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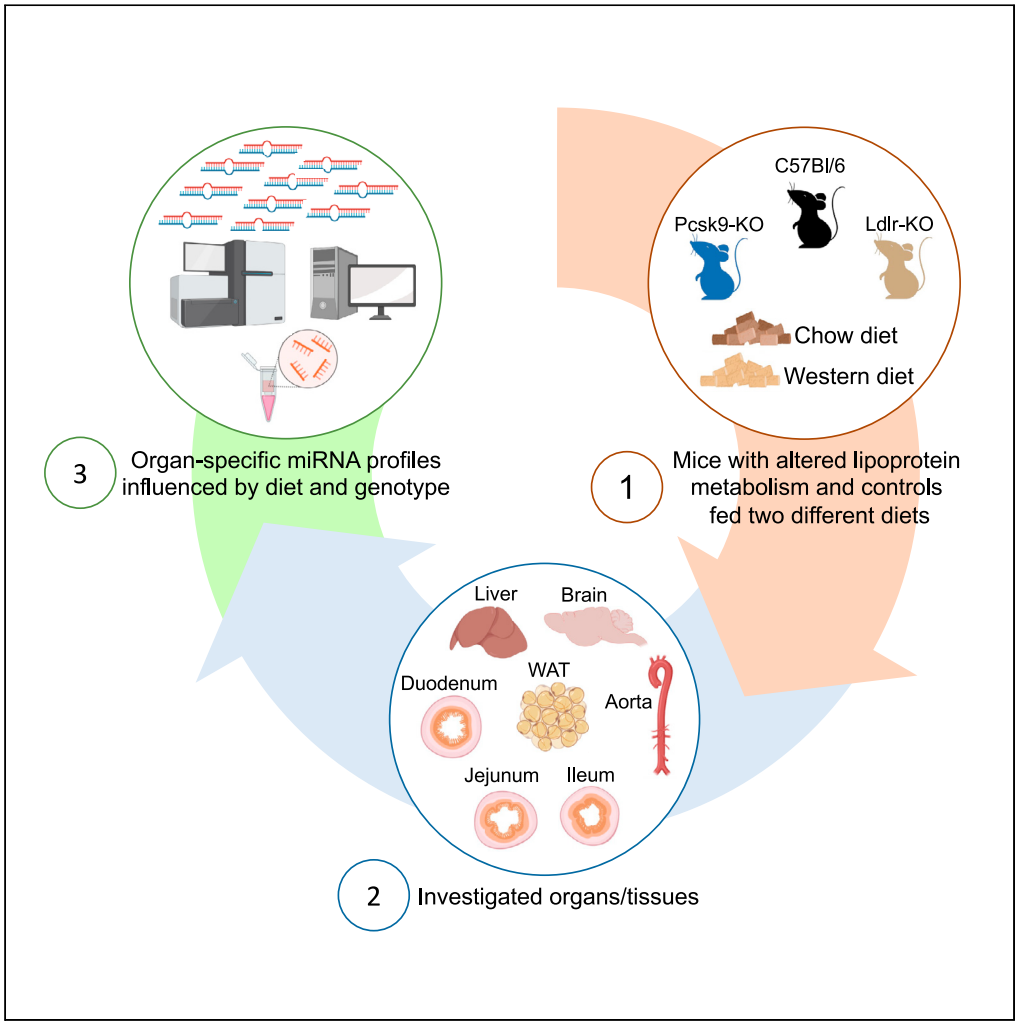

Marco Busnelli, Stefano Manzini, Alice Colombo, Elsa Franchi, ..., Gaia Zaffaroni, David Horner, Giulia Chiesa

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Highlights

Diet and genotype impact on the mouse miRNome in an organ-specific manner

No miRNAs vary consistently in the organism with different diets or genotype

Dietary lipids are more effective than genotype in driving miRNA expression changes

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Effect of diet and genotype on the miRNome of mice with altered lipoprotein metabolism

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SUMMARY

The molecular mechanism by which lipid/lipoprotein biosynthesis is regulated in mammals involves a very large number of genes that are subject to multiple levels of regulation. miRNAs are recognized contributors to lipid homeostasis at the post-transcriptional level, although the elucidation of their role is made difficult by the multiplicity of their targets and the ability of more miRNAs to affect the same mRNAs. In this study, an evaluation of how miRNA expression varies in organs playing a key role in lipid/lipoprotein metabolism was conducted in control mice and in two mouse models carrying genetic ablations which differently affect low-density lipoprotein metabolism. Mice were fed a lipid-poor standard diet and a diet enriched in cholesterol and saturated fat. The results obtained showed that there are no miRNAs whose expression constantly vary with dietary or genetic changes. Furthermore, it appears that diet, more than genotype, impacts on organ-specific miRNA expression profiles.

INTRODUCTION

Lipids are molecules with a paramount role in energy storage, cell membrane makeup, and signaling. The major physiological roles played by lipids and the catastrophic consequences resulting from a dysregulation in their biosynthesis prompts how the expression of a vast number of genes coding for transcription factors, proteins, enzymes, and receptors involved in lipid metabolism is finely regulated at the transcriptional level.[1,2] Of note, gene expression can be also controlled post-transcriptionally.

MicroRNAs (miRNAs) are a class of small (~22 nucleotides) noncoding single stranded RNAs that act as post-transcriptional regulators of gene expression.[3] miRNAs typically control the expression of their target genes by imperfect base pairing to the 3′ untranslated regions (3′ UTRs) of messenger RNAs (mRNAs), thereby inducing repression of the target mRNA through transcript destabilization and/or translational inhibition.

Single miRNAs can achieve modest effects on their mRNA targets and thus are able to slightly modulate protein expression. However, a single miRNA can have multiple target sites in the 3′ UTRs of a particular mRNA, thereby increasing repression efficiency.[2]

Predictions based on sequence similarity suggest that each miRNA can hypothetically target more than 100 different mRNAs. Indeed, human miRNAs are predicted to modulate the activity of more than 60% of all protein-coding genes.[4]

miRNA genes are localized within intergenic regions, or within introns or exons of noncoding RNAs, as well as introns of protein coding genes. Their expression can depend on the same promoter of their host genes or transcription unit, or on their independent promoter.[5,6]

Several studies have highlighted a role of miRNAs in the regulation of lipid metabolism and in the development of atherosclerosis, a pathological condition affecting the arterial wall, mostly triggered by alterations of plasma lipid levels. miR-33 has been shown to be involved in the homeostatic regulation of genes playing a role in cholesterol trafficking, such as ABCA1 (ATP binding cassette subfamily A member 1) and ABCG1 (ATP binding cassette subfamily G member 1). In states of cholesterol depletion, miR-33 expression rises, while it decreases in cholesterol enrichment, in both hepatocytes and macrophages. *In vivo* delivery of miR-33 in mice decreases hepatic ABCA1 expression and reduces circulating HDL (high-density lipoprotein). In turn, inhibition of miR-33 increases HDL levels.[7–9] The up-regulation of miR-122 in mice increases cholesterol biosynthesis whereas in the absence of miR-122 the expression of several genes important for cholesterol biosynthesis is decreased: miR-122 knockout mice display decreased circulating cholesterol levels, liver cholesterol and fatty acid synthesis and increased fatty acid oxidation.[10] Finally, miR-145, the most abundant miRNA in arteries, is significantly downregulated in atherosclerotic arteries of mice and humans.[11]

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Despite the promise of these early studies, our understanding of the impact of miRNAs in regulating lipid metabolism is still limited. Many miRNAs are expressed in a tissue-specific manner, thereby greatly contribute to physiological, cell-type specific profiles of protein expression. In this study, mice with different genotypes and fed diets with a different lipid content were enrolled with the aim of setting up an atlas of miRNA expression levels in different organs with a relevant role in lipid/lipoprotein metabolism. Specifically, three genotypes were investigated: C57Bl/6 mice as controls, together with mice knock-out (KO) for LDLr (low-density lipoprotein receptor) and for PCSK9 (proprotein convertase subtilisin/kexin type 9). LDLr and PCSK9 are both involved in LDL turnover, the former mediating LDL clearance,[12] the latter causing the degradation of the LDLr protein.[13] As a result, LDLr-KO mice, because of the impaired LDL catabolism, are hypercholesterolemic and prone to atherosclerosis development, particularly when fed high-fat, cholesterol-containing diets.[14,15] On the contrary, Pcsk9-KO mice, characterized by an accelerated LDL catabolism, are hypocholesterolemic and atherosclerosis resistant.[16] miRNA expression was investigated in liver, intestine, aorta, white adipose tissue and brain of mice on both standard and Western diet.

RESULTS

Western diet significantly increased body weight and cholesterolemia in all genotypes and dramatically worsened atherosclerosis in Ldlr-KO mice only

In each mouse line, Western diet led to a higher body weight compared to Chow diet. No significant differences in body weight were observed at sacrifice among Bl6, Ldlr-KO and Pcsk9-KO fed Chow diet, as well as Western diet (Figure 1A).

On Chow diet, liver weight was comparable in the three genotypes. In Bl/6 only, Western diet caused a significant increase with respect to all other conditions (Figure 1B). Accordingly, the ratio of liver weight to body weight was also significantly higher in Bl/6 receiving the Western diet (Figure 1C).

TC and TG plasma concentrations were much higher in Ldlr-KO with respect to the other genotypes, under both Chow and Western. On Chow diet, Bl/6 were characterized by higher plasma TC and TG than Pcsk9-KO, but no differences were observed between the two genotypes on Western diet (Figures 1D–1G).

In each mouse line, Western diet-fed mice had higher TC and TG plasma concentrations than Chow-fed mice, with the only exception of TG in Bl/6, whose plasma concentration was not affected by the diet (see Figure S1).

In Bl/6 and Pcsk9-KO mice fed Chow diet, FPLC analysis showed that cholesterol accumulated almost exclusively in the HDL (high-density lipoprotein) fractions. In contrast, in Ldlr-KO mice, a significant amount of cholesterol was also observed in VLDL (very-low-density lipoprotein) and LDL (low-density lipoprotein) fractions (Figure 1H). On Western diet, the profiles indicated increased cholesterol accumulation in the HDL fractions of all genotypes, with a concomitant dramatic increase in the VLDL and LDL fractions of Ldlr-KO mice only (Figure 1I).

Histological analysis at the aortic sinus of Bl/6 and Pcsk9-KO mice did not reveal, as expected, any atherosclerosis development with both dietary treatments. In contrast, in Ldlr-KO, a modest plaque formation was observed on Chow diet, whereas a massive atherosclerosis formation was found after Western diet (Figure 2).

The maximum modulatory effect of diet on miRNA expression occurred in Bl/6 mice

The overall assessment of DE miRNAs in the different comparisons, evaluated in 7 organs/tissues, revealed that the experimental condition in which miRNA expression was mostly affected was the comparison Bl/6 Chow vs. Bl/6 Western diet. This specific comparison showed the highest number of DE miRNAs in liver, WAT, brain, duodenum and ileum (Figure 3). The aorta showed a completely different pattern, since the highest number of DE miRNAs was found in the comparison Bl/6 vs. Ldlr-KO at Chow diet, followed by Ldlr-KO Chow vs. Ldlr-KO Western diet (Figure 3B).

A comprehensive list of all miRNAs differentially expressed in all comparisons between diets and genotypes is available in Table S1.

Western diet affected specific miRNAs depending on the murine genotype

By comparing patterns of expression of miRNAs in different organs after the switch from Chow to Western, it was found that, in Bl/6 mice, the most affected miRNA was mmu-miR-423-5p, whose expression was lower in liver, WAT, jejunum and ileum on Western diet (Figures 4A and 4B). Then mmu-miR-30a-3p showed a significantly higher expression in brain, duodenum, jejunum and ileum on Western diet (Figures 4C and 4D). Finally, the expression of mmu-miR-298-5p was much higher in WAT on Western diet, but lower with the same diet in duodenum, jejunum and ileum (Figures 4E and 4F).

In Ldlr-KO the most affected miRNAs were mmu-miR-21a-3p, whose expression was higher in aorta on Western diet and lower in brain, jejunum, ileum with the same diet (Figures 4G and 4H), and mmu-miR-21a-5p, which similarly showed higher expression in the aorta on Western diet and a concomitantly lower expression in duodenum, jejunum and ileum (Figures 4I and 4J).

In Pcsk9-KO the most affected miRNA was mmu-miR-146a-5p, whose expression was lower with Western diet in duodenum, jejunum and ileum (Figures 4K and 4L).

Each individual organ/tissue responded with a unique miRNA expression pattern to western diet administration

Liver

Considering that the Western diet greatly increased liver weight in Bl/6 mice only, it was evaluated if there were miRNAs varying their expression uniquely in this experimental group. Indeed, mmu-miR-205-5p and mmu-let-7b-5p were found to have dramatically higher expression in the liver of Bl/6 mice at Western, compared to the other two genotypes (Figure 5A).

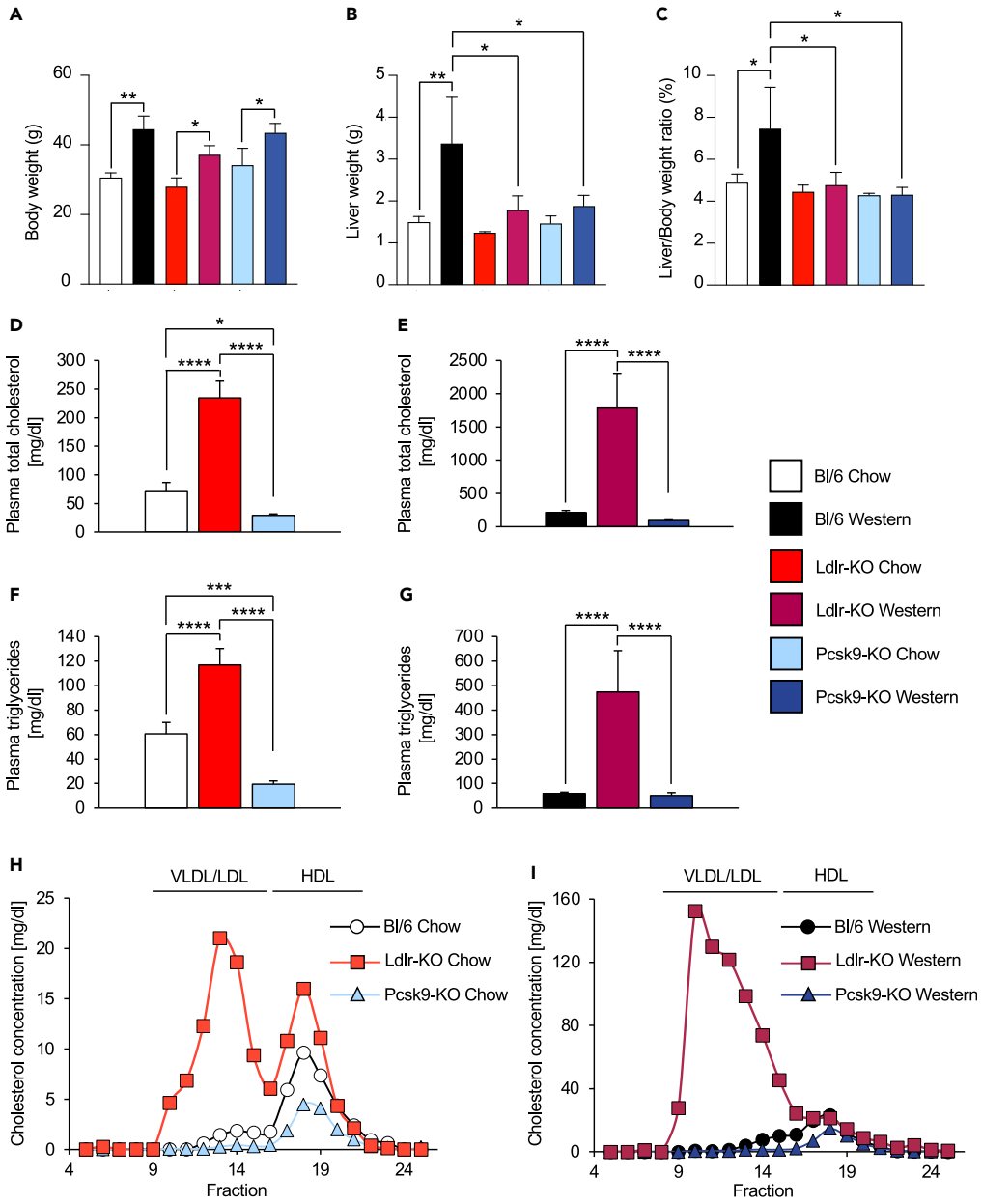

Figure 1. Body weight, liver weight and plasma lipids

(A–I) Body weight (A), liver weight (B) and liver/body weight ratio % at the end of the dietary treatments (C) are shown. Western diet increased body weight in all genotypes. Liver weight was increased only in Bl/6 mice fed Western diet. On Chow diet, Ldlr-KO mice had significantly higher cholesterolemia and triglyceridemia vs. both Bl/6 and Pcsk9-KO mice. Pcsk9-KO mice had significantly lower lipid levels than Bl/6 mice (D and F). On Western diet, Ldlr-KO mice maintained significantly higher cholesterolemia and triglyceridemia. Bl/6 and Pcsk9-KO mice were comparable to each other (E and G). Cholesterol distribution among lipoproteins by FPLC in the 3 genotypes fed Chow (H) and Western diet (I) is shown. In Chow fed Bl/6 and Pcsk9-KO mice, cholesterol accumulated in the HDL fractions only, whereas in Ldlr-KO mice a consistent amount of cholesterol was also observed in VLDL and LDL (H). On Western diet, all genotypes showed a cholesterol increase in the HDL fractions and a dramatic cholesterol accumulation in VLDL-LDL fractions was observed in Ldlr-KO mice only (I). Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by ANOVA followed by Tukey's post-hoc test.

In addition, there were 11 miRNAs whose expression varied significantly in all genotypes when comparing Chow and Western diets. Of these, 9 had lower expression levels on Western diet (mmu-miR-7a-5p, mmu-miR-381-3p, mmu-miR-96-5p, mmu-miR-136-5p, mmu-miR-434-3p, mmu-miR-409-3p, mmu-miR-300-3p, mmu-miR-127-3p, mmu-miR-431-5p), whereas 2 were more highly expressed (mmu-miR-582-5p, mmu-miR-34a-5p) (Figure 5B).

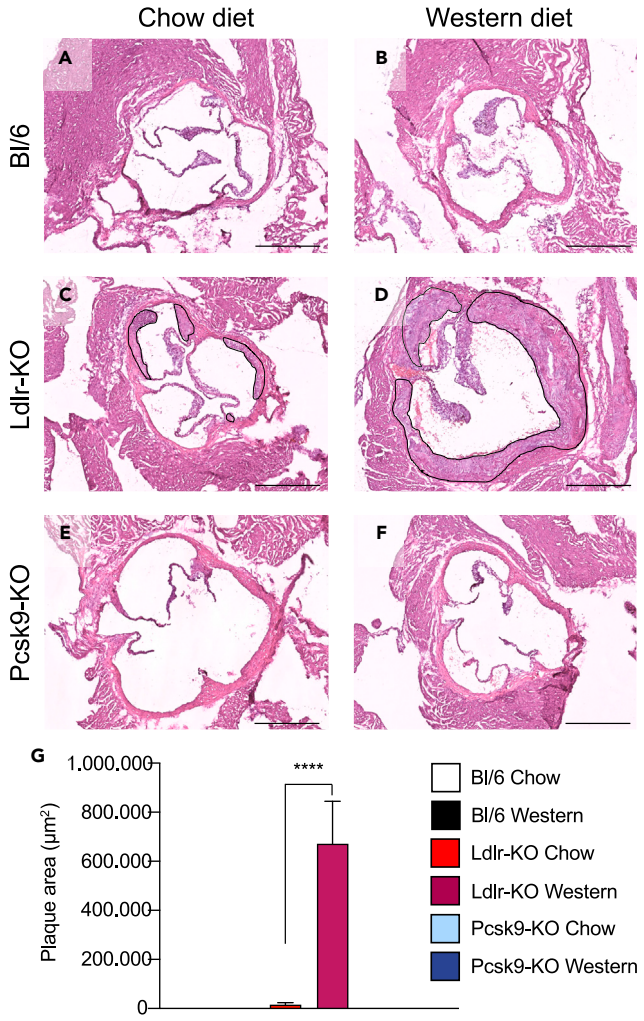

Figure 2. Histological evaluation of atherosclerosis development

(A–G) Representative hematoxylin and eosin staining of atherosclerotic plaques in the aortic sinus of Bl/6 (A and B), Ldlr-KO (C and D) and Pcsk9-KO (E and F). Only a modest plaque formation was observed in Ldlr-KO on Chow diet, whereas massive atherosclerosis was detected after Western diet (G). Bar length = 500 µm. Data are shown as mean ± SD. ****$p < 0.0001$. Significant differences were determined by unpaired t-test.

The evaluation of the predicted targets of these 11 miRNAs indicated that 7944 genes/mRNAs in total were modulated. Of these, 92 transcripts were related to lipid/lipoprotein metabolism. The individual miRNAs had a different ability in simultaneously targeting several lipid-related mRNAs, with the maximum being achieved by mmu-miR-300-3p (55 mRNAs out of 92) and mmu-miR-434-3p (60 mRNAs out of 92) (Figure S2; Table S2).

Among the mRNAs, 810 were synergistically modulated by at least 3 miRNAs (Figure S3A; Table S2). These genes/mRNAs were involved in the regulation of metabolic processes and gene expression, signaling, and protein ubiquitination (GO Term Biological Processes were considered) (Figure S3B). By specifically scanning for genes/mRNAs involved in lipid/lipoprotein metabolism, it was noted that 41 transcripts were simultaneously targeted by at least 3 miRNAs (Figure S3C).

A special feature of this study was the enrollment of Ldlr-KO and Pcsk9-KO mice, representing genetic opposites in LDL metabolism. The most interesting finding obtained from the analysis of hepatic miRNA expression between these two genotypes was the lack of expression of miRNA mmu-miR-103-3p (mmu-miR-103-3p_ID = MIM0000546_MI0000587_mmu-mir-103-1) in the liver of Pcsk9-KO mice (Figure 5C). This was the only miRNA whose expression differed significantly between Pcsk9-KO and Ldlr-KO mice on Chow diet. In contrast, following Western diet administration, 19 miRNAs, including mmu-miR-103-3p, differed between the two genotypes (Figure 5C). These include the well-known miR-33-5p and miR-146b-5p whose expression was respectively lower and higher in Western-fed Ldlr-KO compared to Pcsk9-KO mice (Figure 5C).

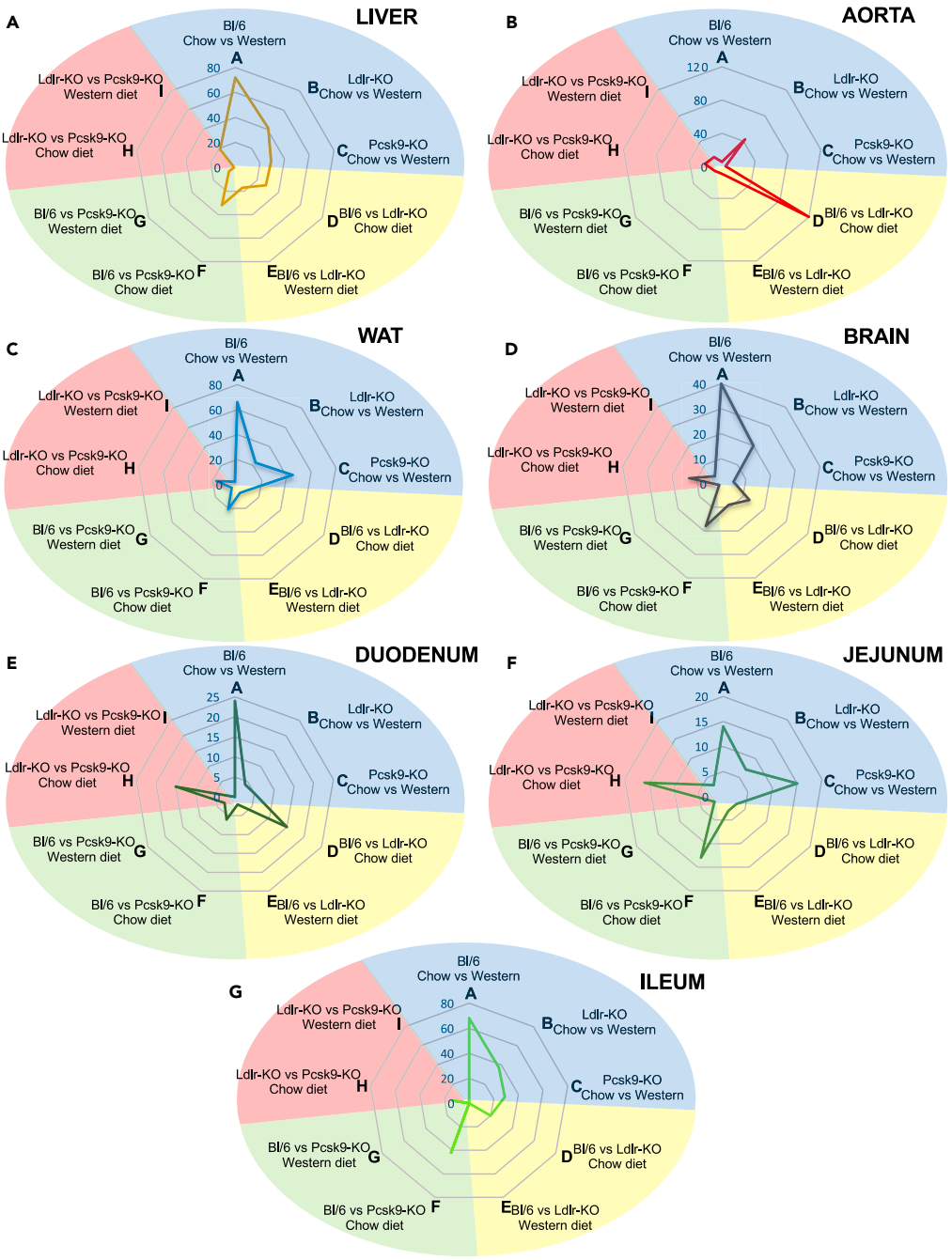

Figure 3. miRNome radar plot

(A–G) Radar plot showing how differentially expressed miRNAs are distributed in liver (A), aorta (B), WAT (C), brain (D), duodenum (E), jejunum (F), ileum (G). Blue area: comparison between Chow and Western diet in the three genotypes. Yellow area: comparison between Bl/6 and Ldlr-KO mice after the two dietary treatments. Green area: comparison between Bl/6 and Pcsk9-KO mice after the two dietary treatments. Red area: comparison between Ldlr-KO and Pcsk9-KO mice after the two dietary treatments.

Aorta

The comparison of Western diet-fed Ldlr-KO mice, where massive atherosclerosis developed, with the other genotypes on Western diet, in which plaque development was absent, showed that 7 miRNAs had consistently higher expression in mice with diseased aorta. These miRNAs include members of the miR-34 family (mmu-miR-34a, −34c-3p, −34c-5p), the paralogous miRNAs mmu-miR-221 and -222, mmu-miR-155 and mmu-miR-342 (Figure 6A).

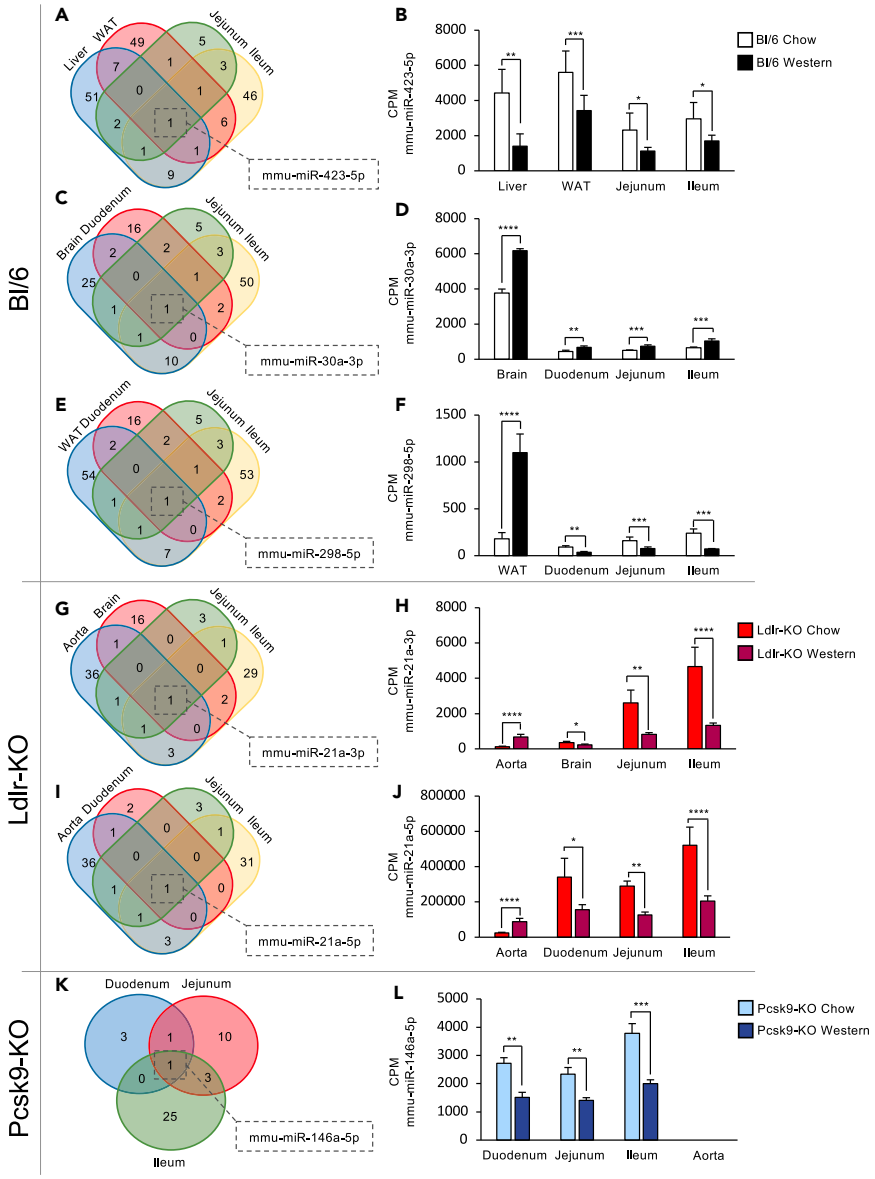

Figure 4. miRNAs affected by dietary treatment in multiple organs

(A–L) Venn diagrams show the miRNAs whose expression was modulated by diet in the highest number of organs in Bl/6 (A–F), Ldlr-KO (G–J) and Pcsk9-KO mice (K and L). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by unpaired T test.

Although plaque development in Ldlr-KO mice on Chow diet was minimal, the animals belonging to this group showed significant differences in the expression of 12 miRNAs compared to Bl/6 and Pcsk9-KO mice fed the same diet. Of these, 6 had increased expression in Ldlr-KO (mmu-miR-501-3p, mmu-miR-323-3p, mmu-miR-1948-3p, mmu-miR-409-3p, mmu-miR-425-3p, mmu-miR-540-3p), while 6 had decreased expression (mmu-miR-1191, mmu-miR-5121, mmu-miR-540-5p, mmu-miR-153-3p, mmu-miR-141-3p, mmu-miR-194-5p) (Figure 6B).

WAT

The body weight of the three genotypes was significantly higher on Western diet compared to Chow diet and the expression of 3 microRNAs was increased in all Western diet-fed mice, regardless of the genotype (mmu-miR-298-5p, mmu-miR-142-5p, mmu-miR-1983) (Figure 7A).

These three miRNAs shared 189 common targets, of which 32 were involved in adipogenesis (Figure S4; Table S2).

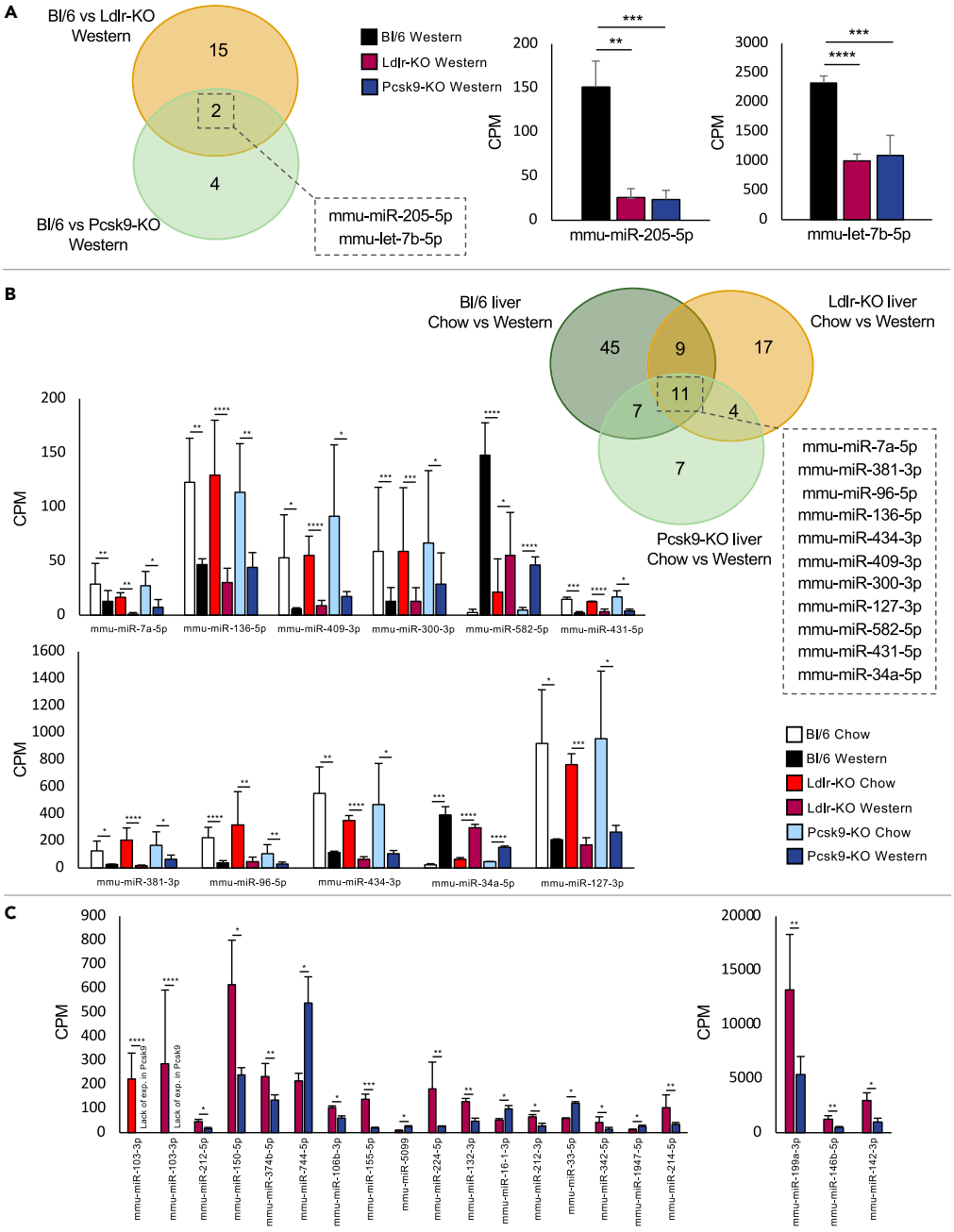

Figure 5. Top-rated miRNAs in liver

(A–C) On Western diet, the expression of mmu-miR-205-5p and mmu-let-7b-5p emerged in the liver of Bl/6 mice compared to the other experimental groups (A). The administration of the Western vs. Chow diet influenced the expression of 11 miRNAs in a genotype-independent manner (B). The specific comparison between Ldlr-KO and Pcsk9-KO mice globally showed 1 miRNA differentially expressed between the two genotypes on Chow diet and 19 miRNAs differentially expressed on Western diet. It was remarkable the trend shown by mmu-miR-103-3p whose expression was negligible in the liver of Pcsk9-KO after both Chow and Western diet (C). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by ANOVA followed by Tukey's post-hoc test or by unpaired t-test.

The expression of 4 miRNAs was significantly different in the WAT of Ldlr-KO mice compared with the other two genotypes on Chow diet (mmu-miR-378d, mmu-miR-423-5p, mmu-miR-744-5p, mmu-miR-199b-5p). Of note, the expression of mmu-miR-378d in WAT of Ldlr-KO at Chow was about a hundred times lower than in Bl6 and Pcsk9-KO (Figure 7B).

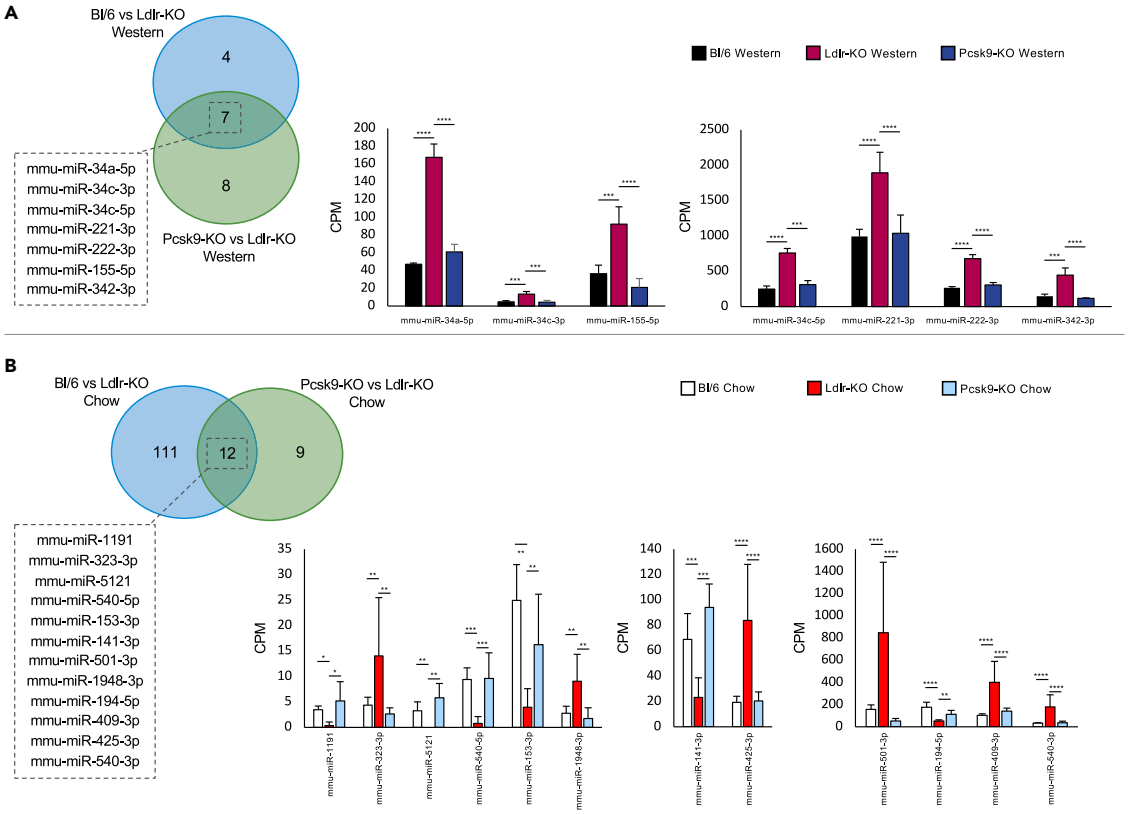

Figure 6. Top-rated miRNAs in aorta

(A and B) Seven miRNAs had significantly higher expression exclusively in aortas in which atherosclerotic plaques had developed (Ldlr-KO) (A). Also on Chow diet, 12 miRNAs had significantly altered expression in the Ldlr-KO athero-prone aortas compared with the other two genotypes (B). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by ANOVA followed by Tukey's post-hoc test.

Brain

In brain, there were no common miRNAs whose expression varied significantly between Chow and Western diet in all the genotypes (Figure 7C). Of note, DE miRNAs in Pcsk9-KO were restricted to this genotype, whereas Bl/6 and Ldlr-KO shared two miRNAs whose expression was higher on Western diet (mmu-let-7e-5p, mmu-let-7c-5p) (Figure 7C).

Intestine

The expression of miRNAs in the small intestine was assessed separately in each intestinal segment (duodenum, jejunum and ileum). In the three genotypes, the three segments responded differently to the two dietary treatments. In particular, the three genotypes did not share any DE miRNA in duodenum and jejunum (Figures 8A and 8B). In contrast, in the ileum there were 4 miRNAs whose expression was significantly modulated by diet: the levels of miR-29a-5p, miR-223-3p, miR-98-3p were always lower on Western diet, whereas that of mmu-miR-467d-5p was higher in Western-diet fed mice (Figure 8C).

The evaluation on their predicted targets indicated that 583 genes/mRNAs were modulated by at least 3 miRNAs simultaneously (Figure S5A; Table S2). As in liver, these genes/mRNAs were part of metabolic processes, gene expression and signaling (GO Term Biological Processes were considered) (Figure S5B). Twenty-two among the 583 genes/mRNAs were involved in intestinal lipid metabolism and all were modulated by mmu-miR-98-3p (Figure S5C).

Comparison of miRNA profiles in the three intestinal segments within each genotype, showed a handful of miRNAs whose expression was influenced by diet in all the three districts: in Bl/6, mmu-miR-298-5p showed a lower expression on Western diet, whereas mmu-miR-30a-3p had a consistently higher expression in Western-fed Bl/6; mmu-miR-21a-5 expression was lower in Western-fed Ldlr-KO, whereas mmu-miR-146a-5p had a lower expression in Western-fed Pcsk9-KO (Figure 9).

DISCUSSION

It is well recognized that the administration of a high-fat diet affects a wide variety of pathophysiological parameters in mouse models of atherosclerosis.[17,18] Indeed, in our study, Western diet significantly increased both the body weight and total cholesterolemia of all enrolled

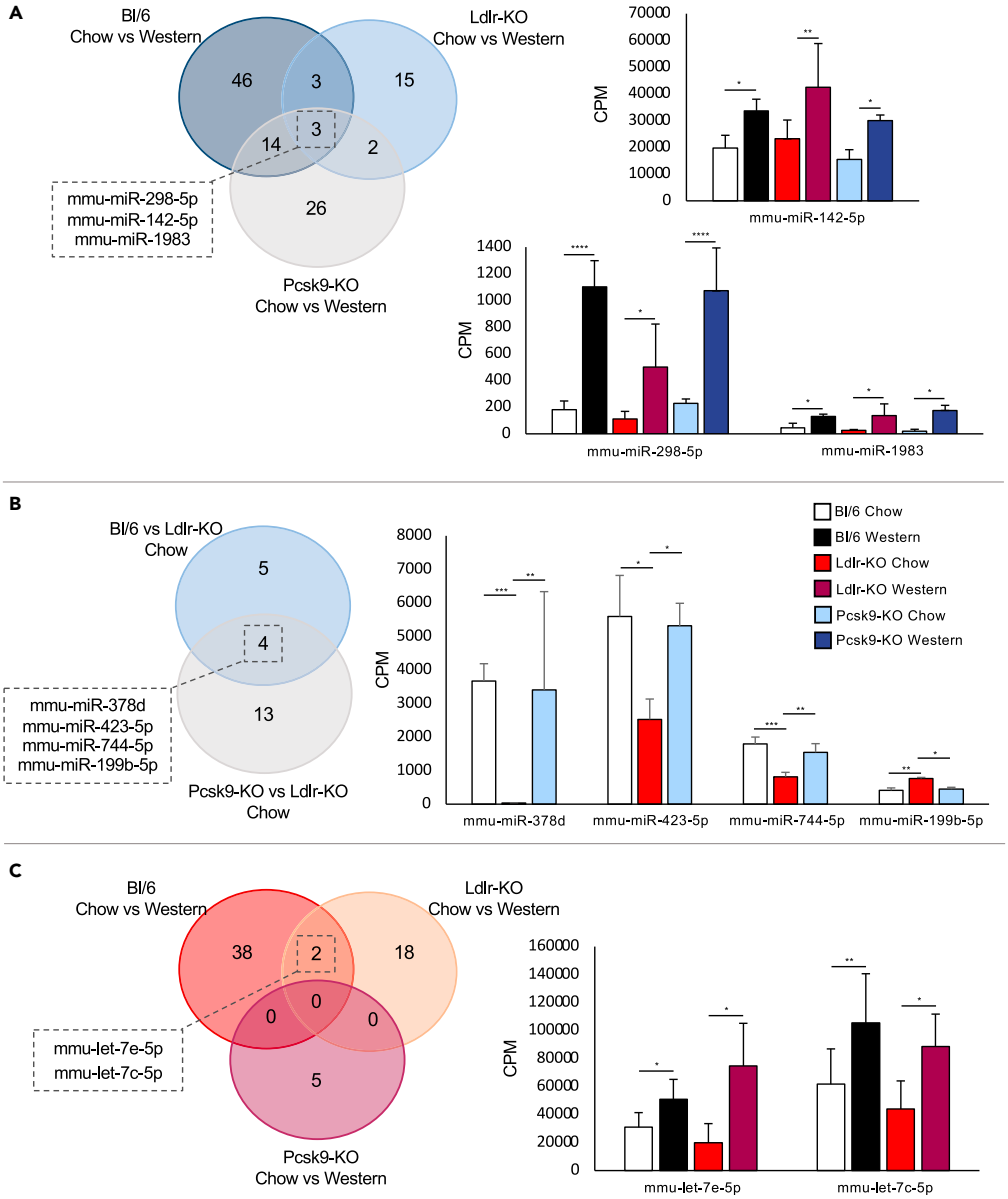

Figure 7. Top-rated miRNAs in WAT and brain

(A–C) In WAT, Western diet influenced the expression of 3 miRNAs in a genotype-independent manner (A). In Ldlr-KO fed Chow diet, the expression of 4 miRNAs differed significantly vs. Bl6 and Pcsk9-KO (B). In brain, no miRNAs were found, whose expression varied significantly between Chow and Western diet in all the genotypes (C). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by ANOVA followed by Tukey's post-hoc test.

murine models. In addition, it also significantly worsened lesion development in the only genotype prone to atherosclerosis development (Ldlr-KO).

Several studies have shown that variations in dietary components, including lipids, modulate host metabolism by affecting miRNA expression, both in humans and in experimental models.[19–23] The present study confirmed that a high-fat diet (Western) in place of a standard low-fat diet (Chow) determines a significant alteration in the expression of several microRNAs. In addition, it demonstrated that the diet-driven effects overcome those of very different genotypes/phenotypes also in the organs mostly involved in lipid/lipoprotein metabolism.

Evaluation of how miRNAs varied in the liver showed that, in the Bl/6 genotype only, switching to the Western diet abnormally increased the expression of miR-205-5p and mmu-let-7b-5p. Possible let-7b-5p-mediated effects on liver or lipid metabolism are not reported. Instead, miR-205-5p is involved in hepatic lipid metabolism, having among its targets acyl-CoA synthetase long-chain family member 1 (Acsl1) and

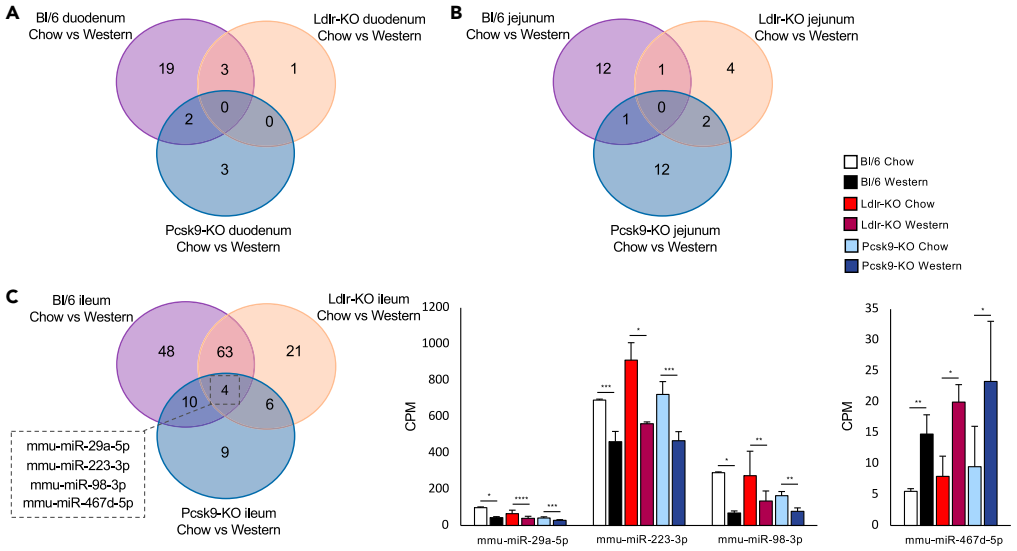

Figure 8. Top-rated miRNAs in duodenum, jejunum and ileum

(A–C) In duodenum (A) and jejunum (B), Western diet did not affect miRNA expression in a genotype-independent manner vs. Chow diet (A and B). On the contrary, in the ileum, Western diet administration influenced the expression of 4 miRNAs in a genotype-independent manner (C). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by ANOVA followed by Tukey's post-hoc test.

acyl-CoA synthetase long-chain family member 4 (Acsl4) mRNAs.[24,25] A reduced hepatic Acsl1 expression determines a marked elevation of total cholesterolemia and hepatic cholesterol accumulation. Moreover, a reduced expression of Acsl1 represses the expressions of several key enzymes involved in bile acid biosynthesis, consequently leading to reduced liver bile acid levels and altered bile acid compositions.[26] In hyperlipidemic mice, Acsl4 plays a role in triglyceride and glucose metabolism and in the hepatic synthesis of phospholipids.[27]

The expression of 11 miRNAs was altered in the liver of Western diet-fed mice of all three genotypes. Evaluation of the predicted mRNA targets indicated that several among these miRNAs were capable of synergistic action against multiple mRNAs/genes involved in lipid/lipoprotein metabolism. In particular, Zinc finger and BTB domain-containing protein 20 (Zbtb20) appeared to be affected by the modulation operated by 9 of these 11 miRNAs. Zbtb20 is a key regulator of lipid homeostasis being involved in the hepatic *de novo* lipogenesis that converts carbohydrates into triglycerides. Specifically, it has been observed that liver-specific deletion of Zbtb20 in mice ameliorates hepatic steatosis and insulin resistance induced by a high-carbohydrate diet.[28] To our knowledge, a possible miRNA-mediated modulation of hepatic Zbtb20 in the regulation of lipid metabolism has never been demonstrated. A better understanding of how this important player is regulated post-transcriptionally could make it a valuable therapeutic target for the treatment of dyslipidemia and fatty liver disease.

Among the miRNAs whose expression was decreased in Western diet-fed mice regardless of genotype, that of miR-96-5p was in accordance with previously published results showing decreased expression levels in high-fat diet-induced non-alcoholic fatty liver.[29,30] Likewise, a reduced hepatic expression of miR-409-3p in Western-fed mice is consistent with previous reports.[31]

The reduced expression of miR-434-3p in Western-fed mice is of particular interest because a previous study indicated that a prolonged treatment with a high-fat diet promoted the release of hepatocyte-derived exosomes enriched in this miRNA. However, data on the hepatic concentration of this miRNA were not reported.[32] These observations certainly require further investigation to better understand how much of the cargo found on extracellular vesicles is representative of the state of the organ from which they originate.

In the liver of mice fed the Western diet, an increased expression of miR-34a was also observed. This result is in line with previous findings indicating that miR-34a is a player in metabolic disorders and a biomarker of NAFLD, since NAFLD/NASH patients showed elevated levels of this miRNA in blood and liver.[33–36]

Additionally, several miRNAs with an involvement in hepatocyte proliferation were modulated by high-fat feeding: those promoting cell proliferation (miR-431-5p, miR-136-5p, miR-127-3p),[37–39] were upregulated, whereas the expression of miR-582-5p, a miRNA involved in the inhibition of hepatocellular carcinoma proliferation, was downregulated.[40]

Of note, the hepatic expression of miR-103-3p was totally abrogated in Pcsk9-KO mice both fed Chow and Western. The precursor (pre-miRNA) sequence of miR-103-3p is found in intron 5–6 of Pank2, the gene encoding for pantothenate kinase, the rate limiting enzyme in the biosynthesis of coenzyme A, although the expression of the mature miRNAs is not synchronized with that of the host gene.[41] Interestingly, a validated target gene for miR-103-3p is Acox1, the gene coding for acyl-CoA oxidase 1, the first and rate-limiting enzyme of the peroxisomal β-oxidation pathway.[42] Although there are no reports pointedly describing that a Pcsk9 deficiency leads to altered peroxisomal beta-oxidation, one article shows that a vaccination able to inhibit Pcsk9 activity resulted in increased Acox1 protein levels.[43] This may represent indirect

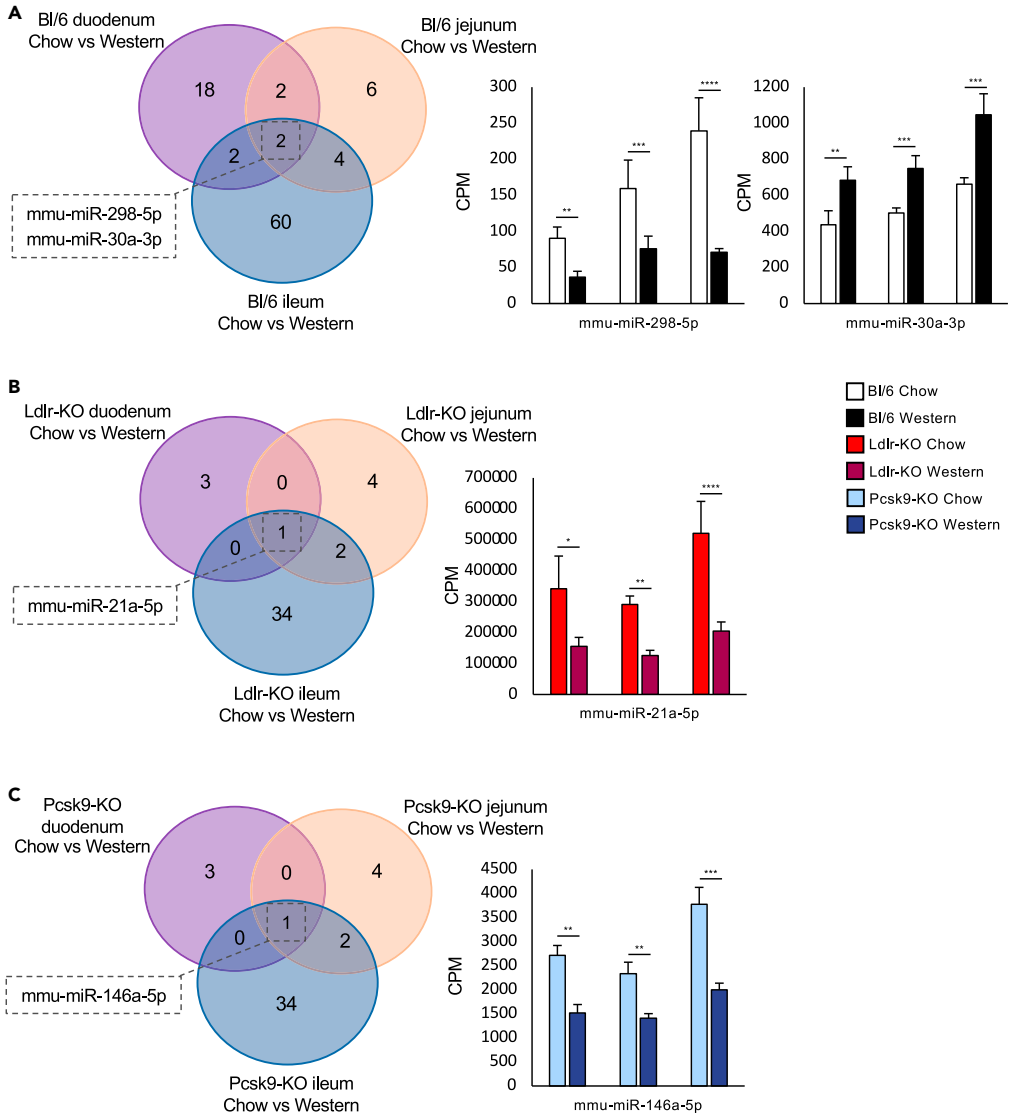

Figure 9. miRNAs modulated by diet throughout the small intestine

(A–C) Western diet administration modified the expression of some miRNAs in the three intestinal segments in a genotype-specific manner: Bl/6 (A), Ldlr-KO (B) and Pcsk9-KO (C). CPM, Counts Per Million. Data are shown as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Significant differences were determined by unpaired T test.

evidence that, regardless of how it is obtained, the lack of Pcsk9 biological action determines a reduced expression of miR-103-3p that, in turns, promotes an increase in Acox1 protein levels.

One of the highlights of the present work was a direct comparison between Ldlr-KO mice and Pcsk9-KO mice, which are - from the standpoint of lipoprotein biochemistry - the opposite of each other.

The comparison of liver miRNA profiling obtained from these two genotypes after Western diet administration showed that miR-33-5p was significantly more expressed in Pcsk9 than in Ldlr-KO mice. Since mir-33 reduces the expression of the ABCA1 transporter, a concomitant reduction in the ABCA1-dependent cholesterol efflux would be expected. This hypothesis was not investigated in the present work but a trend toward a lower ABCA1-mediated efflux was previously demonstrated in hypercholesterolemic subjects after treatment with PCSK9 inhibitors.[44]

The evaluation of aortic miRNA profile indicated that, when the arterial wall was affected by the presence of atherosclerotic plaques, the expression of seven miRNAs (miR-34a, miR-34c-3p, miR-34c-5p, miR-221, miR-222, miR-155 and miR-342) was upregulated. Corroborating the robustness of the result obtained, it is remarkable that the expression of each one of these miRNAs was higher during atherosclerosis development. In detail, it has been observed that macrophage-derived miR-155 and miR-342 are both upregulated in murine atherosclerotic plaques and downregulated during atherosclerosis regression.[45,46] The expression of miR-34 was found to be significantly upregulated in human

atherosclerotic plaques compared to healthy arteries in the Tampere Vascular Study.[47] This enhanced expression could depend on the contribution of immune cells since increased levels of miR-34 in human peripheral blood mononuclear cells are associated with the presence and extent of atherosclerosis in both coronary arteries and other arterial segments.[48] The expression of miR-221/miR-222 induces endothelial cell dysfunction[49]; consistently, it has been shown that arterial miR-221/miR-222 expression is increased in diabetic mice showing intimal thickening.[50] However, the biology of miR-221/miR-222 in atherosclerosis still needs to be fully elucidated, since it was recently observed that the expression of these miRNA in the shoulder regions of plaques undergoing rupture is decreased.[51]

Twelve miRNAs showed significantly altered expression in Ldlr-KO mice compared to both Bl/6 and Pcsk9-KO even after Chow diet administration and early plaque development. To our knowledge, there is no report showing any involvement in atherosclerosis development or arterial wall biology for none of these miRNAs.

Western diet administration significantly increased the expression of three miRNAs in WAT in a genotype-independent manner sharing several potential target mRNAs involved in adipose tissue homeostasis and conversion from white to brown adipose tissue. Among them, miR-142-5p is involved in the regulation of adipogenesis by modulating the expression of Bmp-4 and Fgf10 target genes.[52]

Interestingly, in WAT of Ldlr-KO mice fed Chow diet, the expression of miR-378d was dramatically downregulated compared with that observed in Bl/6 and Pcsk9-KO. Mir-378d originates from the first intron of the peroxisome proliferator-activated receptor gamma, coactivator 1 beta (*Ppargc1b*) gene coding for PGC-1beta,[53] representing an important regulator of brown adipose tissue differentiation and activity.[54]

The analysis of the small intestine showed that, limited to the ileum, in all three genotypes the administration of the Western diet instead of Chow impaired the expression of 4 miRNAs. Similar to what was observed in the liver, some among their possible target mRNAs play a key role in intestinal lipid metabolism and could be modulated by the simultaneous action of multiple miRNAs. In particular, the expression of miR-29a-5p was significantly reduced by the Western diet. miR-29a regulates SLC5A8 expression in intestinal epithelial cells.[55] SLC5A8 is expressed in ileum and colon where it mediates the active absorption of short-chain fatty acids.[56] A second miRNA whose expression was reduced in the ileum of mice fed Western diet, regardless of genotype, was miR-223-3p. Previous studies have shown that a reduced miR-223-3p expression is associated with inflammatory bowel conditions.[57] Since it is known that a high-fat diet can induce intestinal low-grade inflammation associated to increased intestinal permeability and oxidative stress,[58] it is possible that a reduced miR-223-3p expression may contribute to these features.

In addition, the results obtained revealed that the expression of certain miRNAs (mmu-miR-298-5p, mmu-miR-30a-3p, mmu-miR-21a-5p, mmu-miR-146a-5p) varied, along the whole small intestine, by changing dietary lipid content, in a genotype-specific manner. These miRNAs have never been identified as playing a role in intestinal pathophysiology, but, interestingly, they exhibited a role in the regulation of several metabolites during the pathogenesis of NAFLD.[59–61] Whether these miRNAs also play a role in the regulation of small intestinal lipid metabolism certainly deserves further investigations.

In conclusion, the present study aimed to generate an atlas of miRNA expression levels in several organs involved in lipid/lipoprotein metabolism by studying mice with three different genotypes characterized by a very different lipoprotein metabolism, fed two diets with distinct lipid content.

According to the results obtained, our study demonstrates that: (1) diet- and genotype-determined dyslipidemic conditions impact on the miRNome in an organ-specific manner; (2) there is no miRNA whose expression consistently varies throughout the organism with variations of dietary lipid content or genotype; (3) dietary lipids represent a much more effective driver than genotype in altering miRNA expression.

Limitations of the study

Our study design privileged the diversity and variety of the experimental conditions over the number of biological replicates. However high the number of biological replicates, high-throughput approaches always deal with a number of features far exceeding the number of samples. In the present study, we used a limited number of samples; nonetheless, we applied robust statistical methods specifically designed to manage this kind of data and applied strict thresholds to mitigate these issues.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107615.

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AUTHOR CONTRIBUTIONS

Conceptualization: S.M., M.B., and G.C.; Software: M.C. G.F., and D.H.; Formal Analysis: M.B., S.M., and M.C.; Investigation: S.M., M.B., E.F., and A.C.; Resources: G.C.; Writing – Original draft: M.B.; Visualization: M.B.; Supervision: G.C.; Project Administration: G.C.; Funding Acquisition: S.M., M.B., and G.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	Merial	N01AB06
Haematoxtlin	Bio-Optica	05–06002
Eosin	Bio-Optica	05–10003
Trizol	Invitrogen	Cat. no.: 15596026
Critical commercial assays		
Cholesterol quantification kit	HORIBA	A11A01634
Triglycerides quantification kit	HORIBA	A11A01640
NucleoSpin RNA columns	Macherei-Nagel	740955.50S
NucleoSpin miRNA	Macherei-Nagel	FC140971L
TaqMan® MicroRNA Assay: hsa-miR-34a	ThermoFisher Scientific	Cat. no.: 4427975 ID: 000426
TaqMan® MicroRNA Assay: hsa-miR-127	ThermoFisher Scientific	Cat. no.: 4427975 ID: 002229
TaqMan® MicroRNA Assay: mmu-miR-434-3p	ThermoFisher Scientific	Cat. no.: 4427975 ID: 002604
TaqMan® MicroRNA Assay: mmu-miR-136	ThermoFisher Scientific	Cat. no.: 4427975 ID: 002511
TaqMan® MicroRNA Assay: snoRNA234	ThermoFisher Scientific	Cat. no.: 4427975 ID: 001234
TaqMan™ MicroRNA Reverse Transcription Kit	ThermoFisher Scientific	Cat. no.: 4366596
TaqMan™ Fast Advanced Master Mix for qPCR	ThermoFisher Scientific	Cat. no.: 4444556
TruSeq small RNA sample Preparation kit	Illumina	RS-200-0012
Deposited data		
Fastq files	NCBI GEO	GSE227578
Experimental models: Organisms/strains		
Mouse: C57BL6/J	JAX/Charles River Laboratories	Strain: #:000664; RRID:MGI:5655668
Mouse: Ldlr-KO	JAX/Charles River Laboratories	Strain #:002207; RRID:MGI:4443062
Mouse: Pcsk9-KO	JAX/Charles River Laboratories	Strain #:005993; RRID:MGI:3577440
Software and algorithms		
cutAdapt	(Martin, 2011)[62]	https://cutadapt.readthedocs.io/en/stable/
bowtie	(Langmead, 2010)[63]	https://bowtie-bio.sourceforge.net/index.shtml
DESeq2	(Love et al. 2014)[64]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
Prism 9	GraphPad	https://www.graphpad.com/
NDP.view2	Hamamatsu Photonics	https://www.hamamatsu.com/jp/en/product/life-science-and-medical-systems/digital-slide-scanner/U12388-01.html
Other		
Diet: standard rodent chow	Mucedola	4RF21
Diet: Western-type	Envigo	TD.88137

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Marco Busnelli (marco.busnelli@unimi.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- RNAseq data have been deposited to Gene Expression Omnibus (GEO) and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals and diets

Procedures involving animals and their care were conducted in accordance with institutional guidelines, in compliance with national (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). The experimental protocol was approved by the Italian Ministry of Health.

C57BL/6J (Bl/6, RRID:MGI:5655668) male mice, Low-Density Lipoprotein receptor gene knock-out (Ldlr-KO, RRID:MGI:4443062) and Proprotein convertase subtilisin/kexin 9 gene knock-out (Pcsk9-KO, RRID:MGI:3577440) male mice in the C57BL/6 background were purchased from Charles River Laboratories (Calco, Italy). Mice (n = 3 per group) were housed at constant temperature and relative humidity. From the sixth week from birth, mice had free access to either standard rodent chow containing 3% fat (4RF21, Mucedola, Settimo Milanese, Italy) or Western-type diet containing 0.2% cholesterol and 21% fat (TD.88137, Envigo, Bresso, Italy) and tap water to 22 weeks of age.

METHOD DETAILS

Plasma and tissue harvesting

After an overnight fast, blood was collected from the retro-orbital plexus, under 2% isoflurane anesthesia (Forane, Abbot Laboratories Ltd, Illinois, USA), into tubes containing 0.1% (w/v) EDTA and centrifuged for 10 min at 5,900 × g at 4°C.

At the end of the dietary treatments, mice were anesthetized with 2% isoflurane and blood was removed by perfusion with PBS. Aorta was rapidly harvested.[65] Liver, abdominal white adipose tissue (WAT), brain, duodenum, jejunum and ileum were snap-frozen in liquid nitrogen for subsequent molecular analyses.

Biochemical analyses

Plasma total cholesterol (TC) and triglycerides (TG) were measured with an enzymatic method (ABX Diagnostics, Montpellier, France; COD: CPA11 A01634 and CPA11 A01640, respectively). Cholesterol distribution among lipoproteins was analyzed by fast protein liquid chromatography (FPLC).[66]

Histology of the aortic sinus

Hearts were removed, fixed in 10% formalin, and processed.[67] Serial cryosections (7 micron thick) of the aortic sinus were cut and stained with haematoxylin and eosin (H&E, Bio-Optica, Italy) to detect plaque area. The Nanozoomer S60 (Hamamatsu Photonics, Japan) scanner was used to acquire digital images that were subsequently processed with the NDP.view2 software (Hamamatsu Photonics, Japan). Two operators blinded to dietary treatments quantified histological results.[68]

RNA extraction

Total RNA and miRNAs were extracted simultaneously with NucleoSpin miRNA columns (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions following Trizol protocol for tissue lysis (Life Technologies/Invitrogen, Monza, Italy) and quantified with Nanodrop ND-1000 (Marshall Scientific). Large and small RNA samples were checked by gel electrophoresis, and by calculating the absorbance ratios at 260 nm/280 nm and 260 nm/230 nm; acceptable ranges were considered 1.95–2.10 and 1.90–2.3, respectively.

miRNA retrotranscription and qPCR

The sequencing results were validated on 4 selected hepatic miRNAs (Figure S6). 1000 ng of total RNA (containing the small RNA fraction) were retrotranscribed using the TaqMan MicroRNA Reverse Transcription Kit (Cat. no. 4366596, Life Technologies, Segrate, Italy), according to the manufacturer's modified protocol "Custom Reverse Transcription Pools" (publication number 4465407, revision E). Briefly, 10 μL of each TaqMan MicroRNA Assay 5× RT primer of interest (hsa-miR-34a, hsa-miR-127, mmu-miR-434-3p, mmu-miR-136 and snoRNA234, with assay IDs 000426, 002229, 002604, 002511 and 001234, respectively) were pooled to a total volume of 1000 μL of H_2O. Each RT reaction was assembled with 6.0 μL RT primer pool, 0.31 μL dNTPs, 3.0 μL Reverse Transcriptase, 1.50 μL 10× RT Buffer, 0.19 μL RNase inhibitor, 4 μL of total RNA (250 ng/μL), totalling 15 μL. Thermal conditions were 30 min at 16°C, 30 min at 42°C, 5 min at 85°C.

20 ng cDNA were used to quantify by qPCR (CFX Connect, Bio-Rad, Segrate, Italy), in triplicate, the abundance of miRNAs relative to snoRNA234, chosen as reference. Fold changes were calculated with the ΔΔCt method.[69] Each reaction was assembled with 0.5 μL TaqMan MicroRNA Assay 20× primers and probe, 5.0 μL TaqMan Fast Advanced Master Mix (Cat. no. 4444556, Life Technologies, Segrate, Italy), 0.5 μL water and 4 μL cDNA (5 ng/μL), totalling 10 μL. Thermal conditions were 20 s at 95°C, followed by 3 s at 95°C, 30 s at 60°C for 40 cycles. RT no-template control and water were used as negative qPCR controls.

miRNA sequencing and data processing

Indexed cDNA libraries from the RNA fraction at low molecular weight were prepared using the TruSeq small RNA sample Preparation kit (Illumina, San Diego, CA) according to the manufacturer's protocol and recommendations. Single end sequencing (1 × 50 bp), after fluorimetric quantification, was performed on an Illumina Hiseq II platform.

Raw sequence data were processed with cutAdapt[62] with default parameters to remove adapters from inserts of between 14 and 38 nt in length. Trimmed reads were mapped to human mature miRNA sequences, downloaded from miRBase (v21), using bowtie[63] with the following parameters: -n 0 -L 17 –best -strata. Only miRNAs with a read count higher than 10 in at least 1 sample were considered in the differential expression analysis. A detailed quality metrics of the libraries, including total number of reads, total number of trimmed reads, total number of reads assigned to miRNA, and the breakdown of trimmed reads by size is reported in Table S3. TargetScanMouse Release 8.0 was used to predict mRNA targets of differentially expressed miRNAs.[70]

QUANTIFICATION AND STATISTICAL ANALYSIS

Differential expression analyses were performed by DESeq2 using default parameters.[64] An FDR (False Discovery Rate) threshold of 0.05 or lower was applied to identify miRNAs showing a statistically significant differences. Beyond those related to miRNA expression, statistical analyses were performed with GraphPad Prism 9 and significant differences were determined by ANOVA followed by Tukey's post-hoc test or by unpaired T test, according to the check of normality of residuals.