGA	100.64	100.32	78.00	96.12
hsa-miR-4283	MIMAT0016914	UGGGGCUCAGCGAGUUU	109.27	113.05	90.08	106.86
hsa-miR-4284	MIMAT0016915	GGGCUCACAUCACCCCAU	116.91	115.06	111.99	132.55
hsa-miR-4285	MIMAT0016913	GCGGCGAGUCCGACUCAU	104.07	100.75	117.25	113.19
hsa-miR-4286	MIMAT0016916	ACCCCACUCCUGGUACC	145.18	148.60	124.41	100.02
hsa-miR-4287	MIMAT0016917	UCUCCCUUGAGGGCACUUU	93.46	104.11	99.47	101.62
hsa-miR-4288	MIMAT0016918	UUGUCUGCUGAGUUUCC	72.19	80.86	73.85	82.87
hsa-miR-4289	MIMAT0016920	GCAUUGUGCAGGGCUAUCA	116.75	111.36	95.52	109.93
hsa-miR-429	MIMAT0001536	UAAUACUGUCUGGUAAAACCGU	55.60	50.10	77.25	54.10
hsa-miR-4290	MIMAT0016921	UGCCCUCCUUUCUUCCCUC	66.51	91.83	103.97	117.29
hsa-miR-4291	MIMAT0016922	UUCAGCAGGAACAGCU	78.45	92.41	84.12	106.26
hsa-miR-4292	MIMAT0016919	CCCCUGGGCCGGCCUUGG	130.76	155.15	110.90	126.01
hsa-miR-4293	MIMAT0016848	CAGCCUGACAGGAACAG	67.87	68.18	69.39	75.56
hsa-miR-4294	MIMAT0016849	GGGAGUCUACAGCAGGG	125.63	113.84	118.91	87.86
hsa-miR-4295	MIMAT0016844	CAGUGCAAUGUUUUCCUU	137.45	114.79	123.87	97.44
hsa-miR-4296	MIMAT0016845	AUGUGGGCUCAGGCUCA	75.55	66.15	105.33	109.67
hsa-miR-4297	MIMAT0016846	UGCCUUCCUGUCUGUG	121.08	86.27	108.24	137.95
hsa-miR-4298	MIMAT0016852	CUGGGACAGGAGGAGGAGGCAG	126.37	110.67	131.11	123.09
hsa-miR-4299	MIMAT0016851	GCUGGUGACAUGAGAGGC	121.17	129.71	93.41	112.01
hsa-miR-4300	MIMAT0016853	UGGGAGCUGGACUACUUC	110.02	102.74	102.83	89.05
hsa-miR-4301	MIMAT0016850	UCCCACUACUUCACUUGUGA	104.07	115.83	112.81	98.95

hsa-miR-4302	MIMAT0016855	CCAGUGUGGCUCAGCGAG	105.21	123.41	66.19	84.93
hsa-miR-4303	MIMAT0016856	UUCUGAGCUGAGGACAG	80.28	89.25	31.88	36.02
hsa-miR-4304	MIMAT0016854	CCGGCAUGUCCAGGGCA	104.02	94.62	105.87	115.26
hsa-miR-4305	MIMAT0016857	CCUAGACACCUCCAGUUC	69.24	86.27	99.71	82.85
hsa-miR-4306	MIMAT0016858	UGGAGAGAAAGGCAGUA	81.00	62.90	92.53	100.55
hsa-miR-4307	MIMAT0016860	AAUGUUUUUUCCUGUUUCC	99.34	79.64	99.25	90.75
hsa-miR-4308	MIMAT0016861	UCCCUGGAGUUUCUUCUU	88.96	94.77	111.49	90.36
hsa-miR-4309	MIMAT0016859	CUGGAGUCUAGGAUUCCA	86.23	83.70	101.73	94.59
hsa-miR-4310	MIMAT0016862	GCAGCAUUCAUGUCCC	107.48	82.42	111.73	99.48
hsa-miR-4311	MIMAT0016863	GAAAGAGAGCUGAGUGUG	105.13	105.57	111.09	111.17
hsa-miR-4312	MIMAT0016864	GGCCUUGUUCCUGUCCCCA	66.28	62.63	104.02	92.29
hsa-miR-4313	MIMAT0016865	AGCCCCCUGGCCCCAAACCC	122.86	124.31	115.82	109.18
hsa-miR-431-3p	MIMAT0004757	CAGGUCGUCUUGCAGGGCUUCU	79.00	65.89	112.33	117.31
hsa-miR-4314	MIMAT0016868	CUCUGGGAAAUGGGACAG	102.07	105.03	74.31	75.51
hsa-miR-4315	MIMAT0016866	CCGCUUUCUGAGCUGGAC	93.75	95.28	117.61	85.53
hsa-miR-431-5p	MIMAT0001625	UGUCUUGCAGGCCGUCAUGCA	74.01	53.57	98.76	84.88
hsa-miR-4316	MIMAT0016867	GGUGAGGCUAGCUGGUG	29.73	76.16	93.96	81.14
hsa-miR-4317	MIMAT0016872	ACAUUGCCAGGGAGUUU	95.23	112.38	100.09	95.03
hsa-miR-4318	MIMAT0016869	CACUGUGGGUACAUGCU	114.25	120.62	84.61	93.80
hsa-miR-4319	MIMAT0016870	UCCCUGAGCAAAGCCAC	89.02	84.88	96.87	97.03
hsa-miR-4320	MIMAT0016871	GGGAUUCUGUAGCUUCCU	60.75	79.16	82.40	84.46
hsa-miR-4321	MIMAT0016874	UUAGCGGUGGACCGCCCUGCG	116.14	112.44	103.96	132.55
hsa-miR-4322	MIMAT0016873	CUGUGGGCUCAGCGCGUGGGG	103.84	95.01	109.07	99.86
hsa-miR-4323	MIMAT0016875	CAGCCCCACAGCCUCAGA	93.46	80.25	120.27	118.78
hsa-miR-432-3p	MIMAT0002815	CUGGAUGGCUCCUCCAUGUCU	119.74	129.95	127.80	98.41
hsa-miR-4324	MIMAT0016876	CCCUGAGACCCUAACCUUAA	119.99	121.16	102.49	100.51
hsa-miR-4325	MIMAT0016887	UUGCACUUGUCUCAGUGA	81.50	93.15	96.38	112.87
hsa-miR-432-5p	MIMAT0002814	UCUUGGAGUAGGUCAUUGGGUGG	137.46	138.43	101.47	88.82
hsa-miR-4326	MIMAT0016888	UGUUCCUCUGUCUCCCAGAC	42.06	47.72	92.00	84.46
hsa-miR-4327	MIMAT0016889	GGCUUGCAUGGGGGACUGG	130.76	110.70	94.46	79.92
hsa-miR-4328	MIMAT0016926	CCAGUUUUCCCAGGAUU	95.66	98.78	72.21	87.93
hsa-miR-4329	MIMAT0016923	CCUGAGACCCUAGUUCCAC	127.11	119.40	78.44	87.47
hsa-miR-433	MIMAT0001627	AUCAUGAUGGGCUCCUCGGUGU	113.56	98.09	89.06	98.09
hsa-miR-4330	MIMAT0016924	CCUCAGAUCAGAGCCUUGC	81.77	60.69	91.75	103.70
hsa-miR-448	MIMAT0001532	UUGCAUAUGUAGGAUGUCCCAU	99.73	111.45	114.07	110.13
hsa-miR-449a	MIMAT0001541	UGGCAGUGUAUUGUUAGCUGGU	21.82	27.24	45.59	62.93
hsa-miR-449b-3p	MIMAT0009203	CAGCCACAACUACCCUGCCACU	103.77	94.79	131.84	107.89
hsa-miR-449b-5p	MIMAT0003327	AGGCAGUGUAUUGUUAGCUGGC	36.17	26.71	65.57	60.88
hsa-miR-449c-3p	MIMAT0013771	UUGCUAGUUGCACUCCUCUCUGU	145.70	142.40	101.73	105.67
hsa-miR-449c-5p	MIMAT0010251	UAGGCAGUGUAUUGCUAGCGGCUGU	91.29	66.05	94.37	84.42
hsa-miR-450a-5p	MIMAT0001545	UUUUGCGAUGUGUUCCUAAUAU	169.53	177.24	156.37	178.96

hsa-miR-450b-3p	MIMAT0004910	UUGGGAUCAUUUUGCAUCCAUA	95.41	92.40	98.49	107.28
hsa-miR-450b-5p	MIMAT0004909	UUUUGCAAUAUGUUCCUGAAUA	36.30	34.18	86.13	94.62
hsa-miR-451a	MIMAT0001631	AAACCGUUACCAUUACUGAGUU	80.71	89.15	96.72	133.15
hsa-miR-452-3p	MIMAT0001636	CUCAUCUGCAAAGAAGUAAGUG	86.91	92.39	101.40	84.50
hsa-miR-452-5p	MIMAT0001635	AACUGUUUGCAGAGGAAACUGA	136.07	110.20	85.24	87.48
hsa-miR-453	MIMAT0001630	AGGUUGUCCGUGGUGAGUUCGCA	102.07	99.83	116.00	128.83
hsa-miR-454-3p	MIMAT0003885	UAGUGCAAUAUUGCUUAUAGGGU	133.80	134.07	102.83	106.86
hsa-miR-454-5p	MIMAT0003884	ACCCUAUCAAUAUUGUCUCUGC	129.91	129.36	143.18	158.56
hsa-miR-455-3p	MIMAT0004784	GCAGUCCAUGGGCAUAUACAC	129.22	126.39	86.78	115.88
hsa-miR-455-5p	MIMAT0003150	UAUGUGCCUUUGGACUACAUCG	94.45	75.34	126.30	112.87
hsa-miR-466	MIMAT0015002	AUACACAUACACGCAACACACAU	102.26	111.18	87.04	88.59
hsa-miR-483-3p	MIMAT0002173	UCACUCCUCUCCUCCCGUCUU	128.29	126.11	96.63	95.38
hsa-miR-483-5p	MIMAT0004761	AAGACGGGAGGAAAGAAGGGAG	87.08	90.20	119.87	107.38
hsa-miR-484	MIMAT0002174	UCAGGCUCAGUCCCCUCCCGAU	150.67	155.24	100.29	108.68
hsa-miR-485-3p	MIMAT0002176	GUCAUACACGGCUCUCCUCUCU	117.43	134.25	94.49	131.44
hsa-miR-485-5p	MIMAT0002175	AGAGGCUGGCCGUGAUGAAUUC	68.03	76.05	111.06	125.66
hsa-miR-486-3p	MIMAT0004762	CGGGGCAGCUCAGUACAGGAU	96.58	87.82	111.05	115.17
hsa-miR-486-5p	MIMAT0002177	UCCUGUACUGAGCUGCCCCGAG	157.32	116.04	109.60	104.30
hsa-miR-487a	MIMAT0002178	AAUCAUACAGGGACAUCCAGUU	101.88	117.04	95.51	92.10
hsa-miR-487b	MIMAT0003180	AAUCGUACAGGGUCAUCCACUU	137.84	139.42	114.89	120.07
hsa-miR-488-3p	MIMAT0004763	UUGAAAGGCUAUUUCUUGGUC	109.93	95.32	102.79	110.63
hsa-miR-488-5p	MIMAT0002804	CCCAGAUAAUGGCACUCUCAA	86.25	100.83	108.17	88.82
hsa-miR-489	MIMAT0002805	GUGACAUCACAUAUACGGCAGC	115.96	87.66	107.82	93.01
hsa-miR-490-3p	MIMAT0002806	CAACCUGGAGGACUCCAUGCUG	120.76	107.21	106.88	117.51
hsa-miR-490-5p	MIMAT0004764	CCAUGGAUCUCCAGGUGGGU	57.96	61.77	101.37	97.88
hsa-miR-491-3p	MIMAT0004765	CUUAUGCAAGAUUCCCUUCUAC	159.71	132.00	88.08	101.90
hsa-miR-491-5p	MIMAT0002807	AGUGGGGAACCCUUCCAUGAGG	117.07	88.40	102.32	99.78
hsa-miR-492	MIMAT0002812	AGGACCUGCGGGACAAGAUUCUU	92.16	105.77	95.89	109.37
hsa-miR-493-3p	MIMAT0003161	UGAAGGUCUACUGUGUGCCAGG	153.63	201.98	117.11	123.28
hsa-miR-493-5p	MIMAT0002813	UUGUACAUGGUAGGCUUUCAUU	101.74	92.99	109.45	100.99
hsa-miR-494	MIMAT0002816	UGAAACAUACACGGGAAACCUC	82.37	70.77	137.80	136.08
hsa-miR-495-3p	MIMAT0002817	AAACAAACAUGGUGCACUUCUU	92.70	86.00	102.44	81.54
hsa-miR-496	MIMAT0002818	UGAGUAUUACAUGGCCAAUCUC	105.48	110.99	120.69	107.03
hsa-miR-497-3p	MIMAT0004768	CAAACCACACUGUGGUGUUAGA	91.66	85.42	112.75	72.08
hsa-miR-497-5p	MIMAT0002820	CAGCAGCACACUGUGGUUUGU	63.98	64.95	70.39	75.90
hsa-miR-498	MIMAT0002824	UUUCAAGCCAGGGGGCGUUUUUC	62.30	70.60	96.28	109.01
hsa-miR-499a-3p	MIMAT0004772	AACAUCACAGCAAGUCUGUGCU	89.25	69.32	117.05	97.13
hsa-miR-499a-5p	MIMAT0002870	UUAAGACUUGCAGUGAUGUUU	119.13	111.85	85.46	88.41
hsa-miR-500a-3p	MIMAT0002871	AUGCACCUGGGCAAGGAUUCUG	95.38	101.98	98.84	119.47
hsa-miR-500a-5p	MIMAT0004773	UAAUCCUUGCUACCUGGGUGAGA	177.47	179.25	120.91	117.15
hsa-miR-500b	MIMAT0016925	AAUCCUUGCUACCUGGGU	90.69	104.32	97.29	99.74

hsa-miR-501-3p	MIMAT0004774	AAUGCACCCGGGCAAGGAUUCU	160.99	168.08	89.47	97.61
hsa-miR-501-5p	MIMAT0002872	AAUCCUUUGUCCCUGGGUGAGA	113.18	101.18	86.50	92.84
hsa-miR-502-3p	MIMAT0004775	AAUGCACCUGGGCAAGGAUUCA	85.83	87.74	120.13	121.16
hsa-miR-502-5p	MIMAT0002873	AUCCUUGCUAUCUGGGUGCUA	82.88	82.25	77.36	80.68
hsa-miR-503-5p	MIMAT0002874	UAGCAGCGGGAACAGUUCUGCAG	57.78	71.44	67.78	77.43
hsa-miR-504	MIMAT0002875	AGACCCUGGUCUGCACUCUAUC	83.53	86.10	105.04	95.19
hsa-miR-505-3p	MIMAT0002876	CGUCAACACUUGCUGGUUUCCU	108.66	99.59	115.06	121.02
hsa-miR-505-5p	MIMAT0004776	GGGAGCCAGGAAGUAUUGAUGU	89.48	77.48	111.52	103.18
hsa-miR-506-3p	MIMAT0002878	UAAGGCACCCUUCUGAGUAGA	133.97	129.18	143.52	148.45
hsa-miR-507	MIMAT0002879	UUUUGCACCUUUUGGAGUGAA	58.04	68.11	78.87	76.11
hsa-miR-508-3p	MIMAT0002880	UGAUUGUAGCCUUUUGGAGUAGA	102.10	144.70	109.86	74.53
hsa-miR-508-5p	MIMAT0004778	UACUCCAGAGGGCGUCACUCAUG	32.03	46.16	75.53	59.94
hsa-miR-509-3-5p	MIMAT0004975	UACUGCAGACGUGGCAAUCAUG	71.69	63.10	134.57	107.57
hsa-miR-509-3p	MIMAT0002881	UGAUUGGUACGUCUGUGGGUAG	111.32	105.74	114.21	114.76
hsa-miR-509-5p	MIMAT0004779	UACUGCAGACAGUGGCAAUCA	35.04	32.29	129.14	130.42
hsa-miR-510	MIMAT0002882	UACUCAGGAGAGUGGCAAUCAC	36.15	43.58	87.51	115.88
hsa-miR-511	MIMAT0002808	GUGUCUUUUGCUCUGCAGUCA	139.11	125.92	116.77	97.31
hsa-miR-512-3p	MIMAT0002823	AAGUGCUGUCAUAGCUGAGGUC	156.83	167.92	95.63	99.30
hsa-miR-512-5p	MIMAT0002822	CACUCAGCCUUGAGGGCACUUUC	116.50	85.27	76.32	59.02
hsa-miR-513a-3p	MIMAT0004777	UAAAUUUCACCUUUCUGAGAAGG	121.07	100.43	107.66	69.81
hsa-miR-513a-5p	MIMAT0002877	UUCACAGGGAGGUGUCAU	73.99	79.16	85.07	83.39
hsa-miR-513b	MIMAT0005788	UUCACAAGGAGGUGUCAUUUAU	82.38	104.36	75.16	98.36
hsa-miR-513c-5p	MIMAT0005789	UUCUCAAGGAGGUGUCGUUUAU	118.20	108.17	129.61	129.02
hsa-miR-514a-3p	MIMAT0002883	AUUGACACUUCUGUGAGUAGA	71.42	66.15	87.06	76.14
hsa-miR-514b-3p	MIMAT0015088	AUUGACACCUCUGUGAGUGGA	89.12	99.83	82.65	87.18
hsa-miR-514b-5p	MIMAT0015087	UUCUCAAGAGGGAGGCAAUCAU	136.03	112.65	109.49	110.03
hsa-miR-515-3p	MIMAT0002827	GAGUGCCUUCUUUUGGAGCGUU	174.45	173.51	119.73	118.78
hsa-miR-515-5p	MIMAT0002826	UUCUCCAAAAGAAAGCACUUUCUG	120.57	88.57	70.82	104.45
hsa-miR-516a-3p	MIMAT0002860	UGCUUCCUUUCAGAGGGU	98.63	99.52	94.91	113.53
hsa-miR-516a-5p	MIMAT0004770	UUCUCGAGGAAAGAAGCACUUUC	121.17	102.34	112.26	97.36
hsa-miR-516b-5p	MIMAT0002859	AUCUGGAGGUAAGAAGCACUUU	118.46	94.27	75.95	67.73
hsa-miR-517-5p	MIMAT0002851	CCUCUAGAUGGAAGCACUGUCU	122.36	98.88	103.33	107.69
hsa-miR-517a-3p	MIMAT0002852	AUCGUGCAUCCCUUUAGAGUGU	76.44	99.01	61.60	42.38
hsa-miR-517b	MIMAT0002857	UCGUGCAUCCCUUUAGAGUGUU	150.89	144.26	107.34	122.39
hsa-miR-517c-3p	MIMAT0002866	AUCGUGCAUCCUUUUAGAGUGU	87.25	85.64	128.09	105.51
hsa-miR-518a-3p	MIMAT0002863	GAAAGCGCUUCCCUUUGCUGGA	99.79	85.50	104.04	90.63
hsa-miR-518a-5p	MIMAT0005457	CUGCAAAGGGAAGCCCUUUC	22.37	17.67	59.23	41.25
hsa-miR-518b	MIMAT0002844	CAAAGCGCUCCCCUUUAGAGGU	109.27	89.65	85.65	92.22
hsa-miR-518c-3p	MIMAT0002848	CAAAGCGCUUCUCUUUAGAGUGU	101.10	133.28	102.83	108.05
hsa-miR-518c-5p	MIMAT0002847	UCUCUGGAGGGAAGCACUUUCUG	107.03	91.11	95.78	97.19
hsa-miR-518d-3p	MIMAT0002864	CAAAGCGCUUCCCUUUGGAGC	84.89	113.87	98.93	67.30

hsa-miR-518d-5p	MIMAT0005456	CUCUAGAGGGAAGCACUUUCUG	144.45	145.19	120.18	127.57
hsa-miR-518e-3p	MIMAT0002861	AAAGCGCUUCCCUUCAGAGUG	116.49	98.12	118.83	117.92
hsa-miR-518e-5p	MIMAT0005450	CUCUAGAGGGAAGCGCUUUCUG	92.67	76.46	82.22	112.60
hsa-miR-518f-3p	MIMAT0002842	GAAAGCGCUUCUCUUUAGAGG	100.65	89.88	80.88	113.61
hsa-miR-518f-5p	MIMAT0002841	CUCUAGAGGGAAGCACUUUCUC	106.81	113.45	85.45	87.29
hsa-miR-519a-3p	MIMAT0002869	AAAGUGCAUCCUUUUAGAGUGU	125.97	131.43	137.97	161.83
hsa-miR-519b-3p	MIMAT0002837	AAAGUGCAUCCUUUUAGAGGUU	134.15	120.33	115.46	124.00
hsa-miR-519c-3p	MIMAT0002832	AAAGUGCAUCUUUUUAGAGGAU	113.20	121.58	114.13	116.31
hsa-miR-519d	MIMAT0002853	CAAAGUGCCUCCCUUUAGAGUG	80.76	86.29	124.42	114.57
hsa-miR-519e-3p	MIMAT0002829	AAGUGCCUCCUUUUAGAGUGUU	99.19	122.33	82.26	76.10
hsa-miR-519e-5p	MIMAT0002828	UUCUCCAAAAGGGAGCACUUUC	108.53	106.16	68.25	73.52
hsa-miR-520a-3p	MIMAT0002834	AAAGUGCUUCCCUUUGGACUGU	137.73	136.43	138.20	151.71
hsa-miR-520a-5p	MIMAT0002833	CUCCAGAGGGAAGUACUUUCU	37.98	45.06	83.22	78.56
hsa-miR-520b	MIMAT0002843	AAAGUGCUUCCUUUUAGAGGG	97.08	118.93	112.27	134.03
hsa-miR-520c-3p	MIMAT0002846	AAAGUGCUUCCUUUUAGAGGGU	106.85	119.01	122.03	103.16
hsa-miR-520d-3p	MIMAT0002856	AAAGUGCUUCUCUUUGGUGGGU	166.82	203.57	132.14	136.45
hsa-miR-520d-5p	MIMAT0002855	CUACAAAGGGAAGCCCUUUC	162.63	147.31	104.33	87.81
hsa-miR-520e	MIMAT0002825	AAAGUGCUUCCUUUUUGAGGG	100.08	101.57	94.08	123.82
hsa-miR-520f	MIMAT0002830	AAGUGCUUCCUUUUAGAGGGUU	165.34	152.49	164.35	140.85
hsa-miR-520g	MIMAT0002858	ACAAAGUGCUUCCCUUUAGAGUGU	125.60	107.31	114.13	118.77
hsa-miR-520h	MIMAT0002867	ACAAAGUGCUUCCCUUUAGAGU	78.13	84.50	89.97	88.43
hsa-miR-521	MIMAT0002854	AACGCACUUCCCUUUAGAGUGU	101.53	90.65	78.01	102.80
hsa-miR-522-3p	MIMAT0002868	AAAAUGGUUCCCUUUAGAGUGU	46.90	31.45	117.11	110.56
hsa-miR-523-3p	MIMAT0002840	GAACGCGCUUCCCUAUAGAGGGU	101.58	100.63	115.05	107.89
hsa-miR-524-3p	MIMAT0002850	GAAGGCGCUUCCCUUUGGAGU	62.30	63.63	81.67	92.34
hsa-miR-524-5p	MIMAT0002849	CUACAAAGGGAAGCACUUUCUC	127.02	106.37	119.06	109.29
hsa-miR-525-3p	MIMAT0002839	GAAGGCGCUUCCCUUUAGAGCG	93.83	117.46	116.10	141.40
hsa-miR-525-5p	MIMAT0002838	CUCCAGAGGGAUGCACUUUCU	111.66	129.42	107.17	95.06
hsa-miR-526b-3p	MIMAT0002836	GAAAGUGCUUCCUUUUAGAGGC	176.36	184.28	86.13	110.03
hsa-miR-526b-5p	MIMAT0002835	CUCUUGAGGGAAGCACUUUCUGU	63.86	92.18	109.07	100.01
hsa-miR-532-3p	MIMAT0004780	CCUCCCACACCCAAGGCUUGCA	112.93	92.18	77.60	75.35
hsa-miR-532-5p	MIMAT0002888	CAUGCCUUGAGUGUAGGACCGU	137.87	163.67	120.42	117.54
hsa-miR-539-5p	MIMAT0003163	GGAGAAAUUAUCCUUGGUGUGU	140.97	167.01	109.60	117.17
hsa-miR-541-3p	MIMAT0004920	UGGUGGGCACAGAAUCUGGACU	64.74	57.94	120.81	124.53
hsa-miR-541-5p	MIMAT0004919	AAAGGAUUCUGCUGUCGGUCCCACU	116.50	97.56	111.06	108.53
hsa-miR-542-3p	MIMAT0003389	UGUGACAGAUUGAUAACUGAAA	58.49	67.39	81.94	95.48
hsa-miR-542-5p	MIMAT0003340	UCGGGGAUCAUCAUGUCACGAGA	94.13	105.24	103.09	106.62
hsa-miR-543	MIMAT0004954	AAACAUUCGCGGUGCACUUCUU	88.46	128.52	103.39	101.72
hsa-miR-544a	MIMAT0003164	AUUCUGCAUUUUUAGCAAGUUC	51.10	50.43	107.64	87.16
hsa-miR-544b	MIMAT0015004	ACCUGAGGUUGUGCAUUUCUAA	92.43	62.61	92.60	80.02
hsa-miR-545-3p	MIMAT0003165	UCAGCAAACAUUUAUUGUGUGC	141.67	113.87	97.44	91.55

hsa-miR-545-5p	MIMAT0004785	UCAGUAAAUGUUUAUUAGAUGA	87.58	97.14	68.67	75.43
hsa-miR-548a-3p	MIMAT0003251	CAAAACUGGCAAUUACUUUUGC	118.09	97.04	134.19	110.36
hsa-miR-548a-5p	MIMAT0004803	AAAAGUAAUUGCGAGUUUUACC	101.58	103.71	108.79	104.72
hsa-miR-548aa	MIMAT0018447	AAAAACCACAAUUACUUUUGCACCA	63.78	75.14	102.08	104.73
hsa-miR-548b-3p	MIMAT0003254	CAAGAACCUCAGUUGCUUUUGU	105.89	84.06	92.80	107.03
hsa-miR-548b-5p	MIMAT0004798	AAAAGUAAUUGUGGUUUUGGCC	45.67	53.01	115.05	102.82
hsa-miR-548c-3p	MIMAT0003285	CAAAAAUCUCAAUUACUUUUGC	97.03	74.89	99.24	83.90
hsa-miR-548c-5p	MIMAT0004806	AAAAGUAAUUGCGGUUUUUGCC	68.64	69.55	113.10	123.73
hsa-miR-548d-3p	MIMAT0003323	CAAAAACCACAGUUUCUUUUGC	116.12	111.10	96.20	115.00
hsa-miR-548d-5p	MIMAT0004812	AAAAGUAAUUGUGGUUUUUGCC	113.16	113.11	77.21	86.91
hsa-miR-548e	MIMAT0005874	AAAAACUGAGACUACUUUUGCA	145.78	153.49	110.18	119.82
hsa-miR-548f	MIMAT0005895	AAAAACUGUAAUUACUUUU	124.25	108.41	106.76	94.04
hsa-miR-548g-3p	MIMAT0005912	AAAACUGUAAUUACUUUUGUAC	105.04	105.26	74.24	91.18
hsa-miR-548h-5p	MIMAT0005928	AAAAGUAAUCGCGGUUUUUGUC	94.87	111.85	97.00	96.21
hsa-miR-548i	MIMAT0005935	AAAAGUAAUUGCGGAUUUUGCC	124.92	105.03	110.61	107.81
hsa-miR-548j	MIMAT0005875	AAAAGUAAUUGCGGUCUUUGGU	86.87	84.89	83.43	76.72
hsa-miR-548k	MIMAT0005882	AAAAGUACUUGCGGAUUUUGCU	87.44	83.31	121.86	127.96
hsa-miR-548l	MIMAT0005889	AAAAGUAUUUGCGGGUUUUGUC	89.33	97.14	100.34	116.48
hsa-miR-548m	MIMAT0005917	CAAAGGUAUUUGUGGUUUUUG	138.79	137.26	117.45	105.50
hsa-miR-548n	MIMAT0005916	CAAAAGUAAUUGUGGAUUUUGU	69.21	64.79	114.78	124.00
hsa-miR-548o-3p	MIMAT0005919	CCAAAACUGCAGUUACUUUUGC	115.90	131.30	108.92	119.18
hsa-miR-548p	MIMAT0005934	UAGCAAAAACUGCAGUUACUUU	53.83	46.66	111.03	92.61
hsa-miR-548q	MIMAT0011163	GCUGGUGCAAAAGUAAUGGCGG	107.40	119.13	116.49	121.05
hsa-miR-548s	MIMAT0014987	AUGGCCAAAACUGCAGUUAUUUU	88.97	92.06	92.33	103.68
hsa-miR-548t-5p	MIMAT0015009	CAAAAGUGAUCGUGGUUUUUG	129.26	134.97	74.13	100.27
hsa-miR-548u	MIMAT0015013	CAAAGACUGCAAUUACUUUUGCG	37.60	43.57	92.51	111.74
hsa-miR-548v	MIMAT0015020	AGCUACAGUUACUUUUGCACCA	114.94	119.66	105.87	112.02
hsa-miR-548w	MIMAT0015060	AAAAGUAACUGCGGUUUUUGCCU	126.02	110.08	104.02	116.45
hsa-miR-548x	MIMAT0015081	UAAAAACUGCAAUUACUUUCA	91.77	90.27	85.77	104.51
hsa-miR-548y	MIMAT0018354	AAAAGUAAUCACUGUUUUUGCC	126.17	116.38	136.58	121.78
hsa-miR-548z	MIMAT0018446	CAAAAACCGCAAUUACUUUUGCA	88.46	93.25	109.52	99.01
hsa-miR-549a	MIMAT0003333	UGACAACUAUGGAUGAGCUCU	93.20	119.07	75.75	82.51
hsa-miR-550a-3p	MIMAT0003257	UGUCUUACUCCCUCAGGCACAU	85.49	106.31	115.03	104.09
hsa-miR-550a-5p	MIMAT0004800	AGUGCCUGAGGGAGUAAGAGCCC	93.78	106.37	81.72	85.83
hsa-miR-550b-3p	MIMAT0018445	UCUUACUCCCUCAGGCACUG	116.06	77.66	98.69	71.76
hsa-miR-551a	MIMAT0003214	GCGACCCACUCUUGGUUUCCA	134.06	119.13	126.79	127.27
hsa-miR-551b-3p	MIMAT0003233	GCGACCCAUACUUGGUUUCAG	120.80	124.02	125.47	120.36
hsa-miR-551b-5p	MIMAT0004794	GAAAUCAAGCGUGGGUGAGACC	110.44	119.87	89.52	80.18
hsa-miR-552	MIMAT0003215	AACAGGUGACUGGUUAGACAA	54.23	63.07	68.29	80.92
hsa-miR-553	MIMAT0003216	AAAACGGUGAGAUUUUGUUUU	145.63	121.75	117.24	96.63
hsa-miR-554	MIMAT0003217	GCUAGUCCUGACUCAGCCAGU	71.42	99.95	88.43	97.82

hsa-miR-555	MIMAT0003219	AGGGUAAGCUGAACCUCUGAU	98.26	117.65	114.53	119.88
hsa-miR-556-3p	MIMAT0004793	AUAUUACCAUUAGCUCAUCUUU	166.97	214.59	139.64	151.56
hsa-miR-556-5p	MIMAT0003220	GAUGAGCUCAUUGUAAUAUGAG	71.76	83.73	105.94	112.97
hsa-miR-557	MIMAT0003221	GUUUGCACGGGUGGGCCUUGUCU	81.77	80.92	107.82	92.22
hsa-miR-558	MIMAT0003222	UGAGCUGCUGUACCAAAAU	94.20	83.14	94.27	91.42
hsa-miR-559	MIMAT0003223	UAAAGUAAAUAUGCACCAAAA	105.62	90.38	132.43	114.57
hsa-miR-561-3p	MIMAT0003225	CAAAGUUUAAGAUCCUUGAAGU	122.98	103.15	117.76	116.60
hsa-miR-562	MIMAT0003226	AAAGUAGCUGUACCAUUUGC	102.21	78.40	115.49	129.94
hsa-miR-563	MIMAT0003227	AGGUUGACAUACGUUUCCC	106.18	95.35	143.68	126.54
hsa-miR-564	MIMAT0003228	AGGCACGGUGUCAGCAGGC	100.47	118.21	103.73	101.09
hsa-miR-566	MIMAT0003230	GGGCGCCUGUGAUCCCAAC	113.13	108.91	104.51	92.30
hsa-miR-567	MIMAT0003231	AGUAUGUUCUUCCAGGACAGAAC	117.48	89.11	121.89	106.66
hsa-miR-568	MIMAT0003232	AUGUAUAAAUGUAUACACAC	94.24	94.35	99.47	114.49
hsa-miR-569	MIMAT0003234	AGUUAAUGAAUCCUGGAAAGU	81.25	81.01	67.65	99.76
hsa-miR-570-3p	MIMAT0003235	CGAAAACAGCAAUUACCUUUGC	49.76	66.94	71.24	77.34
hsa-miR-571	MIMAT0003236	UGAGUUGGCCAUCUGAGUGAG	107.67	78.40	118.41	120.47
hsa-miR-572	MIMAT0003237	GUCCGCUCGGCGGUGGCCCA	88.36	77.57	103.25	103.53
hsa-miR-573	MIMAT0003238	CUGAAGUGAUGUGUAACUGAUCAG	136.29	125.80	125.60	133.26
hsa-miR-574-3p	MIMAT0003239	CACGCUCAUGCACACACCCACA	113.10	125.65	94.22	100.65
hsa-miR-574-5p	MIMAT0004795	UGAGUGUGUGUGUGUGAGUGUGU	103.66	110.34	119.31	99.30
hsa-miR-575	MIMAT0003240	GAGCCAGUUGGACAGGAGC	101.05	119.30	104.48	125.53
hsa-miR-576-3p	MIMAT0004796	AAGAUGUGGAAAAAUUGGAAUC	99.52	77.80	108.49	113.36
hsa-miR-576-5p	MIMAT0003241	AUUCUAAUUUCUCCACGUCUUU	121.62	139.37	98.94	119.51
hsa-miR-577	MIMAT0003242	UAGAUAAAAUAUUGGUACCUG	129.01	118.23	118.17	106.30
hsa-miR-578	MIMAT0003243	CUUCUUGUGCUCUAGGAUUGU	36.24	25.42	92.55	96.92
hsa-miR-579	MIMAT0003244	UUCAUUUGGUAUAAACCGCGAUU	119.91	101.82	88.83	78.31
hsa-miR-580	MIMAT0003245	UUGAGAAUGAUGAAUCAUUAGG	84.13	110.29	83.34	77.92
hsa-miR-581	MIMAT0003246	UCUUGUGUUCUCUAGAUCAGU	119.23	95.48	112.77	117.65
hsa-miR-582-3p	MIMAT0004797	UAACUGGUUGAACAACUGAACC	99.16	114.48	91.38	110.64
hsa-miR-582-5p	MIMAT0003247	UUACAGUUGUUCAACCAGUUACU	189.17	216.00	73.30	89.05
hsa-miR-583	MIMAT0003248	CAAAGAGGAAGGUCCCAUUAC	114.94	109.23	91.18	92.58
hsa-miR-584-5p	MIMAT0003249	UUAUGGUUUGCCUGGGACUGAG	69.99	68.86	79.47	76.33
hsa-miR-585	MIMAT0003250	UGGGCGUAUCUGUAUGCUA	98.12	86.08	97.84	127.84
hsa-miR-586	MIMAT0003252	UAUGCAUUGUAUUUUUAGGUCC	134.76	154.64	101.47	113.44
hsa-miR-587	MIMAT0003253	UUUCCAUAGGUGAUGAGUCAC	106.78	108.51	89.13	67.39
hsa-miR-588	MIMAT0003255	UUGGCCACAAUGGGUUAGAAC	110.85	123.14	93.08	84.88
hsa-miR-589-3p	MIMAT0003256	UCAGAACAAAUGCCGGUUCCCAGA	112.30	105.47	103.22	109.01
hsa-miR-589-5p	MIMAT0004799	UGAGAACCACGUCUGCUCUGAG	96.49	83.97	91.42	97.85
hsa-miR-590-3p	MIMAT0004801	UAAUUUUAUGUAUAAGCUAGU	98.12	98.37	103.94	107.26
hsa-miR-590-5p	MIMAT0003258	GAGCUUAUUCAUAAAAGUGCAG	132.32	132.09	110.04	96.57
hsa-miR-591	MIMAT0003259	AGACCAUGGGUUCUCAUUGU	81.30	78.96	86.34	92.05

hsa-miR-592	MIMAT0003260	UUGUGUCAAUAUGCGAUGAUGU	119.44	112.16	111.90	120.11
hsa-miR-593-3p	MIMAT0004802	UGUCUCUGCUGGGGUUUCU	87.86	115.09	109.29	96.21
hsa-miR-593-5p	MIMAT0003261	AGGCACCAGCCAGGCAUUGCUCAGC	49.59	52.20	123.81	118.36
hsa-miR-595	MIMAT0003263	GAAGUGUGCCGUGGUGUGUCU	131.07	136.90	100.66	100.55
hsa-miR-596	MIMAT0003264	AAGCCUGCCCGGCUCCUCGGG	130.00	95.35	58.61	61.93
hsa-miR-597	MIMAT0003265	UGUGUCACUCGAUGACCACUGU	178.63	153.83	66.09	85.66
hsa-miR-598	MIMAT0003266	UACGUCAUCGUUGUCAUCGUCA	117.10	111.45	99.30	80.76
hsa-miR-599	MIMAT0003267	GUUGUGUCAGUUUAUCAAAC	112.18	124.25	89.86	88.60
hsa-miR-600	MIMAT0003268	ACUUACAGACAAGAGCCUUGCUC	134.72	149.58	103.97	120.53
hsa-miR-601	MIMAT0003269	UGGUCUAGGAUUGUUGGAGGAG	92.80	94.52	86.00	106.79
hsa-miR-602	MIMAT0003270	GACACGGGCGACAGCUGCGGCCC	120.85	119.45	134.62	145.56
hsa-miR-603	MIMAT0003271	CACACACUGCAAUUACUUUUGC	120.21	87.86	99.24	99.90
hsa-miR-604	MIMAT0003272	AGGCUGCGGAAUUCAGGAC	133.97	119.08	137.67	124.68
hsa-miR-605	MIMAT0003273	UAAAUCCCAUGGUGCCUUCUCCU	137.68	135.98	80.57	86.13
hsa-miR-606	MIMAT0003274	AAACUACUGAAAAUCAAAGAU	111.50	105.12	91.75	95.78
hsa-miR-607	MIMAT0003275	GUUCAAAUCCAGAUCUAUAAC	90.25	97.94	94.76	111.40
hsa-miR-608	MIMAT0003276	AGGGGUGGUGUUGGGACAGCUCCGU	41.01	42.25	125.30	122.49
hsa-miR-609	MIMAT0003277	AGGGUGUUUCUCUCAUCUCU	81.87	88.57	100.80	124.03
hsa-miR-610	MIMAT0003278	UGAGCUAAAUGUGUGCUGGGA	167.45	190.86	124.00	131.65
hsa-miR-611	MIMAT0003279	GCGAGGACCCCUCGGGGUCUGAC	116.54	120.62	115.02	95.75
hsa-miR-612	MIMAT0003280	GCUGGGCAGGGCUUCUGAGCUCCUU	70.35	70.08	88.83	94.42
hsa-miR-613	MIMAT0003281	AGGAAUGUUCCUUCUUUGCC	134.37	109.55	91.69	78.40
hsa-miR-614	MIMAT0003282	GAACGCCUGUUCUUGCCAGGUGG	89.95	112.65	119.47	113.59
hsa-miR-615-3p	MIMAT0003283	UCCGAGCCUGGGUCUCCCUCUU	54.98	47.66	115.99	129.44
hsa-miR-615-5p	MIMAT0004804	GGGGGUCCCCGGUGCUCGGAUC	120.93	121.01	99.79	99.48
hsa-miR-616-3p	MIMAT0004805	AGUCAUUGGAGGGUUUGAGCAG	132.32	115.43	123.90	115.96
hsa-miR-616-5p	MIMAT0003284	ACUCAAAACCCUUCAGUGACUU	118.77	112.40	117.82	94.15
hsa-miR-617	MIMAT0003286	AGACUUCCCAUUUGAAGGUGGC	45.95	78.08	83.47	80.71
hsa-miR-618	MIMAT0003287	AAACUCUACUUGUCCUUCUGAGU	97.03	108.61	85.83	105.64
hsa-miR-619	MIMAT0003288	GACCUGGACAUGUUUGUGCCCAGU	118.19	125.35	83.43	123.09
hsa-miR-620	MIMAT0003289	AUGGAGAUAGAUAUAGAAAU	121.70	89.85	96.16	92.90
hsa-miR-621	MIMAT0003290	GGCUAGCAACAGCGCUUACCU	79.47	112.01	113.45	127.01
hsa-miR-622	MIMAT0003291	ACAGUCUGCUGAGGUUGGAGC	94.45	90.18	90.50	101.59
hsa-miR-623	MIMAT0003292	AUCCCUUGCAGGGGCUGUUGGGU	73.99	108.45	101.60	81.24
hsa-miR-624-3p	MIMAT0004807	CACAAGGUAUUGGUAUUACCU	84.73	97.65	104.54	118.42
hsa-miR-624-5p	MIMAT0003293	UAGUACCAGUACCUUGUGUUCA	92.82	83.62	134.99	126.51
hsa-miR-625-3p	MIMAT0004808	GACUAUAGAACUUUCCCCCUCA	110.23	109.75	102.98	112.64
hsa-miR-625-5p	MIMAT0003294	AGGGGGAAAGUUCUAUAGUCC	113.73	87.27	90.08	77.57
hsa-miR-626	MIMAT0003295	AGCUGUCUGAAAAUGUCUU	87.14	87.23	95.28	90.54
hsa-miR-627	MIMAT0003296	GUGAGUCUCUAAGAAAAGAGGA	117.60	124.17	99.75	101.53
hsa-miR-628-3p	MIMAT0003297	UCUAGUAAGAGUGGCAGUCGA	94.97	91.77	100.55	96.31

hsa-miR-628-5p	MIMAT0004809	AUGCUGACAUAUUUACUAGAGG	36.17	37.29	87.81	76.46
hsa-miR-629-3p	MIMAT0003298	GUUCUCCCAACGUAAGCCCAGC	85.17	98.23	75.40	72.00
hsa-miR-629-5p	MIMAT0004810	UGGGUUUACGUUGGGAGAACU	69.99	70.60	87.15	89.40
hsa-miR-630	MIMAT0003299	AGUAUUCUGUACCAGGGAAGGU	168.45	149.05	107.61	75.35
hsa-miR-631	MIMAT0003300	AGACCUGGCCCAGACCUCAGC	113.81	123.69	87.93	94.95
hsa-miR-632	MIMAT0003302	GUGUCUGCUUCCUGUGGGA	107.81	106.51	76.14	107.27
hsa-miR-633	MIMAT0003303	CUAAUAGUAUCUACCACAAUAAA	86.25	85.91	92.53	82.26
hsa-miR-634	MIMAT0003304	AACCAGCACCCCAACUUUGGAC	40.19	67.38	149.97	150.99
hsa-miR-635	MIMAT0003305	ACUUGGGCACUGAAACAAUGUCC	77.40	91.11	83.02	94.48
hsa-miR-636	MIMAT0003306	UGUGCUUGCUCGUCCCGCCCGCA	123.78	106.25	103.68	92.53
hsa-miR-637	MIMAT0003307	ACUGGGGGCUUUCGGGCUCUGCGU	85.38	84.55	95.55	114.24
hsa-miR-638	MIMAT0003308	AGGGAUCGCGGGCGGGUGGCGGCCU	81.94	90.45	108.17	99.90
hsa-miR-639	MIMAT0003309	AUCGCUGCGGUUGCGAGCGCUGU	83.07	69.73	100.30	85.15
hsa-miR-640	MIMAT0003310	AUGAUCCAGGAACCUGCCUCU	91.75	77.92	98.29	80.02
hsa-miR-641	MIMAT0003311	AAAGACAUAGGAUAGAGUCACCUC	87.68	114.19	87.51	99.53
hsa-miR-642a-5p	MIMAT0003312	GUCCCUCUCCAAAUGUGUCUUG	101.22	92.81	121.46	126.07
hsa-miR-642b-3p	MIMAT0018444	AGACACAUUUGGAGAGGGACCC	69.80	85.48	93.46	86.51
hsa-miR-643	MIMAT0003313	ACUUGUAUGCUAGCUCAGGUAG	137.30	103.77	89.86	103.74
hsa-miR-644a	MIMAT0003314	AGUGUGGCUUUCUUAGAGC	63.07	61.89	79.84	77.96
hsa-miR-645	MIMAT0003315	UCUAGGCUGGUACUGCUGA	121.16	103.71	113.91	110.43
hsa-miR-646	MIMAT0003316	AAGCAGCUGCCUCUGAGGC	65.35	84.36	69.58	90.23
hsa-miR-647	MIMAT0003317	GUGGCUGCACUCACUUCCUUC	97.61	97.30	82.19	98.82
hsa-miR-648	MIMAT0003318	AAGUGUGCAGGGCACUGGU	94.90	112.36	105.58	116.13
hsa-miR-649	MIMAT0003319	AAACCUGUGUUGUUCAAGAGUC	71.95	116.54	85.70	107.17
hsa-miR-650	MIMAT0003320	AGGAGGCAGCGCUCUCAGGAC	94.28	81.19	119.84	123.73
hsa-miR-651	MIMAT0003321	UUUAGGAUAAGCUUGACUUUUG	72.23	77.48	85.09	81.03
hsa-miR-652-3p	MIMAT0003322	AAUGGCGCCACUAGGGUUGUG	73.10	105.15	117.80	112.21
hsa-miR-653	MIMAT0003328	GUGUUGAAACAAUCUCUACUG	35.41	45.32	84.29	95.20
hsa-miR-654-3p	MIMAT0004814	UAUGUCUGCUGACCAUCACCUU	165.29	171.83	87.06	82.51
hsa-miR-654-5p	MIMAT0003330	UGGUGGGCCGCAGAACAUGUGC	96.31	70.55	102.90	116.48
hsa-miR-655	MIMAT0003331	AUAAUACAUGGUUAACCUCUUU	37.88	35.70	76.69	62.99
hsa-miR-656	MIMAT0003332	AAUAUUAUACAGUCAACCUCU	109.51	101.46	88.58	81.86
hsa-miR-657	MIMAT0003335	GGCAGGUUCUCACCCUCUCUAGG	103.21	113.15	103.41	131.52
hsa-miR-658	MIMAT0003336	GGCGGAGGGAAGUAGGUCCGUUGGU	98.26	102.80	115.02	104.70
hsa-miR-659-3p	MIMAT0003337	CUUGGUUCAGGGAGGGUCCCCA	91.72	89.94	93.27	91.50
hsa-miR-660-5p	MIMAT0003338	UACCCAUUGCAUAUCGGAGUUG	99.82	110.59	88.72	109.69
hsa-miR-661	MIMAT0003324	UGCCUGGGUCUCUGGCCUGCGCGU	85.17	81.37	77.64	77.33
hsa-miR-662	MIMAT0003325	UCCCACGUUGUGGCCCAGCAG	142.02	148.84	104.51	120.61
hsa-miR-663a	MIMAT0003326	AGGCGGGGCGCCGCGGGACCGC	94.28	108.69	93.54	115.28
hsa-miR-663b	MIMAT0005867	GGUGGCCCGGCCGUGCCUGAGG	109.04	117.53	120.75	105.99
hsa-miR-664a-3p	MIMAT0005949	UAUUCAUUUAUCCCCAGCCUACA	126.57	128.25	137.13	116.48

hsa-miR-664a-5p	MIMAT0005948	ACUGGCUAGGGAAAAUGAUUGGAU	94.37	92.67	90.59	92.90
hsa-miR-665	MIMAT0004952	ACCAGGAGGCUGAGGCCCCU	78.74	78.19	97.22	108.24
hsa-miR-668	MIMAT0003881	UGUCACUCGGCUCGGCCCACUAC	165.38	150.52	102.71	103.56
hsa-miR-670	MIMAT0010357	GUCCCUGAGUGUAUGUGGUG	68.55	62.72	76.27	93.80
hsa-miR-671-3p	MIMAT0004819	UCCGGUUCUCAGGGCUCCACC	105.48	90.56	93.37	70.21
hsa-miR-671-5p	MIMAT0003880	AGGAAGCCCUGGAGGGGCUGGAG	109.95	112.56	103.46	84.10
hsa-miR-675-3p	MIMAT0006790	CUGUAUGCCCUCACCGCUCA	88.75	121.94	77.66	69.22
hsa-miR-675-5p	MIMAT0004284	UGGUGCGGAGAGGGCCCACAGUG	90.28	89.87	94.29	98.64
hsa-miR-676-3p	MIMAT0018204	CUGUCCUAAGGUUGUUGAGUU	101.25	92.18	118.13	121.46
hsa-miR-676-5p	MIMAT0018203	UCUUCAACCUCAGGACUUGCA	112.15	134.47	74.40	74.27
hsa-miR-708-3p	MIMAT0004927	CAACUAGACUGUGAGCUUCUAG	93.41	103.93	114.80	98.90
hsa-miR-708-5p	MIMAT0004926	AAGGAGCUUACAAUCUAGCUGGG	61.24	74.31	49.38	50.05
hsa-miR-711	MIMAT0012734	GGGACCCAGGGAGAGACGUAAG	83.84	97.62	122.59	105.74
hsa-miR-7-1-3p	MIMAT0004553	CAACAAAUCACAGUCUGCCAUA	94.57	117.88	99.91	111.71
hsa-miR-718	MIMAT0012735	CUUCCGCCCCGCCGGGCGUCG	63.19	74.97	86.20	102.11
hsa-miR-720	MIMAT0005954	UCUCGCUGGGGCCUCCA	141.27	151.30	92.18	113.06
hsa-miR-7-2-3p	MIMAT0004554	CAACAAAUCCCAGUCUACCUAA	54.23	63.78	86.13	58.65
hsa-miR-744-3p	MIMAT0004946	CUGUUGCCACUAACCUCAACCU	57.82	61.09	84.11	75.93
hsa-miR-744-5p	MIMAT0004945	UGCGGGGCUAGGGCUAACAGCA	107.81	122.87	105.94	90.05
hsa-miR-758-3p	MIMAT0003879	UUUGUGACCUGGUCCACUAACC	105.83	99.86	80.04	80.50
hsa-miR-759	MIMAT0010497	GCAGAGUGCAAACAAUUUUGAC	82.42	86.01	81.71	81.63
hsa-miR-7-5p	MIMAT0000252	UGGAAGACUAGUGAUUUUGUUGU	110.83	135.51	134.86	155.41
hsa-miR-760	MIMAT0004957	CGGCUCUGGGUCUGUGGGGA	93.26	88.51	94.77	90.46
hsa-miR-761	MIMAT0010364	GCAGCAGGGUGAAACUGACACA	94.41	114.64	106.16	95.78
hsa-miR-762	MIMAT0010313	GGGGCUGGGGCCGGGGCCGAGC	83.84	88.91	109.07	101.17
hsa-miR-764	MIMAT0010367	GCAGGUGCUCACUUGUCCUCCU	39.51	32.29	89.52	98.47
hsa-miR-765	MIMAT0003945	UGGAGGAGAAGGAAGGUGAUG	96.26	110.60	70.10	86.99
hsa-miR-766-3p	MIMAT0003888	ACUCCAGCCCCACAGCCUCAGC	80.97	81.03	57.97	61.65
hsa-miR-767-3p	MIMAT0003883	UCUGCUCAUACCCCAUGGUUUCU	101.25	103.97	97.73	107.04
hsa-miR-767-5p	MIMAT0003882	UGCACCAUGGUUGUCUGAGCAUG	68.52	44.52	101.30	94.95
hsa-miR-768-3p	MIMAT0003947	UCACAAUGCUGACACUCAAACUGCUGAC	71.40	69.62	106.92	122.69
hsa-miR-768-5p	MIMAT0003946	GUUGGAGGAUGAAAGUACGGAGUGAU	98.34	87.37	102.16	91.09
hsa-miR-769-3p	MIMAT0003887	CUGGGAUCUCCGGGGUCUUGGUU	105.62	91.73	97.34	95.00
hsa-miR-769-5p	MIMAT0003886	UGAGACCUCUGGGUUCUGAGCU	75.82	55.14	106.71	96.57
hsa-miR-770-5p	MIMAT0003948	UCCAGUACCACGUGUCAGGGCCA	87.59	97.61	99.33	100.81
hsa-miR-801	MIMAT0004209	GAUUGCUCUGCGUGCGGAAUCGAC	88.20	95.78	94.28	81.16
hsa-miR-802	MIMAT0004185	CAGUAACAAAGAUUCAUCCUUGU	94.61	76.71	89.70	90.71
hsa-miR-873-5p	MIMAT0004953	GCAGGAACUUGUGAGUCUCCU	80.07	95.50	70.08	81.67
hsa-miR-874	MIMAT0004911	CUGCCCUGGCCCGAGGGACCGA	137.68	91.52	79.84	102.48
hsa-miR-875-3p	MIMAT0004923	CCUGGAAACACUGAGGUUGUG	122.38	125.22	115.55	95.51
hsa-miR-875-5p	MIMAT0004922	UAUACCUCAGUUUUAUCAGGUG	37.73	43.79	101.95	83.14

hsa-miR-876-3p	MIMAT0004925	UGGUGGUUUACAAAGUAAUUCA	73.85	78.13	87.32	116.72
hsa-miR-876-5p	MIMAT0004924	UGGAUUUCUUUGUGAAUCACCA	97.12	113.45	60.51	65.37
hsa-miR-877-3p	MIMAT0004950	UCCUCUUCUCCCUCCUCCCAG	117.51	100.18	102.96	99.90
hsa-miR-877-5p	MIMAT0004949	GUAGAGGAGAUGGCGCAGGG	73.02	73.64	108.42	67.22
hsa-miR-885-3p	MIMAT0004948	AGGCAGCGGGGUGUAGUGGAUA	57.89	61.98	86.57	84.46
hsa-miR-885-5p	MIMAT0004947	UCCAUUACACUACCCUGCCUCU	106.79	86.32	89.21	109.80
hsa-miR-886-3p	MIMAT0004906	CGCGGGUGCUUACUGACCCUU	100.35	81.40	109.54	122.87
hsa-miR-887	MIMAT0004951	GUGAACGGGCGCCAUCCCGAGG	62.30	76.71	106.15	98.88
hsa-miR-888-3p	MIMAT0004917	GACUGACACCUCUUUGGGUGAA	108.17	115.69	92.25	78.37
hsa-miR-888-5p	MIMAT0004916	UACUCAAAAAGCUGUCAGUCA	102.82	112.93	92.87	101.02
hsa-miR-889	MIMAT0004921	UUAAUAUCGGACAACCAUUGU	72.63	67.97	93.21	87.02
hsa-miR-890	MIMAT0004912	UACUUGGAAAGGCAUCAGUUG	65.22	88.07	81.41	98.38
hsa-miR-891a	MIMAT0004902	UGCAACGAACCUGAGCCACUGA	92.67	101.13	89.47	94.62
hsa-miR-891b	MIMAT0004913	UGCAACUUACCUGAGUCAUUGA	50.33	53.01	62.65	55.22
hsa-miR-892a	MIMAT0004907	CACUGUGUCCUUUCUGCGUAG	104.75	95.86	82.90	82.43
hsa-miR-892b	MIMAT0004918	CACUGGCUCCUUUCUGGGUAGA	83.79	71.63	110.61	114.82
hsa-miR-920	MIMAT0004970	GGGGAGCUGUGGAAGCAGUA	93.07	123.77	88.24	77.31
hsa-miR-921	MIMAT0004971	CUAGUGAGGGACAGAACCAGGAUUC	91.53	101.11	101.03	115.22
hsa-miR-922	MIMAT0004972	GCAGCAGAGAAUAGGACUACGUC	93.52	97.60	129.61	105.75
hsa-miR-923	MIMAT0004973	GUCAGCGGAGGAAAAGAAACU	118.77	89.12	128.60	112.90
hsa-miR-924	MIMAT0004974	AGAGUCUUGUGAUGUCUUGC	93.20	96.02	92.84	106.62
hsa-miR-92a-1-5p	MIMAT0004507	AGGUUGGGAUCGGUUGCAAUGCU	108.45	114.19	97.01	109.34
hsa-miR-92a-2-5p	MIMAT0004508	GGGUGGGGAUUUGUUGCAUUAC	67.38	85.27	66.47	75.69
hsa-miR-92a-3p	MIMAT0000092	UAUUGCACUUGUCCCGGCCUGU	48.52	38.78	93.53	93.68
hsa-miR-92b-3p	MIMAT0003218	UAUUGCACUCGUCCCGGCCUCC	57.25	59.50	103.66	84.91
hsa-miR-92b-5p	MIMAT0004792	AGGGACGGGACGCGGUGCAGUG	111.46	105.36	99.51	116.88
hsa-miR-933	MIMAT0004976	UGUGCGCAGGGAGACCUCUCCC	65.24	77.59	101.95	123.13
hsa-miR-93-3p	MIMAT0004509	ACUGCUGAGCUAGCACUUCCCG	124.65	82.27	142.11	122.47
hsa-miR-934	MIMAT0004977	UGUCUACUACUGGAGACACUGG	132.53	142.14	108.65	109.76
hsa-miR-935	MIMAT0004978	CCAGUUACCGCUUCCGCUACCGC	187.10	169.72	144.41	140.35
hsa-miR-93-5p	MIMAT0000093	CAAAGUGCUGUUCGUGCAGGUAG	86.70	83.41	116.88	130.70
hsa-miR-936	MIMAT0004979	ACAGUAGAGGGAGGAAUCGCAG	116.68	118.78	103.33	111.71
hsa-miR-937-3p	MIMAT0004980	AUCCGCGCUCUGACUCUCUGCC	119.13	105.37	112.64	111.38
hsa-miR-938	MIMAT0004981	UGCCCUUAAAGGUGAACCCAGU	123.02	111.39	121.89	126.93
hsa-miR-939-5p	MIMAT0004982	UGGGGAGCUGAGGCUCUGGGGGUG	110.59	108.45	112.27	130.58
hsa-miR-9-3p	MIMAT0000442	AUAAAGCUAGAUAACCGAAAGU	107.51	105.23	100.13	105.42
hsa-miR-940	MIMAT0004983	AAGGCAGGGCCCCCGCUCCCC	77.40	57.21	96.75	119.53
hsa-miR-941	MIMAT0004984	CACCCGGCUGUGUGCACAUGUGC	136.56	146.05	119.31	108.48
hsa-miR-942	MIMAT0004985	UCUUCUCUGUUUUGGCCAUGUG	111.97	110.97	90.50	78.62
hsa-miR-943	MIMAT0004986	CUGACUGUUGCCGUCCUCCAG	78.15	118.04	92.26	112.17
hsa-miR-944	MIMAT0004987	AAAUUAUUGUACAUCGGAUGAG	128.29	122.80	110.05	94.11

hsa-miR-95	MIMAT0000094	UUCAACGGGUAUUUAUUGAGCA	136.14	127.26	109.80	86.46
hsa-miR-9-5p	MIMAT0000441	UCUUUGGUUAUCUAGCUGUAUGA	55.86	71.68	75.75	83.11
hsa-miR-96-3p	MIMAT0004510	AAUCAUGUGCAGUGCCAAUAUG	100.66	77.09	116.55	104.68
hsa-miR-96-5p	MIMAT0000095	UUUGGCACUAGCACAUUUUUGCU	114.35	112.02	120.88	109.32
hsa-miR-98-5p	MIMAT0000096	UGAGGUAGUAAGUUGUAUUGUU	22.99	38.54	68.25	57.76
hsa-miR-99a-3p	MIMAT0004511	CAAGCUCGCUUCUAUGGGUCUG	122.63	101.32	88.53	92.63
hsa-miR-99a-5p	MIMAT0000097	AACCCGUAGAUCCGAUCUUGUG	102.71	82.64	104.46	91.93
hsa-miR-99b-3p	MIMAT0004678	CAAGCUCGUGUCUGUGGGUCCG	140.73	160.55	117.33	98.37
hsa-miR-99b-5p	MIMAT0000689	CACCCGUAGAACCGACCUUGCG	116.91	134.23	93.36	101.82

Chapter 4

Discussion:

Overview of present thesis study

Noncoding RNA, the dark matter of the genome, comprise 90 % of the whole transcriptome (1). It is believed that the main function of non-coding RNA is to regulate the expression or activity of protein coding genes. MicroRNA is a class of small non-coding RNA with the size of 21-23 nt. The seed sequence of a miR is commonly considered as a probe interacting with the 3′-UTR of target genes to enhance the posttranscriptional degradation of mRNA. Different from other small RNA interference, the short 6-8nt targeting sequence makes it possible for one miR to have multiple, but specific, target genes and biological processes, which could achieve more beneficial phenotypes and avoid side effects. The long term aim for this study is to find out miRs that could modulate hepatic lipoprotein secretion and potentially be used to treat dyslipidemia and atherosclerosis.

In the present thesis, I identified two novel miRs, 548p and 1200, for their roles in regulating apolipoprotein secretion and lipid synthesis reactions. Liver is one of the major organs to produce lipoproteins in the circulation. Aiming to find out miRs that could potentially regulate apoB to modulate apoB-containing lipoprotein secretion, I hypothesized that miR-548p could reduce apoB expression by interacting with the predicted targeting sites in the 3′-UTR of apoB. Indeed, miR-548p significantly decreases apoB secretion from human hepatoma Huh-7 cells. Among the two predicted interaction sites, only seed and supplementary sequences in Site I are required for miR-548p to directly target apoB 3′-UTR. In addition, the expression levels of genes encoding for key enzymes, Hmgcr and ACSL4, in the cholesterol and fatty acid synthesis reactions are significantly reduced by miR-548p. Luciferase reporter plasmids harboring the 3′-UTR of ACSL4 or HMGCR were used to confirm the direct miR-mRNA interaction. MiR-548p may interact with 3′-UTR of apoB mRNA to reduce secretion of apoB-containing lipoproteins. Future studies may be planned to determine if miR-548p might be a better agent than mipomerson (ASO apoB) in treating Familial Hyperlipidemia and overcome the fatty liver side effect.

To test the hypothesis that miRs exist in nature to simultaneously regulate apoB and apoAI secretion from the liver, I performed a functional screening using a human miR mimic library in Huh-7. Through first and second screening, miR-1200 was discovered as a novel miR that has a capacity to differentially modulate apoB and apoAI secretion. Mechanistic studies revealed that, 1) miR-1200 reduced apoB secretion by directly targeting apoB 3′-UTR and promoting its posttranscriptional degradation; 2) MiR-1200 increased apoAI secretion by elevating apoAI promoter activity. I further identified a novel transcriptional repressor of apoAI, BCL11B, which was suppressed by miR-1200 to promote apoAI *de novo* transcription; 3) MiR-1200 overexpression also significantly decreased lipid synthesis, which may help avoid hepatosteatosis induced by the blockage of apoB-containing lipoprotein. By acting through these three different pathways miR-1200 may be a promising biologic for the treatment of dyslipidemia, atherosclerosis, or fatty liver.

Previous findings and the Contribution of the present thesis

Numerous miRs have been well studied for their regulatory functions on plasma lipid metabolism and tissue lipid homeostasis. MiR-122, the most abundant endogenously expressed miR in the liver, has been shown to affect hepatic fatty acid and cholesterol synthesis, as well as plasma cholesterol levels (2, 3). The expression levels of several genes involved in lipid synthesis are regulated by miR-122 inhibition, such as Hmgcr, SLC7A1, etc. Inhibition of endogenous miR-122 resulted in the reduction of cholesterol levels in both LDL and HDL. In other words, anti-miR-122 administration decreases both atherogenic bad cholesterol and vascular-protective good cholesterol. MiR-30c and miR-33 target the biosynthesis of LDL and HDL, respectively. MiR-30c targets MTP to inhibit the production of VLDL and decrease plasma LDL cholesterol levels without affecting HDL. In addition, miR-30c also inhibits triglyceride synthesis by lowering the expression levels of LPGAT1, which overcomes the induction of hepatosteatosis, the common adverse effect of apoB-containing lipoprotein production inhibition. Inhibition of miR-33 elevates plasma HDL cholesterol levels by up-regulating ABCA1, an important transporter in the process of cholesterol efflux from macrophages to lipid-poor HDL particles. Besides targeting the direct proterin players in the biosynthesis and assembly of lipoproteins, miR-34 has been shown to reduce plasma cholesterol levels by targeting a transcription activator of both apoB and MTP, HNF4a. However, the downregulation of VLDL production leads to lipid accumulation in the liver. Recently, miR-148a is identified as a dual regulator of LDL and HDL from a LDLR activity functional screening. MiR-148a targets LDLR and ABCA1 to decrease LDL clearance from the circulation and HDL biosynthesis. Therefore, the inhibition of miR-148a achieves the differential modulation of plasma LDL and HDL levels.

It should be pointed out that all the miRs discussed above are well-conserved in different organisms through evolution. Therefore, mouse models were used extensively to investigate the physiological subsequences of overexpression or inhibition of these miRs. The conservation of miRs in different organisms through evolution is often interpreted as a highlight of the importance of these miRs in specific biological pathways. However, from the genome-wide screening in the present study, many primate specific miRs have been shown to have beneficial/valuable effects, such as simultaneously differentially regulating apoB and apoAI secretion, and potently modulating apoB or apoAI secretion, which might result in identification of potential therapeutic drugs for the treatment of CVD. Therefore, it is reasonable to speculate that humans have developed and selected some miRs through evolution to precisely modulate plasma lipid distribution and levels. In the future, the physiological effect of miR-1200 and 548p can be evaluated in non-human primate animal.

MiR-548p is the first miR identified to directly target apoB mRNA to reduce apoB-containing lipoprotein secretion. To our knowledge, the screening in this study is the first large-scale functional miR screening for studying apolipoprotein secretion. To get a genome-wide view of miRs that could regulate apoB or apoAI secretion from the liver, I performed a large-scale screening of a human miR library in Huh-7 cell line. Instead of looking for a miR, which could potentially regulate a gene of interest, this unbiased screening will provide us the information to compare the potency of different miRs. In addition, this can also give us the opportunity to explore unknown function of genes. From the first and second screening, 75 novel miRs are discovered as potent regulators of apolipoprotein secretion, which provides huge amount of information for further investigation and development of these miRs as the therapeutic biologics.

Both miR-548p and miR-1200 have the potential to be developed as new drugs in treating hyperlipidemia and atherosclerosis while avoiding adverse effects. These studies may result in the identification of new pathways that regulate plasma and tissue lipid homeostasis. Overall, these studies provide strong evidence for the evaluation of miR-548p and miR-1200 in translational and preclinical research for future drug development.

In vivo miR delivery system

To translate the basic science discoveries on miR to therapeutics, the *in vivo* delivery methods of miRNA and anti-miRNA are being rapidly developed. Viral vector is a traditional but limited clinical delivery system, due to its toxicity and immunogenicity (4). The non-viral delivery systems include lipid-based (liposome) delivery system, polymer-based polyethylenimine (PEI)-based delivery system, poly (amidoamine)(PAMAMs)dendrimers, and poly(lactide-co-glycolide) (PLGA) Particles, silica/gold-based nanoparticles, etc (5-8). On the other hand, to stabilize anti-miRs, the oligonucleotides can be chemically modified, such as locked nucleic acid (LNA) oligonucleotides, 2'-O-methoxyethyl-oligonucleotides (2'-O-MOE) (6, 9). Therefore the delivery of anti-miR can be achieved by subcutaneous or intraperitoneal injection of saline anti-miR solution (10, 11). The *in vivo* delivery of miR for overexpression is more difficult. Because of the abundant nucleases in the circulation, miRNA is very easy to be degraded. In nature, mature microRNA bound to RISC complex is in the form of single-strand. The most common form of synthetic miR is double-stranded miR mimic harboring chemical modification, such as cholesterol, to enhance the cellular uptake. As RISC protein complex need to recognize the miR targeting strand, then the modification on the targeting strand to increase its stability is limited. The modification of 2'-fluoro (2'-F) can effectively enhance miR stability while keeping the capacity of RISC loading (12). MiR-34 and miR-122 are the two miR-based therapeutic agents on the market so far. MiR-34a is delivered as liposome-encapsulated mimic through intravenous infusion to target oncogene expression (13-15). The inhibitor of miR-122, RG-101 is used to suppress hepatitis C virus (HCV) infection by subcutaneous dosing (16, 17). Liver is one of the major target organs to control hyperlipidemia, atherosclerosis, and metabolic diseases. Due to the filtering function of liver in the circulation, modulating gene expression in the liver is highly feasible by using either miR mimic for replacement or anti-miR inhibition therapy. The present thesis study provides numerous candidate miRs to be further studied in the future for modulating the plasma cholesterol levels in different lipoprotein fractions in human.

References

1. Ling H, Girnita L, Buda O, and Calin GA. Non-coding RNAs: the cancer genome dark matter that matters! *Clin Chem Lab Med.* 2016.
2. Wen J, and Friedman JR. miR-122 regulates hepatic lipid metabolism and tumor suppression. *J Clin Invest.* 2012;122(8):2773-6.
3. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, Watts L, Booten SL, Graham M, McKay R, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 2006;3(2):87-98.
4. Glover DJ, Lipps HJ, and Jans DA. Towards safe, non-viral therapeutic gene expression in humans. *Nat Rev Genet.* 2005;6(4):299-310.
5. Zhang Y, Wang Z, and Gemeinhart RA. Progress in microRNA delivery. *J Control Release.* 2013;172(3):962-74.
6. Li Z, and Rana TM. Therapeutic targeting of microRNAs: current status and future challenges. *Nat Rev Drug Discov.* 2014;13(8):622-38.
7. Babar IA, Cheng CJ, Booth CJ, Liang X, Weidhaas JB, Saltzman WM, and Slack FJ. Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. *Proc Natl Acad Sci U S A.* 2012;109(26):E1695-704.
8. Ben-Shushan D, Markovsky E, Gibori H, Tiram G, Scomparin A, and Satchi-Fainaro R. Overcoming obstacles in microRNA delivery towards improved cancer therapy. *Drug Deliv Transl Res.* 2014;4(1):38-49.
9. van Rooij E, and Kauppinen S. Development of microRNA therapeutics is coming of age. *EMBO Mol Med.* 2014;6(7):851-64.
10. Stenvang J, Petri A, Lindow M, Obad S, and Kauppinen S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence.* 2012;3(1):1.
11. Cheng CJ, Bahal R, Babar IA, Pincus Z, Barrera F, Liu C, Svoronos A, Braddock DT, Glazer PM, Engelman DM, et al. MicroRNA silencing for cancer therapy targeted to the tumour microenvironment. *Nature.* 2015;518(7537):107-10.
12. Grunweller A, and Hartmann RK. Chemical modification of nucleic acids as a key technology for the development of RNA-based therapeutics. *Pharmazie.* 2016;71(1):8-16.
13. Daige CL, Wiggins JF, Priddy L, Nelligan-Davis T, Zhao J, and Brown D. Systemic delivery of a miR34a mimic as a potential therapeutic for liver cancer. *Mol Cancer Ther.* 2014;13(10):2352-60.

14. Agostini M, and Knight RA. miR-34: from bench to bedside. *Oncotarget*. 2014;5(4):872-81.
15. Bader AG, Brown D, and Winkler M. The promise of microRNA replacement therapy. *Cancer Res.* 2010;70(18):7027-30.
16. Jopling CL. Regulation of hepatitis C virus by microRNA-122. *Biochem Soc Trans.* 2008;36(Pt 6):1220-3.
17. Garcia-Sastre A, and Evans MJ. miR-122 is more than a shield for the hepatitis C virus genome. *Proc Natl Acad Sci U S A.* 2013;110(5):1571-2.

Appendices

MicroRNAs regulating apolipoprotein B-containing lipoprotein production

Liye Zhou, Sara Irani, Alaa Sirwi, M. Mahmood Hussain

Contribution: As the first author, I wrote **the Introduction**, **Regulation of apoB –containing lipoprotein secretion**, **Inhibition of miR-122 reduces plasma lipids**, **MiR-34a inhibits VLDL production by targeting HNF4α**, and **Perspectives** sections.

Nitrated apolipoprotein AI/apolipoprotein AI ratio is increased in diabetic patients with coronary artery disease.

Chen X, Bakillah A, **Zhou L**, Pan X, Hoepfner F, Jacob M, Jiang XC, Lazar J, Schlitt A, Hussain MM

Contribution: As the third author, I performed the cholesterol efflux assay from macrophages using plasma samples from 67 subjects and generated Fig 3.

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MicroRNAs regulating apolipoprotein B-containing lipoprotein production☆

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ABSTRACT

MicroRNAs (miRs) are small, non-coding RNAs that regulate gene expression and have been implicated in many pathological conditions. Significant progress has been made to unveil their role in lipid metabolism. This review aims at summarizing the role of different miRs that regulate hepatic assembly and secretion of apolipoprotein B (apoB)-containing lipoproteins. Overproduction and/or impaired clearance of these lipoproteins from circulation increase plasma concentrations of lipids enhancing risk for cardiovascular disease. So far, three miRs, miR-122, miR-34a, and miR-30c have been shown to modulate hepatic production of apoB-containing low density lipoproteins. In this review, we will first provide a brief overview of lipid metabolism and apoB-containing lipoprotein assembly to orient readers to different steps that have been shown to be regulated by miRs. Then, we will discuss the role of each miR on plasma lipids and atherosclerotic burden. Furthermore, we will summarize mechanistic studies explaining how these miRs regulate hepatic lipid synthesis, fatty acid oxidation, and lipoprotein secretion. Finally, we will briefly highlight the potential use of each miR as a therapeutic drug for treating cardiovascular diseases. 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.

1. Introduction

Lipids are hydrophobic molecules that are transported in the blood stream as lipid-protein micelles called lipoproteins. Lipoproteins can be classified as apoB-containing lipoproteins such as chylomicrons, very low density lipoprotein (VLDL), and low density lipoprotein (LDL) and non-apoB-containing high density lipoprotein (HDL). ApoB-containing lipoproteins are synthesized and secreted from the liver and small intestine. Microsomal triglyceride transfer protein (MTP) is a critical chaperone that assists in their assembly (Fig. 1). It transfers neutral lipids to newly translated apoB peptides in the endoplasmic reticulum (ER) and physically interacts with apoB [1,2]. In the absence of MTP, nascent apoB peptides are directed to proteasomal degradation [2–5]. In this review, we will concentrate on the regulation of apoB-containing lipoproteins production; a process highly regulated at different levels, including transcriptional, posttranscriptional, and post-translational [6–9].

ApoB-containing lipoproteins deliver lipids from the liver and small intestine to other peripheral tissues. Excess accumulation of these lipoproteins in the plasma contributes to atherosclerosis as these lipoproteins are modified and taken up by macrophages [10–12]. Atherosclerosis, the thickening of arterial wall caused by the accumulation of lipids, is the main contributor of cardiovascular disease. High plasma cholesterol, hypercholesterolemia, is one of the well-known risk factors of cardiovascular diseases.

According to the Center for Disease Control and Prevention, 71 million adults over 20 years old in the US have high LDL-cholesterol levels ("bad cholesterol") [13]. Significant progress has been made by researchers and pharmaceutical companies to lower plasma lipids using two different approaches; enhancing clearance of plasma lipoproteins and reducing lipoprotein production. Statins are the most popular therapeutic drugs to reduce plasma LDL cholesterol. They inhibit cholesterol synthesis, upregulate LDL receptors, and increase clearance of apoB-containing lipoproteins. These drugs decrease the disease by 30–40% [14]. However, approximately 20% of the population is intolerant to statins [15–20]. PCSK9 (proprotein convertase subtilisin/kexin type 9) antibodies have also emerged as a potential drug to lower plasma cholesterol levels [21]. PCSK9 binds to LDL receptor and enhances intracellular degradation in lysosomes. Therefore, inhibition of PCSK9 leads

Abbreviations: ApoB, apolipoprotein B; ALT, alanine aminotransferase; HDL, high density lipoproteins; HMGCR, HMG-CoA reductase; HNF4, hepatocyte nuclear factor 4; LDL, low density lipoproteins; LNA, locked nucleic acids; miR, microRNA; MTP, microsomal triglyceride transfer protein; PCSK9, proprotein convertase subtilisin/kexin type 9; VLDL, very low density lipoproteins.

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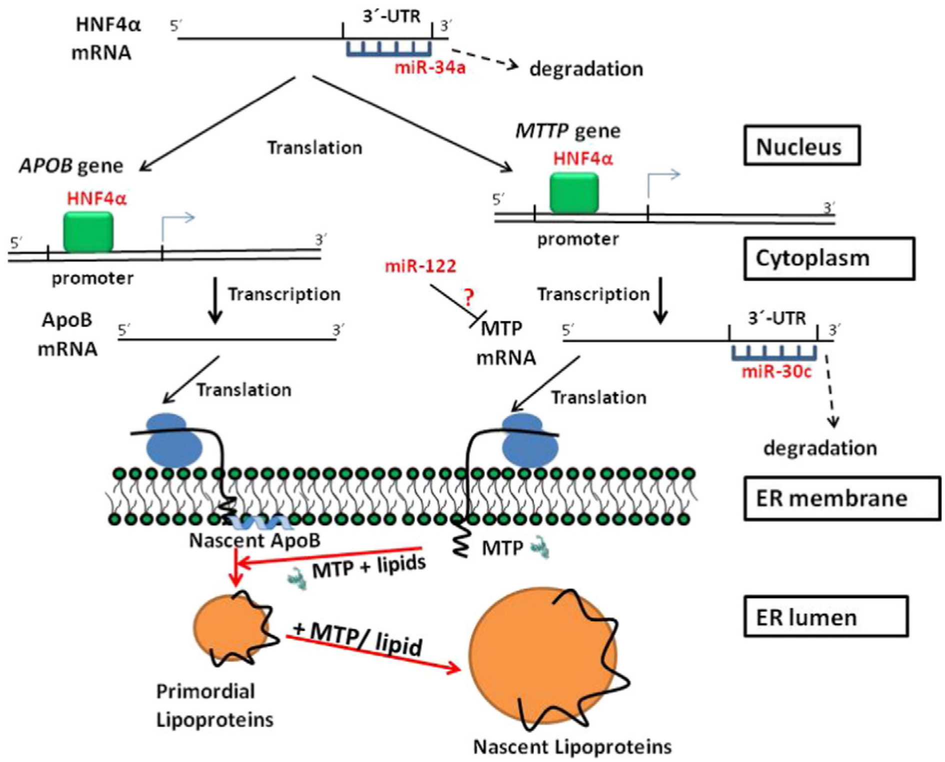

Fig. 1. Role of HNF4α, apoB and MTP in assembly of apoB-containing lipoproteins and their regulation by different microRNAs: HNF4α is a transcription factor. In the nucleus, HNF4α binds to the promoter regions of *APOB* and *MTTP* genes to increase their transcription. MiR-34a decreases HNF4α levels leading to reduced expression of apoB and MTP. ApoB and MTP mRNA are translated by ribosomes on the rough ER membrane. Newly synthesized apoB is translocated across the ER membrane and interacts with the inner leaflet of the ER membrane. Newly synthesized MTP interacts with protein disulfide isomerase to form a functionally active MTP heterodimer. In the ER lumen, MTP transfers lipids to nascent apoB peptide to form primordial lipoproteins which are converted to nascent lipoproteins via a second step of core expansion. In the cytoplasm, miR-30c binds to 3′-UTR of MTP mRNA and induces post-transcriptional degradation resulting in decreased assembly of apoB-containing lipoproteins. MiR-122 reduces the expression of MTP by unknown mechanism.

to reduced receptor degradation, increased LDL receptor expression levels, increased clearance of apoB-containing lipoproteins, and decreased LDL concentrations in plasma [22–24]. Since both statins and PCSK9 antibodies reduce plasma cholesterol by modulating LDL receptor expression, they are ineffective in homozygous familial hypercholesterolemia subjects with <2% of the LDL receptor activity [17,19,25,26]. In addition, many patients experience unmanageable side effects with statins [15] and PCSK9 inhibitor therapy [27].

Second, plasma cholesterol levels can be lowered by reducing production of apoB-containing lipoproteins. This has been achieved by reducing the expression of apoB using antisense oligonucleotide (ASO), mipomersen, and by the use of small molecules that inhibit MTP activity, lomitapide [28–30] in familial hypercholesterolemia subjects. However, both lomitapide and mipomersen cause adverse side effects such as increases in plasma transaminases and hepatic steatosis [31]. Thus, there is a need to find novel therapeutic agents that are well tolerated and beneficially modulate plasma lipids without causing adverse events.

MicroRNAs (miRs) are a class of small non-coding RNAs (~22 nt) that modestly regulate gene expression. Usually miRs regulate gene expression by binding to 3′-untranslated region (3′-UTR) of a target mRNA via seed, and in some cases additionally with supplementary sequences, to destabilize the mRNA or block its translation [32]. In most of the cases (higher than 70%), miR mediated regulation is via mRNA degradation [33]. miRs regulate multiple genes in diverse biological pathways. This makes it possible to use miRs or their inhibitors, anti-miRs, as therapeutic agents to reduce hyperlipidemia and atherosclerosis without causing steatosis or other adverse effects. Currently, miR-34 and miR-122 based drugs are in phase 2 clinical trials [34–36], illustrating the feasibility of miR-based therapy. MiR-30c and miR-33 have been shown to decrease LDL and HDL, respectively [37,38]. Different groups have reported different effects of miR-33 and its inhibition on plasma triglyceride and on the production of apoB-containing lipoproteins (Table 1). The reasons for discrepancies in the literature have been adequately discussed [46]. These might be related to different diets, concentrations of miRs, and models used in different studies by various groups. Due to lack of consensus about the effect of miR-33 on plasma triglyceride and VLDL production, we have not discussed this in detail. In this review, we will summarize the latest research regarding miRs, miR-34a, miR-122 and miR-30c, which have been identified to modulate assembly and secretion of apoB-containing lipoproteins.

2. Regulation of apoB-containing lipoprotein secretion

2.1. Transcriptional regulation: transcription factor

A major transcription factor that regulates both apoB and MTP expressions is the hepatocytes nuclear factor 4α (HNF4α, NR2A1). It is involved in lipoprotein secretion, insulin production, hepatocyte proliferation, differentiation and inflammation [47–49]. Single-nucleotide polymorphisms in HNF4α have been associated with high serum cholesterol and triglyceride levels, as well as diabetic phenotypes [50]. HNF4α forms a homodimer and interacts with direct repeat element 1 separated by one or two nucleotides present in the promoters of several target genes to enhance their transcription [48,51]. It is a member of a large family of nuclear receptors that interact with different ligands and modulate gene expression. HNF4α binds to several different fatty acids; however, fatty acids do not appear to modulate its function. Instead, HNF4α interacts with several co-activators and repressors and these interactions alter its activity [51,52].

Ablation of HNF4α gene is embryonic lethal underscoring its importance in embryogenesis. Major phenotypic effects of liver specific HNF4α gene ablation are significant reductions in serum lipids, increases in serum bile acids, and accretions of lipids in the liver [53].

Table 1
Effect of miR-33 inhibition on plasma cholesterol and triglyceride in mice and monkeys. Except for one study, miR-33 inhibition consistently increased plasma HDL-cholesterol. In all studies, there were no significant changes in LDL-cholesterol. Thus, miR-33 mainly increases plasma HDL cholesterol without affecting LDL cholesterol levels. In contrast, reported changes in total plasma cholesterol and triglyceride levels in these studies are variable. Four studies reported increases in total cholesterol and the other four studies reported no change. In five studies, there were no significant changes in total plasma triglyceride. Two studies each reported increases and decreases in total plasma triglyceride. One study reported increased hepatic VLDL production [39].

Mice	Diet	HDL-cholesterol	LDL/VLDL-cholesterol	Total cholesterol	Total triglyceride	Ref
Wild type C57BL/6	Chow diet	↑				[37]
Wild type C57BL/6	Chow diet	↑	↔	↑	↔	[40]
Wild type C57BL/6	Chow diet			↑	↑	[39]
Wild type C57BL/6	High fat diet	↑		↑	↑	[40]
Wild type C57BL/6	High fat diet			↑	↓	[41]
LDLr$^{-/-}$	Chow diet	↑			↔	[42]
LDLr$^{-/-}$	Western diet switched to chow diet	↑		↔	↔	[43]
LDLr$^{-/-}$	Western diet	↔	↔	↔	↔	[42]
miR-33$^{-/-}$*Apoe*$^{-/-}$ vs *Apoe*$^{-/-}$	Chow diet	↑	↔	↔	↔	[44]
Non-human primates	Chow switched to a high carbohydrate, moderate cholesterol diet	↑	LDL-cholesterol↔ VLDL-cholesterol ↓	↔	↓	[45]

↑, increase; ↓, decrease; ↔, no change.

Significant lower plasma lipids are due to less VLDL production secondary to reduce expression of both *Apob* and *Mttp* genes. The binding site of HNF4α in the *APOB* promoter has been identified [54]. Different groups have reported that HNF4α binds to the *MTTP* promoter and enhances transcription [55–57]. Hepatic lipid accretions are also presumably secondary to decreased VLDL production as acute shRNA mediated knockdown of HNF4α significantly reduces plasma lipids and increases hepatic lipids [58]. In contrast, overexpression of HNF4α lowered plasma cholesterol and hepatic triglyceride levels. Thus, these genetic manipulation studies have established that HNF4α regulates genes involved in lipoprotein assembly and secretion, and is critical for the maintenance of plasma and hepatic lipid homeostasis.

2.2. Posttranscriptional regulation: microRNAs

miR assisted mRNA degradation has emerged as a novel mode of post-transcriptional regulation of gene expression. miRs associate with Argonaute proteins to form miR-induced silencing complexes (miRISCs) [33]. These complexes interact with target mRNA to inhibit their translation or induce degradation. The final outcome of both these scenarios is reduced protein synthesis. miRs degrade mRNA by 5′-to-3′ mRNA decay pathway. In this pathway, mRNA degradation is initiated by the removal of polyadenylate tail at the 3′-end of the mRNA. Following deadenylation, the 5′ cap is removed. Decapping exposes 5′-end of mRNA to degradation by exonucleases. Recently, miRs have been identified that regulate plasma apoB-containing lipoproteins by modulating HNF4α and MTP mRNA levels. These are discussed in detail.

2.3. Posttranslational regulation: MTP and lipid availability

A major mechanism of regulation of apoB-containing lipoproteins secretion is co- and post-translational degradation of apoB [5,59]. Newly translated apoB is translocated across the ER membrane via Sec61 containing translocon and interacts with the inner leaflet of the ER membrane (Fig. 1). MTP is critical for the assembly of apoB containing lipoproteins. In the absence of MTP, apoB is retro-translocated and degraded by proteasomes in the cytosol via ER associated degradation pathway. During sufficient availability of MTP, nascent apoB is lipidated resulting in the formation of a primordial particle. These particles receive more lipids and are expanded into larger nascent lipoproteins that are secreted by hepatocytes and enterocytes. Secretion of these particles is further subjected to quality control. Particles with abnormal lipid composition are degraded by autophagy in a process referred to as post ER presecretory proteolysis. This process may also be regulated by hormones such as insulin [60].

Secretion of apoB-containing lipoproteins can also be modulated by lipid availability [5,61–64]. Lipid availability regulates the intracellular degradation of apoB. The rate of apoB secretion from human hepatoma cells can be stimulated by incubating cells with oleic acid and inhibiting the proteasomal degradation of apoB [61–65]. Hamsters fed a diet containing the inhibitor of fatty acid synthesis produce less VLDL [66]. These studies suggest that triglyceride and fatty acid syntheses in hepatocytes augment secretion of apoB-containing lipoproteins. Besides triglyceride and fatty acids, whether the biosynthesis rate of cholesterol has an effect on secretion of apoB-containing lipoprotein is still controversial. The effects of dietary cholesterol on VLDL production have been investigated in rats [7]. Cholesterol feeding increased VLDL and LDL lipids in plasma compared to control rats on cholesterol-free diet. HMG-CoA reductase (HMGCR) is a key rate-limiting enzyme in cholesterol biosynthesis. Statins, which were identified as inhibitors of HMGCR, reduce hepatic cholesterol synthesis and lower plasma cholesterol levels. Atorvastatin which inhibits HMGCR reduces apoB100 secretion in human HepG2 hepatoma cells [67]. In contrast, the inhibition of HMGCR activity in human intestinal cells and hamster hepatocytes had no effect on the secretion of apoB and apoAI [68,69]. In summary, lipids regulate assembly and secretion of apoB-containing lipoproteins via inhibiting intracellular degradation of nascent apoB.

3. Inhibition of miR-122 reduces plasma lipids

MiR-122 is the most abundant liver-specific miR, which accounts for more than 70% of miRs in the liver [70]. To elucidate the physiological function of miR-122, chemically modified antisense oligonucleotides (antagomirs) complementary to miR-122 (anti-miR-122) were developed to inhibit endogenous miR-122 [71,72]. Intravenous injection of anti-miR-122 in wildtype C57BL/6J adult mice reduced endogenous hepatic miR-122 levels most likely by inducing miR degradation compared with mismatch control group [72]. Gene expression analysis revealed that anti-miR-122 reduced the expression of more than ten genes involved in cholesterol synthesis, particularly HMGCR. Further, HMGCR enzyme activity was significantly reduced in the livers of mice injected with anti-miR-122. Contrarily, overexpression of miR-122 using adenoviruses increased the expression of some of these genes. Analysis of plasma lipids showed significant reductions in plasma cholesterol in anti-miR-122 injected mice with no effect on plasma triglyceride, bile acids, glucose and alanine aminotransferase (ALT). Therefore anti-miR-122 suppressed plasma cholesterol by down-regulating cholesterol synthesis.

In a separate study, inhibition of miR-122 for 4 weeks in wild type chow fed mice resulted in significant reductions in plasma cholesterol, apoB and triglyceride levels with no change in glucose levels [71]. Microarray analysis revealed significant reductions in the expression of genes involved in cholesterol and fatty acid synthesis. In accordance with the changes in these mRNA levels, fatty acid and sterol syntheses were significantly reduced in hepatocytes isolated from mice injected with anti-miR-122. Additionally, increased fatty acid oxidation was noted in these hepatocytes. Inhibition of miR-122 in diet-induced wild type obese mice resulted in reduced plasma cholesterol. Lipoprotein analysis revealed reductions in both LDL and HDL cholesterols. Plasma apoB100, but not apoB48, levels were reduced. In a different series of studies, hepatic triglyceride levels in anti-miR-122 injected *ob/ob* mice were significantly low and these changes were accompanied by reduced mRNA levels of key lipogenic enzymes. Reduction in hepatosteatosis was further substantiated by histological analysis and associated trend towards reductions in plasma transaminases. These studies concluded that miR-122 is a key regulator of cholesterol and triglyceride metabolism and suggested that anti-miR-122 reduces plasma cholesterol and triglyceride by lowering hepatic cholesterol and triglyceride synthesis as well as increasing fatty acid oxidation.

Effect of miR-122 inhibition has also been studied in non-human primates. Injection of locked-nucleic-acid-modified (LNA) anti-miR-122 reduced hepatic miR-122 levels in the livers of female African green monkeys in dose-dependent manner [73]. Three daily injections of the LNA-anti-miR-122 caused sustained reductions in plasma cholesterol and these levels returned to normal levels after 3 months. Plasma cholesterol levels were lower due to reductions in both LDL and HDL. No significant changes in ALT, aspartate aminotransferase, bilirubin, creatine phosphokinase or creatinine were observed indicating no hepatic and renal toxicity. In a different study, LNA-anti-miR-122 was weekly injected for 12 weeks in chimpanzees chronically infected with Hepatitis C Virus and these animals were followed for additional 17 weeks [74]. Antagonism of miR-122 in chimpanzees markedly reduced plasma cholesterol (44% at week 14) due to significant reductions in both LDL and HDL. No adverse effects were reported. Thus, inhibition of miR-122 reduces plasma cholesterol in rodents and non-human primates by reducing both LDL and HDL.

Subsequently, two independent groups have reported the phenotype of global and liver specific miR-122 knockout mice providing critical information about probable long-term outcomes of chronic anti-miR-122 therapy [75,76]. In both studies, miR-122 deficiency was associated with significant reductions in plasma cholesterol and triglyceride due to reductions in both LDL and HDL. In contrast to acute anti-miR-122 injection studies, miR-122 gene ablation studies showed significant increases in hepatic lipids and plasma ALT and alkaline phosphatase. Thus, chronic deficiency of miR-122 is associated with hepatosteatosis. Moreover, these mice developed hepatocellular carcinoma with age. The effects of miR-122 deficiency were mainly due to its effects on hepatic lipid metabolism as liver-specific ablation of miR-122 recapitulated phenotype seen in global miR-122 knockout mice. Mechanistic studies identified two independent mechanisms by which miR-122 controls plasma and liver lipids. First, there were significant increases in the expression of hepatic genes involved in triglyceride synthesis and lipid storage, such as Agpat1, Agpat3, Cidec, and this could contribute to hepatosteatosis [76]. These genes were shown to be direct targets of miR-122 (Table 2) using luciferase reporter assays and by site-directed mutagenesis of 3′-UTR of Agpat1 and Cidec mRNAs [76]. Second, there was a significant reduction in hepatic *Mttp* gene expression in miR-122 knockout mice explaining reduced production of VLDL and plasma lipids [75]. The proximal role of MTP in the deregulation of lipid metabolism in miR-122 knockout mice was validated by over expressing MTP in the livers of these mice. Ectopic expression of MTP in miR-122 knockout mice ameliorated hepatosteatosis and restored plasma lipid levels [75]. It is unknown how miR-122 regulates MTP expression as it is not a predicted target. Thus, miR-122 might regulate hepatic and plasma lipids by regulating hepatic triglyceride synthesis and lipoprotein production. However, long-term inhibition of miR-122 may lead to hepatosteatosis due to higher hepatic triglyceride synthesis and reduced VLDL production. These studies call for caution in the long-term use of anti-miR-122 oligonucleotides as this might lead to reductions in both LDL and HDL cholesterol and cause hepatosteatosis that could progress to hepatocellular carcinoma.

4. MiR-34a inhibits VLDL production by targeting HNF4α

HNF4α is a transcription factor that is expressed in the liver, kidney, intestine and pancreatic β cells [77–79]. It controls the expression of many genes involved in cell proliferation, bile acid, lipid, glucose, and drug metabolism [80–82]. Loss-of-function mutations in human HNF4α cause early onset of diabetes in young adults, and decrease plasma triglyceride and cholesterol levels [58,83–85]. High plasma glucose levels are secondary to reduced insulin secretion from β-cells. Reduced plasma triglyceride and cholesterol are secondary to reduced hepatic

Table 2
Genes targeted by miR-30c, miR-122 and miR-34a and their functions.

microRNA	Target	Function	Ref
miR-30c overexpression	MTTP[a] LPGAT1[b]	↓ VLDL secretion ↓ Atherosclerosis ↓ Lipid synthesis ↓ Hepatic steatosis	[38,88]
miR-122 inhibition	GYS1[a] SLC7A1[a] ALDOA[a] CCNG1[a] P4HA1[a] PMVK[b] ACC1[b] ACC2[b] SCD1[b] ACLY[b] HMGCR[b] MTTP[b]	↓ Plasma cholesterol ↓ VLDL secretion ↓ Fatty acid synthesis ↓ Cholesterol synthesis ↑ Fatty acid oxidation ↑ Triglyceride synthesis ↑ Steatohepatitis ↑ Hepatic fibrosis	[71,72,76]
miR-34a overexpression	HNF4α[a] MTTP[b] ApoB[b] Srebp-1c[b] Acc1[b] Acc2[b] HMGCR[b]	↓ Plasma lipids ↓ VLDL secretion ↑ Atherosclerosis ↑ Hepatic steatosis	[86]

[a] Genes that are proven to be direct targets by site-directed mutagenesis experiments.
[b] Genes that are shown to be regulated by the miRs via qRT-PCR.

lipoprotein production as it is an important positive regulator for the expression of apoB, MTP and several other genes involved in lipid and lipoprotein biosynthesis. Besides these changes in plasma lipid and glucose levels, HNF4α deficiency is also associated with hepatosteatosis and non-alcoholic fatty liver disease (NAFLD). In mice, loss-of-function of HNFα results in fatty liver and hypolipidemia [47,86].

An inverse relationship has been reported in the hepatic expression of HNF4α and miR-34a in several metabolic disorders affecting both glucose and lipid metabolism in humans and mice [86,87]. Further, hepatic lipids are high and HNF4α levels are low in non-alcoholic steatohepatitis patients [86,87]. Additionally, livers of these subjects have >2-fold higher levels of miR-34a compared to normal livers. In diabetic (*ob*/*ob*, *db*/*db*, and streptozotocin treated) and high-fat diet induced obese mice HNF4α levels are reduced by 75–85% and miR-34a levels are increased by 10-fold.

Two well-conserved interacting sites of miR-34a were predicted in the 3′-UTRs of human and mouse HNF4α mRNA. The interaction between miR-34a and HNF4α 3′-UTR was confirmed using site-directed mutagenesis, suggesting that HNF4α is directly targeted by miR-34a (Table 2). To further discern relationship between miR-34a and HNF4α, miR-34a was either overexpressed or deleted in mice. Adenovirus-mediated overexpression of miR-34a reduced HNF4α expression, decreased plasma triglyceride and cholesterol, and increased hepatic triglyceride in wild type mice compared with a control group transduced with scrambled (Scr) miR. Mechanistic studies showed that the production rates of triglyceride-rich VLDL were reduced in miR-34a overexpressing mice. Thus, overexpression of miR-34a reduces hepatic HNF4α and plasma lipids by reducing VLDL production.

In contrast to miR-34a overexpression, *Mir34a*$^{-/-}$ mice had increased plasma levels of triglyceride and cholesterol, decreased hepatic triglycerides, and augmented HNF4α expression. Further, inhibition of hepatic miR-34a using anti-miR-34a in *ob*/*ob* and high fat diet fed mice resulted in significant increases in HNF4α expression. Similar gain and loss of miR-34a function studies in hepatoma cells also resulted in significant changes in HNF4α expression. These studies indicated that miR-34a might reduce HNF4α expression to regulate lipid metabolism. Indeed, further studies revealed that acute and chronic deficiency of HNF4α reduced plasma lipids and atherosclerosis in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice and increased hepatic triglyceride, indicating that the effect of miR-34a was due to reduced HNF4α expression. These studies established that miR-34a regulates HNF4α to modulate plasma and hepatic lipid homeostasis.

Since miR-34a modulates plasma apoB-containing lipoproteins, it is anticipated that it would affect atherosclerosis. In *Ldlr*$^{-/-}$ mice on a Western diet, adenoviral-mediated hepatic overexpression of miR-34a reduced atherosclerotic burden in association with lower plasma triglyceride, cholesterol and apoB levels. MiR-34a reduced VLDL/LDL cholesterol but had no effect on HDL cholesterol. Hepatic overexpression of miR-34a was again associated with increased hepatic triglyceride levels. Thus, the miR-34a-HNFα pathway regulates plasma apoB-containing lipoprotein metabolism and the development of atherosclerosis [86]. Even though miR-34 can reduce plasma lipids and atherosclerosis, it also increases hepatic triglyceride. Hence, it may not be a suitable agent to treat hyperlipidemia.

5. MiR-30c reduces apoB-containing lipoprotein production, lipid synthesis, and atherosclerosis

Our group has recently identified miR-30c as a regulator of lipid metabolism [38,88,89]. MiR-30c belongs to the miR-30 family that consists of five evolutionarily conserved members (miR-30a-e). All members have high sequence similarity and share identical seed sequence (GUAAACA). MiR-30c is a product of two genes: *MIR30C-1* and *MIR30C-2*. *MIR30C-1* gene resides in intron 5 of the nuclear transcription factor Y subunit C (NFY-C) gene (NM-014,223) at chromosome 1 and chromosome 4 in human and mouse, respectively. *MIR30C-2* gene is intronic to a processed transcript (*C6orf155*) that has not yet been shown to produce a protein, and is located on chromosome 6 and chromosome 1 in human and mouse, respectively [38,90,91].

We have demonstrated that miR-30c regulates assembly and secretion of apoB-containing lipoproteins by modulating MTP activity. TargetScan predicted that the miR-30 family could potentially interact with MTP mRNA [38]. Moreover, the predicted miR-30 seed sequence interacting site in MTP mRNA was highly conserved among different species. To evaluate whether miR-30 family members regulate MTP expression, different miR-30 family members (miR-30b, c, and e) were transfected in human hepatoma, Huh-7, cells. MiR-30c significantly reduced and its corresponding anti-miR elevated MTP activity and apoB secretion [38]. Mechanistic studies revealed that miR-30c decreases MTP activity by inducing posttranscriptional degradation of MTP mRNA [38]. This mRNA degradation requires interactions between miR-30c and MTP mRNA involving both seed and supplementary sequences [88]. Other members of the family do not target MTP as they do not form the same supplementary interactions. This might explain why only miR-30c, but not the other miR-30 family members, targets MTP.

To investigate whether miR-30c regulates MTP activity and lipoprotein production in vivo, male C57BL/6 mice were transduced with lentiviruses expressing Scr control, miR-30c, or anti-miR-30c, and started on a Western diet to induce hyperlipidemia. Overexpression of miR-30c significantly reduced hepatic MTP expression and activity, plasma cholesterol levels, and production rate of triglyceride-rich VLDL as compared to control group [38]. Neither hepatic lipids nor plasma transaminases were elevated in miR-30c transduced group demonstrating that miR-30c can lower plasma lipids with no obvious deleterious effects. Further experiments revealed that miR-30c avoids hepatic steatosis by targeting genes involved in lipid synthesis, such as lysophosphatidylglycerol acyltransferase 1 (LPGAT1). Additionally, Western diet-fed *Apoe*$^{-/-}$ mice overexpressing miR-30c showed significant reductions in plasma cholesterol, fasting plasma triglycerides, and plasma apoB levels. Moreover, atherosclerotic lesions were fewer and smaller in miR-30c-expressing *Apoe*$^{-/-}$ mice [38]. These studies demonstrate that miR-30c reduces hepatic lipoprotein production and lipid synthesis to lower plasma lipids and avoid hepatic steatosis.

6. Perspectives

Assembly and secretion of apoB-containing lipoproteins are important steps to mobilize dietary and endogenous lipids to different tissues through circulation. Inhibition of this biosynthetic pathway reduces plasma lipid levels. However, available agents that can efficiently inhibit secretion of apoB-containing lipoproteins, such as MTP inhibitors (lomitapide) and apoB anti-sense oligonucleoide (mipomersen) carry warning labels for hepatic steatosis. To avoid this common adverse effect, a combination therapy involving reductions in hepatic lipid accretions is needed. These strategies may include inhibiting hepatic lipid synthesis or activating consumption of lipids, such as fatty acid oxidation in the liver. Since miRs regulate multiple biological processes, they can be excellent agents for combination therapy. Hence, more efforts are needed to identify additional miRs that could affect multiple pathways in a beneficial way to reduce plasma lipids and atherosclerosis without causing unwanted side effects.

As discussed above, inhibition of miR-122 and overexpression of miR-34 can decrease plasma lipids; however, they also promote hepatic steatosis. On the other hand, miR-30c reduces secretion of apoB-containing lipoproteins and triglyceride synthesis simultaneously, resulting in less plasma lipids and absence of hepatic lipid accretions. Therefore, miR-30c can be a potential therapeutic agent to reduce hyperlipidemia and atherosclerosis. Since viral delivery to human would be formidable in the therapeutic context, further studies using chemically modified double-stranded miR-30c mimics are needed to test whether miR-30c is a safe and efficacious therapeutic agent to treat cardiovascular disease.

For miR therapy to be successful, methods are needed for their efficient delivery via less invasive approaches. This can perhaps be achieved by increasing the stability of miRs by introducing different modifications and utilizing receptor-mediated delivery systems. To avoid invasive approaches, novel formulations can be invented for subcutaneous, nasal, dermal and other routes of delivery. If successful, miR therapy can be the leading modality for treating various diseases.

Declaration of interests

Authors declare no conflicts of interest.

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References

[1] M.M. Hussain, et al., Multiple functions of microsomal triglyceride transfer protein, Nutr. Metab. (Lond.) **9** (2012) 14.
[2] M.M. Hussain, J. Shi, P. Dreizen, Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly, J. Lipid Res. 44 (1) (2003) 22–32.
[3] S. Wang, et al., The microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells, J. Biol. Chem. 271 (24) (1996) 14124–14133.
[4] F. Benoist, T. Grand-Perret, Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. Synchronized translation studies on HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein, J. Biol. Chem. 272 (33) (1997) 20435–20442.
[5] E.A. Fisher, H.N. Ginsberg, Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins, J. Biol. Chem. 277 (20) (2002) 17377–17380.
[6] T.M. Mason, The role of factors that regulate the synthesis and secretion of very-low-density lipoprotein by hepatocytes, Crit. Rev. Clin. Lab. Sci. 35 (6) (1998) 461–487.
[7] T.V. Fungwe, et al., Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol, J. Lipid Res. 33 (2) (1992) 179–191.
[8] D. Gruffat, et al., Regulation of VLDL synthesis and secretion in the liver, Reprod. Nutr. Dev. 36 (4) (1996) 375–389.
[9] S. Tiwari, S.A. Siddiqi, Intracellular trafficking and secretion of VLDL, Arterioscler. Thromb. Vasc. Biol. 32 (5) (2012) 1079–1086.
[10] A. Trpkovic, et al., Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases, Crit. Rev. Clin. Lab. Sci. (2014) 1–16.
[11] Y. Huang, et al., An abundant dysfunctional apolipoprotein A1 in human atheroma, Nat. Med. 20 (2) (2014) 193–203.
[12] K.J. Moore, F.J. Sheedy, E.A. Fisher, Macrophages in atherosclerosis: a dynamic balance, Nat. Rev. Immunol. 13 (10) (2013) 709–721.
[13] Centers for Disease, C. and Prevention, Vital signs: prevalence, treatment, and control of high levels of low-density lipoprotein cholesterol–United States, 1999–2002 and 2005–200, MMWR Morb. Mortal. Wkly Rep. **60** (4) (2011) 109–114.
[14] Q. Gu, et al., Prescription Cholesterol-lowering Medication Use in Adults Aged 40 and Over: United States, 2003–2012, NCHS Data Brief 177 (2014) 1–8.
[15] Z. Ahmad, Statin intolerance, Am. J. Cardiol. **113** (10) (2014) 1765–1771.
[16] R.S. Blumenthal, Statins: effective antiatherosclerotic therapy, Am. Heart J. **139** (4) (2000) 577–583.
[17] M.A. Cornier, R.H. Eckel, Non-traditional dosing of statins in statin-intolerant patients—is it worth a try? Curr. Atheroscler. Rep. **17** (2) (2015) 475.
[18] M.R. Ehrenstein, E.C. Jury, C. Mauri, Statins for atherosclerosis—as good as it gets? N. Engl. J. Med. 352 (1) (2005) 73–75.
[19] J.B. Finkel, D. Duffy, 2013 ACC/AHA cholesterol treatment guideline: paradigm shifts in managing atherosclerotic cardiovascular disease risk, Trends Cardiovasc. Med. 25 (4) (2015) 340–347.
[20] J.C. LaRosa, et al., Intensive lipid lowering with atorvastatin in patients with stable coronary disease, N. Engl. J. Med. 352 (14) (2005) 1425–1435.
[21] R.M. Stoekenbroek, J.J. Kastelein, R. Huijgen, PCSK9 inhibition: the way forward in the treatment of dyslipidemia, BMC Med. **13** (2015) 258.
[22] E. Stroes, et al., Anti-PCSK9 antibody effectively lowers cholesterol in patients with statin intolerance: the GAUSS-2 randomized, placebo-controlled phase 3 clinical trial of evolocumab, J. Am. Coll. Cardiol. 63 (23) (2014) 2541–2548.
[23] F.J. Raal, et al., PCSK9 inhibition with evolocumab (AMG 145) in heterozygous familial hypercholesterolaemia (RUTHERFORD-2): a randomised, double-blind, placebo-controlled trial, Lancet 385 (9965) (2015) 331–340.
[24] J.G. Robinson, et al., Efficacy and safety of alirocumab in reducing lipids and cardiovascular events, N. Engl. J. Med. 372 (16) (2015) 1489–1499.
[25] J.B. Finkel, D. Duffy, 2013 ACC/AHA cholesterol treatment guideline: paradigm shifts in managing atherosclerotic cardiovascular disease risk, Trends Cardiovasc. Med. (2014).
[26] F.J. Raal, D.J. Blom, Anacetrapib in familial hypercholesterolaemia: pros and cons, Lancet 385 (9983) (2015) 2124–2126.
[27] M.J. Lipinski, et al., The impact of proprotein convertase subtilisin-kexin type 9 serine protease inhibitors on lipid levels and outcomes in patients with primary hypercholesterolaemia: a network meta-analysis, Eur. Heart J. (2015).
[28] M. Cuchel, D.J. Rader, Microsomal transfer protein inhibition in humans, Curr. Opin. Lipidol. 24 (3) (2013) 246–250.
[29] D.J. Rader, J.J. Kastelein, Lomitapide and mipomersen: two first-in-class drugs for reducing low-density lipoprotein cholesterol in patients with homozygous familial hypercholesterolemia, Circulation 129 (9) (2014) 1022–1032.
[30] D.N. Ricotta, W. Frishman, Mipomersen: a safe and effective antisense therapy adjunct to statins in patients with hypercholesterolemia, Cardiol. Rev. 20 (2) (2012) 90–95.
[31] M. Cuchel, D.J. Blom, M.R. Averna, Clinical experience of lomitapide therapy in patients with homozygous familial hypercholesterolaemia, Atheroscler. Suppl. 15 (2) (2014) 33–45.
[32] M.A. Valencia-Sanchez, et al., Control of translation and mRNA degradation by miRNAs and siRNAs, Genes Dev. 20 (5) (2006) 515–524.
[33] E. Izaurralde, Gene Regulation. Breakers and blockers—miRNAs at work, Science 349 (6246) (2015) 380–382.
[34] A. Bouchie, First microRNA mimic enters clinic, Nat. Biotechnol. 31 (7) (2013) 577.
[35] Z. Qiu, Y. Dai, Roadmap of miR-122-related clinical application from bench to bedside, Expert Opin. Investig. Drugs 23 (3) (2014) 347–355.
[36] E. van Rooij, S. Kauppinen, Development of microRNA therapeutics is coming of age, EMBO Mol. Med. 6 (7) (2014) 851–864.
[37] K.J. Rayner, et al., MiR-33 contributes to the regulation of cholesterol homeostasis, Science 328 (5985) (2010) 1570–1573.
[38] J. Soh, et al., MicroRNA-30c reduces hyperlipidemia and atherosclerosis in mice by decreasing lipid synthesis and lipoprotein secretion, Nat. Med. 19 (7) (2013) 892–900.
[39] R.M. Allen, et al., Control of very low-density lipoprotein secretion by N-ethylmaleimide-sensitive factor and miR-33, Circ. Res. 115 (1) (2014) 10–22.
[40] L. Goedeke, et al., Long-term therapeutic silencing of miR-33 increases circulating triglyceride levels and hepatic lipid accumulation in mice, EMBO Mol. Med. 6 (9) (2014) 1133–1141.
[41] D. Karunakaran, et al., Therapeutic inhibition of miR-33 promotes fatty acid oxidation but does not ameliorate metabolic dysfunction in diet-induced obesity, Arterioscler. Thromb. Vasc. Biol. 35 (12) (2015) 2536–2543.
[42] N. Rotllan, et al., Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr−/− mice—brief report, Arterioscler. Thromb. Vasc. Biol. 33 (8) (2013) 1973–1977.
[43] K.J. Rayner, et al., Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis, J. Clin. Invest. 121 (7) (2011) 2921–2931.
[44] T. Horie, et al., MicroRNA-33 deficiency reduces the progression of atherosclerotic plaque in ApoE−/− mice, J. Am. Heart Assoc. 1 (6) (2012), e003376.
[45] K.J. Rayner, et al., Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides, Nature 478 (7369) (2011) 404–407.
[46] X. Sun, M.W. Feinberg, MicroRNA-management of lipoprotein homeostasis, Circ. Res. 115 (1) (2014) 2–6.
[47] J.A. Bonzo, et al., Suppression of hepatocyte proliferation by hepatocyte nuclear factor 4alpha in adult mice, J Biol Chem 287 (10) (2012) 7345–7356.
[48] J.P. Babeu, F. Boudreau, Hepatocyte nuclear factor 4-alpha involvement in liver and intestinal inflammatory networks, World J. Gastroenterol. 20 (1) (2014) 22–30.
[49] A. Panayiotou, et al., ApoB/ApoA1 ratio and subclinical atherosclerosis, Int. Angiol. 27 (1) (2008) 74–80.
[50] D. Weissglas-Volkov, et al., Common hepatic nuclear factor-4alpha variants are associated with high serum lipid levels and the metabolic syndrome, Diabetes 55 (7) (2006) 1970–1977.
[51] W.W. Hwang-Verslues, F.M. Sladek, HNF4alpha—role in drug metabolism and potential drug target? Curr. Opin. Pharmacol. 10 (6) (2010) 698–705.
[52] C.P. Martinez-Jimenez, et al., Hepatocyte nuclear factor 4alpha coordinates a transcription factor network regulating hepatic fatty acid metabolism, Mol. Cell. Biol. 30 (3) (2010) 565–577.
[53] G.P. Hayhurst, et al., Hepatocyte nuclear factor 4alpha (nuclear receptor 2 A1) is essential for maintenance of hepatic gene expression and lipid homeostasis, Mol. Cell. Biol. 21 (4) (2001) 1393–1403.
[54] J.A. Ladias, et al., Transcriptional regulation of human apolipoprotein genes ApoB, ApoCIII, and ApoAII by members of the steroid hormone receptor superfamily HNF-4, ARP-1, EAR-2, and EAR-3, J. Biol. Chem. 267 (22) (1992) 15849–15860.
[55] K. Dai, M.M. Hussain, NR2F1 disrupts synergistic activation of the MTTP gene transcription by HNF-4alpha and HNF-1alpha, J. Lipid Res. 53 (5) (2012) 901–908.
[56] V. Sheena, et al., Transcriptional regulation of human microsomal triglyceride transfer protein by hepatocyte nuclear factor-4alpha, J. Lipid Res. 46 (2) (2005) 328–341.
[57] H. Hirokane, et al., Bile acid reduces the secretion of very low density lipoprotein by repressing microsomal triglyceride transfer protein gene expression mediated by hepatocyte nuclear factor-4, J. Biol. Chem. 279 (44) (2004) 45685–45692.
[58] L. Yin, et al., Hepatic hepatocyte nuclear factor 4alpha is essential for maintaining triglyceride and cholesterol homeostasis, Arterioscler. Thromb. Vasc. Biol. 31 (2) (2011) 328–336.
[59] J.L. Brodsky, E.A. Fisher, The many intersecting pathways underlying apolipoprotein B secretion and degradation, Trends Endocrinol. Metab. 19 (7) (2008) 254–259.

[60] M.E. Haas, A.D. Attie, S.B. Biddinger, The regulation of ApoB metabolism by insulin, Trends Endocrinol. Metab. 24 (8) (2013) 391–397.
[61] J.L. Dixon, S. Furukawa, H.N. Ginsberg, Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B, J. Biol. Chem. 266 (8) (1991) 5080–5086.
[62] R.A. Davis, J.R. Boogaerts, Intrahepatic assembly of very low density lipoproteins. Effect of fatty acids on triacylglycerol and apolipoprotein synthesis, J. Biol. Chem. 257 (18) (1982) 10908–10913.
[63] K. Bostrom, et al., Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells, J. Biol. Chem. 261 (29) (1986) 13800–13806.
[64] R.A. Borchardt, R.A. Davis, Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion, J. Biol. Chem. 262 (34) (1987) 16394–16402.
[65] J.B. Moberly, et al., Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally, Biochim. Biophys. Acta 1042 (1) (1990) 70–80.
[66] C.M. Arbeeny, et al., Inhibition of fatty acid synthesis decreases very low density lipoprotein secretion in the hamster, J. Lipid Res. 33 (6) (1992) 843–851.
[67] T. Funatsu, et al., Prolonged inhibition of cholesterol synthesis by atorvastatin inhibits apo B-100 and triglyceride secretion from HepG2 cells, Atherosclerosis 157 (1) (2001) 107–115.
[68] E. Pau, et al., Inhibition of hydroxymethylglutaryl coenzyme A reductase activity does not affect the secretion rate of apolipoproteins B and AI by CaCo-2 cells, Biochem. Cell Biol. 73 (1–2) (1995) 81–90.
[69] Z. Zhang, K. Cianflone, A.D. Sniderman, Role of cholesterol ester mass in regulation of secretion of ApoB100 lipoprotein particles by hamster hepatocytes and effects of statins on that relationship, Arterioscler. Thromb. Vasc. Biol. 19 (3) (1999) 743–752.
[70] M. Lagos-Quintana, et al., Identification of tissue-specific microRNAs from mouse, Curr. Biol. 12 (9) (2002) 735–739.
[71] C. Esau, et al., miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting, Cell Metab. 3 (2) (2006) 87–98.
[72] J. Krutzfeldt, et al., Silencing of microRNAs in vivo with 'antagomirs', Nature 438 (7068) (2005) 685–689.
[73] J. Elmen, et al., LNA-mediated microRNA silencing in non-human primates, Nature 452 (7189) (2008) 896–899.
[74] R.E. Lanford, et al., Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection, Science 327 (5962) (2010) 198–201.
[75] W.C. Tsai, et al., MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis, J. Clin. Invest. 122 (8) (2012) 2884–2897.
[76] S.H. Hsu, et al., Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver, J. Clin. Invest. 122 (8) (2012) 2871–2883.
[77] A.E. Kel, et al., Genome wide prediction of HNF4alpha functional binding sites by the use of local and global sequence context, Genome Biol. 9 (2) (2008) R36.
[78] J. Eeckhoute, et al., Critical role of charged residues in helix 7 of the ligand binding domain in hepatocyte nuclear factor 4alpha dimerisation and transcriptional activity, Nucleic Acids Res. 31 (22) (2003) 6640–6650.
[79] L.W. Harries, J.E. Brown, A.L. Gloyn, Species-specific differences in the expression of the HNF1A, HNF1B and HNF4A genes, PLoS One 4 (11) (2009), e7855.
[80] J. Lausen, et al., Naturally occurring mutations in the human HNF4alpha gene impair the function of the transcription factor to a varying degree, Nucleic Acids Res. 28 (2) (2000) 430–437.
[81] W. Zhou, et al., SUMOylation of HNF4alpha regulates protein stability and hepatocyte function, J. Cell Sci. 125 (Pt 15) (2012) 3630–3635.
[82] C. Walesky, et al., Hepatocyte nuclear factor 4 alpha deletion promotes diethylnitrosamine-induced hepatocellular carcinoma in rodents, Hepatology 57 (6) (2013) 2480–2490.
[83] A. Amara, et al., Frequency of HNF4A-P.I463V variant in the Tunisian North-African population and its relation with diabetes mellitus, Iran J. Public Health 44 (3) (2015) 396–403.
[84] C. Wang, et al., Prediabetes is associated with HNF-4 alpha P2 promoter polymorphism rs1884613: a case–control study in Han Chinese population and an updated meta-analysis, Dis. Markers 2014 (2014) 231736.
[85] L.W. Harries, et al., The diabetic phenotype in HNF4A mutation carriers is moderated by the expression of HNF4A isoforms from the P1 promoter during fetal development, Diabetes 57 (6) (2008) 1745–1752.
[86] Y. Xu, et al., A metabolic stress-inducible miR-34a-HNF4alpha pathway regulates lipid and lipoprotein metabolism, Nat. Commun. **6** (2015) 7466.
[87] S.E. Choi, et al., Elevated microRNA-34a in obesity reduces NAD+ levels and SIRT1 activity by directly targeting NAMPT, Aging Cell 12 (6) (2013) 1062–1072.
[88] J. Soh, M.M. Hussain, Supplementary site interactions are critical for the regulation of microsomal triglyceride transfer protein by microRNA-30c, Nutr. Metab. (Lond.) 10 (1) (2013) 56.
[89] S. Irani, M.M. Hussain, Role of microRNA-30c in lipid metabolism, adipogenesis, cardiac remodeling and cancer, Curr. Opin. Lipidol. 26 (2) (2015) 139–146.
[90] M. Karbiener, et al., MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2, RNA Biol. 8 (5) (2011) 850–860.
[91] P. Alexiou, et al., miRGen 2.0: a database of microRNA genomic information and regulation, Nucleic Acids Res. 38 (Database issue) (2010) D137–D141.

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Nitrated apolipoprotein AI/apolipoprotein AI ratio is increased in diabetic patients with coronary artery disease

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Aims/hypothesis: Recent studies have suggested that determination of HDL function may be more informative than its concentration in predicting its protective role in coronary artery disease (CAD). Apolipoprotein AI (apoAI), the major protein of HDL, is nitrosylated *in vivo* to nitrated apoAI (NT-apoAI) that might cause dysfunction. We hypothesized that NT-apoAI/apoAI ratio might be associated with diabetes mellitus (DM) in CAD patients.
Methods: We measured plasma NT-apoAI and apoAI levels in 777 patients with coronary artery disease (CAD) by ELISA. Further, we measured plasma cholesterol efflux potential in subjects with similar apoAI but different NT-apoAI levels.
Results: We found that median NT-apoAI/apoAI ratio was significantly higher in diabetes mellitus (DM) (n = 327) versus non-diabetic patients (n = 450). Further analysis indicated that DM, thiobarbituric acid-reactive substances and C-reactive protein levels were independent predictors of higher NT-apoAI/apoAI ratio. There was negative correlation between NT-apoAI/apoAI and use of anti-platelet and lipid lowering drugs. The cholesterol efflux capacity of plasma from 67 individuals with differing NT-apoAI but similar apoAI levels from macrophages *in vitro* was negatively correlated with NT-apoAI/apoAI ratio.
Conclusions: Higher NT-apoAI/apoAI ratio is significantly associated with DM in this relatively large German cohort with CAD and may contribute to associated complications by reducing cholesterol efflux capacity.

1. Introduction

Diabetes mellitus (DM), a well known risk factor of atherosclerosis, is responsible for a three to four fold increase in cardiovascular events [1]. It is known that hyperglycemia stimulates the production of advanced glycosylated end products, activates protein kinase C, and enhances the polyol pathway leading to increased superoxide anion formation [2,3]. Superoxide anion interacts with nitric oxide to form a strong oxidant peroxynitrite, which reacts with tyrosines in various proteins to generate nitrotyrosine (NTyr) residues. Therefore, levels of total plasma NTyr provide a composite index of reactive nitrogen species. Another

Abbreviations: ABCA1, ATP-binding cassette family A protein 1; ACS, acute coronary syndrome; ANOVA, analysis of variance; apoAI, apolipoprotein AI; BMI, body mass index; BSA, bovine serum albumin; CAD, coronary artery disease; CRP, C-reactive protein; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; LSD, least significant difference; NTyr, nitrotyrosine; NT-apoAI, nitrated apoAI; NYHA, New York Heart Association; PAD, peripheral artery disease; PBS, phosphate buffer saline; PBST, phosphate buffered saline containing Tween; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TG, triglyceride; TIA, transient ischemic attack.

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pathway of the NTyr production involves reaction of nitrogen dioxide ($\cdot NO_2$) with tyrosine residues [4]. $\cdot NO_2$ is generated by myeloperoxidase from hydrogen peroxide (H_2O_2). Several small population studies have shown that NTyr levels are increased in DM [5–10] and coronary artery disease (CAD) [11,12].

Of various proteins present in plasma, apolipoprotein AI (apoAI) has been shown to be a preferred substrate for modification by reactive nitrogen species in human artery wall [13]. Circulating nitrated apoAI (NT-apoAI) levels are 7-70-fold higher in comparison with other nitrated proteins [13,14]. In the apoAI molecule, tyrosine residues 166 and 192 have been shown to be the predominant sites of nitration by myeloperoxidase [15–17]. *In vitro* nitration of apoAI impairs lipid binding and ATP-binding cassette family A protein 1 (ABCA1)-dependent cholesterol efflux functions [16]. However, physiologic effects of *in vivo* NT-apoAI are unknown.

In a small case–control study, the circulating NT-apoAI/apoAI ratio was significantly increased in diabetic patients compared with controls [18]. In addition, higher levels of NT-apoAI were found in patients with cardiovascular disease in both the circulation and in atheroma [13,14,16]. Recently, Vazquez et al. reported a significantly higher level of circulating NT-apoAI in obese women [19]. Although there appears to be a tendency towards higher plasma NT-apoAI levels in diabetes and CAD subjects, the studies were small and used gas chromatography-mass spectrometry methods that are not suitable for larger cohort studies. Previously, we developed a novel [20] enzyme-linked immunosorbent assay (ELISA), which was confirmed by both Western blot analysis [20] and mass spectrometry [19]. This sensitive and specific ELISA method is suitable to quantify human NT-apoAI levels in a large population. In the present study, we hypothesized that NT-apoAI would be increased in diabetic patients with CAD.

2. Research design and methods

2.1. Study population

A total of 777 subjects with CAD admitted to the Department of Medicine III (Angiology and Cardiology) and the Department of Heart and Thoracic Surgery, University Clinic Halle, Germany between January, 2008 and December, 2010 were prospectively studied. As expected, very few normal subjects were admitted during this period precluding comparison of different parameters between normal and CAD patients. The data presented include a retrospective analysis of measured NT-apoAI and apoAI levels. CAD was diagnosed as the presence of a luminal diameter stenosis $\geq$50% in at least one major coronary artery (left anterior descending, left circumflex or right coronary artery or their major branches) by angiography or a history of myocardial infarction, among which 380 patients had stable angina pectoris and 397 had acute coronary syndrome (ACS). Multi-vessel CAD was defined as a disease stage in which at least two of the major coronary arteries is involved with atherosclerosis of significant severity. Within this cohort, 327 patients had DM and 450 patients had no DM. DM was diagnosed at the time of admission by past clinical history of a fasting blood glucose level >7.0 mmoL/L (>125 mg/dL) and/or the two-hour value in the 75 g oral glucose tolerance test (OGTT) > 11.1 mmoL/L (200 mg/dL) or use of hypoglycemic medications. HbA1c and insulin levels were not determined. At the time of admission, the history of current smoking, hypertension, dyslipidemia, peripheral artery disease (PAD), previous stroke or transient ischemic attack (TIA), New York heart association (NYHA) functional classification, renal failure, and use of anti-platelet drugs and lipid lowering drugs was collected. Hypertension was defined as patients receiving antihypertensive treatment or having a previous diagnosis of hypertension (blood pressure $\geq$ 140/90 mmHg). The study was approved by the ethics committee of the medical faculty of the Martin Luther-University Halle-Wittenberg.

2.2. Laboratory evaluation

Subjects were instructed to fast for at least eight hours prior to blood sampling. Serum samples were collected and stored at −80 °C prior to use. Laboratory tests were performed at the time of hospital admission for triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C) and C-reactive protein (CRP) as described before [21]. For the evaluation of renal function, estimated glomerular filtration rate (eGFR) was calculated by the abbreviated Modification of Diet in Renal Disease (MDRD) [22] Study equation: $186 \times (\text{Creatinine}/88.4) - 1.154 \times (\text{Age}) - 0.203 \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$. Plasma glucose levels were measured (Autokit glucose, Wako, #439-90901) in 71% (550 subjects) of the population, among which 249 patients had DM and 308 patients had no DM.

2.3. Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation in human plasma was quantified by measuring the formation of TBARS using a kit. Plasma (10 μL) was mixed with 10 μL SDS solution. Then 125 μL TBARS solution (0.53 g trichloroacetic acid dissolved in 100 ml of sodium hydroxide and 100 ml of sodium dodecyl sulfate) was added to the sample. After incubation at 95 °C for 60 min and keeping at 4 °C for 15 min, the sample was centrifuged at 3000 rpm for 15 min. The amount of TBARS produced was measured at 532 nm. Malondialdehyde was used to construct a standard curve in parallel.

2.4. Standards used for the quantification of NT-apoAI and apoAI

Purified human HDL (MyBioSource, # MBS173147, total protein 40 mg/ml, standard curve range 1–12 ng/ml) was used as standard for the determination of plasma concentrations of apoAI. For NT-apoAI standard, purified human apoAI (Biomedical Technologies, # BT-927) was nitrated with sodium nitrite [23,24]. Briefly, purified apoAI (~1 mg/ml) was adjusted to pH 3.5 and mixed with 200 mM sodium nitrite and then incubated at 37 °C for 24 h under low agitation. Samples were dialyzed against phosphate buffer saline (PBS) for 24 h at 4 °C, and protein concentration was determined by the Coomassie Reagent (Thermo Scientific, #23236) and bovine serum albumin (BSA) as a standard [25]. The amounts of nitrated tyrosines in the modified apoAI were determined using a commercially available NTyr ELISA kit based on nitrated albumin (Cell Biolabs, # STA-305) as described by the manufacturer. The nitrated apoAI (0.7 mg/ml protein) contained 2000 nM NT-apoAI giving a concentration of 80 mmol of nitrated tyrosine per mol of apoAI.

2.5. ELISA procedure for the measurement of NT-apoAI and apoAI

Serum NT-apoAI was measured by a sandwich ELISA as described before [20]. Briefly, microtiter plates were coated with capturing monoclonal anti-NTyr antibodies (1 μg/mL, Upstate, # 05-233, clone 1A6) in PBS overnight at 4 °C. The non-specific sites were blocked by incubating the wells with 200 μL of phosphate buffered saline containing 0.05% Tween (PBST) and 3% BSA for 1 h at room temperature. After washing three times with PBST, patient samples (1:25,000 dilution in PBST containing 3% BSA) or standard controls (10–100 ng/mL, nitrated human apoAI) were added to each well and incubated for 1 h at 37 °C, enabling the binding of all the nitrated proteins to immobilized monoclonal antibodies. The wells were washed three times with PBST and then incubated for 1 h at 37 °C with 100 μL of goat anti-human apoAI (1:1000 dilution in PBST containing 3% BSA; Academy Bio-Medical Company, # 11S-G2) to specifically identify NT-apoAI. After washing three times with PBST, 100 μL of swine anti-goat IgGs conjugated to alkaline phosphatase (1:1000 dilution in PBST containing 3% BSA; Southern Biotech, # 6300-04) were added to each well and incubated for 1 h at 37 °C, followed by the addition of p-nitrophenyl phosphate solution (1 mg/mL; 150 μL/well). After 10–20 min incubation at 37 °C, the absorbance was measured at 405 nm and background values obtained from wells incubated without serum or standards were subtracted from the total values. The standard curve was linear between 0 and 100 ng/mL. Total apoAI levels were also measured in these plasma samples by ELISA (see below) and the NT-apoAI/apoAI ratios are reported as mmol/mol of apoAI.

Total apoAI levels were measured by the ELISA procedure [26,27]. This method is similar to that described above for the measurement of NT-apoAI except that the coating antibody used for apoAI was anti-human apoAI monoclonal antibody 4H1 (University of Ottawa Heart Institute, # A13). Serum samples were diluted 1:250,000 for apoAI quantifications in PBST containing 3% BSA. The quantification of apoAI was linear within 1–12 ng/mL.

2.6. Cholesterol efflux from macrophage cell line

To study the effect of plasma NT-apoAI levels on the cholesterol efflux capacity of plasma, we selected 67 subjects with a narrow range of apoAI levels (1.4–1.6 mg/dL) and widely variable NT-apoAI levels. Plasma from these subjects was used to measure cholesterol efflux according to the established procedures [28]. Briefly, J774 macrophages were plated in 24-well plates. After 16 h, cells were loaded with 0.2 μCi/ml [^{3}H]-cholesterol in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum for 24 h in 37 °C, 5% CO_2. After labeling, cells were washed three times with serum free DMEM containing 0.2% BSA and incubated with 0.2% BSA in DMEM for 1 h to equilibrate. Cells were then incubated with different human plasma samples (2%, v/v) in serum free DMEM with 0.2% BSA. Radioactivity was measured in both medium and cells after 4 h using a scintillation counter (Beckman Coulter, LS 6500). The % cholesterol efflux was calculated as follows: radioactivity in medium/(radioactivity in medium + cells) × 100.

2.7. Statistical methods

Continuous variables were reported as means ± standard deviation (SD). For variables that did not show normal distribution, median values with their 25–75th percentiles were reported. Normally distributed variables were compared by Student's t-test. The Kolmogorov–Smirnov test was used to test for normality. Comparisons among more than two groups were performed by analysis of variance (ANOVA), followed by least significant difference (LSD) test for multiple comparisons. For skewed distribution variables, the differences among groups were performed by non-parametric Kruskal Wallis test. The Pearson's χ^2 test was used to assess statistical differences in dichotomous and categorical variables. For univariate analyses, Spearman's correlation was used to determine correlation of NT-apoAI/apoAI ratio with various clinical parameters. As NT-apoAI/apoAI ratio and cholesterol efflux in selected 67 subjects were both normally distributed by Kolmogorov–Smirnov test, their relationship was analyzed by Pearson correlation. To further investigate the relationship between NT-apoAI/apoAI ratio and clinical characteristics or laboratory parameters, multivariate linear regression analysis was performed after $\log_{10}$ transformation of skewed distributed continuous variables, using $\log_{10}$ transformed NT-apoAI/apoAI ratio as the dependent variable and gender, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III-IV, renal failure, multi-vessel CAD, current aspirin-use, current statin use, as well as $\log_{10}$ transformed age, BMI, TG, TC, LDL-C, HDL-C, and CRP levels as independent variables. Attempts were also made to predict DM by multivariate logistic regression analysis, using DM as the dependent variable, and age, gender, BMI, smoking, hypertension, dyslipidemia, CRP, TG, TC, LDL-C, HDL-C, TBARS and NT-apoAI/apoAI ratio as independent variables. P values < 0.05 were considered statistically significant. All analyses were done using SPSS version 16 analytical software (SPSS Inc., Chicago, IL).

3. Results

3.1. Clinical characteristics and parameters

Among 777 subjects with CAD, 327 had DM. Plasma apoAI was normally distributed ($p > 0.05$) and was reported as means ± SD. The distributions of age, BMI, glucose, TC, TG, HDL-C, LDL-C, CRP, eGFR, TBARS, and NT-apoAI were skewed ($p < 0.05$). Clinical characteristics and parameters of the two groups and significant differences are shown in Table 1. The DM group was older, had greater BMI, higher incidence of hypertension, PAD, previous stroke or TIA, NYHA functional classification III-IV, multi-vessel CAD, renal failure, higher TG, glucose, CRP, and lower TC, HDL-C, LDL-C, eGFR, and a lower rate of anti-platelet drugs use than the non-DM group.

There was a trend towards lower apoAI in the diabetic group ($p = 0.096$). NT-apoAI levels were not significantly different in these two groups ($p = 0.452$) but NT-apoAI/apoAI ratio was significantly higher in the DM group ($p = 0.016$) (Table 1, Fig. 1).

3.2. Correlations between NT-apoAI/apoAI and different clinical characteristics or parameters

Correlations between NT-apoAI/apoAI and the various clinical parameters are shown in Table 2. Although significant ($p = 0.016$), the correlations ($r = 0.087$) between NT-apoAI/apoAI and DM, male gender, previous stroke or TIA, TBARS, and use of anti-platelet and lipid lowering drugs in the entire cohort were weak. In non-DM subjects, NT-apoAI/apoAI ratio was significantly associated with male gender, hypertension, use of anti-platelet and lipid lowering drugs and TBARS. The NT-apoAI/apoAI ratio was significantly correlated in DM subjects with PAD and with previous stroke or TIA (Table 2). When analyzed for association with different genders, significant correlations were found between NT-apoAI/apoAI and DM ($r = 0.121$, $p = 0.005$), hypertension, previous stroke or TIA, renal failure, use of anti-platelet drugs, and TBARS in the male subjects (Table 3). Such associations, however, were not seen in the females perhaps due to smaller number. These studies indicated that NT-apoAI/apoAI is correlated with DM in male subjects.

3.3. Lower plasma NT-apoAI/apoAI ratio in subjects using aspirin and statins

There was a significant negative correlation between NT-apoAI/apoAI and use of anti-platelet or lipid-lowering medications (Fig. 2). Post-hoc analyses showed significant heterogeneity in NT-apoAI/apoAI ratio in subjects taking four specific drugs (Fig. 2A), with aspirin use is appearing to have the lowest plasma NT-apoAI/apoAI. Similarly, lipid lowering drug classes showed heterogeneity with regard to plasma NT-apoAI/apoAI (Fig. 2B). In particular, subjects taking statins had significantly lower NT-apoAI/apoAI than those not taking lipid-lowering drugs.

Table 1
Group comparisons for different variables between Non-DM and DM groups: Values for variables that show normal distribution are given as means ± SD and evaluated using Student t-test. Values for variables that do not show normal distribution are provided as median (25–75th percentiles) and evaluated using Kruskal Wallis test. Dichotomous and categorical variables are given as percentages and compared using Pearson's χ^2 test.

Variable	Non-DM (n = 450)	DM (n = 327)	p Value
Male (N%)	307 (68.2%)	220 (67.3%)	0.781
Age (Years)	68 (61–75)	71 (65–76)	0.001†
BMI (kg/m^2)	26.7 (24.4–29.7)	28.7 (26.0–32.4)	<0.0001†
Smoking (N%)	82 (18.5%)	45 (14.1%)	0.111
Clinical complications or conditions			
Hypertension (N%)	381 (85.2%)	311 (95.1%)	<0.0001†
Dyslipidemia (N%)	296 (67.4%)	224 (69.1%)	0.616
PAD (N%)	62 (13.8%)	71 (21.3%)	0.004†
Previous stroke or TIA (N%)	46 (10.3%)	52 (15.9%)	0.020†
NYHA functional classification III ~ IV (N%)	106 (24.4%)	116 (36.7%)	<0.0001†
Multi-vessel CAD (N%)	302 (69.1%)	253 (79.8%)	0.001†
ACS (N%)	237 (52.8%)	160 (49.1%)	0.308
Renal Failure (N%)	96 (23.7%)	138 (45.7%)	<0.0001†
Anti-platelet drugs			
Aspirin (N%)	131 (29.2%)	88 (27.2%)	0.050
Clopidogrel (N%)	40 (8.9%)	30 (9.3%)	
Aspirin and Clopidogrel (N%)	211 (47.0%)	133 (41.0%)	
Lipid lowering drugs			
Statins (N%)	373 (83.1%)	266 (81.8%)	0.153
Ezetimibe (N%)	25 (5.6%)	12 (3.7%)	
Other lipid lowering drugs (N%)	18 (4.0%)	10 (3.1%)	
Laboratory parameters			
TC (mmol/L)	4.4 (3.8–5.2)	4.3 (3.5–5.1)	0.036†
TG (mmol/L)	1.3 (1.0–1.8)	1.5 (1.1–2.2)	0.001†
HDL-C (mmol/L)	1.0 (0.8–1.3)	0.9 (0.7–1.1)	<0.0001†
LDL-C (mmol/L)	2.7 (2.1–3.4)	2.7 (1.9–3.2)	0.044†
CRP (mg/L)	6.95 (5.0–23.53)	8.9 (5.0–31.3)	0.019†
Glucose (mg/dL)	113.5 (87.2–138.9)	137.9 (106.4–182.0)	<0.0001†
eGFR (mL/min·1.73m^2)	74.6 (52.6–94.1)	68.5 (45.9–89.6)	0.007†
ApoAI (mg/mL)	1.33 ± 0.68	1.24 ± 0.72	0.096
TBARS (nmol/mL)	1.17 (0.51–2.57)	1.26 (0.50–2.87)	0.314
NT-apoAI (nmol/L)	1868 (1039–3050)	1951 (1131–2966)	0.452
NT-apoAI/apoAI (mmol/mol)	46.74 (24.26–70.90)	53.58 (25.38–88.08)	0.016†
Cholesterol efflux* (%)	20.77 ± 3.71	19.15 ± 3.64	0.09

DM, diabetes mellitus; BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances; ApoAI, apolipoprotein AI; NT-apoAI, nitrated apoAI. *Cholesterol efflux was measured in 67 subjects with similar apoAI level (1.4–1.6 mg/dL), among which 24 subjects had DM and 43 subjects had non-DM. †p < 0.05.

3.4. TBARS are an independent predictor of higher NT-apoAI/apoAI ratio

TBARS were an independent predictor of higher NT-apoAI/apoAI ratio after adjusting for gender, age, BMI, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III–IV, renal failure, multi-vessel CAD, current aspirin use, current statin use, TG, TC, LDL-C and HDL-C levels (Table 4). However, multivariate logistic regression analysis showed that TBARS were not independently associated with DM (Table 5). These data suggest that TBARS are predictors of NT-apoAI/apoAI but not DM.

3.5. DM is an independent predictor of higher NT-apoAI/apoAI ratio

Using multivariate linear regression analysis, DM, TBARS and CRP levels were independently associated with NT-apoAI/apoAI ratio in a positive manner. Subjects with previous stroke or TIA ($B = 0.100$, $p = 0.051$) or taking aspirin ($B = -0.076$, $p = 0.067$) had a trend towards a higher or a lower NT-apoAI/apoAI ratio, respectively (Table 3).

3.6. NT-apoAI/apoAI ratio is independently associated with DM

Using multivariate logistic regression analysis, variables that were independently associated with DM were: age, BMI, smoking, hypertension, plasma TG, TC and NTyr-AI/apoAI ratio (Table 4).

3.7. Plasma samples containing high NT-apoAI/apoAI ratio show reduced cholesterol efflux from macrophages

To understand physiological consequences of high NT-apoAI/apoAI ratio, we hypothesized that this might affect basal cholesterol efflux capacity of plasma from macrophages based on the report that *in vitro* nitration of apoAI reduces its capacity for cholesterol efflux [16]. As apoAI levels affect cholesterol efflux, we wanted to study the effect of NT-apoAI independent of apoAI effects. Therefore, we thought that the analyses of all the 777 samples would not provide information about NT-apoAI independent of apoAI. Hence, we selected plasma samples that had similar apoAI levels (1.4–1.6 mg/dL). There were 67 subjects (non-DM, n = 43; DM, n = 24) that had apoAI levels in this narrow range. In these subjects NT-apoAI/apoAI ratio ranged from 4.9 to 121.5 mmoL/mol and was significantly correlated with cholesterol efflux in a negative manner ($r = -0.704$, $p < 0.0001$) in both non-DM and DM groups (Table 2, Fig. 3, Table 6).

4. Discussion

In the present study, we found that NT-apoAI/apoAI ratio was positively associated with DM and DM was an independent predictor of higher NT-apoAI/apoAI ratio even after adjusting for

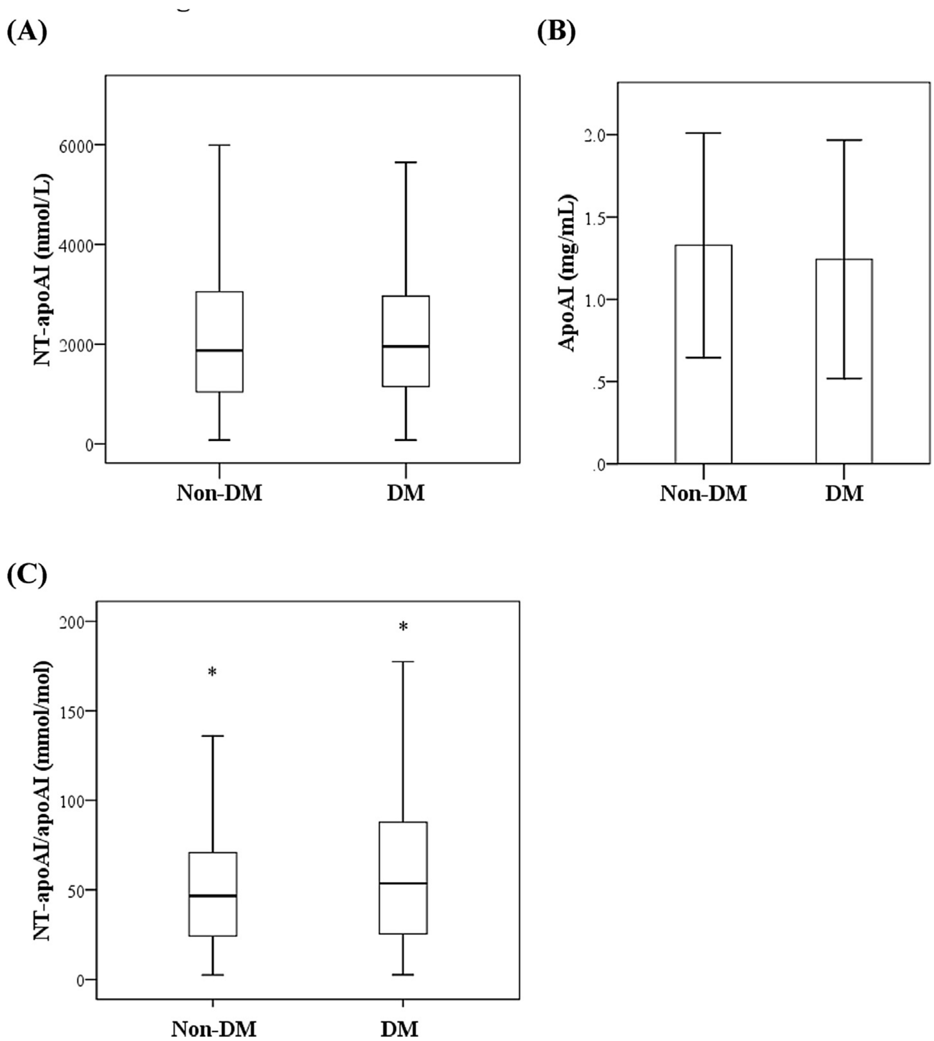

Fig. 1. NT-apoAI, apoAI and NT-apoAI/apoAI levels in non-DM (n = 327) and DM (n = 450) subjects. (A) Median NT-apoAI levels were similar in non-DM and DM subjects (p = 0.452). (B) Mean apoAI levels were not significantly different between non-DM and DM subjects (p = 0.096). (C) Median NT-apoAI/apoAI levels were significantly elevated in DM group than non-DM group (*p = 0.016).

several clinical characteristics and parameters in this relatively large cohort with CAD. Moreover, this study demonstrated a negative association between NT-apoAI/apoAI ratio and cholesterol efflux from macrophages independent of total apoAI levels. Additionally, we observed a positive correlation between TBARS and NT-apoAI/apoAI.

Few studies have found increased total (free and protein associated) NTyr residues in diabetic subjects [7,9,29]. Significant correlations have also been observed between NTyr immunostaining intensity in microvasculature and fasting blood glucose or hemoglobin A1C [30]. However, NT-apoAI/apoAI ratio seems to be better correlated with DM than the total NTyr. One small study, which used a semi-quantitative Western blot assay to measure NT-apoAI, showed that NT-apoAI/apoAI ratio, glucose, HbA1c, lipoperoxides and advance glycation end products were significantly higher in DM patients with different complications than healthy controls [18]. We also found that, as expected, DM subjects had more prevalent clinical conditions such as hypertension, PAD, previous stroke or TIA, renal failure, severe heart failure, or multi-vessel CAD. Further, DM subjects had significantly higher NT-apoAI/apoAI ratios in their plasma. Although there was a trend towards lower apoAI in the DM group in comparison to non-DM ($p = 0.096$), neither NT-apoAI nor apoAI was significantly different in these two groups, which was consistent with the previous report [18]. However, multivariate linear regression showed DM was independently associated with NT-apoAI/apoAI ratio in this cohort of patients with CAD.

The reasons for an association between NT-apoAI/apoAI ratio and DM are unknown. However, we tested the possibility that this ratio might indicate HDL dysfunction and that it may contribute to DM and other clinical conditions. Indeed, we observed that plasma from individuals that have higher amounts of NT-apoAI with similar apoAI levels is defective in basal cholesterol efflux. It is known that decreased cholesterol efflux is correlated with the risk of DM [31,32] and CAD [33,34]. In our study of 67 selected CAD subjects with similar apoAI but differing NT-apoAI/apoAI, cholesterol efflux was significantly associated with NT-apoAI/apoAI ratio (Table 2). Comparison between DM and non-DM subjects showed a trend towards a lower cholesterol efflux in DM ($p = 0.09$) (Table 1). Further, cholesterol efflux showed similar co-morbidities and laboratory parameters including NT-apoAI/apoAI ratio (Table 6) between non-DM and DM group in these selected subjects. Thus, NT-apoAI/apoAI ratio is associated with lower cholesterol efflux in both non-DM and DM groups and that this ratio may contribute to

Table 2
Univariate correlates of NT-apoAI/apoAI (mmol/mol) among total cohort, non-DM group and DM group: Spearman's correlation was used to determine correlation of NT-apoAI/apoAI with various clinical parameters.

Variable	Total population		Non-DM		DM	
	r	p	r	p	r	p
Male gender	−0.073	0.042†	−0.117	0.013†	−0.010	0.856
Age (years)	0.008	0.831	0.051	0.284	−0.704	0.180
BMI (kg/m^2)	−0.049	0.181	−0.07	0.142	−0.065	0.247
Smoking	−0.01	0.784	−0.042	0.376	0.051	0.367
Hypertension	−0.055	0.124	−0.109	0.021†	0.005	0.935
Dyslipidemia	−0.013	0.721	−0.077	0.109	0.066	0.236
PAD	0.038	0.295	−0.081	0.088	0.152	0.006†
Previous stroke or TIA	0.097	0.007†	0.065	0.172	0.120	0.030†
NYHA functional classification III ~ IV	−0.001	0.974	−0.049	0.311	0.031	0.586
Renal Failure	0.049	0.196	0.019	0.696	0.044	0.443
ACS	−0.051	0.156	−0.052	0.275	−0.045	0.422
Multi-vessel CAD	0.005	0.881	−0.012	0.807	0.009	0.879
Anti-platelet drugs	−0.103	0.004†	−0.183	<0.0001†	0.011	0.844
Lipid lowering drugs	−0.085	0.018†	−0.098	0.037†	−0.060	0.281
TC (mmol/L)	0.034	0.398	0.061	0.254	0.020	0.749
TG (mmol/L)	−0.017	0.683	−0.056	0.303	−0.005	0.942
HDL-C (mmol/L)	0.027	0.515	0.057	0.292	0.035	0.583
LDL-C (mmol/L)	0.047	0.256	0.097	0.073	0.004	0.949
CRP (mg/L)	0.059	0.102	0.054	0.255	0.046	0.409
Glucose (mg/dL)	−0.006	0.887	−0.01	0.858	−0.042	0.511
eGFR (mL/min·1.73 m^2)	−0.05	0.168	−0.061	0.202	−0.018	0.746
TBARS (nmol/mL)	0.140	<0.0001†	0.168	<0.0001†	0.102	0.069
Cholesterol efflux* (%)	−0.704	<0.0001†	−0.611	<0.0001†	−0.839	<0.0001†

BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances. *Cholesterol efflux was measured in 67 subjects with similar apoAI level (1.4–1.6 mg/dL), among which 24 subjects had DM and 43 subjects had non-DM. †$p < 0.05$.

Table 3
Univariate correlates of NT-apoAI/apoAI (mmol/mol) grouped by gender: Spearman's correlation was used to determine correlation of NT-apoAI/apoAI with various clinical parameters grouped by gender.

Variable	Female (n = 250)		Male (n = 527)	
	r	p	r	p
Age (years)	0.023	0.713	−0.015	0.723
BMI (kg/m2)	−0.104	0.106	−0.021	0.631
Smoking	0.062	0.337	−0.026	0.548
Hypertension	0.011	0.857	−0.086	0.048†
Dyslipidemia	−0.007	0.916	−0.009	0.830
DM	0.013	0.841	0.121	0.005†
PAD	−0.016	0.807	0.069	0.114
Previous stroke or TIA	0.074	0.246	0.112	0.010†
NYHA functional classification III ~ IV	0.011	0.860	−0.011	0.798
Renal failure	−0.006	0.927	0.093	0.042†
ACS	−0.019	0.771	−0.068	0.117
Multi-vessel CAD	0.035	0.586	0.015	0.732
Anti-platelet drugs	−0.095	0.132	−0.102	0.019†
Lipid lowering drugs	−0.086	0.173	−0.082	0.062
TC (mmol/L)	0.103	0.149	−0.011	0.830
TG (mmol/L)	0.001	0.987	−0.019	0.711
HDL-C (mmol/L)	0.052	0.469	−0.019	0.697
LDL-C (mmol/L)	0.105	0.143	0.016	0.747
CRP (mg/L)	0.081	0.209	0.048	0.273
Glucose (mg/dL)	−0.126	0.095	0.044	0.392
eGFR (mL/min 1.73 m^2)	−0.046	0.475	−0.033	0.458
TBARS (nmol/mL)	0.092	0.153	0.159	<0.0001†
Cholesterol efflux* (%)	−0.719	0.001†	−0.610	<0.0001†

BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances. * Cholesterol efflux was measured in 67 subjects with similar apoAI level (1.4–1.6 mg/dL) (female, n = 18; male, n = 49). †$p < 0.05$.

several co-morbidities seen in CAD subjects.

In this study, we used a subset of 67 individuals with similar levels of apoAI but different levels of NT-apoAI to measure cholesterol efflux. Comparison between DM and non-DM subjects showed a trend towards a lower cholesterol efflux in DM ($p = 0.09$; Table 1). Lack of significant association between DM and non-DM subgroups might be due to small size of the two groups. This trend may become significant if all the samples were analyzed for cholesterol efflux measurement.

Besides DM, we also found history of previous stroke or TIA had an associative trend with a higher NT-apoAI/apoAI ratio ($p = 0.051$). High levels of NTyr were detected during the ischemia and reperfusion of brain in animal experiments [35,36] and in plasma of cerebrovascular ischemia patients after stenting/angioplasty [37]. Although the number of subjects with previous stoke or TIA in our CAD cohort was relatively small (n = 98), it provided an indication that NT-apoAI/apoAI ratio might be associated with severe systemic atherosclerosis including cerebrovascular and cardiovascular disease. On the contrary, PAD was not an independent predictor of NT-apoAI/apoAI in our study perhaps due to underestimation of PAD in comparison with severe cerebrovascular or cardiovascular disease.

Plasma CRP levels were previously found to be associated with NTyr levels [38]. We found that CRP levels were also independent predictors of higher NT-apoAI/apoAI ratio. Elevated CRP was found in patients with DM and those with cardiovascular disease [39], and it carries prognostic value for cardiovascular events in CAD patients with DM [40]. Further investigations are needed to evaluate whether NT-apoAI/apoAI ratio can be an inflammatory biomarker in cardiovascular disease or inflammation contributes to higher NT-apoAI/apoAI ratio.

TBARS, biomarkers of oxidative stress, have been shown to be higher in pre-diabetic and diabetic patients than in normal subjects [41–44]. Since we did not have control subjects we could not evaluate whether TBARS found in our CAD subjects are higher than normal values. However, our study did not show any significant

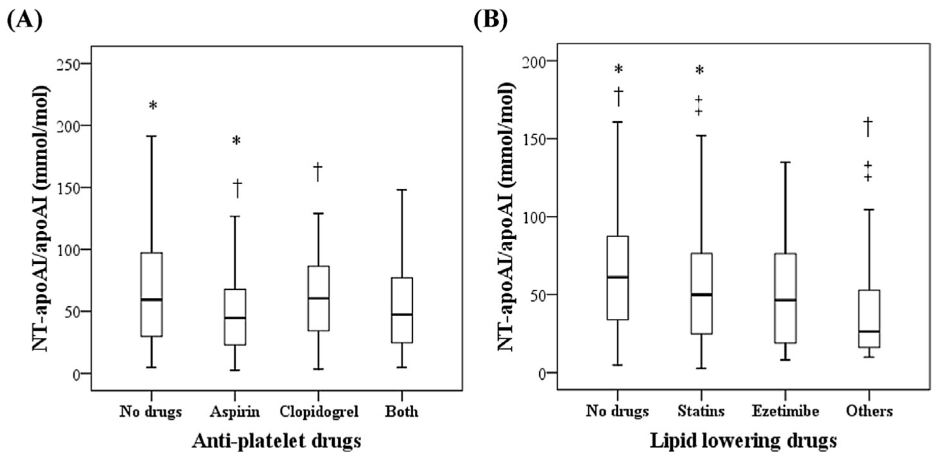

Fig. 2. Comparisons of NT-apoAI/apoAI levels in patients taking different drugs. (A) Anti-platelet drugs: There were 140, 219, 70 and 344 individuals taking no anti-platelet drugs, aspirin, clopidogrel, and aspirin + clopidogrel, respectively. Median NT-apoAI/apoAI levels among the 4 groups were significant different by ANOVA test (P = 0.001), indicating significant effects of drugs on plasma NT-apoAI/apoAI levels. By LSD test, there were significant differences between no drugs vs. aspirin (*p = 0.01) and aspirin vs. clopidogrel (†p = 0.007). (B) Lipid lowering drugs: subjects were divided into four groups. There were 70 individuals who did not take any lipid lowering drugs, whereas 639 and 37 individuals were on statins and ezetimibe, respectively. There were 28 individuals on other drugs. Median NT-apoAI/apoAI levels among the 4 groups were significant different by ANOVA test (p = 0.008), indicating significant effects of drug use on plasma NT-apoAI/apoAI levels. Sub-group comparisons by LSD test showed significant differences between no drugs and statins (*p = 0.029), no drugs and other drugs (†p = 0.001), and statins and other drugs (‡p = 0.014).

difference in TBARS levels between DM and non-DM subjects. This might be partly explained as our subjects, both DM and non-DM, had CAD, which is associated with elevated levels of TBARS [45]. Nevertheless, CRP and TBARS were found to be a predictor of higher NT-apoAI/apoAI ratio. Therefore, inflammatory response and oxidative stress might contribute to increases in NT-apoAI/apoAI ratio.

Table 4
Predictors of $\log_{10}$NT-apoAI/apoAI by multivariate linear regression: Multivariate linear regression analysis was performed by using $\log_{10}$ transformed NT-apoAI/apoAI ratio as the dependent variable, and gender, smoking, hypertension, dyslipidemia, PAD, previous stroke or TIA, ACS, NYHA functional classification III-IV, renal failure, multi-vessel CAD, current aspirin use, current statin use, as well as $\log_{10}$ transformed age, BMI, TG, TC, LDL-C, HDL-C and CRP levels as independent variables.

Variables	p value	B	95% confidence interval for B
Age* (years)	0.929	0.026	(−0.552 to 0.604)
Male gender	0.139	−0.057	(−0.134 to 0.019)
BMI* (kg/m^2)	0.618	−0.125	(−0.616 to 0.366)
Smoking	0.941	0.004	(−0.104 to 0.112)
DM	0.011†	0.097	(0.022 to 0.172)
Hypertension	0.237	−0.074	(−0.196 to 0.048)
Dyslipidemia	0.937	−0.003	(−0.081 to 0.074)
PAD	0.947	−0.003	(−0.093 to 0.087)
Previous stroke or TIA	0.051	0.100	(0.0001 to 0.020)
ACS	0.814	−0.009	(−0.082 to 0.065)
NYHA functional classification III ~ IV	0.659	−0.017	(−0.093 to 0.059)
Renal failure	0.545	0.024	(−0.055 to 0.104)
Multi-vessel CAD	0.827	0.009	(−0.071 to 0.089)
Aspirin taking	0.067	−0.076	(−0.158 to 0.005)
Statins taking	0.625	0.023	(−0.071 to 0.117)
CRP* (mg/L)	0.008†	0.108	(0.028 to 0.188)
TG* (mmol/L)	0.995	<0.0001	(−0.223 to 0.221)
TC*(mmol/L)	0.844	0.105	(−0.941 to 1.150)
LDL-C*(mmol/L)	0.793	0.082	(−0.533 to 0.698)
HDL-C* (mmol/L)	0.888	−0.024	(−0.363 to 0.314)
TBARS* (nmol/mL)	<0.0001†	0.179	(0.106 to 0.252)

BMI, body mass index; DM, diabetes mellitus; PAD, peripheral artery disease; TIA, transient ischemic attack; ACS, acute coronary syndrome; NYHA, New York Heart Association; CAD, coronary artery disease; CRP, C-reactive protein; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TBARS, thiobarbituric acid-reactive substances. *after $\log_{10}$ transformation †$p < 0.05$.

Another observation made in this study was the negative correlation between NT-apoAI/apoAI and anti-platelet or lipid lowering drug use. To our knowledge, there are no prior reports as to the association between cardiovascular medications and NT-apoAI/apoAI ratio. Our analyses identified that aspirin and statins consuming subjects had significantly lower NT-apoAI/apoAI ratio than those not on any drugs (Fig. 2). It is known that long-term administration of aspirin significantly reduces vascular inflammation and atherosclerotic lesions by lowering macrophage-foam cell content [46–48]. Aspirin, predominantly existing in the form of salicylic acid in plasma, might play an anti-inflammatory role by scavenging free radicals [49,50]. Statins, besides their inhibitions on HMG-CoA reductase to lower serum cholesterol levels, have been shown to display anti-thrombotic, anti-inflammatory, and antioxidant activities [51–53]. Statins have been shown to be related to lower total NTyr [54,55]. It is likely that anti-inflammatory and antioxidant effects of aspirin and statins might have contributed to

Table 5
Logistic regression predictors of DM: l006Fgistic regression analysis was performed by using DM as the dependent variable, and age, gender, BMI, smoking, hypertension, dyslipidemia, CRP, TG, TC, LDL-C, HDL-C, TBARS and NT-apoAI/apoAI ratio as independent variables.

Variables	p value	aOR	95.0% C.I. for aOR
Age (years)	<0.0001*	1.051	(1.027 to 1.075)
Male gender	0.770	0.937	(0.608 to 1.446)
BMI (kg/m^2)	<0.0001*	1.081	(1.037 to 1.126)
Smoking	0.009*	2.238	(1.219 to 4.110)
Hypertension	0.002*	3.401	(1.559 to 7.418)
Dyslipidemia	0.860	0.962	(0.662 to 1.486)
CRP (mg/L)	0.456	1.002	(0.996 to 1.008)
TG (mmol/L)	0.002*	1.680	(1.215 to 2.323)
TC(mmol/L)	0.019*	0.441	(0.223 to 0.875)
LDL-C (mmol/L)	0.068	1.943	(0.952 to 3.966)
HDL-C (mmol/L)	0.585	0.822	(0.408 to 1.658)
TBARS (nmol/mL)	0.105	1.123	(0.976 to 1.292)
NT-apoAI/apoAI (mmol/mol)	0.007*	1.005	(1.001 to 1.008)

BMI, body mass index; CRP, C-reactive protein; TG, triglyceride; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TBARS, thiobarbituric acid-reactive substances; NT-apoAI/apoAI, nitrated apolipoprotein AI/apolipoprotein AI ratio; aOR, adjusted odds ratio; C.I., confidence intervals. *$p < 0.05$.

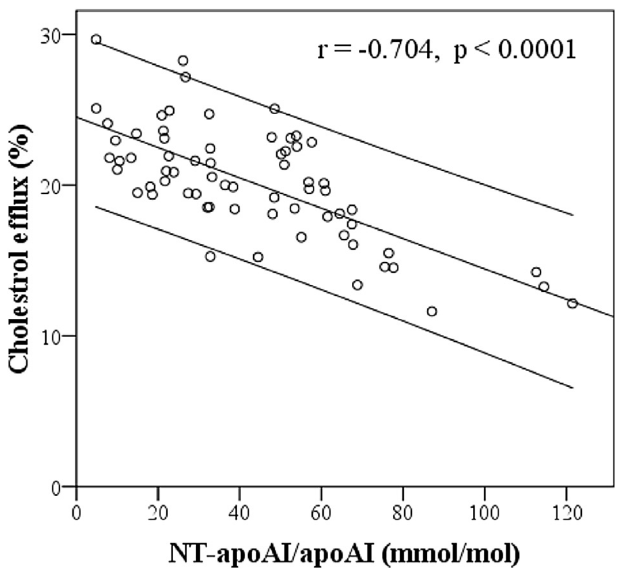

Fig. 3. Relationship between cholesterol efflux and NT-apoAI/apoAI ratio. Linear regression analysis showed a negative correlation between cholesterol efflux and NTyr-apoAI/AI levels in 67 subjects with similar apoAI levels (1.4–1.6 mg/dL).

lower NT-apoAI/apoAI ratio in individuals that were taking these drugs. However, neither aspirin (trend, $p = 0.067$) nor statin ($p = 0.625$) use was an independent predictor of NT-apoAI/apoAI ratio on multivariate analysis. This might be secondary to the high prevalence of subjects taking aspirin ($n = 563$) and statins ($n = 639$) as well as the use of other cardiovascular medications that might have limited statistical significance in detecting such differences.

This cross-sectional study provides a glimpse about different parameters at a given time point. It does not provide understanding about the progressive and/or causal relationships. A prospective study may overcome this shortcoming. This study was based on patients admitted to the clinic and therefore does not include normal subjects. Instead, this study allows differentiating between DM and non-DM subjects that were admitted to hospital for CAD. A major outcome of this study, therefore, is that it establishes a relationship between NT-apoAI/apoAI levels and DM in a relatively large German cohort with CAD.

In conclusion, in this relatively large German cohort with CAD, we report that (1) increased circulating NT-apoAI/apoAI ratio is associated with DM; (2) DM is an independent predictor of higher NT-apoAI/apoAI ratio and this ratio is independently associated with DM; (3) higher NT-apoAI/apoAI ratio is associated with higher CRP levels and TBARS levels; (4) there was a negative correlation between NT-apoAI/apoAI ratio with the use of anti-platelet and lipid-lowering drugs; and (5) higher NT-apoAI/apoAI ratio from subjects with similar apoAI levels is associated with reduced

Table 6
Group comparisons for different variables between Non-DM and DM groups in 67 selected subjects with similar apoAI level (1.4–1.6 mg/dL): Values for variables that show normal distribution are given as means ± SD and evaluated using Student t-test. Values for variables that do not show normal distribution are provided as median (25–75th percentiles) and evaluated using Kruskal Wallis test. Dichotomous and categorical variables are given as percentages and compared using Pearson's χ^2 test.

Variable	Non-DM (n = 43)	DM (n = 24)	p value
Male (N%)	34 (79.1%)	15 (62.5%)	0.142
Age (years)	65.35 ± 13.41	68.13 ± 8.49	0.363
BMI (kg/m^2)	27.49 ± 5.51	29.72 ± 5.08	0.126
Smoking (N%)	10 (23.3%)	5 (22.7%)	0.962
Clinical complications or conditions			
Hypertension (N%)	36 (83.7%)	22 (91.7%)	0.360
Dyslipidemia (N%)	24 (57.1%)	18 (75.0%)	0.147
PAD (N%)	8 (18.6%)	5 (20.8%)	0.825
Previous stroke or TIA (N%)	4 (9.5%)	4 (16.7%)	0.392
NYHA functional classification III ~ IV (N%)	12 (30.8%)	4 (18.2%)	0.283
Multi-vessel CAD (N%)	27 (67.5%)	19 (79.2%)	0.315
ACS (N%)	20 (46.5%)	14 (58.3%)	0.353
Renal failure (N%)	11 (26.8%)	12 (52.2%)	0.043†
Anti-platelet drugs			
Aspirin (N%)	17 (39.5%)	2 (8.3%)	0.008†
Clopidogrel (N%)	1 (2.3%)	3 (12.5%)	
Aspirin and Clopidogrel (N%)	20 (46.5%)	11 (45.8%)	
Lipid lowering drugs			
Statins (N%)	38 (88.4%)	21 (87.5%)	0.758
Ezetimibe (N%)	3 (7.0%)	1 (4.2%)	
Laboratory parameters			
TC (mmol/L)	4.53 ± 1.16	4.31 ± 1.15	0.543
TG (mmol/L)	1.30 (0.90–1.93)	1.10 (0.80–1.95)	0.684
HDL-C (mmol/L)	1.0 (0.8–1.3)	1.1 (0.8–1.3)	0.698
LDL-C (mmol/L)	2.82 ± 1.11	2.49 ± 0.96	0.301
CRP (mg/L)	8.9 (5.0–30.7)	7.3 (5.0–26.9)	0.461
Glucose (mg/dL)	106.50 ± 33.02	142.05 ± 68.34	0.014†
eGFR (mL/min·1.73m^2)	83.40 ± 39.55	68.76 ± 33.61	0.131
ApoAI (mg/mL)	1.50 ± 0.05	1.47 ± 0.05	0.036†
TBARS (nmol/mL)	1.86 ± 1.46	1.62 ± 1.32	0.547
NT-apoAI (nmol/L)	2164.7 ± 1242.4	2499.9 ± 1603.8	0.343
NT-apoAI/apoAI (mmol/mol)	40.30 ± 23.47	47.52 ± 30.36	0.282
Cholesterol efflux* (%)	20.77 ± 3.71	19.15 ± 3.64	0.09

DM, diabetes mellitus; BMI, body mass index; PAD, peripheral artery disease; TIA, transient ischemic attack; NYHA, New York Heart Association; CAD, coronary artery disease; ACS, acute coronary syndrome; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; TBARS, thiobarbituric acid-reactive substances; ApoAI, apolipoprotein AI; NT-apoAI, nitrated apoAI.

cholesterol efflux capacity from macrophages in vitro.

Conflict of interest

None.

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References

[1] J.A. Beckman, M.A. Creager, P. Libby, Diabetes and atherosclerosis: epidemiology, pathophysiology, and management, J. Am. Med. Assoc. 287 (2002) 2570–2581.

[2] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.

[3] A. Ceriello, New insights on oxidative stress and diabetic complications may lead to a “causal” antioxidant therapy, Diabetes Care 26 (2003) 1589–1596.

[4] J.P. Gaut, J. Byun, H.D. Tran, et al., Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry, Anal. Biochem. 300 (2002) 252–259.

[5] R.D. Hoeldtke, K.D. Bryner, D.R. McNeill, et al., Nitrosative stress, uric Acid, and peripheral nerve function in early type 1 diabetes, Diabetes 51 (2002) 2817–2825.

[6] U. Cakatay, A. Telci, S. Salman, et al., Oxidative protein damage in type I diabetic patients with and without complications, Endocr. Res. 26 (2000) 365–379.

[7] A. Ceriello, F. Mercuri, L. Quagliaro, et al., Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress, Diabetologia 44 (2001) 834–838.

[8] A. Ceriello, Nitrotyrosine: new findings as a marker of postprandial oxidative stress, Int. J. Clin. Pract. Suppl. (2002) 51–58.

[9] A. Ceriello, L. Quagliaro, B. Catone, et al., Role of hyperglycemia in nitrotyrosine postprandial generation, Diabetes Care 25 (2002) 1439–1443.

[10] L.K. Mandal, S. Choudhuri, D. Dutta, et al., Oxidative stress-associated neuroretinal dysfunction and nitrosative stress in diabetic retinopathy, Can. J. Diabetes 37 (2013) 401–407.

[11] M.H. Shishehbor, R.J. Aviles, M.L. Brennan, et al., Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy, J. Am. Med. Assoc. 289 (2003) 1675–1680.

[12] C.S. Chu, K.T. Lee, K.H. Cheng, et al., Postchallenge responses of nitrotyrosine and TNF-alpha during 75-g oral glucose tolerance test are associated with the presence of coronary artery diseases in patients with prediabetes, Cardiovasc. Diabetol. 11 (2012) 21.

[13] S. Pennathur, C. Bergt, B. Shao, et al., Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species, J. Biol. Chem. 279 (2004) 42977–42983.

[14] L. Zheng, B. Nukuna, M.L. Brennan, et al., Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease, J. Clin. Invest. 114 (2004) 529–541.

[15] B. Shao, C. Bergt, X. Fu, et al., Tyrosine 192 in apolipoprotein A-I is the major site of nitration and chlorination by myeloperoxidase, but only chlorination markedly impairs ABCA1-dependent cholesterol transport, J. Biol. Chem. 280 (2005) 5983–5993.

[16] L. Zheng, M. Settle, G. Brubaker, et al., Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages, J. Biol. Chem. 280 (2005) 38–47.

[17] J.A. DiDonato, K. Aulak, Y. Huang, et al., Site-specific nitration of apolipoprotein A-I at tyrosine 166 is both abundant within human atherosclerotic plaque and dysfunctional, J. Biol. Chem. 289 (2014) 10276–10292.

[18] R. Hermo, C. Mier, M. Mazzotta, et al., Circulating levels of nitrated apolipoprotein A-I are increased in type 2 diabetic patients, Clin. Chem. Lab. Med. 43 (2005) 601–606.

[19] E. Vazquez, A.A. Sethi, L. Freeman, et al., High-density lipoprotein cholesterol efflux, nitration of apolipoprotein A-I, and endothelial function in obese women, Am. J. Cardiol. 109 (2012) 527–532.

[20] A. Bakillah, Nitrated apolipoprotein A-I, a potential new cardiovascular marker, is markedly increased in low high-density lipoprotein cholesterol subjects, Clin. Chem. Lab. Med. 47 (2009) 60–69.

[21] J. Schroder, U. Muller-Werdan, S. Reuter, et al., Are the elderly different? Factors influencing mortality after percutaneous coronary intervention with stent implantation, Z Gerontol. Geriatr. 46 (2013) 144–150.

[22] A.S. Levey, J.P. Bosch, J.B. Lewis, et al., A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of diet in Renal Disease Study Group, Ann. Intern. Med. 130 (1999) 461–470.

[23] H. Ohshima, M. Friesen, I. Brouet, et al., Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins, Food Chem. Toxicol. 28 (1990) 647–652.

[24] J.C. ter Steege, L. Koster-Kamphuis, E.A. van Straaten, et al., Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA, Free Radic. Biol. Med. 25 (1998) 953–963.

[25] P. Wong, A. Barbeau, A.D. Roses, A method to quantitate coomassie blue-stained proteins in cylindrical polyacrylamide gels, Anal. Biochem. 150 (1985) 288–293.

[26] A. Bakillah, Z. Zhou, J. Luchoomun, et al., Measurement of apolipoprotein B in various cell lines: correlation between intracellular levels and rates of secretion, Lipids 32 (1997) 1113–1118.

[27] M.M. Hussain, Y. Zhao, R.K. Kancha, et al., Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 485–494.

[28] A.V. Khera, M. Cuchel, M. de la Llera-Moya, et al., Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis, N. Engl. J. Med. 364 (2011) 127–135.

[29] A. Ceriello, C. Taboga, L. Tonutti, et al., Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: effects of short- and long-term simvastatin treatment, Circulation 106 (2002) 1211–1218.

[30] C. Szabo, A. Zanchi, K. Komjati, et al., Poly(ADP-Ribose) polymerase is activated in subjects at risk of developing type 2 diabetes and is associated with impaired vascular reactivity, Circulation 106 (2002) 2680–2686.

[31] J.Z. Guan, N. Tamasawa, H. Murakami, et al., HMG-CoA reductase inhibitor, simvastatin improves reverse cholesterol transport in type 2 diabetic patients with hyperlipidemia, J. Atheroscler. Thromb. 15 (2008) 20–25.

[32] H.N. Yassine, A. Belopolskaya, C. Schall, et al., Enhanced cholesterol efflux to HDL through the ABCA1 transporter in hypertriglyceridemia of type 2 diabetes, Metabolism 63 (2014) 727–734.

[33] P. Linsel-Nitschke, H. Jansen, Z. Aherrarhou, et al., Macrophage cholesterol efflux correlates with lipoprotein subclass distribution and risk of obstructive coronary artery disease in patients undergoing coronary angiography, Lipids health Dis. 8 (2009) 14.

[34] P. Pajunen, M. Syvanne, G. Castro, et al., Cholesterol efflux capacity in vitro predicts the severity and extent of coronary artery disease in patients with and without type 2 diabetes, Scand. Cardiovasc. J. SCJ 35 (2001) 96–100.

[35] S. Takizawa, N. Fukuyama, H. Hirabayashi, et al., Dynamics of nitrotyrosine formation and decay in rat brain during focal ischemia-reperfusion, J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 19 (1999) 667–672.

[36] H. Hirabayashi, S. Takizawa, N. Fukuyama, et al., Nitrotyrosine generation via inducible nitric oxide synthase in vascular wall in focal ischemia-reperfusion, Brain Res. 852 (2000) 319–325.

[37] D.F. Bas, M.A. Topcuoglu, Y. Gursoy-Ozdemir, et al., Plasma 3-nitrotyrosine estimates the reperfusion-induced cerebrovascular stress, whereas matrix metalloproteinases mainly reflect plasma activity: a study in patients treated with thrombolysis or endovascular recanalization, J. Neurochem. 123 (Suppl. 2) (2012) 138–147.

[38] T.P. Misko, M.R. Radabaugh, M. Highkin, et al., Characterization of nitrotyrosine as a biomarker for arthritis and joint injury, osteoarthritis and cartilage/OARS, Osteoarthr. Res. Soc. 21 (2013) 151–156.

[39] W. Swardfager, N. Herrmann, S. Cornish, et al., Exercise intervention and inflammatory markers in coronary artery disease: a meta-analysis, Am. Heart J. 163 (2012), 666-676 e661–663.

[40] J.J. Karjalainen, A.M. Kiviniemi, A.J. Hautala, et al., Determinants and prognostic value of cardiovascular autonomic function in coronary artery disease patients with and without type 2 diabetes, Diabetes Care 37 (2014) 286–294.

[41] M. Bandeira Sde, S. Guedes Gda, L.J. da Fonseca, et al., Characterization of blood oxidative stress in type 2 diabetes mellitus patients: increase in lipid peroxidation and SOD activity, Oxidative Med. Cell. Longev. 2012 (2012) 819310.

[42] H.M. Turk, A. Sevinc, C. Camci, et al., Plasma lipid peroxidation products and antioxidant enzyme activities in patients with type 2 diabetes mellitus, Acta Diabetol. 39 (2002) 117–122.

[43] Y. Su, X.M. Liu, Y.M. Sun, et al., The relationship between endothelial

dysfunction and oxidative stress in diabetes and prediabetes, Int. J. Clin. Pract. 62 (2008) 877–882.
[44] R. Thiyagarajan, S.K. Subramanian, N. Sampath, et al., Association between cardiac autonomic function, oxidative stress and inflammatory response in impaired fasting glucose subjects: cross-sectional study, PloS One 7 (2012) e41889.
[45] E.F. Schisterman, D. Faraggi, R. Browne, et al., TBARS and cardiovascular disease in a population-based sample, J. Cardiovasc. Risk 8 (2001) 219–225.
[46] T. Cyrus, Y. Yao, L.X. Tung, et al., Stabilization of advanced atherosclerosis in low-density lipoprotein receptor-deficient mice by aspirin, Atherosclerosis 184 (2006) 8–14.
[47] H. Rus, F.I. Niculescu, Inflammation, aspirin, and the risk of cardiovascular disease, N. Engl. J. Med. 337 (1997) 423 author reply 423–424.
[48] A. Paul, L. Calleja, J. Camps, et al., The continuous administration of aspirin attenuates atherosclerosis in apolipoprotein E-deficient mice, Life Sci. 68 (2000) 457–465.
[49] L. Scheier, Salicylic acid: one more reason to eat your fruits and vegetables, J. Am. Diet. Assoc. 101 (2001) 1406–1408.
[50] N. Santanam, S. Parthasarathy, Aspirin is a substrate for paraoxonase-like activity: implications in atherosclerosis, Atherosclerosis 191 (2007) 272–275.
[51] F. Franzoni, A. Quinones-Galvan, F. Regoli, et al., A comparative study of the in vitro antioxidant activity of statins, Int. J. Cardiol. 90 (2003) 317–321.
[52] S. Wassmann, U. Laufs, K. Muller, et al., Cellular antioxidant effects of atorvastatin in vitro and in vivo, Arterioscler. Thromb. Vasc. Biol. 22 (2002) 300–305.
[53] E. Barone, G. Cenini, F. Di Domenico, et al., Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer disease: a novel mechanism of action, Pharmacol. Res. 63 (2011) 172–180.
[54] C.V. de Oliveira, V.R. Funck, L.M. Pereira, et al., Atorvastatin withdrawal elicits oxidative/nitrosative damage in the rat cerebral cortex, Pharmacol. Res. 71 (2013) 1–8.
[55] U. Singh, S. Devaraj, I. Jialal, et al., Comparison effect of atorvastatin (10 versus 80 mg) on biomarkers of inflammation and oxidative stress in subjects with metabolic syndrome, Am. J. Cardiol. 102 (2008) 321–325.