MODULATION OF MIR-33 AND MIR-122 BY DIETARY POLYPHENOLS

Laura Baselga Escudero

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PhD DOCTORAL THESIS

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FEM CONSTAR que aquest treball titulat "**Modulation of miR-33 and miR-122 by dietary polyphenols**", que presenta Laura Baselga Escudero per a l'obtenció del títol de Doctor, ha estat realitzat sola la nostra dirección al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requeriments per poder optar a Menció Internacional.

Tarragona, 14 de maig de 2013

La directora de la tesi doctoral

Dra. M. Cinta Bladé Segarra

La codirectora de la tesis doctoral

Dra. Anna Arola Arnal

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A mis padres y

a Jaume

"La ciencia es la estética de la inteligencia"

Gastón Bachelard

CONTENT

ABCA1: ATP-binding cassette transporter A1

BA: Bile acid

CE: Cholesterol ester

CM: Chylomicrons

CPT1: Carnitine palmitoyl transferase 1

CVD: Cardiovascular disease

ER: Endoplasmatic reticulum

FA: Fatty acid

FFA: Free fatty acid

FAS: Fatty acid synthase

FXR: Farnesoid X receptor

GSPE: grape seed proanthocyanidin extract

HDL: High Density Lipoprotein

LDL: Low Density Lipoprotein

LPL: Lipoprotein Lipase

LXR: Liver X receptors

miRNA: microRNA

miR-122: microRNA-122

miR-33: microRNA-33

NEFAs: Non-esterified fatty acids

PA: Proanthocyanidin

PPAR: Peroxisome Proliferator-Acvtivated Receptor

PPARα: Peroxisome Proliferator-Acvtivated Receptor alpha

PPARγ: Peroxisome Proliferator-Acvtivated Receptor gamma

PPARδ: Peroxisome Proliferator-Acvtivated Receptor omega

PL: Phospholipids

RCT: Reverse cholesterol transport

SHP: Small heterodimer partner

SREBP: Sterol regulatory element-binding protein

SREBP-1a: Sterol regulatory element-binding protein 1a

SREBP-1c: Sterol regulatory element-binding protein 1c

SREBP-2: Sterol regulatory element-binding protein 2

TG: Triacylglycerides

VLDL: Very Low Density Lipoprotein

1. Overview of miRNAs

MicroRNAs (miRNAs) are small single stranded non-coding RNAs of 18-25 nucleotides in length that regulate gene expression at post-transcriptional level [1]. The study of miRNAs and their effects on translation is one of the most novel and active areas of epigenetic research. These small molecules were first discovered in the nematode *Caenorhabditis elegans* in 1993 by Ambros V et al. [2]. Since then, new miRNAs are continuously identified in the genomes of most plants, viruses and animals [1, 3, 4]. To date, more than 25,141 mature miRNAs sequences have been recorded in the miRBase database, from which 2,246 are from human and 954 from rats (www.mirbase.org/ v.19), May 2013). miRBase is the central online repository for miRNA nomenclature, sequence data, annotation and target prediction [5]. Regarding to miRNAs nomenclature, the numbering of miRNA genes is simple sequential identifiers and the 3-4 letters prefix of the annotation refers to the specie (e.g.; hsa- for *Homo sapiens* or rno- for *Rattus norvegicus*). The mature miRNA is designated "miR", whereas "mir" refers to the precursor hairpirins. Different precursor sequences or genomic loci that express identical mature sequences have numbered suffixes of the form has-mir-121-1 and has-mir-121-2, whereas lettered suffixes (e.g.; hsa-miR-121a and hsa-miR-121b) denote closely related mature sequences that differ only in few positions. Moreover, when two sequences of miRNAs are originated from the same predicted precursor and the relative abundances indicate which is the predominantly miRNA, the mature sequences are assigned names of the form miR-121 (the predominant product) and miR-121* (from the opposite arm of the precursor). However, when the data are not sufficient to determine which sequence is the predominant, these are named like miR-121-5p (from the 5' arm) and miR-121-3p (from the 3' arm). However, there are several exceptions, such as let-7 and lin-4 [6].

2. Biogenesis of miRNAs in animals

miRNAs are transcribed from the nucleus from individual miRNA genes that are located in intra- or inter- regions of protein-coding genes [1] through canonical or non-canonial pathways.

The canonical pathway is driven by RNAse enzymes and generates the majority of animal miRNAs [7] (Figure 1). From the miRNA gene, mainly the RNA polymerase II produces the primary miRNA (pri-miRNA) that adopt a hairpin RNA structure of hundreds of nucleotides long and, it is possible that various pri-miRNA hairpins are

encoded by the same transcript. The pri-miRNAs is further processed in the nucleus by the nuclear ribonuclease III enzyme Drosha. Specifically, the dsRNA binding protein DGCR8 recognizes and interacts to the proximal ~10 bp of stem of the pri-miRNA

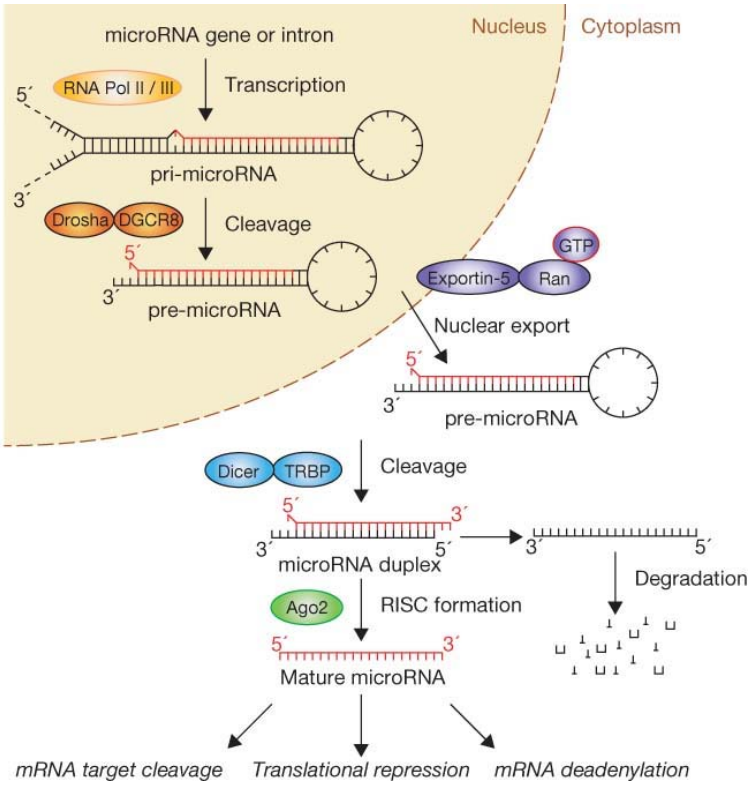

Figure 1. *miRNA biogenesis through canonical pathway* [7].

hairpin, positioning the catalytic sites of Drosha [8]. The Drosha cleavage releases a pre-miRNA hairpin of approximately 70-100 nucleotides stem-loop, which are subsequently transported from the nucleus to the cytoplasm via Exportin-5 (Exp-5). Once in the cytoplasm, the pre-miRNA is subsequently cleaved by the ribonuclease III endonuclease Dicer to produce an approximately 22 nt duplex [7, 9]. After, this short RNA duplex exclude the complementary miRNA* strand, which is generally degraded, while the other strand, known as the mature miRNA, is incorporated onto a RNA-induced silencing complex (RISC) associated to Argonaute (Ago) [7, 9], which guide mature miRNAs binding to its target gene, thereby mediating the mRNA

degradation or inhibition of its protein transcription. As previously mentioned, miRNAs genes can be located intra- or inter-genetics. When the miRNAs are intra-genetics are mainly hosted in introns of protein-coding genes. These intronic miRNAs are known as mirtrons and, in this situation a non-canonical biogenesis pathway (Figure 2) can occur.

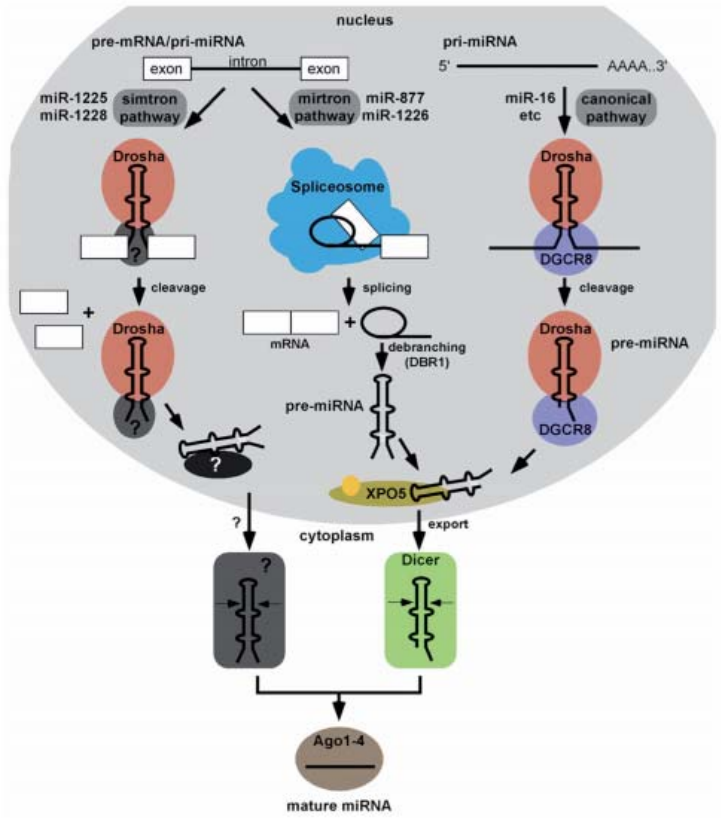

Figure 2. *miRNA biogenesis through non-canonical VS canonical pathway [10]. Left: simtron pathway, Middle: mirtron pathway, Right: canonical miRNA pathway.*

Mirtrons are processed from the host gene without cleavage by Drosha neither DGCR8, but by a splicing that generates a non-linear intermediate that must be resolved by the lariat debranching enzyme before the hairpin structure can be adopted. At this step, mirtron products appear as pre-miRNA mimics and enter the canonical biogenesis pathway as Exp-5 and Dicer substrates [10]. Recently, another alternative miRNA processing pathway, known as the simtron (splicing-independent mirtron-like miRNAs) pathway, has been described. miRNAs processed by the simtron pathway are bound and processed by Drosha and another unknown protein similar to DGCR8, and does not requires splicing, neither the multiprotein complex, Dicer nor Ago [11].

Both simtrons and mirtrons pathways produce mature miRNAs and function in silencing of target transcripts.

3. Cellular functions of miRNAs

Mature miRNA binds to its target genes guided by RISC and Ago controlling the expression of their target genes by mainly acting as sequence specific inhibitors of messenger RNAs (mRNAs) [12]. In animals, the base paring is between approximately 8 nt of the 5′ sequence of mature miRNAs (known as seed sequence) and the recognition element in the 3′UTR (untranslated region) of their target mRNA [1, 9, 12]. Depending on the degree of complementarity between miRNA-mRNA, the miRNA will induce mRNA degradation, traductional repression or deadenylation (Figure 1). Specifically, if there is a high or total miRNA-mRNA complementarity the mRNA will be degraded [11], whereas if the complementarity is insufficient there will be a traductional inhibition. However, recent studies have demonstrated that miRNAs can also silence mRNA targets through binding to other regions, including 5'UTRs or protein-coding exons [13-15]. This, together with the partially complementary binding between the miRNA seed sequence and the mRNA involves that a single miRNA can have multiple binding sites on several mRNAs and that one mRNA can be bound by more than one miRNA. Therefore, considering that to date thousands of miRNAs have been discovered in humans, miRNAs are thought to modulate more than 60% of human genes [16]. Hence, it is not surprising that these tinny molecules demonstrate to have important regulatory roles in a variety of biological processes and thus, that an aberrant deregulation of some miRNAs has been related to metabolic disorders and other diseases. However, the specific role of each miRNA in controlling metabolic pathways is still unknown, and most studies have focused on lipid metabolism. Moreover, apart from lipid metabolism there are several miRNAs that influence glucose and amino acid metabolisms [17, 18]. Other miRNAs do not directly affect metabolism, but instead target nuclear receptors; for example, miR-613 targets the nuclear liver X receptor (LXR) [19].

Hence, intracellular miRNAs, mainly expressed in a tissue- or a developmental stage-specific manner [20], have showed to have a crucial role in cell functionality, but interestingly, extracellular miRNAs have also been identified stable in most biological fluids including blood, urine and saliva. These circulating miRNAs are exported selectively to recipient cells where these tinny molecules are functional (i.e.; alter the genes and functions of recipient cells). miRNAs circulating in the plasma are remarkably stable because they circulate packed inside microparticles (microvesicles,

exosomes and apoptotic bodies) [21, 22] or associated with RNA-binding proteins [23] or lipoprotein complexes [24]. Because miRNAs circulate with microparticles, they may function in cell-to-cell communication. However, the intercellular communication of miRNAs is still not totally understood [25]. Furthermore, miRNAs can be transmitted from one species to another, inducing post-transcriptional gene silencing in distant species, even in a cross-kingdom fashion [26]. Specifically, exogenous plant miRNAs in food have been demonstrated to regulate the expression of target genes in mammals. For example, it has been described that miR-168a, which is the most abundant miRNA in rice, can bind to the human and mouse LDL receptor adapter protein 1 mRNA, inhibiting its expression in liver [27].

Considering all these together, it is not surprising that increasing evidence shows that miRNAs are involved in almost all biological processes and affect most metabolic pathways. Hence, aberrant deregulation of some miRNAs has been related to metabolic disorders and other diseases such as human immunodeficiency virus, cancer, hepatitis C, obesity, cardiovascular diseases (CVD), nonalcoholic fatty liver diseases (NAFLD), and type 2 diabetes (T2D). In this sense, miRNAs are emerging as potential biomarkers of numerous pathologies and therefore, as new therapeutic targets.

CVD is the leading cause of human morbidity and mortality in industrialized countries. These diseases are associated with genetic mutations or deregulation of genes essential for cardiac function, which can also be regulated by miRNAs. Multiple miRNAs important for cardiovascular regulation have been identified and are recognized to control a considerable number of cardiac functions. Furthermore, miRNAs are emerging as potential targets for the diagnosis, prevention, and treatment of CVD (reviewed in [28, 29]). Although specific patterns of miRNA expression correlate well with cardiovascular disorders (e.g.; cardiac hypertrophy, myocardial infarction and cardiac fibrosis) [30-32], the mechanisms and alteration of CVD are complex, and it is unclear which miRNAs are important. However, evidence is mounting that some specific miRNAs have a major role in cardiac pathologies, including miR-1 and miR-133 in cardiac hypertrophy [33] and the miR-29 family in cardiac fibrosis [34]. One of the most common and important cardiovascular health problems is hypertension, which is defined as a constant elevation of systemic blood pressure. Many characteristics of hypertension development at the molecular level are still unknown, but it is evidently a multifactorial disease that involves several genes. In this sense, miRNAs are likely to have a potential role in regulating these main genes [35]. Evidence suggests that specific miRNAs are involved in vascular endothelial pathogenesis in hypertension (e.g.; miR-126), acting as pro-/antiangiogenic factors [35], interacting with the renin-angiotensin-aldosterone system (e.g.; miR-155) [36] or targeting vascular smooth muscle cells (e.g.; miR-143 and miR-145) [37, 38]. Some miRNAs have also been shown to be related to the nitric oxide and atrial natriuretic peptide pathways in vascular smooth muscle cells [39].

T2D, which has reached epidemic levels worldwide, is a metabolic disorder that is characterized by hyperglycemia in the context of reduced insulin sensitivity and insulin resistance. T2D is a complex disease whose disorders are not fully understood. However, it appears that insulin resistance has a major role in the development of this pathology. Moreover, insulin resistance and β-cell dysfunction are mainly developed because of deregulation of adipose tissue function and lipid metabolism [40]. Recently, several studies have shown that miRNAs play major roles in insulin production and secretion, insulin resistance, pancreatic islet development, and β-cell dysfunction (reviewed in [41]). Furthermore, miRNAs are also involved in glucose homeostasis and lipid metabolism related to T2D. Most studies have been based on the miRNA microarray analysis of insulin-resistant tissues, such as skeletal muscle, liver, adipose tissue, and pancreatic β-cells, in animal models of spontaneous T2D. In these studies, various miRNAs were shown to be deregulated, but it is still not clear which specific miRNAs are important for T2D and what their roles are. However, some research in this area has been reported, including the deregulation of miR-335 in the adipose tissue of obese mice, which has been correlated to adipocyte differentiation and maturation [42]. The deregulation of miR-27b and miR-335 in the liver of T2D rats has been suggested to contribute to fatty liver and associated pathologies [42, 43]. Some miRNAs are also involved in the adjustment of skeletal muscle to insulin resistance and T2D. For example, a decrease in miR-24 or miR-126 may help muscles to increase insulin-dependent glucose uptake; miRNAs therefore participate in the adaptation of muscle to high glucose levels [44, 45]. Finally, miR-375 and miR-34a may have an important role in T2D in islets [46].

Obesity, characterized by increased fat mass and energy storage in adipose tissue, has reached pandemic proportions in recent years. This pathology is related to diseases such as T2D, hypertension, CVD, and cancer [47]. miRNAs are important regulators of the development and function of adipose tissue and metabolic functions and therefore have potential roles in obesity and their associated diseases (reviewed in [48]). Several studies have demonstrated that miRNAs acts as central modulators of normal white and brown adipose tissue differentiation and biology. Many miRNAs that are downregulated in obesity are upregulated during adipogenesis and vice versa [49]. In this sense, several miRNAs regulate, enhance, and inhibit adipogenesis (e.g.; miR-143), suggesting that miRNAs have a potential role in controlling adipocyte number and size. However, miRNAs govern not only mass size but also the metabolic consequences of obesity and adipose tissue metabolism [48]. More evidence for the role of miRNAs in obesity-related diseases is necessary to understand their regulatory roles in modulating energy balance, adipose biology, and their potential contribution to obesity [49].

NAFLD is characterized by fat accumulation in the liver without significant alcohol consumption [50]. Clinical manifestations of this pathology include dyslipidemia,

hypertension, and insulin resistance. Recently, the involvement of miRNAs in NAFLD has been described [51, 52]. It has been demonstrated that miRNAs are able to modify lipid droplet accumulation in hepatocytes, which is characteristic of NAFLD [53]. Other studies have showed that some miRNAs target the proliferator-activated receptor (PPAR) α (PPARα), a key molecule for NAFLD [54]. Studies in humans have demonstrated altered hepatic miRNAs profiles in non-alcoholic steatohepatitis (NASH), in which miR-122 is remarkably downregulated [55].

Besides of being involved on chronic diseases, miRNAs are good candidate biomarkers of diseases because they are stable, conserved, tissue specific, pathology specific, and detectable in serum, plasma, and other biological fluids [56]. Therefore, a major challenge for chronic disease research is the identification of reliable biomarkers that can be measured in a noninvasive way using accessible samples such as plasma or serum. Therefore, miRNAs in plasma and serum are beginning to be studied as biomarkers for chronic disease, and altered circulating miRNAs profiles have already been correlated to several diseases states. Circulating liver-specific miR-122 was found to be a good biomarker for hepatic injuries such as NAFLD and NASH [57-59]. Deregulation of circulating miR-223 was correlated with atherosclerosis [24], miR-126 to T2D [60], Let-7a to hypertension [61], and circulating miR-499-5p was postulated to be a sensitive biomarker for non ST-elevation myocardial infarction [62]. Other examples are miR-17-5p and miR-132, which are differentially expressed in obese and non-obese subjects in peripheral blood, suggesting their potential role as novel metabolic biomarkers [63]. Increased levels of miR-122 and miR-370 in plasma were found in patients with coronary artery disease in hyperlipidaemia [64].

4. Control of lipid metabolism

4.1. Lipid metabolism

Lipids are essential for the correct function of the organism, energy homeostasis, for cellular biology and for organ physiology. The human body has developed a system to transport lipids, by lipoproteins, to deliver cholesterol and fatty acids (FA) to the periphery. Lipoproteins are composed of triglycerides (TG), cholesterol esters (CE), phospholipids (PL), and apolipoproteins, which modulate lipoprotein catabolism. The main characteristics of each lipoprotein are described in Table 1. The liver synthesizes various lipoproteins involved in transporting cholesterol and TG throughout the body [65]. Very low density lipoproteins (VLDL) are secreted by the liver to transport TG throughout the body. As VLDL particles are stripped of TG, they become denser and are transformed to low density lipoproteins (LDL). These LDL particles deliver

cholesterol to cells in the body, where it is used in membranes or for the synthesis of steroid hormones. Moreover, the liver, and also the intestine, synthesize the precursor of high density lipoprotein (HDL), which travels in the circulation where it gathers cholesterol to form mature HDL. Then, it returns cholesterol to the liver in a process known as reverse cholesterol transport (RCT). The liver removes lipoproteins from the circulation by receptor-mediated endocytosis [66].

Other important organ in secreting lipoproteins is the intestine, which secretes, mainly, chylomicrons (CMs) and VLDLs [67]. VLDL assembly occurs constitutively, being the predominant lipoproteins during the fasting state [68]. However, in the postprandial state and in response to dietary fat, which is the situation mostly evaluated in this thesis, CMs are the principal lipoproteins secreted by the intestine [68]. After the secretion of CMs into the intestinal lymph and their entrance into the systemic circulation, the TG moiety of CMs is promptly hydrolyzed by LPL, resulting in the production of CMs remnants, which are taken up by the liver via CMs remnant receptors [68]. Furthermore, several studies have demonstrated that non-fasting TG levels are associated with increased risk of cardiovascular events [69, 70], being better predictor of CVD than the fasting levels [69].

Table 1. *Classification and composition of lipoproteins*

	Chylomicrons	***VLDL***	***LDL***	***HDL***
Density (g/mL)	<0,95	0,95-1,006	1,006-1,063	1,062-1,21
Diameter (nm)	>70	30-90	18-22	5-12
Lipids (%)	98	92	78	50
Triglycerides (%)	86	55	6	4
Phospholipids (%)	7	18	22	22
Cholesterol free (%)	2	7	8	4
Cholesterol ester (%)	3	12	42	20
Proteins (%)	A-I,CA-II, A-VI	B-100	B-100	A-I, A-II
	B-48	C-I, C-II		C-I, C-II
	C-I, C-II	C-III		C-III
	C-III, E	E		D, E

In the human body, high levels of TG in the bloodstream have been linked to atherosclerosis and, by extension, to the risk of heart disease and stroke. Despite intestine has an important role secreting TG-rich lipoproteins after meals, liver plays an essential role modulating plasma TG levels. Three main sources of FAs contribute to TG synthesis in liver: dietary TGs delivered by apoE-enriched TG-rich lipoprotein remnant particles, plasma non-esterfied FAs (NEFAs) and *de novo* synthesis.

The liver is the major organ for clearance of apoE-enriched TG-rich lipoprotein remnant particles. TGs from mature TG-rich lipoproteins are hydrolyzed extracellularly by the action of LPL in peripheral tissues, especially in the adipose tissue [71] and once TG stores are almost removed, the apoE-enriched TG-rich lipoproteins become remnants. ApoE allows the liver to identify the remnants to take them up for endocytosis [68].

Furthermore, liver obtains NEFAs from the bloodstream, which enters cells via transporters or by diffusion. In hepatocytes, long chain FAs are bound to FA binding protein (FABP), which plays a crucial role in intracellular FA trafficking, and are activated by fatty acyl-CoA synthetases before their oxidation. FABP and acyl-CoA binding protein also transport NEFAs to intracellular compartments for metabolism or the nucleus to interact with transcription factors [72].

The last main source of FAs that contribute to liver TG is *de novo* synthesis. *De novo* lipogenesis is a key metabolic pathway for energy homeostasis in higher animals and it is strongly controlled by hormonal and nutritional conditions. High carbohydrate diets induce lipogenesis whereas fasting or fat feeding inhibits it. In this sense, the FA synthase (FAS) is the responsible for *de novo* lipogenesis. FAs are produced mainly in the liver, where they are exported through VLDL, and in the adipose tissue, contributing to fat deposition and energy storage [72].

FAS protein exists as a homodimer of 273 kDa subunit although it is only enzymatically active in the dimeric form [73]. Each monomer contains seven protein domains required for FA synthesis: acyl carrier, acyl transferase, β-ketoacyl synthase, β-ketoacyl reductase, β-hydroxylacyl dehydratase, enoyl reductase, and thioesterase. FAS is expressed in almost all tissues with highest levels in the liver, adipose tissue, and lungs [74, 75], being its substrates acetyl-CoA, which acts as a primer for the reaction, and malonyl-CoA, which donates two carbons in each cycle of condensation to elongate the acetyl-CoA; NADPH provides reducing equivalents. The FA is elongated from the initial acetyl-CoA by repeated condensations with malonyl-CoA. Hence, palmitate synthesis, which is the primary product of the FAS, requires seven cycles of malonyl-CoA addition to an acetyl-CoA primer to yield a saturated, 16-carbon FA [76]. The main function of hepatic FAS is to synthesize FAs that can be used for energy storage, membrane assembly and repair, and secretion in the form of lipoprotein-TG particles.

In humans, the contributions of *de novo* lipogenesis to produce VLDL-TG particles in low fat and high carbohydrate diets is higher than in high fat diets [77, 78]. Thus, it seems that the influence of *de novo* lipogenesis depends on dietary conditions.

Besides the key role of FAS in *de novo* lipogenesis, FAS is also considered a signaling enzyme because can affect FA oxidation through the peroxisome proliferator-activated receptor (PPAR) α [79]. PPARα modulates metabolism, inflammation and promotes lipid uptake and catabolism of FAs through β-oxidation to produce ketone bodies during fasting [80, 81]. PPARα null mice shows hypoglycemia, low serum ketone levels, hepatic steatosis and deficient hepatic FA oxidation [81]. The phenotype of these null mice is similar to that of liver-specific Fas knockout mice, whose were not protected against hepatic lipid accumulation, developing severe hepatic steatosis [82]. The deficient activation of PPARα when the FAS is knocked out let to the affirmation that FAS participate on the activation in liver of PPARα. Moreover, FAS appears to contribute to PPARα activity by promoting the synthesis of its ligands, such as the phosphatidylcholine species 16:0/18:1-glycerophosphocholine [83].

Regarding to hepatic TG synthesis, the partition of FAs between oxidation and TG synthesis has a fundamental role. The FA oxidation is a key mechanism to obtain energy in form of ATP, although in liver the oxidation of FAs produces ketone bodies, which are important fuels for extra hepatic organs. The β-oxidation of activated FAs occurs within the mitochondrial matrix and is catalyzed by the sequential action of four enzyme families: acyl-CoA dehydrogenase, anoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, each with different substrate specificity for short-, medium- and long-chain acyl-CoAs [84]. Long-chain FAs are activated on the mitochondria outer membrane. The inner membrane is not permeable to these acyl-CoAs thus, the carnitine dependent transport is essential before β-oxidation. This transport consists of three proteins, carnitine palmitoyltransferase 1 and 2 (CPT-1, CPT-2) and carnitine: acylcarnitine translocase [85]. CPT-1 is the rate-limiting step of mitochondrial FA β-oxidation and controls the transport of long-chain acyl-CoA into the mitochondria [85]. Moreover, when the rate of FA synthesis is high, high levels of malonyl-CoA prevent the oxidation of FAs by inhibiting CPT-1. Alternatively, when acetyl-CoA levels are reduced or when the acetyl-CoA carboxylase (ACC) activity is diminished and malonyl-CoA levels decrease, CPT-1 is activated [86]. Hence, FAS and CPT-1 function at a metabolic crossroads between energy storage (anabolism) and consumption of stored energy (catabolism).

TGs are synthesized through two major pathways, the glycerol phosphate pathway and the monoacylglycerol pathway [87]. The monoacylglycerol pathway functions predominantly in small intestine to generate TG from monoacylglycerol derived from dietary fat [88], whereas the glycerol phosphate pathway is the major pathway utilized by most cell types [89] including the liver. The first step in TG synthesis via the glycerol

phosphate pathway (Figure 3) is the acylation of glycerol 3-phosphate by glycerol-3-phosphate acyltransferase, which reside in the endoplasmic reticulum (ER) and mitochondria [90].

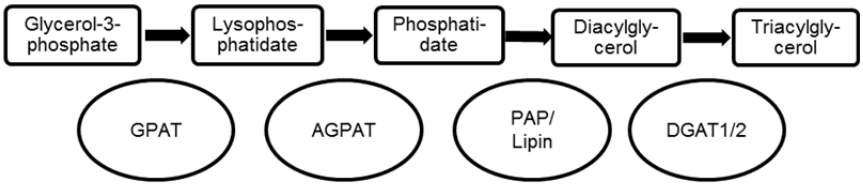

Figure 3. *Glycerolipid biosynthesis in liver.*

An additional FA is subsequently transferred to lysophosphatidic acid by the family of 1-acylglycerol-3-phosphate acyltransferases to produce phosphatidate [91]. Phosphatidate can serve as a precursor of acidic PLs or diacylglycerol (DAG). Finally, the resulting DAG is converted to TG through the action of diacylglycerol acyltransferase (DGAT) [87]. The resulting TGs are used in several metabolic processes, including synthesis of VLDL or energy storage as lipid droplets. Hepatic lipid droplet pools provide up to 70% of the TG for the assembly of VLDL with the remainder coming from *de novo* synthesis [73]. The main apolipoprotein forming the VLDL is the apoB-100, which provides the structural stability of the nascent VLDL particles [92]. In humans, apoB-100 is expressed in liver and is the key structural protein of VLDL, the intermediate-density lipoprotein and LDL, whereas apoB-48 is mainly synthesized by the intestine, forming part of CMs [93], although rodent's liver synthesized both, apoB-100 and apoB-48 [94]. The biogenesis of the VLDL starts in the lumen of the ER and this process is accomplished in two steps [95]. Firstly, it is necessarily the translocation of newly translated of apoB-100, which is partially lipidated (if not the apoB-100 gets degraded) to form the primordial lipid-poor VLDL particle [96]. This step is facilitated by the microsomal TG transfer protein, transferring both neutral and polar lipids to the initial VLDL particle [97]. Secondly, the VLDL particle fuses with TG-rich particles and move from the ER to the *cis*-Golgi in a specialized vesicle, the VLDL transport vesicle (VTV) [98]. Once nascent VLDLs are derived to the Golgi lumen by VTVs, the VLDL particles suffer several modifications, such as phosphorylations [99] and finally, the mature VLDLs are transported across the cytoplasm to the plasma membrane and are secreted in the circulatory system [100]. Enhanced production of VLDLs and their eventual secretion into the circulatory system constitute one of the major risk factors

for the development of atherosclerosis [101], because of higher concentrations of VLDLs in the blood are often translated into higher levels of atherogenic particles, LDLs.

Cholesterol is an important component of mammalian cell membranes and has a key role in membrane trafficking, transmembrane signaling processes and in cell proliferation [102, 103]. It is also the precursor molecule for the synthesis of steroid hormones (reviewed at [104]) and bile salts [105], between other functions. However, elevated levels of cholesterol in the bloodstream have been linked to atherosclerosis and, by extension, to a high risk of heart disease and stroke, being an important risk factor to develop of CVD.

Cholesterol is derived from the diet or synthesized within the body, mainly in the liver and in the central nervous system [105]. Cholesterol derived from diet circulates in CM remnants that are removed by the liver after their binding to the LDL receptor-like protein [106]. The liver also exports cholesterol, mainly secreted in VLDL particles. However, a certain amount of cholesterol is also secreted from the liver via de ATP-binding cassette transporter (ABC) A1 (ABCA1) to join small, newly secreted HDL particles [107]. Moreover, there is also some direct hepatic secretion in LDL in pathological states such as familial hypercholesterolemia [108]. Once VLDLs are in circulation, they are avid acceptor of cholesteryl ester from HDL and LDL. This transfer occurs because the presence of the cholesteryl ester transfer protein in human plasma, although this protein is not present in all species, such as in rats, which have low levels of circulating LDL. The human cholesterol metabolism is also different from those in rats because cholesterol is secreted from the human liver as unsterified cholesterol, whereas in rats cholesterol is esterified before secretion [109]. During its circulation VLDL, as it occurs with the CMs, suffer the progressive removal of TG by the LPL forming the LDL particles, which supply the tissues with cholesterol. Finally, the LDL is taken up by the LDL receptor (LDLR), which recognizes apoB-100, by endocytosis [66].

As previously mentioned, apart from being derived from diet, cholesterol is also synthesized in the body. In this sense, cholesterol is synthesized in the ER and cytoplasm from acetyl-CoA through the mevalonic-pathway [110]. The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is the rate limiting enzyme of this pathway, which catalyzes the conversion of HMG-CoA to mevalonic acid. Following synthesis, cholesterol leaves the ER and is targeted to the plasma membrane, or may access other sites, such as endosomes [111]. In order to prevent over-accumulation of free cholesterol in the plasma and in intracellular membranes, cholesterol is converted to CEs, primary by the enzyme acyl-CoA acyltransferase, which are stored as cytosolic lipid droplets [111].

Mammalian cells respond to cholesterol excess by inhibiting cholesterol biosynthesis and uptake, and by increasing cellular cholesterol efflux by the ABCA1 and the ABC

subfamily G1 (ABCG1). ABCA1, which is widely expressed in almost all tissues with relatively high expression levels in the liver, intestine, adrenal glands, lung, brain and macrophages, mediates the transport of excess cholesterol from cells to lipid-poor apoA-I. It has been reported that hepatic and peripheral ABCA1 have distinct and specific roles in the regulation of HDL levels. Hepatic ABCA1 is critical for the lipidation of nascent apoA-I, while extrahepatic ABCA1 mediates the cholesterol transfer to nascent HDL particles. Moreover, ABCG1, which is highly expressed in macrophages, mediates the efflux of cellular cholesterol to HDL or to lipidated apoA-I but not to lipid-free apoA-I (reviewed in [112]).

RCT is a multi-step process resulting in the net movement of cholesterol from peripheral tissues back to the liver for its excretion to bile (Figure 4).

In humans, there are two pathways for the returning of HDL-cholesterol to the liver: the direct HDL uptake by scavenger receptor B1 or through CE transfer protein exchange of HDL-cholesteryl ester for TG in apoB-containing lipoproteins, followed by hepatic uptake of these apoB-containing particles by the LDLR [113]. High levels of HDL have been associated with reduced incidence of CVD, and low levels of HDL with increased risk of CVD in multiple epidemiological studies [114]. However, newly studies suggest that HDL concentration does not always predict the CVD risk; rather, the amount of cholesterol efflux from cells seems a better predictor [115].

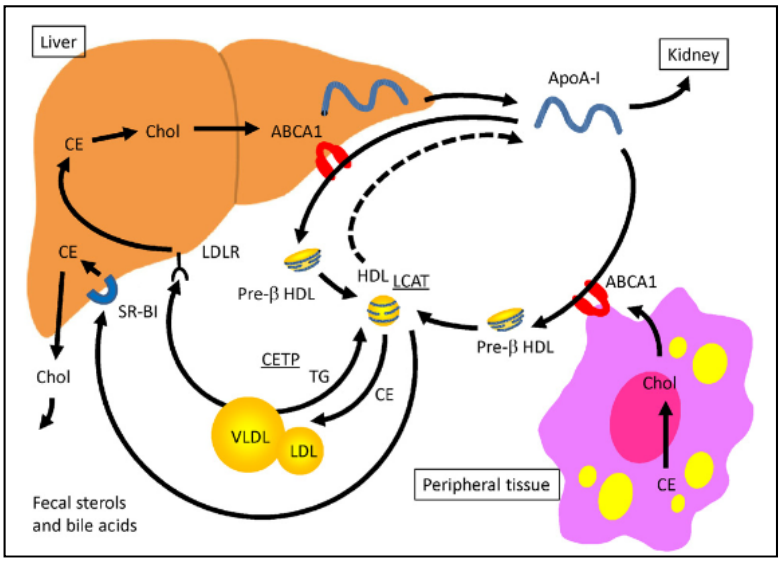

Figure 4. *Schematic diagram of RCT* [116].

The ABCA1 and ABCG1 play crucial roles in the efflux of cellular cholesterol to HDL. The ABCA1 gene was identified over a decade ago as the molecular basis for Tangier disease, a rare disorder of HDL deficiency in which cellular cholesterol efflux is strongly reduced. The prevalence of ABCA1 mutations in subjects with HDL deficiency is estimated to be approximately 10-20% [117]. Moreover, recent genome-wide association studies have identified commons variants in ABCA1 as a significant source of variation in plasma HDL levels across multiple ethnics groups [118] establishing ABCA1 as a major gene influencing HDL levels in humans. Mice overexpressing abca1 in liver and macrophages have increased plasma HDL-cholesterol and apoB levels [119] whereas an overexpression of abca1 in the liver of LDLR-KO mice leads to accumulation of pro-atherogenic lipoproteins and enhanced atherosclerosis [120]. Focusing on the liver, there are several approaches to determine the contribution of hepatic ABCA1 to plasma HDL levels because it is reasonable to expect that liver has a major role in producing nascent-HDL, therefore modulating plasma HDL levels. An overexpression of human ABCA1 in mouse liver [121, 122] produced an increase on plasma HDL and apoA-I levels, with an elevation of preβ-HDL particles and, in a liver specific Abca1 knotout mice the plasma levels of HDL and apoA-I were decreased, with an increase on the HDL catabolism [123]. These studies strongly suggest that the liver is one of the most important sites producing and modulating plasma HDL particles.

The liver provides a mechanism to eliminate the excess of cholesterol from the body, through the bile either directly, as free cholesterol, or after its conversion into bile acids (BAs) [124]. BA secretion is considered the final step on the RCT pathway and the HDL particles are considered the preferential contributor for cholesterol secreted into bile [125]. BAs are physiological detergents that facilitate absorptions, transport, and distribution of dietary fats, sterols, and lipid-soluble vitamins, and disposal of toxic metabolites and xenobiotics. BAs are also signaling molecules that activate the nuclear Farnesoid X Receptor (FXR) [126], thus regulating lipid, glucose, and energy homeostasis. Alterations in BA signaling may contribute to the changes in lipid and glucose metabolism that are linked to diseases, such as CVD and T2D.

Lipid metabolism is regulated by several nuclear receptors and transcription factors.

Sterol regulatory element-binding protein (SREBP) transcription factors were the first defined system controlling lipid metabolism. SREBPs bind to sterol regulatory elements and activate the expression of genes required for cholesterol, FA, TG, and PL uptake and synthesis. In mammals, there are three isoforms of SREBPs: SREBP-1a and SREBP-1c, encoded by *Srebp-1*, and SREBP-2, encoded by *Srebp-2* [127]. They differ in their tissue-specific expression, their target gene selectivity, and the relative potencies of their trans-activation domains. SREBP-1a and SREBP-2 are the predominant isoforms in most cultured cell lines; whereas SREBP-1c and SREBP-2 are predominate in the liver [128]. At normal levels of expression, SREBP-1c favors the

FA biosynthetic pathway and SREBP-2 the biosynthesis of cholesterol. The SREBP-1c target genes include those for ATP citrate lyase (ACLY), ACC and FAS [129]. Other SREBP-1c target genes encode a rate-limiting enzyme of the FA elongase complex, which converts palmitate to stearate [130]; the stearoyl-CoA desaturase, which converts stearate to oleate, and the enzyme glycerol-3-phosphate acyltransferase, which is the first enzyme in TG and PL synthesis [131]. Moreover, the isoform SREBP-2 is responsible of the activation of genes involved in cholesterol biosynthesis (reviewed in [132, 133]), and it is controlled in a negative feedback mechanism by downstream products of the biosynthetic cholesterol pathway [132]. When intracellular cholesterol levels are low, the SREBP-2 becomes active and it is translocated to the nucleus where it activates the expression of cholesterol related genes, such as HMGCR and the LDLR [133].

LXR is a nuclear receptor important in the control of lipid metabolism that acts as cholesterol sensor. Natural ligands of LXR are cholesterol derivatives, including oxysterols. One of the best-characterized effects of LXR is to promote RCT. LXR has two isoforms, LXRα (NR1H3) and LXRβ (NR1H2) [134] and controls genes related to cholesterol and BA metabolism, leading to a conversion of cholesterol to BA. LXRs activate the transcription of genes involved in cholesterol efflux, such as ABCA1, ABCG1, and ABC subfamily G5 and 8 [135]. LXR and SREBP-2 transcriptional pathways work in a coordinated and reciprocal fashion to maintain cellular and systemic cholesterol homeostasis. SREBP-2 is activated in response to low cellular cholesterol levels, whereas LXRs are activated by elevated cholesterol levels. In addition to modulating cholesterol metabolism, another major function of LXR in the liver is the promotion of *de novo* lipogenesis. LXR stimulates lipogenesis mainly through the induction of SREBP-1c [136].

Similarly to LXRs, the FXR (NR1H4) has also been implicated in the regulation of lipid metabolism. Broadly, FXR can be viewed as acting in a complementary and often reciprocal fashion to LXR in the control of lipid metabolism. FXR inhibits whereas LXR promotes BA production and lipogenesis. FXR is a BA sensor, being its natural ligands the BA [137]. The activation of FXR downregulates the expression of genes that codify for cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1), the key controller of BA synthesis pathway. Also, the administration of FXR agonists lowers plasma TG levels by means of the inhibition of hepatic SREBP-1c expression through a mechanism that might involve inhibition of LXR and an activation of SREBP-1c [137]. Additional studies suggest that the increase of FA oxidation might also contribute to TG lowering effect of FXR activation [138, 139]. Furthermore, recent data show the implication of FXR in the glucose metabolism [140]. A special feature of FXR is that, in addition to inducing gene expression directly, FXR mediates the repression of a number of genes indirectly through the regulation of the nuclear receptor Small Heterodimer Partner (SHP, NR0B2).

The PPAR subfamily of nuclear receptors consists of three members, PPAR-α (NR1C1), PPAR-γ (NR1C2) and PPAR-δ (NR1C3). The three PPARs, by acting as FA sensors, are major metabolic regulators in the body and together they control almost every aspect of FA metabolism [141]. They have important implications for human metabolic diseases, as evidenced by the fact that PPARγ and PPARα are respectively molecular targets for the T2D drug thiazolidinediones and dyslipidemia drug fibrates.

Specifically, PPAR-α is mostly expressed in the liver and its activation results in the upregulation of genes involved in the uptake and β-oxidation of FAs, reducing serum TG levels. Moreover, PPARα is induced by fasting and is required for ketogenesis (reviewed in [142]).

PPAR-γ, which has three isoforms; PPAR-γ1, 2 and 3, is a master regulator of adipogenesis. These three mRNA transcripts give rise to only two PPARγ proteins, PPARγ1 and PPARγ2, due to the fact that PPARγ3 mRNA is translated into a protein that is identical to PPARγ1 [143]. PPARγ protein is expressed mainly in adipose tissue [143] and its activation induces expression of an array of genes for FA transport and storage, as well as promotes *de novo* adipogenesis. In this way, activation of PPARγ results in increased FAs uptake by adipose tissue, lowering circulating FFAs, thereby improving insulin resistance in the liver and skeletal muscle [137].

The last member of the PPARs family is the PPAR-δ, which is ubiquitously expressed [137]. PPARδ has a higher expression in the skeletal muscle and is more abundant in the oxidative fibers. Treatment of skeletal muscle cells with the PPARδ agonists *in vitro* induces the expression of genes for FA catabolism and promotes FA oxidation. Recent data obtained from transgenic mouse models, have implicated PPAR-δ as an important regulator of energy expenditure as well as glucose and lipid metabolism, highlighting the potential use of PPAR-δ modulators as therapeutic agents for T2D, obesity and atherosclerosis [137].

4.2. Regulation of lipid metabolism by miRNAs

As previously mentioned in section 3, miRNAs have been shown to play important roles in most metabolic pathways and recently emerged as key regulators of lipid metabolism, playing major roles in regulating FA and cholesterol metabolism [144]. The importance of miRNAs in regulating lipid homeostasis and lipoprotein metabolism opens new avenues for treating dyslipidemias and cardiometabolic disorders.

Although the specific role of each miRNA in controlling lipid metabolism is still unknown, particular miRNAs are emerging as regulators of lipid metabolism. miR-758, miR-26 and miR-106b regulates cellular cholesterol efflux by targeting ABCA1 [145-147]. Another miRNA involved in lipid metabolism is miR-370, which directly targets Cpt1a, thereby reducing FA β-oxidation. Moreover, miR-378/378* also regulates FA metabolism [148] showing to increases the expression of FA binding protein 4 and FAS, between others [148]. However, the miR-378/378* targets that regulate the expression of these genes are unknown. Other miRNAs have also been linked to adipocyte differentiation, such as let-7, miR-143, miR-335, miR-27 and miR-103/107 [42, 149-152], involved in the modulation of TG accumulation at an early stage of adipogenesis.

More recently, additional miRNAs were shown to be associated with liver and pancreatic lipid metabolism in animal's models. miR-216 and miR-302a expression is decreased in the liver of LDLR-KO mice [153]. On the contrary, miR-217 expression is induced in the liver of ethanol fed mice [154] and finally, miR-21 influences the maturation of pancreatic exocrine and endocrine tissue by regulating Srebp-1 and sterol metabolism [155]. However, miR-33 and miR-122 are the most well-studied, which are known to have crucial roles in regulation of lipid metabolism.

4.2.1. miR-33

miR-33 is a well characterized intronic miRNA that is formed of two isoforms, miR-33a and miR-33b which are located within the Srebp-2 and Srebp-1 genes, respectively [156-158]. Moreover, miR-33a and miR-33b are co-transcribed with their host genes, SREBP-2 and SREBP1-c, respectively [156-158]. Although miR-33a and miR-33b share their target activity, they differ in their pattern of evolutionary conservation. miR-33a is encoded within the intron 16 of the human Srebp-2 gene and was highly conserved during evolution being present in the genomes from flies to vertebrates [156]. However, the conservation of miR-33b, which is found within intron 17 of the human Srebp-1 gene, is lost in many species such as rodents and rabbits [156].

Several studies indicate that miR-33 is involved in atherosclerosis (Figure 5), influencing lipid metabolism, inflammatory response [159], insulin signaling [160-162], glucose/energy homeostasis [49, 160, 163], between other functions. In particular to lipid metabolism miR-33 influences cholesterol homeostasis and HDL biogenesis [156, 161-166], FA oxidation [160, 163], PL [163], TG [160, 161] and BA [168] metabolism.

miR-33 has been identified putative binding sites in the 3'UTR for mouse Abca1, Abcg1 and the endolysosomal transport protein Niemann-Pick C1 (NPC1) [156, 163, 164,

[166]. In regards to ABCA1, the 3'UTR of mouse and human ABCA1 contains 3 binding sites for miR-33.

Several *in vivo* and *in vitro* studies in liver confirmed that ABCA1, NPC1 and ABCG1 are direct targets of miR-33 and, whereas ABCA1 and NPC1 are conserved targets for miR-33, ABCG1 is only a target in mouse [164, 166]. Specifically to ABCA1, which is further studied in this thesis, it has been shown that an inhibition of miR-33 in animal liver, including mice or monkeys, produce an increase expression of liver ABCA1, cholesterol efflux to apoA-1 and an increase of plasma HDL particles favoring the RCT and, thus, improving an atherogenic profile [156, 161, 163-166].

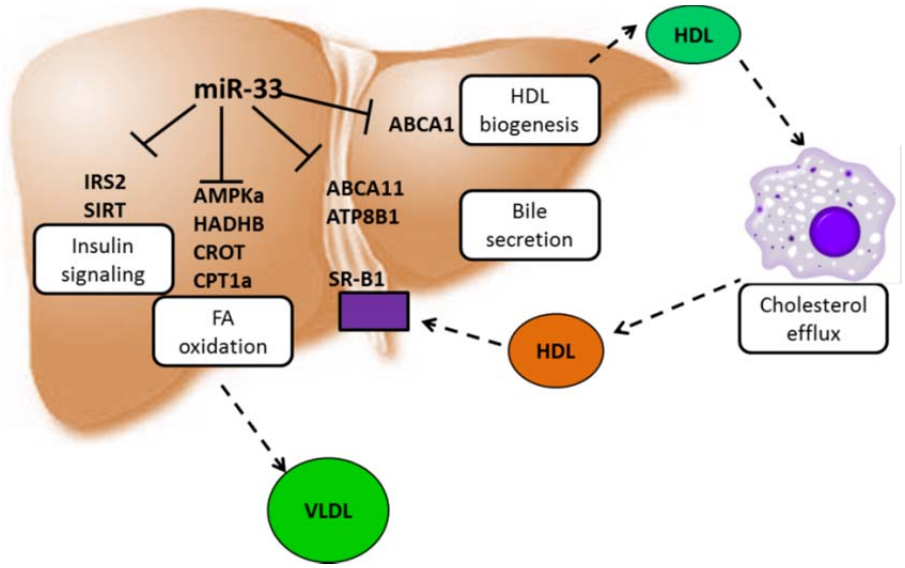

Figure 5. *Main targets of miR-33 in liver, adapted from* [169]

However, further studies are needed to elucidate the adverse effects of suppressing miR-33 *in vivo* and to completely understand the complex roles of ABCA1. A part from liver, the role of miR-33 in other tissues, such as in the brain, is under investigation because ABCA1 also plays an important role in Aβ clearance and, its expression is associated with diseases such as Alzheimer [170]. Interestingly, recent data revealed that ABCA1 and ABCG1 could function as anti-inflammatory factors, suggesting that ABCA1 and ABCG1 may be the molecular basis for the interaction between inflammation and RCT [171, 172]. Otherwise, the lack of ABCA1 and ABCG1 leads to an increase in the expression of CHOP, which plays an important role in ER stress, β-cell dysfunction and apoptosis in animal model of T2D [173]. Thus, miR-33 may regulate ER stress and apoptosis by targeting ABCA1 and ABCG1 which could

be a new therapy against CVD. Also, NPC1 protein was regulated by miR-33 in mouse and human [166]. NPC1 is a lysosomal protein that facilitates the transport of cholesterol from lysosomes to other parts of the cell [174] and it may act in concert with ABCA1 to promote cellular cholesterol efflux to apo A-I.

In addition to the role of miR-33 modulating cholesterol metabolism, is also important the contribution of miR-33 in controlling FA metabolism. This is highlighted when endogenous inhibition of miR-33 demonstrated to increase the degradation of Fas [160, 163], reduce the plasma levels of VLDL, by increasing the expression of miR-33 target genes involved in FA oxidation (o-octanyl transferase, CTP1a, hydroxyacyl-coenzyme A dehydrogenase-3-ketoacyl-coenzime A thiolase-enoyl-coenzyme A hydratase (trifunctional protein) β-subunit (HADHB) and AMP-activates kinase (AMPKα)) and reducing the expression of genes involved in FA synthesis (SREBP-1, FAS, ACLY and ACC alpha) [160, 163]. Also, due to the important regulatory role of miR-33 in the expression of ABCA1 and ABCG1, which mediate the active efflux of PL to apoA-I and HDL, respectively [175, 176], miR-33 may play a role in regulating PL efflux. This is evidenced in several studies, as the co-transfection of miR-33 that dramatically reduced the promoter activity of the PL flippase ATP8B1 3'UTR in reported assays [163]. Additionally, excess of miR-33 results in increased intracellular TG levels as well as an increased lipid droplets formation in human hepatoma cells [160], targeting the histone deacetylase sirtuin-6. Moreover, miR-33 is also predicted to target nuclear receptor-interacting protein 1 [159], which plays a crucial role in TG/lipid metabolism, although it has not been largely investigated.

miR-33 has also been demonstrated to regulate BA metabolism, which is an essential component of RCT pathway and modulate hepatic lipid and glucose metabolism [177]. In this sense, overexpression of miR-33 decrease hepatic expression of biliary transporters, ABC subfamily B11 and ATP8B1, decreasing BA secretion, while silencing of miR-33 increases their expression and increases BA secretion [168].

Besides to the regulation of FA, PL, TG and BA metabolisms, miR-33 has also been shown to control the expression of Ampkα1 [160], which is involved in the regulation of lipid metabolism regulating lipogenic enzymes, such as HMGCR and ACC. Thus, inhibition of AMPKα1 by miR-33 could increase HMGCR and ACC activity to improve intracellular levels of cholesterol and FAs. Apart from the regulation of AMPKα1 by miR-33, the insulin receptor substrate 2 (Irs2), an adaptor protein that controls insulin signaling in the liver, has also been shown to be a miR-33 target, thereby affecting proteins such as protein kinase B (AKT) [160].

4.2.2. miR-122

The most widely studied miRNA is miR-122, also known as miR-122a, and it was the first described for its role in regulating total plasma cholesterol and hepatic metabolism [178].

miR-122 is a highly evolutionary conserved mature miRNA [179] that derives from a liver-specific noncoding RNA precursor transcribed from the helix coiled-coil rod homolog (*hcr)* gene [179]. miR-122 is the mostly expressed miRNA in liver; it was discovered during a cloning study of tissue-specific small RNAs in mice, in which miR-122 accounted for the 72% of all cloned miRNAs in liver [180]. It is mainly studied by its role in controlling the hepatitis C virus (HCV) infection, including its stimulation of the replication and expression of HCV [181]. However, there are also a plethora of studies using *in vivo* gene silencing, *in vitro* experimentation, and transcriptome profiling that demonstrate how miR-122 regulates genes that control lipid metabolism [178, 182-184], cell differentiation [185], hepatic circadian regulation [186], and systemic iron homeostasis. Moreover, pathogenic repression of miR-122 has been observed in NASH [55], liver cirrhosis [187], and hepatocellular carcinoma [187]. In relation to lipid metabolism (Figure 6), several groups have shown that miR-122 is important for regulating liver lipid metabolism by using antisense methods to suppress this miRNA [178, 182, 183]. Direct miR-122 targets that modulate lipid metabolism are essentially unknown and most of the defined target genes of this miRNA, such as Fas, are indirectly modulated.

Inhibition of miR-122 is expected to result in upregulation of its indirect targets however, the exact connection between inhibition of miR-122 and downregulation of these genes is not clear. One possibility is that a transcriptional inhibitor of these genes is negatively regulated by miR-122 and therefore upregulated following miR-122 inhibition. Also, it could be consequences for RISC formation and activity of other miRNAs after miR-122 inhibition. Moreover, genes such as FAS and HMGCR, which are regulated by miR-122, do not contain target sequences for miR-122, which involves that this miRNA is acting through a novel mechanism, although it is still not known [188].

Specifically, knockdown of miR-122 in mice [178, 182] and in non-human primates [183] liver, decreased expression of several genes related to cholesterol biosynthesis, such as HMGCR, and reduced cholesterol plasma and liver levels, decreased FA synthesis and increased FA oxidation with a reduction on plasma TGs, by downregulating genes such as FAS, ACC1/2 and stearoyl-Coenzyme A desaturase 1 (SCD1) [178, 182, 183].

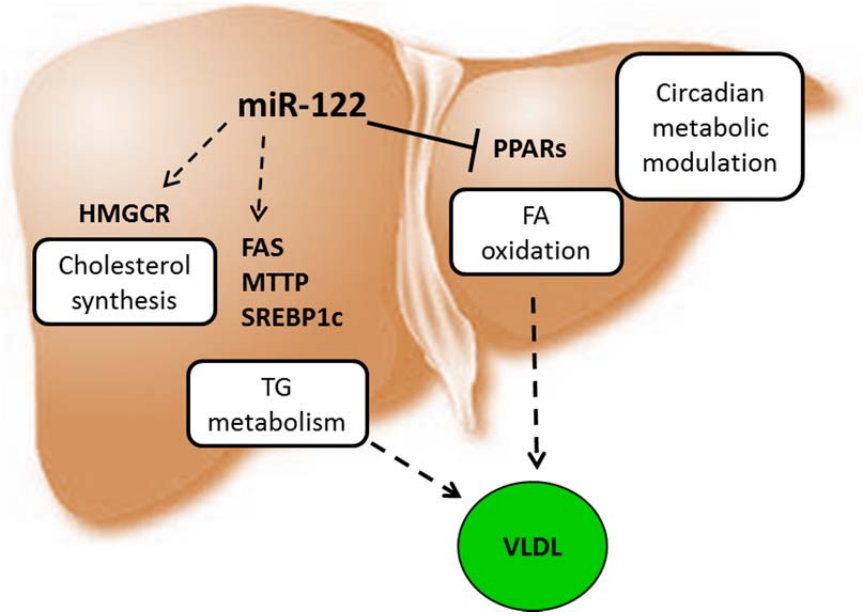

Figure 6. *Main targets of miR-122 in liver.*

Inhibition of miR-122 in non-human primates reduced plasma cholesterol levels without any apparent liver toxicity or histopathological changes [183, 189], suggesting miR-122 inhibition as a feasible therapeutic approach in humans. Surprisingly, the reduction of cholesterol levels persisted for several weeks after the end of the treatment, suggesting that inhibition of miR-122 using antagomirs has prolonged effects on hepatic gene expression and cholesterol metabolism. Recently, miR-122 liver-specific knockout have been shown to have a significant reduction in total serum cholesterol and TG levels [184, 190], whereas adenoviral overexpression of miR-122 increased cholesterol biosynthesis [182]. Furthermore, under certain conditions miR-122 may reduce CYP7A1 mRNA stability to inhibit BA synthesis, and miR-122 antagomirs may stimulate BA synthesis to reduce serum cholesterol and TG levels [191].

Interestingly, it was found in miR-122 germline knockout mice a downregulation of the Mttp, which is essential for the assembly of VLDL [184] although, the MTTP gene is also an indirect target for miR-122, and the mechanism by which miR-122 regulates its expression is unclear. Altogether, these results demonstrated that miR-122 plays an important role in regulating plasma cholesterol and TG levels by controlling cholesterol biosynthesis and VLDL secretion in liver.

Recently, it has been identified PPARα, β, and γ and the PPARα-coactivator (Smarcd1/Baf60a), as novel direct targets of miR-122. This also suggests an

involvement of the circadian metabolic regulators of the PPAR family in miR-122-mediated metabolic control [186].

In addition to the miR-122 importance for liver fat metabolism, there is also some evidences that demonstrated that dysregulation of miR-122 expression might underlie some diseases pathologies including NAFLD as already mentioned in section 3. Further studies are therefore necessary to clarify this apparent inconsistency with the effect of inhibiting miR-122.

5. miRNA modulation by food components

Numerous dietary components have been shown to modulate miRNA expression, such as vitamins, oligoelements, polyphenols, isoflavones, indoles, isothiocyanates, PL, saponins, anthraquinones and polyunsaturated FAs (reviewed in [192]). Between them, polyphenols and ω-3 polyunsaturated FAs (ω-3 PUFAs) are known to have potent hypolipidemic activities. Therfore, in this section the modulation of miRNAs by polyphenols and ω-3 PUFAs as two hypolipidemic natural agents is further detailed. For this reason, we select polyphenols and ω-3 PUFAs to know whether natural hypolipidemic agents could modulate the expression of miRNA related to lipid metabolism.

5.1. Polyphenols

Several hundred unique polyphenols have been identified as secondary metabolites in edible plants. The main classes of polyphenols are classified according to the nature of their carbon skeleton: phenolic acids, stilbenes, flavonoids and lignans (Figure 7). Phenolic acids, which are found generally in low concentrations in foods, are divided in two classes: derivatives of benzoic acid (protocatechuic and gallic acids) and derivatives of cinnamic acid (coumaric, caffeic and ferulic acids) [193]. Stilbenes are not widespread in food plants although one of them, resveratrol, has received great attention for its anticarcinogenic properties [193]. Lignans are formed of two phenylpropane units and the only foods that contain considerable quantities of lignans are flaxseed and flaxseed oil [193]. Flavonoids, mainly proanthocyanidins (PAs), are the most abundant polyphenols in our diet and are the main polyphenols studied in the present thesis.

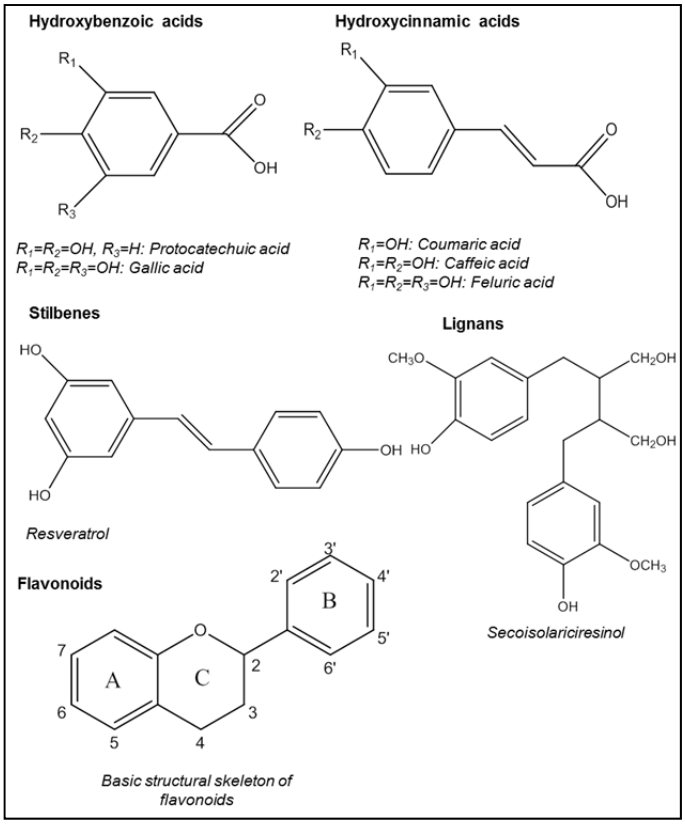

Figure 7. *Chemical structure of polyphenols*

In latest years, the role of flavonoids as protective dietary components in the area of human nutrition has become an interest of research. Moreover, there is increasing evidence that lower long-term intakes of flavonoids may modulate human metabolism for the prevention or reduction in the risk of chronic and degenerative diseases, such as CVD, diabetes, cancer and obesity [194]. To date, more than 6,000 different flavonoids have been described [195].Flavonoids are divided into six subclasses (Figure 8) according to the degree of oxidation of the oxygen heterocycle: flavonols (kaempferol, myricetin and quercetin), flavones (luteolin and apigenin), isoflavones (daidzein and genistein), flavanones (naringenin, eriodictyol and hesperitin), anthocyanidins (pelargonidin, cyanidin, delphinidin, petunidin and malvidin), and flavanols (monomeric catechins and oligomeric PAs) [193]. Moreover, the basic flavonoid skeleton can have several substituents, such as glycosylation and methylation, between others [196] and, the degree of polymerization increases the

diversity of flavonoid compounds.PAs, which are also known as condensed tannins, are the oligomeric or polymeric forms of flavanols.

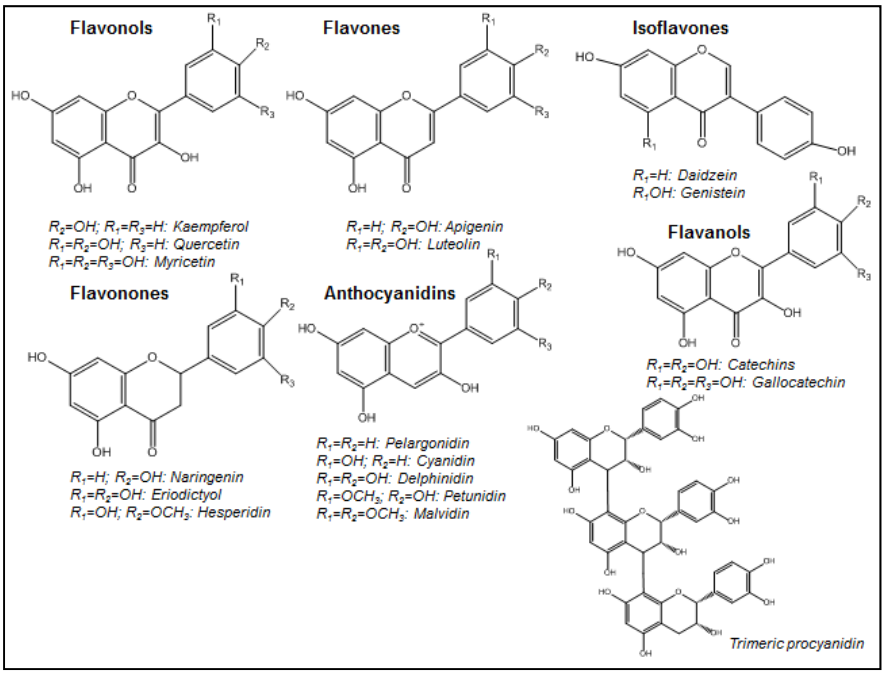

Figure 8. *Chemical structure of flavonoids*

Unlike other classes of flavonoids, which exist in plants primarily in glucoside forms, flavanols are usually present in the aglycone form as monomers and oligomers or are esterified with gallic acid [197]. The most studied PAs are based on the flavanols (+)-catechin and (-)-epicatechin, although (+)-gallocatechin, (-)-epigallocatechin, and (-)-epigallocatechin gallate are other important flavanols. Moreover, PAs can have a variety of structures due to: (i) different hydroxylation patterns; (ii) different degree of polymerization; (iii) different stereochemistries at the three chiral centers and (iv) different locations and types of interflavan linkage [198]. PAs are classified according to their hydroxylation pattern into several subgroups, including procyanidins, which is the most common group, propelargonidins, prodelphinidins, prorobinetinidins, proguibourtinidins, profisetinidins, proteracacinidins and promelacacinidins [199].

PAs are one of the most abundant flavonoid in the human diet, mainly provided by fruits, beans, nuts, cocoa, tea and wine [200]. They are present in relatively high amounts

in the diets of European and US populations, who have a daily intake of PAs, in the context of adults, of 95, 121 and 187 mg/day in U.S.A. [200], Finland [201] and Spain [202], respectively.

PAs are considered to be bioactive compounds because influence physiological and cellular processes and, therefore, can have an effect on health acting as antimicrobial and antioxidants compounds [203], antigenotoxic [204], anti-cancer [205] and anti-inflammatory agents (reviewed in [206]) with cardioprotective properties [207-212]. One of the cardioprotectan activities of PAs is acting as hypolipidemic agents. This hypolipidemic effect of PAs is further detailed, as this thesis is focus in lipid metabolism.

Despite the antioxidant and anti-inflammatory properties of PAs, one of the mechanisms by which PAs exert their cardiovascular protection is improving lipid homeostasis. Animal studies demonstrate that PAs reduce the plasma levels of atherogenic apoB-TG-rich lipoproteins and LDL-cholesterol but increase antiatherogenic HDL-cholesterol [213]. Nevertheless, the effect on the TG metabolism is less clear in the case of human subjects. Several authors conclude that the intake of PAs may be positive [214], neutral [215] or negative [216]. These contradictory conclusions may be explained by different doses and, principally, the different composition of extracts or food administered.

Lipid and lipoprotein levels in blood are the consequence of many biochemical and physiological processes. Therefore, the hypolipidemic effect of PAs can result from the action of these flavonoids on lipid digestion, absorption and CM synthesis in the intestine, VLDL production in the liver, TG uptake by extra-hepatic tissues, uptake of LDL and remnant-like lipoprotein by the liver, and/or HDL metabolism and cholesterol elimination from the body.

The effect of PA-rich foods on CM secretion in humans is conflicting because some studies indicate a decrease [217] whereas others not observed any effect [218, 218]. However, PAs repress CM secretion in animals [211] and apoB48 secretion in Caco-2 cells [219]. Impaired lipid availability in enterocytes seems to be the first cause of the reduction in intestinal CM secretion by PAs. Grape seed PAs extracts [220, 221] and apple PAs [222] inhibit the activity of pancreatic lipase, suggesting limited dietary TG absorption. As far as cholesterol absorption is concerned, elevated excretion of neutral steroids and BAs has been described in rats supplemented with grape [223], apple [224] and cacao [225] PAs, suggesting that PAs inhibit the absorption of cholesterol and BAs, probably by decreasing micellar cholesterol solubility.

Although PAs exert some of their hypolipidemic effect by inhibiting the absorption of dietary lipids and diminishing CM secretion by enterocytes, the repression of VLDL secretion by the liver also has an important role in reducing plasma lipids [211]. In this

sense, grape seed PAs (GSPE) significantly repressed the secretion of VLDL-TG in rats [211] and *de novo* synthesis of TGs and cholesterol, as well as their secretion, in HepG2 cells [226]. Moreover, GSPE modulate the hepatic expression of several genes related to FA, TG and cholesterol metabolism. Thus, the expression of genes related to TG synthesis is strongly repressed in liver by GSPE. mRNA levels of Lipin and Dgat 2 in liver are reduced in mouse [226, 227] and in hyperlipidemic rats [212], respectively. However, the effect of PAs on the expression of several genes depends on animal species and the lengths and doses of PAs. For instance, the expression of HMGCR is reduced in mice liver and increased in rat liver by an acute dose of GSPE, whereas a chronic administration of GSPE not modifies its liver expression in hyperlipidemic rats [212].

The lipid uptake by extra hepatic tissues also is affected by PAs. LPL mRNA is reduced after an acute treatment with GSPE in extrahepatic tissues, and is increased in muscle in normal rats [228] suggesting that TGs are directed preferentially to energy production by the muscle instead of the energy storage by the adipose tissue. Contrary, this effect was not observed in chronic treatments in hyperlipidemic rats [212]. Moreover, apo C-III, which form part of the VLDL and determines the clearance of TG-rich lipoproteins, is repressed in rat liver by GSPE [228]. Furthermore, apo A-V, which accelerates VLDL catabolism [229], is overexpressed by GSPE in mouse liver [226, 227]. Thus, it appears that PAs probably modified extra hepatic TG uptake.

Otherwise, PAs also induces the expression of ABCA1, which is involved it the transference of free cholesterol to lipid poor apoA-I (HDL) in the RCT, in macrophages cultured with oxidized LDL [230]. ApoA-I exert the strongest influence on HDL concentration and moderate red wine consumption increases plasma apoA-I in healthy men [231, 232], improving cholesterol efflux. Moreover, polyphenols from red wine increases LDLR expression and activity in human HepG2 cells [233], but PAs not change LDLR expression in liver either in normal [228] or hyperlipidemic [212] rats. The last step in RCT is the synthesis of BAs by the liver. CYP7A1 mRNA, which controls BA synthesis, is increased by GSPE [228], increasing cholesterol elimination.

To sum up, the hypolipidemic effects of PAs involve: a delay in fat and cholesterol absorption and a reduction in CM secretion by the intestine; repression of VLDL liver secretion; modulation of the lipid uptake by extra hepatic tissues and improving the RCT.

Otherwise, one of the molecular mechanisms described by which PAs exert their hypolipidemia effect is by an induction of FXR with a subsequent upregulation of SHP. The FXR, as it was explained before, plays an important role in lipid metabolism as its activation inhibits hepatic *de novo* lipogenesis. It has been described that the hypotriglyceridemic effect induced by GSPE is dependent on FXR [227]. Using FXR-null mice, the effect reducing TGs by PAs was annulled compared to wild-type mice.

However, it is not known if the activation of FXR by PAs is by a direct interaction or by an indirect mechanism. Moreover, the expression of the SHP is induced by FXR. Using SHP-null mice, the hypotriglyceridemic effect induced by GSPE was annulled and, the involvement of SHP in the effects of PAs was confirmed in HepG2 cells [227]. Thus, it seems that PAs activate FXR and up-regulate SHP, which in turn repress the expression of genes related to lipogenesis and TG synthesis. Interestingly, one of the genes that GSPE down-regulate in an FXR- and SHP-dependent manner is SREBP-1 [226, 227] and, the protein level of SREBP-1 is reduced in the liver of mice with T2D by PAs [234]. Concordant with SREBP-1 reduction, several SREBP-1 target genes involved in lipogenesis and genes involved in TG synthesis are also repressed in the liver of mice and in dislypidemic rats [212]. As a result, PAs decrease plasma TGs by activation of FXR, transient up-regulation of SHP expression and subsequent repression of SREBP-1. Otherwise, it has been demonstrated that GSPE activate the insulin receptor and key targets of insulin signaling pathway, including phosphorylation of AKT [235]. Moreover, PAs also reduce plasma FFAs in normolipidemic [228] and hyperlipidemic [212] rats. Thus, this insulin-like effect of PAs, in conjunction with the activation of FXR and the reduction in the availability of FFAs could repress VLDL secretion and make the hypotriglyceridemic effect of PAs.

A new mechanism of action by which PAs could make their effects is acting as epigenetics agents, modulating miRNA expression, that will be further described in the next section.

5.2. Polyphenols and miRNAs

Polyphenols have beneficial properties in almost all chronic diseases and, recently, polyphenols extracts and pure polyphenols have been shown to modulate the expression of some miRNAs (Table 2). Epigallocatechin gallate (EGCG) was evaluated in HepG2 cells using a range of times and concentrations. Using 50 μM EGCG and a 5-h cell treatment, 5 miRNAs were downregulated by EGCG, miR-30b*, miR-453, miR-520e, miR-629 and miR-608 [236] and, using 100 μM EGCG and a 24-h treatment, 13 miRNAs were upregulated, such as let-7a, miR-16, and miR-221, and 48 miRNAs were downregulated, such as miR-18a, miR-34b, miR-193b ,miR-222 , and miR-342 [237]. Therefore, the number and types of miRNAs deregulated by EGCG depends on the time and polyphenol concentration of the treatment. Furthermore, EGCG treatment of other cell lines, such as lung cancer cells, showed deregulation of other miRNAs, such as miR-210 [238]. Moreover, EGCG and epigallocatechin (EGC) were evaluated in human malignant neuroblastoma cells and it was shown that after

treatment 3 oncogenic miRNAs were decrease, miR-92, -93 and -106b, and 3 tumor suppressors miRNAs were increase, miR-7-1, -34a and -99a [239].

Table 2. *miRNAs deregulated by polyphenols and involved in chronic diseases and metabolic control*

miRNA	Polyphenol	Up/down Regulation	Experi-mental condition	Metabolic pathway	Chronic disease
Let-7a	EGCG [237]	up	100 µM, 24h HepG2 cells	Glucose metabolism Insulin sensitivity	Hypertension [243] Heart hypertrophy [243]
	Ellagitannin [244]	up	15 µg/mL, 6h, HepG2 cells		
Let-7b	EGCG [237]	up	100 µM, 24h HepG2 cells	Glucose metabolism Insulin sensitivity	Diabetes [245] Obesity [41]
Let-7c	Resveratrol [246]	down	50 µM, 24h prostate cancer cells	Glucose metabolism Insulin sensitivity	Heart failure [247] Diabetes [248]
	EGCG [237]	up	100 µM, 24h HepG2 cells		
miR-23a	Resveratrol [249]	down	50 µM 14h, SW480 colon cancer cells	Insulin-dependent glucose transport	Heart failure [247] Cardiac hypertrophy [28]
miR-27a	Resveratrol [250]	up	5 mg/kg/day , 21 days. Ischemic heart of rat	TG storage in adipocytes	HBV-related HCC [251] (adipocyte hypertrophy) Obesity [48, 252] Cardiac hypertrophy [253] Diabetes [248]

miR-29a	Ellagitannin [244]	up	15 µg/mL, 6h, HepG2 cells	Lipoprotein lipase Insulin-dependent glucose transport	Liver fibrosis [251] T2D [41, 248] Obesity [245] Insulin resistance [254] Cardiac hypertrophy [248]
miR-103	Polyphenol extract (Hibiscus sabdariffa) [255]	up	28.6 mg/Kg/day, 10 weeks. Liver hyperlipi-daemic mice	TG storage in adipocytes Insulin sensitivity	Obesity [41, 48, 245, 252] Diabetes [41, 49, 60, 248] Insulin resistance [254]
miR-107	Polyphenol extract (Hibiscus sabdariffa) [255]	up	28.6 mg/Kg/ day, 10 weeks	Insulin sensitivity	Obesity [245, 252] T2D [41] Diabetes [49, 60]
	Ellagitannin [244]	down	15 µg/mL, 6h, HepG2 cells		
miR-122	Polyphenol extract (Hibiscus sabdariffa) [255]	Down	28.6 mg/Kg /day, 10 weeks. Liver of hyperlipi-daemic mice	Cholesterol synthesis Bile acid biosynthesis FA oxidation.	NAFLD and NASH [251, 254]
	Quercetin [241]	Up	2 mg/g diet, 6 weeks. Mice liver		
	Cofee polyphenols [293]	up	0.5 to 1.0%, 2-15 weeks. Mice liver		

	Resveratrol [249]	down	50 µM 14h, SW480 colon cancer cells		
miR-146a	Polyphenol extract (yaupon holly leaves) [258]	up	50 _M, 14h, SW480 colon cancer cells	LDL uptake	ALD/NAFLD [251] T2D [41] Heart failure [256] Apoptosis [60] Inflammation [257]
	Ellagitannin [244]	up	15 µg/mL, 6 h, HepG2 cells		
	Resveratrol [259]	down	50 µM, 14h Human THP-1 monocytic cells and human blood monocy-tes		
miR-155	Quercetin[240]	up	10 µM, 6h, murine RAW264. 7 macropha ges	Glycolysis LDL uptake	Inflammation [251, 257] Hypertension [36]
	Isorhamne-tin [240]	up	10 µM, 6h, murine RAW264. 7 macropha-ges		

miR-206	Resveratrol [242, 249]	down	120 µM, 24h A549 human non-small cell lung cancer cell	Related to protein synthesis in muscle	Obesity [48, 252] Diabetes [245] Apoptosis [41]
		up	50 µM 14h, SW480 colon cancer cells		
miR-210	EGCG [237]	down	100 µM, 24h HepG2 cells	TG storage in adipocytes	T2D [248] Obesity [41]
		up	50 µM, 9h, human and mouse lung cancer cells		
	Ellagitannin [244]	up	15 µg/mL, 6h, HepG2 cells		
miR-223	Ellagitannin [244]	down	15 µg/mL, 6h, HepG2 cells	GLUT4 in myocytes	Diabetes [245]
miR-370	Ellagitannin [244]	down	15 µg/mL, 6h, HepG2 cells	FA oxidation TG synthesis	NAFLD [260]
miR-422	EGCG [237]	down	100 µM, 24h HepG2 cells	Bile acid biosynthesis	Obesity [48, 254] T2D [248]

Quercetin, which is a major representative of the flavonol subclass of flavonoids, also has been reported to modulate miRNAs. Specifically, quercetin and isorhamnetin,

upregulate miR-155 levels in macrophages activated by lipopolysaccharides. However, quercetin metabolites, such as quercetin-3-glucoronide, do not [240]. *In vivo* studies showed that miR-122 and miR-125b are upregulated in the livers of mice fed with quercetin-enriched diets (2 mg quercetin per gram diet), at 61% and 48%, respectively [241].

Resveratrol is the phenolic compound that has been most studied regarding its relationship to miRNAs. In human non-small cell lung cancer cells, line A549, the number of miRNAs that resveratrol modifies depends on the concentration. Some of the miRNAs showed more than a 20-fold change, such as miR-299-5p, miR-194*, miR-338-3p, miR-758, miR-582-3p, and miR-92a-2* [242]. Likewise, in a human colon cancer cell line, SW480, resveratrol decreased the levels of oncogenic miRNAs, such as miR-17, miR-21, miR-25, miR-26a, miR-92a-2, miR-103-2, and miR-181a2. Moreover, resveratrol increased

the levels of the tumor-suppressor miR-663 [249] and, in a transformed human bronchial epithelial cell line, 16HBE-T, miR-622 was upregulated by resveratrol [261]. These and other studies in cancer cells provide evidence that resveratrol can modulate miRNA expression by downregulating oncomiRs and upregulating tumour-suppressors miRs in cancer cells. In contrast, in a monocytecell line, THP-1, resveratrol upregulates miR-663 and impairs the upregulation of the proinflammatory miR-155 [259]. Interestingly, resveratrol also modulates heart and skeletal muscle functions through miRNAs, such as miR-20b, miR-149, miR-133, miR-21, and miR-27b [250]. Recently, resveratrol was also shown to modulate 46 miRNAs in myoblast cells, upregulating 26 and downregulating 20 and two of the most important miRNAs downregulated by resveratrol were miR-20b and -133, those involved in the stimulation of myoblast differentiation [262].

Another polyphenolic compound that it has been studied for its effects on miRNAs is the ellagitannin BJA3121. This ellagitannin modulated 25 miRNAs; 17 were upregulated and 8 were downregulated in HepG2 cells. Surprisingly, seven of the 17 upregulated miRNAs (i.e.; miR-526b, miR-373*, miR-518f*-526a, miR-525, miR-519e*, miR-518c* and miR-512-5p) were located in the same cluster. Moreover, three of the 8 downregulated miRNAs (i.e.; let-7a, let-7f and let-7a) were also located in another cluster. This result suggests that ellagitannin acts on the regulatory region of these gene clusters [244].

A *Hibiscus sabdariffa* phenolic extract was observed to modulate the expression of miRNAs expression in the livers of mice deficient in the LDLR. Interestingly, the continuous administration of this extract reversed the effect of a high fat diet, increasing the expression of miR-103 and miR-107. However, miR-122, which was not affected by the diet, was repressed by the polyphenol extract [255]. In contrast, a polyphenol extract from *Ilex vomitoria* leaves upregulates miR-146a, which is a

negative regulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), in human colon fibroblast [263].[263]

Finally, PAs extracts from cocoa and from grape seed deregulates different miRNAs in HepG2 cells under the same experimental conditions, and only miR-30b* was downregulated and miR-1224-3p, miR-197, and miR-532-3p were upregulated by both extracts [236]. Polyphenols from red wine were tested in human colon myofibroblast cells and it was found an increase in miR-126 expression, an anti-inflammatory miRNA [264]. Following with polyphenolic extracts, GSPE was administrated in a chronic treatment in rats and it was shown 4 miRNAs significantly modulated in pancreatic islets, miR-1249, -483 and -30c-1* that were downregulated and miR-3544 upregulated [265]. It is not well-know the role of these miRNAs in pancreatic islets, but miR-486 is related to be a malignance marker in adrenocortical tumors in humans [266] and miR-30c-1* is associated with the recurrence of non-small cell lung cancer cells [266]. These results suggest that each polyphenol extract may influence particular miRNAs. Polyphenols can have a variety of structures and the characteristic composition of an extract varies based on its botanical origin [193]. Therefore, a specific polyphenol, or a specific interaction between compounds, may affect a specific miRNA.

Altogether, these results provide evidence of the ability of dietary polyphenols to influence miRNA expression, suggesting a new mechanism of action for polyphenols. However, further studies are needed in humans to elucidate the effects of polyphenolic extracts on miRNAs and the metabolic pathways affected by these small molecules, showing a cause-effect relationship.

5.3. ω-3 polyunsaturated fatty acids (PUFAs)

The health benefits of fish oil have emerged since 1970s when epidemiological studies on Greenland Inuits established that this population had reduced rated of myocardial infarction [267]. It was found that the deepwater fish consumed by the Eskimos were abundant in ω-3 PUFAs [268]. The findings from the studies carried out after this revelation led health professionals to encourage the population to consume more ω-3 PUFAs. Nowadays there are several food products, such as milk and eggs, enriched with ω-3 PUFAs.

The most important ω-3 PUFAs in human physiology are alpha-linolenic acid (18:3, *n*−3; ALA), eicosapentaenoic acid (20:5, *n*−3; EPA), and docosahexaenoic acid (22:6, *n*−3; DHA) (Figure 9).

Figure 9. *Chemical structure of ω-3 PUFAs*

In the organism, ALA could be converted to EPA and DHA [269], but this conversion is limited. Thus, it is recommendable to obtain them from diet and are known essential FAs. ALA is mainly found in linseed oil, but also in soybean, wheat germ and nuts, in some vegetable, such as spinach; meat and milk are also a good font of ALA. EPA and DHA are found mainly in fish oil and in blue fish [270]. Most scientific and medical societies, namely those operating in the cardiovascular area, recommend intakes of ω-3 PUFAs of >500mg/day [271]. In addition, a recent FDA's peer-reviewed draft report on the net effect eating fish on brain development and heart health concluded that every 20g of fish consumed per day beyond the current average levels reduces the risk of CVD by 7% [272].

ω-3 PUFAs have been related to be benefitial in health. Due to their physicochemical characteristics, ω-3 PUFAs alter the fluidity of cell membranes, in addition to altering specific areas such as lipid rafts and caveolae [273]. In terms of cardioprotection, ω-3 PUFAs reduce platelet aggregability [274], have direct anti-inflammatory [271], anti-arrhythmic [271] and anti-oxidant [275] activity and modulate the lipid profile.

ω-3 PUFAs reduce the risk for CVD partly by improving the blood lipid profile. One of the most consistent effects of ω-3 PUFAs is the reduction of serum TGs and FFAs in fasting and postprandial conditions [276]. In this sense, the supplementation with EPA, DHA or both, between 1-4 g/day, reduces TG levels in normal and in hyperlipidemic subjects [277-279]. Otherwise, daily supplementation with 20–50 g ALA-rich flaxseed reduced total cholesterol and LDL-cholesterol concentrations in normolipidemic [280] as well as hypercholesterolemic patients [281]. Moreover, DHA increases HDL-cholesterol

levels in overweight, hypercholesterolemics and treated hypertensive subjects with T2D [278, 282, 283]. Therefore, EPA and DHA are equally effective at reducing serum TGs, but only DHA raises HDL-cholesterol (specifically the HDL2 fraction). Furthermore, the supplementation with DHA increases significantly the LDL particle size [284]. Since small, dense LDL particles are associated with an increased risk of coronary artery disease [285] these changes might be expected to contribute to a reduction in atherogenic risk.

Moreover, ω-3 PUFAs are important regulators of critical transcription factors and nuclear receptors that control lipid homeostasis. They have been reported to bind to PPARα and PPARγ, thus promoting β-oxidation and adipogenesis [286]. ω-3 PUFAs also inhibit the conversion of SREBPs to its active form, altering the transcription of a variety of genes involved in cholesterol, TGs and FA synthesis, leading a decrease in plasma TGs concentration [287, 288]. In liver, ω-3 PUFAs promote a change in metabolism through FA oxidation and away from FA synthesis and storage. This shift in metabolism alters hepatic VLDL composition, which in turn affects extrahepatic lipid composition [289].

5.4. ω-3 PUFAs and miRNAs

It is known that fish oil, which is rich in ω-3 PUFAs, has a chemopreventive effect against certain cancer types [290] and several experiments have been done to determine the implication of miRNAs in this protection.

Rats feed with carcinogenic compounds presented a downregulation in rat colon of five tumor suppressors miRNAs, let-7d, miR-15b, -107, -191,and -324-5p. This downregulation was reversed by the addition of fish oil to the rat diet, suggesting that the chemopreventive action of fish oil is dependent on the changes in miRNA expression induced by its consumption [290]. Moreover, miR-34, -25, -17, -26a and -29c have been implicated in the tumoricidal action in gliomas exerted by certain ω-3 PUFAs, including EPA and DHA. [291]. Also, fish oil, and its bioactive compound DHA, inhibits the expression of miR-21 in breast tumor. *In vivo* and *in vitro* resulting [292] increased abundance of the tumor suppressor protein PTEN, which negatively regulates the transcription of CSF-1 (colony stimulating factor-1), via Akt kinase. To date, it does not know whether the effects of ω-3 PUFAs on miRNA expression are the result of a direct effect or are mediated by other metabolites arising from essential fatty acids.

In synthesis, different miRNAs appears to be differentially expressed by fish oil supplementation. However, further studies need to be performed in order to elucidate the effects of essential fatty acids on miRNAs.

6. References

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7. Compendium of miRNAs, polyphenols and chronic diseases

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REVIEW

miRNAs, polyphenols, and chronic disease

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MicroRNAs (miRNAs) are small noncoding RNAs, approximately 18–25 nucleotides in length, that modulate gene expression at the posttranscriptional level. Thousands of miRNAs have been described, and it is thought that they regulate some aspects of more than 60% of all human cell transcripts. Several polyphenols have been shown to modulate miRNAs related to metabolic homeostasis and chronic diseases. Polyphenolic modulation of miRNAs is very attractive as a strategy to target numerous cell processes and potentially reduce the risk of chronic disease. Evidence is building that polyphenols can target specific miRNAs, such as miR-122, but more studies are necessary to discover and validate additional miRNA targets.

Keywords:
Cardiovascular / Insulin / Lipid metabolism / MicroRNA / Polyphenols

1 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 21–23 nucleotides in length, that modulate gene expression by suppressing translation and/or reducing the stability of their target mRNAs [1–4]. These miRNAs are transcribed from DNA as part of longer precursors (primary transcripts or pri-miRNAs) that fold back on themselves to form distinctive hairpin structures. Pri-miRNAs are cleaved in the nucleus into miRNA precursors (pre-miRNAs) by the Drosha complex. These pre-miRNAs, approximately 70 nucleotides, are exported to the cytoplasm and cleaved by Dicer ribonuclease to generate functional miRNAs. Through association with Argonaute proteins, mature miRNAs are included in the RNA-induced silencing complex that binds to the 3′-untranslated region of target mRNA [5–7]. The binding of mature miRNA to the 3′-untranslated region of target mRNA depends on the interaction of a six- to eight-nucleotide seed sequence at the 5′ end of the miRNA with miRNA response elements in the target mRNA [8].

Most of the miRNAs described to date regulate crucial cell processes such as proliferation, differentiation, and apoptosis. Thus, RNAs are involved in normal human development as well as in the initiation of various cancers, where miRNAs have been found to be significant prognostic and predictive markers [9–11]. Furthermore, miRNAs have been reported to regulate several metabolic pathways including insulin secretion and carbohydrate and lipid metabolism [12]. miRNA may influence almost all genetic pathways by targeting transcription factors, secreted factors, receptors, and transporters [4]. Moreover, as an epigenetic mechanism, miRNAs may mediate the effects of nutrition and may be causal in the development of many common chronic diseases [13]. Current data indicate that a wide range of dietary factors, including micronutrients and nonnutrient dietary components such as polyphenols, can modify expression of miRNA [14, 15].

Several hundred unique polyphenols have been identified as secondary metabolites in edible plants. Polyphenols are classified into different groups as a function of their molecular structure: phenolic acids, flavonoids, stilbenes, and lignans. Flavonoids are divided into six subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (monomeric catechins and oligomeric proanthocyanidins) [16].

Epidemiological studies suggest that high dietary intake of polyphenols is associated with a decreased risk of a range of diseases including cardiovascular disease (CVD) and some cancers and neurodegenerative diseases [17]. Flavonoids improve endothelial function, lipid metabolism, and glucose homeostasis, and can reduce oxidative stress and blood pressure [18, 19]. Most of the health effects of flavonoids have been attributed to the alteration of gene expression that codes key metabolic proteins. These gene modifications can result from the interaction of polyphenols with signaling cascades and/or with epigenetic factors such as miRNAs.

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Abbreviations: ABCA1, cholesterol efflux transporter ATP-binding cassette transporter A1; **CVD**, cardiovascular disease; **EGCG**, epigallocatechin gallate; **FA**, fatty acid; **FAS**, fatty acid synthase; **NAFLD**, nonalcoholic fatty liver diseases; **oxLDL**, oxidized LDL; **SREBP1**, sterol response element binding protein 1; **TG**, triglyceride; **T2D**, type 2 diabetes

Thousands of miRNAs have been described, and it is thought that they regulate more than 60% of all human cell transcripts that modulate metabolism and are implicated in various diseases [20]. Polyphenolic modulation of miRNAs is very attractive as a strategy to modulate numerous cell processes and reduce the risk of chronic disease. The aim of the present paper is to review miRNAs that are targeted by polyphenols and to discuss the implication of miRNAs in the beneficial health effects of polyphenols on metabolic disease.

2 miRNAs and metabolic control

miRNAs are known to modulate more than 60% of genes [20] and should therefore be implicated in almost all metabolic pathways. However, the specific role of each miRNA in controlling metabolic pathways is still unknown, and most studies have focused on lipid metabolism. For instance, in the last 2 years, the key roles of miR-33 and miR-122 in lipid metabolism control have emerged. Briefly, miR-122 is expressed primarily in the liver and was the first miRNA to be linked to the regulation of lipid metabolism [21]. It is recognized as vital to hepatitis C virus infection [21]. Another important miRNA in lipid metabolism is miR-33, which has been studied extensively and targets genes involved in cholesterol efflux, fatty oxidation, and VLDL triglycerides (TGs) [22, 23]. Interestingly, two isoforms of miR-33, miR-33b and miR33a [22, 23], have been identified. These miRNAs are intronic of the sterol response element binding protein 1 (SREBF1) and 2 (SREBF2) genes, respectively [24]. SREBF1 and SREBF2 code for the transcription factors SREBP1 and SREBP2, which regulate all SREBP-responsive genes in both the cholesterol and fatty acid (FA) biosynthetic pathways [25]. Therefore, miR-33 and the SREBP host genes cooperate to control cholesterol homeostasis [26]. Other miRNAs do not directly affect metabolism, but instead target nuclear receptors; for example, miR-613 targets the nuclear liver X receptor (LXRα) [27]. Additional studies have implicated other miRNAs in the regulation of lipid metabolism [22].

2.1 Lipid metabolism

Different miRNAs are implicated in the control of each key point in cholesterol homeostasis. For instance, only miR-122 is related to the biosynthesis of cholesterol; miR-122 inhibition in normal mice resulted in reduced plasma cholesterol levels and a decrease in cholesterol synthesis rates [28]. Sequestration of miR-122 represses 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the key controller of cholesterol biosynthesis, and decreases the pathway activity in liver cells [29]. However, the regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by miR-122 is postulated to be indirect [29].

No studies of the role of miRNAs regulating native LDL uptake by cells have yet been published. Intriguingly, exogenous plant miRNAs in food have been described to target genes related to LDL uptake in humans. Rice miR-168a, which is abundant in the sera of Chinese subjects, targets the human and mouse LDL receptor adapter protein 1 mRNA, inhibits LDL receptor adapter protein 1 expression in liver, and consequently decreases LDL removal from mouse plasma [30]. However, some miRNAs have been implicated in the regulation of scavenger receptors for oxidized LDL (oxLDL) in cells activated with oxLDL. The lectin-like oxLDL receptor-1 (LOX-1) contains a let-7g binding site, and the transfection of let-7g inhibits LOX-1 expression in macrophages [31]. In addition, miR-29a regulates the expression of the scavenger receptor for oxLDL on dendritic cells [32], and miR-146a significantly reduces intracellular LDL cholesterol accumulation in macrophages [33].

The cholesterol efflux transporter ATP-binding cassette transporter A1 (ABCA1) is crucial for reversing cholesterol transport. This transporter is involved in both HDL biogenesis in the liver and in cholesterol efflux to HDL in extrahepatic cells such as macrophages [34]. Its expression is under the control of miR-33. In this sense, miR-33 represses ABCA1 expression in liver [35, 36] and extrahepatic tissue, such as pancreatic islets [37] and macrophages [26, 36, 38]. Interestingly, inhibition of miR-33 increases the expression of ABCA1, enhancing HDL biogenesis, and increasing HDL-cholesterol levels in nonhuman primates [35] and mice [36]. In addition, miR-33 also controls other cholesterol transporters implicated in cholesterol efflux, such as ATP-binding cassette sub-family G member 1 and Niemann-Pick C1 (NCP1) [39]. Recently, miR-758 has also been implicated in the repression of ABCA1 levels in several cell types, including macrophages [40]. The key enzyme of bile acid synthesis, 7-alpha-hydroxylase (CYP7A1), is controlled by miR-122a and miR-422a in hepatic cells [41].

In addition to cholesterol, miRNAs control FA and TG metabolism. Key enzymes of FA oxidation are targeted by miR-33 including carnitine O-octaniltransferase, carnitine palmitoyltransferase 1A, and hydroxyacyl-CoA-dehydrogenase in the liver [42]. In this sense, overexpression and inhibition of endogenous miR-33 reduces and increases, respectively, FA oxidation in hepatic cell lines [42]. FA synthesis activity in the liver is also controlled by miR-33, which increases the expression of SREBF1 and genes codifying key enzymes of lipogenesis, such as fatty acid synthase (FAS), ATP citrate lyase , and acetyl-CoA carboxylase alpha [35]. As a result of the inverse effects of miR-33 on FA synthesis and oxidation in the liver, miR-33 antagonism has been reported to significantly reduce the plasma levels of VLDL-associated TGs in a nonhuman primate model [35]. Two other miRNAs controlling FA and TG metabolism in the liver are miR-370 and miR-122. Of these, miR-122 inhibition increases hepatic FA oxidation and decreases hepatic FA synthesis rates in normal mice [28]. Transfection of human liver hepatocellular carcinoma cell lines (HepG2) with sense or antisense miR-370 or miR-122 upregulated and downregulated, respectively, SREBP-1c and the enzymes diacylglycerol

acyltransferase-2, FAS, and acetyl-CoA carboxylase 1 [43]. On the other hand, miR-370 targets carnitine palmitoyltransferase 1A and decreasing the rate of beta oxidation. Interestingly, because miR-370 upregulates the expression of miR-122, it has been suggested that some of the effects of miR-370 on FA and TG metabolism in liver are mediated by miR-122 [43].

TG utilization by tissue and its storage in adipose tissue are also governed by miRNAs. Lipoprotein lipase, which catalyses the delivery of TG from the TG-rich lipoprotein, is a direct target of miR-29a [32]. Several miRNAs control TG storage in adipocytes. For example, overexpression of miR-378/378*, miR-9*, miR-143, miR-103, or miR-210 induces TG accumulation in adipose cells by increasing adipogenesis [44–46]. Specifically, miR-378/378*, an intronic miRNA located within the peroxisome proliferator-activated receptor gamma coactivator-1 alpha, increases the expression of fatty acid binding protein 4, FAS, and stearoyl-coenzymeA desaturase in adipocytes [45]. In addition, some studies have focused on miRNAs that appear to act as negative regulators of adipocyte differentiation and TG accumulation [46]. For instance, miR-27a targets peroxisome proliferator-activated receptor gamma (PPARγ) and represses PPARγ protein levels, thus inhibiting adipocyte differentiation.

2.2 Glucose metabolism

Although glucose metabolism has not been studied as extensively as lipid metabolism, some miRNAs have been linked to glucose homeostasis and insulin sensitivity. For example, miR-375, which is highly expressed in pancreatic islets, is required for normal glucose homeostasis. Mice knockouts for miR-375 are hyperglycemic and exhibit reduced pancreatic beta-cell mass, increased fasting and fed-plasma glucagon levels, and increased gluconeogenesis and hepatic glucose output [47].

Several miRNAs regulate cell insulin sensitivity by acting on various components of the insulin signaling pathway. For instance, the Let-7 family of miRNAs regulates glucose metabolism in multiple organs because it mediates the repression of multiple components of the insulin signaling pathway, including insulin-like growth factor 1 receptor, insulin receptor, and insulin receptor substrate 2 [48]. Therefore, Let-7 overexpression in mice resulted in impaired glucose tolerance and reduced glucose-induced pancreatic insulin secretion [49]. Let-7 knockdown mice, however, are protected from glucose tolerance impairment in obesity induced by diet [49]. Both miR-143 [50] and miR-33 [42] target components of the insulin signaling pathway; in contrast, miR-103 and miR-107 regulate insulin sensitivity by targeting caveolin-1, a component that stabilizes the insulin receptor [51].

Another key molecule in glucose homeostasis is GLUT4, the insulin-dependent glucose transporter, which is under the control of several miRNAs. GLUT4 expression is repressed by miR-9* and miR-143 in adipocytes [46] and by miR-133 in cardiomyocytes [52], whereas miR-223 increases GLUT4 expression in cardiomyocytes [53]. Moreover, insulin-dependent glucose uptake is regulated by miR-29a and miR-23a in muscle [54].

Studies of the control of glycolysis by miRNAs have only been performed in cancer cells. Hexokinase 2, a hexokinase isoform expressed in cancer cells, is the target of miR-143 and acts as a negative regulator [55, 56]. The miRNA miR-155 upregulates hexokinase 2 by repressing miR-143 [57].

2.3 Amino acid metabolism

A few studies have focused on the role of miRNAs in controlling amino acid metabolism and protein synthesis and degradation, but only miR-23 has been directly implicated in the regulation of amino acid metabolism. In cancer cells, miR-23 targets glutaminase mRNA, inhibiting glutamine utilization [58].

Ingestion of essential amino acids, which stimulates muscle protein synthesis, increases miR-499, -208b, -23a, -1, and pri-miR-206 levels in human muscle [59]. Moreover, miR-23a has been associated with protection against muscle atrophy [60]. Further studies are needed to determine whether these miRNA are implicated in amino acid metabolism and/or protein synthesis.

3 miRNA signature in chronic diseases

As previously mentioned increasing evidence shows that miRNAs are involved in almost all biological processes and affect most metabolic pathways. Hence, aberrant deregulation of some miRNAs has been related to metabolic disorders and other diseases such as human immunodeficiency virus, cancer, hepatitis C, obesity, CVD, nonalcoholic fatty liver diseases (NAFLD), and type 2 diabetes (T2D). In this sense, miRNAs are emerging as potential biomarkers of numerous pathologies and therefore as new therapeutic targets. The present review examines the miRNAs involved in some of the major chronic diseases: CVD, obesity, T2D, and NAFLD.

3.1 miRNAs in CVD

CVD is the leading cause of human morbidity and mortality in industrialized countries. These diseases are associated with genetic mutations or deregulation of genes essential for cardiac function, which can also be regulated by miRNAs. Multiple miRNAs important for cardiovascular regulation have been identified and are recognized to control a considerable number of cardiac functions. Furthermore, miRNAs are emerging as potential targets for the diagnosis, prevention, and treatment of CVD. Review articles that describe the role of miRNAs in CVD [61–65] have recently appeared in the literature. Although specific patterns of miRNA

expression correlate well with cardiovascular disorders (e.g. cardiac hypertrophy, heart failure, myocardial infarction, cardiac fibrosis, arrhythmia, angiogenesis, and vascular remodeling) [66–73], the mechanisms and alteration of CVD are complex, and it is unclear which miRNAs are important. However, evidence is mounting that some specific miRNAs have a major role in cardiac pathologies, including miR-1 and miR-133 in cardiac hypertrophy [74–76] and the miR-29 family in cardiac fibrosis [70].

One of the most common and important cardiovascular health problems is hypertension, which is defined as a constant elevation of systemic blood pressure. Many characteristics of hypertension development at the molecular level are still unknown, but it is evidently a multifactorial disease that involves several genes. In this sense, miRNAs are likely to have a potential role in regulating these main genes [77–81]. Evidence suggests that specific miRNAs are involved in vascular endothelial pathogenesis in hypertension (e.g. miR-126), acting as pro-/antiangiogenic factors [82], interacting with the renin-angiotensin-aldosterone system (e.g. miR-155) [83] or targeting vascular smooth muscle cells [84] (e.g. miR-143 and miR-145) [73, 85]. Some miRNAs have also been shown to be related to the nitric oxide and atrial natriuretic peptide pathways in vascular smooth muscle cells [86].

3.2 miRNAs in T2D

T2D, which has reached epidemic levels worldwide, is a metabolic disorder that is characterized by hyperglycemia in the context of reduced insulin sensitivity and insulin resistance. T2D is a complex disease whose disorders are not fully understood. However, it appears that insulin resistance has a major role in the development of this pathology. Moreover, insulin resistance and β-cell dysfunction are mainly developed because of deregulation of adipose tissue function and lipid metabolism [87]. Recently, several studies have shown that miRNAs play major roles in insulin production and secretion, insulin resistance, pancreatic islet development, and β-cell dysfunction (reviewed in ref. [88–90]). Furthermore, miRNAs are also involved in glucose homeostasis and lipid metabolism related to T2D. Most studies have been based on the miRNA microarray analysis of insulin-resistant tissues, such as skeletal muscle, liver, adipose tissue, and pancreatic β-cells, in animal models of spontaneous T2D. In these studies, various miRNAs were shown to be deregulated, but it is still not clear which specific miRNAs are important for T2D and what their roles are. However, some research in this area has been reported, including the deregulation of miR-335 in the adipose tissue of obese mice, which has been correlated to adipocyte differentiation and maturation [91]. The deregulation of miR-27b and miR-335 in the liver of T2D rats has been suggested to contribute to fatty liver and associated pathologies [91, 92]. Some miRNAs are also involved in the adjustment of skeletal muscle to insulin resistance and T2D. For example, a decrease in miR-24 or miR-126 may help muscles to increase insulin-dependent glucose uptake; miRNAs therefore participate in the adaptation of muscle to high glucose levels [93, 94]. Finally, miR-375 and miR-34a may have an important role in T2D in islets [47, 95].

3.3 miRNAs in adipogenesis and obesity

Obesity, characterized by increased fat mass and energy storage in adipose tissue, has reached pandemic proportions in recent years. This pathology is related to diseases such as T2D, hypertension, CVD, and cancer [96]. miRNAs are important regulators of the development and function of adipose tissue and metabolic functions and therefore have potential roles in obesity and their associated diseases (reviewed in ref. [46, 97, 98]). Several studies have demonstrated that miRNAs acts as central modulators of normal white and brown adipose tissue differentiation and biology. Many miRNAs that are downregulated in obesity are upregulated during adipogenesis and vice versa [44, 99]. In this sense, several miRNAs regulate, enhance, and inhibit adipogenesis (e.g. miR-143), suggesting that miRNAs have a potential role in controlling adipocyte number and size. However, miRNAs govern not only mass size but also the metabolic consequences of obesity and adipose tissue metabolism [98]. More evidence for the role of miRNAs in obesity-related diseases is necessary to understand their regulatory roles in modulating energy balance, adipose biology, and their potential contribution to obesity [12].

3.4 miRNAs in NAFLD

NAFLD is characterized by fat accumulation in the liver without significant alcohol consumption [100]. Clinical manifestations of this pathology include dyslipidemia, hypertension, and insulin resistance. Recently, the involvement of miRNAs in NAFLD has been described (reviewed in ref. [101, 102]). It has been demonstrated that miRNAs are able to modify lipid droplet accumulation in hepatocytes, which is characteristic of NAFLD [103]. Other studies have showed that some miRNAs target PPARα, a key molecule for NAFLD [104]. Studies in humans have demonstrated altered hepatic miRNAs profiles in non-alcoholic steatohepatitis (NASH), in which miR-122 is remarkably downregulated [105].

3.5 Circulating miRNAs as biomarkers for chronic diseases

miRNAs are good candidate biomarkers of diseases because they are stable, conserved, tissue specific, pathology specific, and detectable in serum, plasma, and other biological fluids [106]. miRNAs circulating in the plasma are remarkably stable because they circulate packed inside microparticles (microvesicles, exosomes, and apoptotic bodies) [80, 107] or

associated with RNA-binding proteins (Argonaute2) [108] or lipoprotein complexes (HDL) (reviewed in ref. [109, 110]). Because miRNAs circulate with microparticles, they may function in cell-to-cell communication, as suggested by several studies [80, 111]. Because circulating miRNAs are transported from donor cells to surrounding tissue, they alter the genes and functions of recipient cells and therefore have a role in endocrine and paracrine communication (reviewed in ref. [12]). Furthermore, miRNAs can originate from exogenous sources, such as ingested plants [30].

A major challenge for chronic disease research is the identification of reliable biomarkers that can be measured in a noninvasive way using accessible samples such as plasma or serum. Therefore, miRNAs in plasma and serum are beginning to be studied as biomarkers for chronic disease, and altered circulating miRNAs profiles have already been correlated to several diseases states. Circulating liver-specific miR-122 was found to be a good biomarker for hepatic injuries such as NAFLD and NASH [112–114]. Deregulation of circulating miR-223 was correlated with atherosclerosis [109], miR-126 to T2D [115], Let-7a to hypertension [116], and circulating miR-499-5p was postulated to be a sensitive biomarker for non ST-elevation myocardial infarction [117]. Other examples are miR-17-5p and miR-132, which are differentially expressed in obese and nonobese subjects in peripheral blood, suggesting their potential role as novel metabolic biomarkers [118]. Increased levels of miR-122 and miR-370 in plasma were found in patients with coronary artery disease in hyperlipidaemia [119].

4 Modulation of miRNA levels by polyphenols

Polyphenols have beneficial properties in almost all chronic diseases, and recently polyphenol extracts and polyphenols such as quercetin or resveratrol have been shown to modulate the expression of miRNAs.

Epigallocatechin gallate (EGCG) was evaluated in HepG2 cells using a range of times and concentrations. Using 50 μM EGCG and a 5-h cell treatment, 5 miRNAs were downregulated by EGCG, miR-30b*, miR-453, miR-520e, miR-629, and miR-608 [15]. Using 100 μM EGCG and a 24-h treatment, 13 miRNAs were upregulated, such as let-7a, miR-16, and miR-221, and 48 miRNAs were downregulated, such as miR-18a, miR-34b, miR-193b, miR-222, and miR-342 [120]. Therefore, the number and types of miRNAs deregulated by EGCG depends on the time and polyphenol concentration of the treatment. Furthermore, EGCG treatment of other cell lines, such as lung cancer cells, showed deregulation of other miRNAs, such as miR-210 [121].

Quercetin, which is a major representative of the flavonol subclass of flavonoids, also has been reported to modulate miRNAs. Specifically, quercetin and isorhamnetin, upregulate miR-155 levels in macrophages activated by LPS. However, quercetin metabolites, such as quercetin-3-glucoronide, do not [122]. In vivo studies showed that miR-122 and miR-125b are upregulated in the livers of mice fed with quercetin-enriched diets (2 mg quercetin per gram diet), at 61% and 48%, respectively [123].

Resveratrol is the phenolic compound that has been most studied regarding its relationship to miRNAs. In human nonsmall cell lung cancer cells, line A549, the number of miRNAs that resveratrol modifies depends on the concentration. Some of the miRNAs showed more than a 20-fold change, such as miR-299-5p, miR-194*, miR-338-3p, miR-758, miR-582-3p, and miR-92a-2* [124]. Likewise, in a human colon cancer cell line, SW480, resveratrol decreased the levels of oncogenic miRNAs, such as miR-17, miR-21, miR-25, miR-26a, miR-92a-2, miR-103-2, and miR-181a2. Moreover, resveratrol increased the levels of the tumor-suppressor miR-663 [125]. In a transformed human bronchial epithelial cell line, 16HBE-T, miR-622 was upregulated by resveratrol [126]. These and other studies in cancer cells provide evidence that resveratrol can modulate miRNA expression by downregulating oncomiRs and upregulating tumour-suppressors miRs in cancer cells. In contrast, in a monocyte cell line, THP-1, resveratrol upregulates miR-663 and impairs the upregulation of the proinflammatory miR-155 [127]. Interestingly, resveratrol also modulates heart and skeletal muscle functions through miRNAs, such as miR-20b, miR-149, miR-133, miR-21, and miR-27b [128].

Another polyphenolic compound that it has been studied for its effects on miRNAs is the ellagitannin BJA3121. This ellagitannin modulated 25 miRNAs; 17 were upregulated and eight were downregulated in HepG2 cells. Surprisingly, seven of the 17 upregulated miRNAs (i.e. miR-526b, miR-373*, miR-518f*-526a, miR-525, miR-519e*, miR-518c*, and miR-512-5p) were located in the same cluster. Moreover, three of the eight downregulated miRNAs (i.e. let-7a, let-7f, and let-7a) were also located in another cluster. This result suggests that ellagitannin acts on the regulatory region of these gene clusters [129].

A *Hibiscus sabdariffa* phenolic extract was observed to modulate the expression of miRNAs expression in the livers of mice deficient in the LDL receptor. Interestingly, the continuous administration of this extract reversed the effect of a high fat diet, increasing the expression of miR-103 and miR-107. However, miR-122, which was not affected by the diet, was repressed by the polyphenol extract [130]. In contrast, a polyphenol extract from *Ilex vomitoria* leaves upregulates miR-146a, which is a negative regulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), in human colon fibroblast [131]. Finally, proanthocyanidin extracts from cocoa proanthocyanidin extract and grape seed (grape seed proanthocyanidin extract) deregulates different miRNAs in HepG2 cells under the same experimental conditions, and only miR-30b* was downregulated and miR-1224-3p, miR-197, and miR-532-3p were upregulated by the two extracts [15]. Recently, we have shown that grape seed proanthocyanidin extract downregulated miR-122 and miR-33 and modulated their target gene FAS and ABCA1, respectively,

in liver cells, both in vivo and in vitro [132]. These results suggest that each polyphenol extract may influence particular miRNAs. Polyphenols can have a variety of structures and the characteristic composition of an extract varies based on its botanical origin [16]. Therefore, a specific polyphenol, or a specific interaction between compounds, may affect a specific miRNA.

Altogether, these results provide evidence of the ability of dietary polyphenols to influence miRNA expression, suggesting a new mechanism of action for polyphenols. However, further studies are needed in humans to elucidate the effects of polyphenolic extracts on miRNAs and the metabolic pathways affected by these small molecules, showing a cause–effect relationship.

5 Relationship between polyphenol miRNA modulation and their health effects on chronic diseases

The majority of studies involving miRNAs as mediators of polyphenol effects in cells have been performed in cancer cells, and only a few studies have been centerd on metabolic diseases. As we indicated in the last section, several research groups have studied the ability of polyphenols to modulate miRNAs using microarray technology. In Table 1, we present those miRNAs that are deregulated by polyphenols and are known to regulate metabolism and be involved in chronic diseases other than cancer. From all of the miRNAs described as targets of polyphenols in the literature, only 16 miRNAs are clearly involved in metabolic control and chronic diseases. Furthermore, the referenced polyphenols (or polyphenols extract) do not target all 16 miRNAs, and some of them are only targeted by one polyphenol. This is not surprising because the experimental conditions (e.g. cell line, tissues, etc.) and treatment conditions (e.g. time, dose, etc.) are different between studies.

Although the identity of miRNAs that are common targets for all polyphenols seems unclear, the liver-specific miR-122 is a clear putative target of polyphenols. miR-122 is targeted by different types of polyphenols (i.e. a polyphenol extract from *H. sabdariffa*, quercetin, coffee polyphenols, and grape seed proanthocyanidins) in mice livers. miR-122 controls cholesterol and bile acid biosynthesis and FA oxidation in liver as well as being related to NAFLD. Interestingly, polyphenol extracts from *H. sabdariffa* [130], quercetin [133], and coffee polyphenols [134] prevent diet-induced liver steatosis in mice. Moreover, these polyphenols repress the expression of SREBP-1c [133, 134], acetyl-CoA carboxylase 1 [134], CYP7A1 [135], and FAS [132], which are also under the control of miR-122 in the liver. Clinical manifestations of NAFLD include dyslipidemia, hypertension, and insulin resistance. Therefore, the health benefits of polyphenols for dyslipidemia, hypertension, and insulin resistance partially could be caused by improvement of the liver metabolism, resulting from the targeting of miR-122. Relating to lipid metabolism proanthocyanidins also repress miR-33, which plays a crucial role in cholesterol homeostasis and lipoprotein levels.

In addition to miR-122, other miRNAs are influenced by specific polyphenols in hepatic cell lines. Specifically, EGCG and ellagitannin modulate the expression of some components of the Let-7 family and miR-210 in HepG2, and both of them are related to insulin sensitivity. The Let-7 family regulates glucose metabolism in multiple organs as result of these miRNAs mediating the repression of several components of the insulin signaling pathway [48, 49]. In contrast, miR-210 is upregulated in the liver of diabetic rats [88] and downregulated in the fat of obese humans [90].

In addition, the modulation of miRNAs by polyphenols has been studied in monocytes and macrophages. All of the polyphenols studied in macrophages, including resveratrol, quercetin, and isorhamnetin, target miR-155. Levels of miR-155 in serum are proposed as a biomarker of CVD [110], and this miRNA is linked to inflammatory responses in macrophages [136]. The inflammatory response of macrophages is a key feature in the pathogenesis of atherosclerosis, and interestingly, quercetin metabolites are accumulated in human atherosclerotic lesions but not in the normal aorta [137, 138]. Activated macrophages show the accumulation of quercetin metabolites, suggesting that this accretion underlies the antiatherosclerotic activity of this polyphenol [138]. miR-155 has also been related to hypertension through targeting the renin-angiotensin-aldosterone system [81]. Protection from atherosclerosis and hypertension is a generalized effect of polyphenols [139–142]. However, further studies with other polyphenols are necessary to confirm miR-155 as a real target of polyphenols.

The modulation of miRNAs in the heart by polyphenols has recently been studied using resveratrol, which targets miR-27a. miR-27a is upregulated during cardiac hypertrophy [143]. Furthermore, miR-27a controls the phosphoinositide 3-kinase pathway that regulates physiological hypertrophy and cardiac protection [61]. Resveratrol reduces cardiac hypertrophy in hypertensive animals [144], and several signaling pathways affected by resveratrol (or its analogues) in the heart have been described to influence this effect, including the protein kinase B/phosphoinositide 3-kinase pathway [144, 145].

6 Concluding remarks

A single miRNA can regulate the expression of multiple target mRNAs, and a particular transcript can be modulated by multiple miRNAs. To date, thousands of miRNAs have been discovered, and it is thought that these small molecules may regulate more than 60% of all cell transcripts [20]. Hence, the fact that dietary compounds modulate miRNAs suggests new functions of polyphenols and provides insights into the mechanisms by which these compounds improve health and protect from diseases. However, the information in human is poor and scarce and most of the evidence is only observational and do not show a cause–effect relationship. Therefore,

Table 1. miRNAs deregulated by polyphenols and involved in chronic diseases and metabolic control

miRNA	Polyphenol	Up-/down-regulation	Experimental condition	Metabolic pathway	Chronic disease
Let-7a	EGCG [120]	Up	100 μM, 24 h HepG2 cells	Glucose metabolism, insulin sensitivity	Hypertension [61] Heart hypertrophy [61]
	Ellagitannin [129]	Up	15 μg/mL, 6 h, HepG2 cells		
Let-7b	EGCG [120]	Up	100 μM, 24 h, HepG2 cells	Glucose metabolism, insulin sensitivity	Diabetes [88] Obesity [90]
Let-7c	Resveratrol [148]	Down	50 μM, 24 h, prostate cancer cells	Glucose metabolism, insulin sensitivity	Heart failure [149]
	EGCG [120]	Up	100 μM, 24 h, HepG2 cells		Diabetes [88]
miR-23a	Resveratrol [125]	Down	50 μM, 14 h, SW480 colon cancer cells	Insulin-dependent glucose transport	Heart failure [149] Cardiac hypertrophy [64]
miR-27a	Resveratrol [128]	Up	5 mg/kg/day for 21 days. Ischemic heart of rat	TG storage in adipocytes	HBV-related HCC [150] (adipocyte hypertrophy) Obesity [46, 98] Cardiac hypertrophy [143] Diabetes [88]
miR-29a	Ellagitannin [129]	Up	15 μg/mL, 6 h, HepG2 cells	Lipoprotein lipase, insulin-dependent glucose transport	Liver fibrosis [150] T2D [88, 90] Obesity [89] Insulin resistance [97] Cardiac hypertrophy [88]
miR-33	Grape seed proanthocyanidins [132]	Down	250 mg/kg for 1 h, mice liver 25 mg/L for 1 h, FAO cells	Cholesterol efflux, HDL biogenesis, and VLDL levels. Fatty acid metabolism and insulin signaling	Atherosclerosis [36]
miR-103	Polyphenol extract (*Hibiscus sabdariffa*) [130]	Up	28.6 mg/kg/day, 10 weeks Liver hyperlipidaemic mice	TG storage in adipocytes Insulin sensitivity	Obesity [46, 89, 90, 98] Diabetes [12, 88, 90, 115] Insulin resistance [97]
miR-107	Polyphenol extract (*Hibiscus sabdariffa*) [130]	Up	28.6 mg/kg/day, 10 weeks liver hyperlipidaemic mice	Insulin sensitivity	Obesity [46, 89] T2D [90] Diabetes [12, 115]
	Ellagitannin [129]	Down	15 μg/mL, 6 h, HepG2 cells		
miR-122	Polyphenol extract (*Hibiscus sabdariffa*) [130]	Down	28.6 mg/kg/day, 10 weeks Liver of hyperlipidaemic mice	Cholesterol synthesis Bile acid biosynthesis Fatty acid oxidation	NAFLD and NASH [97, 150]
	Quercetin [123]	Up	2 mg/g diet, 6 weeks, mice liver		
	Coffee polyphenols [134]	Up	0.5–1.0% for 2-15 weeks, mice liver 2.5 μg/mL, 24 h Hepa 1-6 cells		
	Grape seed proanthocyanidins [132]	Down	250 mg/Kg for 1 h, mice liver 25 mg/L for 1 h, FAO cells		

Table 1. Continued

miRNA	Polyphenol	Up-/down-regulation	Experimental condition	Metabolic pathway	Chronic disease
miR-146a	Resveratrol [125]	Down	50 μM 14 h, SW480 colon cancer cells	LDL uptake	ALD/NAFLD [150] T2D [90] Heart failure [63] Apoptosis [115] Inflammation [136]
	Polyphenol extract (yaupon holly leaves) [131]		50 μM, 14 h, SW480 colon cancer cells		
	Ellagitannin [129]	Up	15 μg/mL, 6 h, HepG2 cells		
miR-155	Resveratrol [127]	Down	50 μM, 14 h, human THP-1 monocytic cells and human blood monocytes	Glycolysis	Inflammation [136, 150] Hypertension [83]
	Quercetin [122]	Up	10 μM, 6 h, murine RAW264.7 macrophages	LDL uptake	
	Isorhamnetin [122]	Up	10 μM, 6 h, murine RAW264.7 macrophages		
miR-206	Resveratrol [124, 125]	Down	120 μM, 24 h, A549 human nonsmall cell lung cancer cell	Related to protein synthesis in muscle	Obesity [46, 98] Diabetes [89] Apoptosis [90]
		Up	50 μM, 14 h, SW480 colon cancer cells		
miR-210	EGCG [120]	Down	100 μM, 24 h, HepG2 cells	TG storage in adipocytes	T2D [88] Obesity [90]
		Up	40 μM, 9 h, human and mouse lung cancer cells		
	Ellagitannin [129]	Up	15 μg/mL, 6 h, HepG2 cells		
miR-223	Ellagitannin [129]	Down	15 μg/mL, 6 h, HepG2 cells	GLUT4 in myocytes	Diabetes [89]
miR-370	Ellagitannin [129]	Down	15 μg/mL, 6 h, HepG2 cells	Fatty acid oxidation TG synthesis	NAFLD [151]
miR-422	EGCG [120]	Down	100 μM, 24 h, HepG2 cells	Bile acid biosynthesis	Obesity [97, 98] T2D [88]

more studies are needed with other polyphenols and different cell types, animal models, and more specifically in humans to establish the target miRNAs of polyphenols. However, evidence of these effects is being uncovered, and some clear targets of polyphenols, such as miR-122, can be identified. This research will be important because the modulation of key miRNAs implicated in chronic diseases by natural products, such as polyphenols, has a great potential for dietary applications.

Currently, the molecular mechanism by which polyphenols modulate miRNAs levels is unknown. However, there is evidence that polyphenols can bind to mRNAs and proteins [146, 147]. Therefore, it is possible that they also bind to miRNAs or to some component involved in miRNA biogenesis, such as Dicer or RNA-induced silencing complex. Additionally, some miRNAs are intronic of genes and polyphenols that modify host gene expression, which would also affect the miRNA levels.

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The research work carried out in this Ph. D. Thesis is part of a general research project developed by the Nutrigenomics Research Group of the Universitat Rovira i Virgili, which deals with the potential beneficial effects of dietary proanthocyanidins which are the most abundant polyphenols in the human diet. Proanthocyanidins improve human health, with cardioprotectant, antigenotoxic, anti-inflammatory, antioxidant and anticarcinogenic activities. Moreover, proanthocyanidins improve several risk factors for cardiovascular disease, such as dyslipidemia and insulin resistance. More specifically, grape seed proanthocyanidins are potent hypolipidemic agents and our research group has previously shown that proanthocyanidins improve lipid metabolism in the liver through a FXR-SHP dependent pathway. However, it is becoming clear that microRNAs (miRNAs) play key roles in the regulation of genes involved in lipid metabolism in the liver, such as miR-33 and miR-122, and the effects of GSPE on these miRNAs is unknown. Recently, it has been reported that dietary polyphenols such as resveratrol, epigallocatechin gallate, and proanthocyanidins from grape seed and cocoa modulate miRNA expression. Thus, we hypothesized that miRNAs could mediate the hypolipidemic effects of proanthocyanidins. Therefore, the main objective of this thesis was to assess whether dietary polyphenols, mainly proanthocyanidins, can modulate miR-33 and miR-122 in rats and evaluate the mechanism by which these compounds affect these miRNAs. For this aim, three specific objectives were proposed:

1. **To determine the ability of proanthocyanidins to modulate miR-33 and miR-122 and their target genes in rat liver (manuscript 1).**

   In order to assess the capacity of pronthocyanidins to modulate these miRNAs two experimental approaches were undertaken: an acute *in vivo* experiment in rats and an *in vitro* experiment in rat hepatoma Fao cells.

2. **To evaluate whether a chronic consumption of proanthocyanidins at dietary doses, which reflect a physiological dietary condition, can modulate miR-33 and miR-122 and their target genes and in different hyperlipidemic status:**

   2.1. In healthy rats in a postprandial state (manuscript 2).

   Nowadays there is increasing evidence that the postprandial state is an important contributing factor to chronic diseases. Therefore, the experimental model approach was the use of a lipid tolerance test after three weeks of chronic proanthocyanidin consumption in healthy rats.

   2.2. In a cafeteria diet induced obese dyslipidemics rats (manuscript 3).

The cafeteria diet provides a robust model of human metabolic syndrome compared to traditional lard-based high-fat diets. Hence, the experimental approach was to first induce dyslipidemia and obesity and thereafter to evaluate the capacity of proanthocyanidins to normalize miRNAs levels in liver.

3. **To compare the capacity of proanthocianidins and ω-3 PUFAS, a well known hypolipidemic agent, to modulate miR-33 and miR-122 in a cafeteria diet induced obese dyslipidemics rats (manuscript 4).**

4. **To verify whether the levels of miR-33 and their target gene in PBMCs reflected the modifications induced in the liver by diet (manuscript 4).**

   In order to validate that PBMCs can reflect the liver levels of miR-33 different dietary approaches were induced: a cafeteria diet, a cafeteria diet supplemented with proanthociandins and/or ω-3 PUFAS.

5. **To determine if different classes of polyphenols have the same ability to modulate miR-33 and miR-122 and their target genes in hepatic cells (manuscript 5).**

   The experimental approach to achieve this objective was *in vitro* in Fao and HepG2 cells. In order to determine the capacity of polyphenols to modulate miRNAs levels, a wide range of different classes of polyphenols that differs in their chemical structure were evaluated.

6. **Moreover, to study some of the potential mechanism by which polyphenols could affect miRNAs levels (manuscript 5).**

   In order to determine the mechanism by which polyphenols modulate miRNAs levels, two approaches were evaluated: the coexpression of the miR-33 and its host gene and the binding capacity of polyphenols to interact with miR-33 and miR-122 by ^{1}H NMR spectroscopy.

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1. Effect of an acute dose of proanthocyanidins on miR-33 and miR-122 expression in rat liver (manuscript 2, published)

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RESEARCH ARTICLE

Grape seed proanthocyanidins repress the hepatic lipid regulators miR-33 and miR-122 in rats

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Scope: One major health problem in westernized countries is dysregulated fatty acid and cholesterol metabolism that causes pathologies such as metabolic syndrome. Previous studies from our group have shown that proanthocyanidins, which are the most abundant polyphenols in the human diet, regulate lipid metabolism and are potent hypolipidemic agents. The noncoding RNAs, miR-33 and miR-122, regulate genes that are involved in lipid metabolism.

Methods and results: Here, we show that grape seed proanthocyanidins rapidly and transiently repressed the expression of miR-33 and miR-122 in rat hepatocytes in vivo and in vitro. Furthermore, the miR-33 target gene ATP-binding cassette A1 and the miR-122 target gene fatty acid synthase were also modulated by proanthocyanidins. Specifically, ATP-binding cassette A1 mRNA and protein levels were increased, and fatty acid synthase mRNA and protein levels were reduced after the miRNA levels were altered.

Conclusion: These results suggest that proanthocyanidin treatment increased hepatic cholesterol efflux to produce new HDL particles by repressing miR-33, and it reduced lipogenesis by repressing miR-122. These results highlight a new mechanism by which grape seed proanthocyanidins produce hypolipidemia through their effects on miRNA modulators of lipid metabolism.

Keywords:
Abca1 / Fas / Flavonoids / Hepatocytes / MicroRNAs

1 Introduction

The most abundant polyphenols in the human diet are the proanthocyanidins, a subclass of flavonoids [1, 2]. These compounds are mainly present in apples, grapes, nuts, red wine, tea, and cocoa [3]. Proanthocyanidins improve human health with cardioprotectant [4], antigenotoxic [5], anti-inflammatory [6, 7], antioxidant [4], and anticarcinogenic [8] activities. Moreover, proanthocyanidins improve several risk factors for cardiovascular disease (CVD), such as dyslipidemia [9] and insulin resistance [10]. More specifically, grape seed proanthocyanidins are potent hypolipidemic agents. A grape seed proanthocyanidin extract (GSPE) was shown to reduce plasma triglycerides (TGs) levels, apo B and LDL cholesterol, as well as to increase the percentage of HDL cholesterol in healthy rats given an acute oral dose of GSPE [11]. The hypolipidemic effects of GSPE were even more obvious in a lipid tolerance test model [12]. Additionally, chronic treatment with GSPE corrects the dyslipidemia associated with dietary obesity in rats [13]. Several mechanisms by which GSPE induces hypolipidemia have already been described [9]. For example, GSPE activates genes that control fatty acid oxidation and represses genes that control lipogenesis and VLDL assembly in the liver [12–14], thus inducing hypolipidemia. However, it is becoming clear that microRNAs (miRNAs) play key roles in the regulation of genes involved in lipid metabolism in the liver, and the effects of GSPE on these miRNAs is unknown [15–17].

miRNAs are a novel class of noncoding RNAs that are 20–25 nucleotides long. miRNAs regulate the expression of specific target genes at the posttranscriptional level, mainly by triggering mRNA cleavage or inhibiting translation [18].

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Abbreviations: Abca1, ATP-binding cassette A1; **CPT1α**, carnitine palmitoyltransferase 1α; **Fas**, fatty acid synthase; **GSPE**, grape seed proanthocyanidin extract; **miRNAs**, microRNAs; **QqQ**, triple-quadrupole; **TGs**, triglycerides

Their effects are mostly mediated by their binding to the 3′ untranslated region of target mRNAs [19], but miRNAs can also bind to other regions, including 5′ untranslated regions and protein-coding exons [20]. miRNAs play important regulatory roles in a variety of biological processes. Specifically, several miRNAs have been correlated with obesity and metabolic syndrome [21] and are proposed to regulate glucose metabolism [22, 23], adipocyte differentiation and adipogenesis [24], and lipid metabolism [15, 16]. Two of the best-studied miRNAs involved in the regulation of lipid metabolism are miR-122 and miR-33 [15]. miR-122 is liver specific and represents 70% of all miRNA expression in liver [25]. The dysregulation of this miRNA has been associated with the dysregulation of genes with key roles in the control of liver lipid metabolism. miR-122 regulates several genes that control fatty acid and TG biosynthesis, such as fatty acid synthase (Fas), acetyl-CoA carboxylase 1, acetyl-CoA carboxylase 2, stearoyl-CoA desaturase 1, diacylglycerol O-acyltransferase 2, ATP citrate lyase, and sterol regulatory element-binding protein 1c, as well as genes that regulate fatty acid β-oxidation, such as carnitine palmitoyltransferase 1α (CPT1α) [15]. A second miRNA, miR-33, plays an important role in the regulation of cholesterol homeostasis, regulating the ATP-binding cassette transporters (ABC transporters), Abca1 and ABCG1, in addition to its role in fatty acid β-oxidation, where it regulates carnitine *O*-octanoyltransferase (CROT), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase β-subunit (HADHB), and CPT1α [15].

Recently, it has been reported that dietary polyphenols such as curcumin [26], resveratrol [27], epigallocatechin gallate [28], ellagitannin [29], isoflavones [30], and proanthocyanidins from grape seed and cocoa [31] modulate miRNA expression. Thus, we hypothesized that miRNAs could mediate the hypolipidemic effects of proanthocyanidins. Here, we test this hypothesis by examining the effects of GSPE on miR-122 and miR-33 levels in hepatic cells, using both in vivo and in vitro models. The results show that GSPE rapidly reduced miR-122 and miR-33 levels in both models. Furthermore, the effects of GSPE on these miRNAs occurred upstream of the identified effects of GSPE on the expression of the miR-33 and miR-122 target genes Fas and Abca1.

2 Materials and methods

2.1 Proanthocyanidin extract

The GSPE used contained: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g) [32]. The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France).

2.2 Analysis of flavanols and their metabolites in plasma

2.2.1 Chemicals and reagents

Methanol (ME03151000, Scharlab S.L., Barcelona, Spain), acetone (34850, Sigma-Aldrich, Madrid, Spain), and glacial acetic acid (131008.1611, Panreac, Barcelona, Spain) were of HPLC analytical grade. Ultrapure water was obtained from a Milli-Q advantage A10 system (Madrid, Spain).

Stock standard solutions of 2000 mg/L in methanol of (+)-catechin, (–)-epicatechin and pyrocatechol (Fluka/Sigma-Aldrich, Madrid, Spain), and a standard solution of 1000 mg/L in methanol of procyanidin B_2 (Fluka/Sigma-Aldrich) were stored in a dark-glass flask at –20°C. A 100 mg/L stock standard mixture in methanol of (+)-catechin, (–)-epicatechin, and procyanidin B_2 were prepared weekly and stored at –20°C. This stock standard solution was diluted daily to the desired concentration using an acetone:water:acetic acid (70:29.5:0.5, v/v/v) solution.

2.2.2 Micro-solid flavanol extraction

Prior to chromatographic analysis, the rat plasma samples were pretreated by off-line μSPE following the methodology previously described by Martí et al. [33] using OASIS HLB μElution Plates of 30 μm (186001828BA, Waters, Barcelona, Spain). Briefly, the micro-cartridges were conditioned sequentially with 250 μL of methanol and 250 μL of 0.2% acetic acid. Plasma or amniotic fluid of 350 μL was mixed with 300 μL of 4% phosphoric acid and 50 μL of pyrocatechol (2000 ppb), and then this mixture was loaded onto the plate. The loaded plates were washed with 200 μL of Milli-Q water and 200 μL of 0.2% acetic acid. The retained procyanidins were eluted with 2 × 50 μL of acetone/Milli-Q water/acetic acid solution (70:29.5:0.5, v/v/v). The eluted solution was directly injected in the LC-QqQ-MS^2 (where QqQ is triple-quadrupole), and the sample volume was 2.5 μL.

2.2.3 Instrumental conditions

A 1200 LC Series coupled to a 6410 QqQ-MS/MS (Agilent Technologies, Palo Alto, USA) was used for the metabolites procyanidins and metabolites quantification. The chromatographic method used was, with a Zorbax C18 (100 mm × 2.1 mm id, 1.8 μm particle size) as chromatographic column, from Agilent Technologies. Mobile phases were 0.2% acetic acid (solvent A) and acetonitrile (solvent B). Flow rate was 0.4 mL/min. Elution gradient was 0–10 min, 5–55% B, 10–12 min, 55–80% B, 12–15 min, 80% B isocratic, 15–16 min 80–5% B. A post run of 10 min was applied.

ESI conditions were at 350°C and 12 L/min of drying gas temperature and flow, respectively, 45 psi of nebulizer gas pressure, and 4000 V of capillary voltage. QqQ operated in

Table 1. Quality parameters of the quantitative method by LC-ESI-QqQ/MS^2

Procyanidin	Determination coefficient (R^2)	Linearity (μM)	Recovery (%)	Precision (%RSD, $n = 3$)	LOD (μM)	LOQ (μM)	MDL[a)] (μM)	MQL[a)] (μM)
Acid gallic	0.9994	0.84–59	49	9	0.25	0.84	0.072	0.240
Catequin	0.9995	0.13–35	97	11	0.04	0.13	0.011	0.038
Epicat	0.9995	0.11–35	95	7	0.03	0.11	0.009	0.031
B2	0.9998	0.03–17	90	15	0.01	0.03	0.003	0.010

a) Method detection and quantification limits in μmol/L of fresh sample, calculated for the analysis of 350 μL of plasma sample.

negative mode. QqQ acquisition was done in MRM mode for procyanidins and their metabolites.

2.2.4 Method validation and samples quantification

For the quantitative method validation, calibration curves, linearity, extraction recovery, precision, sensitivity, and method detection and quantification limits were studied by analysis of standard solutions and blank plasma samples spiked with the standard procyanidins. Calibration curves were obtained by plotting analyte/IS peak abundance ratio and the corresponding analyte/IS concentration ratio. Extraction recovery was evaluated by comparison of the spiked samples response with standard solutions calibration curve. Method precision was determined from RSD in a triplicate analysis of a spiked sample. Sensitivity was evaluated by determining the LOD, defined as the concentration corresponding to three times the signal/noise rate, and the LOQ, defined as the concentration corresponding to ten times the signal/noise rate. Method detection and quantification limits (MDL and MQL, respectively) were calculated for the analysis of 350 μL of sample, following the procedure described in previous paragraphs. Table 1 shows the obtained values for each quality parameter.

In the quantification of samples, spiked blank samples at six different levels of concentration were used to obtain calibration curves, and standard compounds in the samples were quantified by interpoling the analyte/IS peak abundance ratio in these curves. In the case of catechin and epicatechin metabolites, due to the lack of standards, they were tentatively quantified using the standard catechin and epicatechin calibration curves, respectively. In the same way, dimer procyanidins B1 were quantified using the calibration curve of dimer procyanidin B2.

2.3 Cells and cell culture

FAO cells, a rat hepatoma cell line (ECACC, code 85061112), were grown to 80% confluence in Nutrient Mixture F12 Coon's Modification (F6636–10×1L, Sigma-Aldrich) supplemented with gentamicin (50 μg/mL) (LONZA, Basel, Switzerland), polymyxin B (50 μg/mL) (Sigma-Aldrich), and 10% fetal bovine serum (BioWhittaker, Cologne, Germany) in a 95% air, 5% CO_2 atmosphere at 37°C. At 15 h before GSPE treatment, the media was replaced with serum-depleted media (Coon's modified Ham's F12) supplemented with 100 μM oleic acid (MERCK, Germany) and 40 μM BSA (bovine serum albumin, fatty acid free, Sigma-Aldrich). FAO cells were treated with 10, 25, 50, or 100 mg GSPE per liter of media to select the working dose. For kinetic experiments, cells were treated with 25 mg GSPE per liter of media. GSPE was dissolved in ethanol and added to the culture media; the final concentration of ethanol in the media was 0.05%, a nontoxic percentage. miRNAs and mRNAs were extracted at 0, 0.5, 1, 3, and 5 h after GSPE treatment.

2.4 Animals and experimental design

Male Wistar rats weighing 225 g were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of our university approved all procedures (reference number 4249 by Generalitat de Catalunya). Animals were housed in animal quarters at 22°C with a 12 h light/dark cycle (light from 8:00 to 20:00 h) and fed ad libitum with a standard chow diet (Panlab, Barcelona, Spain).

At 9 a.m. on the day of the experiment, the rats (five animals per group) were orally gavaged with lard oil (2.5 mL/kg body weight) (control group) or GSPE (250 mg/kg body weight) dissolved in lard oil (GSPE group).

At 0, 1, or 3 h after treatment, the rats were sedated using a combination of ketamine (70 mg ketamine/kg body weight, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg xylazine/kg body weight, Bayer, Barcelona, Spain). After anesthetization, the rats were exsanguinated from the abdominal aorta. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 *g*, 15 min, 4°C) and stored at –80°C until analysis. The liver was excised, frozen immediately in liquid nitrogen, and stored at –80°C until RNA and lipid extraction.

2.5 RNA extraction

Total RNA containing small RNA species was extracted from frozen liver and from FAO cells using the mi/mRNA extraction kit (miRNA kit, E.Z.N.A., Omega Bio-tek, Norcros, USA) according to the manufacturer's protocol. To isolate both total RNA and miRNA, 1.5 volumes of absolute ethanol were added instead of the recommended 0.33 volumes in

step 5. The washing step was performed according to the isolation of large RNAs.

The hepatocytes were washed twice with PBS before extraction. The quality of the purified RNA was checked using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

2.6 miRNAs quantification by real-time (qRT) PCR

To analyze the expression of each miRNA, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA-specific reverse transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used. The reaction was performed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The final total RNA concentration used was 2.5 ng/μL. We used 1.33 μL of these diluted cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Specific Taqman probes were used for each gene: microRNA-122 (miR-122: hsa-mir-122), 5′-UGGAGUGUGACAAUGGUGUUUG-3′, and microRNA-33 (miR-33: hsa-mir-33), 5′-GUGCAUUGUAGUUGCAUUG-3′. The results were normalized to the expression of the U6 small nuclear RNA, which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS RT-PCR system (Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale according to the equation $2 - \Delta\Delta Ct$, where $\Delta Ct =$ Ct miRNA – Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples – ΔCt untreated controls.

2.7 mRNA qRT-PCR

mRNA levels were evaluated by reverse transcription performed using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (long gene) was used. The reaction was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. The final total RNA concentration used was 25 ng/μL in 125 μL. We used 5 μL of this diluted cDNA solution for subsequent quantitative RT-PCR amplification using TaqMan Universal PCR master mix (Applied Biosystems). Specific Taqman probes were used for each gene: Abca1 (Rn00710172_m1), Fasn (Rn00569117_m1), CPT1α (Rn00580702_m1). The results were normalized to cyclophilin (PPIA: Rn00690933_m1), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS RT-PCR system (Applied Biosystems) with a protocol of 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The fold change in the mRNA level was calculated by the log 2 scale using the equation $2 - \Delta\Delta Ct$, where $\Delta Ct =$ Ct miRNA – Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples – ΔCt untreated controls.

2.8 Western blot analysis

Proteins were extracted using radio-immunoprecipitation buffer (RIPA: 100 nM Tris-Cl pH 7.4 (300 nM NaCl), Tween 10%, Na-Deoxycholate 10%, H_2O Milli-Q). Equal amounts of proteins, 75 μg for cells, were resolved on 7.5% and 5% Tris-glycine polyacrylamide minigels, for Abca1 and Fas, respectively, and transferred to polyvinylidene fluoride (PVDF) membranes (Immun-Blot PVDF Membrane for Protein Blotting, BR05814503, Bio-Rad Laboratories, UK) using a tank-transfer system. Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) and incubated with primary antibodies in TBS containing 0.05% Tween-20 overnight at 4°C. Primary antibodies were used at the following dilutions: Abca1 at 1:1000 (ab18180, abcam, Cambridge, UK), Hsp90 at 1:1000 (610419, BD Biosciences, Franklin Lakes, NJ, USA), Fas at 1:5000 (ab128870, abcam). Secondary antibodies were used at the following dilutions: secondary antibody to mouse IgG-H&L (HRP) (ab6728, abcam) was used at 1:5000 and secondary antibody to rabbit (NA934V, Amersham, Buckinghamshire, UK) was used at 1:10 000 in 5% skimmed milk in TBS containing 0.05% Tween-20. Signals were revealed using an enhanced chemiluminescence reagent (ECL Plus Western Blotting Detection System, RPN2132, Amersham), and digital images were taken with a Chemi XL1.4 Camera (Syngene, Cambridge, UK), which permits the semiquantification of the band intensity. Hsp90 was use as an endogenous protein control.

2.9 Plasma and liver lipid analysis

Plasma total cholesterol and TGs were measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). Liver (0.5 g) lipids were extracted using the Folch method [34]. An aliquot of extract was subjected to gravimetry to measure total lipids. The remaining extract was allowed to evaporate under a nitrogen draft and redissolved in a 2:1 mixture of chloroform (Panreac) and methanol (Panreac) and further diluted with NaCl (Panreac). The TG and cholesterol concentrations in the dissolved extract were measured using QCA enzymatic colorimetric kits (QCA) following the manufacturer's protocols.

2.10 Statistical analysis

The results are reported as the mean ± SEM of three independent in vitro experiments or five animals for in vivo experiments. Group means were compared with an independent samples Student's *t*-test ($p \leq 0.05$) using SPSS software.

Table 2. Flavanols and their metabolites quantified in rat plasma over a 1-h period after ingestion of an acute intake of grape seed procyanidin extract (250 mg/kg) with lard oil. The data are given as the mean (μM) ± SEM ($n = 4$–5)

	Total amount (μM)
Compound	
Catechin	0.07 ± 0.01
Epicatechin	0.39 ± 0.02
Procyanidin dimer B2	0.11 ± 0.01
Procyanidin dimer B1 + B3	0.12 ± 0.01
Gallic acid	0.48 ± 0.04
Metabolite	
Catchin-glucuronide	>6.13 ± 0.00
Epicatechin-glucuronide	>6.13 ± 3.21
Methyl-catechin-glucuronide	2.78 ± 0.37
Methyl-epicatechin-glucuronide	1.18 ± 0.14
Catechin-sulfate	nd
Epicatechin-sulfate	nd
3-o-methyl-epicatechin	0.05 ± 0.00
4-o-methyl-epicatechin	0.05 ± 0.01
Methyl-catechin-o-sulfate	0.24 ± 0.01
Methyl-epicatechin-o-sulfate	0.62 ± 0.06

nd: not detected.

3 Results

3.1 Flavanols and their metabolites in plasma

GSPE mostly contains timeric and dimeric proanthocyanidins, but also the monomeric flavon-3-ol catechin, epicatechin, and epicatechin gallate are abundant [32] (see material and methods section). After 1 h of an acute ingestion of GSPE (250 mg/kg body weight) with lard oil, the free forms of catechin, epicatechin, and dimeric procyanidins were determined in rat plasma. The main conjugation forms of catechin and epicatechin were also determined, being the most abundant the glucuronide forms. Moreover, the methyl and sulfate conjugations of catechin and epicatechin were determined in plasma. However, catechin-sulfate and epicatechin-sulfate were not detected (Table 2). These data show that at a concentration of GSPE of 250 mg/kg, the main flavanols metabolites are present in rat plasma after 1 h of ingestion at concentrations in the micromolar range.

3.2 GSPE decreased TG and cholesterol levels in plasma and liver

In control animals, plasma TG levels increased markedly 3 h after the oral administration of 2.5 mL of lard oil/kg of body weight (Table 3). The simultaneous administration of GSPE (250 mg/kg body weight) significantly blocked the increase of plasma TG levels induced by lard oil. However, the simultaneous administration of GSPE had no significant effect on plasma cholesterol level.

TG, cholesterol, and total lipids were quantified to determine the effect of GSPE on liver lipids (Table 3). At 3 h after the administration of lard oil, the TG and cholesterol contents in the liver increased by approximately 40% with respect to the basal condition. As in the plasma, the simultaneous administration of GSPE significantly prevented the accumulation of lipids in the liver after lard oil treatment. These data show that GSPE improves lipid tolerance, both in the plasma and the liver.

3.3 GSPE repressed miR-122 and miR-33 expression and modulated the expression of their target genes in the liver in vivo

Relative changes in the levels of miR-122 and miR-33 in the rat liver were quantified by RT-PCR at 1 and 3 h after the administration of lard oil alone or in combination with GSPE (Fig. 1). At 1 h after administration, GSPE treatment decreased miR-122 and miR-33 levels by 34% and 60%, respectively. At 3 h after GSPE administration, miR-33 levels were similar to those in the livers of rats given lard oil only, whereas miR-122 levels increased by 38%. These results indicate that the repression of miR-122 and miR-33 induced by GSPE was rapid and transient.

To validate the effects of GSPE on miR-122 and miR-33, we quantified the relative changes in the expression levels of several target genes of these miRNAs (Fig. 1). We have chosen Fas for miR-122, Abac1 for miR-33, and CPT1α for both miRNAs. No changes were found in the expression of Fas, CPT1α, or Abca1 after 1 h of GSPE administration. However, Fas was significantly repressed and Abca1 was significantly

Table 3. Triglyceride and cholesterol levels in the plasma and livers of rats fed lard oil with or without proanthocyanidins (grape seed proanthocyanidin extract (GSPE))

	Basal (0 h)	Lard (3 h)	Lard + GSPE (3 h)
Plasma triglycerides (g/100 mL plasma)	71.4 ± 6.0	123.1 ± 6.6	73.4 ± 12.4[a)]
Plasma cholesterol (g/100 mL plasma)	18.5 ± 2.5	31.6 ± 6.1	26.1 ± 3.7
Total liver lipids (g/100 g liver)	6.7 ± 0.5	7.1 ± 0.5	5.5 ± 0.3[b)]
Liver triglycerides (g/100 g liver)	1.7 ± 0.2	2.4 ± 0.2	1.45 ± 0.33[a)]
Liver cholesterol (g/100 g liver)	0.9 ± 0.1	1.3 ± 0.1	0.9 ± 0.01[a)]

Rats fasted for 14 h were orally administered lard oil (2.5 mL/kg) with or without GSPE (250 mg/kg). Lipids were quantified before treatment (0 h) and at 3 h after GSPE administration. The values are the means of five animals per group.
a) Significant difference between the lard group and the lard + GSPE group.
b) Significant difference between the basal group and the lard + GSPE group ($p < 0.05$; Student's t test).

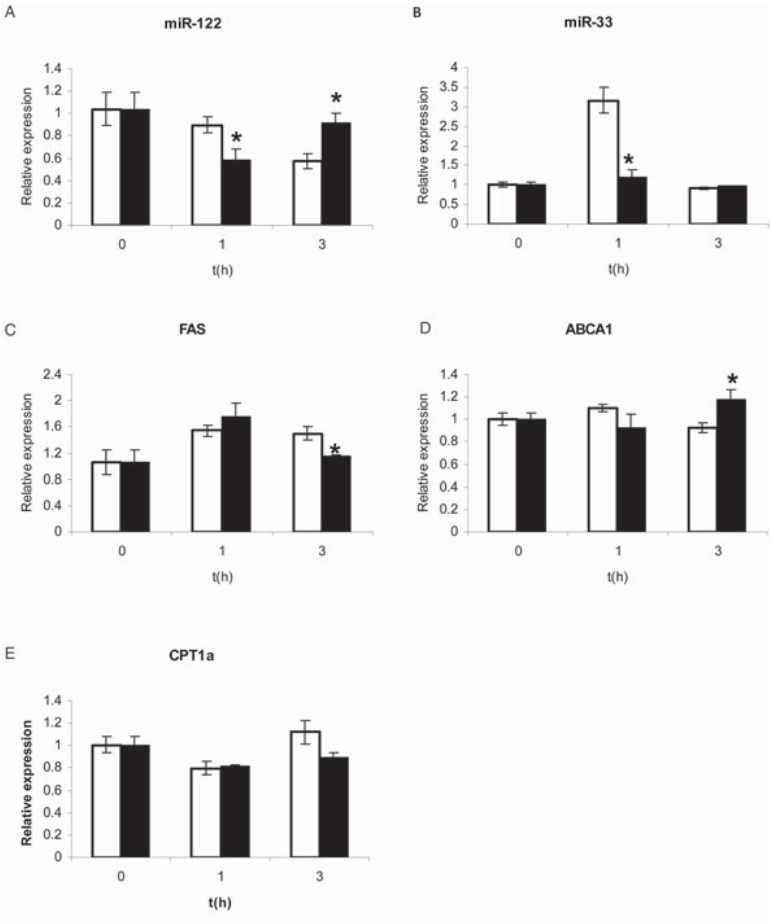

Figure 1. Levels of miR-122 (A), miR-33 (B), and their target mRNAs (C–E) in the livers of rats fed on lard oil with or without proanthocyanidins (grape seed proanthocyanidin extract [GSPE]). Rats were fasted for 14 h and then orally administered lard oil (2.5 mL/kg) with or without GSPE (250 mg/kg). RNAs were quantified prior to treatment (0 h) and at 1 and 3 h after GSPE administration. miRNA levels were normalized to U6 small nuclear RNA. The values shown are the means of five animals per group. White bars, control group; black bars, GSPE treated group. *Significant difference between the lard group and the lard + GSPE group ($p < 0.05$; Student's *t* test).

overexpressed at 3 h after GSPE administration. Additionally, CPT1α tended to be repressed at this time. These results show that GSPE modulated the expression of miR-122 and miR-33 prior to that of their target genes, Fas, CPT1α, and Abca1.

3.4 GSPE repressed miR-122 and miR-33 expression and modulated their target genes at mRNA and protein levels in FAO cells

To assess whether GSPE repressed miR-122 and miR-33 directly, we studied the effect of GSPE treatment in vitro using the rat hepatoma FAO cell line. To select the working dose of GSPE, FAO cells were treated with different doses of GSPE, and changes in miR-122 and miR-33 levels were analyzed after 1 h of treatment (Fig. 2). The expression of both miRNAs was decreased significantly at a dose of 25 mg/L of GSPE. However, higher doses of GSPE (50 and 100 mg/L) did not increase the repression of either miR-122 or miR-33. Consequently, we chose to use 25 mg/L of GSPE in the kinetic experiments.

Figure 3 shows the expression kinetics of miR-122 and miR-33 from 0 to 5 h after GSPE treatment in FAO cells. No changes in miRNAs levels were observed after 30 min of treatment. However, after 1 h of treatment, GSPE decreased the levels of miR-122 and miR-33 by 34% and 39%, respectively. miR-33 expression was further decreased (up to 50%) at 3 h and returned to the baseline value after 5 h of treatment. In contrast, miR-122 expression remained low until 5 h of treatment (62% decrease). Thus, GSPE directly modulates the expression of miR-122 and miR-33 in hepatic

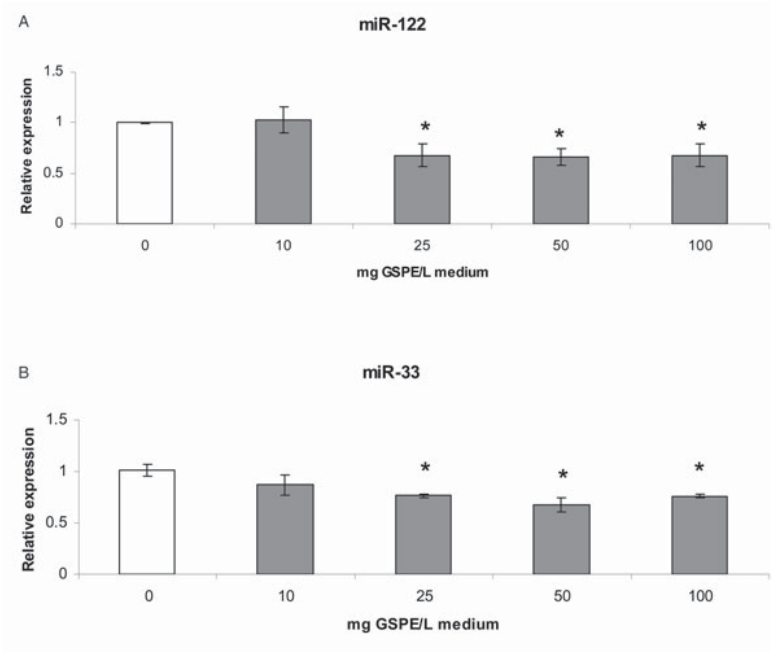

Figure 2. The effect of increasing doses of proanthocyanidin extract on miR-122 (A) and on miR-33 (B) levels in FAO cells. FAO cells were treated with the corresponding concentrations of GSPE for 1 h. miRNA levels were determined by RT-qPCR and normalized to U6 small nuclear RNA levels. All values shown are the means of three independent experiments. *Significant difference between control cells (0 mg/L) and treated cells ($p < 0.05$; Student's *t* test).

cells. GSPE repressed miR-122 and miR-33 in FAO cells with kinetics similar to those observed in vivo (1 h), but compared with the in vivo system, the repression was constant and not transient. Moreover, both in vivo and in vitro systems show that miR-33 was repressed to a greater degree than miR-122.

Abca1 and Fas expression was also analyzed in FAO cells (Fig. 3). Abca1 was overexpressed after 1 and 3 h of GSPE treatment. Fas was repressed only after 5 h of treatment. Moreover, after 5 h of treatment the protein levels of these target genes were analyzed (Fig. 4). According to mRNA levels, the Abca1 protein was increase and Fas was decrease.

4 Discussion

Flavonoid consumption is associated with lower risk of death from CVD. More specifically, five subclasses of flavonoids, including proanthocyanidins, are individually associated with lower risk of fatal CVD [35]. One of the mechanisms by which proanthocyanidins exert their cardiovascular protection is by reducing postprandial hypertriglyceridemia and LDL cholesterol [9]. Previously, we showed that proanthocyanidins repress TG secretion in the liver through an farnesoid X receptor- and small heterodimer partner-dependent pathway [36, 37]. However, lipid metabolism in liver is controlled by diverse signaling pathways, including miR-122 and miR-33, working in concert [15, 17]. We have used microarray analyses to show that proanthocyanidins from grape seed and cacao are able to modulate miRNAs levels in HepG2 cells after 5 h of treatment [31]. However, we did not observe any significant modulation of miRNAs related to lipid metabolism at 5 h. Therefore, because GSPE hypolipidemic action is very fast, we hypothesized whether miRNA regulators of lipid metabolism, such as miR-122 and miR-33, could be modulated by proanthocyanidins at earlier timepoints. Our results show that proanthocyanidin treatment significantly reduced miR-122 and miR-33 levels in rat hepatic cells after 1 h of GSPE treatment, both in vivo and in vitro.

Previously, to determine the effect of GSPE on miRNAs levels in liver in vivo, we performed a lipid tolerance test to confirm the hypolipidemic effects of GSPE administration. The administration of lard oil concomitant with GSPE produced a clearly hypolipidemic effect, reducing plasma TG by a 40%, plasma cholesterol by 18%, and liver TG and cholesterol by 40% relative to the levels in animals given lard oil alone. The hypolipidemic effect of GSPE was similar to those observed previously using the same animal model [12]. Therefore, the livers of these animals were a good model in which to study the roles of miR-122 and miR-33 in the hypolipidemic effects of GSPE.

GSPE decreased miR-122 and miR-33 levels in liver very rapidly (within 1 h), but after 1 h of GSPE ingestion the concentration of flavanols and their metabolites were already abundant in plasma in the micromolar range. miR-122 inhibition reduces plasma cholesterol levels in normal mice

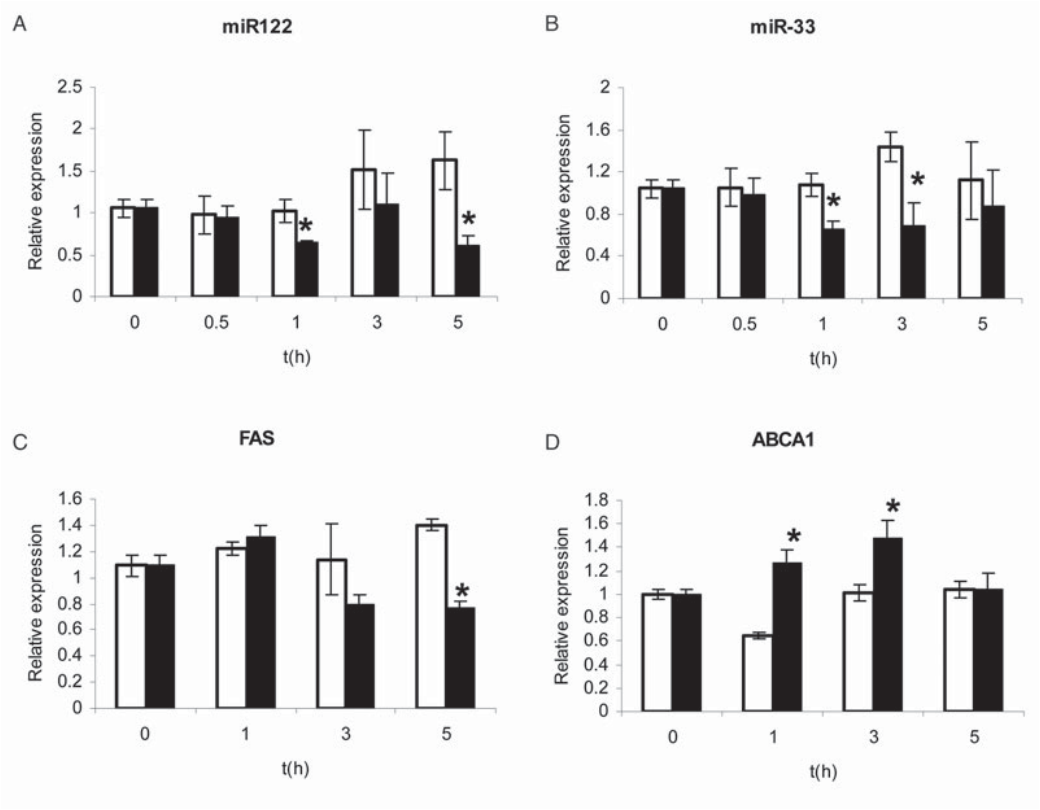

Figure 3. The effects of proanthocyanidin extract on the levels of miR-122 (A), miR-33 (B), and their target mRNAs (C, D) in FAO cells. FAO cells were treated with 25 mg GSPE per liter for 0.5, 1, 3, or 5 h. miRNA levels were determined by RT-qPCR and normalized to U6 small nuclear RNA. All values shown are the means of three independent experiments. White bars, control group; black bars, GSPE treated group. *Significant difference between control cells and treated cells ($p < 0.05$; Student's *t* test).

and decreases plasma cholesterol and hepatic TG levels in a mouse model of diet-induced obesity [38]. Moreover, miR-33 inhibition lowers VLDL-TG in nonhuman primates [39]. Therefore, these miRNAs may be considered putative mediators of the hypolipidemic effect of GSPE.

The repression of miR-122 and miR-33 induced by GSPE in the liver in vivo could be secondary to hormonal changes and/or to variations in the nutrient supply to the liver as a result of the action of GSPE in other organs, such as the intestines. For this reason, we studied the effect of GSPE in hepatic cells in vitro, in which the growth conditions and nutrient composition of the media were the same for control and treated cells and could be strictly controlled. Using this experimental approach, GSPE repressed miR-122 and miR-33 in FAO cells, similar to its effects on the liver in vivo. Therefore, the repression of miR-122 and miR-33 is a direct outcome of GSPE activity in hepatic cells. Moreover, the repression of miR-33 is greater than the repression of miR-122 in both hepatocyte models in vivo and in vitro. This finding suggests that the hypolipidemic effects of GSPE are more strongly mediated by miR-33 than by miR-122.

In contrast to the transitory effects of GSPE on miRNAs in vivo, the repression of miR-122 and miR-33 by GSPE in FAO cells was persistent. The pharmacokinetics of proanthocyanidins in vivo could be responsible of this discrepancy. In vivo, proanthocyanidin monomers and dimers are absorbed very rapidly, picking at 1–2 h after consumption, and then eliminated from the body [32]. A concentration of 25 mg/L is needed in vitro to reach the significant reduction of miRNAs. A global estimation of the total concentration of catechin and epicatechin (free and conjugated forms) in plasma indicated that in vivo the concentration was five times higher than

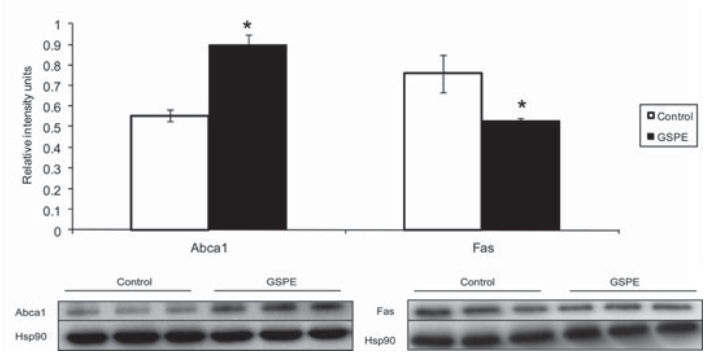

Figure 4. The effects of proanthocyanidin extract on the protein levels of Abca1 and Fas in FAO cells. FAO cells were treated with 25 mg GSPE per liter for 5 h. Proteins were extracted with radioimmunoprecipitation (RIPA) buffer and analyzed with Western blot technique. Proteins were normalized with an endogenous protein, Hsp90. Relative intensity units were obtained dividing the intensity band of the protein problem between the intensity band of the endogenous protein. All values are the means of two independent experiments. *Significant difference between control cells and treated cells ($p < 0.01$; Student's *t* test).

catechin and epicatechin in media at 25 mg GSPE per liter. Although the in vivo and in vitro conditions are not exactly comparable, it will be possible that a lower GSPE concentration in vivo could be also effective repressing miRNAs levels. The used dose of 250 mg of GSPE per kilogram body weight in rats is equivalent to 40.5 mg of GSPE per kilogram body weight in humans [40]. Therefore, for a 70 kg man, this dose corresponds to an intake of 2.8 g of GSPE. Hence, the used dose in this work is six times higher to the estimated proanthocyanidin intake in the high quintile of U.S. population [35]. Therefore, a proanthocyanidin-rich diet could be enough to modulate miRNAs in humans.

The effects of GSPE on the mRNA levels of Fas and Abca1 were consistent with those induced by miR-33 and miR-122. Abca1 mRNA, which is repressed by miR-33 [41], was overexpressed following GSPE treatment. In contrast, Fas mRNA was repressed by GSPE, consistent with the observation that mRNAs involved in lipogenesis tend to be downregulated when miR-122 is inhibited [25, 38]. The Abca1 and Fas protein levels in FAO cells were changed by GSPE correlating with the mRNA levels changes and miRNAs effects. Moreover, GSPE modulated miR-122 and miR-33 expression before effects on Fas and Abca1 were observed, both in vivo and in vitro. Taken together, these data reinforce the hypothesis that miR-122 and miR-33 mediate the hypolipidemic effect of GSPE.

In the liver, Abca1 activity is a rate-limiting step in the formation of HDL and a key determinant of circulating HDL levels [42]. Abca1 mediates cholesterol efflux from hepatic cells for apolipoprotein A-I lipidation, decreasing the hepatic pool of cholesterol [43]. Therefore, the reduction of liver cholesterol induced by GSPE could be explained by an increase of cholesterol transport to form and stabilize nascent HDL via an increase of Abca1 expression levels mediated by miR-33. Moreover, the reduction of plasma and liver TG levels after GSPE administration could be a result of decreased fatty acid synthesis due to Fas repression mediated by miR-122. Also, both miR-33 and miR-122 regulate genes that control fatty acid β-oxidation, and both target CPT1α [44, 45]. But, CPT1α mRNA levels in liver in vivo did not change, even though this gene is modulated by both of these miRNAs.

Little is known about the mechanisms that regulate the expression of miR-122 in the liver. However, the regulation of miR-33 is somewhat better understood. There are two isoforms of miR-33, miR-33a and miR-33b, that are intronic of Srebf2 and Srebf1 genes, respectively [41]. The molecular mechanism by which proanthocyanidins modulate miRNAs levels is unknown. However, there is evidence that polyphenols can bind to mRNAs and proteins [46, 47]. Therefore, it is possible that they also bind to miRNAs or to some component involved in miRNA biogenesis, such as DICER or RISC. More studies will be necessary to identify the mechanism by which GSPE modulate miR-33 and miR-122 levels in liver.

In conclusion, GSPE represses miR-33 and miR-122 in rat hepatic cells, both in vivo and in vitro. The repression of these miRNAs by GSPE is rapid and transient. Moreover, GSPE represses Fas and promotes the expression of Abca1, both of which are target genes of these miRNAs. These data suggest that GSPE increases liver cholesterol efflux to stabilize and promote HDL formation and reduce fatty acid synthesis. Therefore, the repression of miR-122 and miR-33 can be considered a new mechanism of action through which proanthocyanidins exert hypolipidemic effects in the liver.

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2. Effect of different chronic doses of proathocyanidins on miR-33 and miR-122 expression in postprandial and dyslipidemic status in rat liver

2.1. Effect of chronic doses of proathocyanidins on miR-33 and miR-122 expression in postprandial state in healthy rats (manuscript 3, submitted)

Chronic supplementation of proanthocyanidins reduces postprandial lipemia and liver miR-33a and miR-122 levels in a dose-dependent manner in healthy rats.

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Running title: Proanthocyanidins represses miRNAs dose-dependently

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Keywords: flavonoids, microRNAs, Fas, Abca1, triglycerides, cholesterol

Abstract

Elevated postprandial triglycerides are associated with an increased risk of cardiovascular disease. Acute proanthocyanidin supplementation improves postprandial lipemia. Therefore, in this study, we evaluated whether a chronic treatment (3 weeks) of grape seed proanthocyanidins (GSPE) improves tolerance to lipid overload and represses liver miRNA-33a and miRNA-122 and their target genes as a mechanism to soften the elevated postprandial triglycerides in healthy rats. Additionally, the minimal GSPE chronic dose required to alter miRNA levels was determined by means of a dose-response experiment using 5, 15, 25, or 50 mg of GSPE/kg body weight. GSPE repressed miR-33a and miR-122 liver expression and reduced postprandial lipemia in a dose-dependent manner. Significant effects were only observed at high levels of proanthocyanidin consumption, but moderate doses of proanthocyanidins were still able to modulate miRNA expression. Therefore, it can be suggested that a population with a normal intake of proanthocyanidin-rich foods can benefit from the modulation of miRNA expression. At the molecular level, this action can confer homeostatic robustness and will thus exert subtle changes in lipid metabolism, thereby reducing the risk associated with postprandial hyperlipemia.

Introduction

The postprandial state is a dynamic period of metabolic trafficking, biosynthesis and oxidative metabolism of absorbed substrates, such as glucose, lipids, proteins and other dietary constituents. During this period, the organism responds with compensatory and adaptive mechanisms and manages short-term disturbances to restore homeostasis. In developed societies, the modern lifestyle usually favors an excessive intake of energy by eating several times a day with limited energy expenditure, which results in prolonged metabolic, oxidative and immune imbalance, causing cellular dysfunction and disease [1]. Moreover, elevated postprandial triglycerides (TG) are associated with an increased risk for cardiovascular diseases (CVD) and can be more accurate in assessing high-risk atherogenic conditions than by measuring fasting TG concentrations [2, 3]. One strategy to treat CVD is by using dietary compounds such as flavonoids, and specifically proanthocyanidins, which have been reported to improve the risk factors for CVD, such as dyslipemia [4] and insulin resistance [5]. However, the mechanism by which proanthocyanidins realize their beneficial effects is still unclear, although it has been attributed to potentially altering key proteins at the gene expression level. This alteration can result from the interaction of polyphenols with signaling cascades or from epigenetic factors such as microRNAs (miRNAs). miRNAs constitute a conserved class of post-transcriptional regulators of gene expression [6-8], and their effects are primarily mediated through binding to the 3' untranslated region (3'UTR) of target mRNAs [7]. miRNAs are known to modulate more than 60% of all human genes [9] and have been reported to regulate several metabolic pathways, including lipid metabolism [10]. In the latter, miR-33 [10, 11] and miR-122 [10] have emerged as key regulators of lipid metabolism in the liver and have been related to metabolic disease [12]. miR-122 expression is liver specific and plays a critical role in maintaining liver homeostasis [13, 14]; its inhibition has been associated with the dysregulation of genes involved in liver lipid metabolism, such as fatty acid synthase (Fas), as well as genes that regulate fatty acid β-oxidation [13, 15-18]. miR-33 plays an important role in the regulation of cholesterol homeostasis, regulating the ATP-binding cassette transporters (ABC-transporters) Abca1 and Abcg1, in addition to its role in fatty acid β-oxidation [15]. Interestingly, two isoforms of miR-33 have been identified, miR-33a and miR-33b [15, 19], but isoform b is not found in rodents. As an epigenetic mechanism, miRNAs may mediate the effects of nutrition and may contribute to the development of many common chronic diseases [20]. Current data indicate that a wide range of dietary factors, such as polyphenols, can modify the expression of miRNA [21-23]. We have previously shown that an acute pharmacological dose of a grape seed proanthocyanidin extract (GSPE) represses miR-33a and miR-122 in rat liver after 1 h [23] and counteracts the increase of TG and total cholesterol (TC) induced by a saturated fat overload [24, 25]. However, from a nutritional perspective, it is interesting to know if a dietary dose of a regular GSPE ingestion could also improve postprandial dyslipemia as well as the

implication of miR-33 and miR-122 in the improvement of postprandial dyslipemia. Therefore, the main objective of this study was to determine whether a chronic treatment of GSPE could improve tolerance to lipid overload and repress liver miR-33a and miR-122 and their target genes as a mechanism to reduce elevated postprandial TG in healthy rats. Furthermore, the minimal chronic GSPE dose required to alter miRNAs levels was determined using a dose-response and a kinetic experiment.

Methods and Materials

Grape seed proanthocyanidins extract

The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The composition of the GSPE used in this study was previously analyzed by Quiñones et al [26] and is described in Table 1.

Animal design

miRNA Kinetic experiment

Thirty-nine six-week-old male Wistar rats weighing 150 g were purchased from Charles River (Barcelona, Spain). The rats were double-caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 8:00 to 20:00 pm) and were fed ad libitum with a standard chow diet (STD, Panlab 04, Barcelona, Spain) and tap water. The Animal Ethics Committee of our university approved all of the procedures. After one week of adaptation, the rats were randomly divided into 13 groups (n=3). The first group was sacrificed before treatment, at 0 hours; the other 12 groups were divided into 6 groups orally administered with 1 mL of tap water (control groups) and 6 groups with 250 mg of GSPE/kg of bw dissolved in 1 mL of tap water. Both the control and treated groups were sacrificed by decapitation at 0.5, 1, 3, 6, 12 and 24 hours after treatment. The liver was excised, frozen immediately in liquid nitrogen and stored at -80ºC until RNA and lipid extraction.

Dose-response experiment

Fifty six-week-old male Wistar rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain). The rats were singly caged in animal quarters at 22ºC with a 12 h light/dark cycle (light from 8:00 to 20:00 pm) and were fed ad libitum with a standard chow diet (STD, Panlab 04, Barcelona, Spain) and tap water. After one week of adaptation, the animals were randomly divided into five groups (n = 10) and supplemented with 0 (control group), 5, 15, 25 or 50 mg of GSPE/kg body weight for 3 weeks. GSPE was dissolved in sugary milk (100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 1175 kJ) at appropriate concentrations such that the same volume of milk (750 µL) was always administered to the animals. Before supplementation, all of the rats were trained to voluntarily lick the milk, and all groups were administered with the same volume of sugary milk for 21 days. Treatment was administered every day at 9:00 am.

After 21 days of supplementation, the rats were fasted overnight. At 9:00 am, the rats were orally gavaged with lard oil (2.5 mL/kg of body weight) with or without (control groups) the adequate dose of GSPE (5, 15, 25 or 50 mg/kg body weight). After 3 h, the rats were sedated using a combination of ketamine (70 mg/kg body weight, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg/Kg body weight, Bayer, Barcelona, Spain). After anesthetization, the rats were exsanguinated from the abdominal aorta. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 g, 15 min, 4ºC) and stored at -80ºC until analysis. The liver was excised and frozen immediately in liquid nitrogen and stored at -80ºC until RNA and lipid extraction.

RNA extraction

Total RNA, including small RNA species, was extracted from the frozen liver using a mi/mRNA extraction kit (miRNA kit, E.Z.N.A., Omega Bio-tek, Norcros, U.S.A.) according to the manufacturer's protocol. To isolate both total RNA and miRNA, 1.5 equivalents of absolute ethanol were added instead of the recommended 0.33 equivalents in step 5. The washing step was performed according to the protocol for isolating large RNAs. The concentration of the purified RNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

MicroRNA quantification by real-time qRT-PCR

To analyze the expression of each miRNA, reverse transcription was performed using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA-specific reverse transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems, Madrid, Spain). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used; the reaction was performed at 16ºC for 30 min, 42ºC for 30 min and 85ºC for 5 min. The final total RNA concentration used was 2.5 ng/µL in 7 µL. We used 1.33 µL of the resulting diluted cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). The following specific Taqman probes were used for each gene: microRNA-122a (miR-122a: hsa-mir-122a), 5'UGGAGUGUGACAAUGGUGUUUG-3' and microRNA-33 (miR-33: hsa-mir-33), 5'- GUGCAUUGUAGUUGCAUUG-3'. The results were normalized to the expression of the U6 small nuclear RNA (U6 snRNA), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated by a log 2 scale according to the equation $2-\Delta\Delta Ct$, where ΔCt = Ct miRNA - Ct U6 and $\Delta\Delta Ct$ = ΔCt treated samples - ΔCt untreated controls.

mRNA quantification by real-time qRT-PCR

mRNA levels were evaluated by reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used. The reaction was performed at 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 sec. The final total RNA concentration used was 25 ng/µL in 125 µL. We used 5 µL of the resulting diluted cDNA solution for subsequent quantitative RT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain). The following specific Taqman probes were used for each gene: Abca1 (Rn00710172_m1), Fasn (Rn00569117_m1). The results were normalized to cyclophilin (PPIA: Rn00690933_m1), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) with a protocol of 50ºC for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The fold change in the mRNA level was calculated by the log 2 scale using the equation $2-\Delta\Delta Ct$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct$= ΔCt treated samples- ΔCt untreated controls.

Plasma and liver lipid analysis

Plasma TC and TG were measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). Liver lipids (0.5 g) were extracted using the Folch method [32]. An aliquot of extract was subjected to gravimetric analysis to measure total lipids. The remaining extract was allowed to evaporate under a nitrogen flow and dissolved in 1 mL of LPL buffer, containing PIPES disodium salt (P3768, Sigma-Aldrich, Madrid, Spain), $MgCl_2 \cdot 6H_2O$ (M9272, Sigma-Aldrich, Madrid, Spain), albumin free fatty acids (A8806, Sigma-Aldrich, Madrid, Spain) and 0.1% SDS (L3771, Sigma-Aldrich, Madrid, Spain). The TG and cholesterol concentrations in the dissolved extract were measured using QCA enzymatic colorimetric kits (QCA, Barcelona, Spain) following the manufacturer's protocols.

Statistical analysis

The results are reported as the mean ± S.E.M. of 10 animals per group. Group means were compared with one-way ANOVA ($p \leq 0.05$) using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Acute administration of GSPE maintained low levels of miR-33a and miR-122 in liver for 24 hours.

We have previously shown that an acute GSPE treatment significantly repressed miR-33a and miR-122 in rat liver very shortly after its administration (1 h). Therefore, prior to a chronic administration of GSPE, we evaluated whether the repression of miR-33a and miR-122, found at 1 h after a single dose of GSPE administration, can be

maintained over a longer period of time. For this evaluation, a kinetic experiment from 0.5 to 24 h was performed and the relative changes in the levels of miR-33a and miR-122 in the rat liver were quantified by RTq-PCR at 0.5, 1, 3, 6, 12 and 24 h after the administration of GSPE (Fig. 1). The experiment was conducted with only 3 rats per group; therefore, no statistical analyses were performed. miR-33a was highly down-regulated at 1 h, with a transient up-regulation at 6 h, and down-regulated again at 12 and 24 h after GSPE treatment. miR-122 levels remained down-regulated after GSPE treatment throughout the full 24 h. Therefore, because the GSPE effect of a single dose on decreasing these miRNAs levels was effective for a full 24 h, the chronic administration of this extract was selected to be a single dose of GSPE administered in the morning.

Chronic GSPE administration decreases miR-33a and miR-122 levels and modulates the expression of their target genes in a dose-dependent manner in the liver of healthy rats.

Rats were treated for 3 weeks with condensed milk, with or without GSPE, at 5, 15, 25 and 50 mg of GSPE/kg of body weight. To determine if chronic GSPE treatment modulates miR-33a and miR-122 expression in the liver of healthy rats, the animals were subjected to a lipid tolerance test by an oral overload of saturated fat 3 h before sacrifice. The chronic supplementation with GSPE decreased miR-33a and miR-122 in the rat liver in a dose-dependent manner compared to rats overloaded with fat alone. Moreover, miR-33a was significantly repressed by a dose of 15 mg of GSPE/kg of body weight, whereas the repression of miR-122 was significant with a dose of 25 mg of GSPE/kg of body weight. Consequently, miR-33a appears to be more sensitive to GSPE than miR-122.

We examined the effect of GSPE treatment on miR-33a target genes (Fig. 2C). Three weeks of pretreatment with GSPE up-regulated Abca1 in a dose-dependent manner, and the increased levels of Abca1 were significant with a dose of 25 mg of GSPE/kg of body weight. However, Fas mRNA expression, an indirect target gene of miR-122, was down-regulated from 25 mg of GSPE/kg of body weight, without any effect at lower doses. Therefore, 25 mg of GSPE/kg of body weight was the minimal dose required to efficiently modulate both miRNAs and their target genes.

GSPE treatments improved postprandial hyperlipemia in healthy rats.

One of the most well understood consequences of GSPE administration is its hypolipidemic effect. Therefore, we determined which doses of GSPE were actually physiologically effective at reducing plasma and liver lipids in a postprandial state.

GSPE supplementation was effective in reducing plasma lipid levels after the lipid tolerance test (Table 2). GSPE administration clearly reduced the plasma levels of TC, LDL-C and the ratio of TC/HDL-C in a dose-dependent manner, with a significant reduction at 50 mg of GSPE/kg of body weight. However, the plasma TG levels were only significantly reduced at 25 mg of GSPE/kg of body weight, while GSPE

administration increased the plasma HDL-C levels with a similar intensity (by 20-25%, not significant) for all of the doses tested.
GSPE supplementation for 3 weeks did not have any effect on the liver weight and did not result in lipid accumulation in the liver. In contrast, there was an improvement of the TG and TC levels following GSPE supplementation (Table 3).

Discussion

There is a continually increasing body of evidence that the postprandial state is an important contributing factor to chronic disease [27-29]. Our research group has previously reported that an acute pharmacological dose of a GSPE markedly blocks the increase of plasma TG after an overload of saturated fat [24]. However, it is interesting to determine whether regular ingestion of proanthocyanidin-rich foods could also improve postprandial dyslipemia and modulate miR-33a and miR-122.
Two important aspects were considered when designing the experiment: first, the dose of GSPE, and second, the time of day and how many times GSPE should be administered. Previous results in our laboratory [23] have shown that an acute administration of GSPE significantly represses miR-33a and miR-122 in the liver a very short time after its administration (1 h). Thus, we performed a 24 h kinetic experiment, administering a high dose of GSPE (250 mg of GSPE/kg of body weight) once a day (9:00 a.m.). The miR-33a and miR-122 levels remained mostly down-regulated for 24 h. Therefore we decided to administer GSPE as a single dose in the morning (9:00 a.m.) for the chronic experiment. The prolonged down-regulation could be explained because of the absorption kinetics of proanthocyanidins and their flora metabolites, with parental compounds absorption at early time points and microbial metabolites until 24 h [30].
In the chronic experiment, GSPE was administered once a day at 5, 15, 25 or 50 mg of GSPE/kg of body weight. These doses, using a translation of animal to human doses [31] and estimating the daily intake for a 70 kg human, correspond to an intake of 57, 171, 284 and 560 mg of GSPE/day. These GSPE intakes are representative for humans with a healthy diet. For example, the average proanthocyanidin intake of U.S. adults over 19 years of age is 95 mg/day [32]. In Finnish adults, the total dietary intake of polyphenols was 863±415 mg/day [33], 14% of which was of proanthocyanidins; in Spanish adults, the mean dietary flavonoid intake was 313.26 mg/day, with proanthocyanidins comprising 60.1% [34]. Therefore, the chronic experiment was designed to use two doses of GSPE (i.e., 15 and 25 mg/kg in rats), a lower dose (i.e., 5 mg/kg in rats) and a higher dose (i.e., 50 mg/kg in rats), to simulate human dietary intake.
After 3 weeks of GSPE treatment in healthy rats, GSPE decreased the levels of miR-33a and miR-122 in rat liver at all the doses tested with a dose-response, although miR-33a was more sensitive to GSPE than miR-122. Moreover, 25 mg of GSPE/kg of body weight was the minimal dose required to efficiently modulate both miRNAs. The levels of miR-33a and miR-122 target mRNAs, Abca1 and Fas, respectively, were also

modulated by GSPE with the same pattern of miR-33a and miR-122 repression. These data suggest that GSPE increases liver cholesterol efflux to HDL formation and reduces fatty acid synthesis from a dose of 25 mg of GSPE/kg of body weight, improving the postprandial state. Therefore, a proanthocyanidin rich diet will be necessary to obtain a beneficial effect on miRNAs and their target genes.

In concordance with the GSPE dose-dependent effect on miRNAs, a dose-dependent effect was also observed in reducing TC and LDL-C in plasma, despite the fact that the GSPE dose for a significant effect was 50 mg of GSPE/kg of body weight. The effect on plasma TG was observed from only 25 mg of GSPE/Kg of body weight. Taken together, this result shows that 3 weeks of GSPE supplementation improved plasma lipid parameters in the postprandial state, indicating that GSPE is able to reduce plasma lipids with non-pharmacological doses in healthy animals.

It has been reported that the consumption of even relatively small amounts of flavonoid-rich foods may be beneficial for reducing the risk of fatal CVD, and proanthocyanidins are also associated with a lower CVD risk [35]. Moreover, epidemiological observations have revealed that some French populations suffer a relatively low incidence of coronary heart disease (CHD), despite having a relatively high dietary intake of saturated fatty acids but with a high intake of red wine, which is a proanthocyanidin- rich food. This phenomenon is named the *French paradox* [36]. It has been reported that wine drinkers had a significantly lower risk for death from CHD in a dose-response manner [37], and therefore, is in accordance with the dose-response effect observed in this study with lipids and miRNAs levels. Moreover, the protective effect of wine consumption is only observed when the drinking pattern is constant, analogous to this study.

In conclusion, the chronic consumption of proanthocyanidins modulates miR-33a and miR-122 liver expression and lipemia with a dose-dependent profile in healthy rats in a postprandial state. Therefore, the repression of miR-33a and miR-122 in liver could be one of the mechanisms of action through which proanthocyanidins exert their hypolipidemic effects. Although the significant effects were only observed at high levels of proanthocyanidin consumption, moderate doses of proanthocyanidins are able to modulate miRNA expression. Therefore, even though animal studies cannot be directly applied to humans, it can be suggested that a population with a normal intake of proanthocyanidin-rich foods can benefit from a low modulation of miRNA expression. This action at the molecular level can confer homeostatic robustness, and in turn, will exert subtle changes in lipid metabolism, reducing the risks associated with postprandial hyperlipemia.

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Table 1. Amounts of the individual flavanols and phenolic acids of the grape seed proanthocyanidin extract (GSPE) used in this study.

Compound	Amount (mg compound/g extract)
Gallic acid	17.7 ± 2.0
Protocatechuic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Procyanidin dimer[a]	144.2 ± 32.2
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
p-coumaric acid	0.1 ± 0.0
Dimer gallate[a]	39.7 ± 7.1
Epigallocatechin gallate	0.4 ± 0.1
Procyanidin trimer[a]	28.4 ± 2.0
Procyanidin tetramer[a]	2.0 ± 0.2
Epicatechin gallate[b]	55.3 ± 1.5
Quercetin-3-O-galactoside	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0
Quercetin	0.3 ± 0.0

(Adapted from Quiñones et al [26]) Phenolic components were determined by reverse-phase HPLC-MS. The results are expressed as the mean ± SD (n =3). [a]Quantified using the calibration curve of procyanidin B2. [b]Quantified using the calibration curve of epigallocatechin gallate [b]Quantified using the calibration curve of epigallocatechin gallate.

Table 2. Plasma lipids in rats fed with a standard chow diet, with or without different doses of GSPE (5, 15, 25 or 50 mg of GSPE/kg of body weight), in chronic treatments.

Plasma parameters	STD	5 mg GSPE/Kg bw	15 mg GSPE/Kg bw	25 mg GSPE/Kg bw	50 mg GSPE/Kg bw
TG (mg/dL)	93.3±7.5a	58.7±14.3ab	77.2±17.5ab	42.4±10.0b	58.1±6.8ab
TC (mg/dL)	75.5±3.5a	69.2±2.7ab	66.2±2.7ab	63.7±3.9b	60.6±1.2b
HDL-C (mg/dL)	33.1±2.2	41.1±1.6	41.6±1.0	39.5±2.8	38.4±1.9
LDL-C (mg/dL)	16.2±1.1a	11.9±1.6ab	11.1±1.6ab	10.7±2.0ab	9.5±1.1b
HDL-C/ LDL-C	2.4±0.2	3.5±0.3	3.5±0.4	3.5±0.7	4.6±0.7
TC/ HDL-C	2.0±0.2ab	1.8±0.01a	1.5±0.1ab	1.6±0.1ab	1.6±0.0b

Abbreviations: STD, standard diet group; bw, body weight; GSPE, grape seed proanthocyanidin extracts; TG, triacylglycerides; TC, total cholesterol. Rats were fed with a standard chow diet (STD group) or supplemented with 5, 15, 25 or 50 mg of GSPE per kg of body weight for 3 weeks. Each value is the mean ± S.E.M. of 10 rats per group. Letters denote a significant difference between groups ($p<0.05$; One-way ANOVA).

Table 3. Liver weight and liver lipids in rats fed with a standard chow diet, with or without different doses of GSPE (5, 15, 25 or 50 mg of GSPE/kg of bw), in chronic treatments.

Liver parameters	STD	5 mg GSPE/Kg bw	15 mg GSPE/Kg bw	25 mg GSPE/Kg bw	50 mg GSPE/Kg bw
Liver weight (% bw)	2.58±0.05	2.61±0.08	2.54±0.06	2.52±0.02	2.64±0.03
Total lipids (g/100g liver)	6.49±0.64	5.14±0.60	5.87±0.46	5.27±0.16	4.64±0.19
TG (g/100g liver)	1.31±0.06a	1.24±0.12ab	1.10±0.07ab	1.02±0.03b	1.07±0.04ab
TC (g/100g liver)	0.44±0.03a	0.42±0.06ab	0.34±0.02b	0.28±0.02b	0.32±0.00b

Abbreviations as in table 2.

Figure 1. miR-33a and miR-122 liver expression in rats treated with an acute dose of GSPE (250 mg of GSPE/Kg of body weight) over 24 h.

Rats were treated with 1 mL of tap water with or without (control group) 250 mg of GSPE/kg of body weight (treated group). miRNAs were quantified prior to treatment (0 h) and at 0.5, 1, 3, 6, 12 and 24 h after GSPE administration. miRNA levels were normalized to U6 small nuclear RNA. The values shown are the means of 3 animals per group and time point.

Figure 2. Levels of miR-33a, miR-122 and their target mRNAs, Abca1 and Fas, respectively, in the liver of healthy rats treated with different doses of GSPE (5, 15, 25 or 50 mg of GSPE/kg of body weight) in chronic treatments.

Rats were fed with a standard chow diet (STD group) or with STD plus 5, 15, 25 or 50 mg of GSPE/Kg of body weight for 3 weeks. miRNA and mRNA levels were normalized to U6 small nuclear RNA and PPIA, respectively. The values shown are the means of ten animals per group.

Letters denotes a significant difference between groups ($p<0.05$; One-way ANOVA).

Figure 1.

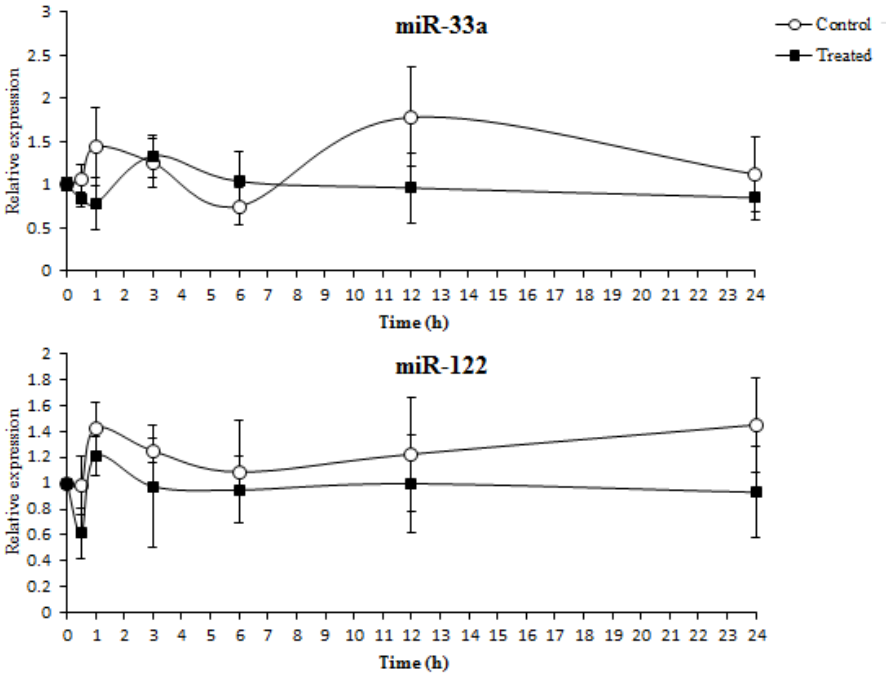
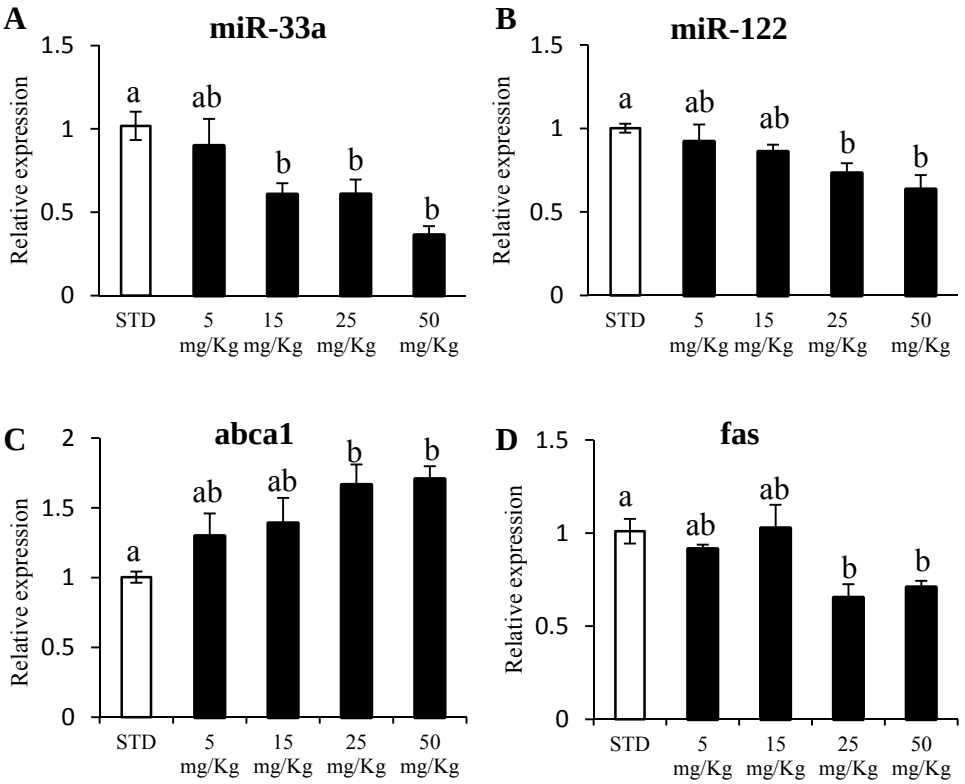

miR-33a
Control
Treated
Relative expression
Time (h)
miR-122
Relative expression
Time (h)

Figure 2.


A
miR-33a
Relative expression
1.5
1
0.5
0
a
ab
b
b
b
STD
5 mg/Kg
15 mg/Kg
25 mg/Kg
50 mg/Kg
B
miR-122
Relative expression
1.5
1
0.5
0
a
ab
ab
b
b
STD
5 mg/Kg
15 mg/Kg
25 mg/Kg
50 mg/Kg
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abca1
Relative expression
2
1.5
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15 mg/Kg
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1.5
1
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STD
5 mg/Kg
15 mg/Kg
25 mg/Kg
50 mg/Kg

2.2. Effect of chronic doses of proanthocyanidins on miR-33 and miR-122 expression in dyslipidemic obese rats (manuscript 4, submitted)

Chronic low doses of grape proanthocyanidins normalize miR-33a and miR-122 levels in obese rats.

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Keywords: Flavonoids, microRNAs, obesity, abca1, fas
Running title: miR-33a and miR-122 expression in obese rats.

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Abstract

Background: Deregulation of miR-33 and miR-122 has been related to obesity and metabolic syndrome.

Objective: The objective of this work was to determine whether the chronic consumption of dietary proanthocyanidins could effectively normalize the expression of miR-33a and miR-122 in rats made obese by a high-fat diet (HFD).

Design and Methods: Rats were fed a HFD for 15 weeks. Thereafter, they were divided into 4 groups and maintained on the HFD with or without supplementation with a grape seed proanthocyanidin extract (GSPE) at different doses. Plasma and liver lipid levels were measured by colorimetric and gravimetric analyses. The expression of miRNAs and their target genes were measured by real-time RT-PCR.

Results: 3 weeks of supplementation with GSPE normalized the overexpression of miR-33a and miR-122 in the liver of obese rats for all of the doses studied, with no dose-dependent outcome. GSPE supplementation reduced the levels of plasma and liver lipids in a dose-dependent manner.
Conclusion: A low chronic dose of proanthocyanidins, lower than the estimated mean intake for a European population, is enough to normalize miR-33a and miR-122 levels in the livers of obese rats.
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Abbreviations: GSPE, grape seed proanthocyanidin extract; TG, triglycerides; TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; HFD, high fat diet; STD, standard diet; miRNAs, microRNAs.

Introduction

Obesity and the associated metabolic syndrome represent major health problems in developed countries (1) because of their related co-morbidities, such as coronary artery disease, hypertension and type 2 diabetes mellitus. Metabolic syndrome is characterized by elevated fasting plasma triglyceride (TG) levels, high levels of low density lipoproteins (LDL) and low levels of high density lipoproteins (HDL), which increase cardiovascular risk. The presence of small dense LDLs, postprandial hyperlipidemia and hepatic overproduction of apoB-containing lipoproteins are novel lipid risk factors associated with obesity (2).

Generally, patients have difficulties following a long-term diet and exercise program to combat obesity and improve metabolic syndrome symptoms. Thus, food components that ameliorate the risk factors associated with these diseases can facilitate dietary-based therapies. Dietary polyphenols have positive effects on several risk factors associated with obesity; specifically, proanthocyanidins, a subclass of flavonoids, improve dyslipidemia (3), insulin resistance (4) and inflammation (5, 6). More specifically, chronic treatment with a grape seed proanthocyanidin extract (GSPE) normalizes plasma TG levels and LDL cholesterol (LDL-C) in obese rats (7). Moreover, transcriptomic (7) and proteomic (8) studies have shown that the chronic administration of GSPE represses lipogenesis and the assembly of very low density lipoproteins in the rats fed a high-fat diet (HFD). These gene modifications can result from the interaction between polyphenols and signaling cascades and/or epigenetic factors such as mi(cro)RNAs (9, 10, 11).

miRNAs constitute an abundant and evolutionarily conserved class of post-transcriptional regulators of gene expression (12, 13, 14). miRNAs play important regulatory roles in a variety of biological processes, are known to modulate more than 60% of human genes (15) and have been reported to regulate several metabolic pathways including insulin secretion and carbohydrate and lipid metabolism (16). miR-33 (16, 17) and miR-122 (16) have emerged as key regulators of lipid metabolism in the liver, and they have been related to metabolic diseases such obesity and metabolic syndrome. miR-122 is liver-specific and plays a critical role in liver homeostasis (18, 19). Its inhibition has been associated with the deregulation of genes with key roles in the control of lipid metabolism in the liver, such as fatty acid synthase (FAS), as well as genes that regulate fatty acid β-oxidation (18, 20, 21, 22, 23). miR-33 plays an important role in the regulation of cholesterol homeostasis, regulating the ATP-binding cassette transporters (ABC-transporters) ABCA1 and ABCG1, and plays a role in fatty acid β-oxidation (20). Interestingly, two isoforms of miR-33 have been identified, miR-33b and miR-33a (20, 24). miR-33b and miR-33a reside in the intronic region of the sterol response element binding protein 1 (SREBF1) and 2 (SREBF2) genes, respectively (25), and the b isoform is only found in humans and non-human primates.

We have previously shown that an acute dose of GSPE repressed miR-33a and miR-122 expression in the livers of healthy rats 1 hour after its oral administration (10). Thus, the aims of this article were to determine whether the chronic consumption of proanthocyanidins was able to normalize miR-33a and miR-122 deregulation in the livers of rats made obese by a HFD and to determine the dose of proanthocyanidins required to reverse deregulation.

MATERIALS AND METHODS

2.2 Grape seed proanthocyanidins extract

The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The following GSPE composition used in this study has been previously analyzed (26): 17.7 ± 2.0 mg Gallic acid/g extract, 1.0 ± 0.1 mg Protocatechuic acid/g extract, 0.1 ± 0.0 mg Vanillic acid/g extract, 144.2 ± 32.2 mg Procyanidin dimmer/g extract, 90.7 ± 7.6 mg Catechin/g extract, 55.0 ± 0.8 mg Epicatechin/g extract, 0.1 ± 0.0 mg p-coumaric acid/g extract, 39.7 ± 7.1 mg Dimer gallate/g extract, 0.4 ± 0.1 mg Epigallocatechin gallate/g extract, 28.4 ± 2.0 mg Procyanidin trimer/g extract, 2.0 ± 0.2 mg Procyanidin tetramer/g extract, 55.3 ± 1.5 mg Epicatechin gallate/g extract, 0.2 ± 0.0 mg Quercetin-3-O-galactoside/g extract, 0.1 ± 0.0 mg Naringenin-7-glucoside/g extract, 0.1 ± 0.0 mg Kaempferol-3-glucoside/g extract and 0.3 ± 0.0 mg Quercetin/g extract.

Animal design

Thirty-six-week-old, female Wistar rats (Crl: WI (Han)) were purchased from Charles River (Barcelona, Spain). The rats were individually caged in the animal quarters at 22ºC with a 12 h light/dark cycle (light from 8:00 to 20:00 pm) and were fed *ad libitum* a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After 1 week of acclimation, the rats were fed *ad libitum* a standard chow diet for the entire experimental procedure (standard diet group, STD, n = 6) or were fed *ad libitum* a cafeteria diet, the HFD model, for 15 weeks. The gross composition of the standard diet was 60.5% carbohydrate, 2.9% lipid and 15.4% protein, and the gross composition of the cafeteria diet was 35.2% carbohydrate, 23.4% lipid and 11.7% protein (27).

After 15 weeks, the animals fed the HFD were randomly divided into 4 groups (n = 6) and maintained on this diet with GSPE supplementation at 5, 25 or 50 mg of GSPE/kg body weight (HFD-low GSPE, HFD-medium GSPE and HFD-high GSPE groups, respectively) or without GSPE (HFD control group, HFD) for 3 additional weeks. The GSPE was dissolved in sugary milk at the appropriate concentrations, and a consistent milk volume (750 µl) was administered to the animals. At week 14, all of the rats were trained to voluntarily consume the milk, and both of the control groups, STD and

HFD, were fed the same volume of sugary milk for 3 weeks. The treatment was administered every day at 9:00 am.

The rats were fasted for 12 hours and were anesthetized with ketamine (70 mg/kg body weight, Parke-Davis, Grupo Pfizer, Madrid, Spain) plus xylazine (5 mg/kg body weight, Bayer, Barcelona, Spain) at 8:00 am. The rats were exsanguinated from the abdominal aorta. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The plasma was obtained by centrifugation (1500 g, 15 min, 4ºC) and stored at -80ºC until analysis. The liver was excised and frozen immediately in liquid nitrogen and stored at -80ºC until RNA and lipid extraction.

RNA extraction

The total RNA containing small RNA species was extracted from the frozen liver using the mi/mRNA extraction kit (miRNA kit, E.Z.N.A., Omega Bio-tek, Norcros, Georgia, U.S.A.), according to the manufacturer's protocol. To isolate both total RNA and miRNA, 1.5 volumes of absolute ethanol were added instead of the recommended 0.33 volumes in step 5. The washing step was performed according to the isolation protocol for large RNAs. The quality of the purified RNA was checked using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

microRNA quantification by real-time qRT-PCR

To analyze the expression of each miRNA, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA-specific reverse-transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems, Madrid, Spain). A My Gene L Series Peltier Thermal Cycler (Long Gene) was used for reverse transcription. The reaction was performed at 16ºC for 30 min, 42ºC for 30 min and 85ºC for 5 min. The final total RNA concentration used was 2.5 ng/µl in 7 µl. We used 1.33 µl of the diluted cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Specific Taqman probes were used for each gene: 5'UGGAGUGUGACAAUGGUGUUUG-3' for microRNA-122a (miR-122a: hsa-mir-122a) and 5'- GUGCAUUGUAGUUGCAUUG-3' for microRNA-33 (miR-33: hsa-mir-33). The results were normalized to the expression of U6 small nuclear RNA (U6 snRNA), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The fold change in the miRNA level was calculated in the log 2 scale according to the equation $2^{-\Delta\Delta Ct}$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct$ = ΔCt treated samples-ΔCt untreated controls.

mRNA quantification by real-time qRT-PCR

mRNA levels were evaluated by reverse transcription performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). A My Gene L Series Peltier Thermal Cycler (Long Gene) was used for reverse transcription. The reaction was performed at 25ºC for 10 min, 37ºC for 120 min and 85ºC for 5 sec. The final total RNA concentration used was 25 ng/µl in 125 µl. We used 5 µl of the diluted cDNA solution for a subsequent quantitative RT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain). Specific Taqman probes were used for each gene: Abca1 (Rn00710172_m1) and Fasn (Rn00569117_m1). The results were normalized to cyclophilin (PPIA: Rn00690933_m1), which was used as an endogenous control. The amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) with a protocol of 50ºC for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The fold change in the mRNA level was calculated in the log 2 scale using the equation $2-\Delta\Delta Ct$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct = \Delta Ct$ treated samples- ΔCt untreated controls.

Plasma and liver lipid analysis

Plasma total cholesterol and TGs were measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). Liver (0.5 g) lipids were extracted using the Folch method [32]. An aliquot of extract was subjected to gravimetry to measure total lipids. The remaining extract was evaporated under a nitrogen draft and dissolved in 1 ml of LPL buffer, containing PIPES disodium salt (P3768, Sigma-Aldrich, Madrid, Spain), $MgCl_2x6H_2O$ (M9272, Sigma-Aldrich, Madrid, Spain), albumin free fatty acids (A8806, Sigma-Aldrich, Madrid, Spain) and 0.1% SDS (L3771, Sigma-Aldrich, Madrid, Spain). The TG and cholesterol concentrations in the dissolved extract were measured using QCA enzymatic colorimetric kits (QCA, Barcelona, Spain), following the manufacturer's protocols.

Statistical analysis

The results are reported as the mean ± S.E.M. of 6 animals per group. The group means were compared using Student's t-test ($p \leq 0.1$) for lipids and a one-way ANOVA ($p \leq 0.05$) for miRNAS and mRNAs with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

RESULTS

Chronic GSPE administration normalized the expression of miR-33a, miR-122 and their target genes.

The expression of the lipid regulators miR-33a and miR-122 in the liver was deregulated significantly in the obese rats for 18 weeks compared to the STD (Figure 1A and B). Specifically, the HFD increased miR-33a and miR-122 levels by 165% and 23%, respectively. Thus, miR-33a was highly sensitive to the dietary fat content. Concomitant with miR-122 overexpression, fas, an indirect target gene of miR-122, was significantly overexpressed when the rats were fed a HFD. However, abca1 expression, a target gene of miR-33a, remained unaltered.

3 weeks of GSPE administration counteracted the overexpression of miR-33a and miR-122 in the liver of the HFD fed obese rats at all of the doses analyzed, without a dose-dependent effect (Figure 1A and B). Specifically, compared to the obese rats only fed a HFD, miR-33a was reduced by 60%, 48% and 60% after treatment with 5, 25 and 50 mg of GSPE/kg of body weight, respectively. In addition, miR-122 was reduced by 45%, 65% and 34%, after treatment with 5, 25 and 50 mg of GSPE/kg of body weight, respectively. miR-33a and miR-122 target mRNA were also modulated by supplementation with GSPE, reflecting the effects of GSPE on miRNAs levels (Figure 1C and D). abca1 mRNA, the target of miR-33a, was upregulated, whereas fas mRNA, the indirect target gene of miR-122, was downregulated. For both target genes, all of the doses studied significantly affected the mRNA levels without a dose-dependent effect on the miRNAs levels.

GSPE treatments improved the lipid profile in dietary obese rats.

One of the best described consequences of GSPE administration is its hypolipidemic outcome. Therefore, we determined which doses of GSPE effectively reduced the levels of plasma and liver lipids. Although the rats were fasted 12 hours prior to sacrifice, there was a large standard deviation for the lipid parameter means in the 4 groups of obese rats. Therefore, we applied the Student's t-test to analyze these parameters to uncover trends.

Rats fed a HFD for 18 weeks had a body weight 38% higher than the rats fed the standard diet (360 g for the HFD group and 260 g for the STD group). Moreover, these obese rats had plasma TG and LDL-C levels significantly increased, thus negatively affecting the atherosclerosis risk ratio HDL-C (cholesterol from HDL)/LDL-C (Table 1). GSPE administered simultaneously with the HFD (from week 15 to week 18) improved the dislypidemia induced by the HFD (Table 1). The administration of GSPE reduced the body weight by 6.5%, 3% and 6.5% after treatments with 5, 25 and 50 mg of GSPE/kg of body weight, respectively. GSPE also reduced plasma TG and total cholesterol (TC) levels in a dose-dependent manner; although, any of the doses normalized the plasma TG and TC levels. GSPE treatment effectively reduced the

levels of LDL-C in the plasma (by 40-50%); although, the reduction was only significant at the high dose.
GSPE administration for 3 weeks also reduced the total lipid and TC content in the livers of the obese rats (Table 2) in a dose-dependent manner. GSPE had a low effect on TG content (8%) in the liver.

DISCUSSION

Bioactive food compounds can aid the success of dietary regimens designed to ameliorate risk factors associated with obesity and metabolic syndrome. Therefore, dietary proanthocyanidins can be useful because their consumption reduces several risk factors associated with obesity, such as dyslipidemia. Moreover, proanthocyanidins have the ability to repress the expression of miR-33a and miR-122 (10), two of the most well-known miRNAs that are key controllers of lipid metabolism. Thus, the aims of this article were to determine whether chronic consumption of proanthocyanidins was able to normalize miR-33a and miR-122 deregulation in the livers of dietary obese rats and to estimate the dose of proanthocyanidins required for this reversion.

We chose the cafeteria diet as our obesogenic diet model because this diet provides a robust model of human obesity and metabolic syndrome compared to traditional lard-based high-fat diets (28). In this model, animals are allowed free access to a standard diet and water and are concurrently offered highly palatable, energy-dense, unhealthy human foods *ad libitum*. This diet promotes voluntary hyperphagia that results in rapid weight gain and increases fat pad mass and prediabetic parameters, such as glucose and insulin intolerance and dyslipemia (28).

GSPE was administered each day at a dose of 5, 25 or 50 mg of GSPE/kg of body weight. These doses, using a translation of animal to human doses (29) and estimating the daily intake for a 70 kg human, are equivalent to an intake of 57, 284 and 560 mg of proanthocyanidins/day, which are realistic intakes for humans with a habitual diet. For example, the mean proanthocyanidin intake in adults has been estimated as 95, 121 and 187 mg/day in the U.S.A. (30), Finland (31) and Spain (32), respectively. Therefore, the chronic experiments were designed to use a GSPE dose similar to the dietary proanthocyanidin intake in humans (i.e.; 25 mg/kg in rats). However, we also evaluated a GSPE dose below (i.e.; 5 mg/kg in rats) and exceeding (i.e.; 50 mg/kg in rats) the dietary intake of humans.

In this experiment, the rats fed a HFD exhibited obesity and an atherogenic lipid profile and lipid accumulation in the liver. The lipid regulators miR-33a and miR-122 and the target gene fas in the liver were significantly overexpressed after 18 weeks on this diet. All of these features indicated deregulated lipid homeostasis in this organ,

such as increased fatty acid synthesis, which resulted in dyslipidemia and fatty liver. Of these two miRNAs, miR-33a was especially sensitive to the HFD.

3 weeks of HFD supplemented with GSPE resulted in a normalization of miR-33a and miR-122 overexpression at all of the doses tested; thus, GSPE was a very powerful agent reversing the overexpression of these miRNAs induced by the HFD. The levels of the miR-33a and miR-122 target mRNAs, abca1 and fas, respectively, were also modulated by supplementation with GSPE in a pattern reflecting miR-33a and miR-122 repression. These data indicate that chronic consumption of GSPE, even at a low dose, may result in increased HDL biogenesis by the liver and reduced fatty acid synthesis in obese rats. Collectively, these results clearly show that the rats made obese by feeding a HFD are very sensitive to proanthocyanidins due to changes in the expression of miRNAs and their target genes in the liver.

The plasma and liver lipid levels were reduced in a dose-dependent pattern, which reflected the GSPE-induced repression of miR-33a and miR-122. However, despite the normalization of these miRNAs in the liver, any of the tested doses of GSPE were able to completely restore the levels of plasma lipids. Reinforcing our results, the intake of beverages rich in proanthocyanidins, such as apple polyphenols (33, 34), grape juice (33, 34) or red wine (35), did not significantly reduce plasma lipid levels in obese or overweight humans. However, the consumption of even relatively small amounts of flavonoid-rich foods, specifically proanthocyanidin-rich foods, has been beneficial for reducing the risk of cardiovascular disease in humans (36). Moreover, epidemiological observations demonstrated that some French populations experienced a relatively low incidence of coronary heart disease (CHD), despite a relatively high dietary intake of saturated fatty acids but with a high intake of red wine, which is a food rich in proanthocyanidins (37). Therefore, although proanthocyanidin-rich foods and beverages do not significantly reduce plasma lipid levels, they can confer phenotypic robustness at the molecular level to repress miR-33 and miR-122 to exert subtle changes in lipid metabolism in obesity.

The normalization of miR-33 levels in the liver by proanthocyanidins, with respect to obesity and metabolic syndrome, could extend the beneficial effects of proanthocyanidin-rich foods to other risks associated to these pathologies, such diabetes. In addition to the regulation of lipid metabolism, miR-33 has also been shown to control the expression of AMP-activated kinase (Ampkα1) and sirtuin 6 (Sirt6), which are involved in the regulation of both lipid and glucose metabolism (38). Moreover, miR-33 controls insulin secretion by the pancreas and the expression of ISR2, which is a component of the insulin signaling cascade in the liver (38).

In conclusion, the chronic administration of proanthocyanidins normalized miR-33a and miR-122 expression in the livers of obese rats. This normalization was significant at doses lower than the estimated mean intake for the European population. Therefore, although dietary proanthocyanidins may not significantly reduce plasma lipids, they can confer homeostatic robustness by acting at the molecular level to repress miR-33

and miR-122 improving lipid metabolism in obesity thus reducing the risk of comorbidities. The modulation of specific miRNAs is considered a promising strategy to treat metabolic diseases; therefore, proanthocyanidin-rich food can be a good candidate to reduce the risks associated with obesity. Furthermore, given that apples, grapes, nuts, red wine, tea and cocoa are very rich in proanthocyanidins (39), it will be easy to introduce proanthocyanidin-rich food into the diet of obese subjects.

Conflicts of Interest

The authors have declared no conflicts of interest.

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LB, AA, CB designed the experiments and carry out the data analyses. LB, EC, AR carried out experiments. LA, MJ, CB, AA, LB made the literature research. All the authors were involved in writing the paper and had the final approval of the submitted and published version

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Table 1. Plasma lipids levels of lean rats fed standard diet (STD) or obese rats fed high-fat diet (HFD) with or without different doses of a grape seed proanthocyanidin extract (5, 25 or 50mg of GSPE/kg of body weight).

Plasma parameters	STD	HFD	HFD-low GSPE	HFD-medium GSPE	HFD-high GSPE
TG (mg/dL)	95.89±4.5	249.1±70.5†	218.0±60.7†	180.1±35†	173.7±37
TC (mg/dL)	59.94±1.9	83.28±15.5	93.76±17.5	79.88±12.5	70.01±6.9
HDL-C (mg/dL)	34.83±1.9	33.50±2.5	35.57±5.7	30.71±2.5	35.98±3.2
LDL-C (mg/dL)	7.54±1.3	22.48±4.8†	12.70±5.9	13.44±4.1	11.0±0.8*
HDL-C/ LDL-C	5.28±0.3	2.06±4.5†	8.24±1.2	3.00±0.3†	3.40±1.5*†
TC/ HDL-C	1.71±0.1	1.98±0.1	1.96±0.2	2.06±0.2	1.90±0.1

Rats were fed with standard diet (STD group) or with standard chow plus cafeteria diet (HFD group) for 15 weeks. Thereafter, rats fed with standard chow plus HFD were orally treated with 5, 25 or 50 mg of GSPE per Kg body weight for 3 weeks simultaneously with the HFD. Each value is the mean ± S.E.M. of six rats.*denotes significant difference versus HFD group ($p<0.1$; Student-t test) † denotes significant difference versus STD group ($p<0.1$; Student-t test).

Table 2. Liver weight and liver lipids of lean rats fed standard diet (STD) or obese rats fed high-fat diet (HFD) with or without different doses of a grape seed proanthocyanidin extract (5, 25 or 50mg of GSPE/kg of body weight).

Liver parameters	STD	HFD	HFD-low GSPE	HFD-medium GSPE	HFD-high GSPE
Liver weight (% bw)	1.90±0.07	1.82±0.09	1.86±0.07	1.73±0.05	1.91±0.1
Total lipids (g/100g liver)	7.61±0.36	9.26±0.37†	8.23±0.54	7.93±0.21*	6.60±0.42*
TGs (g/100g liver)	1.36±0.07	1.54±0.12	1.36±0.07	1.35±0.08	1.35±0.11
TC (g/100g liver)	0.36±0.07	0.62±0.10†	0.40±0.14	0.39±0.08*	0.37±0.08*

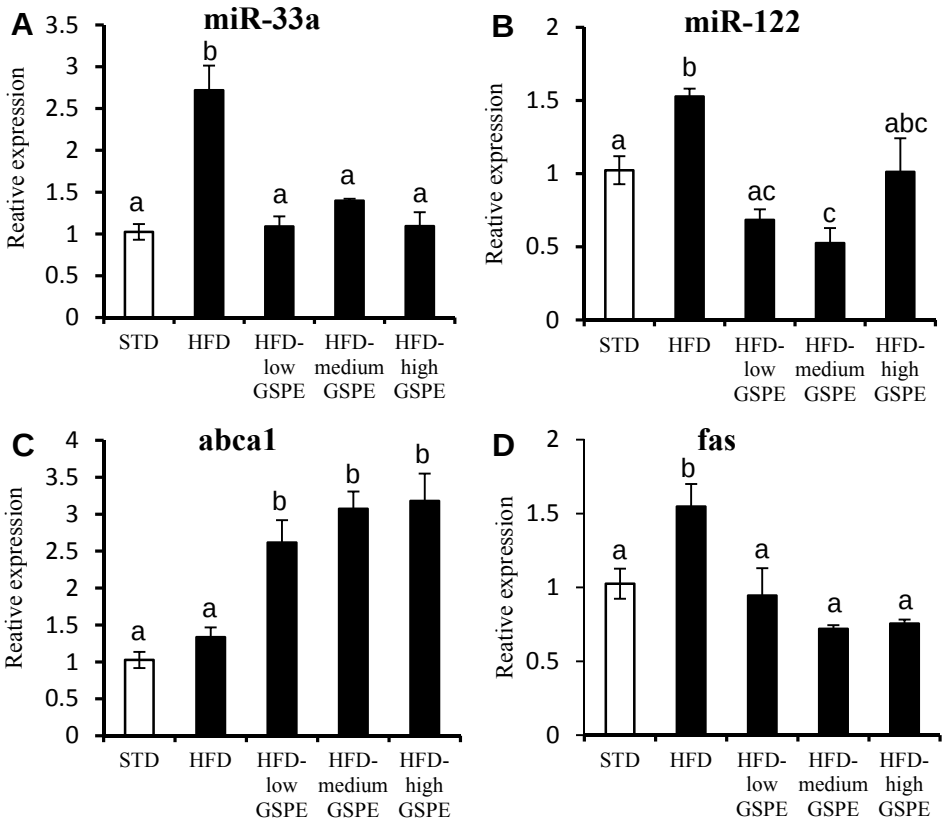
Rats were fed with standard diet (STD group) or with standard chow plus cafeteria diet (HFD group) for 15 weeks. Thereafter, rats fed with standard chow plus HFD were orally treated with 5, 25 or 50 mg of GSPE per Kg body weight for 3 weeks simultaneously with the HFD. Each value is the mean ± S.E.M. of six rats.*denotes significant difference versus HFD group ($p<0.1$; Student-t test) † denotes significant difference versus STD group ($p<0.1$; Student-t test).

Figure legends

Figure 1. Levels of miR-33a, miR-122 and their target mRNAs in the livers of lean rats fed a standard diet or obese rats fed a high-fat diet with or without varying doses of a grape seed proanthocyanidin extract (5, 25 or 50 mg of GSPE/kg of body weight).

The rats were fed a standard chow diet (STD group) or a standard chow plus cafeteria diet (HFD group) for 15 weeks. Thereafter, the rats fed a standard chow plus CD were orally treated with 5, 25 or 50 mg of GSPE per kg body weight for 3 weeks in conjunction with the CD. miRNA and mRNA levels were normalized to U6 small nuclear RNA and PPIA, respectively. The mean ± S.E.M. of 6 rats is shown. The letters denote a significant difference between the groups ($p < 0.05$; One-way ANOVA).

Figure 1


A
miR-33a
Reative expression
3.5
3
2.5
2
1.5
1
0.5
0
a
b
a
a
a
STD
HFD
HFD-low GSPE
HFD-medium GSPE
HFD-high GSPE
B
miR-122
Reative expression
2
1.5
1
0.5
0
a
b
ac
c
abc
STD
HFD
HFD-low GSPE
HFD-medium GSPE
HFD-high GSPE
C
abca1
Reative expression
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
b
b
b
STD
HFD
HFD-low GSPE
HFD-medium GSPE
HFD-high GSPE
D
fas
Reative expression
2
1.5
1
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a
b
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a
a
STD
HFD
HFD-low GSPE
HFD-medium GSPE
HFD-high GSPE

3. Comparative effect of chronic administration of proanthocyanidins and/or ω-3 PUFAs on the modulation of miR-33 and miR-122 in rat liver and PBMCs (manuscript 5, accepted, Plos One)

Chronic Administration of Proanthocyanidins or Docosahexaenoic Acid Reversess the Increase of mir-33a and mir-122 in Dyslipidemic Obese Rats

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Abstract

miR-33 and miR-122 are major regulators of lipid metabolism in the liver, and their deregulation has been linked to the development of metabolic diseases such as obesity and metabolic syndrome. However, the biological importance of these miRNAs has been defined using genetic models. The aim of this study was to evaluate whether the levels of miR-122 and miR-33a in rat liver correlate with lipemia in nutritional models. For this purpose, we analyzed the levels of miRNA-33a and miR-122 in the livers of dyslipidemic cafeteria diet-fed rats and of cafeteria diet-fed rats supplemented with proanthocyanidins and/or ω-3 PUFAs because these two dietary components are well-known to counteract dyslipidemia. The results showed that the dyslipidemia induced in rats that were fed a cafeteria diet resulted in the upregulation of miR-33a and miR-122 in the liver, whereas the presence of proanthocyanidins and/or ω-3 PUFAs counteracted the increase of these two miRNAs. However, srebp2, the host gene of miR-33a, was significantly repressed by ω-3 PUFAs but not by proanthocyanidins. Liver mRNA levels of the miR-122 and miR-33a target genes, fas and pparβ/δ, cpt1a and abca1, respectively, were consistent with the expression of these two miRNAs under each condition. Moreover, the miR-33a and abca1 levels were also analyzed in PBMCs. Interestingly, the miR-33a levels evaluated in PBMCs under each condition were similar to the liver levels but enhanced. This demonstrates that miR-33a is expressed in PBMCs and that these cells can be used as a non-invasive way to reflect the expression of this miRNA in the liver. These findings cast new light on the regulation of miR-33a and miR-122 in a dyslipidemic model of obese rats and the way these miRNAs are modulated by dietary components in the liver and in PBMCs.

Keywords: microRNA, cafeteria diet, lipid metabolism, proanthocyanidins, omega-3 fatty acids, PBMCs, DHA.

Abbreviations: miRNA, microRNA; **TG**, triglyceride; **TC**, total cholesterol; **FA**, fatty acid; **CD**, cafeteria diet; **STD**, standard chow diet; **Abca1**, ATP-binding cassette A1; **Fas**, fatty acid synthase; **GSPE**, grape seed proanthocyanidins extract; **DHA-OR**, oil-rich in docosahexaenoic acid; **PBMCs**, peripheral blood mononuclear cells.

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Introduction

In the last decade, mi(cro)RNAs have emerged as a novel class of non-coding RNAs that are 20-25 nucleotides long and that regulate the expression of specific target genes at the post-transcriptional level, mainly by binding to the 3' untranslated region (3'UTR) of target mRNAs. This triggers mRNA cleavage or inhibits translation (1-3). miRNAs are known to modulate more than 60% of human transcripts and thus play important regulatory roles in a variety of biological processes and are implicated in almost all metabolic pathways (4). Moreover, there is much evidence that the deregulation of miRNAs is related to the development of chronic diseases (5). Specifically, miR-33 and miR-122 are known as major regulators of lipid metabolism in the liver, and their deregulation may contribute to the development of metabolic diseases such as obesity and metabolic syndrome (5,6). miR-122 plays a critical role in liver homeostasis by regulating genes with key roles in the synthesis of triglycerides (TGs) and fatty acids (FAs), such as FA synthase (FAS) and sterol regulatory element-binding protein 1c (SREBP1c), as well as genes that regulate FA β-oxidation (7,7,8). Moreover, gene silencing of miR-122 in mice (9,10), African green monkeys (11) and chimpanzees (12), using either antagomirs or antisense oligonucleotides, significantly decrease plasma cholesterol and TG levels. Additionally, miR-33 plays an important role in the regulation of cholesterol homeostasis in the liver, regulating the ATP-binding cassette transporters (ABC transporters) ABCA1 and ABCG1 in addition to its role in FA β-oxidation by targeting the carnitine palmitoyltransferase 1a (CPT1a) (13). Interestingly, miR-33 has two isoforms:miR-33b, which is present in a subset of species, such as dogs, pigs, non-human primates and humans, but not in rodents and miR-33a, which is highly conserved from humans to Drosophila. miR-33b and miR-33a isoforms are found in introns in the sterol response element binding protein 1 (SREBF1) and 2 (SREBF2) genes, respectively (13-15). SREBF1 and SREBF2 code for the transcription factors SREBP1 and SREBP2, which regulate all SREBP-responsive genes in both the cholesterol and FA biosynthetic pathways (16). Furthermore, the silencing of miR-33 by knockout or antisense techniques in mice results in an improvement in the plasma lipid profile, increasing plasma high density lipoprotein-cholesterol (HDL-C) levels (15,17,18). Moreover, the inhibition of miR-33 in African green monkeys increased hepatic abca1 expression and the HDL-C plasma levels and decreased plasma very low density lipoprotein (VLDL) levels (19).

Aberrant lipid homeostasis, which is implicated in conditions such as dyslipidemia, is associated with obesity and metabolic syndrome. These pathologies represent an important health problem for developed and developing countries (20,21). The use of dietary compounds that reduce the health complications related to these pathologies is appearing as a new strategy. It has been reported that polyunsaturated fatty acids (PUFAs) and polyphenols have the capacity to affect cardiovascular diseases, improving lipid homeostasis (22,23). Specifically, a grape seed proanthocyanidin

extract (GSPE) was shown to reduce plasma TG, apo B and low density lipoprotein-cholesterol (LDL-C) and increase the percentage of HDL-C in healthy rats given an acute oral dose of GSPE (24) and in dyslipidemic rats given chronic administration (25). Additionally, the intake of ω-3 PUFAs is helpful in the prevention of cardiovascular disease (26). Hence, the beneficial effects of ω-3 PUFAs on the treatment of hyperlipidemia have been extensively studied in both humans (27) and animals (28,29), showing a potent hypolipidemic effect. Regarding the mechanism of action of both proanthocyanidins and ω-3 PUFAs, the evidence has recently shown that these dietary compounds can modulate miRNAs expression. Specifically, different FAs have been shown to exert certain biological effects through the direct modulation of miRNAs expression (30-32). Moreover, there are several examples in the literature that demonstrate the capacity of polyphenols to modulate miRNAs (33); for example, we recently reported the ability of grape seed proanthocyanidins to downregulate liver miR-33a and miR-122 in rats (34).

Regardless of the well-defined roles of miR-33 and miR-122 in controlling lipid metabolism in genetic models, the association of these miRNAs with lipemia in pathophysiological conditions is not well understood. Therefore, the aim of this study was to evaluate whether the levels of miR-122 and miR-33 in the liver correlate well with lipemia that has been nutritionally (non-genetically) induced in different rat models. For this purpose, we analyzed liver miRNA-33a and miR-122 levels in dyslipidemic cafeteria diet- (CD) fed rats and in rats fed a CD supplemented with proanthocyanidins and/or ω-3 PUFAs, two dietary components that are known to counteract dyslipidemia. While determining miRNAs levels in tissues remains important, peripheral blood mononuclear cells (PBMCs) are becoming a non-invasive way to study gene and miRNAs expression because they can be easily collected from the blood and reflect, as biomarkers, the pathological and physiological state of the organism (35,36). Therefore, we also evaluated whether miR-33a is expressed in PBMCs and if the changes in liver miR-33a and abca1 expression are reflected in these cells.

Materials and methods

Grape seed proanthocyanidins extract

The GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The following GSPE composition used in this study has been previously analyzed (37): catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g).

Oil rich in docosahexaenoic acid

The DHA-OR was kindly provided by MarteK DHA^{TM} –S**.** The nutritional oil used in the experiment is derived from the marine alga, *Schizochytrium* sp., a rich source of ω-3 DHA with sunflower lecithin and rosemary extract (flavoring). The fatty acid profile of DHA-OR was 5.6% of 14:0, 16.1% of 16:0, 0.9% of 18:0, 15% of 18:1n-9, 1.4% of 18:2n-6, 0.5% of 20:4n-6 (ARA), 1.1% of 20:5n-3 (EPA), 16.2% of 22:5n-6 (DPA), 38.8% of 22:6n-3 (DHA) and 0.5% of others. The oil also contained tocopherols and ascorbyl palmitate as antioxidants to provide stability.

Ethic statements

All procedures involving the use and care of animals were reviewed and approved by The Animal Ethics Committee of our university (reference number 4249 by Generalitat de Catalunya).

Animals

Male Wistar rats weighing 150 g were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of our university approved all procedures. The animals were housed in animal quarters at 22 ºC with a 12 h light/dark cycle (light from 08:00 hours to 20:00 hours) and were fed a standard chow diet (STD) *ad libitum* (Panlab, Barcelona, Spain). After one week, the rats were divided into 5 groups (n=7): the STD control group, where rats were fed STD *ad libitum,* and 4 other groups that were fed a STD plus a CD as a high fat model which had 23.4% lipids (0.05% cholesterol), 35.2% carbohydrates and 11.7% protein. The CD consisted of the following foods: cookies with foie-gras and cheese triangles, bacon, biscuits, carrots and sugary milk. After 10 weeks, rats feeding on the CD were trained to lick arabic gum (1 mL) (G9752, Sigma-Aldrich, Madrid, Spain), which was used as the vehicle; were divided into 4 groups and were fed a CD plus treatments for 3 more weeks. The first group was supplemented every day with 25 mg GSPE/kg bw dissolved in arabic gum (CD-GSPE group). The second group was supplemented every day with a dose of DHA-OR equivalent to 515 mg ω3-PUFAs/kg bw dissolved in arabic gum (CD-DHA-OR group). The third group was supplemented every day with 25 mg GSPE/kg bw plus a dose of DHA-OR equivalent to 515 mg ω-3 PUFAs/kg bw dissolved in arabic gum (CD-GSPE+ DHA-OR group). The fourth group received the same volume of arabic gum (CD control group). All treatments were administered at the same time point (7 p.m.). After 3 weeks of treatments, rats were killed at 9 a.m. by anesthetizing them with 50 mg/kg bw of sodium pentobarbital (0804118, Fagron Iberica, Terrasa, Spain), and they were sacrificed by bleeding. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 x *g*, 15 min, 4 ºC) and stored at -80 ºC until analysis. The livers were excised, frozen immediately in liquid nitrogen and stored at -80 ºC until RNA and lipids could be extracted.

RNA extraction

Total RNA containing small RNA species was extracted from frozen liver and PBMCs using a mi/mRNA extraction kit (miRNA kit, E.Z.N.A., Omega Bio-tek, Norcros, U.S.A.) according to the manufacturer's protocol. To isolate both total RNA and miRNA, 1.5 volumes of absolute ethanol was added instead of the recommended 0.33 volumes in step 5. The washing step was performed according to the isolation of large RNAs. The quantity of the purified RNA was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

microRNA quantification by real-time qRT-PCR

To analyze the expression of each miRNA, reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA-specific reverse-transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems, Madrid, Spain). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used. The final total RNA concentration used was 2.5 ng/µL. The reaction was performed at 16 ºC for 30 min, 42 ºC for 30 min and 85 ºC for 5 min. We used 1.33 µL obtained cDNAs in a subsequent quantitative qRT-PCR amplification using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) and the associated specific probe provided in the TaqMan® MicroRNA Assay Kit (Applied Biosystems). Specific Taqman probes were used for each gene: microRNA-122a (miR-122a: hsa-mir-122a), 5'UGGAGUGUGACAAUGGUGUUUG-3' and microRNA-33 (miR-33: hsa-mir-33), 5'- GUGCAUUGUAGUUGCAUUG-3'. The results were normalized to the expression of the U6 small nuclear RNA (U6 snRNA), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95°C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fold change in the miRNA level was calculated by the log 2 scale according to the equation $2^{-\Delta\Delta Ct}$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct$= ΔCt treated samples- ΔCt untreated controls.

mRNA quantification by real-time qRT-PCR

mRNA levels were evaluated by reverse transcription performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). For the reverse transcription, a My Gene L Series Peltier Thermal Cycler (Long Gene) was used. The final total RNA concentration used was 25 ng/µL. The reaction was performed at 25 ºC for 10 min, 37 ºC for 120 min and 85 ºC for 5 sec. We used 5 µL obtained cDNA solution for subsequent quantitative RT-PCR amplifications using the TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain). Specific Taqman probes were used for each gene: Abca1 (Rn00710172_m1), Fasn (Rn00569117_m1), Cpt1a (Rn00580702_m1), Pparβ/δ (Rn00565707_m1) and Srebp2 (Rn01502638_m1). The results were normalized to cyclophilin (PPIA:

Rn00690933_m1), which was used as an endogenous control. Amplification was performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) with a protocol of 50 ºC for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fold change in the mRNA levels was calculated by the log 2 scale using the equation $2-\Delta\Delta Ct$, where ΔCt = Ct miRNA-Ct U6 and $\Delta\Delta Ct$= ΔCt treated samples- ΔCt untreated controls.

Plasma and liver lipid analysis

Plasma total cholesterol and TG were measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). Liver (0.05 g) was used to extract total lipids using gravimetric analysis. Lipids were dissolved in 1 mL LPL buffer containing: PIPES disodium salt (P3768, Sigma-Aldrich, Madrid, Spain), $MgCl_2x6H_2O$ (M9272, Sigma-Aldrich, Madrid, Spain), albumin free FAs (A8806, Sigma-Aldrich, Madrid, Spain) and 0.1% SDS (L3771, Sigma-Aldrich, Madrid, Spain). The TG and cholesterol concentrations in the dissolved extract were measured using QCA enzymatic colorimetric kits (QCA, Barcelona, Spain) following the manufacturer's protocols.

Isolation of primary rat monocyte-derived macrophages

Rat PBMCs (peripheral blood mononuclear cells) were isolated from total blood by density gradient centrifugation using Histopaque®-1077 (10771, Sigma-Aldrich, Madrid, Spain) according to the manufacturer's protocol. Histopaque®-1077 is a solution of polysucrose (5.7 g/dL) and sodium diatrizoate (9 g/dL) adjusted to a density of 1.077+/−0.001 g/mL. The isolated PBMCs were dissolved in a lysis buffer to obtain total RNA.

Statistical analyses

The results are reported as the mean ± S.E.M. of seven animals for the group. Groups were compared with one-way ANOVA ($p \leq 0.05$) using SPSS software.

Results

GSPE and oil-rich in docosahexaenoic acid treatments improve the atherogenic lipid profile induced by a CD.

Feeding rats a CD for 13 weeks significantly increased their body weight (bw) by 20% (443 to 531 g bw) and increased plasma TG, total cholesterol (TC) and LDL-C levels compared with those of rats fed a standard chow diet (STD), thus worsening the atherosclerosis risk ratio, HDL-C/LDL-C (table 1). Once obesity and dyslipidemia were induced in rats (i.e., after 10 weeks of CD), rats were fed GSPE and/or oil-rich in docosahexaenoic acid (DHA-OR) for 3 more weeks. The results showed that GSPE administration for 3 weeks returned plasma TG and LDL-C levels to normal values, whereas it induced only a slight reduction of TC. Moreover, rats treated with GSPE

showed a ratio of HDL-C/LDL-C that was even higher than that of rats fed a STD. On the other hand, 3 weeks of DHA-OR treatment did not significantly reduce plasma TG levels, but rather normalized the levels of plasma TC and LDL-C and the HDL-C/LDL-C ratio. When GSPE and DHA-OR were simultaneously administered for 3 weeks, this corrected all the dyslipidemic effects induced by the CD because plasma TG, LDL-C, TC and the ratio of HDL-C/LDL-C were normalized.
Liver lipid parameters were analyzed by gravimetric analysis (table 2). The CD control group showed an increase in the total lipid content, increasing TG and TC levels. However, no difference was observed in the liver weight compared to livers from rats that were fed a STD. Nevertheless, after the GSPE treatment, the total liver lipids were reduced with a decrease in TG that was comparable to the levels in the CD control. After the DHA-OR treatment, there was no effect on the liver lipid content. Moreover, when GSPE and DHA-OR were simultaneously administered, TC levels were decreased. Therefore, our results demonstrated that GSPE and DHA-OR treatments improved the atherogenic lipid profile caused by a CD.

CD increased miR-33a and miR-122 levels in the liver and modulated the expression of their target genes.

The CD was able to alter the levels of lipid metabolism-related miRNAs (Figure 1 and 2). Specifically, a supplemental CD plus a STD for 13 weeks resulted in an increase in miR-33a levels by 60% and a significant reduction in abca1 target mRNA, with no modification of Cpt1a target mRNA, compared to rats fed only a STD. Similarly, miR-122 expression was upregulated by 47%, and fas target mRNA levels significantly increased, whereas pparβ/δ was no modified, when rats were fed a CD.

GSPE and DHA-OR reversed the increase in miR-122 and altered their target genes induced by a CD in rat liver.

After 3 weeks of GSPE and/or DHA-OR treatments in rats that were fed a CD, the expression of miR-122 in the liver was normalized (Figure 1A). GSPE reduced miR-122 expression by 53%. However, DHA-OR was more effective and reduced miR-122 expression in the liver by 59%. Moreover, when GSPE and DHA-OR were administered simultaneously, the reduction in miRNA expression was greater than when they were administered separately, with reduction in miR-122 expression of 68%. The direct and indirect targets mRNAs levels for miR-122 were also modulated by the three treatments according to their effects on miRNAs levels (Figure 1B and 1C). Therefore, the expression of Fas and Pparβ/δ mRNAs, an indirect and a direct target of miR-122, respectively, were modified according to miR-122 levels. Therefore, fas mRNA was downregulated after treatments, with a greater effect when GSPE and DHA-OR were administered simultaneously. Moreover, Pparβ/δ mRNA levels were increased after treatments, but not significantly.

GSPE and DHA-OR reversed the increase in miR-33a induced by a CD, whereas only DHA-OR repressed significantly srebp2 in liver.

After 3 weeks of GSPE and/or DHA-OR treatments in rats that were fed a CD, the expression of miR-33a in the liver was normalized (Figure 2B). However, the srebp2 mRNA, which encodes miR-33a and are known to be cotranscribed, was no significantly modified by GSPE. Moreover, similar to miR-122, DHA-OR was more effective reducing miR-33a expression in the liver than GSPE. Moreover, DHA-OR also repressed significantly srebp2 comparing with CD. Furthermore, when GSPE and DHA-OR were administered simultaneously, the reductions of miR-33a and srebp2 expressions were greater than when they were administered separately. The targets mRNAs levels for miR-33a were also modulated by the three treatments according to their effects on miRNA level (Figures 2C, 2D). Therefore, Abca1 and Cpt1a mRNAs were upregulated after GSPE and DHA-OR treatments, and when GSPE and DHA-OR were administered simultaneously, the increase in Abca1 mRNA was greater than when they were administered separately.

PBMCs reflected the liver miR-33a and abca1 mRNA levels

Figure 3 shows the levels of miR-33a and abca1 in the PBMCs of rats that were fed a CD and rats that were fed a CD supplemented with GSPE and/or DHA-OR. The abca1 mRNA expression in PBMCs reflected that of the liver profile: abca1 mRNA was downregulated by 76% in rats that were fed a CD compared to rats that were fed a STD. As in the liver, GSPE and DHA-OR reversed the effect of the CD, increasing abca1 expression by 54% and 37%, respectively. When the two treatments were administered simultaneously, the effect on abca1 was increased by 66%, as it was in the liver. Moreover, miR-33a expression in the PBMCs of CD-fed rats was upregulated by 81%, which was an even greater increase than in the liver. GSPE and DHA-OR reversed the effect of a CD, reducing miR-33a levels by 62% and 74%, respectively. A greater reduction (80%) was observed when the two treatments were administered simultaneously.

Discussion

miRNAs have been described as regulators of gene expression, and the deregulation of several miRNAs that are related to chronic diseases has been reported (5). Specifically, miR-122 and miR-33 play key roles in lipid metabolism. It is well-known that miR-122 is involved in the regulation of several genes in the cholesterol biosynthesis pathway (9) and that it modulates TG metabolism (10). Additionally, miR-33 plays a crucial role in the regulation of cholesterol metabolism (15,18,38,39).

Although the deregulation of miR-33 and miR-122 has been related to the development of risk factors associated to metabolic diseases, such as obesity and metabolic syndrome, most of the studies on the regulation of lipid metabolism by these two miRNAs have been performed using knockout or antisense models (9-12,17-19,40). The purpose of this work was to study whether the levels of miR-122 and miR-33a correlate with nutritionally induced lipemia in different rat models; this reflects a more natural physiological state. Therefore, we have analyzed these miRNAs in the livers of dyslipidemic CD-fed rats and in those of rats that were fed a CD supplemented with proanthocyanidins and/or ω-3 PUFAs, which are two dietary components known to counteract dyslipidemia (22,23).

The CD provides a robust model of human metabolic syndrome compared to traditional lard-based high-fat diets (41). In this model, animals are allowed free access to a STD and water and are concurrently offered highly palatable, energy dense, unhealthy human foods *ad libitum*. This diet promotes voluntary hyperphagia that results in rapid weight gain and increases fat pad mass and prediabetic parameters, such as glucose and insulin intolerance and dyslipemia (41). In this experiment, rats fed a CD showed a 20% increase in bw and an atherogenic lipid profile demonstrating a significant increase in TG, TC and LDL-C levels in the plasma. Moreover, rats fed a CD showed increased liver lipids due to the accumulation of both TG and TC.

Interestingly, in association with the atherogenic lipid profile and fatty liver, the levels of miR-122, miR-33a and srebp2, the host gene of miR-33a, were increased in the livers of rats who were fed a CD. In contrast to our results, a downregulation of miR-33 has been described in mice fed a high fat diet (HFD) (38). However, the effect of a HFD on srebp2 expression is controversial, with studies that demonstrate a downregulation (38) or an overexpression (42) of this gene in mice. Moreover, it should be stated that the severity of hepatic steatosis and inflammation induced by a CD is stronger than that induced by a HFD, with a malignant progression from non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) in animals fed a CD (41). Interestingly, NASH patients have increased expression of srebp2 in liver (43). Thus, it seems that the type of diet conditions an over- or down-regulation of srebp2 and consequently miR-33 levels.

In relation to miR-122, our results are supported by evidence that plasma miR-122 is increased in patients with hyperlipidemia (44). However, as with miR-33a, this increase in hepatic miR-122 in rats that were fed a CD has not been observed using other HFD. For instance, mice fed a diet of 58%-60% fat (45), with or without 30% w/v of fructose in the drinking water (46), show a downregulation of miR-122 expression in the liver. Additionally, a reduction in liver miR-122 expression was found in genetically obese ob/ob mice (47). However, mice that were deficient in LDL receptors and that were fed a diet of 22% fat and 0.32% cholesterol do not show any change in miR-122 expression in the liver (48). Therefore, like for miR-33a, it seems that diet and genetic modification influence liver miR-122 expression in

different ways. Thus, further studies of liver miR-122 and miR-33 expression using different types of diets are warranted.

Direct miR-122 targets that modulate lipid metabolism are essentially unknown and most of the defined target genes of this miRNA, such as Fas, are indirectly modulated. However, recently it has been identified PPARα, β, and γ and the PPARα-coactivator (Smarcd1/Baf60a), as direct targets of miR-122 (49). For this reason, we have quantified a direct, pparβ/δ, and an indirect, fas, target gene of miR-122. The liver expression of target genes of miR-122 and miR-33a indicate that these rats had increased lipogenesis, decreased fatty acid oxidation and reduced HDL biosynthesis in the liver. Consequently, the increase in miR-122 and miR-33a levels in the liver could explain, to some extent, the dyslipemic effect of a CD.

We used two well-known dietary components that improve lipid metabolism, proanthocyanidins and ω-3 PUFAs (GSPE and DHA-OR, respectively) to determine whether the improvement in the atherogenic profile induced by these compounds is associated with a reduction in liver miR-122 and miR-33a, which were previously increased by the CD. Our results show that a chronic treatment of GSPE in CD-fed rats improved the atherogenic lipid profile caused by the CD, normalizing plasma TG and LDL-C levels and reducing liver TGs and total liver lipids. In contrast, a chronic treatment of DHA-OR in rats that were fed a CD normalized TC and LDL-C plasma levels without an effect on the lipid content of the liver. There are some studies that show that polyphenols and ω-3 PUFAs have a synergistic effect (50). Thus, we evaluated the hypolipidemic effect of a simultaneous administration of GSPE and DHA-OR. In this case, liver TC and plasma TG, TC and LDL-C levels were decreased to a similar degree to that found when administering the compounds separately, and the effect was not additive or synergistic, but complementary. To confirm the protective effect of GSPE and DHA-OR against cardiovascular diseases, the TC/HDL-C ratio, known as the atherogenic or Castelli index, and the HDL-C/LDL-C ratio were calculated because they are two important indicators of vascular risk with greater predictive value than the isolated parameters (51). Both treatments resulted in an increase in the HDL-C/LDL-C ratio, reversing the decrease of this index that results from a CD.

Interestingly, these dietary treatments also counteract the overexpression of miR-122, miR-33a that is induced by the CD. Both GSPE and DHA-OR treatments repressed miR-122, miR-33a in the liver, reaching the levels found in rats that were fed a STD. Moreover, when the two treatments were orally administered together, the repression of these miRNAs was greater than when the treatments were administered separately. Previously, we have shown that GSPE represses miR-122 and miR-33a liver expression in rats treated with an acute dose, which also induced postprandial hypolipidemia in normal rats (34). There is increasing evidence that in the presence of ω-3 PUFAs, different PUFAs, such as DHA, araquidonic acid and gamma linoleic acid, can exert certain biological effects - for example, on the apoptosis pathway - through the direct modulation of miRNAs (32). However, to our knowledge, there is

not any study showing the influence of ω-3 PUFAs on lipid regulator miRNAs such as miR-33 and miR-122. The repression of rat liver miR-122 and miR-33a, induced by GSPE and DHA-OR, was clearly associated with the improvement in the plasma lipid profile that was induced by these two treatments. Therefore, as both treatments repress these miRNAs in the liver and normalize plasma lipids, it could be suggested that the modulation of miR-122 and miR-33a could be one of the molecular mechanisms used by proanthocyanidins and ω-3 PUFAs to improve the plasmatic atherogenic profile that was induced by a CD.

The inhibition of miR-122 by antisense oligonucleotides reduces the biosynthesis of cholesterol and FAs and increases the oxidation of FAs in the livers of normal mice, and it reduces both hepatic cholesterol accumulation and the development of a fatty liver during the development of diet-induced obesity in mice (10). But, despite the fact that both treatments repressed the expression of miR-33a and miR-122 in the liver to a similar degree, only the GSPE treatment was effective in reducing the liver lipid content. However, it has been reported that rats fed a high fat diet combined with ω-3 PUFA supplementation were protected against steatosis (52). Nevertheless, our results do not show any modification of lipids in the livers of rats that were fed a CD and treated with DHA-OR. Thus, it should be stressed that the reduction in hepatic levels of miR-122 and miR-33a was associated with the reduction of lipids in the plasma but not in the liver.

In order to elucidate the mechanism by which GSPE and DHA-OR treatments can repress miR-33a in the liver, the expression of the host gene of miR-33, srebp2, was also evaluated. Analyzing the percentage of decrease of srebp2 and miR-33 levels it seems that the mechanisms repressing miR-33 by DHA-OR or GSPE are not exactly the same for each treatment. The percentage of repression of srebp2 (55%) accounted for the percentage of repression of miR-33a (45%) with DHA-OR supplementation, hence indicating that DHA-OR repress miR-33a by repression of its host-gene. On the contrary, the percentage of repression of miR-33a (33%) was higher than the percentage of repression of srebp2 (20%) with GSPE supplementation, suggesting that the repression of srebp2 only partially contributed to miR-33a downregulation.

The effects of GSPE and DHA-OR on the levels of fas, pparβ/δ, cpt1a and abca1 mRNA in the liver were consistent with the repression of miR-33a and miR-122 that was induced by these treatments. The abca1 and cpt1a mRNA, which are repressed by miR-33a (53), were overexpressed with a synergistic effect in abca1 mRNA levels after GSPE and DHA-OR treatments, increasing abca1 expression when the two treatments were orally administered simultaneously. This synergistic effect on abca1 overexpression agrees with the increased ability of the combination of GSPE and DHA-OR to repress miR-33a expression over that of each treatment alone. The fas mRNA was repressed by GSPE and DHA-OR is consistent with the observation that mRNAs involved in lipogenesis tend to be downregulated when miR-122 is inhibited (10,54), whereas pparβ/δ mRNA levels were no modified significantly. Once again, there was a powerful effect on fas repression when the two treatments were orally

administered simultaneously, a situation that was more effective in repressing miR-122 in the liver than was administrating the compounds separately. Therefore, in the liver, there was a direct association between miR-33a and miR-122 levels and the expression of their target genes: abca1 and fas, respectively.

PBMCs are being used in gene expression studies because they can be easily collected from blood and can reflect the pathological and physiological state of an organism in a non-invasive way (35,36). Therefore, miR-33a and its target gene, abca1, were analyzed in PBMCs from rats, demonstrating the expression of miR-33a in PBMCs. Furthermore, in all studied groups of rats, miR-33a and abca1 expression in PBMCs reproduced the changes in expression in the liver. Therefore, PBMCs reflected the modifications in the liver that were induced by diet and treatments. Notably, the response of PBMCs to these nutritional interventions was magnified for miR-33a; the degree to which miR-33a was downregulated in PBMCs was always greater than in the liver. This increased response was also observed for abca1 expression in rats that were fed a CD. However, abca1 mRNA levels in PBMCs from rats in the treated groups were similar to or less than those observed in the liver. Our data give evidence that PBMCs can enhance both liver miR-33a and abca1 expression. Therefore, analyzing miR-33a in PBMCs could provide information about the state of miR-33a expression in the liver in a non-invasive way and therefore give information about the physiological state of the patient.

More studies are necessary to elucidate the exact mechanism by which GSPE and DHA-OR repress miR-122 and miR-33a. miR-122 is the most abundant miRNA in the liver and is expressed as a unique miRNA within a single transcript, *hcr* (54), whereas both miR-33a and b are intronic miRNAs located within Srebf2 and Srebf1, respectively (53). These loci encode the membrane-bound transcription factors SREBP1 and SREBP2, which activate FA synthesis and cholesterol synthesis and uptake (55). Therefore, it could be possible that proanthocyanidins and ω-3 PUFAs modulate miR-33a by controlling host gene expression.

In conclusion, the levels of miR-122 and miR-33a in the liver correlate with a state of lipemia in physiological rat models that were nutritionally induced by a cafeteria diet or a cafeteria diet plus hypolipidemic dietary compounds (i.e., GSPE and/or ω-3 PUFAs). Furthermore, we demonstrated that miR-33a is expressed in PBMCs and correlates well with miR-33a liver expression. Hence, the PBMCs could be used as a non-invasive diagnostic or therapeutic biomarker for the levels of liver miR-33.

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Table 1. Plasma lipids levels of rats fed with STD or CD with or without a GSPE and/or DHA-OR in chronic treatments.

Plasma parameters	STD	CD control	CD+GSPE	CD+DHA-OR	CD+GSPE+ DHA-OR
TG (mg/dL)	64.1±6.4a	104.3±12.7b	66.3±6.8a	84.1±10.5ab	66.7±6.6a
TC (mg/dL)	50.6±2.7a	72.4±1.9b	67.2±1.0b	51.5±2.3a	54.7±3.9a
HDL-C (mg/dL)	33.2±2.2	40.7±0.5	49.4±1.8	32.8±1.4	34.7±2.2
LDL-C (mg/dL)	5.5±0.3a	13.1±1.4b	5.5±1.1a	6.0±1.0a	6.9±1.5a
HDL-C/ LDL-C	6.2±0.6	3.9±0.5	8.0±1.4	6.6±1.2	3.9±0.8
TC/ HDL-C	1.5±0.0	1.6±0.1	1.5±0.1	1.5±0.0	1.6±0.1

Abbreviations: GSPE, grape seed proanthocyanidin extracts; CD, cafeteria-diet; STD, standard chow diet; DHA-OR, oil-rich in docosahexaenoic acid; TG, triacylglyceride; TC, total cholesterol. Rats were fed with STD (STD group) or with STD plus CD for 10 weeks. After 10 weeks, rats fed a STD plus a CD were orally treated with 25 mg GSPE/ kg bw (CD+GSPE), 515 mg PUFAs/ kg bw (CD+DHA-OR), 25 mg GSPE and 515 mg ω-3 PUFAs/kg bw (CD+GSPE+DHA-OR group) or vehicle (CD control group) for 3 weeks simultaneously with the CD. Each value is the mean ± s.e.m. of seven rats. Letters denotes a significant difference between groups ($p<0.05$; One-way ANOVA)

Table 2. Liver weight and liver lipids in rats fed a STD or CD with or without a GSPE and/or DHA-OR in chronic treatments

Liver parameters	STD	CD control	CD+GSPE	CD+DHA-OR	CD+GSPE+ DHA-OR
Liver weight (% bw)	3.01±0.01a	3.06±0.06ab	2.94±0.05a	3.23±0.04b	3.19±0.04b
Total lipids (g/100 g liver)	2.73±0.2a	6.94±0.5b	3.79±0.1c	7.00±0.8b	6.50±0.2b
TG (g/100 g liver)	0.44±0.03a	1.59±0.1b	0.85±0.08c	2.03±0.4bc	1.60±0.2bc
TC (g/100 g liver)	0,15±0.02a	0.69±0.1bc	0.31±0.02b	0.65±0.1bc	0.66±0.05c

Abbreviations: GSPE, grape seed proanthocyanidin extracts; CD, cafeteria-diet; STD, standard chow diet; DHA-OR, oil-rich in docosahexaenoic acid; TG, triacylglyceride; TC, total cholesterol. Experimental details and symbols as in Table 2.

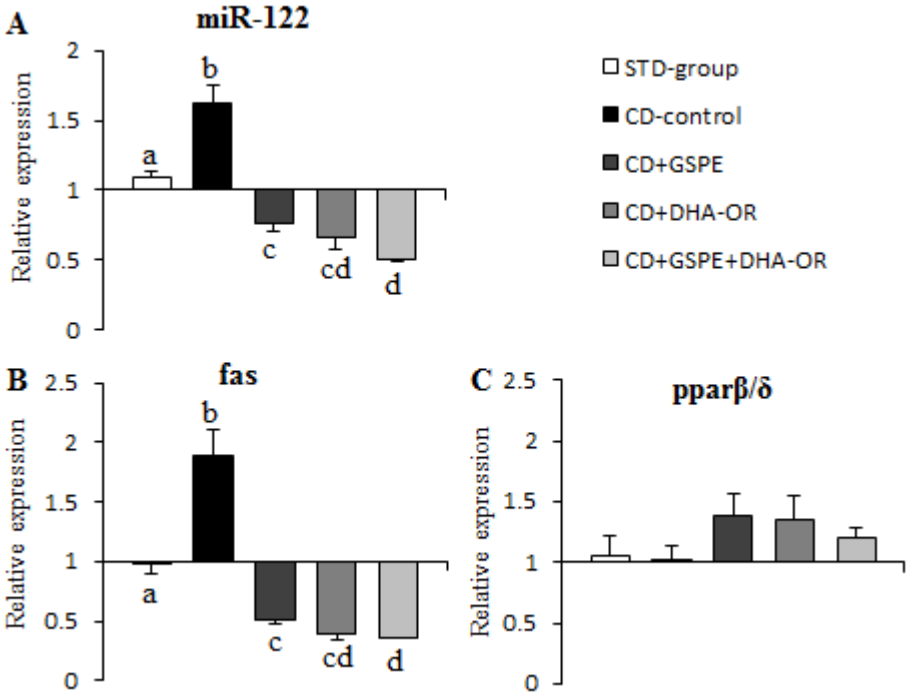
Figure legends

Figure 1. Liver miR-122 and their target mRNA levels.
Rats were fed a STD (STD group) or a STD plus CD for 10 weeks. After 10 weeks, rats fed a STD plus CD were orally treated with 25 mg GSPE/kg bw (CD-GSPE group), 515 mg ω-3 PUFAs/kg bw (CD- DHA-OR), 25 mg GSPE and 515 mg ω-3 PUFAs/kg bw (CD-GSPE- DHA-OR group) or vehicle (CD control group) for 3 weeks simultaneously with the CD. Each value is the mean ± s.e.m. of seven rats. Letters denotes a significant difference between groups ($p<0.05$; One-way ANOVA).

Figure 2. Liver srebp2 and miR-33a and their target mRNA levels.
Experimental details and symbols as in Figure 1.

Figure 3. miR-33a and abca1 mRNA levels in peripheral blood mononuclear cells (PBMCs)
Experimental details and symbols as in Figure 1.

Figure 1.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miR-122
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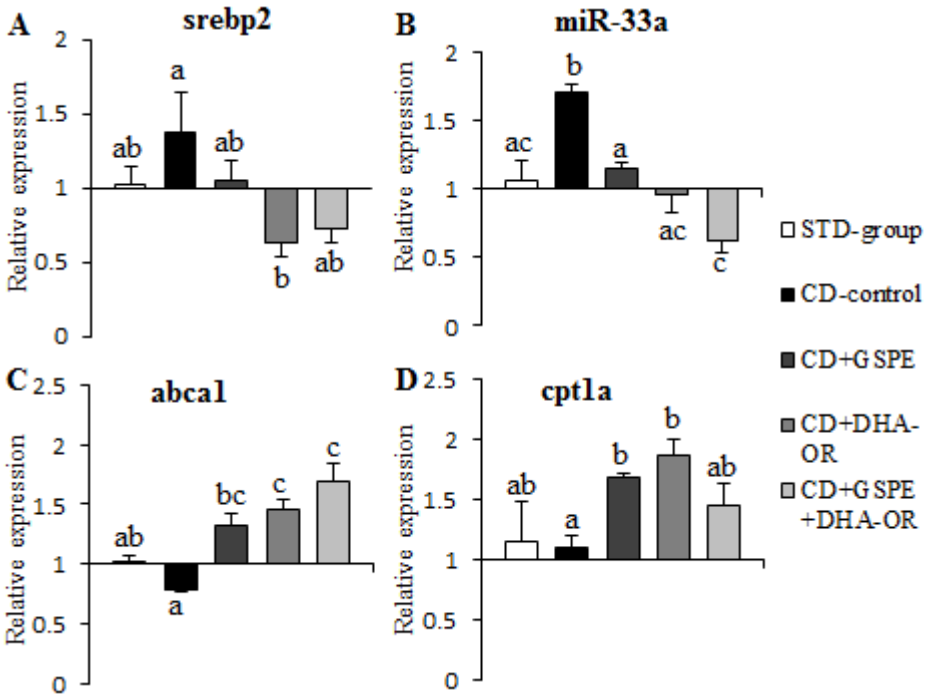
1.5
1
0.5
0
a
b
c
cd
d
STD-group
CD-control
CD+GSPE
CD+DHA-OR
CD+GSPE+DHA-OR
B
fas
Relative expression
2.5
2
1.5
1
0.5
0
a
b
c
cd
d
C
pparβ/δ
Relative expression
2.5
2
1.5
1
0.5
0

Figure 2.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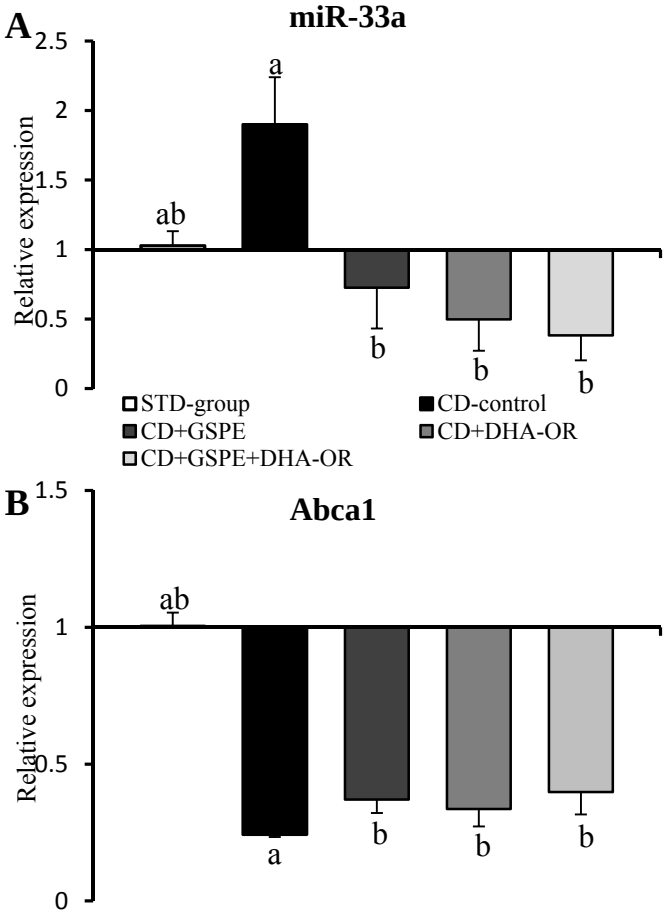
srebp2
B
miR-33a
C
abca1
D
cpt1a
Relative expression
STD-group
CD-control
CD+GSPE
CD+DHA-OR
CD+GSPE +DHA-OR

Figure 3.


A
miR-33a
Relative expression
2.5
2
1.5
1
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ab
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b
b
STD-group
CD-control
CD+GSPE
CD+DHA-OR
CD+GSPE+DHA-OR
B
Abca1
Relative expression
1.5
1
0.5
0
ab
a
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b

4. Effect of different classes of polyphenols on miR-33 and miR-122 expression in hepatic cells and potential mechanism of action (manuscript 6, submitted)

Resveratrol and EGCG directly bind to miR-33a and miR-122 and modulate their expression in hepatic cell lines.

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Abstract

Modulation of miR-33 and miR-122 has been proposed to be a promising strategy to treat dyslipidemia and insulin resistance associated with obesity and metabolic syndrome. Moreover, dietary polyphenols modulate the expression of these mi(cro)RNAs. The aim of this study was to elucidate the effect of polyphenols of different chemical structure on miR-33a and miR-122 expression in hepatic cells and to determine whether direct binding of the polyphenol to the mature miRNAs is a plausible mechanism of action. The effect of two grape proanthocyanidin extracts, their fractions, and pure polyphenol compounds on miRNA expression was evaluated using hepatic cell lines. The ability of resveratrol (RSV) and EGCG to bind miR-33a and miR-122 was measured using ^{1}H NMR spectroscopy. Extracts and pure polyphenols differentially modulate miRNA expression depending on the size of their components and the chemical structure of their monomeric constituents. RSV and EGCG were the most effective at modulating miRNAs with opposite effects. Specifically, RSV increased whereas EGCG decreased miRNA levels. These effects were consistent with the nature of the binding of these compounds to the miRNAs; although both compounds bound miR-33a and miR-122, RSV bound the miRNAs more strongly and through a different type of interaction than EGCG.

Key words: Fao cells, GSPE, HepG2 cells, lipid metabolism, microRNAs, polyphenols, proanthocyanidins, ABCA1, FAS.

Introduction

Mi(cro)RNAs are short (22 nt) double-stranded regulatory noncoding RNAs and have emerged as critical regulators of gene expression at the posttranscriptional level [1, 2]. To date, thousands of miRNAs have been discovered, and it is thought that these small molecules may regulate more than 60% of all gene transcripts [3]. Specifically, miR-33a/b [4, 5] and miR-122 [4] have emerged as key regulators of genes involved in lipid metabolism in liver, and they have been implicated in metabolic diseases such obesity and metabolic syndrome. miR-122 is liver specific and plays a critical role in liver homeostasis [6, 7], and its inhibition has been associated with the deregulation of genes playing key roles in the control of liver lipid metabolism, such as fatty acid synthase (FAS). miR-33a/b plays an important role in the regulation of cholesterol homeostasis, regulating the ATP-binding cassette transporters (ABC transporters) ABCA1 and ABCG1, in addition to its role in fatty acid β-oxidation [8]. miR-33a and b are intronic of the sterol response element protein 2 (SREBF2) and 1 (SREBF1) genes, respectively, and they are simultaneously cotranscribed [9]. Increasing evidence indicates that deregulation of these miRNAs is related to metabolic diseases. Moreover, the modulation of miR-33 and miR-122 has been proposed to be a promising strategy to treat dyslipidemia and insulin resistance associated with obesity and metabolic syndrome [10]. Our research group has demonstrated that dietary polyphenol extracts can modulate the expression of these miRNAs [11].

The basic structural skeleton of flavonoids contains 12 carbons composing two aromatic rings connected by a pyrone ring. Flavonoids are divided into 6 subclasses depending on the oxidation state of the pyrone ring and the connection of one aromatic ring with the pyrone ring: flavonols (quercetin, Q), flavanones, flavones, anthocyanidins, isoflavones and flavanols. Flavanols, such as epicatechin (EC), catechin (C) and epigallocatechin (EGG) can exist in a variety of oligomeric structures that differ in chain lengths, hydroxylation pattern, stereochemistries, interflavan linkages and whether they are esterified with gallic acid (GA) [12]. Stilbenoids represent another type of polyphenols and include resveratrol (RSV), which is produced *de novo* by plants in response to stress factors, such as pathogen attack. The basic structural skeleton of RSV features a central carbon–carbon double bond conjugated with two phenolic moieties [13].

The effect of each individual polyphenol and the exact mechanism by which these dietary compounds modulate miRNA expression remains unclear. Hence, the aim of this study was to elucidate the influence of the polyphenolic chemical structure on miR-33a and miR-122 expression in hepatic cells and to determine whether polyphenols can directly bind to mature miRNAs.

Results

Grape skin, seeds and stems contain higher polyphenolic content than grape seeds alone.

Two different grape proanthocyanidin extracts (grape seed proanthocyanidin extract, GSPE; and grape pomace extract, GPE) and their monomeric (MF), oligomeric (OPA) and condensed tannin (CT) fractions were used to evaluate their effect on miR-33a and miR-122 expression. GSPE is a proanthocyanidin-rich extract from grape seeds, whereas GPE was obtained from skins, seeds and a small amount of stems. The extracts and fractions were characterized using reversed-phase HPLC-MS (Table 1). The results indicated that GPE is richer than GSPE in nearly all of the analyzed phenolic compounds. For example, GPE contains more monomeric forms, such as approximately 46% more C and 10% more EC, than GSPE; GPE also contains 30% more gallate dimer. Regarding other types of flavonols, such as Q, GPE contains approximately 100% more Q than GSPE; GA and gallate forms of flavanols, such as epigallocatechin gallate (EGCG), are also present in GPE at levels that are 40 and 60% higher, respectively, than those in GSPE; GPE also contains RSV, whereas GSPE does not. Conversely, GSPE is richer in vanillic acid (VA), containing 85% more VA than GPE; GSPE is 16% richer in procyanidin B2 (B2) and 68% richer in epicatechin gallate. Moreover, the MF is rich in the monomeric flavanols C and EC; the OPA fraction is rich in dimeric and trimeric procyanidins; and the CT fraction is rich in condensed tannins with a polymerization degree greater than 4.

The different composition of grape extracts and their fractions differentially influence miR-33a and miR-122 expression in hepatic cells.

The effect of GSPE, GPE and their MF, OPA and CT fractions on miR-33a and miR-122 expression in rat (Fao cells; Figs. 1A and 1B) and human (HepG2 cells; Figs. 2A and 2B) hepatic cancer cell lines was investigated after 1 h of treatment. The effects on miR-122 and miR-33a exerted by the two extracts were similar in Fao and HepG2 cells. However, although GSPE reduced both miR-122 and miR-33a levels, GPE reduced miR-122 but increased miR-33a expression. The expression of miR-122 and miR-33a was not altered by the OPA fraction in either Fao or HepG2 cells. Conversely, the CT fraction displayed a similar effect as GPE, decreasing miR-122 and increasing miR-33a levels in both cell lines. Although the OPA and CT fractions demonstrated a similar effect on miR-33a and miR-122 expression in Fao and HepG2 cells, the MF fraction demonstrated a different effect on miR-122 expression, which depended on the cell type. Hence, the MF fraction reduced miR-122 levels in HepG2 cells, whereas the expression in Fao cells was not altered. Nevertheless, miR-33a expression was increased by the MF in both cell lines. These results demonstrated that the different composition of the grape extracts differentially influence miR-33a and

miR-122 expression in hepatic cells, indicating that the molecular structure of each polyphenol demonstrates different effects on the expression of these miRNAs.

Flavonoids repressed and RSV increased miR-33a and miR-122 expression in hepatic cells.

To elucidate which polyphenol or class of polyphenol from the extracts is primarily responsible for modulating miR-33a and miR-122 expression, Fao and HepG2 cells were treated with different phenolic compounds present in GSPE and/or GPE for 1 h (Figs. 1C and 1D and Figs. 2C and 2D). The flavanol C and the flavonol Q were evaluated as two compounds present in GPE at higher levels than in GSPE, whereas the flavanols EC, EGCG and B2 were evaluated as three different compounds present in both GSPE and GPE at a similar concentration. Additionally, the stilbene RSV was evaluated as a compound only present in GPE and not in GSPE. All compounds demonstrate a similar effect on Fao and HepG2 cell lines, demonstrating an identical tendency in modulating these two miRNAs. Regarding the four studied flavanols, although C did not demonstrate any effect on miR-33a and miR-122 expression, EC and EGCG decreased miR-33a and miR-122 levels. Finally, B2 also decreased miR-122 expression but did not alter miR-33a expression. Similarly, Q repressed miR-33a expression but did not affect miR-122 expression. Interestingly, RSV increased the levels of these two miRNAs in contrast to all of the other evaluated compounds. In summary, all evaluated flavonoids, with the exception of C, repressed miR-33a and miR-122 expression in a similar fashion as GSPE. In contrast, the stilbene RSV, which is only present in GPE, increased the expression of these two miRNAs, which is more similar to the upregulation of miR-33a expression by GPE.

Microbial metabolites of polyphenolic extracts demonstrated a different modulation of miR-33a and miR-122 expression in Fao cells compared with HepG2 cells.

We analyzed four different phenolic acids as colonic microbial metabolites of proanthocyanidins. For this purpose, the effect of GA, VA, 3-hydroxyphenylacetic acid (m-HPAA) and 3-hydroxybenzoic acid (HBA) was investigated in Fao and HepG2 cells after 1 h of treatment (Figs. 1E and 1F; Figs. 2E and 2F). In contrast to the nonmetabolized compounds, the microbial metabolite phenolic acids demonstrated a different effect on Fao cells compared with HepG2 cells. All compounds repressed miR-122 expression in HepG2 cells, whereas no effect was observed in Fao cells. In contrast, miR-33a expression was not affected by the metabolites in HepG2 cells, whereas m-HPAA and GA downregulated miR-33a expression in Fao cells. Therefore, it appears that the HepG2 cells are more sensitive to microbial metabolites than Fao cells.

RSV, EGCG, Q and GA correspondingly modulated ABCA1 and FAS mRNA and protein levels in HepG2 cells with the modulation of miR-33a and miR-122, respectively.

Of the phenolic compounds that affected miR-33a and miR-122 expression, we selected RSV, EGCG, Q and GA as representative of each class of polyphenol. Moreover, each of these selected compounds demonstrated a different effect on miRNA expression. Of the selected compounds, we analyzed whether the target genes of miR-33a and miR-122, ABCA1 and FAS, respectively, were also modulated by treating HepG2 cells with these compounds for 1 h for mRNA analyses and 5 h to determine protein levels (Figs. 3 and 4). The results indicated that RSV, which increased miR-33a and miR-122 expression, did not alter ABCA1 mRNA levels but significantly decreased ABCA1 protein levels at 5 h. Consistent with increased miR-122 expression, RSV treatment produced an increase in FAS mRNA and protein levels. Conversely, EGCG treatment, which decreased miR-33a and miR-122 levels, increased ABCA1 mRNA and protein levels. However, the level of FAS mRNA was not altered by EGCG, which only slightly decreased FAS protein levels, although this was not significant. Treatment with Q, which only decreased miR-33a expression, increased ABCA1 mRNA and protein levels without any modification of FAS mRNA and protein levels. In contrast, GA treatment, which decreased miR-122 expression, displayed a decrease in FAS mRNA, with no significant modification in FAS protein levels. Therefore, flavonoids, RSV and phenolic acids can correspondingly modulate the target genes ABCA1 and FAS with their modulation of miR-33a and miR-122 expression, respectively, in HepG2 cells.

Polyphenols modulate miR-33a levels directly without deregulation of the SREBP2 host gene.

To establish the mechanism by which polyphenols modulate miRNA levels, we studied two potential approaches. First, we evaluated whether miR-33a is indirectly modulated by the deregulation of its host gene SREBP2 because they are coexpressed. For this purpose, we studied the expression of SREBP2 in HepG2 cells after 1-h treatment with RSV, EGCG, Q and GA. The results indicated that the mRNA levels of SREBP2 were not modified by any of these compounds (Fig. 5).

RSV and EGCG directly bind to miR-33a and miR-122 as evidenced by ^{1}H NMR analysis

The second approach to elucidate the mechanism of action of polyphenols was to evaluate whether polyphenols can directly and specifically bind to miRNAs. ^{1}H NMR spectroscopy is a useful technique to probe the interactions of polyphenols with miRNAs. As RSV and EGCG demonstrated major and opposite effects on the modulation of the miRNAs, we selected these compounds to characterize their binding

to the miRNAs. For this purpose, a ^{1}H NMR titration experiment was performed with an increasing concentration of either RSV or EGCG in the presence of each miRNA. The results exhibited ^{1}H chemical shift displacements ($\Delta\delta Hz=\delta_{free}-\delta_{complex}$) and broad signals for most of the protons of both RSV and EGCG in solution with miR-33a or miR-122 and thus indicate a direct interaction of RSV and EGCG with both miR-33a and miR-122 (Table 2). Interestingly, when either of these miRNAs was present in solution with RSV, all of the protons were deshielded, whereas with EGCG, the protons were shielded (Fig. 6). Furthermore, the chemical shift displacements were larger in the presence of RSV than in the presence of EGCG. Specifically, when RSV was present in solution with either miR-33a or miR-122, the deshielding effect and broad signals were similar for both miRNAs. At a 0.3:1 RSV:miRNA-33a ratio, all RSV protons were significantly deshielded, whereas at a 0.6:1 ratio, the signals were not further displaced downfield. Interestingly, at a 1:1 ratio, the protons were displaced upfield compared with those in the spectrum for a 0.6:1 ratio and resembled the free RSV spectrum. The most deshielded protons were H2, H6 and H4. When EGCG was present in solution with miR-122, the chemical shift displacement upfield was larger than with miR-33a. Furthermore, upon increasing concentrations of EGCG relative to miR-33a, a larger shielding effect was observed, whereas with miR-122 at a 0.3:1 EGCG:miR-122 ratio, the shielding effect was identical to that observed for a 1:1 ratio (Table 2). Collectively, these results indicate a stronger interaction of EGCG with miR-122 than with miR-33a. In contrast to RSV, all of the protons of EGCG demonstrated a similar chemical shift displacement and broad signals.

Discussion

Polyphenols and polyphenol-rich extracts have been found to modulate miRNA expression [11, 14]. Furthermore, some of the miRNAs whose expression was deregulated by these dietary compounds serve important functions in regulating metabolism (reviewed in [15]). Because plant extracts are complex mixtures of different polyphenolic compounds of different chemical structures, which range from monomers to oligomeric forms, we attempted to elucidate whether specific compounds in the extracts were able to deregulate specific miRNAs and/or a synergistic effect of different compounds is necessary. For this purpose, we studied the expression of miR-33a and miR-122 in HepG2 and Fao cells after treatment with two grape proanthocyanidin extracts of different polyphenolic composition. We demonstrated that GSPE or GPE induced a different modulation of miR-33a and miR122 expression, most likely due to their different composition of polyphenols. Furthermore, when cells were treated with MF, OPA and CT fractions, miRNAs were differentially deregulated, hence, indicating that the polyphenol size differentially influenced the expression of the miRNAs. Moreover, to elucidate whether the

chemical structure of the small constituent moieties also differentially influenced the expression of these miRNAs, we treated HepG2 and Fao cells with a range of pure polyphenolic compounds. Although different types of flavonoids repressed or did not affect miR-33a and miR-122 expression, RSV increased the expression of the miRNAs in hepatic cells. Flavonoids consist of two aromatic rings connected to a pyrone ring, which can be esterified with a GA or other multiple substitutions generating different classes of flavonoids. In contrast, RSV features a central carbon–carbon double bond conjugated with two phenolic moieties. Hence, it could be suggested that the chemical structure of the polyphenols influence the modulation of miR-33a and miR-122 expression in hepatic cells. As RSV was present in GPE but not in GSPE, it might be hypothesized that the upregulation of these two miRNAs by GPE may be due to the presence of RSV. However, the MF and CT fractions, which did not contain RSV, also increased miR-33 expression, which thus indicates that the combination of different polyphenols in the extract produced a synergistic effect.

After absorption, dietary polyphenols undergo phase II enzymatic conjugations in the small intestine and/or in the liver [16]. Furthermore, most dietary polyphenols can reach the colon in which the enzymes produced by the gut bacteria can convert them into different low molecular weight metabolites such as phenolic acids that later can be absorbed *in situ* [17]. Therefore, we analyzed four different phenolic acids, GA, VA, m-HPAA and HBA, as colonic microbial metabolites of proanthocyanidins. Although VA and GA are products of proanthocyanidin metabolism, these compounds can also be present in proanthocyanidin extracts, as is the case in GSPE and GPE. In contrast to nonmetabolized compounds, which demonstrated an identical tendency to modify the miRNA levels in HepG2 cells compared with Fao cells, a different effect of microbial metabolites was observed in Fao cells compared with HepG2 cells. Thus, it appears that the HepG2 cells are more sensitive to microbial metabolites than Fao cells. Reinforcing our results, Fao cells were found to be more sensitive than HepG2 cells to an inhibitory effect of RSV on the proliferation of hepatic cell lines [18].

We recently established a new mechanism by which GSPE exerts its hypolipidemic effect by reducing TG and TC, downregulating miR-33a and miR-122 and correspondingly increasing the miR-33a target gene ABCA1 and repressing the miR-122 indirect target gene FAS in the liver [11]. In this study, we demonstrated that most classes of polyphenols also correspondingly modify ABCA1 and FAS mRNA and protein levels in HepG2 cells with modulation of miR-33a and miR-122 levels, respectively. Therefore, these results indicate that polyphenols can alter lipid metabolism by miRNA modulation.

Currently, the molecular mechanism by which polyphenols modulate miRNA levels is unknown. However, there is evidence that polyphenols can bind to mRNAs and proteins [19, 20]. Therefore, it is possible that polyphenols also bind to miRNAs or to

some component involved in miRNA biogenesis, such as Dicer or the RNA-induced silencing complex. Additionally, some miRNAs are intronic of genes, and polyphenols that modify host gene expression would also affect the miRNA levels. Herein, we demonstrated that polyphenols modify miR-33a expression without altering the levels of its host gene SREBP2, although they are simultaneously coexpressed. We also provide evidence that RSV and EGCG directly bind to miR-33a and miR-122 using ^{1}H NMR spectroscopic studies and that they differ in their binding and modulation of miRNA expression in hepatic cells. Specifically, RSV binds equally to miR-33a and miR-122 primarily through an A ring interaction, whereas EGCG binds more strongly to miR-122 than to miR-33a through an interaction with all of the rings in the molecule. Moreover, RSV binds more strongly to these miRNAs than EGCG and through a different type of interaction because the protons in RSV were deshielded, whereas the protons in EGCG were shielded. These results are consistent with the opposing effects of RSV and EGCG on miRNA modulation.

In conclusion, the binding of polyphenols to miRNAs is very specific, and the molecular structure of the polyphenol will determine the nature of the binding to the specific miRNA. Therefore, these results suggest that the interaction of polyphenols with miRNAs influences the polyphenol functionality by altering the binding of the miRNAs to the seed sequence of their target genes.

Materials and methods

Grape proanthocyanidin extracts and pure compounds

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France), and its composition is described in Table 1. GPE and the MF, OPA and CT fractions were kindly provided by Miguel Torres S.A. (Vilafranca del Penedès, Spain), and their compositions are also described in Table 1. (+)-C, (-)-EC, Q dehydrate, RSV, EGCG, B2, GA, VA, m-HPAA and HBA were purchased from Sigma-Aldrich (Madrid, Spain). Organic solvents (high-performance liquid chromatography grade) were obtained from Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany).

Analysis of phenolic compounds and derivatives by reversed-phase chromatography coupled to mass spectrometry

Major phenolic compounds of the studied extracts were quantified by HPLC-MS following the method described by Quiñones et al [21]. For further details, see the supplementary data and Table S1.

Cells and cell culture

Fao cells, which are a rat hepatoma cell line (ECACC, code 85061112), were cultured as described by *Baselga-Escudero et al* [11]. The culture conditions for HepG2 cells are specified in the supplementary material.

RNA extraction from Fao and HepG2 cells

Total RNA containing small RNA species was extracted from Fao and HepG2 cells using the mi/mRNA extraction kit (E.Z.N.A. miRNA kit, Omega Bio-Tek, Norcross, Georgia, U.S.A.) following the procedure described by *Baselga-Escudero et al* [11].

Quantification of microRNA using real-time RT-PCR

To quantify miR-122 and miR-33a expression, we followed the procedure described by *Baselga-Escudero et al* [11].

Quantification of mRNA using real-time qRT-PCR

mRNA levels were evaluated following the procedure described by *Baselga-Escudero et al* [11].

Western blot analysis

Western blot analysis was performed according to the procedure described by *Baselga-Escudero et al* [11].

^{1}H NMR

NMR measurements are described in the supplementary material.

Statistical analysis

The results are reported as the mean ± S.E.M. of three independent experiments for the *in vitro* gene expression analyses. Group means were compared with Student's t-test for independent samples ($p \leq 0.05$) using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

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Table 1. Identified phenolic compounds and derivatives determined by reversed-phase HPLC-MS. Values are expressed as mg compound/g extract.

Phenolic compound	**GSPE**	**GPE**	**MF**	**OPA**	**CT***
Gallic acid	15.1 ± 3.5	24.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Vanillic acid	0.2 ± 0.05	0.03 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Procyanidin dimer[a]	82.2 ± 13.1	81.1 ± 5.5	5.0 ± 0.5	406.6 ± 50.9	0.0 ± 0.0
**Dimer B2	30.8 ± 8.3	25.8 ± 3.0	2.1 ± 0.5	133.5 ± 24.9	0.0 ± 0.0
Catechin	51.8 ± 14.9	97.5 ± 28.7	746.5 ± 30.1	10.4 ± 0.1	0.7 ± 0.02
Epicatechin	33.6 ± 12.7	37.7 ± 7.1	234.2 ± 5.4	4.2 ± 0.1	0.2 ± 0.0
Gallate dimer[a]	23.9 ± 7.4	34.5 ± 2.0	0.0 ± 0.0	107.7 ± 8.6	8.9 ± 2.4
Epigallocate-chin gallate	0.1 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0
Trimer C1	4.4 ± 1.0	4.8 ± 0.4	0.0 ± 0.0	19.4 ± 7.9	0.5 ± 0.1
Epicatechin gallate[b]	10.3 ± 5.5	3.3 ± 2.9	0.0 ± 0.0	17.2 ± 8.1	0.0 ± 0.0
Quercetin	0.2 ± 0.0	5.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Resveratrol	0.0 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

* Fraction enriched in procyanidin oligomers with a mean degree of polymerization of 3.7, mean molecular weight of 1232 and percentage galloylation of 31%, as estimated by thioacidolysis and HPLC and described by *Torres et al* (22).

[a] Quantified using the calibration curve of procyanidin B2.

[b] Quantified using the calibration curve of epigallocatechin gallate.

Table 2. ^{1}H chemical shifts of RSV and EGCG in the presence and absence of miR-33a and miR-122.

		Polyphenol:miR-33a			**Polyphenol:miR-122**		
RSV protons	**δ (ppm) free**	**Δδ (Hz) 0.3:1**	**Δδ (Hz) 0.6:1**	**Δδ (Hz) 1:1**	**Δδ (Hz) 0.3:1**	**Δδ (Hz) 0.6:1**	**Δδ (Hz) 1:1**
2', 6'	7.29	-36.01	-36.01	-18.00	-36.01	-36.01	-24.00
α'	6.95	-30.01	-30.01	-18.00	-24.00	-24.00	-12.00
α	6.73	-54.01	-54.01	-30.01	-54.01	-54.01	-30.01
3', 5'	6.60	-90.03	-90.03	-60.02	-90.03	-90.03	-66.02
2, 6	6.30	-114.0	-114.03	-72.02	-114.0	-114.0	-78.02
4	5.98	-108.0	-108.03	-66.02	-114.0	-114.0	-72.02
EGCG protons							
2'', 6''	6.85	6.00	12.00	18.00	48.01	48.01	48.01
2', 6'	6.46	0	6.00	6.00	42.01	42.01	42.01
8	6.03	6.00	12.00	18.00	36.01	36.01	36.01
6	5.99	0	6.00	18.00	42.01	42.01	42.01
3'	5.47	12.00	18.00	24.00	-42.01	-42.01	-42.01
2	5.02	6.00	12.00	18.00	n. d.	n. d.	n. d.

$\Delta\delta Hz=\delta_{free}-\delta_{complex}$. The concentration of the miRNAs was 50 μM, and the concentrations of the polyphenols were 15 μM, 30 μM and 50 μM to obtain polyphenol:miRNA ratios of 0.3:1; 0.6:1; and 1:1, respectively.

Abbreviations: EGCG, epigallocatechin gallate; and RSV, resveratrol.

Figure legends

Figure 1. Effect of polyphenolic extracts, pure compounds and microbial metabolites on the levels of miR-33a and miR-122 in rat hepatoma Fao cells.

Fao cells were treated with 25 mg/L of polyphenolic extracts and fractions (A, B), 50 μM of pure compounds (C, D) and 50 μM of microbial metabolites (E, F) for 1 h. miRNA levels were determined by qRT-PCR and normalized to U6 snRNA levels. All values represent the mean of three independent experiments. White bars represent the control group (CNT), and black bars represent the treated group. An * denotes a significant difference between control cells and treated cells ($p<0.05$; Student's t-test).

Abbreviations: CNT, control cells; GSPE, grape seed proanthocyanidin extract; GPE, grape pomace extract; MF, monomeric fraction; OPA, oligomeric proanthocyanidins; CT, condensed tannins; Q, quercetin; C, catechin; EC, epicatechin; RSV, resveratrol; EGCG, epigallocatechin gallate; B2, dimer B2; HBA, 3'-hydroxybenzoic acid; m-HPAA, m-hydroxyphenylacetic acid; VA, vanillic acid; and GA, gallic acid.

Figure 2. Effect of polyphenolic extracts, pure compounds and microbial metabolites on the levels of miR-33a and miR-122 in human hepatoma HepG2 cells.

HepG2 cells were treated with 25 mg/L of polyphenolic extracts and fractions (A, B), 50 μM of pure compounds (C, D) and 50 μM of microbial metabolites (E, F) for 1 h. Experimental details, symbols and abbreviations are as in Figure 1.

Figure 3. Effect of RSV, EGCG, Q and GA on the mRNA and protein levels of FAS in HepG2 cells.

HepG2 cells were treated with 50 μM of RSV (Fig. 3A), EGCG (Fig. 3B), Q (Fig. 3C) and GA (Fig. 3D) for 1 h to analyze FAS mRNA levels and for 5 h to analyze FAS protein levels. mRNAs levels were determined by qRT-PCR and normalized to PPIA mRNA levels. Proteins were extracted with radio-immunoprecipitation (RIPA) buffer and analyzed by Western blot. Protein levels were normalized to an endogenous protein, Hsp90. Relative intensity units were obtained by dividing the intensity of the band of the target protein by that of the endogenous protein. All of the values represent the means of two independent experiments. An * denotes a significant difference between control cells and treated cells ($p<0.05$; Student's t-test).

Abbreviations: EGCG, epigallocatechin gallate; GA, gallic acid; Q, quercetin; and RSV, resveratrol.

Figure 4. Effect of RSV, EGCG, Q and GA on the mRNA and protein levels of ABCA1 in HepG2 cells.

HepG2 cells were treated with 50 μM of RSV (Fig. 4A), EGCG (Fig. 4B), Q (Fig. 4C) and GA (Fig. 4D) for 1 h to analyze ABCA1 mRNA levels and for 5 h to analyze ABCA1 protein levels. Experimental details, symbols and abbreviations are as in Figure 3.

Figure 5. SREBP2 mRNA levels after 1h RSV, EGCG, Q and GA treatment in HepG2 cells.

HepG2 cells were treated with 50 μM of RSV, EGCG, Q and GA for 1h to analyze mRNA levels of SREBP2. mRNAs levels were determined by RTqPCR and normalized to PPIA mRNA levels. All the values are the means of three independent experiments. *denotes significant difference between control cells and treated cells ($p<0.05$; Student's t test).

Abbreviations: RSV, resveratrol; EGCG, epigallocatechin gallate; Q, quercetin; GA, gallic acid.

Figure 6. ^{1}H NMR spectra of RSV and EGCG in the presence of miR-33a and miR-122.

A) The chemical structure of RSV. B) ^{1}H NMR spectra of RSV alone or in solution with mature miR-33a. C) ^{1}H NMR spectra of RSV alone or in solution with mature miR-122. D) The chemical structure of EGCG. E) ^{1}H NMR spectra of EGCG alone or in solution with mature miR-33a. F) ^{1}H NMR spectra of EGCG alone or in solution with mature miR-122. The miRNA concentration was 50 μM, and the concentrations of the polyphenols were 15 μM, 30 μM and 50 μM to obtain the polyphenol:miRNA ratios of 0.3:1; 0.6:1; and 1:1, respectively.

Abbreviations: EGCG, epigallocatechin gallate; and RSV, resveratrol.

Figure 1

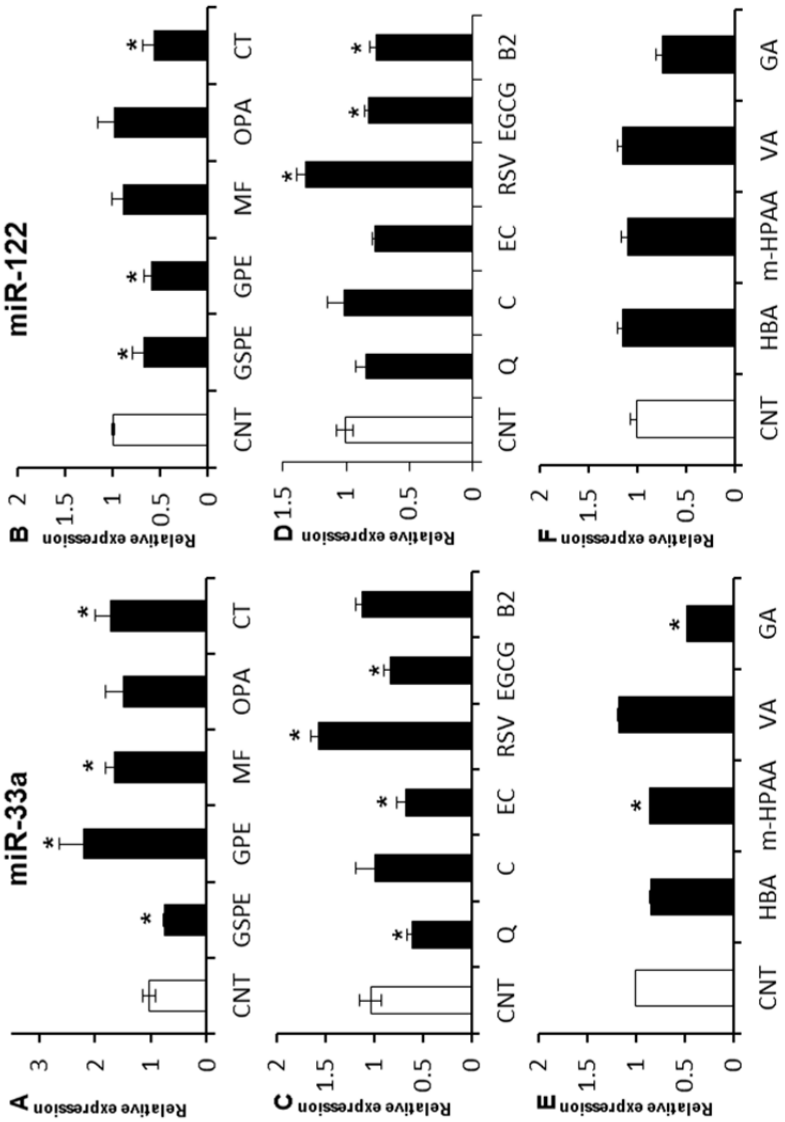

miR-33a
miR-122
A
B
C
D
E
F
Relative expression
CNT
GSPE
GPE
MF
OPA
CT
Q
C
EC
RSV
EGCG
B2
HBA
m-HPAA
VA
GA

Figure 2

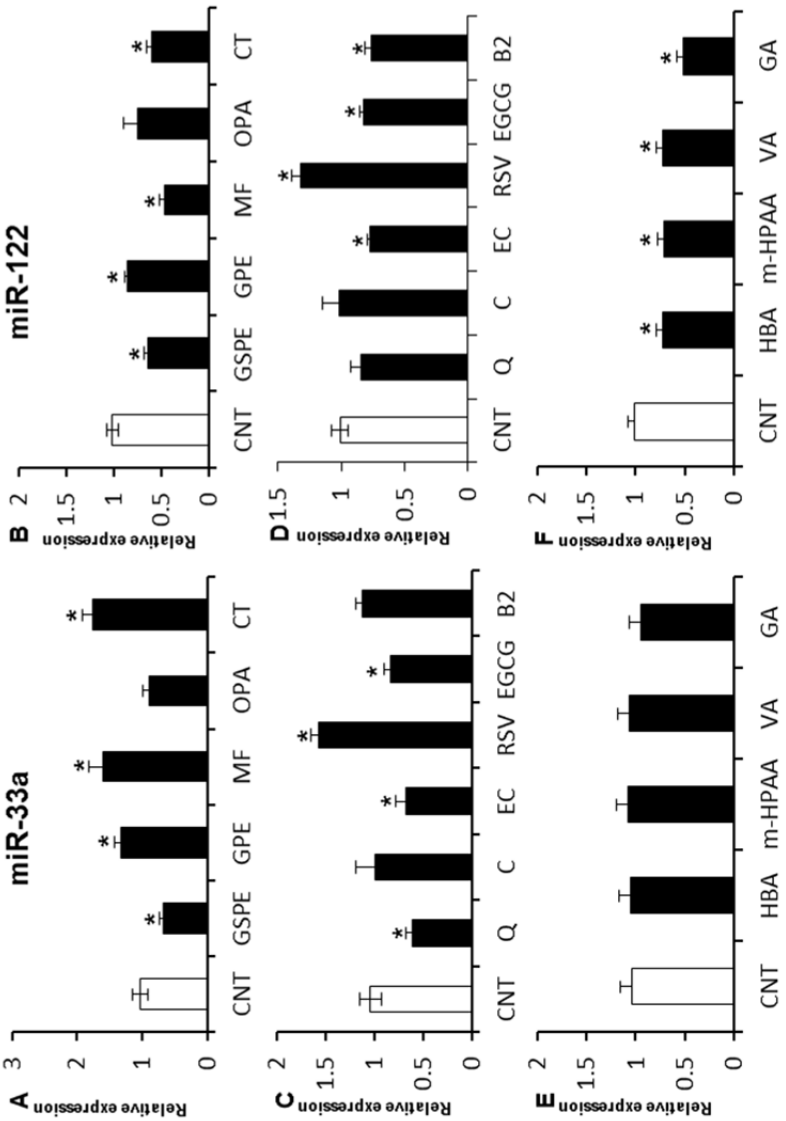

miR-33a
miR-122
A
B
C
D
E
F
Relative expression
CNT
GSPE
GPE
MF
OPA
CT
Q
C
EC
RSV
EGCG
B2
HBA
m-HPAA
VA
GA

Figure 3

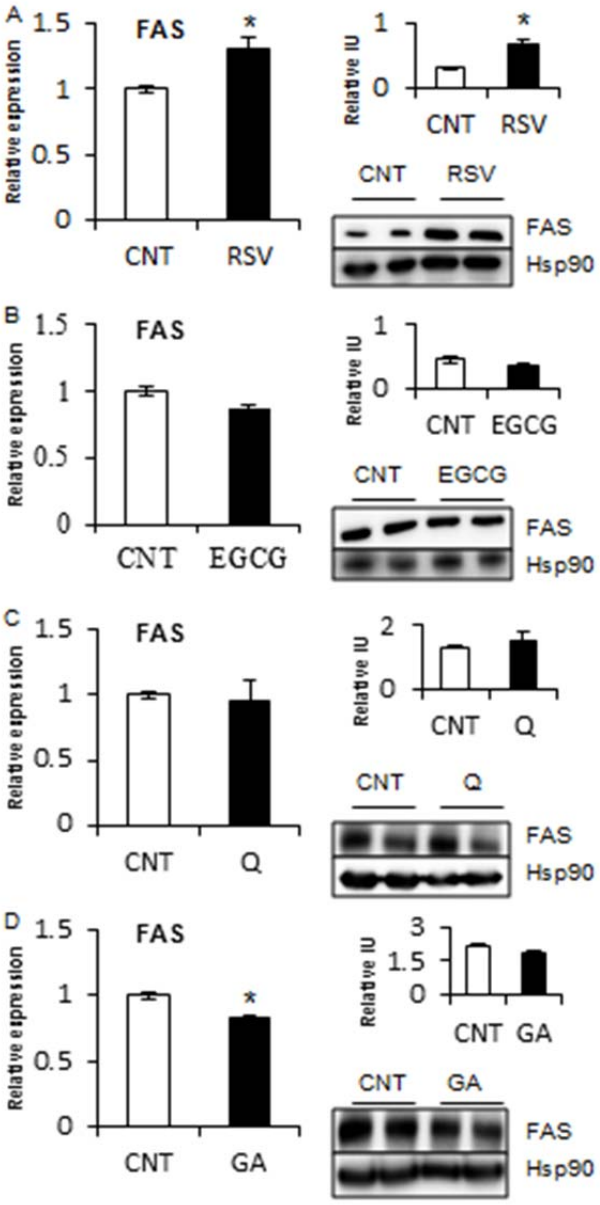

A
FAS
Relative expression
1.5
1
0.5
0
CNT
RSV
*
Relative IU
1
0
CNT
RSV
*
CNT
RSV
FAS
Hsp90
B
FAS
Relative expression
1.5
1
0.5
0
CNT
EGCG
Relative IU
1
0
CNT
EGCG
CNT
EGCG
FAS
Hsp90
C
FAS
Relative expression
1.5
1
0.5
0
CNT
Q
Relative IU
2
0
CNT
Q
CNT
Q
FAS
Hsp90
D
FAS
Relative expression
1.5
1
0.5
0
CNT
GA
*
Relative IU
3
1.5
0
CNT
GA
CNT
GA
FAS
Hsp90

Figure 4

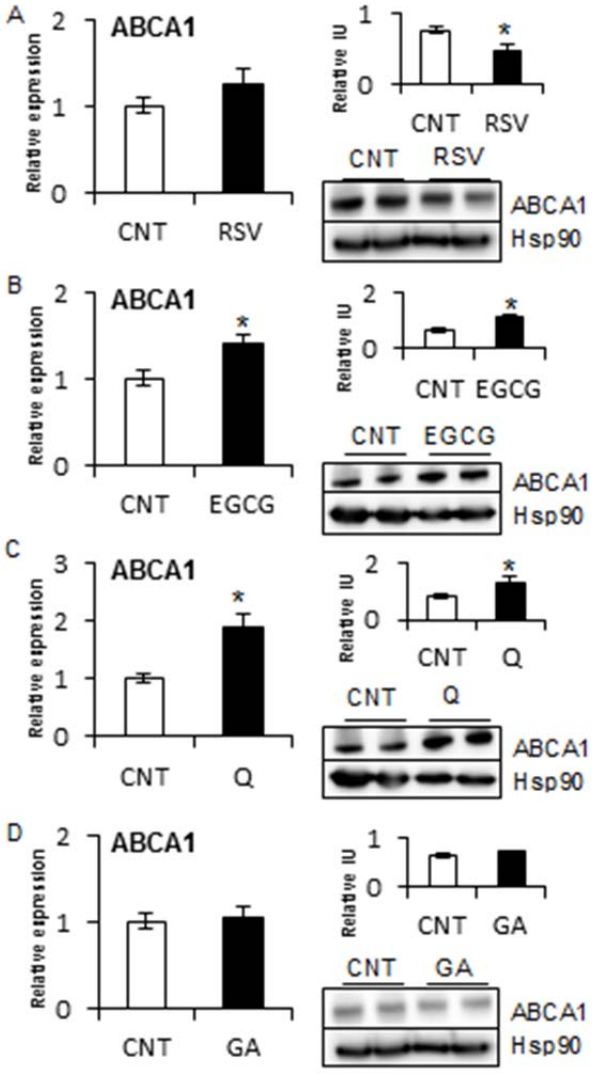

A
ABCA1
Relative expression
CNT
RSV
Relative IU
CNT
RSV
ABCA1
Hsp90
B
ABCA1
Relative expression
CNT
EGCG
Relative IU
CNT
EGCG
ABCA1
Hsp90
C
ABCA1
Relative expression
CNT
Q
Relative IU
CNT
Q
ABCA1
Hsp90
D
ABCA1
Relative expression
CNT
GA
Relative IU
CNT
GA
ABCA1
Hsp90

Figure 5.

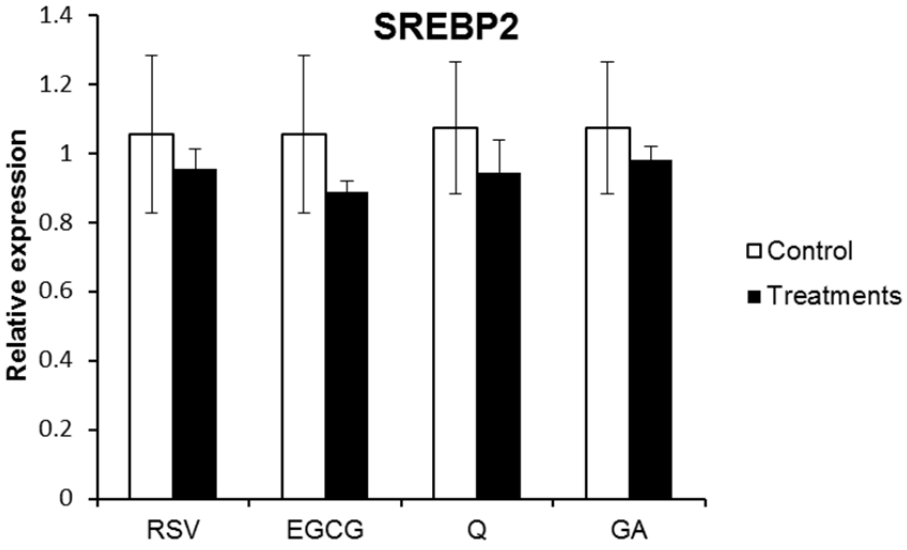
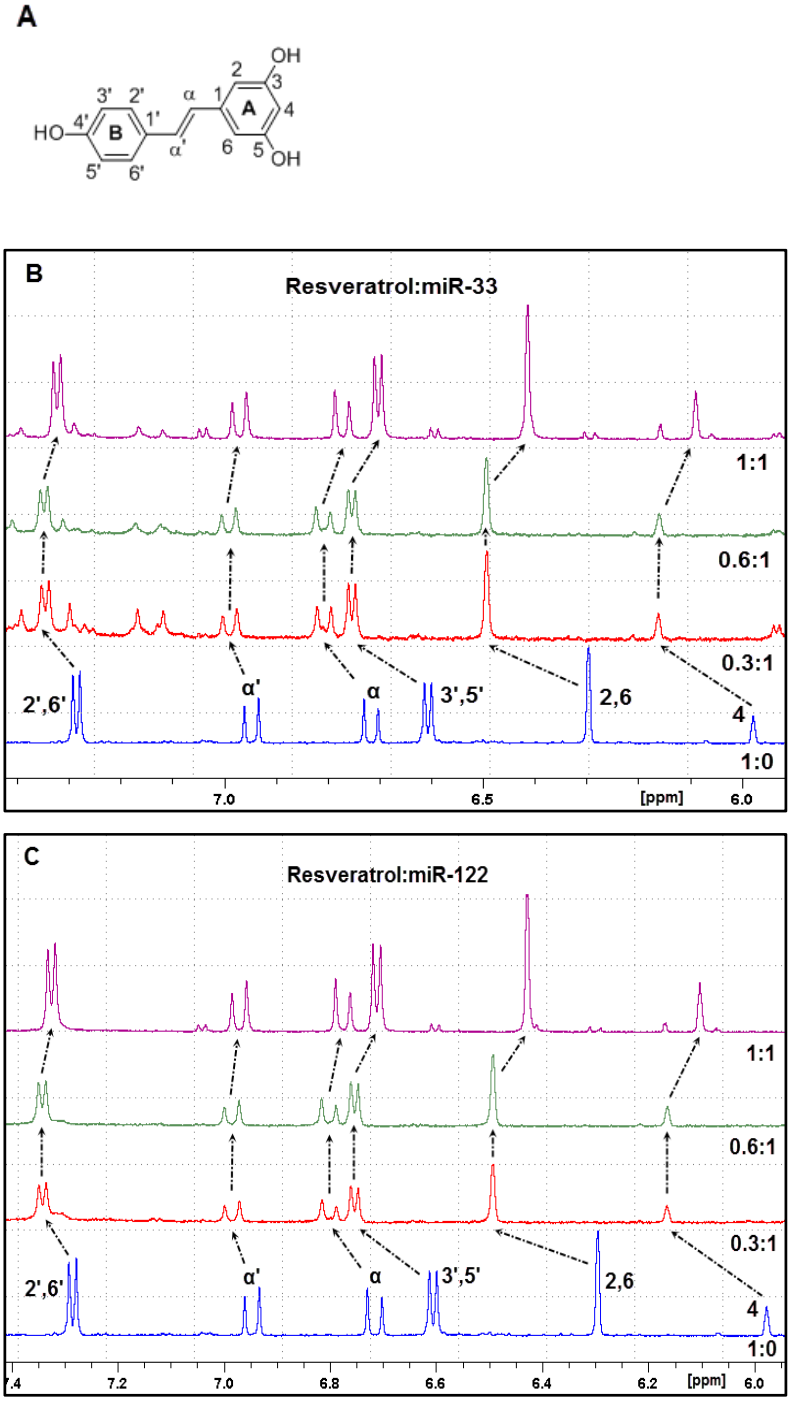
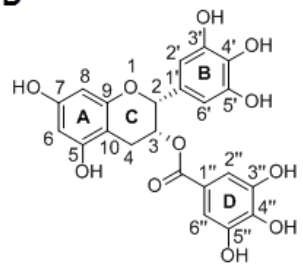
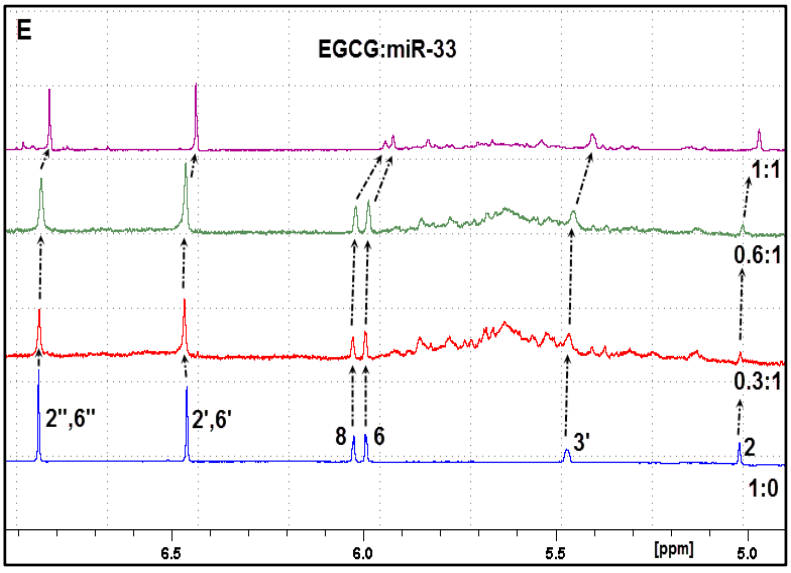

SREBP2
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
RSV
EGCG
Q
GA
Control
Treatments

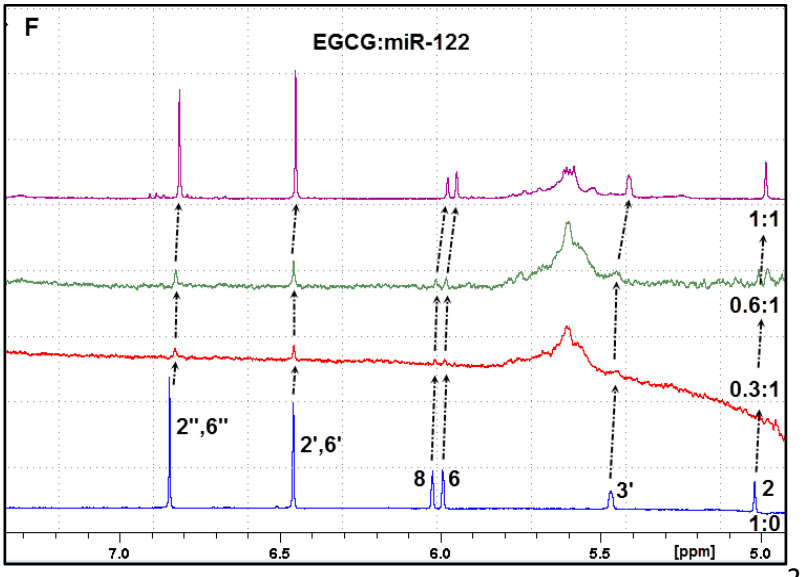
Figure 6


A
OH
HO
OH
B
A
B
Resveratrol:miR-33
1:1
0.6:1
0.3:1
1:0
2',6'
α'
α
3',5'
2,6
4
7.0
6.5
[ppm]
6.0
C
Resveratrol:miR-122
1:1
0.6:1
0.3:1
1:0
2',6'
α'
α
3',5'
2,6
4
7.4
7.2
7.0
6.8
6.6
6.4
6.2
[ppm]
6.0

D


OH
3'
4' OH
2'
B
1
HO 7
8
9
O
2
1'
5' OH
6'
A
C
6
10
4
3
O
5
OH
2"
1"
3"OH
D
4"
6"
5"
OH
OH


E
EGCG:miR-33
1:1
0.6:1
0.3:1
1:0
2",6"
2',6'
8
6
3'
2
6.5
6.0
5.5
[ppm]
5.0


F
EGCG:miR-122
1:1
0.6:1
0.3:1
1:0
2",6"
2',6'
8
6
3'
2
7.0
6.5
6.0
5.5
[ppm]
5.0

Supporting material

Supporting information

Materials and methods.

Analysis of phenolic compounds and derivatives by reversed-phase chromatography coupled to mass spectrometry. Major phenolic compounds of the studied extracts were quantified by HPLC-MS following the method described by Quiñones et al (21). Instrumental equipment included an Agilent 1200 HPLC series coupled to a 6120 TOF mass detector (Agilent Technologies). Chromatographic separation was performed using a Zorbax SB-Aq column (3.5 µm, 150 mm x 2.1 mm internal diameter [i.d.]) equipped with a Zorbax SB-C18 pre-Column (3.5 µm, 15 mm x 2.1 mm i.d.), both from Agilent. Data analysis was performed using MassHunter software. Individual compounds were quantified by means of a six-point calibration curve using standards obtained from commercial suppliers unless otherwise stated (Table S1).

Cells and cell culture. Fao cells, which are a rat hepatoma cell line (ECACC, code 85061112), were cultured as described by *Baselga-Escudero et al* (11). HepG2, which is a human hepatocarcinoma cell line (ATCC, LGC Promochen, HB8065, Salisbury, United Kingdom), were grown to 80% confluence in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Barcelona, Spain) supplemented with 10% fetal bovine serum (Lonza, Barcelona, Spain), 0.1 mM nonessential amino acids (Sigma, Madrid, Spain), 100 U/ml penicillin, 100 mg/ml streptomycin (Lonza, Barcelona, Spain), 2 mM glutamine (Lonza, Barcelona, Spain), and 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma, Madrid, Spain) in a humidified incubator with 5% CO_2 at 37ºC.

At 15 h before the treatments, the media was replaced with serum-depleted media supplemented with 100 µM oleic acid (MERCK, Germany) and 40 µM BSA (bovine serum albumin, fatty acid-free, Sigma-Aldrich, Madrid, Spain). Cells were treated with 25 mg/L of GSPE, GPE and MF, OPA, and CT fractions; 50 µM of the pure compounds Q, C, EC, RSV, EGCG and B2; and 50 µM of the polyphenol microbial metabolites HBA, m-HPAA, VA and GA.

Each compound was dissolved in ethanol and added to the culture media; the final concentration of ethanol in the media was 0.05%, which is nontoxic. miRNAs were extracted after 1 h of the treatments. mRNA and proteins were extracted after 5 h of the treatment.

^{1}H NMR. For NMR measurements, the compounds were reconstituted in 600 µL of D_2O/H_2O or deuterated methanol, CD_3OD. 1H NMR spectra were measured at a

600.20 MHz frequency using an Avance III-600 Bruker spectrometer equipped with an inverse TCI 5 mm cryoprobe®. For the ^{1}D aqueous spectra, one-dimensional (1D) nuclear Overhauser effect spectroscopy with a spoil gradient (noesygppr1d) was used. Solvent presaturation was applied during recycling delay (RD=5 s) and mixing time (tm =100 ms) to suppress residual water. A total of 256 transients were collected across a 12 kHz spectral width at 300 K into 64 k data points, and exponential line broadening of 0.3 Hz was applied before Fourier transformation. The sequences of the two miRNAs used were as follows: hsa-miR-122, 5'-ugg agu gug aca aug gug uuu g-3', and hsa-miR-33a, 5'-gug cau ugu agu ugc auu gca -3', which were purchased from Biomers (Ulm, Germany). The concentration of the miRNAs was 50 μM, and the concentrations of the polyphenols were 15 μM, 30 μM and 50 μM to obtain stoichiometric ratios of 0.3:1; 0.6:1; and 1:1 polyphenol:miRNA, respectively. The data were processed with TopSpin 2.1 (Bruker BioSpin, Fallanden, Switzerland).

Table S1. Retention time (Rt, min), m/z, linearity and calibration curves of the studied compounds using HPLC-TOF-MS.

Phenolic compound	**m/z**	**Rt (min)**	**Linearity**	**Calibration curve**
Gallic acid	169.01	7.7	0.0-19.8	y = 854865x
Vanillic acid	167.03	24.9	0.0-18.1	y = 866985x
Procyanidin Dimer B2	577.13	28.9	0.0-14.9	y = 520296x
Catechin	289.07	25.2	0.0-18.0	y = 1313205x
Epicatechin	289.07	29.5	0.0-18.0	y = 1512281x
Epigallocatechin gallate	457.08	33.5	0.0-19.8	y = 696675x
Resveratrol	227.07	50.9	0.0-19.8	y = 1971255x
Quercetin	301.03	58.1	0.0-19.9	y = 1600691x

Our research group has previously shown that PAs repress TG secretion in the liver through an FXR-SHP dependent pathway [1, 2]. However, lipid metabolism in liver is controlled by diverse mechanisms, including miR-33 and miR-122, working in concert (reviewed in [3, 4]). As mentioned in the introduction section, the inhibition of miR-122 has been associated with the deregulation of key genes that control lipid metabolism in liver like FAS [5-8]. Moreover, miR-33 plays an important role in the regulation of cholesterol homeostasis regulating genes such as ABCA1 [9-13]. Therefore, the objective of this thesis was to study the modulation of miR-33 and miR-122 by dietary polyphenols, especially PAs, as one of the mechanisms from which PAs improve lipid metabolism.

As GSPE hypolipidemic action is very fast (3-5h), we first studied whether miR-33a and miR-122 were modulated by GSPE at earlier timepoints both *in vivo* and *in vitro*. To this end, once we confirmed the hypolipidemic effect of GSPE in male wistar rats with an overload of saturated fat, we evaluated the levels of miR-33a and miR-122 in liver. The results showed a significant reduction of both miRNAs at 1h after GSPE administration, confirming that the miRNAs modulation by PAs was rapid and transient. Moreover, the mRNA levels of Abca1 and Fas, were modulate later at 3h according to the miRNA levels. The repression of miR-122 and miR-33a induced by GSPE in the liver *in vivo* could be secondary to hormonal changes and/or variations in the nutrient supply to the liver as a result of the action of GSPE in other organs, such as the intestines. For this reason, we confirmed the results *in vitro*, using rat hepatoma Fao cells showing that the PAs effects on miRNAs were a direct, rapid effect in liver.

As an acute treatment does not reflect a physiological dietary condition, we next tested if a chronic treatment of dietary doses of GSPE in male wistar rats were able to reduce miR-33a and miR-122 levels in liver. Furthermore, recent research shows increasing evidence that the postprandial state is an important contributing factor to chronic diseases [14-16]. Therefore, the experiment was performed in a postprandial state. Two important aspects had been taken into account for designing the experiment. Firstly, the dose of GSPE and secondly, when and how often GSPE should be administered. Thus, we carried out a 24h kinetic experiment, administering a high dose of GSPE once a day (9:00 a.m.). Mostly miR-33a and miR-122 levels remained down-regulated for 24h. Therefore, we decided to administrate GSPE as a single dose in the morning (9:00 a.m.) at 5, 15, 25 and 50 of GSPE/Kg of body weight for 3 weeks. These doses correspond, using a transnational animal model [17] to human intake, to 57, 171, 284 and 560 mg of GSPE/day. These GSPE intakes are representative for humans with a healthy diet. For example, the total proanthocyanidin intake of U.S. adults is 95 mg/day [18], in Finnish adults the total dietary intake of polyphenols was 863±415 mg/day [19], from which 14% was of PAs and in Spanish adults the mean dietary flavonoids intake is 313.26 mg/day [20], with PAs representing 60.1% of this. One of the doses was low (5 mg of GSPE/Kg of body weight), two were normal doses (15 and 25

mg of GSPE/Kg of body weight) and a higher dose (50 mg of GSPE/Kg of body weight) than the normal intake were administered. Results showed a dose-response effect of GSPE modulating miRNA and mRNA expression. However, only the higher doses (25 and 50 mg of GSPE/Kg of body weight) significantly repressed liver miR-33a and miR-122. This data suggests that GSPE increases liver cholesterol efflux to HDL formation and reduces FA synthesis improving the postprandial state. Therefore, a PA-rich diet would be necessary to obtain a beneficial effect on miRNAs and their target genes. In concordance with the GSPE dose-response effect on miRNAs, a dose-response effect was also observed reducing LDL-cholesterol and total cholesterol plasma levels despite the fact that the GSPE dose for a significant effect was from 50 mg of GSPE/Kg of body weight. Otherwise, the effect on plasma TG was observed from 25 mg of GSPE/Kg of body weight. All this together, shows that 3 weeks of GSPE supplementation improves plasma lipid parameters at the postprandial state, showing that GSPE is able to recover plasma lipids with non-pharmacological doses in healthy animals.

Bioactive food compounds can aid the success of dietary regimens designed to ameliorate risk factors associated with obesity and metabolic syndrome. Therefore, dietary PAs can be useful because their consumption reduces several risk factors associated with obesity, such as dyslipidemia, and in a postprandial state they repress miR-33a and miR-122. Therefore, we determined whether chronic consumption of PAs was able to normalize miR-33a and miR-122 deregulation in the livers of obese and dyslipidemic rats fed an HFD and to estimate the dose of PAs required for this reversion. To this purpose, dyslipidemic and obese female rats were treated with the same doses of GSPE as in the previous experiment. The HFD produced a hyperlipidemic profile and an increase of miR-33a and miR-122 liver expression. After 3 weeks of HFD supplemented with GSPE, miR-33a and miR-122 overexpression was normalized at all the doses tested and, the levels of abca1 and fas were also modulated in a pattern reflecting miRNA repression. However, despite the normalization of these miRNAs in the liver, none of the tested doses of GSPE were able to completely restore the levels of plasma lipids. Reinforcing our results, the intake of beverages rich in PAs, such as apple polyphenols [21, 22], grape juice [21, 22] or red wine [23], did not significantly reduce plasma lipid levels in obese or overweight humans, even though the consumption of even relatively small amounts of flavonoid-rich foods reduces the risk of CVD in humans [24]. Therefore, although PA-rich foods and beverages do not significantly reduce plasma lipid levels, they can confer phenotypic robustness at the molecular level to repress miR-33a and miR-122 to exert subtle changes in lipid metabolism. The normalization of miR-33a levels in the liver by PAs, with respect to obesity and metabolic syndrome, could extend the beneficial effects of PA-rich foods to other risks associated with these pathologies, such as diabetes. In addition to the regulation of lipid metabolism, miR-33 has also been shown to control the expression of AMP-activated kinase (Ampkα1) and sirtuin 6

(Sirt6), which are involved in the regulation of both lipid and glucose metabolism [25]. Moreover, miR-33 controls insulin secretion by the pancreas and the expression of ISR2, which is a component of the insulin signaling cascade in the liver [25].
Comparing both chronic experiments, although the gender of the animals was different, we are able to say that dyslipidemic obese rats were more sensitive to GSPE modulating miRNAs than lean animals. In this sense, in obese rats, the normalization of miRNAs was significant at doses lower than the estimated mean intake for the European population. However, in healthy rats a PA-rich diet is needed to modulate miRNAs.

Due to the effectiveness of PAs in normalizing miRNAs levels in obesity, we evaluated whether ω-3 PUFAs, which are also effective at improving dyslipidemia, can also normalize miR-33 and miR-122 levels. In this experiment dyslipidemic obese male rats were treated for 3 weeks with 25 mg of GSPE/Kg of body weight and/or with 515 mg of ω-3 PUFAS /Kg of body weight. Once again, although being male, these rats also became dyslipidemic and obese and had increased levels of miR-33a and miR-122 with a cafeteria diet (CD) as a HFD. Interestingly, these dietary treatments also counteract the overexpression of miR-33a and miR-122 that was induced by the CD, reaching the levels found in rats that were fed with standard chow diet (STD). Moreover, when both treatments were orally administered together, the repression of these miRNAs was greater than per separate. The repression of rat liver miR-33a and miR-122, induced by GSPE or ω-3 PUFAS, was clearly associated with the improvement in the plasma lipid profile. Therefore, among both males and females the HFD increased both miRNAs and chronic doses equivalent to a normal human diary intake of PAs normalized miR-33a and miR-122 liver levels.

In order to be able to carry out epidemiological studies with dietary components that modulate miRNAs in liver, it is necessary to analyze samples in a non-invasive way such as peripheral blood mononuclear cells (PBMCs). We demonstrated that the levels of miR-33 and Abca1 in PBMCs reflected the modifications in the liver that were induced by diet and treatment. Therefore, analyzing miR-33a in PBMCs could provide information about the state of miR-33a expression in the liver in a non-invasive way and give information about the physiological state of the patient.

miR-33 has two isoforms, miR-33a and miR-33b, which reside in the intronic region of the SREBP2 and SREBP1 [9-11, 26], respectively, and are reported to be cotranscribed [9-11, 26]. When miRNAs are introns of genes, such as miR-33, it is important to evaluate whether the changes of the miRNAs levels by dietary compounds, such as PAs or ω-3 PUFAS, are due to the modulation of the host gene. ω-3 PUFAS modified both Srebp2 and miR-33a, suggesting that ω-3 PUFAS repress miR-33a levels though the modulation of the host gene expression. On the contrary, GSPE modified miR-33a expression without altering the levels of its host gene, Srebp2, although they are

simultaneously coexpressed. As there is evidence that polyphenols can bind to mRNAs and proteins [27, 28], we speculated that polyphenols also bind to miRNAs or to some component involved in miRNA biogenesis, such as Dicer or the RISC. In this thesis we provide evidence that polyphenols, like resveratrol and EGCG, directly bind to miR-33a and miR-122 and that they differ in their binding, suggesting a specific binding. Resveratrol and EGCG are two polyphenols, present or not in grape extracts, that have different chemical structures and we showed that these compounds modify differently miR-33a and miR-122 in hepatic cells. These results suggest that the interaction of polyphenols with miRNAs (i) alters their functionality by shifting the binding of the miRNAs to the seed sequence of their target mRNAs and/or (ii) increases their degradation. The effect of the polyphenols chemical structure on modulating miRNAs levels was confirmed, evaluating the capacity of a range of different polyphenols, phenolic acid metabolites, extracts and their fractions that showed a different behavior. Furthermore, we demonstrated that most classes of polyphenols also correspondingly modify ABCA1 and FAS mRNA and protein levels in HepG2 cells with modulation of miR-33a and miR-122 levels, respectively. Therefore, these results indicate that polyphenols can alter lipid metabolism by miRNA modulation.

In summary, the modulation of specific miRNAs is considered a promising strategy to treat metabolic diseases; therefore, proanthocyanidin-rich food can be a good candidate to reduce the risks associated with dyslipidemias in obesity and postprandial state. Moreover, these results suggest that the modulation of miR-33a and miR-122 could be one of the molecular mechanisms used by proanthocyanidins and ω-3 PUFAs to improve the plasma atherogenic profile induced by a CD.

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1) **An acute dose of proanthocyanidins directly repress miR-33a, miR-122 in rat liver rapidly and transiently. Moreover, the mRNA levels of their target genes, abca1 and fas, were modulated after the miRNA.**

2) **A moderate chronic consumption of proanthocyanidins modulates the levels of liver miR-33a, miR-122 and their target genes in a postprandial state with a dose-response profile in healthy rats.**

3) **Cafeteria diet administration, as a model of high fat diet, resulted in increased miR-33a and miR-122 levels in liver together with an atherogenic lipid profile. miR-33 was more sensitive to dietary fat than miR-122.**

4) **A low chronic consumption of proanthocyanidins counteracted the increase of liver miR-33a and miR-122 levels and improved the atherogenic lipid profile induced by a cafeteria diet, and also modulated abca1 and fas mRNA levels in accordance with miRNA expression.**

5) **Despite the different genders used in chronic studies, dyslipidemically obese rats were more sensitive to GSPE modulating miRNAs than lean animals.** Different doses of GSPE in normolipidemic lean rats modulated miR-33a and miR-122 with a dose-response profile, whereas in dyslipidemically obese rats all tested doses were effective.

6) **Other dietary components rather than proanthocyanidins, such as ω-3 PUFAs, also counteracted the increase of miR-33a and miR-122 produced by a cafeteria diet in chronic treatment, with an additive effect when rats were chronically administered GSPE+ ω-3 PUFAs.**

7) **The levels of miR-33a and abca1 in PBMCs reflected the modifications induced in the liver by diet and treatment.**

8) **ω-3 PUFAS repressed both miR-33 and its host gene SREBP2. On the contrary, GSPE modified miR-33a expression without altering the levels of its host gene, although they are cotranscribed.**

9) **Resveratrol and EGCG directly bound to miR-33a and miR-122 and showed a different type of interaction.**

10) **The miR-33a and miR-122 modulation by polyphenols depended on the chemical structure, size and extract composition in hepatic cells**