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Involvement of DNA-methylation and microRNAs in the delipidating effects of stilbenes in white adipose tissue and liver

Ana Gracia Jadraque

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TESIS DOCTORAL

Departamento de Farmacia y Ciencias de los Alimentos

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GRACIAS

3´UTR: three prime untranslate region

ACC: acetyl-CoA carboxilase

AMPK: AMP-activated protein kinase

ATGL: adipocyte triglyceride lipase

ATP: adenosine triphosphate

BAT: brown adipose tissue

BP: base pair

cAMP: cyclic adenosine 3´-5´-monophosphate

CACT: carnitine/acyl carnitinetranslocase

CCL2: CC Motif Chemokine ligand 2

C/EBP: CCAAT/enhancer binding protein

CGI-58: comparative gene identification 58

CPT I: carnitine pamitoyltransferase I

CPT II: carnitine pamitoyltransferase II

ChREBP: carbohydrate Response Element Binding Protein

CpG: cytosine nucleotide followed by Guanine nucleotide. 5'-C-phosphate-G-3'

DHFR: dihydrofolate reductase

DMEM: dulbecco´s Modified Eagle Medium

DNA: desoxiribonucleotid Acid

DNMT: DNA methyltransferase

EPI: epididymal fat

FABP: fatty acid binding protein

FAS: fatty acid synthase

FBS: fetal bovine serum

FPGS: folylpolyglutamate synthase

FOXO1: transcription factor forkhead O1

G6PDH: glucose-6-phosphate dehydregenase

HEXB: hexosaminidase subunit beta

HSL: hormone sensitive lipase

IBAT: interscapular fat

IL-6: interleukine 6

LDL: low-density lipoproteins

LncRNAs: long non coding RNAs

LPL: lipoprotein lipase

LXR: liver X receptors

MCP1: monocyte chemoattractant protein-1

ME: malic enzyme

MGL: monoglyceride lipase

MiRISC: miRNA-induced silencing complex

MiRNAs: microRNAs

MS: mesenteric fat

MTHFD1: methylenetetrahydrofolate 1

NADPH: nicotinamide adenine dinucleotide phosphate

NcRNAs: non-coding RNAs

Nt: nucleotides

PiRNAs: piwi-interacting RNAs

PKA: protein kinase A

PPAR: peroxisome proliferator-activated receptor

PR: perirenal fat

Pri-miRNA: primary miRNA

Pre-miRNA: precursor miRNA

RNA: ribonucleic acid

RT-PCR: real Time- Polymerase Chain Reaction

RXR: retinoid X receptor

SAM: S-Adenyl Methionine

SC: subcutaneous fat

SLC19A1: Solute Carrier Family 19 Member 1

SiRNAs: short inhibitory RNAs

Sp1: transcription factor Sp1

SREBP: sterol regulatory binding protein

SRNAs: small non-coding RNAs

TNFα: tumor necrosis factor alpha

WAT: white adipose tissue

INTRODUCTION

TRIGLYCERIDE METABOLISM

Triglycerides consist of a molecule composed of three fatty acids, each in ester linkage with a single glycerol. Because of their water insolubility, triglycerides are transported in the plasma in combination with phospholipids and proteins, as well as with cholesterol and cholesterol esters, in complex macromolecules known as lipoproteins (1). In mammals, excess energy is stored primarily as triglycerides, which are mobilized when energy demands appear. In other words, the triglyceride store reflects the net balance between fat deposition and fat mobilisation. Triglyceride metabolism involves several pathways of fat storage and mobilization that are, at least in part, inter-dependent and cross-regulated (2, 3). Blood plays an important role in the triglyceride metabolism as well as other organs and tissues such as adipose tissue, liver and muscle.

Adipose tissue is defined as a connective tissue consisting in part of fat cells (adipocytes), specialized in the synthesis and storage of fat, within a structural network of fibres. Only one third of the tissue is constituted by adipocytes, the rest is represented by fibroblasts, preadipocytes, macrophages, monocytes and stromal cells (4). This tissue is classified in two types, white adipose tissue (WAT) and brown adipose tissue (BAT). While the function of WAT is to serve as an energy store, BAT function is to generate heat within the thermogenic process (5). Adipocytes play a critical role in energy homeostasis by hydrolysis of their triglyceride reserves to provide fatty acids, which are important oxidative fuels for other tissues during periods of energy deprivation (6). Lipolysis is the biochemical pathway responsible for this catabolism. Due to the importance of this pathway, lipolysis occurs in essentially all tissues and cell types, however it is most abundant in white and brown adipose tissues (7). In addition, adipose tissue is one of the main sites of *de novo* lipogenesis. Although the key enzymes involved in fatty acid synthesis are present in human adipose tissue, its contribution to whole-body lipogenesis is considered very low and less

than in liver. However, in rats, *de novo* lipogenesis occurs in similar extent in adipose tissue and liver (8).

Liver is an organ that performs numerous biochemical functions necessary for metabolic homeostasis. In addition to *de novo* lipogenesis, fatty acid β-oxidation is a process that also takes place in liver. This metabolic pathway degrades fatty acids derived from adipose tissue triglycerides or an excessive accumulation of intrahepatic triglycerides in the mitochondria (9).

Skeletal muscle is also involved in triglyceride metabolism. In lipid overload situation, both this tissue as well as liver could store fat (10). Moreover, in fasted state, fatty acid oxidation is the main metabolic activity of this tissue (11).

In this Introduction, only those aspects of the triglyceride metabolism addressed in this thesis, are included.

De novo LIPOGENESIS

De novo lipogenesis is the process that allows tissues the synthesis of fatty acids from acetyl-CoA, a precursor coming from glucose metabolism, amino acid degradation or even fatty acids. Besides acetyl-CoA, nicotinamide adenine dinucleotide phosphate (NADPH), a substrate with reducing power, is also necessary for fatty acid synthesis. NADPH comes from biochemical reactions catalysed by the enzymes glucose-6-phosphate dehydregenase (G6PDH), malate dehydrogenase, malic enzyme (ME) and NADP isocitrate dehydrogenase.

The process starts with the transformation of acetyl-CoA to malonyl-CoA by the action of acetyl-CoA carboxylase (ACC) in the presence of adenosine triphosphate (ATP). Malonyl-CoA is the substrate used by a multi-enzyme complex called fatty acid synthase (FAS), which generates long-chain fatty acids (palmitate), in a repetitive sequence reaction using the reducting power (NADPH). The activity of this enzyme, FAS, together with the activity of ACC, limit fatty acid biosynthesis (12).

Acetyl-CoA required for fatty acid synthesis is present in the mitochondria, while biosynthesis takes place in the cytosol. The shuttle citrate system is an essential transport mechanism, which allows acetyl-CoA transportation to the cytosol (12) (Figure 1).

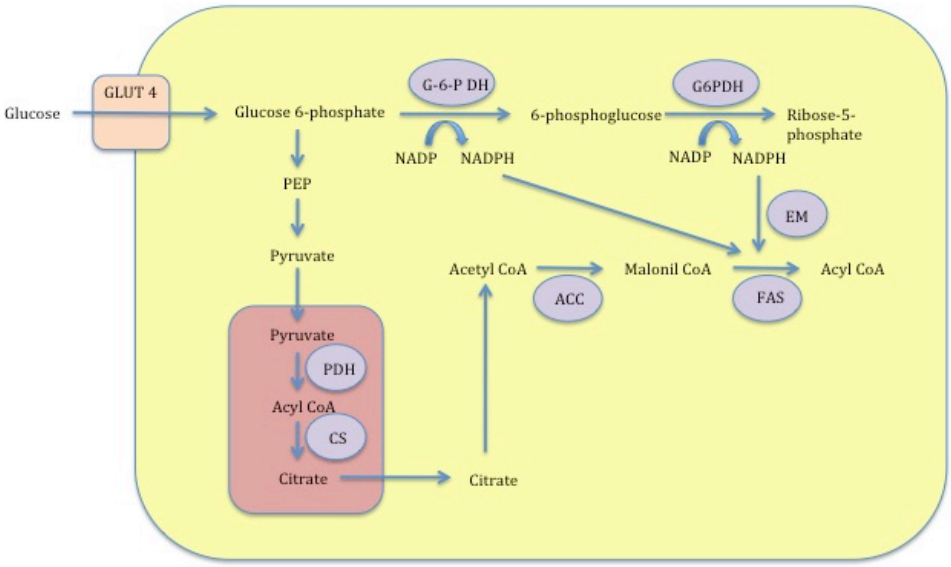

Figure 1. *De novo* lipogenesis pathway.

ACC and FAS are mostly controlled by the modulation of their transcription, but they also can be regulated by post-transcriptional mechanisms. There are two principal transcriptional regulators, both present in adipose tissue and liver, which are widely expressed: sterol response element binding protein 1c (SREBP 1c) and carbohydrate response element binding protein (ChREBP). ACC activity is also controlled by phosphorylation/dephosphorylation of serine, and the amount of enzyme is regulated by different hormones such as insulin or growth hormone. Similarly, hormones like insulin and glucagon can regulate FAS. Insulin and citrate availability activate the enzyme while glucagon and catecholamines inhibit its activity via cyclic adenosine monophosphate dependent phosphorylation (3, 13-15).

SREBP 1c is an isoform of the SREBP family induced in response to insulin. The nutritional status is also a regulator of SREBP. The expression of this transcription factor decreases with fasting and increases after feeding high-

carbohydrate diets, as a result of increased blood glucose and insulin. This transcription factor promotes the expression of genes encoding the lipogenic enzymes, due to the interacting with sterol response elements located in the gene promoter (8, 16). It is known that SREBP 1c is negatively regulated by AMP-activated protein kinase (AMPK). This kinase is an energy sensor that regulates cellular metabolism. Activated AMPK stimulates ATP-producing catabolic pathways, such as fatty acid oxidation, and inhibits ATP consuming processes, such as lipogenesis. Therefore, AMPK activation suppresses the expression of ACC and FAS via down-regulation of SREBP 1c (17) (Figures 2). In addition to this regulation, the activation of AMPK also inhibits directly the expression of ACC by the phosphorylation of a serine residue (mostly SER_{79}) in the N-terminal region of the enzyme.

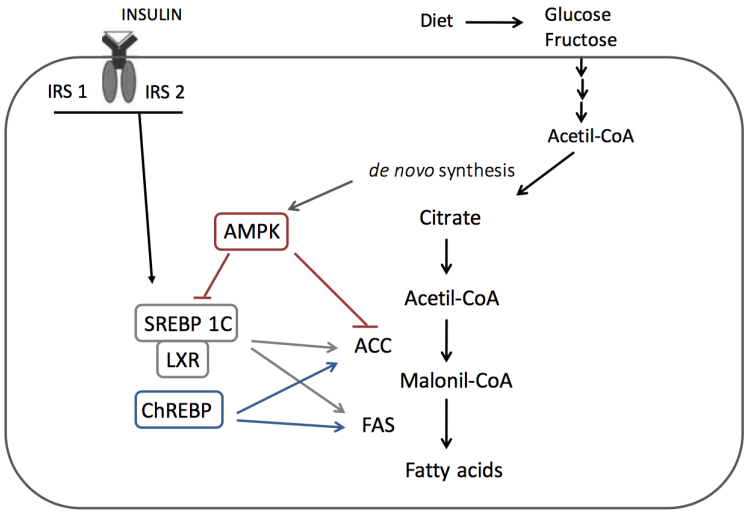

Figure 2. De *novo* synthesis of fatty acids and regulation of lipogenic genes by ChREBP, LXR, SREBP 1c and AMPK.

The transcriptional activity of SREBP 1c can also be stimulated or inhibited by cofactors such as Sp1 transcription factor (SP1) (16, 18). SP1 binds in Guanine-Citosine-rich elements, which are common regulatory elements in the promoter region of numerous genes. Although its main activity has been described as activator, it can also act as a repressor. Its behaviour depends on the promoter and the region of each promoter its binds to. In addition, the binding to different co-regulators also has an influence on its behaviour (19).

ChREBP is another transcription factor, first identified as a glucose-responsive element, which regulates glycolytic, gluconeogenic and lipogenic gene expression. Transcriptional targets of ChREBP encode enzymes in lipogenic pathway such as FAS and ACC (20) (Figure 2).

Liver X receptor (LXR) is also a key regulator of the lipogenic pathway. In rodents, there are two isoforms, which form heterodimers with the retinoid X receptor (RXR) to activate their target genes. The effect of this nuclear factor on liver lipogenesis involves direct and indirect mechanisms. LXR can bind gene promoter of FAS, or regulate gene expression via the insulin mediated activation of SREBP 1c (21-23) (Figure 2).

LIPOLYSIS

The major physiological role of WAT is to supply fatty acids as energy substrates to other tissues. This process is known as lipolysis. Triglycerides stored in the adipocyte are hydrolysed into one molecule of glycerol and three fatty acids, which are delivered to the plasma. However, the release of fatty acids and glycerol from fat cells does not occur in the ratio of 3:1 because some fatty acids are re-utilized, mostly in re-esterification of new triglycerides (24, 25).

This lipolytic process is catalysed by at least three adipocyte-specific enzymes. The adipocyte triglyceride lipase (ATGL) catalyses the first and rate-limiting step of triglyceride to diglyceride. Hormone sensitive lipase (HSL) is a multifunctional enzyme which catalyses diglycerides to monoglycerides. Finally, monoglyceride lipase (MGL) efficiently catalyses the break-down of monoglycerides to glycerol and fatty acids (7, 25).

Caloric deprivation, as occurs in fasting or starvation, is accompanied by increased mobilization of fatty acids from fat cells. There is an increase in basal lipolysis and enhanced lipolytic sensitivity to catecholamine behind the increment of the lipolysis during this caloric deficit. Catecholamines, noradenaline and adrenaline, regulate lipolysis via lipolytic beta-adrenoceptors and antilipolytic alpha$_2$-adrenoceptors, and they are the main regulators of this process (24, 25).

ATGL expression and activity could be transcriptionally regulated by numerous effectors or conditions. Peroxisome proliferator-activated receptor gamma (PPARγ) and the insulin responsive transcription factor forkhead O1 (FOXO1) up-regulate ATGL during adipocyte differentiation. Moreover, there are some factors which induce its mRNA expression, such as glucocorticoids, thiazolidinediones and fasting. By contrast, insulin, TNFalpha, mTor complex 1 and feeding repress ATGL mRNA expression. Insulin resistance and obesity have also been correlated with changes in ATGL mRNA or protein levels. ATGL activity is regulated by the ATGL co-activator comparative gene identification 58 (CGI-58). The up-regulation of CGI-58 expression accelerates triglyceride depletion and increases lipolysis and fatty acid oxidation. AMPK is other regulatory factor which induces ATGL phosphorylation (Ser406) increasing the hydrolyse activity in adipocytes (7, 26).

HSL adipose activity is controlled by two distinct mechanisms in response to beta-adrenergic stimulation. First, HSL have at least 5 distinct serine residues that can be phosphorylated (Ser660 important in HSL rat) by cAMP dependent protein kinase A (PKA). This leads to an increase of the intrinsic enzyme activity. Second, phosphorylated HSL interacts with perilipin 1, which itself is a target of PKA phosphorylation. In this context, perilipin 1 can regulate lipolysis by phosphorylation/dephosphorylation events. The phosphorylation of this molecule simultaneously triggers release of CGI-58, binding to HSL and activating the hydrolytic process, and ATGL activation. In addition, perilipin 1 phosphorylation is responsible for structural modifications at the lipid droplet surface, which promote the increase of the lipolytic rate (27).

The third enzyme involved in this lipolytic pathway, MGL, is not subjected to extensive regulation. A high MGL hydrolase activity is constitutively present in adipocytes and other types of cells (7) (Figure 3).

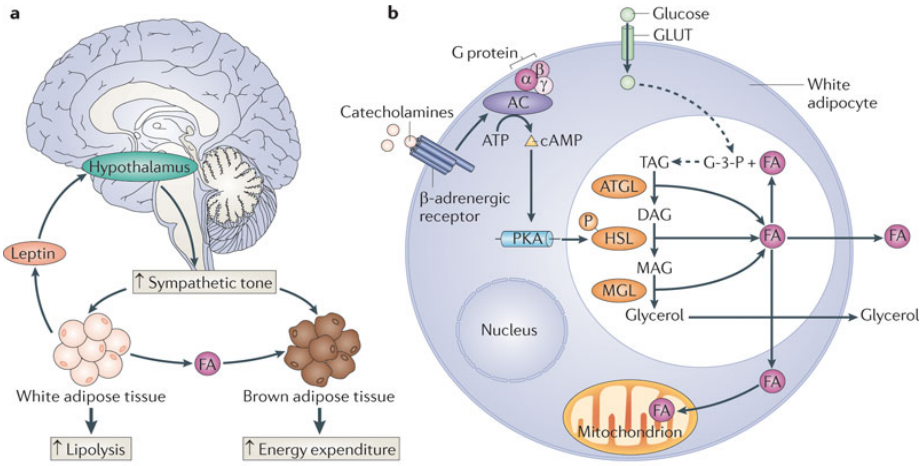

Figure 3. Lipolysis pathway and its regulation (28).

FATTY ACID β-OXIDATION

The main pathway for the degradation of plasma free fatty acids is mitochondrial fatty acid β-oxidation. This pathway, coupled to oxidative phosphorylation is the most important route for the production of metabolic energy during fasting. This process implies several steps, which are necessary before fatty acids are oxidised. Triglycerides are first hydrolysed by the action of endothelium-bound lipoprotein lipase (LPL) in skeletal muscle and the uptake of fatty acids is mediated by membrane proteins or passive uptake (3, 9, 29). The liver takes up free fatty acids from blood via transporters (FATP, FAT and CD36) or by diffusion. These free fatty acids and acyl-CoA bound to the fatty acid binding protein (FABP) and acyl-CoA binding protein, which transport them to intracellular compartments for metabolism, or to nucleus to interact with transcription factors. Non-esterified acyl-CoA maybe oxidised in the mitochondria or in peroxisomes. Once fatty acids are taken up in the tissue, where they are activated to their CoA esters and the complexes are transported into the mitochondria for subsequent β-oxidation. Mitochondrial membrane requires a membrane transporter that uses acylcarnitine instead acyl-CoAs. The initial conversion of acyl-CoA to an acylcarnitine ester, followed by the transport of the acylcarnitine across the inner mitochondrial membrane into the

mitochondrial matrix, and the reformation of acyl-CoA constitutes a carnitine shuttle that requires the concerted action of three proteins: carnitine pamitoyltransferase I (CPT I), carnitine/acylcarnitine translocase (CACT) and carnitine pamitoyltransferase II (CPT II) (Figure 4). Mitochondrial oxidation may be either complete, generating acetyl-CoA that supports gluconeogenesis, or incomplete with the final formation of ketone bodies (3, 30).

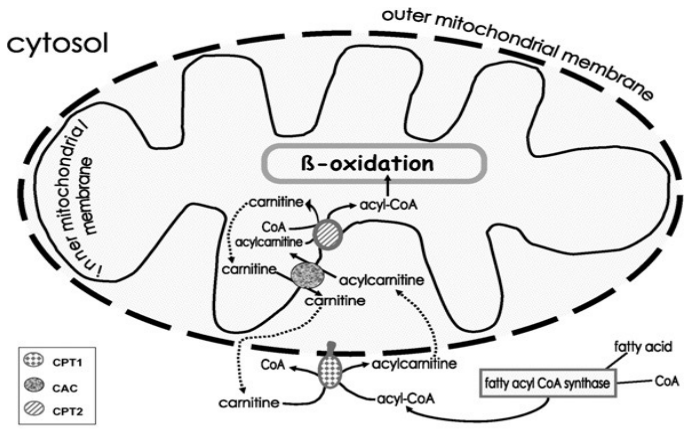

Figure 4. Mitochondrial carnitine shuttle (29).

As mentioned above, although the central site of β-oxidation is the mitochondria, other organelles, such as peroxisomes, contain enzymes capable of oxidizing fatty acids by a similar pathway. The peroxisomal system is much more active on the oxidation of very-long chain fatty acids. Furthermore, other difference between β-oxidation in mitochondria and peroxisomes lies in the first reaction of this process. While acyl-CoA dehydrogenase acts in the mitochondria, acyl-CoA oxidase is involved in the peroxisome, producing hydrogen peroxide (H_2O_2). The energy released in this first oxidation reaction dissipates as heat (31). In addition, the oxidation carried out in these organelles, cannot completely degrade the fatty acids in acetyl-CoA units, so they will be redirected to the mitochondria where they finish their oxidation (32).

Fatty acid metabolism in liver is transcriptionally regulated by two main systems under the control of either liver X receptors (LXRs) or PPARs. PPARα, highly expressed in the liver, regulates the expression of genes involved in

mitochondrial and peroxisomal β-oxidation. PPARα increases transcription and expression of proteins and enzymes necessary to catabolise fatty acids (3, 33). This transcription factor heterodimerises with another nuclear receptor, RXR, by interacting with a specific DNA sequence, peroxisome proliferator responsive element, in the promoter regions of target genes, such as CPT I. This binding induces changes resulting in the enhanced transcription of the genes (34).

CPT I is one of the rate-limiting steps in fatty acid oxidation, and it is regulated by malonyl-CoA. Negative energy balance results in a decrease in malonyl-CoA and in an increase in fatty acid oxidation. The control of CPT I by malonyl-CoA is a way to prevent simultaneous oxidation and synthesis of fatty acids within the hepatocytes (3, 9).

EPIGENETIC AND POST-TRANSCRIPTIONAL REGULATION

The central dogma of molecular biology describes that genetic information is from DNA via mRNA to proteins. Is well known how genes are transcribed and that mRNAs are translated into amino acid chains. However, this pathway is much more complex and can be controlled at different levels involving numerous factors, epigenetic mechanisms and post-transcriptional events such as phosphorylation, methylation, acetylation, non-coding RNAs, attachment of peptides, etc.

EPIGENETIC

Epigenetic is a term that was first introduced by Conrad H. Waddington in 1942 and usually refers to inheritable changes in genetic expression without changing the DNA sequence itself (35). The majority of our cells contains the same genome but there are differences in gene expression which characterizes different tissue. Epigenetic changes are typically reversible and refer to chemical modifications in DNA or chromosomal proteins (histones).

Epigenetic mechanisms establish and maintain tissue and cell type specific gene expression (36). Epigenetic modifications can be passed from one cell generation to the next (mitotic inheritance) and may be passed between generations (meiotic inheritance) (37). The epigenetic machinery is particularly important in organism development where stable and distinct cellular functions must be established from an identical genotype (38).

The major epigenetic mechanisms described are the addition of methyl groups to DNA, called DNA methylation, and post-translational modifications in histone proteins, such as acetylation and methylation (39). Commonly, these epigenetic processes alter the accessibility of the transcriptional machinery to a particular gene.

There is a high degree of complexity in epigenetic processes during prenatal and early-postnatal development. There is evidence that environmental exposures during early life can induce persistent alterations in the offspring

epigenome, which may lead to an increased risk of obesity and metabolic syndrome later in life (40). The epigenetic profiles are also sensitive to the environment in childhood and adult life (37). These epigenetic alterations can be influenced by lifestyle and environmental factors resulting in phenotypic changes, which play a vital role in development and human diseases. Ageing has also been reported to have an epigenetic influence. This theory is a rapidly developing modern concept postulating that non-adaptative epigenetic alterations are fundamental to ageing.

Nutrition is thought to be the most influential of all the external environmental factors (35). Quality of diet can also be crucial for triggering epigenetic changes. Utilizing dietary compounds to target, prevent and even treat certain diseases has become an area of interest. The consumption of certain foods, grapes, soy, cruciferous vegetables or green tea, have been shown to induce epigenetic mechanisms that protect against some diseases. Introduction of these food groups into a normal diet regime could serve as an effective therapeutic strategy for medicinal proposes (Michael Daniel, 2015). Moreover, polyphenols from several sources, such as resveratrol, quercetin or curcumin, have shown a modulator effect on epigenetic mechanisms such DNA methylation, post-transcriptional regulation by miRNAs and histone modifications (41).

This thesis focuses DNA methylation as an epigenetic mechanism and to microRNAs as post-transcriptional regulation. These two mechanisms are going to be described in more depth in the following sections.

DNA methylation

DNA methylation is the major epigenetic mechanism involving direct chemical modifications (42). It refers to the addition of a methyl group to the 5-position of cytosine (Figure 5). The majority of DNA methylation occurs on cytosines that precede a guanine nucleotide, commonly named CpG sites (42). Clusters of CpGs are called CpG islands. Gardiner-Garden and Frommer defined a

CpG island as being a 200 base pair (bp) region of DNA with a high G+C content, greater than 50%, and observed CpC / expected CpG ratio of greater or equal to 0.6. (43-46). The methylation of nucleotides (nt) provides a molecular path to reversibly mark genomic DNA (47). Approximately 70% of the CpG residues in mammalian genome are methylated, but the distribution of CpG is non-random. Certain regions of the genome, which are often clustered at the 5´- ends of genes (promoter region) and in first exons, possess the expected CpG frequency. Curiously CpGs at CpG islands are non-methylated despite their abundance, while the majority of the remaining CpGs across the genome are mostly methylated (48).

DNA methylation is a potent suppressor of gene activity when the methylation process takes place in the promoter region. There are two possible mechanisms proposed for this repression. The first one involves the direct inhibition of binding of sequence specific transcription factors, whose binding sites contain CpG sites, and the second one is mediated by methyl-CpG binding proteins which are specific for methylated DNA (44).

Methylation is performed in three steps involving enzymes that establish, recognize and remove DNA methylation. These enzymes are a family of DNA methyltransferases (DNMTs), DNMT 1, DNMT 3a and DNMT 3b, which directly catalyse the addition of a methyl group from SAM to the fifth carbon of a cytosine residue to form 5-methylcytosine (Figure 5).

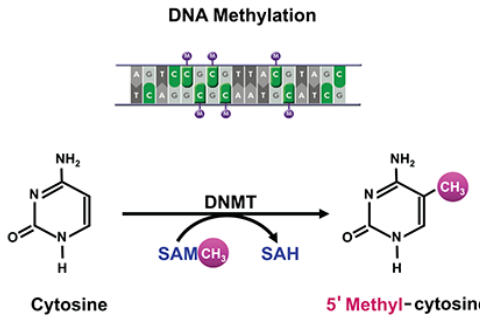

Figure 5. Addition of a methyl group from S-Adenosyl Methionine (SAM) to the fifth carbon of a cytosine residue. Adaptated from(49).

These enzymes share a similar structure with a N-terminal regulatory domain and C-terminal catalytic domain and they have unique functions and expression patterns (42).

DNMT 1, the maintenance methyltransferase, binds to the newly synthesized DNA after replication and methylates it to precisely mimic the original methylation pattern present before the replication. It also has the ability to repair DNA methylation. DNMT 3a and DNMT 3b are called *de novo* DNA methyltransferases because they establish a pattern of methylation that is faithfully maintained through cell division (47). The main difference between these two enzymes is the gene expression pattern. DNMT 3a is expressed ubiquitously while DNMT 3b is poorly expressed by the majority of differentiated tissues (Figure 6).

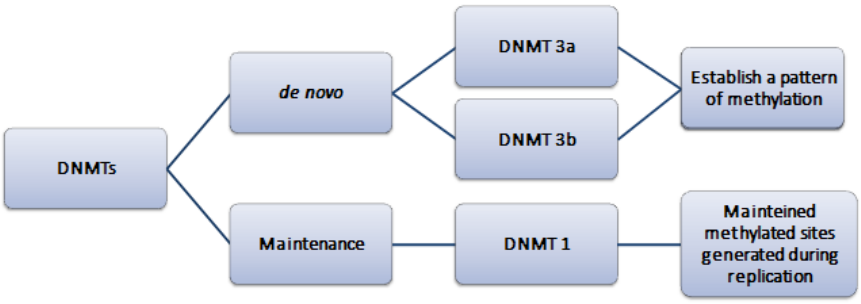

Figure 6. Classification of DNMTs.

There are two more DNA methyltransferases, DNMT2 and DNMT3L, with less relevant functions in DNA methylation. The first one is the smallest mammalian DNMT and its structure suggest that this enzyme participates in the recognition of DNA damage, DNA recombination and mutation repair (50). DNMT3L belongs to DNMT3 family, and is commonly non-functional, but in some cases it has a functional catalytic site, which may act in antagonizing DNMT3A and 3B activities (44).

POST-TRANSCRIPTIONAL REGULATION

Other known regulator mechanisms are non-coding RNAs (ncRNAs). In general, they produce transcripts that function directly as structural, catalytic or regulatory RNAs, rather than expressing mRNAs that encode proteins (51). NcRNAs are historically divided into two groups, small non-coding RNAs (sRNAs) with length <200nucleotides and long non coding RNAs (lncRNAs) with length >200nt. Those ncRNAs that are involved in epigenetic regulation comprise the short ncRNAs (<30nt), such as microRNAs (miRNAs), short inhibitory RNAs (siRNAs), piwi-interacting RNAs (piRNAs) and the long ncRNAs (>200nt) (52). Long ncRNAs play a regulatory role during development and exhibit cell type-specific expression. MiRNAs are the best explored subclass of ncRNAs which play a central role in a post-transcriptional gene silencing mechanism (53).

MicroRNAs

MiRNAs were discovered in 1993 by Victor Ambros, Rosalind C. Lee and Rhonda L. Feinbaum in an experiment in *C.Elegans.* They show that *lin-4* gene codifies not to a protein, but rather as two small transcripts of approximately 22 and 61 nucleotides, nowadays known as the mature miRNA and double-stranded stem precursor respectively. They also observed that this transcript contained sequences complementary to a repeated sequence element in the three prime untranslate region (3´UTR) region in mRNA (54).

MiRNAs are defined as small RNAs of around 23 nucleotides, which can play important regulatory roles in animals and plants, by targeting mRNAs for cleavage or translational repression. These non-coding RNAs comprise one of the more abundant classes of gene regulatory molecules in multicellular organisms and influence the output of many protein-coding genes (55). MiRNAs modulate a variety of important functions including proliferation, differentiation, apoptosis and senescence in animals and plants (56).

Since miRNAs discovery there has been an exponential increase in the number of microRNAs. MiRBase sequence database (http://www.mirbase.org/)

in 2016 contains 28645 entries representing hairpin precursor miRNAs, expressing 35828 mature miRNA products, in 223species.

A few hundred of miRNAs have been confirmed to regulate the 30-80% of genes encoded in the human genome, with each miRNA targeting up to 100 genes, and multiple miRNAs having the potential to act on one gene. A single miRNA can have multiple target sites of a particular mRNA transcript, and a single miRNA family can regulate up to 400 targets (57).

The generation of miRNAs is a process with multiply steps. Some miRNAs genes seem to be solitary and are expressed under the control of their own promoters. Other miRNAs genes are arranged in clusters (Figure 7), and may be co-regulated with other members of the cluster (58).

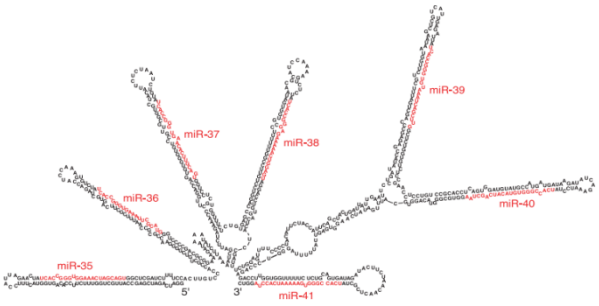

Figure 7. The miRNA-35-miRNA-41 cluster (59).

Over 50% of mammalian miRNAs are located within the intronic regions of annotated protein-coding or non protein-coding genes. These miRNAs use their host gene transcripts as carriers, although some of them actually transcribe separately from internal promoters. Other miRNAs are located in intergenic regions and apparently have their own transcriptional regulatory elements, constituting independent transcription units (60).

In a first step of the biogenesis process, the Polymerase II or III produce one strand of an approximately 33nt double-stranded stem, called primary miRNA (pri-miRNA) with a hairpin structure. This pri-miRNA is cleaved in two steps by endoribonucleolytic enzymes. In the first step, the nuclear endoribonuclease Drosha in a complex with DGCR8 cuts the hairpin stem at the 11th nt, thereby releasing an approximately 70 nt stem-loop precursor miRNA (pre-miRNA) that possesses a 3´ overhang. Afterwards, the pre-miRNA is exported to the cytoplasm via Exportin-5. Once exported, the pre-miRNA is processed by a second endoribonucleolytic reaction, catalyzed by enzyme Dicer, yielding an approximately 22 nt RNA duplex (miRNA/miRNA*) with 30 overhangs at both ends. Subsequently, one strand (miRNA) of the duplex is incorporated into a miRNA-induced silencing complex (miRISC), whereas the other strand (miRNA*) is released and degraded. Nevertheless, miRNA* strands are not always by-products of miRNA biogenesis and can also be loaded into miRISC to function as miRNA (Figure 8) (61, 62).

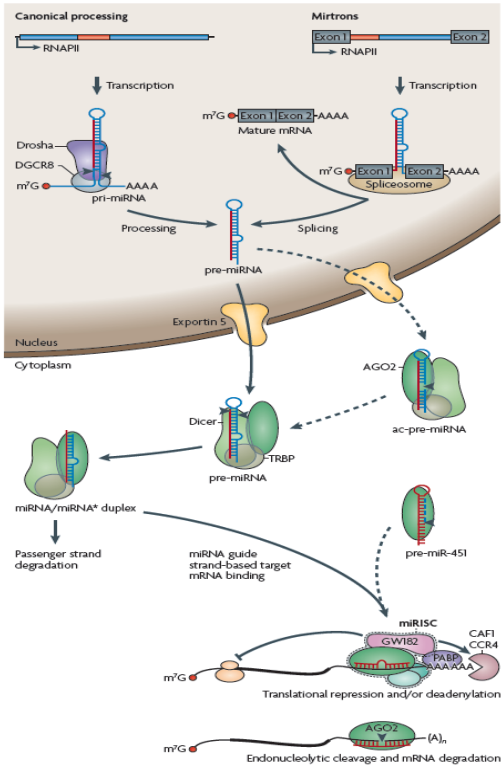

Figure 8. MicroRNA biogenesis (61).

MiRNAs can direct the RISC complex to down-regulate protein expression. To do so, RISC complex guides the miRNA to the specific target by complementary binding to the 3´UTR of target mRNAs (55, 63).

The interaction of base pairing of 2-8 nt needs to occur in the miRNA-mRNA association, also called seed region. RISC complex requires with mRNAs a pairing to the seed region to down-regulate gene expression by of two post-transcriptional mechanisms: either mRNA cleavage or transcriptional repression (55). Perfect complementary interactions between miRNA and target mRNA result in mRNA degradation. In contrast, mismatched interactions between the short RNA and the mRNA can result in translational repression. The majority of these interactions in animals are only partially complementary, therefore translational inhibition process predominates (64).

The regulatory action of miRNAs is complex. These non-coding RNAs can act directly on target mRNA transcripts having repressive effect, or indirectly by first regulating intermediate components such as transcription factors or co-transcription factors which control the expression of downstream genes. Together, various signalling cascades create a network with a few or several nodes (57).

In order to understand the functions of miRNAs in complex biological processes, it is important to experimentally assess the functional relevance of the predicted targeting site(s).

PHENOLIC COMPOUNDS

Phenolic compounds are molecules showing at least one aromatic ring with one or more hydroxyl groups attached. More than 8000 phenolic structures have been described (65). Phenolic compounds occur naturally in healthy plant tissues and are ubiquitous in all plant organs. Studies over the years have shown them to play a role in plant physiology, thus they are involved in growth, reproduction and resistance to pathogens and predators (66). The phenolic profile of vegetables depends on the type of plants, the conditions under which these plants are grown, and the harvest and storage conditions (67).

In the diet, these molecules are present in plant foods such as cereal grains, vegetables, fruits, nuts and berries and their related processed foods (juices, teas and wines) (65). They can be classified into different groups according to the number of phenol rings that they contain and the structural elements that bind these rings to one other. Distinctions are thus made between phenolic acids, flavonoids, stilbenes and lignans (68). Flavonoids are the most abundant polyphenols in our diet. This class of phenolic compounds is divided into a variety of subclasses differing in the level of oxidation and pattern of substitution of the C ring (69).

CONSUMPTION AND BIOAVALABILITY

The intake of polyphenols depends on dietary habits and preferences (70). Their consumption has been related to a reduction in the risk of different diseases, linked to their bioactions such as antioxidant, antinflammatory, antimicrobial and antiproliferative activities (71). However, most of the nutritional interest in phenolic compounds relates to the deleterious effects due to their adsorption into some macromolecules reducing food digestibility (66).

It is important to highlight that storage and culinary preparations can affect the content of polyphenols in foods. Oxidation reactions occur during storage periods and result in the formation of polymerized substances, which could be either beneficial (black tea), or harmful (browning of fruit).

Furthermore, simple peeling of fruits and vegetables can eliminate a significant portion of polyphenols, as these compounds are commonly present in the peel. These two examples are enough to support the idea that a considerable number of factors can modify the concentration of phenolic compounds in foods making the elaboration of reference food-composition tables difficult (68).

The biological properties of dietary phenolic compounds may depend on their absorption in the gut and their bioavailability. The percentage of absorption differs among phenolic compounds. Only those compounds released from the food matrix by the action of the digestive enzymes and bacterial microflora, are suitable for absorption in the gut and therefore potentially bioavailable (68, 72). Aglycones, compounds remaining after the glycosyl group on a glycoside is released, can be absorbed in the small intestine. To do this, these substances must be previously hydrolysed by intestinal enzymes. However, most of these compounds are present in food in the form of esters, glycosides or polymers that cannot be absorbed in their native form.

Phenolic compounds are highly metabolized via a common pathway. After absorption, phenolic compounds are conjugated in the phase II metabolism, in the small intestine and the liver. Those phenolic compounds not absorbed in stomach will reach the colon. Phenolic compound metabolites are more hydrophilic and subsequently they are excreted via feces and urine. In addition, phenolic compounds that are absorbed, metabolized in the liver and excreted in the bile or directly from the enterocyte back to the small intestine, will also reach the colon in a glucuronide form (68, 71) (Figure 9).

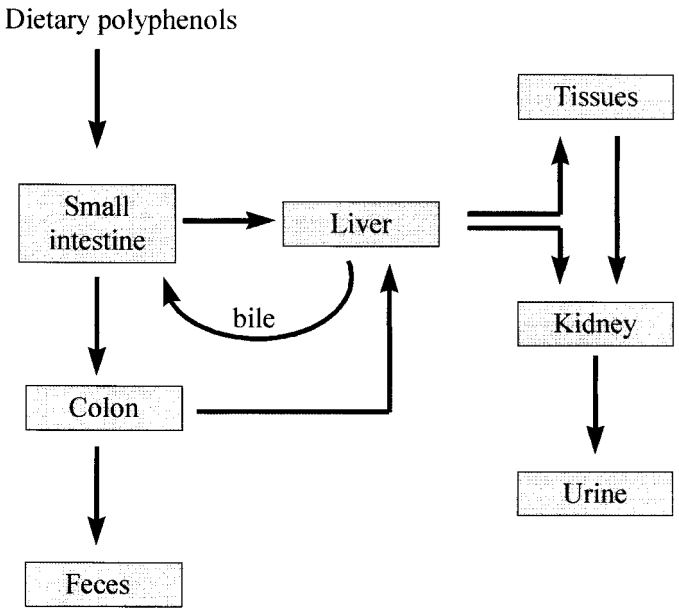

Figure 9. Possible routes for ingested phenolic compounds in humans (70).

RESVERATROL

Resveratrol (3,5,4-trihydroxystilbene) is a natural polyphenol with a stilbene structure. Takaoka, who isolated it from the root of *Veratrum grandiorum*, characterized its chemical structure in 1940 consisting of two phenolic rings bonded together by a double styrene bond. This double bound is responsible for the isomeric *cis*- and *trans*- forms of resveratrol, the second being the most stable from the steric point of view (73) (Figure 10). The property of this polyphenol to inhibit the progress of fungal infection has allowed its inclusion in the class of plant antibiotics known as phytoalexin (74).

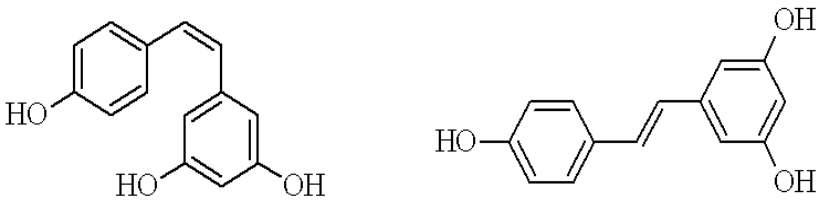

Figure 10. Isomeric *cis*- and *trans*- forms of resveratrol.

Resveratrol biosynthesis is restricted to only a few species commonly used for human consumption, such as peanuts, grapes and berries. However, red

wine is the main source of resveratrol in the Mediterranean diet (Table 1) (73, 75).

Table 1. Resveratrol content in different foodstuffs.

Food source	Total resveratrol amount (μg/kg)	*Trans*-resveratrol amount (μg/kg)	References
Red grape	3100	2500	Zamora-Ros *et al*. 2008
Red wine	8470	1810	Chiva-Blanch *et al*. 2011; Zamora-Ros *et al*. 2008
Chocolate	3325	692	Chiva-Blanch *et al*. 2011; Hurst *et al*. 2008
Grape juice	890	100	Chiva-Blanch *et al*. 2011; Zamora-Ros *et al*. 2008
Berries	80	80	Chiva-Blanch *et al*. 2011; Zamora-Ros *et al*. 2008
Toasted peanuts	55	55	Baur *et al*. 2006
Beer	15	9	Burns *et al*. 2002

*(Adapted from Saioa Gómez-Zorita´s thesis(76).

Although resveratrol exists naturally in both *cis*- and *trans*-isomers this second one appears to be more stable and predominant. *Trans*-resveratrol has often been reported to be the major natural form, even though the *cis*-isomer is also present in wine. *Cis*-isomerization occurs when the *trans*-isoform is exposed to artificial ultraviolet or natural daylight (73, 77).

Resveratrol bioavailability

After ingestion, resveratrol is absorbed by passive diffusion or by forming complexes with membrane transporters. This phenolic compound exhibits lipophilic characteristics, which allow high absorption. Thus in bloodstream, resveratrol can be found in three different forms: free and conjugated forms, glucuronide or sulphate. The free form can be bound to albumin and lipoproteins such as low-density lipoproteins (LDL) (73, 77). Phase II metabolism

of resveratrol leads to the production of conjugated forms in the small intestine and in liver.

Resveratrol is subjected to a metabolism carried out by UDP-glucuronosyl transferase enzymes synthesising resveratrol glucuronides and resveratrol sulphates. The preference sites of glucuronidation in resveratrol molecule, 3´and 4´positions, have been well established after synthesis of the metabolites. Similarly, the major site of sulphate conjugation is 4´position and was also established by synthesis (78). Differences in metabolites plasma levels were found between species. Sulphate metabolites following glucuronide are the main forms in rats, while glucuronide metabolites are predominant in humans (79, 80). The prolonged detection of low plasma levels of these resveratrol metabolites suggests that resveratrol, partly metabolized in the small intestine, is distributed to various tissues mainly in its conjugated forms restricting the bioavailability and reducing the efficacy of this phenolic compound *in vivo* (79, 81). Despite of that, resveratrol shows efficacy *in vivo*. This may be explained by the the entero-hepatic recirculation, when some of the metabolites formed in the liver will again be secreted into the intestine via the bile, where they will be removed to be reabsorbed, or continue to the colon. The gut microbiota forms dihydroresveratrol, which could be reabsorbed and metabolized again (73, 74, 82, 83).

The elimination of resveratrol, as well as that of its metabolites, is mainly produced by urinary route (53-85%). Several molecular forms of resveratrol and its metabolites have been detected in urine. Further, part of resveratrol not metabolized by colon bacteria appears also in feces, ranging from 0.3% to 38% of human dietary intake (78, 84).

Different approaches to enhance resveratrol bioavailability are being carried out. Research programs are currently exploring other methods such as co-administration with metabolism inhibitors, the use of resveratrol analogous or a drug delivery system using nanotechnology (75, 79).

Biological effects of resveratrol

Antioxidant and antinflammatory properties have been attributed to resveratrol. It is also effective in the prevention of several diseases including cardiovascular diseases, diabetes, cancer and recently, obesity (85).

With regard to obesity, this polyphenol reduces lipid accumulation, as it has been reported in different *in vitro* and *in vivo* studies. Differentiation of 3T3-L1 murine preadipocytes in *in vitro* studies induce metabolic pathways including the expression of different specific genes related to lipid metabolism, such as PPARγ, CCAAT/enhancer binding protein (C/EBP), SREBP 1, FAS, LPL and HSL. Resveratrol down-regulates the expression of all these genes, indicating that this polyphenol may alter fat mass by directly affecting biochemical pathways involved in adipogenesis (86). Furthermore, resveratrol treatments in mature adipocytes reduce triglyceride content and lipogenesis and increase fat mobilization, thus reducing fat accumulation (87, 88). As far as *in vivo* studies are concerned, most of them, performed in rats and mice, show a reduction in body weight and/or adipose tissue weight. Different mechanisms have been proposed, such as increased lipid oxidation or lipolysis, or decreased lipogenesis (89-94).

In vivo studies in rodents show a reduction in liver weight and/or lipid content (89, 92-97). The proposed mechanisms for this delipidating effect are decreased lipogenesis and increased lipid oxidation associated to mitochondriogenesis (89, 90, 92, 95, 97).

PTEROSTILBENE

Pterostilbene (3,5- dimethoxy-4´-hydroxystilbene) is a phytoalexin and a natural dimethylated analogue of resveratrol (98). The difference between these two phenolic compounds is that pterostilbene has two hydroxy groups substituted by two methoxy groups (Figure 11).

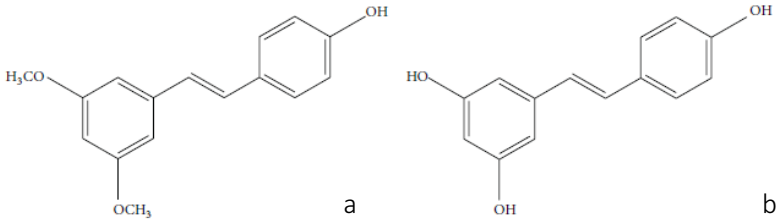

Figure 11. Chemical structure of pterostilbene (a) and resveratrol (b) (99).

This secondary metabolite of plants was originally isolated from heartwood of red sandalwood (98) and is present in different foods and drinks such as grapes, blueberries, nuts and red wine. Nevertheless, in these sources, it is generally found in very low quantities (100). It has been estimated that the content per blueberries varies from 99 ng to 520ng/gram depending on the type of berry ingested (99). Therefore, the amount of daily pterostilbene consumption varies according to dietary intake.

Pterostilbene bioavailability

After oral administration, pterostilbene undergoes a similar metabolism process to resveratrol. However, due to the presence of two methoxy groups, pterostilbene has better lipophylicity, absorption and higher potential for cellular uptake. In animal studies, pterostilbene has been shown to have 80% bioavailability compared to 20% for resveratrol. This fact gives pterostilbene potential advantages as a therapeutic agent (98, 99, 101).

Furthermore, pterostilbene in rats could saturate phase II metabolism reactions. Consequently, plasma levels of these phenolic compound might be higher than expected (102). Equimolar dose of resveratrol and pterostilbene have different profiles in their glucuronide and sulphate conjugates (101).

Biological effects of pterostilbene

As in the case of resveratrol, the multiple benefits of pterostilbene have been attributed to its antioxidant, antiinflammatory and anticarcinogenic properties, leading to improved function of normal cells and inhibition of malignant cells (99). In mouse, pterostilbene has been demonstrated to show

chemopreventive activity. This phenolic compound is cytotoxic to a number of cancer cell lines *in vitro* and it also was shown to have antioxidant and DNA synthesis inhibition activities. Additionally, pterostilbene has shown to significantly decrease plasma glucose levels (100).

Studies showing the delipidating effect of pterostilbene on adipocytes are scarce so far. An *in vitro* study in 3T3-L1 showed that pterostilbene could inhibit cell proliferation and adipogenesis in preadipocytes. These results suggest that pterostilbene has antiadipogenic effect on preadipocytes, differentiating adipocytes and mature adipocytes (103, 104). Moreover, an *in vivo* study performed in our laboratory showed a reduction in body fat accumulation and lipogenesis in adipose tissue and increased fatty acid oxidation in liver, thus demonstrating its antiobesity properties (105).

AIM AND OBJECTIVES

AIM

The aim of the present Doctoral Thesis was to assess changes induced by stilbenes to DNA methylation profile and microRNA expression in white adipose tissue and liver from rats fed an obesogenic diet.

SPECIFIC OBJECTIVES

The specific objectives of this experimental work were:

1. To study the effects of resveratrol and pterostilbene on the DNA methylation pattern of genes involved in triglyceride metabolism in white adipose tissue, and their relationship with obesity (Manuscript 1).

2. To analyse the effects of resveratrol on microRNAs regulation of genes involved in adipose tissue triglyceride metabolism and their relationship with obesity (Manuscript 2).

3. To assess the effect of resveratrol on the expression of the three major hepatic microRNAs (miRNA-103-3p, miRNA-107-3p and miRNA-122-5p), and their relationship with steatosis (Manuscript 3).

MATERIAL AND METHODS

MATERIAL AND METHODS

EXPERIMENT 1

Effects of stilbenes on DNA methylation levels in *fasn*, *pnpla2* and *pparγ* genes in white adipose tissue.

After a 6-day adaptation period, 32 male Wistar rats (6 week-old), were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Buguggiate, Italy) in a temperature controlled room (22±2°C) with 12-12 h light-dark cycles. The experiment was carried out according to the protocol accepted by the Ethics Committee for Animal Experimentation (CEBA CUIED/30/2010) of the University of the Basque Country.

The animals were randomly divided into four experimental groups (Figure 12):

- Control group (Control; n=8)
- High-fat high-sucrose group (HFS; n=8)
- Resveratrol group (RSV; n=8): fed a high-fat high-sucrose diet and treated with 30 mg/kg body weight/d of *trans*-resveratrol
- Pterostilbene group (PT; n=8): fed a high-fat high-sucrose diet and treated with 30 mg/kg body weight/d of pterostilbene

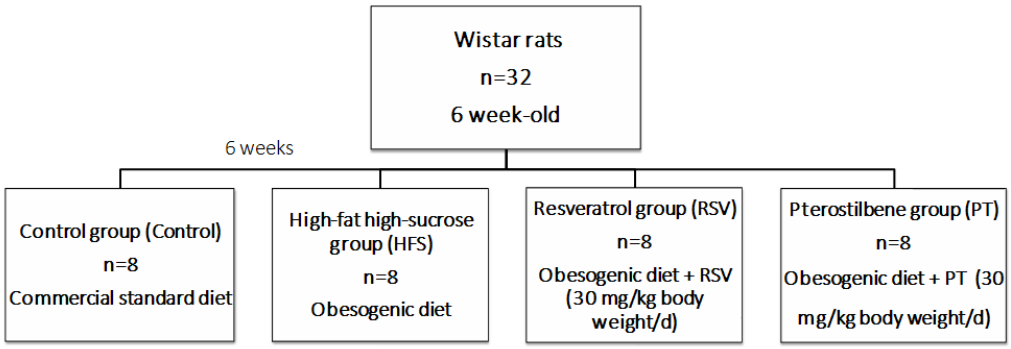

Figure 12. Experimental design of experiment 1.

The control group was fed a commercial standard diet (Harlan Iberica, Barcelona, Spain, TD.06416) which provided 3.7 kcal/g and 10% of calories as fat.

The other three groups, HFS, RSV and PT, were fed an obesogenic commercial diet (high-fat high-sucrose) (Harlan Iberica, Barcelona, Spain, TD.06415), consisting of 245 g/kg casein, 3.5 g/kg L-cysteine, 85g/kg corn starch, 115 g/kg maltodextrin, 200 g/kg sucrose, 195 g/kg lard, 30 g/kg soybean oil, 58 g/kg cellulose, 43 g/kg mineral mix, 3.4 g/kg biphasic calcium phosphate, 19 g/kg vitamin mix and 3g/kg choline bitartrate. The experiment was carried out during 6 weeks and the animals were given free access to food and water. Body weight and food intake were measured daily.

Trans-resveratrol (96.6% purity) (Monteloeder, Elche, Spain) and pterostilbene (>98% purity) (Bertin Pharma, Montigny le Bretonneux, France) were incorporated onto the diet surface daily, dissolved in absolute ethanol and at the beginning of the dark period. The time of the administration was chosen in order to match with the beginning of the rodent activity phase, thus, to ensure an immediate intake.

After 6 weeks of treatment, and after 12-hour-fasting period, animals were sacrificed under anesthesia with chloral hydrate. Sacrifice was performed by cardiac exsanguination. Fat depots from different anatomical locations [subcutaneous (SC), perirenal (PR), epididymal (EPI) and mesenteric (MS)], as well as liver were dissected, weighed and immediately frozen in liquid nitrogen. The samples were stored at -80°C until analysis.

In this experiment the following analyse were carried out:

- Pyrosequencing: *fasn*, *pnpla2* and *pparγ* enzymes were analysed by PyroMark MD pyrosequencer in perirenal adipose tissue.
- Gene expression: *fasn* was measured by RT-PCR (Real time-polymerase chain reaction) in perirenal adipose tissue.
- Nuclear DNMT (DNA methyltransferase) activity assay: EpiSeeker DNMT Activity Quantification Kit was used to measure DNMTs activity involved in the DNA methylation process in perirenal adipose tissue.

The detailed protocols are included in manuscript 1.

EXPERIMENT 2

Changes in microRNA profile induced by resveratrol in white adipose tissue.

After an adaptation period of 6 days, 16 male Wistar rats (6 week-old) were individually housed and treated following the same experimental design as described in the previous study.

The animals were randomly divided into two experimental groups (Figure 13):

- High-fat high-sucrose group (HFS; n=8)
- Resveratrol group (RSV; n=8): fed a high-fat high-sucrose diet treated with 30 mg/kg body weight/d of *trans*-resveratrol.

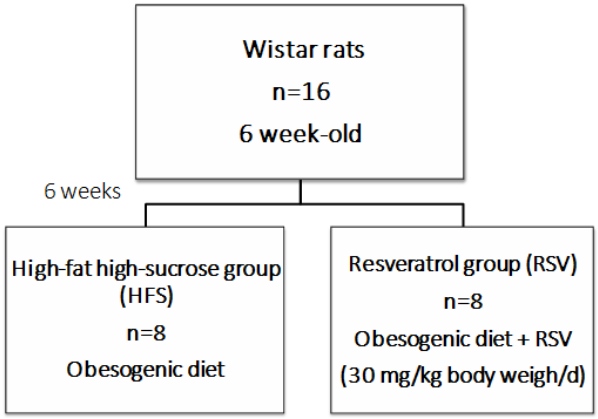

Figure13. Experimental design of experiment 2.

All animals were fed the same obesogenic diet (high fat-high sucrose) mentioned in experiment 1 (Harlan Iberica, Elche, Spain, TD. 06415) and also given free access to food and water. Body weight and food intake were measured daily. Resveratrol administration was performed as explained in the first experiment.

In addition, an *in vitro* study in3T3-L1 cells (American Type Culture Collection) was performed. Cells were cultured in Dulbecco´s Modified Eagle

Medium (DMEM) containing 10% fetal bovine serum (FBS)at 37°C in a humidified 5% CO_2 atmosphere.

In this experiment, the following analyses were carried out:

- MicroRNA array: 719 rat miRNA probes were performed in adipose tissue (ID 046 066 Agilent Technologies, Palo Alto, CA, USA).
- MicroRNA transfection: transfection of mimics mmu-miRNA-539-5p and mmu-miRNA-1224-5p was performed using a DeliverX™ Plus siRNA Transfection Kit in 3T3-L1 adipocytes.
- Protein expression: SP1, HSL, PPARγ and SREBP1 were measured by Western Blotting in perirenal adipose tissue and 3T3-L1 adipocytes.
- Gene expression: *fasn, hsl* and *ppar* were measured by RT-PCR in perirenal adipose tissue.

The detailed protocols are included in manuscript 2.

EXPERIMENT 3

Changes in microRNAs highly expressed in liver induced by resveratrol.

After the adaptation period, 16 male Sprague-Dawley rats (6 week-old) were individually housed and treated following the same experimental design as described in the previous study.

The animals were randomly divided into two experimental groups (Figure 14):

- High-fat high-sucrose group (HFS; n=8)
- Resveratrol group (RSV; n=8): fed a high-fat high-sucrose diet treated with 30 mg/kg body weight/d of *trans*-resveratrol.

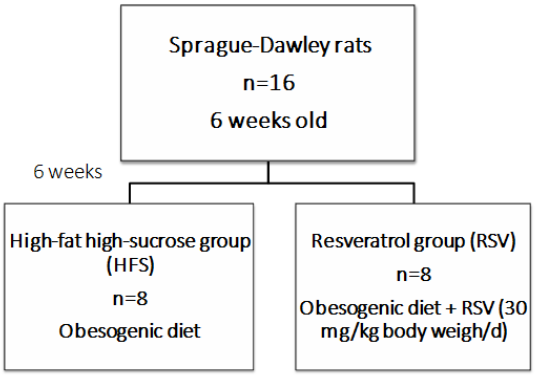

Figure 14. Experimental design of experiment 3.

All animals were fed the same obesogenic diet (high fat-high sucrose) mentioned in the previous experiments (Harlan Iberica, Elche, Spain, TD. 06415) and also given free access to food and water. Body weight and food intake were measured daily. Resveratrol administration was performed as explained in the first experiment.

In addition, an *in vitro* study with AML12 hepatocytes (ATCC CRL-2254) was performed. Cells were cultured 1:1 DMEM/HAM´S F12 glutamax medium containing 10% fetal bovine serum (FBS), 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone and 1%

Penicillin/Streptomycin (10,000 U/mL) at 37°C in a humidified 5% CO_2 atmosphere.

In this experiment the following analyses were carried out:

- MicroRNA analysis: rno-miRNA-103-3p, rno-miRNA-107-3p and rno-miRNA-122-5p expression was measured for qRT-PCR in liver.
- MicroRNA transfection: transfection of mimics mmu-miRNA-103-3p, mmu-miRNA-107-3p and mmu-miRNA-122-5p separately was performed using Lipofectamine RNAiMAX in AML12 hepatocytes.
- Protein expression: FAS, CPT 1aand SREBP1 were measured by Western Blotting in AML12 hepatocytes.

The detailed protocols are included in manuscript 3.

MANUSCRIPTS

MANUSCRIPT 1

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Fatty acid synthase methylation levels in adipose tissue: Effects of an obesogenic diet and phenol compounds

Genes Nutr (2014) 9:411

Abstract

DNA methylation is an epigenetic mechanism that can inhibit gene transcription. The aim of this study was to assess changes induced by an obesogenic diet in the methylation profile of genes involved in adipose tissue triacylglycerol metabolism, and to determine whether this methylation pattern can be altered by resveratrol and pterostilbene. Rats were divided into four groups. The control group was fed a commercial standard diet, and the other three groups were fed a commercial high-fat high-sucrose diet (6 weeks): the high-fat high-sucrose group (HFS), the resveratrol-treated group (RSV; 30 mg/kg/d) and the pterostilbene-treated group (PT; 30 mg/kg/d). Gene expression was measured by RT-PCR and gene methylation by pyrosequencing. The obesogenic diet induced a significant increase in adipose tissue weight. Resveratrol and pterostilbene partially prevented this effect. Methylation pattern of *ppnla2* and *pparγ* genes was similar among the experimental groups. In *fasn*, significant hypomethylation in -90bp position and significant hypermethylation in -62bp position were induced by obesogenic feeding. Only pterostilbene reversed the changes induced by the obesogenic diet in *fasn* methylation pattern. By contrast, the addition of resveratrol to the diet did not induce changes. Both phenolic compounds averted *fasn* up-regulation. These results demonstrate that the up-regulation of *fasn* gene induced by an obesogenic feeding, based in a high-fat high-sucrose diet, is related to hypomethylation of this gene in position -90bp. Under our experimental conditions, both molecules prevent *fasn* up-regulation, but this change in gene expression seems to be mediated by changes in methylation status only in the case of pterostilbene.

Key words: obesogenic diet, resveratrol, pterostilbene, methylation, fatty acid synthase

Running title: Obesogenic diet, phenols and fatty acid synthase methylation

Introduction

Epigenetic processes are those that act to regulate heritable changes in gene activity through remodelling of chromatin, but are not accompanied by changes in the DNA coding sequence. Major epigenetic mechanisms include DNA methylation, nucleosome remodelling and histone modifications. DNA methylation involves the addition of a methyl group to the 5-position of cytosine in the context of CpG dinucleotides, which are underrepresented in DNA. Clusters of CpGs, called CpG islands, are often found in association with genes, most often in the promoters and first exons (Jones and Takai 2001; Takai et al. 2001). Methylation of these CpG islands prevents gene transcription through different mechanisms in mammals. It can directly repress transcription by blocking the binding of transcriptional activators to cognate DNA sequences. Moreover, Methyl-CpG-binding proteins (MBPs) directly recognize methylated DNA and recruit co-repressors to silence transcription and to modify surrounding chromatin (Klose and Bird 2006). Epigenetic mechanisms such as DNA methylation have an implication in gene expression regulation, and they have become an interesting topic in the onset, development and therapy of several diseases. Although epigenetic changes take place mainly during embryogenesis (Morgan et al. 2005), gestation and lactation (Martínez et al. 2012), it has been demonstrated that some environmental changes could also induce variations in DNA methylation profile during adult life (Paternain et al. 2012).

Diet is one of the most determining modifiable environmental factors which affect epigenome (Park and Lee 2013). It has been reported that a wide range of dietary factors, which includes nutrients (folic acid, vitamin B_{12}, choline, methionine, fatty acids) and some bioactive food components (polyphenols) (Campión et al. 2010; Supic et al. 2013) can modify gene methylation. This methylation pattern can also be modified by the percentage of macro-nutrients in the diet (cafeteria diets rich in fat, diets rich in sucrose) (Milagro et al. 2009; Lomba et al. 2010b; Lomba et al. 2010a). With regard to polyphenols, several studies have shown that these molecules can modify gene methylation patterns, mainly in cancer prevention(Link et al. 2010; Henning et al. 2013; Saha et al.

2013), but very scarce data concerning adipocyte and adipose tissue genes have been reported (Boqué et al. 2013; Alberdi et al. 2013).

In previous studies from our laboratory we observed significant reductions in body fat in rats fed an obesogenic diet and treated with resveratrol (Macarulla et al. 2009; Arias et al. 2011; Miranda et al. 2013; Gómez-Zorita et al. 2013) or pterostilbene, a di-methylether derivative of resveratrol (manuscript submitted). Interest in pterostilbene is based on the fact that the substitution of a hydroxy with a methoxy group in polyphenols increases the transport into cells and increases the metabolic stability of the molecule (Wen and Walle 2006). Thus, the low bioavailability showed by resveratrol is increased in the case of pterostilbene (Kapetanovic et al. 2011).

In this context, the aim of the present work was to assess changes induced by an obesogenic diet in the methylation of genes involved in white adipose tissue triacylglycerol metabolism, and to determine whether this methylation pattern can be altered by resveratrol and pterostilbene.

Material and Methods

Animals, diets, and experimental design

The experimental procedure used in the present study followed the guidelines of the Animal Usage of the University of Basque Country (CUEID CEBA/30/2010). Six-week-old male Wistar rats (Harlan Ibérica, Barcelona, Spain) were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy). Animals were housed in a temperature controlled facility (22±2 ºC) and maintained under a light-dark cycle with 12 h of light and 12 h of darkness per day. After a 6-day adaptation period, the animals were randomly divided into four groups (n=8 per group) and fed the experimental diets for 6 weeks. Experimental diets were supplied by Harlan Ibérica (Barcelona, Spain). One group (control group) was fed a commercial standard diet (TD.06416) which provided 3.7 kcal/g and 10% of calories as fat. The other three groups, high-fat high-sucrose group (HFS), resveratrol-treated group (RSV) and pterostilbene-treated group (PT) were fed a commercial high-fat high-sucrose

diet (obesogenic diet) (TD.06415) which provided 4.6 kcal/g and 45% of kcal as fat. In RSV and PT groups, resveratrol or pterostilbene were added to the fresh diet daily, as previously described by Macarulla *et al*. (Macarulla et al. 2009) in amounts that ensured a dose of 30 mg/kg body weight/d.

Body weight and food intake were measured daily. At the end of the experimental period rats were sacrificed after an overnight fast under anaesthesia (chloral hydrate) by cardiac exsanguination. Adipose tissues from epididymal, perirenal, mesenteric and subcutaneous regions were dissected and weighed and then immediately frozen. All samples were stored at -80 ºC until analysis.

Epigenetic Study

Selection of relevant genes. For the epigenetic study, it was decided to include genes that satisfied the following two criteria; (a) genes involved in triacylglycerol metabolism, such as the lipoprotein-lipase (*lpl*), fatty acid synthase (*fasn*), acetyl-CoA-carboxylase (*acaca*), hormone-sensitive-lipase (*lipe*), adipose tissue triglyceride lipase (*pnpla2*), sterol regulatory-element binding transcription factor 1 (*srebf1*) or peroxisome proliferator-activated receptor γ (*pparγ*), and (b) genes with at least one CpG island in the gene promoter or first exon. The CpG Island Searcher Program (http://cpgislands.usc.edu) was used to identify which genes had CpG islands. Based on the fulfillment of these two criteria, only *fasn*, *pnpla2* and *pparγ*genes were selected for methylation analysis.

DNA samples. 150 mg of perirenal adipose tissue samples were used for DNA extraction. Isolation of DNA was performed using QIAamp DNA Investigator Kit (QIAGEN, Valencia, USA; Cat. No. 56504) following the manufacturer's instructions.

Pyrosequencing. For the pyrosequencing analysis, 1 µg of genomic DNA was bisulphyte converted using the Ez DNA Methylation Gold-Kit (Zymo Research; D5005; Irvine, CA, USA) according to the manufacturer’s protocol. Primers for

fasn, pnpla2 and *pparγ* were designed using the Assay Design Software (Qiagen, Valencia, USA) and synthesized by Fisher Scientific (Pittsburgh, PA). For each gene, four, three and two fragments were analysed respectively. PCR amplifications were performed using Qiagen HotStar Taq Plus DNA polymerase kit reagents (Qiagen, Valencia, USA), 7.5 µMbiotinylated primer, 15 µM non-biotinylated primer and 2 µl of bisulphyte-treated DNA (60ng). PCR conditions were as follows: 5 min at 95°C for enzyme activation followed by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 sec at 58°C and extension for 30 s at 72°C, with a final extension of 2 min at 72°C. PCR primer sequences and sequencing primer sequences are given in Table 1. The quality and quantity of the PCR product was confirmed by agarose gel (1%) electrophoresis before the cleanup and pyrosequencing analysis. Pyrosequencing was carried out using the PyroMark Gold Q96 Reagents (Qiagen, Valencia, USA) on a PyroMark MD pyrosequencer (Qiagen, Valencia, USA) and the methylation level was calculated using the Pyro Q CpG software (Qiagen, Valencia, USA).

Functional analysis. ALGGEN-PROMO (http://alggen.lsi.upc.es) bioinformatic program was used to identify potential transcription factors at those CpG sites that showed significant differences in the methylation level among groups.

Table 1. PCR and pyrosequencing primer sequences

GENES	ANALYZED ZONES AND PRIMER SEQUENCES		
fasn		Fwd	5´-GTGTGGAAGTTAGATGATAATT- 3´
	-90 to -62	Rev	5´-BIO-CTTAAAACTCTAATCTTATAACCACCTTAC-3´
		Seq	5´-GTTAGATGATAATTTTTAAGTGGG- 3´
		Fwd	5´-GGTTTGGGATTGGAAAGAGATT – 3´
	+754 to +812	Rev	5´-BIO-ACATATCTAAACTATATCCTTTTTACACCA-3´
		Seq	5´-GGATTGGAAAGAGATTGA -3´
		Fwd	5´-GAATTTTGGAAAATAAATATAGTGAGTGT-3´
	+951 to +997	Rev	5'-BIO-ATAACCCTTAATCCCCAACACT-3'
		Seq	5'-ATTTTGGAAAATAAATATAGTGAG-3'
pnpla2		Fwd	5'-GTAGGGTGTGGTGGAGAT -3'
	-205 to -162	Rev	5'-BIO-TTCTACCCCTCCTACTACACT-3'
		Seq	5'-GGGTGTGGTGGAGAT -3'
		Fwd	5´-BIO-GGTGTTTAGATTTGTATAAAATTTG- 3´
	+150 to +172	Rev	5´-CACCTACTAAACAAAACCATCT-3´
		Seq	5´-TCTCAAACTTTAACTTCTCAT-3´
		Fwd	5´-GATGTTTTTAAGGGAGATTAAGT- 3´
	+349 to +391	Rev	5´-BIO-AACCACTCCAATATAATAAACC-3´
		Seq	5´-GATTAAGTGGAATATTT- 3´
pparg		Fwd	5'-GGTTAGGAGGGTTATAGTGGAGTT -3'
	-431 to -388	Rev	5'-BIO-AACTATCACCAAATCCACACAAT-3'
		Seq	5'-GGTTTTTTTAGAAGGTGTT -3'
		Fwd	5´-BIO-GGTGATAGTTTAAGTAATTTGGT -3´
	-214 to -190	Rev	5´-ATAACCCCATTTTCCTCA-3´
		Seq	5´- CATTTTCCTCACACCTA-3

Fwd: Forward; Rev: Reverse; Seq: Sequence; BIO: Biotinylated; fasn: fatty acid synthase; pnpla2: adipose tissue triglyceride lipase; pparg: peroxisome proliferator-activated receptor γ

RNA extraction for expression analysis

Total RNA was isolated from the perirenal adipose tissue (100 mg) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In a following step DNase treatment (Applied Biosystems, Foster City, CA, USA) was carried out. The quantity of the purified RNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). After checking the suitable integrity (RIN> 7) of RNA (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA, USA), 1.5μg of total RNA were reverse transcribed into complementary DNA (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions.

Quantitative PCR

A 4.75 μL aliquot of each diluted complementary DNA sample was used for PCR amplification in a 12.5 μL reaction volume. The complementary DNA samples were amplified on an iCycler-MyiQ real-time PCR detection system (BioRad, Hercules, CA, USA) in the presence of SYBR® Green master mix (Applied Biosystems, Foster City, CA, USA) and a 300 nM concentration of each of the sense and antisense primers. Real-time PCR condition as well as *fasn* and *β-actin* primers were previously reported (Alberdi et al. 2011). PCR Specific primers were synthesized commercially (Integrated DNA Technologies, Leuven, Belgium): mRNA levels in all samples were normalized to the values of *β-actin* and the results expressed as fold changes of the threshold cycle (Ct) value relative to the controls using the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen 2001). Three technical replicates of each PCR reaction were done for each sample and the specificity of a quantitative PCR assay was confirmed by dissociation curve.

Nuclear DNA methyltransferase (DNMT) activity assay

Nuclear protein was extracted from perirenal adipose tissue (300 mg) using RIPA buffer (100 μL).15 μg nuclear proteins of each sample were used to measure DNMT activity by the EpiSeeker DNMT Activity Quantification Kit (ABCAM, Cambridge, UK; Cat. ab113467) following the manufacturer's protocol with minor modifications. Briefly, first incubation time was increased to 3 hours.

Incubation conditions were 37ºC with constant orbital shaking. The results were calculated as optical density/h/mg according to the manifacturer's instructions.

Statistical analysis

Results are presented as means ± standard error of the means. Statistical analysis was performed using SPSS 19.0 (SPSS Inc. Chicago, Illinois, USA).Analysis of variance (ANOVA) was used to determine the presence or absence of significant differences ($P<0.05$) in the analytical variables among the four groups of animals with different diets. Mixed linear model was used including "diet" as fixed effect and "animal" as random effect. The Tukey test was used as a *post-hoc* test for multiple comparison analyses among the four groups of animals. Correlation analysis was performed using Pearson´s correlation coefficient to determine relationships between *fasn* methylation in different positions and *fasn* expression (expressed as $2^{-\Delta\Delta}$Ct). Statistical significance was set-up at $P< 0.05$.

Results

Body weight and adipose tissue weights

High-fat high-sucrose feeding resulted in increased body weights as compared to control diet fed animals. When obesogenic diet supplemented with resveratrol or pterostilbene were fed to the rats, significantly lower body mass was observed in the resveratrol and pterostilbene offered groups compared to the HFS group. No differences in energy intake among the three HFS fed animals were found, and all these groups showed higher caloric intakes than their standard diet fed counterparts (Table 2).

Table 2. Body weight, energy intake and adipose tissue weights of rats fed control diet or high-fat, high-sucrose diets supplemented or not with resveratrol or pterostilbene for 6 weeks

	Control	HFS	RSV	PT	ANOVA
Final weight (g)	290 ± 4 [a]	403 ± 7 [b]	363 ± 6 [c]	373 ± 9 [c]	*P*<0.001
Energy intake (kcal/d)	65.5 ± 2.3 [a]	73.0 ± 2.1 [b]	69.6 ± 2.6 [b]	72.9 ± 3.1 [b]	*P*<0.05
Adipose tissue weights (g)					
Epididymal	4.0 ± 0.7 [a]	15.3 ± 0.7 [b]	13.0 ± 0.7 [b, c]	10.0 ± 0.8 [c]	*P*<0.001
Perirenal	4.7 ± 0.7 [a]	16.9 ± 0.7 [b]	11.9 ± 0.8 [c]	10.9 ± 0.8 [c]	*P*<0.001
Mesenteric	1.4 ± 0.6 [a]	6.5 ± 0.6 [b]	4.4 ± 0.6 [c]	4.1 ± 0.6 [c]	*P*<0.001
Subcutaneous	5.2 ± 1.1 [a]	20.0 ± 1.5 [b]	13.1 ± 1.9 [c]	10.9 ± 1.1 [c]	*P*<0.001
E + PR + M	10.4 ± 0.3 [a]	37.2 ± 2.0 [b]	30.8 ± 1.2 [c]	25.8 ± 2.1 [d]	*P*<0.001
PR + M	6.5 ± 0.3 [a]	22.4 ± 1.0 [b]	17.7 ± 0.8 [c]	15.7 ± 1.6 [c]	*P*<0.001
Σ Adipose tissues	17.3 ± 0.3 [a]	56.1 ± 2.9 [b]	42.7 ± 1.9 [c]	35.6 ± 4.7 [c]	*P*<0.001

Values are means ± SEM (n = 8). Differences among groups have been determined by ANOVA, post-hoc analysis Tukey. E: epididymal; PR: perirenal; M: mesenteric. HFS: High-fat high-sucrose; RSV: resveratrol; PT: pterostilbene

The obesogenic diet induced a significant increase in adipose tissue weight in all the anatomical locations analyzed. When resveratrol or pterostilbene were included in the diet an important reduction in all the adipose depots, except for epididymal adipose tissue in animals fed RSV-supplemented diet, was observed. These molecules prevented the increase in fat accumulation induced by the obesogenic diet but only partially because adipose tissue weights in RSV and PT groups were higher than those in control rats. When comparing resveratrol- and pterostilbene-treated rats, statistical differences were only detected in the sum of epididymal+ perirenal+ mesenteric adipose depots. PT group presented an additional 16% reduction in the visceral fat (Table 2).

DNA methylation

Taken as a whole, the methylation pattern of *ppnla2* and *pparγ* genes was not significantly different among the four experimental groups (data not shown). With regard to *fasn,* no relevant differences in total methylation status were found among the groups (79-80 % methylation for all the groups) in the perirenal adipose tissue. However, some variations were observed in specific DNA positions. All the changes observed in HFS, RSV and PT groups when

compared with the control group, expressed as percentage, are summarized in Table 3. Only those higher than 5% were considered since this is the sensitivity threshold of the pyrosequencing assay.

Table 3. Changes in the methylation status of the measured positions in *fasn* gene, expressed as percentage

Groups	CpG site (position)								
	-90	**-62**	**+754**	**+791**	**+812**	**+951**	**+956**	**+962**	**+997**
HFS	-11	+6	-1	0	-1	-3	-7	+1	-2
RSV	-10	+6	-2	-2	-3	-1	-2	+2	+2
PT	-1	+3	+2	+2	+1	-4	-3	0	-2

Data for each CpG site represent the mean percentage of methylation change compared to the Control group. HFS: high fat- high sucrose; RSV: resveratrol; PT: pterostilbene

As far as obesogenic feeding is concerned, when rats from HFS group were compared to the control group, significant hypomethylation in -90bp position (P<0.001) and significant hypermethylation in -62bp position (P<0.01) were observed (Figure 1; Table 3). The addition of resveratrol and pterostilbene to the obesogenic diet led to different methylation patterns of *fasn*. In the case of pterostilbene, according to changes in positions -90bp and -62bp, it can be stated that this molecule reversed the changes induced by the obesogenic diet (Figure 1; Table 3). By contrast, when HFS group was compared to the RSV group no significant changes in methylation status were observed (Figure 1; Table 3).

In the four experimental groups methylation status was lower in positions after the TSS (+754bp to +956bp) (average methylation 66%) than in the rest of the promoter region (average percentage 90.5%) (Figure 1). This fits with a canonical pattern of methylation (Davies et al. 2012).

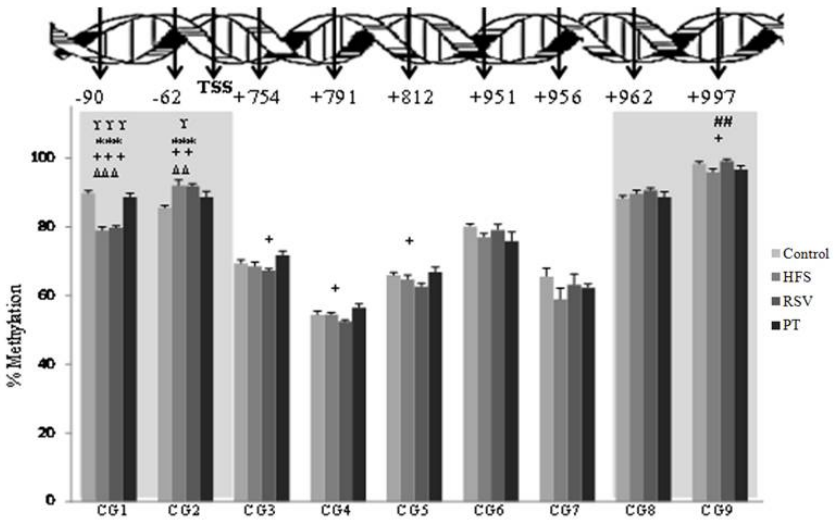

Figure 1

Figure 1. *Fasn* methylation pattern in selected CpG sites in perirenal adipose tissue of rats fed a control diet or high-fat, high-sucrose diets supplemented or not (HFS) with resveratrol (RSV) or pterostilbene (PT) for 6 weeks. Values are presented as means ± standard error of the means. Symbols in bars show statistical differences between groups as follows: Control *vs* HFS: ***P<0.001; Control *vs* RSV: ϒP<0.05; ϒϒϒP<0.001; HFS *vs* RSV: ## P<0.01; HFS *vs* PT; ΔΔ P<0.01; ΔΔΔ P<0.001; PT *vs* RSV; + P<0.05; ++ P<0.01; +++ P<0.001. TSS: transcriptional start site.

Gene expression of fatty acid synthase

Fasn expression was significantly increased (five-fold) in rats fed the obesogenic diet (HFS group) as compared to the control rats. Resveratrol and pterostilbene prevented this effect. Thus, expression values in these two groups were significantly lower than that of the HFS group, but similar to that of control rats (Figure 2).

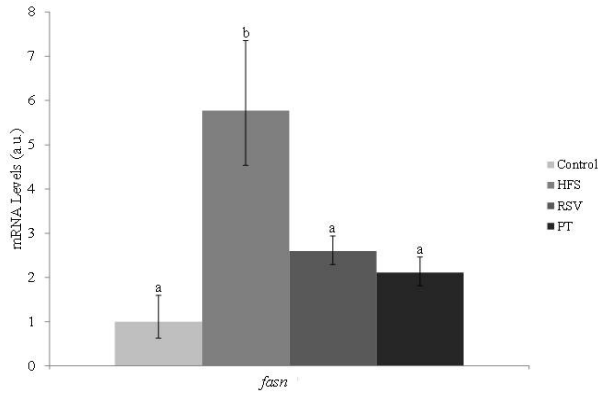

Figure 2

Figure 2. Gene expression of fatty acid synthase in perirenal adipose tissue of rats fed a control diet or high-fat, high-sucrose diets supplemented or not (HFS) with resveratrol (RSV) or pterostilbene (PT) for 6 weeks. Values are presented as means ± standard error of the means. [a,b] Bars not sharing common letter are significantly different (P<0.05). *Fasn*: fatty acid synthase, HFS: high-fat high-sucrose.

Correlation analysis between methylation and expression in fasn gene

Pearson´s correlation coefficients were calculated when significant differences in *fasn* methylation status were found between groups (Figure 1). Significant correlations were only found in -90bp position when control and HFS groups(*P*=0.01; Pearson-coefficient: 0.708), as well as HFS and PT groups (*P*=0.05; Pearson-coefficient: 0.648)were considered(Figure 3).

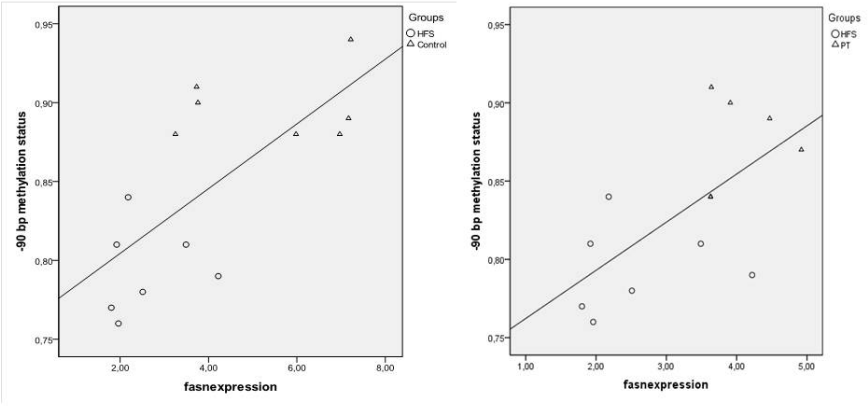

Figure 3. Pearson's correlations between percentage of DNA methylation and gene expression for *fasn* in rats from Control and HFS groups (A) and in rats from HFS and PT groups (B) were compared. Statistical significance was set-up at $P<0.05$. HFS: high-fat high-sucrose; PT: pterostilbene.

Nuclear DNA methyltransferase activity

Nuclear DNA methyltransferase (DNMT) activity showed a decrease in HFS group when compared with the control group, which showed a $P=0.061$. No differences were found among the other three experimental groups (Figure 4).

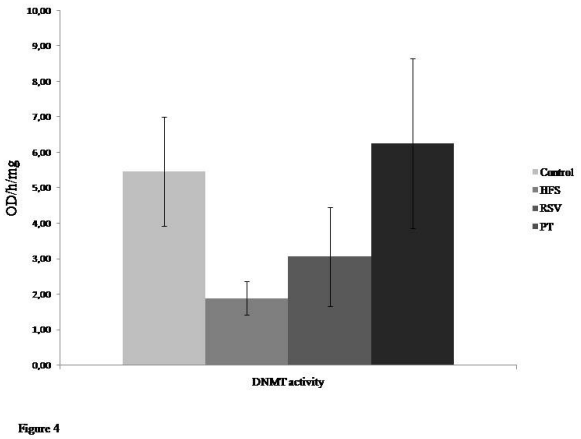

Figure 4. Nuclear DNA methyltransferase activity in perirenal adipose tissue of rats fed a control diet or high-fat high-sucrose diets supplemented or not (HFS) with resveratrol (RSV) or pterostilbene (PT) for 6 weeks. Values are presented as means ± standard error of the means.

Discussion

As expected, feeding a diet rich in fat and sucrose led to increased energy intake, and consequently to increased fat accumulation, as observed when rats from HFS group were compared with rats fed the control diet. The addition of resveratrol or pterostilbene in the HSF diet at a dose of 30 mg/kg/d prevented this effect, but only partially because rats in groups RSV and PT did not reach the values showed by control animals in body weight and adipose tissue weights. In general terms, no important differences were observed between both phenolic compounds.

As explained in the Material and Method section, in the present study we were interested in the methylation of *lpl*, *fasn*, *acaca, lipe, pnpla2, srebf1* and *pparγ* because these are genes play important roles in the control of triacylglycerol metabolism in adipose tissue. Moreover, these genes have been shown to be targets for resveratrol (Rivera et al. 2009; Szkudelska and Szkudelski 2010; Baile et al. 2011; Kim et al. 2011; Alberdi et al. 2011; Gómez-Zorita et al. 2012; Lasa et al. 2012). Nevertheless, when CpG-rich areas were investigated we observed that only *fasn, pnpla2* and *pparγ* genes showed these areas near to the gene promoter. Consequently, only these three genes were studied.

The pattern of methylation showed by *pnpla2* and *pparγ* in control rats was not altered in rats from HFS, RSV and PT groups, suggesting that under the present dietary conditions this epigenetic mechanism was not important in the regulation of these genes. By contrast, several changes were observed in *fasn*. Thus, the following discussions will focus on this gene.

Feeding a high-fat high-sucrose diet led to a significant increase in *fasn* expression. It is well documented that while high-fat diets decrease the expression of this gene (Duran-Montgé et al. 2009; Jiang et al. 2009), diets rich either in simple carbohydrates (sucrose) or carbohydrates with high glycemic index, induce an increase (Kim and Freake 1996; Kabir et al. 1998; Morris et al. 2003). It seems that in the present study, the effect of high sucrose content was greater than that of high fat content and the final effect was an up-regulation of *fasn*. These results agree with those reported by Yang *et al*. (Yang et al. 2012) when using this type of diet. Taking into account that this enzyme catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA, and thus it is one of the rate-limiting enzyme in *de novo* lipogenesis, it can be proposed that the increase in body fat induced by the high-fat, high-sucrose diet was due, at least in part, to increase in fatty acid synthesis.

Obesogenic diet induced significant DNA methylation changes with respect to the controls. We observed a mild but significant methylation increase in -62bp position, while a decrease in -90bp, being only this one significantly correlated with the overexpression of *fasn*. It has been previously reported that small

methylation changes can be associated with gene expression variability that exert significant effects on phenotype (Irizarry et al. 2009), and even if we think that not all the expression change observed for *fasn* should be attributed to this methylation change at position -90bp, we believe that it truly contributed to the down-regulation of *fasn.* Interestingly, nuclear DNMT activity showed a similar pattern of response to DNA methylation level at -90bp, this meaning that it could be a mechanism, among others, which justifies the observed effects of high-fat high-sucrose feeding and pterostilbene on *fasn* methylation.

To better understand the mechanism by which DNA methylation level in the -90bp position of *fasn* could be related to the decrease in gene expression, we searched for consensus response elements around this position -90bp position of *fasn* promoter is a binding site for Sp1, an ubiquitous transcription factor which acts as a glucose sensor (Vaulont et al. 2000). It has been demonstrated that Sp1 is crucial for *fasn* gene promoter activity in adipocytes (Rolland et al., 1996). Consequently, the influence of the observed methylation status changes on the regulation of *fasn* transcription mediated by Sp1 cannot be discarded.

Hypomethylation of *fasn* induced by a high-fat, high-sucrose feeding was previously reported by Uriarte *et al.* (Uriarte et al. 2013). When comparing this study with the present one, two important issues should be underlined. On the one hand, Uriarte *et al.* observed hypomethylation in *fasn* after 20 weeks of feeding a high-fat high-sucrose diet. In the present study, this effect was observed after 6 weeks of the same dietary treatment, meaning that this epigenetic mechanism does not need very long periods to take place. On the other hand, the method used by Uriarte *et al*. to measure DNA methylation (MALDI-TOF mass spectrophotometry) was different from that used in the present study. The fact of finding similar results by using two methods with differences in specificity, sensibility and accuracy reinforces the hypomethylating action of this dietary pattern on *fasn*.

Resveratrol and pterostilbene treatments led to significant decreases in *fasn* expression. mRNA levels found in RSV and PT groups were not different from those found in the control group, meaning that these molecules totally

prevented the alteration induced by the obesogenic diet. This effect was involved, at least in part, in the obesity prevention action showed by these phenolic compounds. As far as resveratrol effects are concerned, the methylation pattern in the investigated regions of *fasn* gene remained unchanged in RSV group when compared with the HSF group, suggesting that gene expression changes were not likely to be associated with modifications in DNA methylation. By contrast, pterostilbene reversed the changes induced by the obesogenic diet in positions -90bp and -62bp, and the percentage of methylation in these regions was similar in PT and control groups. Pearson's correlations revealed that only hypermethylation in -90bp position showed significant correlation with *fasn* expression. These results, together with those obtained when the effects of obesogenic diet were analysed, reinforce the relevance of methylation in -90bp position in the control of *fasn* expression by the diet.

Taken as a whole, the results obtained in the present study demonstrate that the up-regulation of *fasn* gene induced by an obesogenic feeding based in a high-fat high-sucrose diet is related to hypomethylation of this gene in position -90bp. Under our experimental conditions, both resveratrol and pterostilbene prevent *fasn* up-regulation, but this change in gene expression seems to be mediated by changes in methylation status only in the case of pterostilbene.

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MANUSCRIPT 2

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Involvement of miR-539-5p in the inhibition of *de novo* lipogenesis induced by resveratrol in white adipose tissue

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Abstract

The epigenetic mechanisms of action of resveratrol as an anti-obesity molecule have not been fully addressed so far. The aim of the present study was to assess changes produced by resveratrol in microRNA (miRNA) profile in white adipose tissue (WAT) and to relate these changes to those induced in the expression of genes involved in triacylglycerol metabolism. Male Wistar rats were fed (6 weeks) an obesogenic diet: a control group and a group treated with resveratrol (30 mg/kg/d). A miRNA microarray was carried out in perirenal adipose tissue. Overexpression of miR-539-5p and miR-1224-5p was performed in 3T3-L1 cells. Protein expression was analysed by western-blot and gene expression by qRT-PCR. Associations between variables were assessed by Pearson´s correlations. The microarray showed that 3 miRNAs were decreased and 13 were increased after resveratrol treatment. Among those miRNAs increased, miR-129, miR-328-5p and miR-539-5p showed predicted target genes relevant for triacylglycerol metabolism in WAT (pparγ: peroxisome proliferator-activated receptor gamma, hsl: hormone sensitive lipase and sp: SP1 transcription factor) in miRWalk Database. Moreover, the literature shows that miR-1224, another miRNA up-regulated by resveratrol, can also regulate sp1. Among the three targets, only SP1 showed a reduction in protein expression. Correlation and overexpression studies revealed that the decrease in SP1 protein expression was only associated with the increase of miR-539-5p. In addition, significant reductions in SREBP1 protein expression and fasn gene expression were found in resveratrol-treated rats. In conclusion, the up-regulation of miR-539-5p is involved in the inhibition of de novo lipogenesis induced by resveratrol in WAT.

Key words: adipose tissue, *de novo* lipogenesis, microRNAs, resveratrol, SP1.

1 INTRODUCTION

Resveratrol has been shown to elicit anti-obesity properties in animal models such as mice (91, 94, 108), rats (109-111) and primates (112). This polyphenol is a trans-3,5,4'-trihydroxystilbene occurring naturally in various plants, including grapes, berries and peanuts, in response to stress as a defense mechanism against fungal, viral and bacterial infections, and damage from exposure to ultraviolet radiation (113, 114).

The mechanisms of action of resveratrol as an anti-obesity molecule have been studied and reported in the literature: reduction in proliferation and differentiation of pre-adipocytes, increase in apoptosis, increase in lipid mobilization and fatty acid oxidation, and decrease in de novo lipogenesis (88, 115). However, the vast majority of these studies have not addressed this issue at an epigenetic level.

Epigenetics involves the control mechanisms of gene-activity-describing-pathways which are different from those directly attributable to the DNA sequence, and which have an influence on the adaptive response of an organism (116). Epigenetic mechanisms include non-coding RNAs, such as microRNAs (miRNAs) (117).

MiRNAs are small, non-coding RNAs which regulate the expression of specific target gene post-transcriptionally, mainly by suppressing translation and/or reducing the stability of their target mRNAs (55, 118). MiRNAs are essential regulators of diverse biological processes, comprising lipid metabolism and pre-adipocyte differentiation (119-121).

It has been reported that miRNAs can mediate the effects of nutrition. A wide range of dietary factors, which includes micronutrients and non-nutrients such as polyphenols, can modify the expression of miRNAs (122). The effects of several polyphenols on miRNAs involved in cancer have been widely reported (52, 123-132). By contrast, results concerning their effects on miRNAs involved in triacylglycerol metabolism are scarcer, and they have mainly addressed in hepatocytes and liver. Moreover, these studies have not focussed on resveratrol, but on other individual polyphenols such as proanthocyanidins(133, 134),

quercetin, heperidin, naringenin, anthocyanin, catechin, proanthocyanin, caffeic acid, ferulic acid and curcumin in liver (135), or on plant extracts, such as Hibiscus sabdariffa(136). As far as we know, there are no reported studies devoted to assessing the effects of resveratrol on miRNAs involved in triacylglycerol metabolism, and more specifically in adipose tissue.

In this context, the aim of the present study was to assess the changes produced by resveratrol in miRNA profile in white adipose tissue from rats and to explore whether these modifications can be related to changes induced by this polyphenol in triacylglycerol metabolism in this tissue.

2 EXPERIMENTAL

2.1 Animals, diets and experimental design

The experiment was conducted using sixteen six-week-old male Wistar rats purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the institution's guide for the care and use of laboratory animals (CUEID CEBA/30/2010). The rats were individually housed in polycarbonate metabolic cages (TechniplastGazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 $\pm$ 2ºC) with a 12 h light-dark cycle. After a 6-day adaptation period, rats were randomly divided into two dietary groups of eight animals each, namely a control group and a group treated with resveratrol (30 mg/kg/d) fed a commercial obesogenic diet, high in sucrose (20.0%) and fat (22.5%) (Harlan Iberica, TD.06415) for 6 weeks. Resveratrol, supplied by Monteloeder (Elche, Spain), was added to the diet as previously reported (137) in order to ensure a dose of 30 mg resveratrol/kg body weight/d. All animals had free access to food and water. Food intake and body weight were measured daily. This cohort of animals had been previously used in another study reported by our group (138). The parameter food efficiency was calculated as Δbody weight/100 kcal.

At the end of the experimental period, animals were sacrificed under anaesthesia (chloral hydrate) by cardiac exsanguination after a 12-hour fasting period. Adipose tissue from different anatomical locations (perirenal,

epididymal, mesenteric and subcutaneous) was dissected, weighed and immediately frozen.

2.2 MicroRNA microarray

Total RNA containing small RNA species was extracted from perirenal adipose tissue with a microRNA extraction kit (mirVana, Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA quality was assessed in a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) by using a Eukaryote Total RNA Nano and Small RNA assays (Agilent Technologies, Palo Alto, CA, USA). The quantity of the purified RNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

After checking whether the concentration and purity were sufficient for hybridization, four samples from each group were used for microarray analysis. The microarray was performed using a rat miRNA microarray (ID 046 066 Agilent Technologies, Palo Alto, CA, USA) where 719 rat miRNA probes were represented (content sourced from the miRBase database Release 19.0). For labeling, hybridization, washing and scanning the miRNA Microarray System with miRNA Complete Labeling and Hyb KITG v. 2.2 protocols were followed according to manufacture recommendations (Agilent Technologies, Palo Alto, CA, USA).

2.2.1 Validation of miRNAs

The validation process was performed in two steps. The first one, previous to any further analysis, consisted in the random selection of two miRNA among those with low fold change values, miR-211-5p (up-regulated) and miR-511-5p (down-regulated). In a second step, after analyzing changes in gene and protein expression of target genes for the miRNAs significantly modified by resveratrol, miR-539-5p and miR-1224 were also chosen (Figure1). This second step reinforced the validation process. In both steps the validation was carried out with qRT-PCR in all experimental samples (n=8/group).

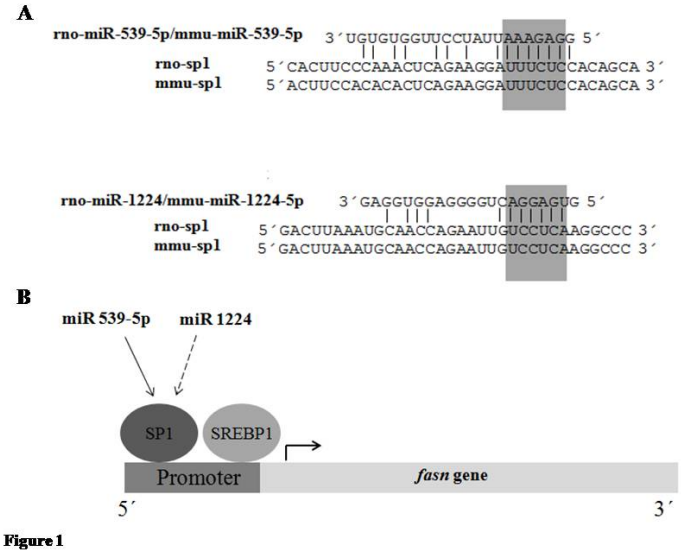

Figure 1. A. Alignment of rno-miR-539-5p/mmu-miR-539-5p and rno-miR-1224/mmu-miR-1224-5p binding sites in 3´ untranslated region (3´UTR) of sp1 transcription factor mRNA. B. MicroRNA-gene and gene-gene regulatory network involved in fatty acid synthase pathway. rno: rattusnorvergicus. mmu: musmusculus. SP1: Sp1 transcription factor, SREBP1: sterol regulatory element-binding protein 1, fasn: fatty acid synthase.

Total RNA (5 ng) was reverse-transcribed using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and the miRNA-specific reverse-transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). For the reverse transcription, iCycler™ Thermal cycler (Applied Biosystems, Foster City, CA, USA) was used with the following conditions: 16°C for 30 min; 42°C for 30 min and 85°C for 5 min. 1.33 µL of miRNA-specific cDNA from this reaction was amplified with the TaqMan® Universal PCR master mix and the respective specific probe provided in the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA, USA). The targeted miRNA assay sequences were as follows:

rno-miR-211-3p	5'-GGCAAGGACAGCAAAGGGGG-3'
rno-miR-1224	5'- GUGAGGACUGGGGAGGUGGAG -3'
rno-miR-511-3p	5'-AAUGUGUAGCAAAAGACAGGA-3'

rno-miR-539-5p 5´-GGAGAAAUUAUCCUUGGUGUGU-3´

PCR was performed in an iCycler™–MyiQ™ Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA). Amplification was performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. U6 small nuclear RNA was used as an endogenous control. All mRNA levels were normalized to the values of U6 snRNA. The results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method (139).

2.2.2 Predicted target genes for miRNAs modified by resveratrol

Sequences of miRNAs up- and down-regulated by resveratrol were obtained in MIRBASE (mirbase.org). In order to obtain the predicted target genes for these miRNAs, a comparative analysis by five algorithms (miRanda, miRDB, miRWalk 1.0, RNA22 and Target Scan) was performed according to the miRWalk Database (140). After checking the function of all these predicted genes in Gene Cards (http://www.genecards.org), only those with a well-documented involvement in the anti-obesity effect of resveratrol were selected.

2.3 Cell culture

3T3-L1 preadipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% fetal bovine serum (FBS). One day after, the cells were stimulated to differentiate with DMEM containing 10% FBS, 10 µg/mL insulin, 0.5 mMisobutylmethylxanthine (IBMX) and 1 µM dexamethasone for 2 days.On day 2, the differentiation medium was replaced by 10% fetal bovine serum/DMEM medium containing 0.2 µg/mL insulin and incubated for 2 days. This medium was changed every two days until the 5 day, when the cells were harvested. All media contained 1% Penicillin/Streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of Biotin and Panthothenic Acid. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere.

2.4 miRNA transfection

Adipocytes in day five of differentiation, and at a confluence status of approximately 90%, were transfected with DeliverX™ Plus siRNA Transfection Kit (Affimetrix, Santa Clara, CA) prepared following manufacturer´s protocol with mirVana miRNA mimics of mmu-miR-539-5p and mmu-miR-1224-5p (homologous to rno-miR-539-5p and rno-miR-1224 respectively) (Figure 1A) (Applied Biosystems, Foster City, CA, USA) at a final concentration of 30 mM. Optimal transfection conditions were determined in previous experiments, and transfection efficiency was assessed using miRNA probes and fluorescent transfection controls. Cell transfection period was established 48 hours. To rule out unspecific effects, control cells were transfected with negative controls.

2.5 Protein expression analysis

2.5.1 Hormone sensitive lipase, peroxisome proliferator-activated receptor gamma and sterol regulatory element-binding protein 1

For hormone sensitive lipase (HSL) protein extraction, 100 mg of perirenal adipose tissue were homogenized in a PBS buffer with protease inhibitors (pH 7.4) and centrifuged (800 g, 5 minutes, 4ºC). In the case of peroxisome proliferator-activated receptor gamma (PPARγ and sterol regulatory element-binding protein 1 (SREBP1), a nuclear protein extraction was carried out with 100 mg of perirenal tissue, as previously described (141).

Immunoblot analyses were performed in all samples (n=8/group) using 40 µg of protein for HSL and 10 µg and 30 µg of protein for PPARγ and SREBP1 respectively, separated by electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred to PVDF membranes. Subsequently, the membranes of the two assays were blocked with casein PBS-Tween buffer for 2 hours. These membranes were incubated overnight at 4ºC with HSL antibody (1:1000), PPARγ antibody (1:1000) or SREBP1 antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) respectively. Afterwards, polyclonal mouse anti-β-actin (1:5000) (Sigma, St. Louis, MO, USA), and rabbit anti-HSL antibody (1:5000) (Sigma, St. Louis, MO, USA) were incubated for 2 hours at room

temperature. Antibodies were visualized by using a chemiluminescent substrate (Thermo Scientific, Wilmington, DE, USA) and quantified by a ChemiDoc MP imaging system (BioRad, USA). After antibody stripping of PPARγandSREBP1, the membranes were blocked and then incubated with a mouse anti-PPARγand mouse anti-SREBP1 (Sigma, St. Louis, MO, USA), and measured again. The measurements were normalized by actin.

2.5.2 SP1 transcription factor

300 mg of perirenal adipose tissue were homogenized in a PBS buffer with protease inhibitors (pH 7.4) and centrifuged (14,000 g, 1 minute, 4ºC). The pellet was resuspended in 100 µL of radioimmunoprecipitation assay buffer (RIPA buffer). The homogenates were centrifuged at 36,000 g for 10 min at 4°C. In the case of 3T3-L1 cells, total protein was extracted with 200 µL of lysis buffer (trisHCl 2mM, sodium chloride (NaCl) 0.1M, Triton 1%, glycerol 10%, sodium orthovanadate (OvNa) 1mM, EDTA 2mM, phenylmethylsulfonyl fluoride (PMSF) 1mM, sodium fluoride (FNa) 2mM and protease inhibitor 1%) and centrifuged (12,000 g, 15 minute, 4ºC). The protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Wilmington, DE, USA).

For the SP1, inmunobloting after immunoprecipitation was performed. A total of 100µg of adipose tissue extracts or 20µg of 3T3-L1 protein extract, were diluted with three volumes of PBS (with added protease inhibitors). SP1 was immunoprecipitated with 1:20 of SP1 antibody (Santa Cruz Biotech, CA, USA) in constant rotation, at 4°C, overnight. Afterwards, 20 µL Protein G Agarose (Santa Cruz Biotech, CA, USA) was added to each sample, and these were rotated for 3 h at 4ºC. The immunoprecipitated samples (n=8/group) were then washed three times with 500 µL PBS buffer. A total of 40 µg of tissue extracts or 20 µg of cell extracts were separated by electrophoresis in a 7.5% SDS–polyacrylamide gel and then transferred to a PVDF membrane. The membranes were incubated overnight at room temperature with SP1 antibody (1:200) (Santa Cruz Biotech, CA, USA). Afterwards, polyclonal rabbit anti-SP1 antibody (1:1000) (Sigma, St. Louis, MO, USA) was incubated for 2 hours at room temperature.Antibody was

visualized by using a chemiluminescent substrate (Thermo Scientific, Wilmington, DE, USA) and quantified by a ChemiDoc MP imaging system (BioRad, USA).

2.6 Gene expression analysis

Total RNA was isolated from the perirenal adipose tissue (100 mg) in all samples (n=8/group) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In a following step DNAse treatment (Applied Biosystems, Foster City, CA, USA) was carried out. The quantity of the purified RNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 1.5 µg of RNA were reverse transcribed into complementary DNA (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions.

2.6.1 Quantitative PCR (qRT-PCR)

A 4.75 µL aliquot of each diluted complementary DNA sample was used for PCR amplification in a 12.5 µL reaction volume. The complementary DNA samples were amplified on an iCycler-MyiQ real-time PCR detection system (BioRad, Hercules, CA, USA) in the presence of SYBR® Green master mix (Applied Biosystems, Foster City, CA, USA) and a 300 nM concentration of the sense and antisense primers. Specific primers were synthesized commercially (Integrated DNA Technologies, Leuven, Belgium). The primer sequences were: hsl; forward: 5´-CCATAAGACCCCATTGCCTG-3´, reverse: 5´-CTGCCTCAGACACACTCCTG-3´, pparγforward: 5'-ATTCTGGCCCACCAACTTCGG-3', reverse: 5'-TGGAAGCCTGATGCTTTATCCCCA-3', fatty acid synthase (fasn); forward: 5'-AGCCCCTCAAGTGCACAGTG-3', reverse: 5'-TGCCAATGTGTTTTCCCTGA-3' and βactin; forward: 5'-ACGAGGCCCAGAGCAAGAG-3', reverse: 5'-GGTGTGGTGCCAGATCTTCTC-3'.

PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 60°C for 30 s. mRNA levels in all samples were

normalized to the values of β-actin and the results expressed as fold changes of the threshold cycle (Ct) value relative to the controls using the $2^{-\Delta\Delta Ct}$method (139).

2.7 Statistical analysis

Results are presented as mean ± S.E.M. Statistical analysis was performed using IBM SPSS Statistics 19.0. Student's t test was used for comparisons between both experimental groups. Significance was assessed at the P value <0.05 level.

Statistical analyses of miRNA microarray results were carried out using the software "R" with the Limma, Marray, AgiMicroRna, GOstats y GSEABase package. An adjusted value of probability was achieved by using the Benjamini-Hochberg method for False Discovery Rate (FDR) correction.

Correlation analysis was performed using Pearson´s correlation coefficient. Statistical significance was set-up at $P < 0.05$.

3 RESULTS AND DISCUSSION

Resveratrol has been reported to show anti-obesity properties in animal models such as mice (91, 94, 108), rats (109-111) and primates (112) by acting on several processes, such as adipogenesis, lipogenesis and lipolysis, which take place in white adipose tissue (108, 142-150). In fact, in the present cohort of animals, resveratrol reduced body weight and the size of epididymal and perirenal adipose tissues, as well as the sum of the four depots dissected (perirenal +epididymal+mesenteric+subcutaneous). These data are not presented in the present report as, since we previously used this cohort of animals for other study, they have been already published (138). Accordingly, food efficiency was calculated for control and resveratrol-treated groups (1.5±0.1g/kcal and 1.3±0.1g/kcal, respectively; P<0.05).

The aim of the present study was to assess the changes produced by resveratrol on miRNA profile in white adipose tissue from rats fed an obesogenic diet, and to explore whether these modifications may be related to changes

induced by this polyphenol in triacylglycerol metabolism in this tissue. For this purpose a miRNA microarray was carried out in perirenal adipose tissue. The results showed that, of a total of 719 analysed miRNAs, 273 were detected (Table S1) and 16 were significantly modified by resveratrol treatment (P<0.05). Among them, 13 were significantly increased and 3 were significantly decreased (Table 1). Four of these miRNAs (miR-211-3p, miR-1224, miR-511-3p and miR-539-5p) were selected for qRT-PCR validation (n=8/group). As shown in Figure 2, this validation confirmed significant changes found in microarray analysis.

Table 1. MicroRNAs differentially expressed between control and resveratrol treated animals (expressed as fold change), categorized by P-value adjusted for multiple comparisons. An adjusted value of probability was achieved by using Benjamini-Hochberg FDR correction for multiple testing.

miRNAs	Fold Change	Intensity	*P*-value	*P*-adjusted value
rno-miR-465-5p	1.368	2.859	0.00011	0.01234
rno-miR-466d	1.396	3.111	0.00014	0.01234
rno-miR-539-5p	1.385	2.984	0.00021	0.01234
rno-miR-206-3p	1.565	3.536	0.00022	0.01234
rno-miR-466b-1-3p	2.430	6.641	0.00023	0.01234
rno-miR-1306-3p	0.835	2.708	0.00042	0.01891
rno-miR-511-3p	-0.681	5.71	0.00058	0.0227
rno-miR-487b-3p	-0.553	2.688	0.00074	0.02332
rno-miR-328a-5p	0.674	3.895	0.00077	0.02332
rno-miR-211-3p	0.688	3.206	0.00087	0.02377
rno-miR-672-5p	1.853	5.158	0.00126	0.02793
rno-miR-466b-5p	2.040	5.026	0.00127	0.02793
rno-miR-188-5p	0.953	3.075	0.00133	0.02793
rno-miR-32-3p	2.637	4.818	0.00182	0.03545
rno-miR-1224	1.439	6.792	0.00204	0.03705
rno-miR-129-2-3p	-0.533	2.319	0.00285	0.04859

[a] Values represent mean ± S.E.M (n=8/group). Significance was assessed as the P- adjusted value < 0.05.

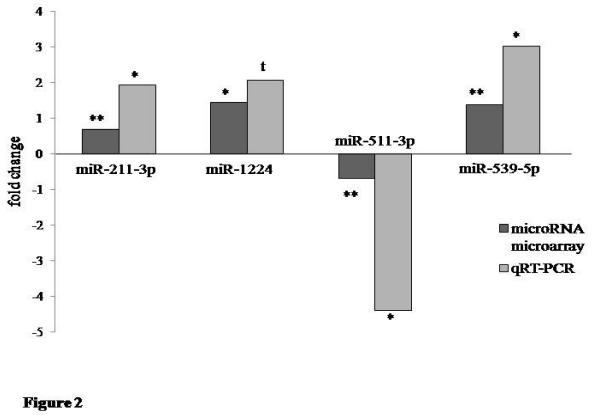

Figure 2

Figure 2. Comparison between the two methods applied to assessing miRNA expression: miRNA microarray (dark columns; n=4) and qRT-PCR (light columns; n=8). The expression of miR-211-3p, miR-1224, miR-511-3p and miR-539-5p, is represented as fold change of resveratrol group with respect to control group at baseline. Data show mean values. Comparisons between groups were made by using the Benjamini-Hochberg method for False Discovery Rate correction (miRNA microarray) and student's t test (qRT-PCR). *, $P<0.05$; **, $P<0.01$; t, $P<0.1$.

Several miRNAs have been reported to be involved in the control of genes related to adipose tissue metabolic pathways responsible for the anti-obesity effect of resveratrol (88, 115): CCAAT/enhancer binding protein alpha (C/EBPα) (miR-27a, miR-31), fasn (miR-378/378), acetyl-CoA carboxylase (acaca) (miR-378/378), pparγ (miR-27a, and miR-130, stearoyl Coenzyme A desaturase (scd1) (miR-378/378) and fatty acid bindig protein4 (fabp4) (miR-143)(151-154). However, in the present study these miRNAs were not modified by this polyphenol.

In view of the miRNAs that were in fact modified by resveratrol (Table 1), we used miRWalk Database to find validated and predicted target genes for these miRNAs (Table 2). There were no validated target genes related to triacylglycerol metabolism in adipose tissue. Among the predicted target genes, fatty acid binding protein 3 (fabp3), sp1, carnitine palmitoyltransferase 1A (cpt1a), lipase hormone sensitive (hsl), uncoupling protein 1 (ucp1), uncoupling protein 3 (ucp3), carnitine palmitoyltransferase1b (cpt1b) and pparγ were

found. For a more detailed study, only those genes relevant to white adipose tissue were selected in the present work: hsl, pparγ and sp1. As mentioned in the Introduction, miRNAs regulate the expression of specific target genes post-transcriptionally by suppressing translation. Consequently, protein expression of the predicted target genes selected was measured.

Table 2. Predicted target genes for the miRNAs significantly modified by resveratrol and related to the triacylglycerol metabolism in white adipose tissue.

miRNA	Sequence	Predicted targets	Algorithms coincidences
rno-mir-465-5p	UAUUUAGAACGGUGCUGGUGUG	No target genes	
rno-mir-466d rno-mir-466b-1-3p rno-mir-466b-5p	UGUGAUGUGUGCAUGUACAUG AUACAUACACACACACAUACAC UAUGUGUGUGUGUAUGUCCAUG	No target genes	
rno-mir-539-5p	GGAGAAAUUAUCCUUGGUGUGU	*Fabp3*: Fatty acid binding protein 3 *Sp1*: Sp1 transcription factor	2 2
rno-mir-206-3p	UGGAAUGUAAGGAAGUGUGUGG	*Cpt1a*: Carnitine palmitoyltransferase 1A	2
rno-mir-1306-3p	GACGUUGGCUCUGGUGGUGAUG	No target genes	
rno-mir-511-3p	AAUGUGUAGCAAAAGACAGGA	No target genes	
rno-mir-487b-3p	AAUCGUACAGGGUCAUCCACUU	No target genes	
rno-mir-328a-5p	GGGGGGCAGGAGGGGCUCA	*Cpt1a* : Carnitine palmitoyltransferase 1[a] *hsl*: hormone sensitive lipase	3 2
rno-mir-211-3p	GGCAAGGACAGCAAAGGGGG	*Cpt1a* : Carnitine palmitoyltransferase 1A *Ucp1*: Uncopling protein 1 *Ucp3*: Uncopling protein 3	3 2 2
rno-mir-672-5p	UGAGGUUGGUGUACUGUGUGUGA	*Cpt1b*: Carnitine palmitoyltransferase 1B	2
rno-mir-188-5p	CAUCCCUUGCAUGGUGGAGGG	*Cpt1a* : Carnitine palmitoyltransferase 1A *Ucp1*: uncopling protein 1	4 2
rno-mir-32-3p	GCAAUUUAGUGUGUGUGAUAUU	Target genes not related to obesity	
rno-mir-1224	GUGAGGACUGGGGAGGUGGAG	No target genes	
rno-mir-129-1-3p rno-mir-129-2-3p	AAGCCCUUACCCCAAAAAG AAGCCCUUACCCCAAAAAGCAU	*Pparγ*: peroxisome proliferator-activated receptor gamma	2
rno-mir-455-3p	GCAGUCCACGGGCAUAUACACU	Target genes not related to obesity	
rno-mir-496-5p	AGGUUGUCCAUGGUGUGUUC	No target genes	
rno-mir-345-5p	UGCUGACCCCUAGUCCAGUGC	No target genes	
rno-mir-3557-3p	ACACAGGACCUGGAGUCAGGAG	No target genes	
rno-mir-3562	UUGGGGCAGUGGCUGGAUGGGA	No target genes	

[a] Fabp3: Fatty acid binding protein 3, sp1: Sp1 transcription factor, cpt 1a: Carnitine palmitoyl transferase 1A, lipe: lipase, hormone sensitive, ucp1: Uncoupling protein 1, ucp3: Uncoupling protein 3, cpt 1b: Carnitine palmitoyl transferase 1B, ppar[redacted]peroxisome proliferator-activated receptor gamma.

[b] Sequences were obtained from miRBase.

[c] Data base for predicted targets were miRWalk (differences between 3p and 5p were not applied in the predicted target searched).

[d] Algorithms used: miRanda, miRDB, miRWalk, RNA22 and Target Scan.

As far as PPARγ and HSL are concerned, no changes were observed in their protein expression (Figure S1). In view of this result, and taking into account that another mechanism of miRNA regulation is the reduction in the mRNA stability of their targets, gene expression of pparγ and hsl was also measured and, once again, no differences were observed between both experimental groups (data not shown). These results show that, although pparγ is a predicted target gene for miR-129-1-3p, miR-129-2-3p and hsl for 328a-5p (Table 2), are not involved in the body fat-lowering effect of this polyphenol.

Sp1 is an important member of the ubiquitously expressed Sp/KLF transcription factor family (155). In miRWalk Database sp1gene is considered as a predicted target gene for miR-539-5p. In the present study, the significant increase observed in gene expression of this miRNA (Table 2) matches well with the significant reduction found in SP1 protein expression (Figure 3C) in resveratrol-treated rats. Although sp1 has not been recorded in miRWalk Database as a predicted or validated target gene for miR-1224, a miRNA which was increased by resveratrol in the present study, several studies in the literature have demonstrated the involvement of this miRNA in the regulation of sp1. Thus, Niu et al. (156) confirmed the decrease in gene and protein expression of sp1 transcription factor after miR-1224 transfection in HEK-293 (Human Embryonic Kidney) and RAW264.7 (Mouse leukaemic monocyte macrophage cell line) cells. Although it is important to highlight that these two cell lines do not have a rat origin, as was the case in our experiment, according to the alignment of potential miR-1224 binding site in 3´ untranslated region (3´UTR) of sp1, this miRNA theoretically could also be able to bind to the rat sp1 mRNA (Figure 1A). In fact, in the present study the significant increase in gene expression of miR-1224 observed in rats treated with resveratrol matched well with the reduction in SP1 protein expression.

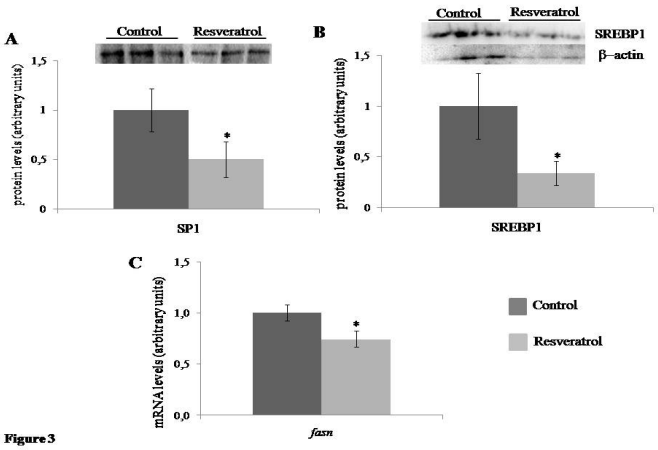

Figure 3. Protein expression of SP1 (A) and SREBP1 (B), and gene expression of fasn (C) in adipose tissue from rats fed a high-fat, high-sucrose diet supplemented with resveratrol (Resveratrol group) or not (Control group) for 6 weeks (n=8). For the SP1, immunobloting after immunoprecipitation was performed. For the SREBP1, immunoblot using β-actin as housekeeping was performed. For fasn gene expression qRT-PCR using a β-actin as housekeeping was performed. Values are presented as means ± standard error of the means. *, P<0.05. SP1: SP1 transcription factor protein, SREBP1: sterol regulatory element-binding protein 1, fasn: fatty acid synthase gene.

In order to obtain further information concerning the regulatory role of miR-539-5p and miR-1224 on SP1 protein Pearson´s correlations between the expression of each miRNA and SP1 protein expression were carried out. This analysis showed that miR-539-5p expression and SP1 protein expression were negatively correlated (R^2= 0.663; P=0.001). By contrast, no significant correlation was found between miR-1224 expression and SP1 protein expression. Additionally, miR-539-5p expression was negatively correlated with "food efficiency" (R^2= 0.514; P=0.002).

In a further step, an *in vitro* miRNA overexpression experiment was conducted in 3T3-L1 cells. When SP1 protein expression was evaluated in cells transfected with miR-539-5p mimics the target protein was not detectable, compared with the control group (Figure 4). By contrast, no significant

differences were detected between control cells and those transfected with miR-1224 mimics. Taken together, these results suggest that the reduction in SP1 protein expression induced by resveratrol can be modulated only by miR-539-5p, and gives experimental support to the prediction provided by miRWalk Database.

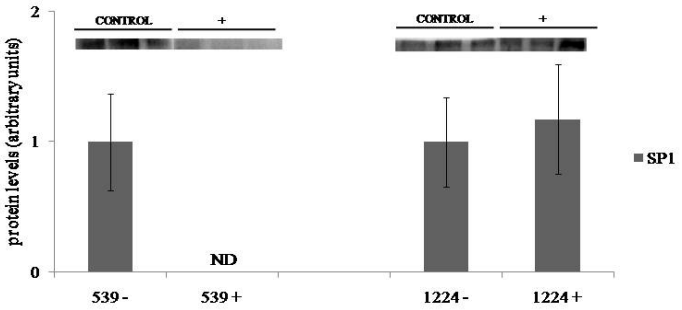

Figure 4

Figure 4. Protein expression of SP1 in 3T3-L1 control cells (n=8) and 3T3-L1 cells overexpressing miR-539-5p and miR-1224-5p (n=8). 539 -: mmu-miR-539-5p control cells, 539 +: cells overexpressing mmu-miR-539-5p, 1224 -: mmu-miR-1224-5p control cells, 1224 +: cells overexpressing mmu-miR-1224-5p. ND: not detectable. SP1: SP1 transcription factor protein.

SP1 acts together with SREBP1 to synergistically activate the promoter of fasn, gene, which codifies for fatty acid synthase, a key enzyme involved in de novo lipogenesis (157) (Figure 1B). In view of these facts, we decided to analyze protein expression of the transcriptional factor SREBP1 and gene expression of fasn. A significant reduction was observed in SREBP1 protein expression in resveratrol-treated rats (Figure 3B). In good accordance with this result, gene expression of fasn was also reduced (Figure 3C). In our previous paper, carried out in this precise cohort of rats, we showed that fatty acid synthase activity was significantly reduced in resveratrol-treated rats (138).

4 CONCLUDING REMARKS

The present results demonstrate for the first time that resveratrol modifies miRNA profile in white adipose tissue. As far as triacylglycerol metabolism is concerned in this tissue, this study shows that miR-539-5p is involved in the inhibition of de novo lipogenesis induced by resveratrol.

Acknowledgements

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Conflict of interest

No conflicts of interest are reported by any of the authors.

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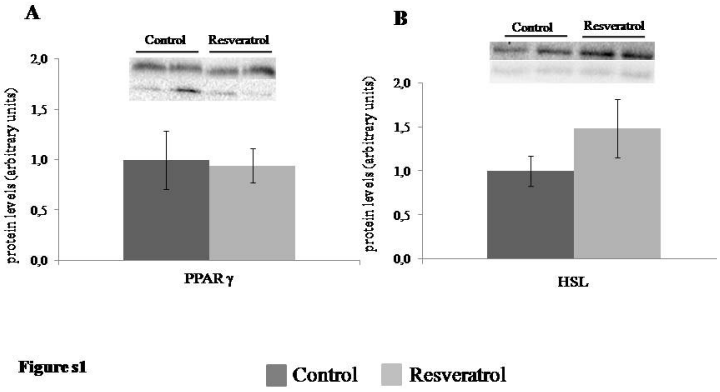

Figure S1. Protein expression of PPAR A) and HSL (B) in adipose tissue from rats fed a high-fat, high-sucrose diet supplemented with resveratrol (Resveratrol group) or not (Control group) for 6 weeks (n=8). Values are presented as means + standard error of the means.

MANUSCRIPT 3

Ana Gracia, Alfredo Fernández-Quintela, Jonatan Miranda, Itziar Eseberri,

Marcela González and María P. Portillo

Are miRNA-103, miRNA-107 and miRNA-122 involved in the prevention of liver steatosis induced by resveratrol?

Nutrients. 2017 Apr 4;9(4) pii: E360

Abstract

The aim of the present study was to determine whether the reduction in liver fat previously observed in our laboratory in a cohort of rats which had been fed an obesogenic diet was mediated by changes in the expression of microRNA (miRNA)-103-3p, miRNA-107-3p and miRNA-122-5p, which represent 70% of total miRNAs in the liver, as well as in their target genes. The expression of the three analysed miRNAs was reduced in rats treated with resveratrol. A reduction in sterol-regulatory element binding protein 1 (SREBP1) and an increase in carnitine palmitoyltransferase 1a (CPT1a) were observed in resveratrol-treated rats. No changes were found in fatty acid synthase (FAS). In cultured hepatocytes, SREBP1 protein was increased after the transfection of each miRNA. FAS protein expression was decreased after the transfection of miRNA-122-5p, and CPT1a protein was down-regulated by the over-expression of miRNA-107-3p. This study provides new evidences which show that *srebf1* is a target gene for miRNA-103-3p and miRNA-107-3p, *fasn* a target gene for miRNA-122-5p and *cpt1a* a target gene for miRNA-107-3p. Moreover, the reduction in liver steatosis induced by resveratrol in rats fed an obesegenic diet is mediated, at least in part, by the increase in CPT1a protein expression and activity, via a decrease in miRNA-107-3p expression.

Key words: miRNA-103, miRNA-107, miRNA-122, steatosis, liver, resveratrol, rat

1. Introduction

Excessive fat accumulation in liver is known as hepatic steatosis, which is the most benign form of non-alcoholic fatty liver disease (NAFLD). It is a major cause of chronic liver disease in Western societies. It encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis; NAFL) to hepatic steatosis with inflammation (non-alcoholic steatohepatitis, NASH) [1]. This disorder is closely associated with obesity and insulin resistance [2]. Although the current treatment of liver steatosis is based on dietary energy restriction and physical activity [3,4], a great deal of attention has been paid in recent years to bioactive molecules, such as phenolic compounds present in foods and plants, which can represent complementary tools.

One of the most widely studied molecules is resveratrol (*trans*-3,5,4′-trihydroxystilbene), a phytoalexin occurring naturally in grapes, berries and peanuts [5,6]. Several studies on rats and mice have shown that resveratrol is able to reduce liver fat accumulation [7,8]. Some authors have also found this effect in human beings [9,10].

The mechanisms of action of resveratrol underlying its effect on liver steatosis are mainly a reduction in lipogenesis and/or an increase in fatty acid oxidation, very commonly associated with enhanced mitochondriogenesis [11]. However, little is known concerning the potential involvement of microRNAs (miRNAs) on changes induced by resveratrol in these metabolic pathways.

MiRNAs are short double stranded RNAs (approximately 22 nucleotides) encoded in the genome that act post-transcriptionally to regulate protein expression. These non-coding RNAs can act directly on target mRNA transcripts binding to complementary target sites in 3′ untranslated regions (3′ UTR) of messenger RNAs (mRNAs), causing translational repression and/or mRNA destabilization. They can also act indirectly by regulating intermediate components, such as transcripts that encode transcription factors which, in turn, control the expression of downstream genes. A single miRNA can have multiple targets, acting simultaneously to regulate the post-transcriptional expression of various genes and physiological processes. Furthermore, each gene can be

regulated by several miRNAs [12,13]. What is more, the expression of these miRNAs can be modified by changes induced either directly in the enzymes involved in their biogenesis process or in miRNA epigenetic modifications, or indirectly via lipoprotein-mediated miRNA delivery to cells, among others [14,15].

It has been reported in the literature that different types of polyphenols, such as proanthocyanidins or a mixture extracted from *Hibiscus sabdariffa*, are able to modify the expression of miRNA-122-5p (a liver specific miRNA and the most abundant one) and the paralogs miRNA-103-3p and miRNA-107-3p in liver [16–19].

In a previous study carried out by our group using this precise cohort of animals, resveratrol treatment did not reduce final body weight or liver weight. No changes were observed in food intake. By contrast, resveratrol treatment induced a significant decrease in hepatic triacylglycerol content. Moreover, when the activity of several enzymes involved in hepatic lipid metabolism was measured, no changes in fatty acid synthase (FAS) activity and an increase in carnitine palmitoyltransferase 1 (CPT1) activity were found, suggesting that the delipidating effect was due, at least in part, to increased fatty acid β-oxidation, which reduces the availability of fatty acids for triacylglycerol synthesis [20].

In this context, the aim of the present study was to determine whether, as in the case of other polyphenols, this reduction in liver fat was mediated by changes in the expression of miRNA-122-5p, miRNA-103-3p and miRNA-107-3p, which represent more than 75% of total miRNAs in the liver [19,21–25], as well as in their target genes.

2. Material and Methods

2.1. Animals and Experimental Design

The experiment was conducted with 16 male Sprague-Dawley rats purchased from Harlan Ibérica (Barcelona, Spain) and took place in accordance with the institution's guide for the care and use of laboratory animals (CUEID CEBA/30/2010). The rats were individually housed in polycarbonate metabolic

cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2 °C) with a 12 h light-dark cycle. After a 6-day adaptation period, rats were randomly divided into two dietary groups of eight animals each, namely a control group (Control) and a group treated with resveratrol (Resveratrol), both fed a commercial obesogenic diet (4.6 kcal/g; 44.8% energy from fat, 36.2% from carbohydrates and 19.0% from proteins) supplied by Harlan Ibérica (TD. 06415) for 6 weeks (Figure S1). Resveratrol, supplied by Monteloeder (Elche, Spain), was added to the diet as previously reported [6]. Briefly, the phenolic compound was dissolved in an ethanolic solution (5 mg/mL) and poured on the surface of the diet. Rats started eating immediately once the diet was daily replaced, and thus they ate all the resveratrol added before it started to degrade (3 h). In order to ensure a dose of 30 mg resveratrol/kg body weight/day, the amounts of resveratrol to be included in the diet for each animal were calculated daily based on their individual body weight. This dose was selected for this experiment because in a previous study we observed that, under our experimental conditions, 30 mg/kg body weight/day was the most effective of the following: 6, 15, 30 and 60 mg/kg body weight/day. Diet for control animals was added with the same amount of ethanolic solution without resveratrol. All animals had free access to food and water. Food intake and body weight were measured daily. This cohort of animals had been previously used in another study reported by our group [20,26].

At the end of the experimental period, animals were sacrificed under anaesthesia by intraperitoneally administering 400 mg chloral hydrate/kg body weight, by cardiac exsanguination, after a 12-h fasting period. The liver was dissected, weighed and immediately frozen at −80 °C.

2.2. Cell Culture

AML12 (Alpha Mouse Liver 12) hepatocytes, supplied by ATCC (ATCC CRL-2254), were cultured in 1:1 DMEM/HAM'S F12 glutamax medium containing 10% fetal bovine serum (FBS), 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, 40 ng/mL dexamethasone and 1% Penicillin/Streptomycin (10,000

U/mL). This medium was changed every two days. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. MicroRNA expression analysis

Total miRNAs were extracted using E.Z.N.A. miRNA kit (R7034-02; Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. Total RNA (9 ng) was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), as previously reported in Gracia et al. [27]. The targeted miRNA assay sequences were as follows (source miRBase):

rno-miRNA-103-3p: 5′-AGCAGCAUUGUACAGGGCUAUGA-3′

rno-miRNA-107-3p: 5′-AGCAGCAUUGUACAGGGCUAUCA-3′

rno-miRNA-122-5p: 5′-UGGAGUGUGACAAUGGUGUUUG-3′

PCR was performed in an iCycler™–MyiQ™ Real-time PCR Detection System (Applied Biosystems, Foster City, CA, USA). Amplification was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. U6 small nuclear RNA was used as an endogenous control. All mRNA levels were normalized to the values of U6 snRNA. The results were expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [28].

2.4. Target Genes for miRNAs

In order to obtain the predicted and validated target genes for these miRNAs, a comparative analysis was carried out in miRecords. This database is an integrated resource for animal miRNA-target interactions, which stores predicted miRNA targets produced by 11 established miRNA target predicted programs [29]. No validated target genes were found. Among the predicted target genes, only those involved in hepatic lipid metabolism (*srebf1, fasn, cpt1a*) were selected (Table 1). *Fasn* codifies for fatty acid synthase, a key enzyme involved in de novo lipogenesis, *srebf1* is the transcription factor that regulates this enzyme, and *cpt1a* codifies for carnitine palmitoyltransferase, a

key enzyme involved in the fatty acid oxidation. In addition, we reviewed the literature and found that several authors had proposed *srebf1* and *fasn* as target genes for miR-122-5p and miR-107-3p, respectively (Table 1).

Table 1. Predicted target genes and validated genes reported in the literature related to triacylglycerol metabolism of the miRNAs studied.

miRNA	Predicted Target Genes (miRecords)	Data from the Literature
rno-miR-103-3p	*Srebf1* *Cpt1a*	
rno-miR-107-3p	*Srebf1* *Cpt1a*	*Fasn*: Bhatia et al. [35]
rno-miR-122-5p	*Fasn*	*Srebf1*: Shibata et al. [33] *Srebf1*: Iliopoulos et al. [32]

Srebf1: sterol regulatory element binding factor 1; *Cpt1a*: carnitine palmitoyltransferase 1a; *Fasn*: fatty acid synthase.

2.5. miRNA Transfection

Hepatocytes in a confluence status of approximately 90%, were transfected with Lipofectamine RNAiMAX (Applied Biosystems, Foster City, CA, USA) prepared following the manufacturer's protocol, with mirVana miRNA mimics of mmu-miRNA-103-3p, mmu-miRNA-107-3p and mmu-miRNA-122-5p (homologous to rno-miRNA-103-3p, rno-miRNA-107-3p and rno-miRNA-122-5p respectively) (Applied Biosystems, Foster City, CA, USA). Each mimic was transfected for 48 h in a final concentration of 25 nM per well. Optimal transfection conditions were determined in previous experiments, and transfection efficiency was assessed using miRNA probes and fluorescent transfection controls. To rule out unspecific effects, control cells were transfected with negative controls.

2.6. Western Blot Analysis

2.6.1. Liver Protein Expression of Fatty Acid Synthase, Sterol Regulatory Element-Binding Protein 1 and Carnitine Palmitoyltransferase 1a

Fatty acid synthase (fas) and sterol regulatory element-binding protein 1 (srebp1) protein extraction was carried out with 100 mg of liver as previously described [30]. The protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Wilmington, DE, USA).

Immunoblot analyses were performed in all tissue samples using 80 µg of protein for FAS and 40 µg of protein for srebp1. Protein were separated by electrophoresis in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. Equal loading of proteins was confirmed by staining the membranes with Coomassie Blue or incubating these membranes with polyclonal mouse β-actin antibody. The membranes of the two assays were blocked with casein phosphate buffered saline (PBS)-Tween buffer for 2 h. These membranes were incubated overnight at 4 °C with mouse origin FAS immunoglobulin G (IgG) (1:1000) and srebp1 IgG monoclonal antibodies (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, in both cases, new incubation with goat- anti-mouse IgG-Horseradish Peroxidase (HRP) antibody (1:5000) (Sigma, St. Louis, MO, USA) was carried out for 2 h at room temperature. Antibodies were visualized by using a chemiluminescent substrate (Thermo Scientific, Wilmington, DE, USA) and quantified by a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA). After stripping, FAS protein-containing membranes were incubated with a polyclonal mouse β-actin antibody (1:5000) followed by goat- anti-mouse IgG-HRP antibody (1:5000) (Sigma, St. Louis, MO, USA), and measured again. The FAS protein measurements were normalized by β-actin.

For carnitine palmitoyltransferase 1a (CPT1a), 100 mg of liver were homogenized in a PBS buffer with protease inhibitors (pH 7.4) and centrifuged (14,000 *g*, 1 minute, 4 °C). The pellet was resuspended in 100 µL of radioimmunoprecipitation assay buffer (RIPA buffer). The homogenates were

centrifuged at 36,000 *g* for 10 min at 4 °C. The protein concentration was measured by BCA protein assay kit (Thermo Scientific, Wilmington, DE, USA).

Immunoblotting was performed after immunoprecipitation. A total of 250 µg of liver extracts were diluted with three volumes of PBS (with added protease inhibitors). CPT1a was immunoprecipitated with 1 µL of monoclonal mouse anti-CPT1a antibody (ABCAM, Cambridge, MS, USA) in constant rotation, at 4 °C, overnight. Afterwards, 20 µL Protein G Agarose (Santa Cruz Biotech, Santa Cruz, CA, USA) was added to each sample, and these were rotated for 3 h at 4 °C. The immunoprecipitated tissue samples were then washed three times with 500 µL PBS buffer. A total of 30 µg of extracts were separated by electrophoresis in a 7.5% SDS–polyacrylamide gel and then transferred to a PVDF membrane. The membranes were incubated overnight at room temperature with mouse anti-CPT1a antibody (1:1000) (ABCAM, Cambridge, MS, USA). Afterwards, polyclonal goat- anti-mouse IgG-HRP antibody (1:2500) (Sigma, St. Louis, MO, USA) was incubated for 2 h at room temperature. Antibody was visualized by using a chemiluminescent substrate (Thermo Scientific, Wilmington, DE, USA) and quantified by a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA).

2.6.2. SREBP1, FAS and CPT1a Protein Expression after Over-Expression in AML12

In the case of AML12 cells, total protein was extracted with 200 µL of lysis buffer as previously reported [27]. Protein concentration was measured by BCA protein assay kit (Thermo Scientific, Wilmington, DE, USA).

For FAS protein, 65 µg of cell protein extract were used to perform the immunoblotting. Protein were separated by electrophoresis in a 7.5% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were blocked with casein PBS-Tween buffer for 2 h. These membranes were incubated overnight at 4 °C with mouse origin FAS IgG (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, new incubation with goat- anti-mouse IgG-HRP antibody (1:5000) (Sigma, St. Louis, MO, USA) was carried out for 2 h at room temperature. Antibodies were visualized by using a chemiluminescent substrate (Thermo Scientific, Wilmington, DE, USA) and quantified by a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA). Coomassie Blue staining of

membranes was used as protein loading control. In case of CPT1a and SREBP1, immunoblotting after immunoprecipitation was performed. A total of 40 μg for CPT1a and 70 μg for SREBP1 of cell extracts were immunoprecipitated. The total amount of protein was used for immunoblotting in both cases and following the same conditions as described above.

2.7. Statistical Analysis

Results are presented as median ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics 24.0 (SPSS Inc., Chicago, IL, USA). All of the parameters are normally distributed according to the Shapiro-Wilk's test. Student's *t*-test was used for comparisons between both experimental groups. Significance was assessed at the $p < 0.05$ value.

3. Results

3.1. Cell Culture Studies

MiRNA-103-3p, miRNA-107-3p and miRNA-122-5p were individually over-expressed in AML12 hepatocytes. Over-expressions were confirmed by measuring each miRNA expression. Protein expression of SREBP1 was significantly increased after transfection of each miRNA ($p < 0.05$) (Figure 1A). In the case of FAS, protein expression was significantly decreased after transfection of miRNA-122-5p ($p < 0.001$) (Figure 1B). Finally, CPT1a protein expression was down-regulated by the over-expression of miRNA-107-3p ($p < 0.001$) (Figure 2).

3.2. In Vivo Study

Body weight gain in rats treated with resveratrol was similar to that observed in control animals (data previously reported in Alberdi et al. 2011 [31]). Similarly, no significant differences were observed in liver weight, expressed as a percentage of final body weight (3.4 ± 0.1 in Control group and 3.5 ± 0.2 in Resveratrol group).

In the present study, we observed that the expression of the three miRNA analysed (miRNA-103-3p, miRNA-107-3p and miRNA-122-5p) was significantly reduced in the liver of rats treated with resveratrol (Table 2). When protein

expression of the target genes for these miRNAs was measured, we observed a significant reduction in SREBP1 ($p < 0.05$) and a significant increase in CPT1a ($p < 0.05$) in resveratrol-treated rats (Figures 3A and 4). No changes were found in FAS protein levels (Figure 3B).

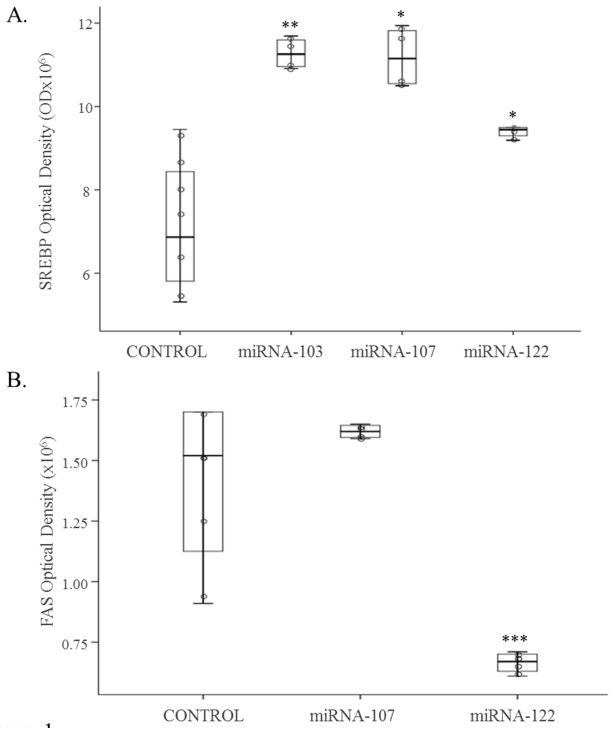

Figure 1. Protein expression of SREBP1 (**A**) and FAS (**B**) in AML12 control cells (n = 6) and AML12 cells over-expressing mmu-miRNA-103-3p, mmu-miRNA-107-3p and mmu-miR-122-5p (n = 6). Scatter dot plots including median and standard deviation were expressed as optical density. Comparisons between each treatment and the controls were analysed by Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. SREBP1: sterol regulatory element-binding protein 1, FAS: fatty acid synthase; AML12: alpha mouse liver 12.

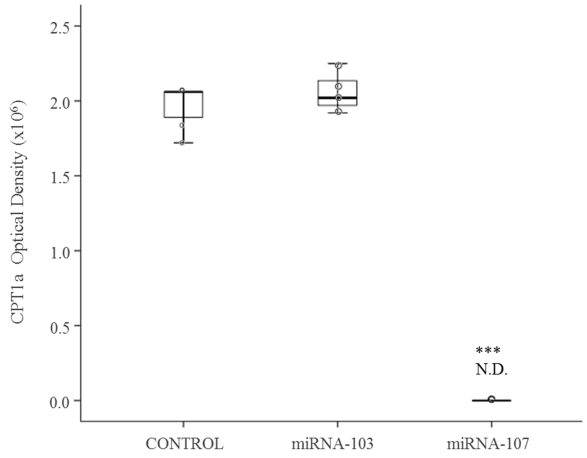

Figure 2. Protein expression of CPT1a in AML12 control cells (*n* = 6) and AML12 cells over-expressing mmu-miRNA-103-3p and mmu-miRNA-107-3p (*n* = 6). Scatter dot plots including median and standard deviation were expressed as optical density. Comparisons between each treatment and the controls were analysed by Student's *t*-test. Coomassie Blue staining was used as protein loading control. ND: not detectable. CPT1a: carnitine palmitoyltransferase 1a; AML12: alpha mouse liver 12.

Table 2. The gene expression fold change of miRNA-103, miRNA-107 and miRNA-122 in the liver of rats fed an obesogenic diet supplemented with resveratrol (Resveratrol group) or not (Control group) for 6 weeks (*n* = 8).

miRNA	**Fold Change (Resveratrol vs. Control)**	*p*
miR-103	−2.49	<0.01
miR-107	−2.08	<0.05
miR-122	−2.59	<0.01

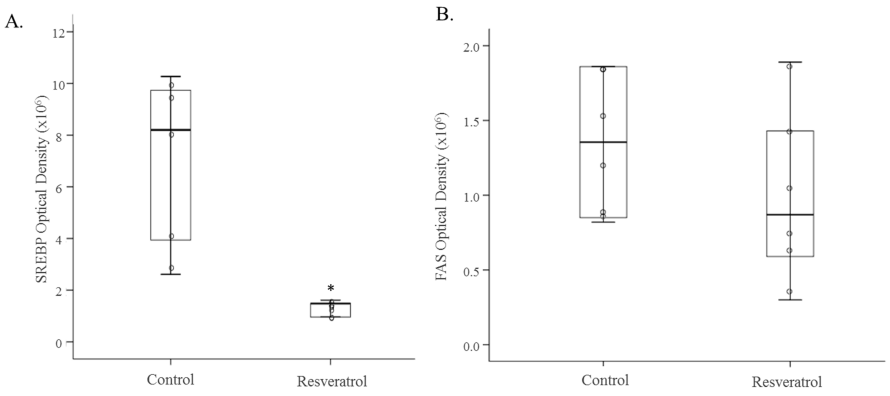

Figure 3. Protein expression of SREBP1 (**A**) and FAS (**B**) in the liver of rats fed an obesogenic diet supplemented with resveratrol (Resveratrol group) or not (Control group) for 6 weeks (*n* = 8). Scatter dot plots including median and standard deviation were expressed as optical density. * $p < 0.05$. Coomassie Blue staining was used as protein loading control for SREBP1 and β-actin for FAS. SREBP1: sterol regulatory element-binding protein 1, FAS: fatty acid synthase.

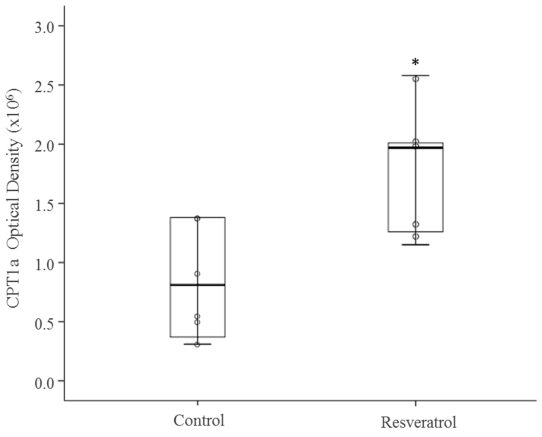

Figure 4. Protein expression of CPT1a in the liver of rats fed an obesogenic diet supplemented with resveratrol (Resveratrol group) or not (Control group) for 6 weeks (*n* = 8). Scatter dot plots including median and standard deviation were expressed as optical density. * $p < 0.05$. Coomassie Blue staining was used as protein loading control. CPT1a: carnitine palmitoyltransferase 1a

4. Discussion

As indicated in the Introduction, in a previous study we had observed that resveratrol was able to partially prevent liver steatosis induced by an obesogenic diet. We found a significant increase in the activity of CPT1a, a rate-limiting enzyme in fatty acid oxidation, in the liver of rats treated with resveratrol, without changes in FAS activity [20]. The present study helps us to gain more insight into the effect of resveratrol on the regulation of these two enzymes.

As far as the lipogenic pathway is concerned, miRecords data base showed that *srebf1* was a predicted target gene for miRNA-103-3p and miRNA-107-3p and *fasn* for miRNA-122-5p. In addition, Iliopoulos et al. [32] showed that the up-regulation of miRNA-122 induced the increased protein expression of SREBP1. Taking into account that miRNA are negative regulators of protein translation and that no miRNA-122-5p binding sites are found in the 3'UTR or the coding region of this gene, the authors suggested that miRNA-122 could regulate other genes that, in turn, could affect the transcription of *srebf1.* They concluded that *srebf1* was an indirect target gene for miRNA-122, but they did not describe the intermediate steps in the signaling cascade that led to the up-regulation of SREBP1. Later on, Shibata et al. [33] reported that silencing miRNA-122 led to decreased SOCS3 expression, which in turn increased STAT3 expression. Therefore, SREBP1 was negatively regulated by STAT3 and, consequently, a decrease in miRNA-122 induced a reduction in SREBP1 expression. In order to obtain more scientific support concerning the involvement of these three miRNAs in SREBP1 regulation, in the present study we over-expressed these miRNAs in AML12 hepatocytes. In all the three cases we observed a significant increase in SREBP1 protein expression.

In rats treated with resveratrol, we found a significantly decreased expression of miRNA-103-3p, miRNA-107-3p and miRNA-122-5p, which was paralleled by a significant decrease in SREBP1 protein expression. As far as miRNA-122-5p is concerned, taking into account the results of our transfection study, and bearing in mind the results reported by Iliopoulos et al. and Shibata et al. [32,33], it can

be proposed that resveratrol decreases the protein expression of the transcription factor SREBP1 indirectly via miRNA-122-5p.

With regard to miRNA-103-3p and miRNA-107-3p, as indicated before in the Discussion section, computational analysis (miRecords) revealed complementarity between these miRNAs and the 3'UTR region of *srebf1,* suggesting that it can be a direct target gene. Usually, miRNAs regulate gene transcription in a negative way, which is to say that they inhibit this process. However, in some cases, the transcription of the RNAs is positively regulated and thus, the up-regulation of some miRNAs increases mRNA levels of their targets [34]. In our in vitro study, the over-expression of miRNA-103-3p and miRNA-107-3p in hepatocytes led to an increased expression of SREBP1, suggesting that in fact they were positive regulators. In the in vivo study, resveratrol induced the down-regulation of miRNA-103-3p and miRNA-107-3p in the liver, which was accompanied by a reduced expression of SREBP1. Taking all that into account, it may be said that miRNA-103-3p and miRNA-107-3p are involved as positive regulators in the effects of this polyphenol on SREBP protein expression [34].

As shown in Table 1, *fasn* was a predicted target gene only for miRNA-122-5p. Bhatia et al. [35] transfected HepG2 hepatocytes with miRNA-107 at various doses and they observed that, using the most common dose in transfection studies (25 nM), no changes in FAS protein expression were observed. When we measured FAS protein expression, we found no change in resveratrol-treated rats. This result is in good accordance with the lack of change in FAS activity observed in our previous study addressed to this cohort of rats. However, it was somewhat surprising because *fasn* is, according to the miRecords data base, a predicted target gene for miRNA-122-5p, which was reduced by resveratrol, and in fact our transfection experiment showed that the over-expression of miRNA-122-5p induced a significant reduction in FAS protein expression. Consequently, increased FAS expression should be expected. On the other hand, SREBP1, which is a transcription factor that regulates FAS, was reduced in resveratrol-treated rats. Thus, it could be hypothesized that the increase in FAS protein expression

expected as a consequence of miRNA-122-5p down-regulation could be compensated by the decrease expected due to the reduction in SREBP1.

According to the miRecords data base, *cpt1a* is a predicted target gene for miRNA-103-3p and miRNA-107-3p. In cultured hepatocytes, we observed that only those over-expressing miRNA-107-3p showed the down-regulation of CPT1a protein expression, suggesting that in fact *cpt1a* is a real target gene for miRNA-107-3p, but not for miRNA-103-3p. In our in vivo experiment, rats treated with resveratrol showed decreased miRNA-107-3p expression and increased CPT1a protein expression. All in all, these results suggest that the increase induced by resveratrol in CPT1a protein expression, which is involved in the liver delipidating effects of this polyphenol, was mediated by a reduction in miRNA-107-3p expression.

5. Conclusions

The present study provides new evidence showing that *srebf1* is a target gene for miRNA-103-3p and miRNA-107-3p and *cpt1a* a target gene for miRNA-107-3p. Furthermore, the reduction in liver steatosis induced by resveratrol under our experimental conditions is mediated, at least in part, by increased CPT1a protein expression and activity, via a decrease in miRNA-107-3p expression.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Diagram of the work-plan for the *in vivo* study.

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Author Contributions: A.G., J.M. and M.G. revised the literature. A.G. (PhD student) measured microRNA expressions and revised the miRecords database. AG and IE performed the in vitro experiments. A.G. and J.M. carried out the

Western blot analysis in in vivo and in vitro samples. A.F.Q. and M.P.P. designed the experiment. M.P.P. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of Interest: The authors declare no competing financial interest.

Abbreviations

3′UTR	3′ untraslated regions
BCA	bicinchoninic acid
CPT1a	carnitine palmitoyltransferase 1a
FAS	fatty acid synthase
FBS	fetal bovine serum
miRNA	microRNA
mRNA	messenger RNA
SREBF1	sterol regulatory element binding factor 1
SREBP1	sterol regulatory element binding protein 1

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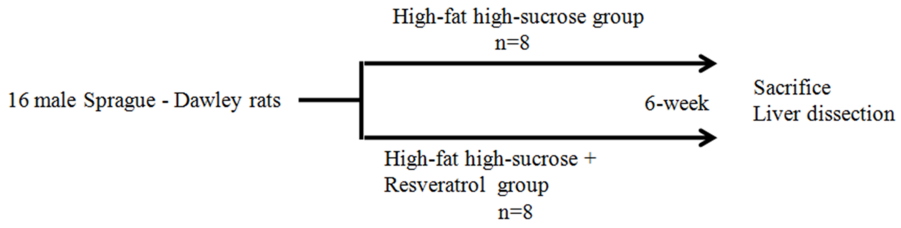
High-fat high-sucrose group
n=8
16 male Sprague - Dawley rats
6-week
Sacrifice
Liver dissection
High-fat high-sucrose +
Resveratrol group
n=8

Figure S1.

SUMMARY

This Doctorial Thesis has been carried out within the research group “Nutrition and Obesity” leaded by Prof. María del Puy Portillo. This group has extensive experience in the study of the effects of functional ingredients on lipid metabolism in the fields of obesity and liver steatosis. Conjugated fatty acids, such as linoleic and linolenic acids, and phenolic compounds, such as, quercetin, resveratrol and pterostilbene have been studied in recent years. Resveratrol and pterostilbene have been chosen for this Doctoral Thesis.

Due to the fact that little is known concerning the effects of phenolic compounds on triglyceride regulation by epigenetic mechanisms and microRNAs, this Doctoral Thesis focuses on these molecular aspects.

Two different approaches were carried out. The first was designed to analyse the possible influence of DNA methylation on triglyceride accumulation in adipose tissue, and the second to establish the involvement of post-transcriptional regulation by microRNAs in adipose tissue and liver fat accumulation.

1. EFFECTS OF RESVERATROL AND PTEROSTILBENE ON DNA METHYLATION PATTERN OF GENES INVOLVED IN TRIGLYCERIDE METABOLISM AND THEIR RELATIONSHIP WITH OBESITY.

Methylation of nucleotides, as an epigenetic mechanism, provides a molecular means to reversibly mark genomic DNA. This epigenetic process can change the functional state of regulatory regions, and is functionally involved in many forms of stable epigenetic repression (47).

In this experiment, we determined the effect of resveratrol and its methoxy derivate, pterostilbene, on methylation of genes involved in triglyceride metabolism. To this end we used Wistar rats divided into four experimental groups: a control group fed a standard diet (control group), a group fed a high-fat high-sucrose diet (high-fat high-sucrose group), and two groups also fed also a high-fat high-sucrose diet but treated with either resveratrol or pterostilbene at a dose of 30 mg/kg/day (resveratrol and pterostilbene group respectively). This dose was used because in previous studies from our laboratory we observed that it was effective in reducing body fat mass (105, 137). As expected, feeding a diet rich in fat and sucrose led to increased energy intake and consequently to increased fat accumulation, when compared with the standard diet. The addition of these two phenolic compounds to the high-fat high-sucrose diet prevented this fattening effect, but only partially, because body weight and adipose tissue weights in these rats did not reach control values.

For the DNA methylation study, we established the following inclusion criteria: a) genes involved in triglyceride metabolism and b) genes with at least one CpG island in the gene promoter or first exon. First, *lpl, fasn, acaca, lipe, pnpla2, srebf1* and *pparγ* genes, were selected due to their role in white adipose tissue triglyceride metabolism. Moreover, these genes have been shown to be altered by resveratrol (86, 92, 110, 111, 115, 146, 182). After the analysis of CpG positions, only *fasn, pnpla2* and *pparγ* had CpG-rich areas near the promoter region.

DNA methylation analysis, performed by pyrosequencing, showed that high-fat high-sucrose feeding or phenolic compounds did not modify *pnpla2* and

pparγ gene methylation patterns. By contrast, several changes were observed in *fasn* gene.

DNA methylation on the promoter regions is a potent suppressor of gene expression. Although changes produced by this process could be small, they may be associated with gene expression modifications that exert significant effects in phenotype (44, 183). As previously mentioned, the high-fat high-sucrose diet induced significant changes in the methylation pattern of *fasn* gene with regard to the controls, a hypermethylation in -62 bp position (6%) and a hypomethylation in -90 bp position (-11%). The addition of the two phenolic compounds led to different methylation patterns. In case of pterostilbene, it reversed the changes induced by the obesogenic diet in -90 bp and -62 bp positions. By contrast, no changes in the methylation status were observed when resveratrol and high-fat high-sucrose groups were compared.

In order to analyse the effect of the obesogenic diet on *fasn*, we measured the gene expression of this enzyme, showing an increase in high-fat high-sucrose diet group. Additionally, no differences in gene expression levels were observed in resveratrol and pterostilbene groups compared with control group, thus these molecules totally prevented the alteration produced by the obesogenic diet. However, regarding the methylation pattern of *fasn* in resveratrol and pterostilbene groups, resveratrol had not effects in any analysed region of the gene when compared with high-fat high-sucrose group. By contrast, pterostilbene reversed the changes induced by the obesogenic diet in -62 bp and -90 bp positions. In addition, methylation percentages of pterostilbene and control groups were similar.

Uriarte *et al.* (184) previously reported a hypomethylation of fasn gene induced by a high-fat high-sucrose diet. This reinforces our hypothesis of the hypomethylating action of an antiobesogenic dietary pattern on *fasn*, although two main differences appear when comparing both studies. On the one hand, Uriarte *el al*. observed hypomethylation in *fasn* gene after 20 weeks of high-fat high-sucrose diet while, in the present study, this effect was observed after 6 weeks. This means that DNA methylation process does not need long time-

periods to take place. Furthermore, similar results were found using different DNA methylation analysis methods (mass spectrophotometry and pyrosequencing).

Concerning *fasn* gene, several authors reported that while a high-fat diet decreases the expression of this gene, diets rich in simple or complex carbohydrates increase *fasn* expression (185-189). In the present study, it seems that the effect of high-sucrose content was greater than that of high-fat content. This result is in good accordance with those reported by Yang; 2012 when using this type of diet.

In order to explore the possibility that changes in -90 bp position could be related to decreased gene expression, a bioinformatic program was performed to identify potential transcription factors binding around this altered -90 bp position. We found that Sp1 transcription factor, which acts as a glucose sensor (190), could bind in this position. It has been demonstrated that Sp1 is crucial for *fasn* gene promoter activity in adipocytes (191), being able to influence the regulation of the gene.

In order to analyse the possible relationship between the methylation status and *fasn* gene expression, Pearson´s correlation coefficients were calculated. The results showed that only the hypomethylation of -90 bp position had a significant correlation with the *fasn* gene expression, which suggests a contribution by this position to the down-regulation of *fasn* gene.

Finally, nuclear DNMT (DNA methyltransferases) activity was measured in order to analyse the behaviour of these enzymes, which directly catalyse the addition of a methyl group cytosine residue. The activity of these enzymes showed a similar pattern of response to DNA methylation level at -90 bp. That means that it could be a mechanism in the modulation activity of these enzymes, which justifies the observed effects of high-fat high-sucrose diet and pterostilbene on *fasn* methylation.

2. INVOLVEMENT OF MIR-539-5p IN THE INHIBITION OF *de novo* LIPOGENESIS INDUCED BY RESVERATROL IN WHITE ADIPOSE TISSUE

Resveratrol is a well-studied phenolic compound, which has been suggested to be effective in preventing the development of several diseases, such as obesity. The mechanisms of action of resveratrol as anti-obesity molecule have been reported in our previous studies and in the literature: reduction in proliferation and differentiation of pre-adipocytes, increase in apoptosis, increase in lipid mobilization and fatty acid oxidation, and decrease in *de novo* lipogenesis (88, 110, 111, 115, 182). However, we want to get in deep in molecular mechanisms of these effects, such as miRNAs, as the vast majority of these studies have not addressed this issue. MiRNAs are small non-coding RNAs which regulate the expression of specific target genes post-transcriptionally, mainly by suppressing translation and/or reducing the stability of their targets mRNAs. It has been reported that several polyphenols, included resveratrol, can modify the expression of miRNAs (122).

This experiment, we determined resveratrol modifications on miRNA profile in adipose tissue and their implications in the modulation of triglyceride metabolism. Rats were divided into two experimental groups: Control group fed a high-fat high-sucrose diet (obesogenic diet) and the other a treated fed the same obesogenic diet supplemented with resveratrol in the amounts needed to reach a dose of 30 mg/kg/day (Resveratrol group). This dose was selected based on the DNA methylation experiment and on previous reported studies from our laboratory which showed that it was effective in reducing body fat mass (137). As expected, resveratrol supplementation reduced body weight and the size of epididymal and perirenal adipose tissues, as well as the sum of the four depots dissected (perirenal + epididymal + mesenteric + subcutaneous).

In order to explore the possible implication of resveratrol in the modification of miRNA profile, a miRNA microarray was carried out in perirenal adipose tissue. Of 719 microRNAs analysed, only 273 were detected and 16 were significantly modified by resveratrol supplementation, with 13 up-regulated and

3 down-regulated. The validation of four of these modified microRNAs by RT-PCR confirmed the significant changes found in the microarray analysis.

According to the literature, some miRNAs are involved in the control of genes related to metabolic pathways responsible for the anti-obesity effect of resveratrol in adipose tissue. Surprisingly, in the present study these miRNAs were not modified by this polyphenol. In view of this fact an analysis was performed according to the miRWalk Database, based on five algorithms, in order to find validated or predicted target genes of the 16 changed miRNAs. While no validated target genes were found after the analysis, *fabp3*, *sp1*, *cpt1a*, *hsl*, *ucp1*, *ucp3*, *cpt1b* and *pparγ* were found as predicted target genes. For this study, among these predicted target genes, only those relevant for triglyceride metabolism in white adipose tissue were selected: *hsl*, predicted target gene of miRNA-328a-5p, *pparγ*, predicted target gene of miRNA-129-1-3p and miRNA-129-2-3p, and *sp1*, target gene of miRNA-539-5p. These three miRNAs were significantly increased by resveratrol treatment.

As indicated before, usually miRNAs act as repressors of protein translation, directly by linking the 3´UTR of the target gene or indirectly targeting a transcription factor or other intermediates which alter the expression of proteins (106). Consequently, protein expression of these three selected genes was measured. As far as HSL, enzyme involved in lipolysis, and PPARγ, a transcription factor which regulates HSL and LPL, are concerned, no changes in protein expression were observed between Control and Resveratrol groups. Taking into account that another mechanism of miRNA regulation is the reduction in the mRNA stability of their target genes, gene expression analysis of these two genes was performed. In the same way, no differences were observed between both experimental groups. Consequently, the results of our study showed that although miRWalk database indicates that *pparγ* is a predicted target gene of miRNA-129-1-3p and miRNA-129-2-3p, and *hsl is* a predicted target gene of miRNA-328-5p, the lack of changes in their gene and protein expressions suggests that in fact they cannot be considered real target genes.

As previously mentioned in Manuscript 1, Sp1 transcription factor is an important member of the ubiquitously expressed family SP/KLF, involved among others, in *fasn* gene regulation. In contrast to HSL and PPARγ results, SP1 protein expression was significantly reduced in resveratrol-treated rats. This effect was in good accordance with the significant increase in miRNA-539-5p expression thus showing a negative way of regulation of this miRNA.

Several studies have reported that miRNA-1224, up-regulated by resveratrol supplementation in the present study, is involved in the regulation of *sp1*. Niu *et al*. (156) showed a decrease in gene and protein expression of *sp1* after miRNA-1224 transfection in human embryonic kidney cells and mouse monocytes macrophage cells. As these cell lines do not have a rodent origin, we performed an alignment of the potential rno-miRNA-1224 binding site in the 3´UTR sequence of *sp1* in rat. This analysis consists in finding possible interaction of base pairing of 2-8 nt in the in the 5´extreme of the microRNA and the 3´UTR region of the mRNA (55). The analysis revealed that miRNA-1224 theoretically can be able to bind rat *sp1* mRNA. Taking these results in mind, although this relationship has not been detected by miRWalk database as predicted or validated target gene, we decided to analyse the possible implication on the regulation of SP1.

Accumulating evidence shows that, in order to identify potential miRNA targets, over-expression or inhibition of microRNAs is a good approach to obtain robust scientific evidence. According with this fact, 3T3-L1 adipocytes were transfected with mmu-miRNA-539-5p and mmu-miRNA-1224. Cells over-expressing miRNA-1224 showed that SP1 protein expression was not altered compared with control group. By contrast, SP1 protein expression was not detectable in cells over-expressing miRNA-539-5p. These results suggest that the reduction of SP1 protein expression induced by resveratrol is modulated by miRNA-539-5p.

In order to obtain further information concerning both analysed miRNAs, Pearson´s correlations between each microRNA expression and SP1 protein

expression were calculated. Results showed that only miRNA-539-5p was negatively correlated with SP1 protein modification.

Magaña *et al*.(18) reported that *sp1* acts together with *srebp1* to synergistically activate the promoter of *fasn* gene, regulating *de novo* lipogenesis pathway. Accordingly, we analysed protein expression of SREBP1 transcription factor and *fasn* gene expression. A significant reduction in SREBP1 protein expression was observed in resveratrol-treated group compared with the control group. In good accordance with these results, gene expression of *fasn* was also reduced. These results show that the body fat-lowering effect of resveratrol is mediated, at least in part, by a reduction in *de novo* lipogenesis.

This study demonstrates for the first time that resveratrol modifies the miRNA profile in white adipose tissue. As far as triglyceride metabolism is concerned in this tissue, this study shows that miRNA-539-5p is involved in the inhibition of *de novo* lipogenesis induced by resveratrol.

3. INVOLVEMENT OF MIRNA-103, MIRNA-107 AND MIRNA-122 IN THE PREVENTION OF LIVER STEATOSIS INDUCED BY RESVERATROL

Previous studies performed in our laboratory, as well as studies reported by other authors, have demonstrated that resveratrol is able to reduce liver steatosis. With regard to the mechanisms, it has been observed in our group that resveratrol treatment could induce increased fatty acid oxidation because the activity of CPT1a, key enzyme in the carnitine-dependent transport across the mitochondrial inner membrane, was enhanced. By contrast, *de novo* lipogenesis was not involved in the effect of resveratrol because FAS activity remained unchanged. As previously mentioned in Summary 2, molecular regulatory pathways of lipid metabolism include miRNAs. It has been reported in the literature that different polyphenols, such as proanthocyanidins or a mixture extracted from *Hibiscus sabdariffa*, are able to modify the expression of miRNA-122-5p (a liver specific miRNA and the most abundant one) and the paralogs miRNA-103-3p and miRNA-107-3p in liver (136, 167-169, 192). In this context, we wanted to determine whether the reduction in liver steatosis induced by resveratrol in rats fed an obesogenic diet was mediated by miRNAs. For this purpose, we analysed miRNA-103, miRNA-107 and miRNA-122, which represent 70% of all miRNAs in liver (136).

The experiment was conducted with Sprague-Dawley rats which were divided in two experimental groups: a control group fed a high-fat high-sucrose diet (obesogenic diet) and the other a treated group fed the same obesogenic diet supplemented with resveratrol in the amounts needed to reach a dose of 30 mg/kg/day (Resveratrol group). This study showed that resveratrol treatment did not reduce final body weight or liver weight. By contrast, resveratrol treatment induced a significant decrease in hepatic triglyceride content (170).

First, we searched possible predicted and validated target genes of these three miRNAs related to triglyceride metabolism in miRecords database and in the literature. As far as lipogenic pathway is concerned, according to miRecords, *srebf1* is a predicted target gene for paralogs miRNA-103-3p and miRNA-107-3p

and *fasn* is a predicted target gene of miRNA-122-5p. Moreover, Iliopoulos *el al.* (178) reported the indirect regulation of *srebf1* by that miRNA. They observed that up-regulation of this miRNA induced increased SREBP1 protein expression. Taking into account that miRNAs are negative regulators of protein translation, and that no miRNA-122-5p binding sites are found in the 3´UTR or in the coding regions of this gene, they concluded that *srebf1* was an indirect target gene for miRNA-122. Unfortunately, they did not know the intermediate steps in the signalling cascade that led to the up-regulation of SREBP1. In good accordance, Shibata *el al.*(179) reported that silencing miRNA-122-5p led to decreased SOCS3 expression, which in turn increased STAT3 expression. Thus, SREBP1 was negatively regulated by STAT3. Similarly, a decrease in miRNA-122 expression induced a decrease in SREBP1 expression. In addition, Bhatia *et al.* (181) reported that miRNA-107 targeted *fasn* and decreased its protein levels by complementary binding to the 3´UTR of target mRNAs.

In order to obtain more scientific support concerning the involvement of these miRNAs in SREBP1 regulation, an over-expression study was performed in AML 12 hepatocytes. In the three cases, we observed a significant increase in SREBP1 protein expression.

In addition, we measured the expression of the three selected miRNAs in liver of rats treated with resveratrol and we observed a significant decrease in resveratrol-treated rats in all cases. Then, we analysed protein expression of SREBP1 and FAS. While SREBP1 was significantly decreased in resveratrol treated-group, no changes were observed in FAS.

With regard to miRNA-122-5p bearing in mind that the results of our *in vitro* study and considering the results reported by Iliopoulus *et al.* and Shibata *et al.* it may be suggested that resveratrol decreases protein expression of SREBP1 indirectly via miRNA-122-5p. In addition, the over-expression of miRNA-103-3p and miRNA-107-3p in AML 12 hepatocytes resulted in increased SREBP1 protein expression. Moreover, in the *in vivo* study, liver from resveratrol-treated rats showed down-regulation of these miRNAs, which was accompanied by a reduced expression of SREBP1. Taking together, these results suggest that these miRNAs are positive regulators of SREBP1.

According to miRecords, *fasn* is a predicted target gene of miRNA-122-5p. Over-expression in AML12 hepatocytes of this miRNA induced a significant reduction of in FAS protein expression. Therefore, increased gene expression should be expected. When we measured FAS protein expression in liver, we found no change in resveratrol-treated rats. This result is in good accordance with the lack of change in FAS activity observed in our previous study addressed in this cohort of animals. This result is surprising because *fasn* is, according to miRecords, a predicted target gene for miRNA-122-5p, which was reduced by resveratrol. In fact, our transfection experiment shows that over-expression of miRNA-122-5p induced a significant reduction in FAS protein expression. On the other hand, SREBP1, a transcription factor that regulates FAS, was reduced in resveratrol-treated rats. Thus, it could be hypothesized that the increase in FAS protein expression expected as a consequence of miRNA-122-5p down-regulation could be compensated by the decrease expected due to the reduction in SREBP1. Moreover, Bathia *et al*. (181) reported transfected HepG2 hepatocytes with miRNA-107 at various doses. They observed that when using the most common dose in transfection studies (25 nM) no changes in FAS protein expression were observed

As far as CPT 1a is concerned, according to miRecords, *cpt 1a* is a predicted target gene for miRNA-103-3p and miRNA-107-3p. Over-expression of miRNA-107-3p in AML12 hepatocytes reduced CPT 1a protein expression. By contrast, miRNA-103-3p did not produce any effect. Furthermore, in the *in vivo* study, liver from resveratrol-treated rats showed down-regulation of these miRNAs. These results are in good accordance with the increase in CPT 1a activity previously reported in this cohort of animals. Accordingly, the increase induced by resveratrol in CPT 1a protein expression, involved in the delipidating effect of this polyphenol, seems to be mediated by the reduction on miRNA-107-3p expression.

CONCLUSIONS

1. Under our experimental conditions, high-fat high-sucrose feeding induces over-expression of *fasn* due to hypomethylation of -90bp position, whereas it does not modify methylation status of *pnpla2* and *pparγ* genes.

2. Pterostilbene prevents *fasn* gene up-regulation induced by an obesogenic diet, due to the hypomethylation in -90 bp position of this gene.

3. Resveratrol also prevents *fasn* gene up-regulation induced by an obesogenic diet, but this effect is not mediated by modifications in DNA methylation pattern.

4. Resveratrol supplementation modifies microRNA profile in white adipose tissue of rats fed an obesogenic diet, but only miRNA-328a-5p, miRNA-129-1-3p, miRNA-129-2-5p and miRNA-539-5p have predicted target genes related to triglyceride metabolism, and thus could be involved in the body-fat lowering effect of this phenolic compound.

5. MiRNA-539-5p, up regulated by resveratrol in white adipose tissue, is a direct regulator of SP1 protein expression.

6. The reduction in protein expression of SP1 and SREBP1 transcription factors induced by resveratrol in white adipose tissue leads to the inhibition of *fasn* gene expression, which in turn is involved in the anti-obesity effect of this phenolic compound.

7. The reduction in SREBP1c protein expression induced by resveratrol in liver is positively mediated by miRNA-103-3p and miRNA-107-3p.

8. Despite the reduction in SREBP1c, FAS protein expression remained unchanged, probably because the effect of this transcription factor is

compensated by the increase expected as a consequence of miRNA-122-5p down-regulation.

9. The increase in CPT1a protein expression induced by resveratrol in liver is negatively mediated by miRNA-107-3p. Consequently, it can be suggested that the prevention of liver steatosis induced by resveratrol is due, at least in part, to increased fatty acid oxidation.

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ANNEX 1

EXPERIMENTAL WORK IN THE INTERNATIONAL STAY. MANUSCRIPT 4.

My international stay, which lasted from March to June 2016, took place in the Unit of Endocrinology of Karolinska University Hospital, led by Dr. Mikael Rydén and in the Lipid Laboratory at NOVUM in Huddinge, led by Professor Peter Arner, who has been studying the role of human adipose tissue in common metabolic disorders for more than four decades. The studies developed in the laboratory span from the study of genetics and molecules in different *in vitro* systems to clinical assessments in human subjects. The aim is to better understand the mechanisms that link changes in fat mass to metabolic disorders.

Obesity is characterized by a chronic low grade inflammation associated with cardiometabolic risk factors such as insulin resistance and type 2 diabetes mellitus. It is proposed that increased release of chemoattractant proteins from white adipose tissue promotes macrophage infiltration, which attenuates adipocyte insulin sensitivity by means of their pro-inflammatory and pro-fibrotic properties (Gökhan S, Hotamisligil *et al*. 2006; Weisberg *et al*. 2003). CC Motif Chemokine ligand 2 (CCL2 or MCP-1) appears to play the major role in this process (Deshmane *et al.* 2009). Thus, increased CCL2 expression in white adipose tissue has been directly linked to the development of insulin resistance in obesity (Kanda *et al*. 2006; Sartipy and Loskutoff *et al*. 2003).

A central epigenetic process is DNA methylation and recent studies in obese and/or type 2 diabetes subjects have demonstrated altered methylation metabolically relevant genes in white adipose tissue and isolated adipocytes (Agha *et al.* 2015; Dick *et al.* 2014; Nilsson *et al.* 2014; Arner *et al.* 2015). DNA methylation is induced by DNMTs that transfer methyl moieties from the methyl donor SAM. Dietary methyl moieties fuel plays an important role in intracellular SAM levels, as does folate (vitamin B9). The folate and methionine cycle are together known as the one carbon cycle (Ducker and Rabinowitz *et al.* 2016; Gueant *et al.* 2013).

Previous studies in this laboratory demonstrated that adipocyte DNA from obese compared with that from non-obese individuals is globally hypermethylated, compared with that from non-obese individuals. Despite this, obese individuals have lower serum folate levels than non-obese subjects. Moreover, genetic variations in the one carbon cycle gene MTHFR are associated with folate deficiency and increased circulating levels of CCL2. Conversely, folate supplementation in humans reduces systemic inflammation as well as plasma CCL2 levels.

The project in which I worked was devoted to analysing the possible implication of folate transporter SLC19A1 expression in the promotion of white adipose tissue inflammation through DNA methylation, by using adipocytes from obese subjects. During the four months I contributed to the development of some parts of the project. Primary cultures of human *in vitro* differentiated adipocytes were set up.

- SLC19A1 silencing by RNAi transfections
- SLC19A1, IL-6, TNFα, DNMT1, DNMT 2A, DNMT 3A, DNMT 3B, HEXB, FPGS, MTHFD1, DHFR gene expression
- SLC19A1 protein analysis
- IL-6, MCP-1 and TNFα quantification
- Transcription factor binding prediction
- Pyrosequencing and methylation status assessment of CCL2

CONCLUSIONS

The results obtained with the present analysis suggest that:

1. Down-regulation of SLC19A1 in obese adipocytes results in DNA hypermethylation of this gene and this increases the expression of different inflammatory genes, highlighting CCL2.

2. The methylation status of CpG 12698626, present in a predicted glucocorticoid receptor binding site, affect the response to cortisol of CCL2 gene in differentiated adipocytes.

MANUSCRIPT 4

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Adipocyte Expression of SLC19A1 Links DNA Hypermethylation to Adipose Tissue Inflammation in Obesity

EMBO Molecular Medicine (Submitted)

Summary

Insulin resistance in obese white adipose tissue (WAT) is linked to local inflammation, an acquired phenotype which may depend on epigenetic mechanisms. Herein, analyses of human adipocytes revealed an obesity-linked global DNA hypermethylation associated with the expression of pro-inflammatory pathways. DNA-methylation is regulated through the one carbon cycle (1CC); several adipocyte-expressed 1CC-genes were altered in obese WAT. The strongest association with insulin resistance and pro-inflammatory genes was observed for a reduction in *SLC19A1,* encoding a cell-membrane folate carrier. *SLC19A1*knockdown in human adipocytes perturbed intracellular 1CC-metabolite levels and induced DNA hypermethylation where methylation of a CpG site (cg12698626) in the promoter of *CCL2* associated positively with CCL2 expression/secretion.cg12698626is situated in a glucocorticoid receptor binding region and the repressive action of cortisol on *CCL2* promoter activity was abrogated upon cg12698626 methylation. Thus, reduced *SLC19A1* expression in obese adipocytes may promote WAT inflammation through an epigenetic mechanism involving cortisol resistance.

Word count (150/150)

Key words

One carbon metabolism, Glucocorticoid, Insulin resistance, Diabetes, Adipose tissue, Adipocyte, Inflammation, CCL2

Introduction

Obesity is characterized by a chronic low grade inflammation associated with cardiometabolic risk factors such as insulin resistance and type 2 diabetes mellitus (T2DM) (Gökhan S. Hotamisligil, 2006; Ouchi et al., 2011; Xu et al., 2003). The current paradigm proposes that increased release of chemoattractant proteins from WAT promotes macrophage infiltration which attenuate adipocyte insulin sensitivity through their pro-inflammatory and pro-fibrotic properties (Gökhan S. Hotamisligil, 2006; Weisberg et al., 2003). Macrophages are attracted by specific cyto- and chemokines secreted from both adipocytes and resident macrophages. Among these, C-C Motif Chemokine Ligand 2 (CCL2; also known as Monocyte Chemoattractant Protein-1)appears to play a major role (Deshmane et al., 2009). Thus, increased *CCL2* expression in WAT has been directly linked to the development of insulin resistance in obesity (Kanda, 2006; Sartipy and Loskutoff, 2003). Although WAT macrophages account for most of the *CCL2* expression, mature adipocytes express substantial levels as well (Dahlman et al., 2005; Meijer et al., 2011). This suggests that the adipocytes themselves may induce inflammation by recruiting macrophages into the tissue. However, despite intense research in animal models, surprisingly little is known about the molecular mechanisms that initiate WAT inflammation. Unraveling the regulation of *CCL2* in adipocytes could therefore provide insights into this process.

Epigenetic regulation links environmental factors to altered gene expression (Vanhees et al., 2014) and a growing body of evidence suggests that epigenetic mechanisms influence the risk of metabolic complications in obesity (Gluckman et al., 2009; Jirtle and Skinner, 2007). A central epigenetic process is DNA methylation and recent studies in obese and/or T2DM subjects have demonstrated altered methylation near metabolically relevant genes in both WAT(Agha et al., 2015; Dick et al., 2014; Nilsson et al., 2014) and isolated adipocytes (Arner et al., 2015). DNA methylation is induced by DNA methyl transferases (DNMTs) that transfer methyl moieties from the methyl donor *S*-adenosyl methionine (SAM). SAM levels are regulated via the methionine cycle

wherein the enzyme methionine adenosyltransferase 2 converts methionine to SAM. Dietary methyl moieties fuel to the methionine cycle in order to regenerate SAM. Thus, folate (vitamin B9, an important dietary methyl source) plays an important role for intracellular SAM levels (Ducker and Rabinowitz, 2016; Guéant et al., 2013; Wolff et al., 1998). The folate and methionine cycle are together known as the one carbon cycle (1CC, summarized in a simplified manner in Figure 1A).

We recently demonstrated that adipocyte DNA from obese compared with non-obese individuals is globally hypermethylated (Arner et al., 2015). Despite this, obese individuals have lower serum folate levels than non-obese subjects (Mojtabai, 2004). Moreover, genetic variations in the 1CC gene *MTHFR* are associated with folate deficiency and increased circulating levels ofCCL2(Hammons et al., 2009). Conversely, folate supplementation in humans reduces systemic inflammation as well as plasmaCCL2levels (Solini et al., 2006). Altogether, these observations suggest a link between the 1CC and inflammation. Nevertheless, despite great progress in the field of WAT epigenetics (Agha et al., 2015; Dick et al., 2014; Gehrke et al., 2013; Keller et al., 2014; Koza et al., 2006; Nilsson et al., 2014)there is still a lack of mechanistic insights into the link between DNA-methylation, gene expression and obesity-induced inflammation.

We hypothesized that WAT inflammation could be influenced by epigenetic mechanisms resulting from perturbed 1CC metabolism in obese adipocytes. Weidentified*SLC19A1*, a gene encoding a cell membrane expressed folate carrier, to be associated with a pro-inflammatory phenotype in WAT of obese individuals. Through a combination of different analyses and assays we demonstrate that reduced adipocyte *SLC19A1* expression results in increased global DNA methylation including a specific site in the *CCL2* promoter. The latter attenuates the repressive action of endogenous cortisol resulting in increased CCL2 secretion. Our data therefore establish a potential epigenetic link between obese adipocytes and the regulation of WAT inflammation.

Results and Discussion

Adipocyte DNA-hypermethylation is associated with an inflammatory transcriptome

In order to identify epigenetic factors regulating adipocyte inflammation we compared the global adipocyte DNA-methylome with WAT gene expression in obese(n=8) and non-obese(n=8) individuals (subgroup of cohort 1, clinical characteristics in Table S3). In line with previous data on global DNA methylation (Arner et al., 2015), we observed a higher methylation of CpGs proximal (defined as <1.5 kb upstream to TSS, in gene bodies, in 5'- or 3'- UTR) to protein-encoding genes in adipocytes from obese vs non-obese individuals (Figure 1B). The genes whose expression correlated with hypermethylated CpGs in obesity were subdivided based on the direction of their expression, *i.e.* increased or decreased upon DNA methylation. Agene ontology (GO) analysis revealed pronounced differences in the cellular processes associated with the two groups (Figure 1C, Supplementary table S1). CpG hypermethylation associated positively with the expression of genes in pro-inflammatory pathways but negatively with metabolic processes, in particular lipid metabolism. A correlation between DNA hypermethylation and increased inflammation has previously been reported also in murine 3T3-L1 adipocytes (Malodobra-Mazur et al., 2014).

DNA methylation has classically been considered to confer gene repression. However, results in recent years have also demonstrated increased gene activation(Suzuki and Bird, 2008). The direction of the association between DNA methylation and gene expression have been proposed to depend on the location of the CpG in relation to the gene, *i.e.* repression when located in the promoter and activation when located in the gene body (Jones, 2012). We found that the location of the CpGs in relation to a CpG island rather than to the gene was linked to the direction of gene expression (Figure S1). Thus, CpG methylation in genes associated with increased expression was overrepresented in open sea regions whereas CpGs in negatively associated genes were more common in shore and shelf regions (Figure S1). That differentially methylated regions (DMR)

in human adipocytes associated with gene expression were more common outside CpG islands is in line with previous reports on DMRs in non-adipose tissues and cancer cells(Irizarry et al., 2009).Altogether, these findings indicate a link between adipocyte inflammation and DNA-hypermethylation, particularly in open sea chromatin.

Expression of 1CC genes in obese adipocytes identifies a strong association between SLC19A1 and insulin resistance

DNA methylation is a dynamic process which is influenced by the availability of specific metabolites and their precursors (e.g. folates, vitamin B12, choline and/or methionine).Another level of regulation is the activity/expression of enzymes and transporters in the 1CC (Ducker and Rabinowitz, 2016; Guéant et al., 2013; Wolff et al., 1998). To determine whether the expression of 1CC genes (n=78) was altered in obese WAT, we probed previously generated transcriptomic data from obese (n=15) and non-obese (n=15) subcutaneous abdominal WAT (cohort 1, Table S3).Approximately half of the folate and one third of the methionine cycle genes were altered in obesity (Figure 1Dand Table S2). Among the genes dysregulated in obesity, those involved in the folate cycle were pronouncedly induced during the later stages of adipogenesis (Figure 1E), indicating that this gene set may be of relevance for adipocyte function in obesity. Seven folate cycle genes, altered in both obesity and induced during adipogenesis, were identified (Table 1). Out of these, *SLC19A1* (Figure 1A, in bold) displayed the strongest association with insulin sensitivity(measured by insulin tolerance test *in vivo*) and expression of pro-inflammatory genes (Table 1,cohort 2,n=56, characteristics in Table S3). The correlation between *SLC19A1* and genes in inflammation pathways stood out also in cohort 1 (p=0.004) however, insulin tolerance tests were not performed in these individuals. *SLC19A1*encodes a cell membrane-expressed folate transporter which is responsible for the uptake of the most abundant circulating form of folate, i.e. the reduced form termed 5-methyltetrahydrofolate (5-CH_3-THF).5-CH_3-THF

constitutes a central substrate in the intersection between the folate and methionine cycles (Guéant et al., 2013)(Figure 1A).

SLC19A1 expression in WAT is enriched in adipocytes and reduced in obesity

The cell-specific expression of *SLC19A1* in human WAT was validated by qPCR in paired samples from 11 individuals of isolated adipocytes and stroma vascular fraction (SVF). In line with the expression during adipogenesis, *SLC19A1* was enriched in the adipocyte fraction (Figure 2A). Moreover, *SLC19A1* was lower in isolated adipocytes from obese (n=13) compared with age-matched lean (n=19) subjects (Figure 2B).These results, together with the link to insulin sensitivity and inflammation described above, suggest that reduced *SLC19A1*expression in obese adipocytes may impact on adipocyte function, pro-inflammatory adipokine secretion and possibly cardiometabolic health. Admittedly, the factors regulating *SLC19A1* expression are not known. We observed no effects on *SLC19A1* mRNA levels in *in vitro* differentiated human adipocytes following incubation with TNFα (50 ng/ml up to 24h) or after exposure to hypoxia (1 and 5% O_2 up to 48h) (data not shown).

Reduced SLC19A1 expression induces global DNA hypermethylation

The functional consequences of attenuated *SLC19A1* expression were determined by gene knockdown in *in vitro* differentiated human adipocytes. The expression of *SLC19A1* was significantly reduced using small interfering RNA (siRNA) at both the mRNA (~75%, Figure 3A) and protein level (~50%, Figure 3B). Attenuated SLC19A1 levels resulted in a lower SAM/SAH ratio (Figure 3C) and a significant increase in global DNA methylation (Figure 3D), establishing a causal link between reduced adipocyte *SLC19A1*expressionand DNA hypermethylation. It may seem counterintuitive that a decrease in folate metabolism leads to an increase in DNA methylation. However, it has been demonstrated in several different cell models that folate deprivation induces DNA-methylation both globally (Farias et al., 2015)as well as in site-specific CpGs (Jhaveri et al., 2001).

Similar observations have been reported in rat colon cells *in vivo* (Sohn et al., 2003). Additionally, Ngo *et. al.* showed that SAH treatment alone in 3T3-L1 cells(resulting in a ~25% decrease of the SAM/SAH ratio, i.e. similar to that observed herein after si*SLC19A1*) induced site specific increases in DNA methylation(Ngo et al., 2014). The mechanisms responsible for these effects are either an increased methylation activity or an attenuated demethylation activity. In *SLC19A1* RNAi-treated adipocytes there were no changes in the gene expression of methyltransferases (*DNMT1, DNMT3A, DNMT3B* and *DNMT3L*) or demethylation enzymes (*TET1, TET2* and *TET3*) compared with control cells(transfected with non-silencing siRNA, data not shown). However, as DNMTs undergo several post-translational modifications (e.g. ubiquitination and sumoylation) which regulate their stability and activity (Kinney and Pradhan, 2011), it is interesting to note that several genes involved in ubiquitination were selectively altered in the knockdown. We also observed an almost 50 % increase in *SUMO1* expression(data not shown) which has been shown to regulate DNMT activity via sumoylational modifications (Lee and Muller, 2009; Ling et al., 2004). Taken together, these findings demonstrate that *SLC19A1* affects DNA methylation through mechanisms that remain to be defined.

Bioinformatic analyses identifies a link between SLC19A1 expression and CpG methylation in the CCL2 promoter

The association between DNA hypermethylation, pro-inflammatory pathways and *SLC19A1* expression prompted us to search for specific CpG loci linking *SLC19A1* to inflammation. To this end, we overlapped three global -transcription and -methylation arrays (Figure 3E). First, we performed transcriptome arrays comparing *SLC19A1*-silenced adipocytes with control-transfected cells. This identified 1400 significantly regulated transcripts (FDR<25 %). In order to select regulated genes of possible pathophysiological relevance, this dataset was compared with the expression of *SLC19A1*in the previously mentioned subjects where both adipocyte DNA-methylation and WAT gene expression was available (sub-cohort 1, Table S3). Genes were filtered by Pearson's r with a threshold of -

0.6>r>0.6 and a direction that was congruent with the regulation *in vitro* (*i.e.* a gene downregulated in *SLC19A1* silenced cells should correlate positively with *SLC19A1 in vivo*).279 genes fulfilled these criteria. To assess whether the expression of these genes were linked to differences in DNA methylation, we correlated their expression with the degree of methylation in proximal CpG loci (defined as above). The criterion in this analysis was that the direction of the correlation between methylation and gene expression should be concordant with the direction of the gene expression in *SLC19A1* silenced adipocytes and the observation that low *SLC19A1* expression leads to a hypermethylated DNA. We identified 107 CpG loci associated with the expression of 46 genes (Table S4). The genes that were annotated in at least one of the inflammatory biological processes (Figure 1B) were selected and are summarized in Table 2. Using this bioinformatic approach, *CCL2* was identified as the top gene that fulfilled all four criteria, i.e. upregulated in *SLC19A1* silenced adipocytes, negative correlation with *SLC19A1* expression in WAT, at least one CpG (cg12698626) where methylation correlated positively with gene expression and annotated in the GO inflammatory processes.

Reduced SLC19A1 expression inducesCCL2 secretion and promoter methylation

A causal link between *SLC19A1* and *CCL2* was demonstrated *in vitro* as si*SLC19A1* treatment resulted in increased *CCL2* expression (by qPCR, graph not shown) as well asCCL2 secretion (Figure 4A). This effect was specific for CCL2as neither TNFα nor adiponectin were affected (Figure 4A). Furthermore, there was a significant negative association between adipose *SLC19A1* expression and *ex vivo* CCL2(but not TNFα or adiponectin) secretion which was independent of BMI in cohort 2(Figure 4B). That epigenetic mechanisms can regulate *CCL2* expression in WAT has previously been shown in a murine study where DNA hypermethylation induced by adipocyte-specific overexpression of the DNA methyl transferase Dnmt3a resulted in an enhanced, high fat diet-induced, expression of *CCL2* and overall WAT inflammation (Kamei et al., 2010). In murine

adipocytes, DNA hypermethylation may also inhibit the expression of adiponectin (Kim et al., 2015), an effect which we did not observe in human cells indicating inter-species differences in adipose epigenetic regulations. Given that *SLC19A1* silencing resulted in an intracellular state that mimics folate-deficiency (Figure 3C), it is interesting to note that a 12-week long folate supplementation in overweight subjects reduced circulating CCL2levels and improved insulin sensitivity (expressed as HOMA-index) (Solini et al., 2006). Admittedly, that study did not determine the *CCL2*expression in WAT and it is possible that the observed effects reflected actions in other cell types including leukocytes and/or endothelial cells as discussed elsewhere(Brown et al., 2006). Furthermore, folic acid supplementation might not be of benefit for everyone and harmful effects cannot be excluded (Smith et al., 2008).

Our combined analysis of different data sets demonstrated an association between *SLC19A1* and cg12698626 in the*CCL2* promoter. To establish a link between *SLC19A1* and *CCL2* expression through cg12698626 methylation, pyrosequencing assays were performed in si*SLC19A1* treated adipocytes. This demonstrated that *SLC19A1* silencing increased the methylation of cg12698626 (Figure 4C). Furthermore, changes in cg12698626 methylation correlated positively with the change in *CCL2* expression (Figure 4D). In primary *in vitro* differentiated adipocytes from various donors there was a clear bimodal distribution in cg12698626 methylation (Figure 4E). Cells with highly methylated cg12698626 displayed a significantly higher secretion of CCL2 than cells with a low degree of methylation (Figure 4F). Although several CpG loci linked to gene expression are conserved between mice and humans (Multhaup et al., 2015), cg12698626 is not among these (data not shown). Moreover, in contrast to observations in humans, diet-induced obesity in mice has been reported to induce hypomethylation of CpGs near inflammatory genes (Multhaup et al., 2015). Taken together, this suggests that *CCL2* is regulated through specific epigenetic mechanism in humans which discouraged us from developing an adipocyte-specific *Slc19a1* knockout mouse.

Methylation of cg12698626 inhibits glucocorticoid repression of CCL2

To identify a molecular mechanism explaining why site-specific methylation increases *CCL2* expression we retrieved publically available ENCODE ChIP-sequencing data to determine whether cg12698626 was in the proximity of potential transcription factor binding sites. The promoter region of *CCL2* (defined as 1.5 kb upstream of the transcription start site) contained a total of seven CpGs (CG1-7, Figure 5A) where the relative importance of cg12698626 (CG4) for WAT *CCL2*expression was demonstrated by correlation analyses between the methylation degree (β-value) of each CpG and *CCL2* expression (sub-cohort 1, Figure 5B). CG4is present in a predicted glucocorticoid receptor (GR) binding site, which was confirmed by electromobility shift assay (EMSA)using a purified GR DNA binding domain (GR-DBD) (Figure S2A). Glucocorticoids are anti-inflammatory (Staab and Maser, 2010) and as expected cortisol rapidly decreased *CCL2* expression in *in vitro* differentiated adipocytes (Figure 5C).To determine whether CG4 methylation could impact on this effect we developed a luciferase reporter assay where different plasmids containing the *CCL2* promoter (with or without CG4) were electroporated in *in vitro* differentiated adipocytes. In agreement with the results in Figure 5C, *CCL2* promoter activity was significantly reduced upon treatment with cortisol, are pressive effect that was fully abrogated when the promoter was methylated (Figure 5D). In similar experiments performed with constructs lacking CG4(Figure 5E),the repressive effect of cortisol was abolished and was independent of DNA methylation status. This suggests that cg12698626 as well as its methylation status affect the response to cortisol. Despite this, EMSA demonstrated that the GR-DBD interacted with cg12698626-containing oligonucleotides irrespective of whether it was unmethylated (CG4/-CH_3) or methylated (CG4/+CH_3) (Figure S2B). However, further investigation using thermal shift assay showed that the GR-DBD required a significantly higher dissociation temperature when bound to CG4/-CH_3 than to CG4/+CH_3 (Figure 5F), supporting the notion thatcg12698626 methylation attenuates GR binding affinity. It must be stressed that the interactions between GR and the *CCL2* promoter were assessed using techniques

that may not fully reflect the complexity in epigenetic modifications of chromatin DNA (Cedar and Bergman, 2009). A direct evaluation of cg12698626would ideally require site-specific modifications using CRISPR/Cas9 linked to a DNA methyltransferase (Vojta et al., 2016),techniques that are not yet well-established in primary human fat cells as they require the establishment of cell clones (Claussnitzer et al., 2015).

In conclusion, the findings of the present study suggest that downregulation of *SLC19A1* in obese adipocytes results in DNA hypermethylation and altered expression of specific genes among which *CCL2* appears to be of particular importance. Although speculative, this may constitute a mechanism through which enlarged fat cells in expanding WAT initiate macrophage infiltration and inflammation, a hypothesis that needs to be tested in future studies. Also, the mechanisms responsible for *SLC19A1* downregulation need to be defined.

Methods

Patient cohorts

Clinical characteristics of the cohorts are detailed in Table S3. Cohort 1 (n=30),including the subgroup (n=16) where adipocyte DNA methylation was available has been described elsewhere (Arner et al., 2015). Cohort 2is described in more detail in (Arner et al., 2012). The study was approved by the regional ethics board and performed according to the statutes in the Declaration of Helsinki. Informed written consent was obtained from all participants.

Adipocyte and SVF fractionation

Mature adipocytes and SVF were isolated from adipose tissue as previously described (Curat et al., 2004). For isolation of RNA from mature adipocytes, samples were obtained from 19 non-obese (age 36 ± 9 years, BMI, 23 ± 1 kg/m^2) and 13 obese (age 33 ± 6 years, BMI 37 ± 4 kg/m^2). For isolation of adipocyte and SVFfrom adipose tissue, WAT was obtained from 11 individuals undergoing plastic surgery for non-malignant diseases.

Cell cultures

Primary cultures of human *in vitro* differentiated adipocytes were set up as described previously (van Harmelen et al.).These cells were used to define inter-individual differences in the effects of *SLC19A1* knockdown. The reporter construct experiments were performed in *in vitro* differentiated adipocytes obtained from adipose-derived stem cells (ASCs) which were expanded *in vitro* and differentiated into adipocytes as reported (Pettersson et al., 2013). The cortisol time course were performed using differentiated ASC adipocytes and adding cortisol 6, 4 and 2 h prior to cell lysis. Cells were cultured on standard 96, 48, 24 and 12 well plates.

SLC19A1 silencing by RNAi

SLC19A1 silencing was performed in primary adipocytes using the ON-TARGET plus Human *SLC19A1* siRNA SMART pool (L-007422-01-0005) and compared to siGENOME Non-Targeting siRNA (D-001206-13-05) purchased from Dharmacon (Little Chalfont, UK). Transfections were performed as previously described (Gao et al., 2014).

RNA isolation, cDNA synthesis and RT-qPCR

All steps were performed as in (Gao et al., 2014) except the cDNA synthesis which was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, Carlifornia, USA). Furthermore RNA from some samples was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). All TaqMan probes were purchased from Applied Biosystems (CA, USA) and targeted *SLC19A1* (Hs00953344_m1)*, CCL2* (Hs00234140_m1) and the housekeeping gene *LRP10* (Hs00204094_m1)

Protein lysis and Western Blot

Protein lysis and Western blotting was performed as described (Ryden et al., 2002). Briefly, an 8% acrylamide gel was cast and 20 µg of total protein were loaded into each well. Anti-SLC19A1 (Atlas antibodies, Stockholm, Sweden, product number HPA024802) was diluted 1:500 , blots were incubated at 4 °C overnight with gentle agitation. SLC19A1 levels were normalized against actin (Sigma Aldrich, product number A2066).

Metabolite measurements

Control- or*SLC19A1*-silenced cells were placed on dry ice directly after medium aspiration and incubated with 80 % pre-cooled (-80°C) methanol for 20 minutes. Cells were harvested, transferred to tubes and centrifuged at 4°C, 14000 *g*for5 minutes. The supernatants were transferred to new tubes and the pellets were

washed with additional methanol followed by an additional round of centrifugation. The samples were stored in -80°C until analysis.

Transcriptome and epigenome arrays

Primary *in vitro* differentiated adipocytes were transfected with non-targeting siRNA or si*SLC19A1* and gene expression was assessed using GeneChip® Human Transcriptome Array 2.0 (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Cells from three individual donors were used. The global WAT transcriptome and adipocyte epigenome of the two clinical cohorts has previously been described (Arner et al., 2012; Arner et al., 2015).

Enzyme-linked immunosorbent assay (ELISA)

Kits from R&D Biosystems (Minneapolis, USA) were used to assess the secretion of CCL2 (catalog number DCP00), TNFα (catalog number QTA00B) and Adiponectin (catalog number DY1065). An ELISA detecting 5-methylcytosines (Sigma Aldrich, catalog number MDQ1) was used to determine global DNA methylation.

DNA isolation, bisulfite conversion and pyrosequencing

DNA was isolated from *in vitro* cultured adipocytes with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). The bisulfite conversion was performed using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA) following the instructions provided by the company. Two hundredng of input DNA was used in 20 µl. The software PyroMark Assay Design 2.0 (Qiagen, Hilden, Germany) was used for primer design of both PCR amplification primers and the sequencing primer. The following primers were used; forward-AGGTAATTAGTTGGAGGATTTGT; biotinylated reverse: CCAAACAACCCTATCCCCAATAAA and sequencing: ATTAGTTGGAGGATTTGTA. The dispensing order was: TAYGTTTTTT TTTAGTAGTA TGTTAGAG, the Y representing

the cg12698626 position. One microliter bisulfite converted DNA was amplified using the PyroMark PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An annealing temperature of 58 °C was used. The size of the PCR product was controlled by gel electrophoresis. Thereafter, 4 µM sequencing primer and sepharose beads (GE Healthcare, Little Chalfont, UK) was mixed with the PCR product and pyrosequenced with the PSQ 96 ID system (Qiagen, Hilden, Germany) using PyroMark Gold Q96 reagents (Qiagen, Hilden, Germany).

Gene Ontology (GO) analysis and transcription factor binding prediction

GO-analyses were performed on gene IDs where CpG loci (<1.5 kb upstream to TSS, in gene bodies, in 5' UTR or in 3' UTR)were hypermethylated in obesity and where the degree of methylation correlated with the expression. The genes that correlated positively and negatively with associated CpG methylation were analyzed in the ToppGene database (https://toppgene.cchmc.org/) to define their role in different biological processes. The transcription factors binding to the *CCL2* promoter were identified using the publicly available ENCODE ChIP-seq data presented in the hg19 UCSC genome browser (https://genome.ucsc.edu/index.html). Thereafter the ALGGEN-PROMO (http://alggen.lsi.upc.es) bioinformatic software was used to predict transcription factor binding that overlapped with cg12698626.

CCL2 promoter luciferase reporter assay

Two constructs containing the upstream region of the*CCL2*promoter with and withoutcg12698626were prepared by PCR using KAPA HotStart ReadyMix (Kapa Biosystems, Wilmington, MA) from human genomic DNA (patient sample) using primers, forward 5´-GAAGATCTACAGAGAGAGGACCCAAGCA-3´(with cg12698626) or forward-766 5´-GAAGATCTACCCTTCTGTGCCTCAGT-3´ (without cg12698626) together with the reverse 5´-

CATGCCATGGTGCGAGCTTCAGTTTGAGAA-3´ (Invitrogen, Carlsbad, CA). Each DNA sequence was sub-cloned into pCpGL-basic vector (kindly provided by prof. M. Rehli, Regensburg, Germany) via BglII and NcoI restriction sites, resulting inpCpGL-CCL2+cg12698626 and pCpGL-CCL2-cg12698626 vectors. For the transformation One Shot PIR1 competent E. coli cells were used (Invitrogen, Carlsbad, CA). Both, LB agar plates and LB growth medium, for colony forming and inoculum preparation, contained Zeocin as a selective antibiotic (Invitrogen, Carlsbad, CA). The correct insertion of each DNA fragment was controlled by sequencing. For transfection into cell cultures, plasmids were isolated and purified using EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). The methylation was performed using SssI methyltransferase (New England Biolabs, Hitchin, United Kingdom) according to the manufacturer's recommendation. The *CCL2* promoter constructs were electroporated in ASC-derived adipocytes at day 8 of differentiation using Neon electroporator (Invitrogen, Carlsbad, CA) according to the instructions provided by the manufacturer. Each electroporation was done with100,000 cells in 10 µl together with 500 ng of plasmid. Electroporation conditions were 1400 Volts, 20 ms width, and 2 pulses. Thereafter, the cells were cultured in 48-well plates, with or without 100 µM cortisol, for 24 h. Luciferase activities were measured in cell lysates using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized to the protein concentrations of each sample using the BCA protein assay (Thermo Fisher, Waltham, Massachusetts, USA).

Thermal shift assay and EMSA

The plasmid encoding the glucocorticoid DNA binding domain (GR-DBD) were kindly provided by Professor JussiTaipale (KarolinskaInstitutet, Sweden) and purified by the Protein Science Facility at Karolinska Institutet/SciLifeLab (http://psf.ki.se). The sequences representing the known GRE (Lundbäck and Härd, 1996) (5´-GCGTCAGAACATGATGTTCTAGGCG-3´), random sequence (5´-

CCCTTAACATATAAGATGTATTTTG-3´) and the sequence surrounding cg12698626 (5´-GACTTGTACA C(+/-CH_3)GTTTCCTTCCA-3´) in both the methylated and unmethylated form were all ordered from Eurofins Genomics (Germany, Luxembourg). The sequences were diluted to 500 µM and annealed to the corresponding reverse sequence by a 15 min incubation in 95 °C following an overnight cooldown. For the gel shift assay 10 µM DNA was mixed with 0, 5, 10 µM (GR-DBD) in the same buffer the protein storage buffer (20mM HEPES, 300mM NaCl, 10% glycerol, 2mM TCEP, pH 7.5). E-Gel® Agarose Gels with SYBR® Safe DNA Gel Stain, 1.2% from Thermo Fischer Scientific (Waltham, MA, USA) were used for gel shift runs. The thermal shift assay was performed by mixing 10 µg GR-DBD with 50 µM DNA and SYPRO® Orange (Thermo Fischer Scientific) in protein storage buffer. The thermal shift was analyzed in a CFX96™ Real-Time System C1000 Touch Thermal Cycler (Bio Rad). Channel 2 (excitation 515-535 nm, detection 560-580 nm) was used to measure fluorescence every 0.2 °C in the 20-80 °C range.

Statistical analysis

Differences between treatments were assessed using paired or unpaired student's t-test and statistical significances are annotated as; *P*-values< 0.05=*, 0.01=** and 0.001=***. Comparison between more than two treatments were assessed with a one-way ANOVA and Fisher's least significant difference post-hoc test. All correlations are presented with Pearson's product-moment correlation coefficient and a *P*-value<0.05 was considered significant; correction for multiple testing were performed when needed. Associations between *SLC19A1* and parameters obtained from the patient cohorts were corrected for BMI in multiple regression analyses. The Benjamini& Hochberg method was used to detect significantly altered genes in the microarray of *SLC19A1*-silenced cells. A false discovery rate of <25% was considered significant. Arbitrary units (a.u.) were used to normalize for inter-individual variances between donors and were calculated by dividing the treated sample with the control sample within each donor. All charts except the box plots represent the mean with whiskers

representing the standard deviation. All statistical tests were performed using the software IBM SPSS Statistics version 22.0.

Author contributions

The study was planned and designed by PP, HG and MR. PP, HG, NM, LB, TC, SN, AG, ST and JB performed the experiments and the data was analyzed and interpreted by PP, HG and MR. The patient material was collected and analyzed by PA, MR and ID. The plastic surgery material was provided by PH. PP and MR wrote the manuscript which was commented on and approved by the other authors. M.R is the guarantor of the work.

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Figure legends

Figure 1.DNA methylation and the one carbon cyclein obese adipocytesA.A simplified overview of the one carbon cycle (1CC) highlighting the folate and methionine cycle, respectively. Protein names are indicated alongside the arrows and the metabolites are written in italics, SLC19A1 is highlighted in bold. Abbreviations are in alphabetical order: ALDH1L1-Aldehyde Dehydrogenase 1 Family Member L1; AHCY-Adenosylhomocysteinase; DNMT-DNA Methyltransferase; MAT2-methionine adenosyltransferase 2; MTHFD1-Methylenetetrahydrofolate Dehydrogenase, Cyclohydrolase And FormyltetrahydrofolateSynthetase 1; MTHFR-Methylenetetrahydrofolate Reductase; MTR-5-Methyltetrahydrofolate-Homocysteine Methyltransferase; MTRR-5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase; SHMT1-Serine Hydroxymethyltransferase 1. **B.**Methylation in CpG loci (β-value) neighboring protein-encoding genes were compared between non-obese and obese individuals in sub-cohort 1.**C.**Based on data from sub-cohort 1, gene ontology-analyses were performed for genes associated with attenuated (left panel) or induced (right panel) expression upon hypermethylation in neighbouring CpGs.**D.** Genes in the 1CC were extracted from gene expression arrays in cohort 1 comparing non-obese and obese individuals and subdivided according to their involvement in the folate (n=25) or methionine (n=53) cycle, respectively. The number of genes regulated or not by obesity is indicated in the pie charts.**E.**The mean gene expression of the obesity-regulated genes in the folate and methionine cycles was measured during *in vitro* differentiation of human adipose derived stem cells. **P*<0.05, ***P*<0.01 and ****P*<0.001

Figure 2. *SLC19A1* expression in human adipocytes.A.*SLC19A1* expression was measured by qPCR in the stroma-vascular fraction (SVF) and isolated mature adipocytes in paired samples from 11 individuals. **B.**The attenuated WAT expression of *SLC19A1* in obesity was confirmed in isolated adipocytes. mRNA levels are expressed in relative units to the reference gene *LRP10*.**P*<0.05, ***P*<0.01 and ****P*<0.001

Figure 3.*SLC19A1* silencing in *in vitro* differentiated human adipocytes A-B.SLC19A1 gene (A) and protein (B) expression were downregulated by siRNA treatment. **C-D.***SLC19A1* silencing resulted in a reduced intracellular SAM/SAH ratio (C) and an increase in global DNA methylation measured using an antibody-based kit (D).**E.** A flowchart summarizing the bioinformatic approach to identify SLC19A1-regulated inflammation genes linked to altered methylation of proximal CpG loci. **P*<0.05, ***P*<0.01 and ****P*<0.001

Figure 4. *SLC19A1* knockdown inducescg12698626 methylation and CCL2expression A. Analyses of protein secretion confirmed thatCCL2was selectively induced *in vitro*upon *SLC19A1* silencing. No effects on TNFα or adiponectin secretion were observed.**B.**In cohort 2,*SLC19A1* expression in subcutaneous WAT associated significantlywithCCL2 secretion*ex vivo*. Standardized beta-coefficients and *P*-valuesare shown for multiple regression analyses correcting for BMI.**C-D.** The methylation of cg12698626 increased significantly upon *SLC19A1* silencing in human adipocytes (C) andthe fold-change in methylation correlated significantly with the fold-change in *CCL2* expression (D). **E.**The absolute methylation of cg12698626in primary *in vitro* differentiated adipocytes from five donors displayed a bimodal distribution and could be subdivided according to "low" or "high" methylation. **F.**CCL2 secretion from adipocytes in E was compared between the groupswith high or low cg12698626 methylation. **P*<0.05, ***P*<0.01 and ****P*<0.001

Figure 5.Functional analysis of cg12698626 in regulating CCL2 expression.A. The promoter (defined as 1.5 kb upstream to the transcription start site (TSS)) region of human *CCL2* contained a total of seven CpGs (CG1-7),cg12698626 (CG4) is located in the middle. Analyses of ENCODE ChIP-seq data predicted a glucocorticoid receptor (GR) binding site overlapping with CG4. **B.**The relative importance of CG4 methylation was suggested by correlatingthe degree of methylation (β-value)in each individual CpG with *CCL2* expression in sub-cohort 1.Pearson's correlation coefficient and *P*-values are shown. **C.***In vitro* differentiated adipocytes were incubated with 100 nMcortisol and *CCL2* expression was measured after the indicated times. **D.** Cortisol-mediated

repression of the *CCL2*promoter was assessed using a luciferase reporter assay containing CG4-7 in the unmethylated (left panel) or methylated (right panel) state.**E.**Similar experiments as in Dwere performed using constructs where CG4 was deleted.**F.** Binding affinity of the GR-DNA binding domain (GR-DBD) was determined by thermal shift assay. Binding to a positive control (GR-Element, GRE) shifted the dissociation temperature significantly while a random oligonucleotide sequence was not different from GR-DBD alone. Binding of GR-DBD to an oligonucleotide containing CG4 showed a significantly higher dissociation temperature in the unmethylated (CG4/-CH_3) compared with the methylated (CG4/+CH_3) state. **P*<0.05, ***P*<0.01 and ****P*<0.001

Table legends

Table 1. Associations between folate cycle genes, insulin sensitivity and WAT inflammation. The relationship between insulin sensitivity *in vivo*(determined by insulin tolerance test) and the WAT mRNA levels (by micro-array) of the seven folate cycle genes altered in obesity and during adipogenesis was determined by multiple regression analysis setting the expression of each gene and BMI as independent regressors. Standardized beta coefficients and *P*-values are shown. The number of pro-inflammatory genes (among a total of 238 included in the GO-term) associating significantly (P<0.01) with each of the 7 folate cycle genes are shown.

Table 2. Inflammation-related genes predicted to be regulated by hypermethylation. An overlap of gene expression arrays in *SLC19A1*-silenced adipocytes and subcutaneous WAT in cohort 1 with an adipocyte methylation array in cohort 1 identified genes that were predicted to be regulated by SLC19A1 through DNA-methylation. The genes that were annotated in at least one of the 10 GO inflammatory biological processes in Figure 1C are listed.

Supplementary figure legends

Figure S1. Location of CpGs in relation to direction of gene expression The degree of methylation in relation to the gene expression was compared between CpG loci according to their location in relation to neighboring protein-encoding genes (upper panel) or their proximity to CpG islands (lower panel).

Figure S2. Interaction between GR-DBD and cg12698626 containing oligonucleotides. Representative gel shift assays displaying the interaction between theGR-DBD and different oligonucleotides.GRE was used as a positive control. The GR-DBD bound to an oligonucleotide representing the 22 base pairs surrounding cg12698626 (CG4) which was in contrast tothe DBD of another unrelated transcription factor (EBF1)(left panel). GR-DBD interacted with oligonucleotides containing cg12698626 in both the unmethylated (CG4/-CH_3) and methylated (CG4/+CH_3) form (right panel).

Supplementary table legends

Table S1 GO-analysis. Genes displaying a positive correlation between expression and methylation in proximal CpG loci subdivided according toGO-pathways detailed in figure 1C (right panel).

Table S2Regulation of 1CC genes in obesity. Genes in the folate and methionine cycle regulated by obesity.

Table S3Patient cohorts. Short description of the cohorts and sub-cohort included in this study.

Table S4SLC19A1-regulated genes linked to altered methylation of proximal CpGs. A list of all CpGs and corresponding proximal genes (defined as <1.5 kb upstream to TSS, in gene bodies, in 5' UTR or in 3' UTR) identified in the array overlap outlined in Figure 3E, except that the fourth filtering step (genes in GO-terms of inflammation) was omitted herein. Thus the list includes all candidate genes that may be epigenetically regulated by SLC19A1.

Table 1.

GeneID	K_{ITT} Std Beta coeff	*P*-value	Number of significant BMI independent correlations with 238 GO annotated inflammation genes (*P*<0.01)
SLC19A1	**0.36**	**0.047**	**81**
ALDH1L1	0.26	0.154	44
MTHFD1	0.15	0.432	31
DHFR	0.05	0.781	9
GCSH	0.05	0.782	6
FPGS	0.02	0.904	41
SHMT1	-0.02	0.900	35

Table 2.

CpG ID	CpG location in relation to CpG island	CpG location in relation to gene	Neighboring gene	Fold change in si*SLC19A1* treated adipocytes (a.u.)	Correlation with *SLC19A1* expression (r-value)	Correlation between CpG methylation and gene expression (r-value)
cg12698626	Open sea	TSS1500	CCL2	2.31	-0.72	0.72
cg03909504	Open sea	Body	LGALS9	1.81	-0.72	0.60
cg00045690	S Shelf	Body	PSMB9	1.75	-0.68	0.71
cg22696542	N Shore	TSS1500	PSMB9	1.75	-0.68	0.61
cg01916918	Open sea	Body	CLU	1.73	-0.72	0.65
cg08198265	S Shelf	Body	BST1	1.66	-0.61	0.70
cg12192566	N Shore	3'UTR	CD14	1.66	-0.72	0.72
cg25099065	Open sea	5'UTR	ANXA4	1.57	-0.72	0.74
cg05681859	Open sea	Body	FLOT1	1.56	-0.73	0.65
cg18695800	N Shelf	Body	FLOT1	1.56	-0.73	0.62
cg19448318	Open sea	Body	FLOT1	1.56	-0.73	0.62
cg04353171	Open sea	Body	FLOT1	1.56	-0.73	0.61
cg23423329	N Shore	Body	FLOT1	1.56	-0.73	0.61
cg24615251	Open sea	1stExon	CCL13	1.49	-0.64	0.65
cg21042139	Open sea	1stExon	CCL13	1.49	-0.64	0.65
cg24436462	Open sea	3'UTR	ARPC5	1.44	-0.78	0.68

Figure 1

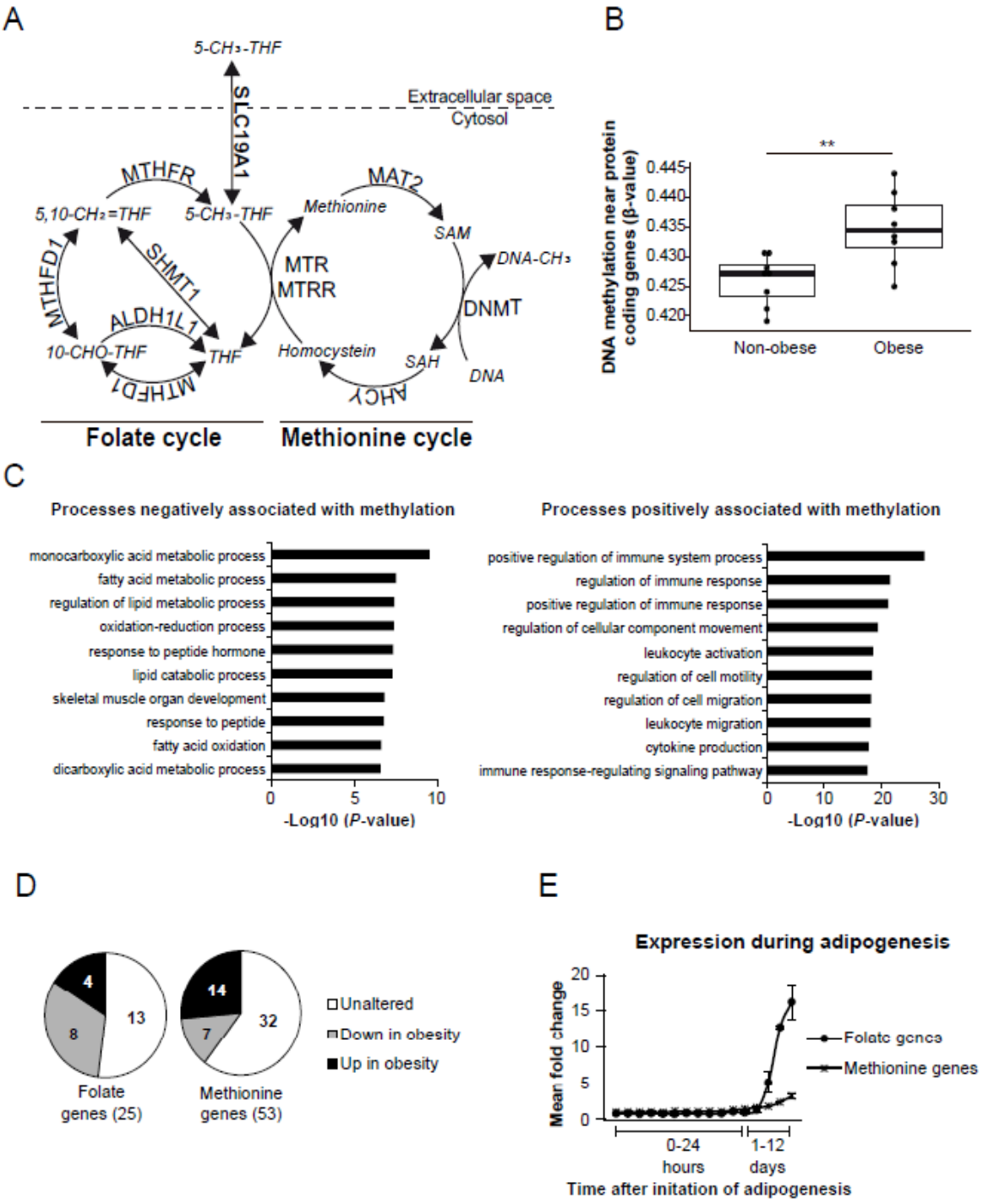

A
5-CH₃-THF
SLC19A1
Extracellular space
Cytosol
MTHFR
5,10-CH₂=THF
5-CH₃-THF
SHMT1
MTHFD1
ALDH1L1
10-CHO-THF
THF
MTHFD1
MTR
MTRR
Methionine
MAT2
SAM
DNA-CH₃
DNMT
DNA
SAH
Homocystein
AHCY
Folate cycle
Methionine cycle
B
DNA methylation near protein coding genes (β-value)
0.445
0.440
0.435
0.430
0.425
0.420
**
Non-obese
Obese
C
Processes negatively associated with methylation
monocarboxylic acid metabolic process
fatty acid metabolic process
regulation of lipid metabolic process
oxidation-reduction process
response to peptide hormone
lipid catabolic process
skeletal muscle organ development
response to peptide
fatty acid oxidation
dicarboxylic acid metabolic process
0
5
10
-Log10 (P-value)
Processes positively associated with methylation
positive regulation of immune system process
regulation of immune response
positive regulation of immune response
regulation of cellular component movement
leukocyte activation
regulation of cell motility
regulation of cell migration
leukocyte migration
cytokine production
immune response-regulating signaling pathway
0
10
20
30
-Log10 (P-value)
D
4
13
8
Folate genes (25)
14
32
7
Methionine genes (53)
Unaltered
Down in obesity
Up in obesity
E
Expression during adipogenesis
Mean fold change
20
15
10
5
0
Folate genes
Methionine genes
0-24 hours
1-12 days
Time after initation of adipogenesis

Figure 2

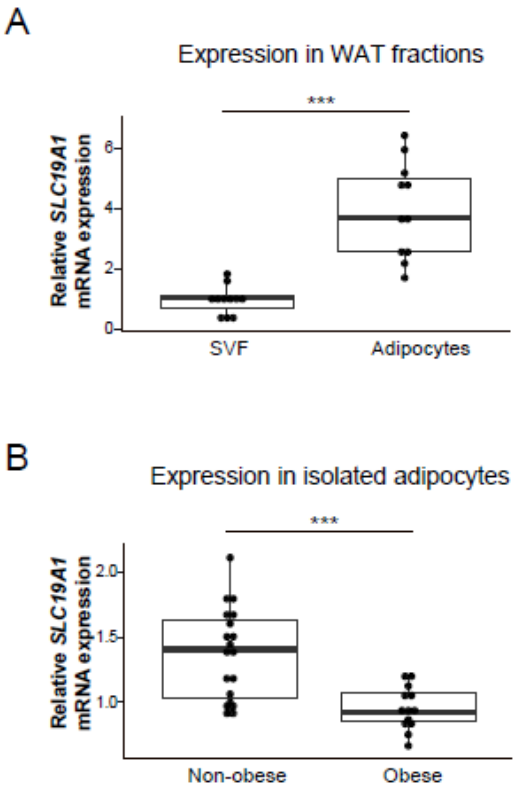

A
Expression in WAT fractions
***
Relative SLC19A1 mRNA expression
0
2
4
6
SVF
Adipocytes
B
Expression in isolated adipocytes
***
Relative SLC19A1 mRNA expression
1.0
1.5
2.0
Non-obese
Obese

Figure 3

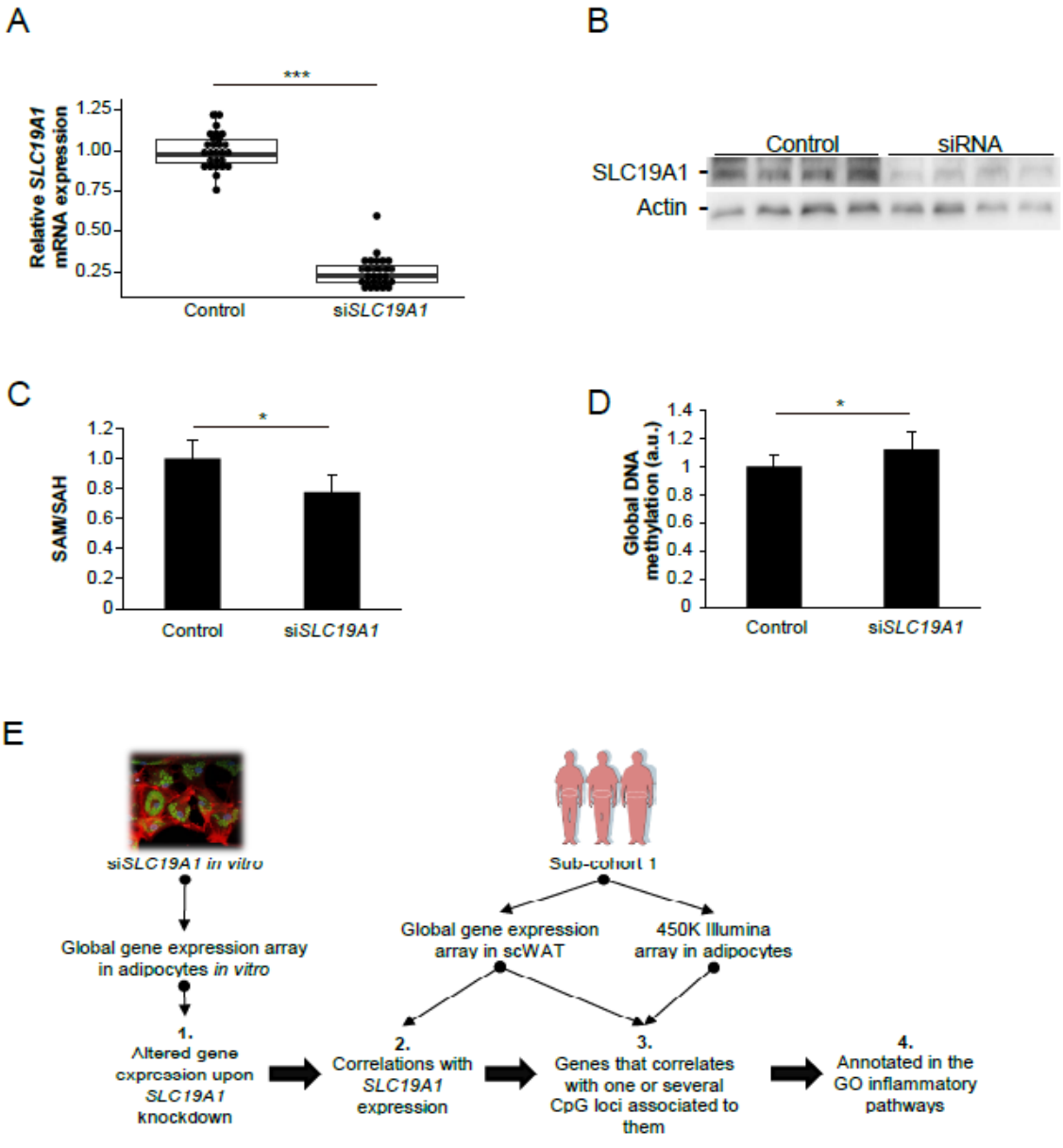

A
***
Relative SLC19A1 mRNA expression
1.25
1.00
0.75
0.50
0.25
Control
siSLC19A1
B
Control
siRNA
SLC19A1
Actin
C
*
SAM/SAH
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
siSLC19A1
D
*
Global DNA methylation (a.u.)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
siSLC19A1
E
siSLC19A1 in vitro
Global gene expression array in adipocytes in vitro
1. Altered gene expression upon SLC19A1 knockdown
Sub-cohort 1
Global gene expression array in scWAT
450K Illumina array in adipocytes
2. Correlations with SLC19A1 expression
3. Genes that correlates with one or several CpG loci associated to them
4. Annotated in the GO inflammatory pathways

Figure 4

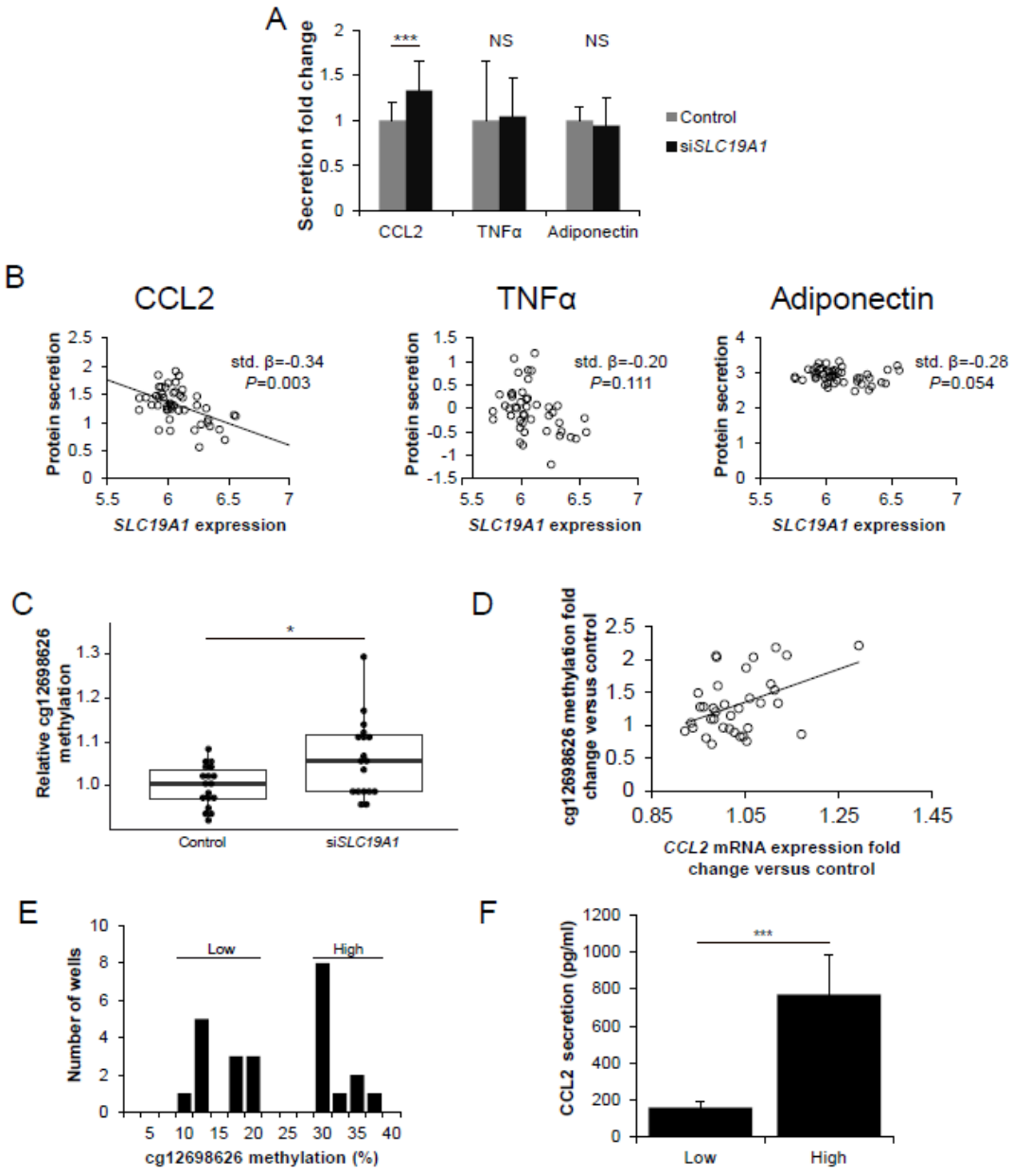
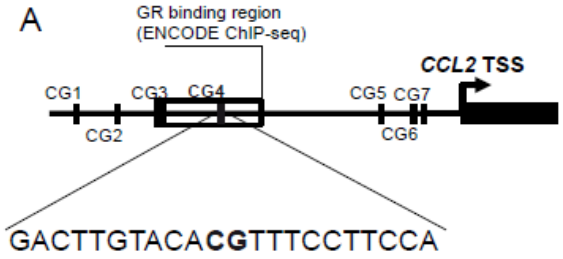

A
Secretion fold change
***
NS
NS
Control
siSLC19A1
CCL2
TNFα
Adiponectin
B
CCL2
TNFα
Adiponectin
Protein secretion
std. β=-0.34
P=0.003
std. β=-0.20
P=0.111
std. β=-0.28
P=0.054
SLC19A1 expression
C
Relative cg12698626 methylation
*
Control
siSLC19A1
D
cg12698626 methylation fold change versus control
CCL2 mRNA expression fold change versus control
E
Number of wells
Low
High
cg12698626 methylation (%)
F
CCL2 secretion (pg/ml)
***
Low
High

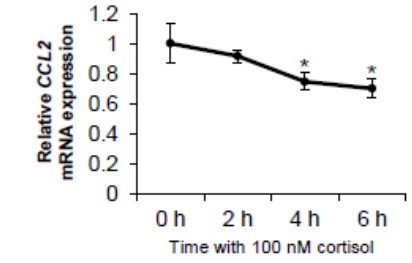
Figure 5

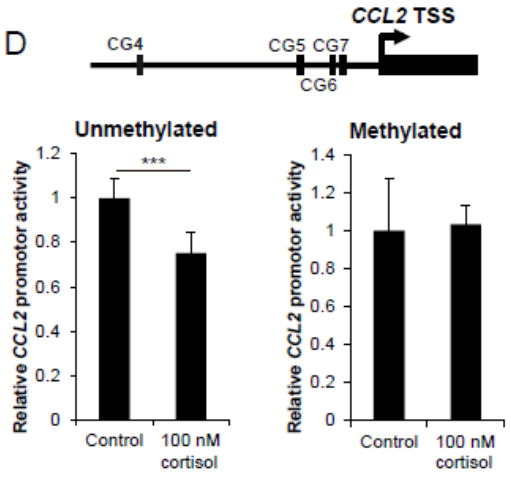


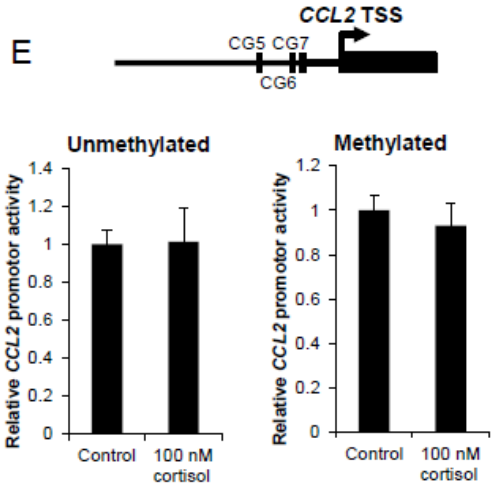
B

Correlations between methylation in *CCL2* promoter CpGs and *CCL2* gene expression

CpG#	cg#	Pearsson's r	*P*-value
CG1	cg21109025	-0.20	0.448
CG2	cg05440824	0.53	0.034
CG3	cg04633676	0.31	0.236
CG4	cg12698626	0.72	0.002
CG5	cg11640275	-0.22	0.416
CG6	cg08540958	0.32	0.221
CG7	cg26079699	-0.30	0.256







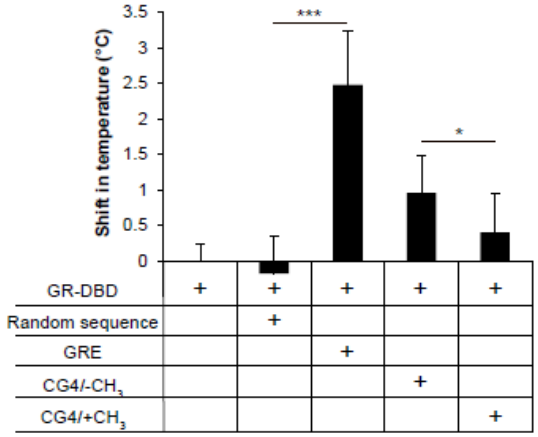


Figure S1

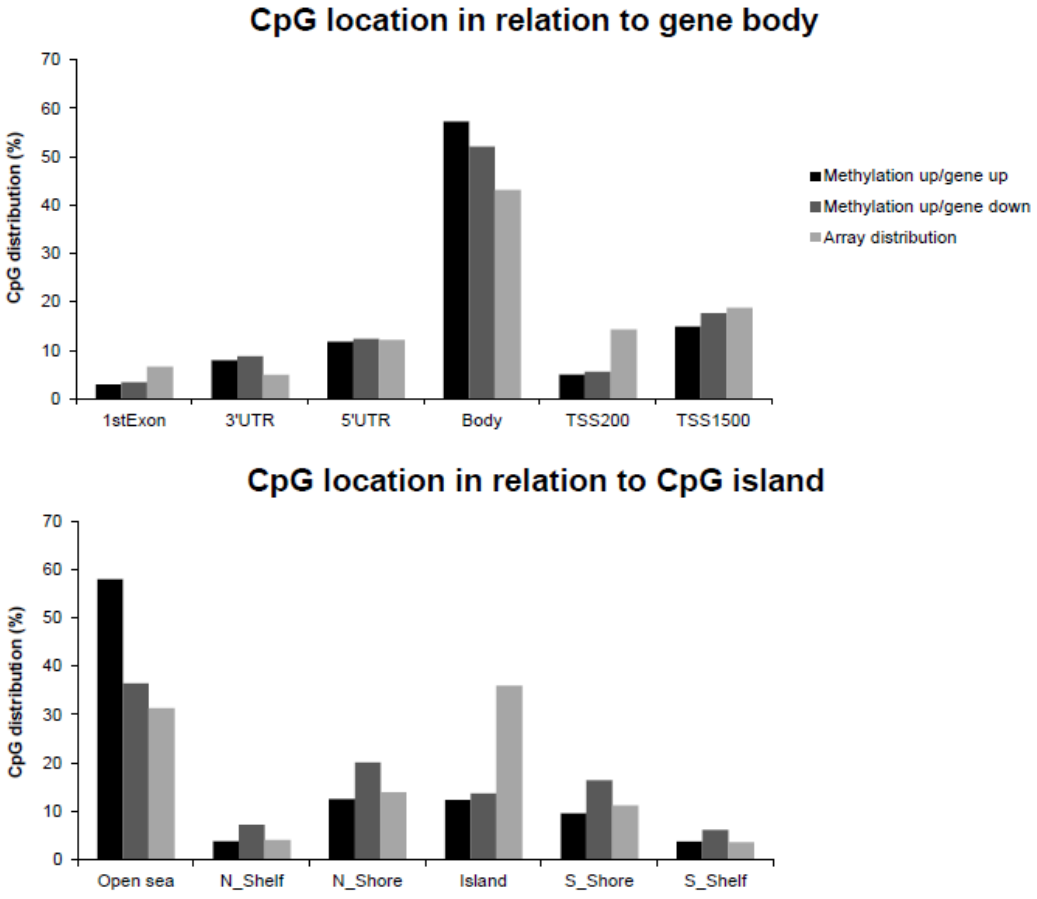
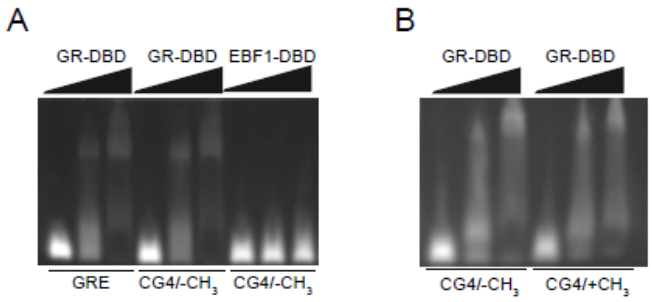


Figure S2



RESUMEN

Esta Tesis Doctoral se ha llevado a cabo dentro del grupo de investigación "Nutrición y Obesidad" dirigido por la Catedrática. María del Puy Portillo. Este grupo tiene una amplia experiencia en el estudio de los efectos de los ingredientes funcionales sobre el metabolismo de los lípidos en los campos de la obesidad y la esteatosis hepática. En los últimos años se han estudiado los ácidos grasos conjugados y los diferentes compuestos fenólicos, como el ácido linoléico conjugado, la quercetina, el resveratrol y el pterostilbeno. De entre ellos, el resveratrol y el pterostilbeno han sido elegidos para el desarrollo de esta Tesis Doctoral.

Debido al escaso conocimiento sobre los efectos de los compuestos fenólicos sobre la regulación de los triglicéridos por mecanismos epigenéticos y microRNAs, esta tesis doctoral se ha centrado en estos dos aspectos moleculares.

Para ello, se llevaron a cabo dos enfoques diferentes. El primero fue analizar la posible influencia de la metilación del ADN en la acumulación de triglicéridos en el tejido adiposo, y el segundo establecer la participación de la regulación postranscripcional por microRNAs en el tejido adiposo y la acumulación de grasa hepática.

Además, dentro del marco de tesis internacional, se realizó una estancia internacional entre los meses de marzo y junio de 2016. Esta estancia tuvo lugar en la Unidad de Endocrinología del Hospital Universitario Karolinska, dirigida por el Dr. Mikael Rydén y en el Laboratorio de Lípidos en NOVUM en Huddinge, dirigido por el profesor Peter Arner. Los estudios desarrollados en este el laboratorio abarcan desde genética y análisis molecular en diferentes sistemas *in vitro* hasta evaluaciones clínicas en sujetos humanos. El objetivo fue comprender mejor los mecanismos que vinculan los cambios en la masa grasa a los trastornos metabólicos.

1. Efectos del resveratrol y el pterostilbeno en el patrón de metilación del ADN de genes involucrados en el metabolismo de los triglicéridos y su relación con la obesidad.

La metilación de los nucleótidos del ADN, como mecanismo epigenético, proporciona una explicación molecular para las marcas que se producen de manera reversible el ADN genómico. Este proceso epigenético puede cambiar el estado funcional de las regiones reguladoras, y está implicado de manera funcional en muchas formas de represión epigenética estable (47).

En este experimento, se determinó el efecto del resveratrol y su metoxi derivado, el pterostilbeno, sobre la metilación de los genes implicados en el metabolismo de los triglicéridos. Para ello se utilizaron ratas Wistar divididas en cuatro grupos experimentales: un grupo control alimentado con una dieta estándar (grupo control), un grupo alimentado con una dieta alta en grasa y alta en sacarosa (grupo alto en grasa con alto contenido en sacarosa), y dos grupos también alimentados con una dieta alta en grasa y alta en sacarosa, pero tratada con resveratrol o pterostilbeno a una dosis de 30 mg / kg / día (resveratrol y pterostilbeno, respectivamente) (105, 137). Como era de esperar, la alimentación de una dieta rica en grasa y sacarosa condujo a un aumento de la ingesta de energía y, en consecuencia, al aumento de la acumulación de grasa, en comparación con la dieta estándar. La adición de estos dos compuestos fenólicos a la dieta alta en grasa y alta en sacarosa evitó este efecto de engorde, pero sólo parcialmente, porque el peso corporal y el peso de los tejidos adiposos en estas ratas no alcanzaron los valores de control.

Para el estudio de metilación del ADN, se establecieron los siguientes criterios de inclusión: a) genes implicados en el metabolismo de los triglicéridos y b) genes con al menos una isla CpG en el promotor del gen o en el primer exón. En primer lugar, debido a su papel en el metabolismo de los triglicéridos del tejido adiposo blanco, se seleccionaron los genes *lpl, fasn, acaca, lipe, pnpla2, srebf1 y pparγ*. Además, todos estos genes han demostrado ser alterados por el resveratrol (86, 92, 110, 111, 115, 146, 182). En segundo lugar y después del

análisis de las posiciones CpG, sólo *fasn, pnpla2 y pparγ* tenían áreas ricas en CpG cerca de la región promotora.

El análisis de metilación del ADN, realizado por pyrosecuenciación, mostró que el alto contenido de grasa y de sacarosa de de la alimentación o la adición de los compuestos fenólicos no modificaron el patrón de metilación de los genes *pnpla2* y *pparγ*. Por el contrario, si se observaron cambios en el gen *fasn*.

El proceso de metilación del ADN en regiones promotoras, es un potente supresor de la expresión génica. Aunque los cambios producidos por este proceso podrían ser pequeños, pueden estar asociados con modificaciones de la expresión génica que ejercen efectos significativos en el fenotipo (44, 183). Como ya ha sido mencionado anteriormente, la dieta con alto contenido en grasa y sacarosa indujo cambios significativos en el patrón de metilación del gen *fasn* con respecto a los controles, una hipermetilación en posición -62 pb (6%) y una hipometilación en posición -90 pb %). Además, la adición de los dos compuestos fenólicos en la dieta produjo diferentes patrones de metilación. En el caso del pterostilbeno, invirtió los cambios inducidos por la dieta obesogénica en posiciones de -90 pb y -62 pb. Por el contrario, no se observaron cambios en el estado de metilación cuando se compararon resveratrol y grupos con alto contenido de grasa y sacarosa.

Con el fin de analizar el efecto de la dieta obesogénica en *fasn*, se midió la expresión génica de esta enzima, mostrando un aumento en el grupo que tomo la dieta con alto contenido en grasa y sacarosa. Además, no se observaron diferencias en los niveles de expresión génica en los grupos resveratrol y pterostilbeno en comparación con el grupo control, por lo que estas moléculas impidieron totalmente la alteración producida por la dieta obesogénica. Sin embargo, con respecto al patrón de metilación del gen *fasn* en los grupos resveratrol y pterostilbeno, el resveratrol no tuvo efectos en ninguna región analizada del gen cuando se comparó con el grupo de alto contenido de grasa y sacarosa. Por el contrario, el pterostilbeno invirtió los cambios inducidos por la

dieta obesogénica en posiciones de -62 pb y -90 pb. De hecho, los porcentajes de metilación de pterostilbeno y grupo de control fueron similares.

Uriarte *et al.* (184) previamente publicaron una hipometilación del gen del *fasn* inducida por una dieta con alto contenido de grasa y alto contenido en sacarosa. Esto refuerza nuestra hipótesis de la acción hipometilante de un patrón dietético antiobesogénico en el gen *fasn*, aunque aparezcan dos diferencias principales al comparar ambos estudios. Por un lado, Uriarte *el al.* observó la hipometilación en el gen del *fasn* después de 20 semanas de dieta con alto contenido de grasa y alto contenido de sacarosa mientras que, en el presente estudio, este efecto se observó después de 6 semanas. Esto significa que el proceso de metilación del ADN no necesita largos períodos de tiempo para tener lugar. Además, se encontraron resultados similares usando diferentes métodos de análisis de metilación del ADN (espectrofotometría de masas y pirosequenciación).

En cuanto a la expresión del gen *fasn*, varios autores informaron que mientras una dieta rica en grasas disminuye la expresión de este gen, las dietas ricas en carbohidratos simples o complejos aumentan la expresión de *fasn* (185-189). En el presente estudio, parece que el efecto del alto contenido de sacarosa fue mayor que el de alto contenido de grasa. Este resultado coinciden con los publicados por Yang *et al*; 2012 cuando se utiliza este tipo de dieta.

Con el fin de explorar la posibilidad de que los cambios en la posición de -90 pb podrían estar relacionados con la disminución de la expresión génica, se realizó un análisis bioinformático para identificar los factores de transcripción vinculantes alrededor de esta posición -90 pb que se ha visto alterada. Encontramos que el factor de transcripción Sp1, que actúa como un sensor de glucosa (190), podría unirse en esta posición. Se ha demostrado que Sp1 es crucial para la actividad promotora del gen *fasn* en los adipocitos (191), pudiendo influir en la regulación del gen.

Para analizar la posible relación entre el estado de metilación y la expresión génica del *fasn*, se calcularon los coeficientes de correlación de Pearson. Los resultados mostraron que sólo la hipometilación de la posición -90 pb tenía una correlación significativa con la expresión génica de *fasn*, lo que

sugiere una contribución de esta posición a la disminución de la regulación del gen *fasn*.

Finalmente, se midió la actividad nuclear de DNMT (ADN metiltransferasas) para analizar el comportamiento de estas enzimas que catalizan directamente la adición de un resto de citosina del grupo metilo. La actividad de estas enzimas mostró un patrón similar de respuesta al nivel de metilación del ADN a -90 pb. Esto sugiere que podría ser un mecanismo en la actividad de modulación de estas enzimas, lo que justifica los efectos observados de la dieta con alto contenido en grasa y sacarosa y pterostilbeno en la metilación del *fasn*.

2. Implicación del miRNA-539-5p en la inhibición inducida por el resveratrol de la lipogénesis *de novo* en el tejido adiposo blanco

El resveratrol es un compuesto fenólico ampliamente estudiado, sugerido como eficaz para prevenir el desarrollo de varias enfermedades, como la obesidad. Los mecanismos de acción del resveratrol como molécula antiobesidad han sido publicados en nuestros estudios anteriores y en la literatura (88, 110, 111, 115, 182). Sin embargo, hemos querido profundizar en los mecanismos moleculares de estos efectos, como son los miRNAs, ya que la gran mayoría de estos estudios no han abordado esta cuestión. Los MiRNAs son pequeños ARN no codificantes que regulan la expresión de genes diana específicos postranscripcionalmente, principalmente suprimiendo la traducción y/o reduciendo la estabilidad de sus mRNAs dianas. Se ha visto que varios polifenoles, incluido resveratrol, pueden modificar la expresión de miRNAs (122).

En este experimento hemos determinado las modificaciones producidas por el resveratrol en el perfil de miRNAS en el tejido adiposo, y sus implicaciones en la modulación del metabolismo de los triglicéridos. En el diseño experimental, las ratas se dividieron en dos grupos: el grupo control alimentado con una dieta alta en grasa con alto contenido de sacarosa (dieta obesogénica) y el otro tratado con la misma dieta obesogénica suplementada con resveratrol en las

cantidades necesarias para alcanzar una dosis de 30 mg / kg / Día (grupo Resveratrol). Esta dosis se seleccionó basándose en el experimento de metilación del ADN y en estudios previos reportados por nuestro laboratorio que demostraron que era eficaz para reducir la masa grasa corporal (137). Como era de esperar, la suplementación con resveratrol redujo el peso corporal y el tamaño de los tejidos adiposos epididimal y perirrenal, así como la suma de los cuatro depósitos de grasa (perirrenal + epidídimo + mesentérico + subcutáneo).

Con el fin de explorar la posible implicación de resveratrol en la modificación del perfil de miRNAs, se llevó a cabo un miRNA microarray en el tejido adiposo perirrenal. De entre los 719 microRNAs analizados, sólo 273 fueron detectados y 16 fueron significativamente modificados por la suplementación de resveratrol. 13 aumentaron su expresión y 3 la disminuyeron. La validación de cuatro de estos microRNAs que se vieron modificados por RT-PCR, confirmó los cambios encontrados en el análisis de microarrays.

Según la literatura, algunos miRNAs están involucrados en el control de genes relacionados con las vías metabólicas responsables del efecto anti-obesidad del resveratrol en el tejido adiposo. Sorprendentemente, en el presente estudio estos miRNAs no se vieron modificados por este polifenol. En vista de este hecho se realizó un análisis de acuerdo con la base de datos miRWalk, con el fin de encontrar genes validados o predichos de los 16 miRNAs que cambiaron. Aunque no se encontraron genes diana validados tras el análisis, se encontraron los genes *fabp3, sp1, cpt1a, hsl, ucp1, ucp3, cpt1b y pparγ* como genes diana predichos. Entre estos, para este estudio, sólo fueron seleccionados los involucrados en el metabolismo de triglicéridos en el tejido adiposo blanco: *hsl*, gen diana predicho de miARN-328a-5p, *pparγ* gen diana predicho de miARN-129-1- 3p y miARN-129-2-3p, y *sp1*, gen diana predicho de miARN-539-5p. Estos tres miRNAs incrementaron significativamente su expresión con el tratamiento con resveratrol.

Como ya se ha indicado anteriormente, los miRNAs actúan como represores de la traducción de proteínas, directamente uniéndose en la región 3'UTR del gen diana o dirigiéndose indirectamente a un factor de transcripción u otros intermedios que alteran la expresión de las proteínas (106). En

consecuencia, se midió la expresión de las proteínas de estos tres genes seleccionados. En cuanto a la HSL, enzima implicada en la lipólisis, y el factor de transcripción PPARγ, que regula HSL y LPL, no se observaron cambios en la expresión proteica entre los grupos Control y Resveratrol. Teniendo en cuenta que otro mecanismo de la regulación miARN es la reducción de la estabilidad del mRNA, se realizó el análisis de la expresión génica de estos dos genes. Del mismo modo, no se observaron diferencias entre ambos grupos experimentales. En consecuencia, los resultados de nuestro estudio mostraron que, aunque miRWalk base de datos indica que *pparγ* es un gen diana predicho de miARN-129-1-3p y miARN-129-2-3p, y *hsl* es un gen diana predicho de miRNA-328- 5p, la falta de cambios en las expresiones de sus genes y proteínas sugiere que en realidad no pueden ser considerados genes diana reales.

Como se mencionó anteriormente en el Manuscrito 1, el factor de transcripción Sp1 es un miembro importante de la familia SP/KLF, involucrado entre otras, en la regulación del gene *fasn*. A diferencia de los resultados de HSL y PPARγ, la expresión de la proteína SP1 se redujo significativamente en las ratas tratadas con resveratrol. Este efecto en relación con el aumento significativo en la expresión de miARN-539-5p, muestra una forma negativa de regulación de este miRNA.

Varios estudios han informado de que el miRNA-1224, regulado por la suplementación de resveratrol en el presente estudio, está involucrado en la regulación de *sp1*. Niu *et al.* (156) mostró una disminución en la expresión génica y proteica de sp1 tras la transfección de miRNA-1224 en células de riñón embrionario humano y células de macrófagos de monocitos de ratón. Como estas líneas celulares no tienen un origen roedor, para nuestro estudio se realizó una alineación de rno-miRNA-1224 y la secuencia 3'UTR de *sp1* en rata. Este análisis consiste en encontrar la posible interacción de emparejamiento de bases de 2-8 nt en el 5'extreme del microRNA y la región 3'UTR del mRNA (55). El análisis reveló que el miARN-1224 teóricamente puede ser capaz de unirse al ARNm de *sp1* de la rata. Teniendo en cuenta estos resultados, y aunque esta relación no ha sido detectada por la base de datos miRWalk como gen diana

predicho o validado, decidimos analizar la posible implicación en la regulación de SP1.

Con el fin de identificar potenciales miRNA diana, la sobreexpresión o la inhibición de microRNAs es un buen enfoque con el fin obtener pruebas científicas sólidas. De este modo, se transfectaron adipocitos 3T3-L1 con mmu-miRNA-539-5p y mmu-miRNA-1224. Las células que sobreexpresaron miRNA-1224 no vieron alterada la expresión proteica de SP1 en comparación con el grupo de control. Por el contrario, no se detectó la expresión proteica de SP1 en las células que sobreexpresaron miRNA-539-5p. Estos resultados sugieren que la reducción de la expresión de la proteína SP1 inducida por resveratrol es modulada por el miRNA-539-5p.

Con el fin de obtener más información sobre ambos miRNAs analizados, se calcularon las correlaciones de Pearson entre cada expresión de microRNA y la expresión de la proteína SP1. Los resultados mostraron que sólo el miRNA-539-5p se correlacionó negativamente con la modificación de la proteína SP1.

Magaña *et al.* (18) observó que *sp1* actúa junto con *srebp1* para activar sinérgicamente el promotor del gen *fasn*, regulando la vía de lipogénesis *de novo*. En consecuencia, se analizó la expresión proteica de SREBP1 y la expresión génica de *fasn*. Se observó una reducción significativa en la expresión de la proteína SREBP1 en el grupo tratado con resveratrol en comparación con el grupo de control. De acuerdo con estos resultados, la expresión génica de *fasn* también se vio reducida. Estos resultados muestran que el efecto reductor de la grasa corporal del resveratrol está mediado, al menos en parte, por una reducción en la lipogénesis *de novo*.

Este estudio demuestra por primera vez que el resveratrol modifica el perfil de miRNAs en el tejido adiposo blanco. En cuanto al metabolismo de triglicéridos en este tejido, este estudio muestra que miRNA-539-5p está involucrado en la inhibición de la lipogénesis *de novo* inducida por resveratrol.

3. Implicación del miRNA-103, miRNA-107 y miRNA-122 en la prevención de la esteatosis hepática inducida por el resveratrol

Estudios realizados previamente en nuestro laboratorio, así como estudios publicados por otros autores, observaron que el resveratrol es capaz de reducir la esteatosis hepática. Con respecto a los mecanismos, se observó en nuestro grupo que el tratamiento con resveratrol podría inducir una mayor oxidación de ácidos grasos debido a la actividad de CPT1a, enzima clave en el transporte dependiente de carnitina a través de la membrana interna mitocondrial. Por el contrario, la lipogénesis *de novo* no se vio implicada en el efecto del resveratrol ya que la actividad de FAS permaneció sin cambios. Como se mencionó en el Resumen 2, las vías de regulación molecular del metabolismo de los lípidos incluyen los miRNAs. Se ha visto en la literatura que diferentes polifenoles, tales como proantocianidinas o una mezcla extraída de *Hibiscus sabdariffa*, son capaces de modificar la expresión de miRNA-122-5p y los parálogos miRNA-103-3p y miRNA-107-3p en el hígado (136, 167 - 169, 192). En este contexto, el objetivo de este tercer estudio fue determinar si la reducción de la esteatosis hepática inducida por resveratrol en ratas alimentadas con una dieta obesogénica fue mediada por miRNAs. Para este propósito, analizamos los miRNA-103, miRNA-107 y miRNA-122, que representan el 70% de todos los miRNAs en el hígado (136).

El experimento se realizó con ratas Sprague-Dawley divididas en dos grupos experimentales: un grupo control alimentado con una dieta alta en grasa con alto contenido de sacarosa (dieta obesogénica) y el otro un grupo tratado alimentado con la misma dieta obesogénica suplementada con resveratrol en las cantidades necesarias para alcanzar una dosis de 30 mg / kg / día (grupo Resveratrol). Este estudio mostró que el tratamiento con resveratrol no redujo el peso corporal final ni el peso del hígado. Por el contrario, el tratamiento con resveratrol indujo una disminución significativa en el contenido de triglicéridos hepáticos (170).

En primer lugar, se buscaron posibles genes diana predichos y validados de estos tres miRNAs, relacionados con el metabolismo de los triglicéridos en la base de datos miRecords y en la literatura. En lo que respecta a la vía lipogénica, según miRecords, *srebf1* es un gen diana predicho para miRNA-103-3p y miRNA-107-3p y *fasn* es un gen diana predicho de miRNA-122-5p. Por otra parte, Iliopoulos *el al.* (178) observó una regulación indirecta de *srebf1* por ese miRNA. La regulación positiva de este miRNA indujo una mayor expresión de la proteína SREBP1. Tomando en cuenta que los miRNAs son reguladores negativos de la traducción de proteínas, y que no se encontraron sitios de unión para miRNA-122-5p en la región 3'UTR de este gen, llegaron a la conclusión de que *srebf1* es un gen diana indirecto para miRNA-122. Desafortunadamente, no conocían los pasos intermedios en la cascada de señalización que conducían a la regulación positiva de SREBP1. Shibata *et al*. (179) observaron que el silenciamiento del miRNA-122-5p conducía a una disminución de la expresión de SOCS3, que a su vez aumentaba la expresión de STAT3. Por lo tanto, SREBP1 era negativamente regulado por STAT3. De manera similar, una disminución en la expresión del miRNA-122 indujo una disminución en la expresión de SREBP1. Además, Bhatia *et al.* (181) observó que el miRNA-107 disminuyó los niveles de proteína de *fasn* por la unión complementaria a la región 3'UTR de su mRNAs.

Con el fin de obtener más apoyo científico en relación con la participación de estos miRNAs en la regulación SREBP1, se realizó una sobreexpresión de los tres miRNAs en hepatocitos AML 12. En los tres casos, se observó un aumento significativo en la expresión de la proteína SREBP1.

Además, se midió la expresión de los tres miRNAs en el hígado de ratas tratadas con resveratrol y observándose una disminución significativa en el grupo tratado con resveratrol en todos los casos. A continuación, se analizó la expresión proteica de SREBP1 y FAS. Mientras que el SREBP1 disminuyó significativamente en el grupo tratado con resveratrol, no se observaron cambios en el FAS.

Con respecto al miRNA-122-5p, teniendo en cuenta que los resultados de nuestro estudio *in vitro* y teniendo en cuenta los resultados informados por Iliopoulos *et al.* y Shibata *et al*. se puede sugerir que resveratrol disminuye la

expresión de la proteína de SREBP1 indirectamente a través de miRNA-122-5p. Además, la sobreexpresión de los miRNA-103-3p y miRNA-107-3p en los hepatocitos AML 12 resultó en un aumento de la expresión de la proteína SREBP1. Asimismo, en el estudio *in vivo*, el hígado de las ratas tratadas con resveratrol mostró una regulación negativa de estos miRNAs, que se vio acompañdo de una expresión reducida de SREBP1. Estos resultados sugieren que estos miRNAs son reguladores positivos de SREBP1.

De acuerdo con miRecords, *fasn* es un gen diana predicho de miRNA-122-5p. La sobreexpresión en los hepatocitos AML12 de este miRNA, indujo una reducción significativa de la expresión de la proteína FAS. Por lo tanto, podríamos esperar un aumento de la expresión génica. Cuando medimos la expresión proteica de FAS en el hígado, no se encontraron cambios en las ratas tratadas con resveratrol. Este resultado coincide con la falta de cambios en la actividad de FAS que se observó en nuestro estudio anterior en esta mism cohorte de animales. Este resultado es sorprendente ya que *fasn* es, según miRecords, un gen diana predicho para miRNA-122-5p, que fue reducido por resveratrol. De hecho, nuestro experimento de transfección muestra que la sobreexpresión del miRNA-122-5p indujo una reducción significativa en la expresión proteica de FAS. Por otro lado, SREBP1, un factor de transcripción regulador de FAS, se vio reducida su expresión en el grupo tratado con resveratrol. Por lo tanto, la hipótesis de que un aumento de la expresión proteica de FAS esperado como consecuencia de disminución de la expresión del miRNA-122-5p, podría ser compensado por la disminución esperada debido a la reducción de SREBP1. Además, Bathia *et al.* (181) transfectaron hepatocitos hepG2 con miRNA-107 a diversas dosis. Ellos observaron que al usar la dosis más común en los estudios de transfección (25 nM) no se veían cambios en la expresión de la proteína FAS.

En cuanto a CPT 1a, según miRecords, gen diana predicho de miRNA-103-3p y miRNA-107-3p la sobreexpresión del miRNA-107-3p en hepatocitos AML12 redujo la expresión proteica de CPT 1a. Por el contrario, el miRNA-103-3p no indujo ningún efecto. Además, en el estudio *in vivo*, en el hígado de las ratas

tratadas con resveratrol se observó una regulación de estos miRNAs. Estos resultados concuerdan con el aumento de la actividad de CPT 1a previamente observada en esta cohorte de animales. Por consiguiente, el aumento inducido por el resveratrol en la expresión proteica de CPT 1a, implicado en el efecto deslipidante de este polifenol, parece estar mediado por la reducción en la expresión del miRNA-107-3p.

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[i] Las referencias están incluidas en el apartado REFERENCES.