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miRNAs as Predictive Biomarkers of Obesity and Weight Loss: Epigenetic Regulation and Target Gene Identification

Marcos García Lacarte

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Marcos García Lacarte

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VºBº Director
Dr. Fermín I. Milagro Yoldi

VºBº Co-Director
Prof. J. Alfredo Martínez Hernández

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“Quien tropieza y no cae, avanza
dos pasos hacia delante”

A mi Famiglia: *Leticia*
Pepe
Francho
Álvaro

Y a ti, *Abuela Lola*

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ABSTRACT

Epigenetics refers to molecular factors and processes around the DNA that regulate genome activity without nucleotide sequence modification. One of these epigenetic factors are miRNAs, a class of small (17-25 nts in length) single stranded non-coding RNAs that post-transcriptionally regulate gene expression in animals, plants and unicellular eukaryotes. Furthermore, miRNAs coding regions may also be regulated by other epigenetic mechanisms, such as DNA methylation. Therefore, a complex interactive epigenetic regulation may occur onto DNA, ultimately controlling gene transcription and translation. Consequently, it has been demonstrated that epigenetics can be involved in the development of obesity, inflammation and metabolic disturbances (type 2 diabetes, hypercholesterolemia, hypertension, or cardiovascular disease). Furthermore, in the era of "personalized nutrition", miRNAs have emerged as promising tools for the prediction, screening, diagnosis and prognosis of obesity and related comorbidities.

In this sense, the present research work has applied different –omics approaches such as methylation/expression microarrays and deep sequencing technologies in order to address the following objectives: 1) to demonstrate the role of miRNA epigenetic regulation in the early stages of childhood obesity; 2) to identify miRNA-type biomarkers for weight loss prediction; and 3) to verify the regulation of selected miRNAs over their predicted target genes.

In the first chapter we performed a methylation microarray in an infant population and identified that DNA methylation levels in miR-1203, miR-412 and miR-216A coding regions significantly correlated with body mass index standard deviation score (BMI-SDS). Moreover, these methylation levels were able to explain up to 40% of the variation of BMI-SDS in childhood obesity. In chapter 2, after combining methylation and expression microarrays data, we observed that miR-612 and miR-1976 were hypomethylated and overexpressed in high responders to a weight loss intervention. Additionally, after construction of specific expression vectors containing the particular 3'-UTR of each gene, we demonstrated that miR-612 and miR-1976 targeted *TP53* and *CD40* genes respectively. Furthermore, miR-1976 was able to down-regulate *CD40* expression levels when transfected into mature human

adipocytes. Lastly, in chapter 3, we noticed that miR-548q and miR-1185-1 also differed in their expression levels between high and low responders. Overexpression miRNA mimic experiments and luciferase reporter vectors showed that both miRNAs were implicated in *GSK3B* mRNA expression regulation, by a direct binding of the miRNA over the 3'-UTR of *GSK3B* in the case of miR-1185-1, and by an indirect regulation in the case of miR-548q.

Overall, the results exhibited in this thesis add new insights into the importance of miRNAs in the management of diseases, especially of obesity and inflammation-related illnesses; presenting different miRNAs involved in obesity development as prognostic biomarkers with potential utility in the personalization of weight loss treatments.

LIST OF ABBREVIATIONS

ADIPOR1	Adiponectin Receptor 1
BMI	Body Mass Index
CAV	Caveolin
CIDEA	DNA fragmentation factor-alpha-like effector A
CIP	Alkaline Phosphatase, Calf Intestinal
circRNAs	Circular RNAs
CVD	Cardiovascular Disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
EWAS	Epigenome-Wide Association Studies
FAS	Fas cell surface deth receptor
FCR1	Fragile X mental Retardation-related protein 1
GENOI	Grupo Navarro de Obesidad Infantil
GSK3	Glycogen synthase kinase-3
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HR	High Responders
IFN	Interferon
IL	Interleukin
IS	Identification Sample
LB	Lysogeni Broth
LEP	Leptin
LEPR	Leptin Receptor
lncRNAs	Long ncRNAs
LR	Low Responders
luc	Luciferase
MCP-1	Monoctye chemoattractant protein-1
MetS	Metabolic Syndrome
miRNAs	microRNAs
ncRNAs	Non-coding RNAs
nts	Nucleotides

O/N	Over-Night
PAI-1	Plasminogen Activator Inhibitor-1
P-bodies	Processing bodies
PLIN	Perilipin
PPGARG	Peroxisome Proliferator-Activated Receptor Gamma
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PTEN	Phosphatase and Tensin Homolog
PUFA	Polyunsaturated Fatty Acids
qPCR	Quantitative PCR
RESMENA	Reducción del Síndrome Metabólico en Navarra
RNAa	RNA activation
RISC	RNA Induced Silencing Complex
SD	Standard Deviation
SDI	Sociodemographic Index
SFA	Saturated Fatty Acids
T2D	Type 2 Diabetes
TNF	Tumor Necrosis Factor
TP53	Tumor suppressor protein p53
UTR	Untranslated Region
WAT	White Adipose Tissue
WBC	White Blood Cell
WHO	World Health Organization

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INTRODUCTION

Obesity

Etiology and Prevalence

Obesity is defined as an abnormal or excessive accumulation of fat mass derived from an energy imbalance, which comprises energy intake, energy expenditure, and energy storage (Kadouh & Acosta, 2017). Although the consumption of excessive calories is key for abnormal storage in adipose tissue, obesity is a heterogeneous disease where several biological, environmental and behavioral obesogenic factors interact to alter energy balance (Apalasamy & Mohamed, 2015).

A fast and convenient method for classifying an individual as lean, overweight or obese is using the body mass index (BMI), a value calculated by dividing body weight (in kilograms) by the square of height (in meters). In adults, it is considered that a BMI between 25 kg/m^2 - 30 kg/m^2 corresponds to an overweight subject; whereas a BMI >30 kg/m^2 is characteristic of an obese individual, regardless of sex (Racette et al., 2003). Despite that BMI is the most commonly used method for obesity classification, it has a major limitation differentiating fat mass and fat-free mass, and therefore BMI may misclassify muscular subjects as overweight (Racette et al., 2003). Epidemiologic studies have identified high BMI as a risk factor of many chronic diseases, including cardiovascular diseases (CVD), diabetes, chronic kidney disease, several cancers, and musculoskeletal disorders.

Indeed, type 2 diabetes (T2D), hypertension and hyperlipidemia are well-known complications of severe obesity, existing a positive correlation between BMI and development of T2D in all ethnic groups (Nguyen et al., 2008). Likewise, up to one-fourth of hypertension events occur due to excess body weight (Wilson et al., 2002). Lipid metabolism is altered in obese individuals with central fat distribution, specifically high serum cholesterol, low-density lipoproteins, and very low-density lipoproteins and triglycerides (Singla et al., 2010). Severe obesity is related as well to heart disease and ischemic stroke (Poirier et al., 2006). Obesity also increases mortality from a number of cancers, such as esophagus, colon, rectum, liver, gallbladder, pancreas and kidney cancers (Jarolimova et al., 2013).

It is estimated that a total of 107.7 million children and 603.7 million adults were obese in 2015 and that the prevalence of obesity has doubled since the 80's according to sociodemographic index (SDI) (Afshin et al., 2017) (Fig 1). Moreover, in 2014, nearly 5% of the deaths worldwide were attributable to obesity, indicating that obesity is a threat to global health in terms of prevalence, incidence or even economic burden. Consequently, in 2014 the global economic impact of obesity was considered to be 2.8% of the global gross domestic product (which is the combined gross national product worldwide) (Tremmel et al., 2017). Besides excess health care expenditure, obesity's costs are also in the form of lost productivity due to illness, mortality or permanent disability (Dee et al., 2014).

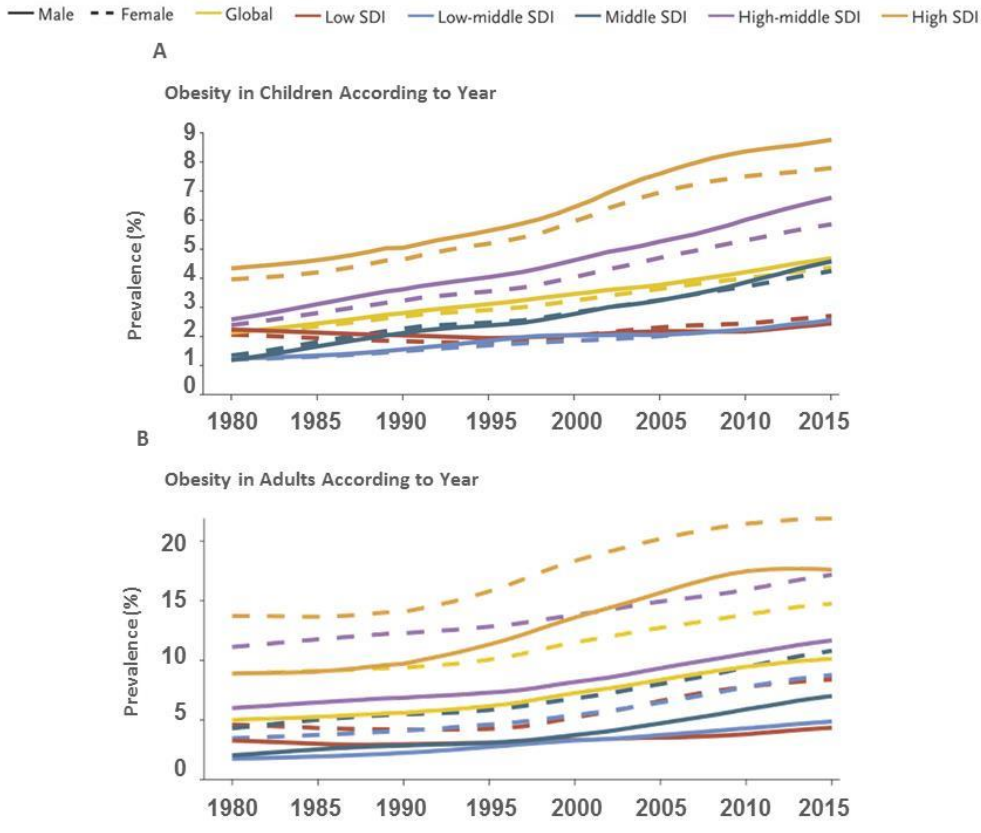

Fig 1. Prevalence of Obesity at the global level, according to sociodemographic index (SDI). As observed, obesity is pervasive and affects people of all SDI. Adapted from Afshin A *et al.* 2017.

Childhood Obesity

Childhood obesity is defined as the proportion of children with weight-for-height z-score values greater than two standard deviations (SDs) from the World Health Organization (WHO) growth standard median (de Onis et al., 2007). According to WHO, the number of overweight and obese infants and young children increased from 32 million to 41 million since 1990 to 2016 (WHO, 2017).

Environmental factors strongly impact the risk of obesity development in childhood. These factors include exposure to gestational diabetes in utero, shorter breastfeeding duration, unhealthy diet and sedentary lifestyles (Bass & Eneli, 2015). Childhood obesity development could have dramatic consequences in the quality of life of children if compared to their lean peers. Children with severe obesity are influenced to develop adult obesity, metabolic syndrome (MetS), T2D, early atherosclerosis, fatty liver disease and premature death (Calcaterra et al., 2008). Furthermore, 39% of children with moderate obesity have at least two cardiovascular risk factors, while 59% of children with severe obesity have at least two cardiovascular risk factors (Freedman et al., 2007).

Obesity and Inflammation

Inflammation is a key feature of obesity. One of the explanations is because adipocytes (particularly those from the visceral depot) are in close proximity to immune cells and have immediate access to blood vessels. This situation may trigger to a meta-inflammation when there is an excess of metabolic surplus (Hotamisligil, 2006).

The greater proportion of adipocytes remains constant in adulthood, being hypothesized that this number is set during childhood and adolescence (Spalding et al., 2008). This situation may explain why three-quarters of obese children develop obesity in adulthood and less than 10% of lean children become obese adults (Freedman et al., 2001).

Adipose tissue on obese subjects is characterized by an increased size of adipocytes (hypertrophy) accompanied also by an increase in the number of adipocytes (hyperplasia). Moreover, there also exists an infiltration of macrophages,

endothelial cell activation and higher vascularization of the tissue (Bourlier et al., 2008, Maury & Brichard, 2010).

Hypertrophic adipocytes begin to abnormally produce pro-inflammatory molecules (cytokines) that activate several inflammatory signaling pathways, generating the characteristic inflammatory status of obese individuals (Wellen & Hotamisligil, 2005). Some of the pro-inflammatory cytokines secreted in excess by obesity-induced hypertrophic adipocytes are interleukin (IL)-6, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor (TNF) -α, or monocyte chemoattractant protein-1 (MCP-1) (Shapouri-Moghaddam et al., 2018). In adipose tissue, TNF-α activates the NF-κB pro-inflammatory pathway, but also inhibits the production of anti-inflammatory cytokines (Wensveen et al., 2015).

As mentioned above, one important event that occurs in obese adipose tissue is infiltration of macrophages. Thus, MCP-1 is a potent chemoattractant that recruits both monocytes and macrophages into the adipose tissue (Maury & Brichard, 2010). As well as by increasing the macrophage number, obesity has also been associated with macrophage polarization. In the lean state, adipose tissue is mainly dwelled by M2 macrophages, that product mostly anti-inflammatory cytokines (e.g., IL-4, IL-10, IL-13). Higher secretion of MCP-1 by adipocytes results in attraction of inflammatory monocytes from the circulation that change to pro-inflammatory cytokine-producers M1 macrophages. It is important to highlight that this shift in the balance of M2/M1 is due to the migration of monocytes from the circulation and not to the conversion of resident M2 macrophages into M1 (Lumeng et al., 2008). Moreover, in an effort to create an effective oxygen supply to the growing adipose tissue, angiogenesis is observed (Fig 2).

Several cytokines have been related with MetS. For example hyperleptinemia has been associated with atherosclerosis, hypertension and MetS (Sierra-Johnson et al., 2007). Resistin levels are increased in obesity and inhibits cellular glucose uptake (Graveleau et al., 2005, Kusminski et al., 2005). Moreover, there exist biomarkers that inform about inflammatory status or insulin sensitivity of an individual. Certainly, circulating levels of TNF-α correlate with BMI, and IL-6 contributes to insulin resistance (Katsareli & Dedoussis, 2014).

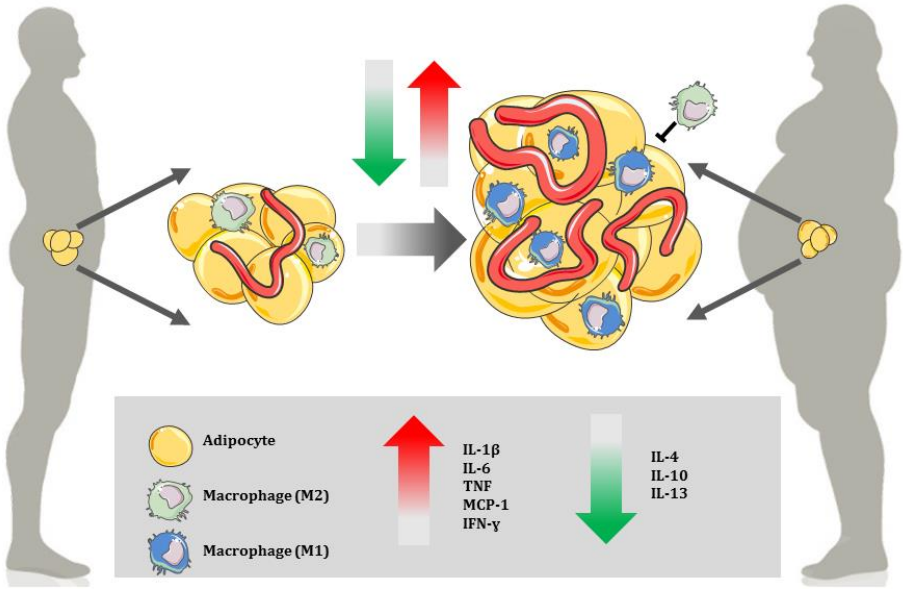

Fig 2. Obesity-related inflammation in white adipose tissue. In a lean state, adipose tissue maintains energy homeostasis through the regulation of regulatory T cells and M2 macrophages, which produce anti-inflammatory cytokines and contribute to maintaining the healthy environment. However, in obesity, adipose tissue expands and adipocytes become hypertrophied. These hypertrophic adipocytes secrete chemoattractants such as MCP-1 that recruit monocytes from the blood, where they become M1 polarized macrophages and produce pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, MCP-1, or Interferon (IFN)-ɣ. These cytokines act in autocrine, paracrine, and endocrine manners and increase inflammation in adipose tissue and other peripheral organs (Samad & Ruf, 2013).

Biomarkers in Obesity Prevention and Management

Obesity treatments are focused in achieving and maintaining weight loss with the aim of reducing the cluster of obesity-related diseases previously commented. To date, there are several strategies to treat obesity, including reduced-energy diets, physical activity, behavior modification, pharmacotherapy and surgery. However, the less invasive and more commonly used method to fight against obesity is nutritional intervention (Racette et al., 2003). It is estimated that a weight loss of around 5%-10% is accompanied by health benefits and represent a clinical success (Racette et al., 2003). In this context, the interest of identifying biomarkers related to obesity is growing, and researchers are looking for measurable "indicators" to facilitate monitoring of disease prevention, detection, treatment and management;

and to focus strategies and resources on subjects who would benefit the most. The term "biomarker" refers to an objective indication of medical state observed from outside the patient, accurately measurable, reproducible and interpretable (Strimbu & Tavel, 2010). In cell biology, a "biomarker" is a molecule detectable and isolable of a particular cell type (Martins-de-Souza, 2013). There are many events that occur during the manifestation of a disease, so biomarkers can be used at any point in this chain of events, at the molecular, cellular, or organ level. Thus, the ideal biomarker is one through which the disease comes about or through which an intervention alters the disease. It is important to mention that biomarker reliability would depend on the biological sample, sample isolation and management, study design, as well as to the desirable application of the biomarker (Dragsted et al., 2017).

Furthermore, genetics and environment could interact and alter the expression of some biomarkers (Musaad & Haynes, 2007). Thus, obesity-related biomarkers may be useful for early identification of susceptible individuals and may add value to the risk of developing cardiovascular disease and other pathologies (Musaad & Haynes, 2007).

Epigenetics

Definition

Epigenetics can be defined as "the molecular factors and processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable" (Skinner, 2011). Several biological factors, such as genetics, brain-gut axis, prenatal determinants, pregnancy, menopause, neuroendocrine disorders, medications, physical disability, gut microbiome, and viruses, have evidence-based associations with obesity (Kadouh & Acosta, 2017). In this context, epigenetics is taking on importance particularly in gene expression regulation.

There are several factors that affect gene expression, such as lifestyle, physical activity, exercise, and mutagenic and carcinogenic toxic chemicals and pesticides, that all influence genomic alignment by affecting gene expression (Abdul et al., 2017).

Epigenetic Mechanisms in the Regulation of Gene Expression

Epigenetic mechanisms are involved in the regulation of a number of cellular activities, from unicellular to eukaryotic organisms, and they are not only considered as transcription repressors, but are also implicated in transcriptional activation. The three mayor epigenetic mechanisms that regulate gene expression are DNA methylation, covalent histone modifications, and non-coding RNAs (Zhang & Pradhan, 2014) (Fig 3).

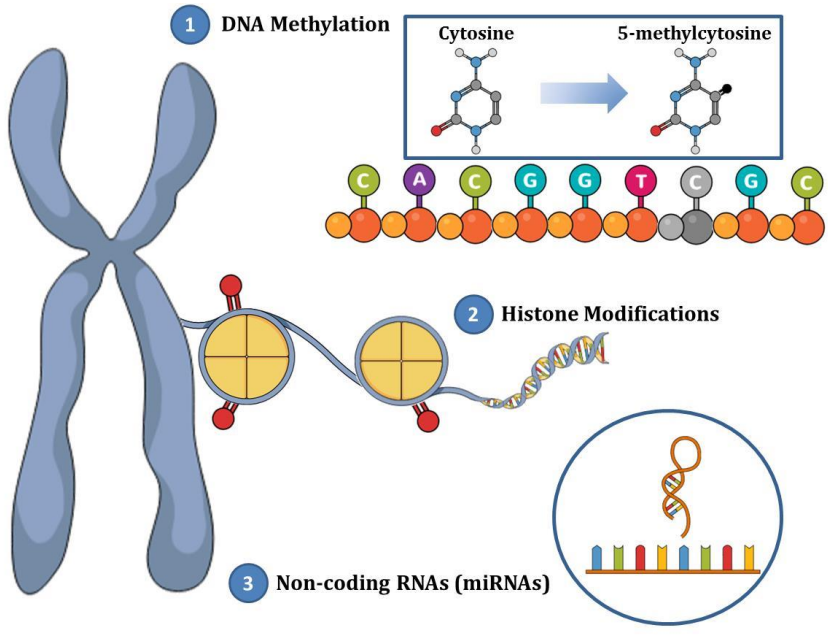

Fig 3. Main epigenetic mechanisms involved in gene expression regulation in mammalian cells.

DNA Methylation

The most studied epigenetic mechanism in mammalian genomes is DNA methylation. This DNA modification consists in the incorporation of a methyl group at the C-5 position of a cytosine adjacent to a guanine resulting in 5-methylcytosine. Although this is the predominant DNA modification in mammals, there exits also another forms of DNA methylation such as 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (Zhang & Pradhan, 2014). The majority of DNA methylation occurs in CpGs dinucleotides (nts) and about 30% of the CpGs

islands (human genome with high density of repeated CpG sites) are located in transcription start sites (Han et al., 2008). The effect of DNA methylation over gene expression could result in three different events: changes in the binding affinity of transcription factors (a) or methylation-specific recognition factors (b); or altering chromatin spatial conformation that alter the accessibility of transcription factors (c) (Siegfried & Simon, 2010).

Normally, DNA methylation compacts heterochromatin making transcription machinery less accessible and thus reducing gene expression. However, methylation not always implies gene repression as studies in zinc finger family transcription factors or in the methyl-CpG-binding domain containing proteins reveal; indicating that CpGs sites could act as insulators for transcription factors binding (Lopez-Serra et al., 2006).

Histone Modifications

Histones are proteins that package and structure DNA into nucleosomes. They can be modified by methylation or demethylation of arginine, lysine and histidine aminoacid residues (Zhang & Pradhan, 2014). Additionally, acetylation in conserved lysine residues is another histone modification that relaxes condensed heterochromatin reducing the histone binding affinity for DNA. Lastly, histones can also be phosphorylated in serine, threonine and tyrosine residues (Zhang & Pradhan, 2014) (Fig 4).

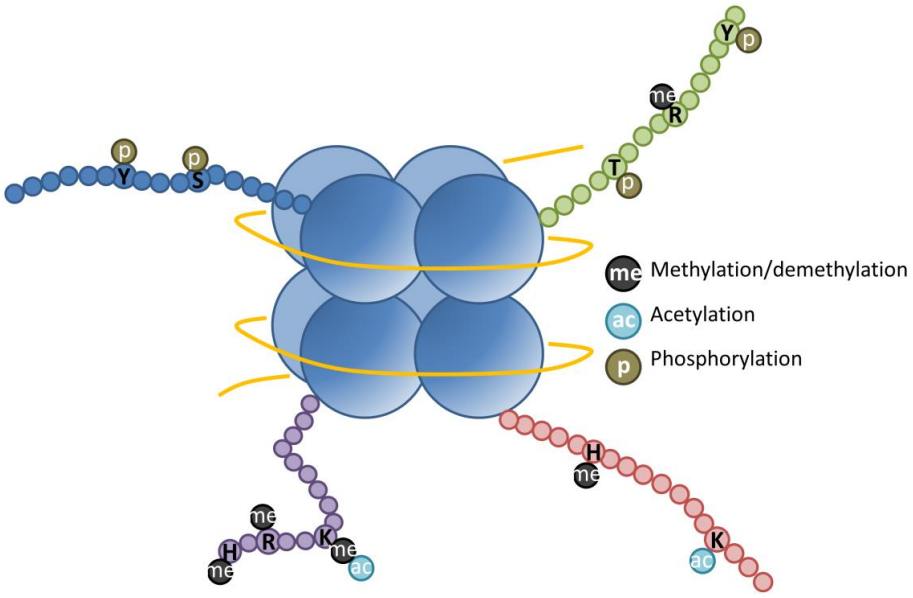

Fig 4. The most common histones modifications. Y: tyrosine, S: serine, T: threonine, R: arginine, H: histidine, K: lysine.

Non-coding RNAs

Non-coding RNAs (ncRNAs) are a large percentage of DNA that are transcribed but not translated into proteins. According to nucleotides length, ncRNAs can be categorized into two classes: small ncRNAs (19-200 nts) and long ncRNAs (lncRNAs) (>200 nts) (Huang et al., 2013, Delpu et al., 2016) (Fig 5). lncRNAs play important roles in chromosome dosage compensation, organ development, and disease progression processes (Nie et al., 2012). On the other hand, microRNAs (miRNAs) are well-known small RNAs that post-transcriptionally regulate gene expression through RNA interference pathways. miRNAs-encoding sequences are dispersed over the genome, although they are usually found clustered. They are generally transcribed from either the coding sequence of their corresponding genes or from the splicing of their introns (Zhang & Pradhan, 2014). Regarding gene expression regulation, miRNAs could act as repressors or enhancers of gene expression, although the most common effect is gene repression (Moutinho & Esteller, 2017).

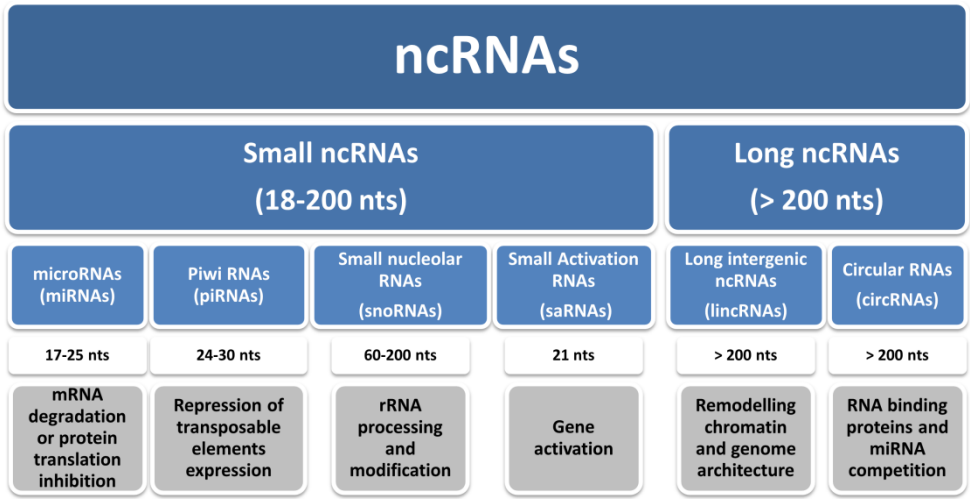

Fig 5. Classification and main role of the different non-coding RNAs according to their length.

Epigenetics and Obesity: Nutriepigenomics

As environmental, lifestyle and other epigenetic-affecting factors, dietary components such as nutrients and bioactive compounds can also induce changes in chromatin organization resulting in an alteration in gene expression. Diet can act alone or by interaction with other environmental factors causing epigenetic changes that may affect gene expression. Nutriepigenomics describes the interactions and effects of nutrients over gene expression through epigenetic modifications (Remely et al., 2015). Nutriepigenomics encompasses different dietary effects on genome stability (DNA damage at molecular and chromosomal level), epigenome alterations (DNA methylation), RNA and miRNA expression (transcriptomics), protein expression (proteomics), and metabolic changes (metabolomics) (Fenech et al., 2011). It is well accepted that nutrients can affect the 1-carbon metabolism altering DNA and histones methylation (Remely et al., 2015).

One nutriepigenomic-affected gene is leptin, since it has been described that supplementation with apple extracts decreases the methylation levels of CpG sites in leptin promoter (Boque et al., 2013), and an overexpression is observed after induction by short chain fatty acids (Remely et al., 2014). Additionally, unsaturated

free fatty acids are enrolled on DNA methylation, by affecting involved enzymes or modifying the availability of the necessary substrates (Milagro et al., 2013).

Different bioactive compounds affect epigenome in different manners. For example, polyphenols can affect DNA methylation, histone marks, or miRNAs expression (Remely et al., 2015). On the other hand, sulforaphane, curcumin, butyrate and genistein among others can modify histone acetyl and deacetyl transferases, changing chromatin condensation and altering gene expression (Vahid et al., 2015).

DNA methylation is the most extensively studied epigenetic-mechanism related to obesity. Several researches have associated global DNA methylation (measured by *LINE-1* and *Alu* methylation levels) with some metabolic disorders. For instance, *LINE-1* promoter methylation levels has been inversely associated with BMI and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) in healthy women (Na et al., 2014), with body fat mass in healthy young adults (Marques-Rocha et al., 2016), with CVD in overweight and diabetic subjects (Cash et al., 2011), with myocardial infarction risk in a case-control cohort (Guarrera et al., 2015) and with metabolic risk markers in the adipose tissue of MetS subjects (Turcot et al., 2012). Furthermore, in women with BMI > 30 kg/m^2 DNA methylation levels of *IL-6* was observed to be higher when compared to normal weight and overweight individuals (Na et al., 2015).

Traditionally, nutrition research has focused on the idea that all individuals have the same nutritional requirements, without distinction by sex, gender or ethnicity (Fenech et al., 2011). The fact is that there is a great diversity in the genome sequence between ethnic groups; people differ in their food preferences depending on cultural, economic and geographic differences; and that genome stability itself may lead to different phenotypes; underpinning personalized nutrition based on biomarkers as an important strategy for optimal health and disease prevention.

Many studies have correlated gene-wide or gene-specific DNA methylation levels with individual response to the development of obesity or related comorbidities, suggesting that baseline DNA methylation levels could be used as epigenetic markers for the prevention of obesity-related disorders (Campion et al., 2009, Wang et al., 2010, Milagro et al., 2011, Remely et al., 2015, van Dijk et al., 2015, Abdul et al., 2017, Nilsson & Ling, 2017, Wahl et al., 2017, Castellano-Castillo et al., 2018).

It is thus conceivable that in a near future epigenetic and genetic markers could be used to detect predisposition for obesity and may improve its prevention and therapy.

Epigenetics and Other Diseases

Epigenetics is involved in the regulation of normal development and biology, and many diseases develop when abnormal epigenetic marks are added at the wrong place at the wrong moment (Portela & Esteller, 2010).

DNA methylation alterations contribute to cancer, both through hypo- or hypermethylation. As DNA hypomethylation leads to DNA instability, this can result in oncogene activation and thus oncogenesis. Moreover, hypermethylation is associated with silencing of tumor suppressor genes (Tollefsbol, 2012). Histone modifications are also important in disease development and progression. Histone acetylation or deacetylation can disrupt gene expression regulation and are associated with several cancers and neurological disorders (Tollefsbol, 2012).

Cancer is one of the most frequently disease affected by epigenetic changes, and DNA methylation alterations have been associated with many tumors, such as glioma, lung, stomach, colorectal, gastrointestinal, renal cell carcinoma, breast, melanoma, or esophageal cancers, among others (Wang & Wu, 2018).

Neurodegenerative diseases such as Alzheimer's disease have been increasingly associated with alterations in epigenetic processes, as observed in changes in amyloid β peptide levels derived from epigenetic modifications (Mastrototaro & Sessa, 2018).

On the other hand, autoimmune disorders have been also associated with aberrations in epigenetic mechanisms such as systemic lupus erythematosus, psoriasis, Crohn's disease, Vitiligo, Grave's disease, diabetes mellitus type I, or celiac disease among others diseases (Javierre et al., 2012).

DNA methylation profile alterations have been also associated with different metabolic diseases. For example, a meta-analysis from Richard *et al.* revealed that blood pressure influences the methylation in several CpGs (Richard et al., 2017). Recent reviews have summarized all evidences relating the associations between

DNA methyhlation and coronary heart disease (Fernandez-Sanles et al., 2017), and with non-alcoholic fatty liver disease (Lee et al., 2017).

miRNAs

Biogenesis

miRNAs are a class of small (17-25 nts in length) single stranded non-coding RNAs that post-transcriptionally regulate gene expression in animals, plants and unicellular eukaryotes (Melo & Melo, 2014). miRNAs were discovered by Victor Ambros in 1993 when identified in *Caenorhabditis elegans* the *lin-4* gene which is essential for the control of post-embryonic developmental events (Lee et al., 1993). Approximately 50% of miRNAs are found in clusters although a significant part of miRNAs are located in introns of coding and/or non-coding transcripts (Rodriguez et al., 2004). It is considered that a single miRNA has different target genes, while one single gene transcript is regulated by several miRNAs, generating an enormous cluster of miRNAs-target gene regulatory networks (Ross & Davis, 2014).

Usually, miRNAs are transcribed by RNA polymerase II, which generates an RNA transcript that contains stem loops and that have, as regular RNA transcripts, a 5' cap and a polyA tail [known as primary-miRNA (pri-miRNA)]. Before regulating gene expression, miRNAs suffer a maturation process that starts in the nucleus. First at all, the RNA transcript (pri-miRNA) is processed by an RNase III enzyme (Drosha) that cleaves the double-stranded stem-loop and generates a 100-700 nts hairpin structure known as pre-miRNA (precursor miRNA) (Han et al., 2006). This pre-miRNA is then transported to the cytoplasm to continue its maturation. The exportation is done by the exportin 5 transporter through the recognition of the 3' overhang and the duplex nature of the pre-miRNA. Once in the cytoplasm, the pre-miRNA is recognized by a second RNAse III enzyme called Dicer. Dicer binds to the 3' overhang and also to the 5' end to correctly positionate the pre-miRNA and cleavage both strands eliminating the loop and generating an intermediate dsRNA product which is 21-23 nts in length (Macrae et al., 2006). The mature miRNA is now assembled into Argonaute multiprotein family and forms the RISC complex (RNA induced silencing complex). Here, a selection of one of the strands occurs,

being the discarded strand degraded. It is thought that the strand whose 5'-end is located at the thermodynamically weaker end of the duplex will be the guide strand and will recognize and bind to the target mRNA (Schwarz et al., 2003). The single-stranded guide-RNA together with Argonaute proteins is now ready to regulate gene expression. A summary of these steps is shown in Fig 6.

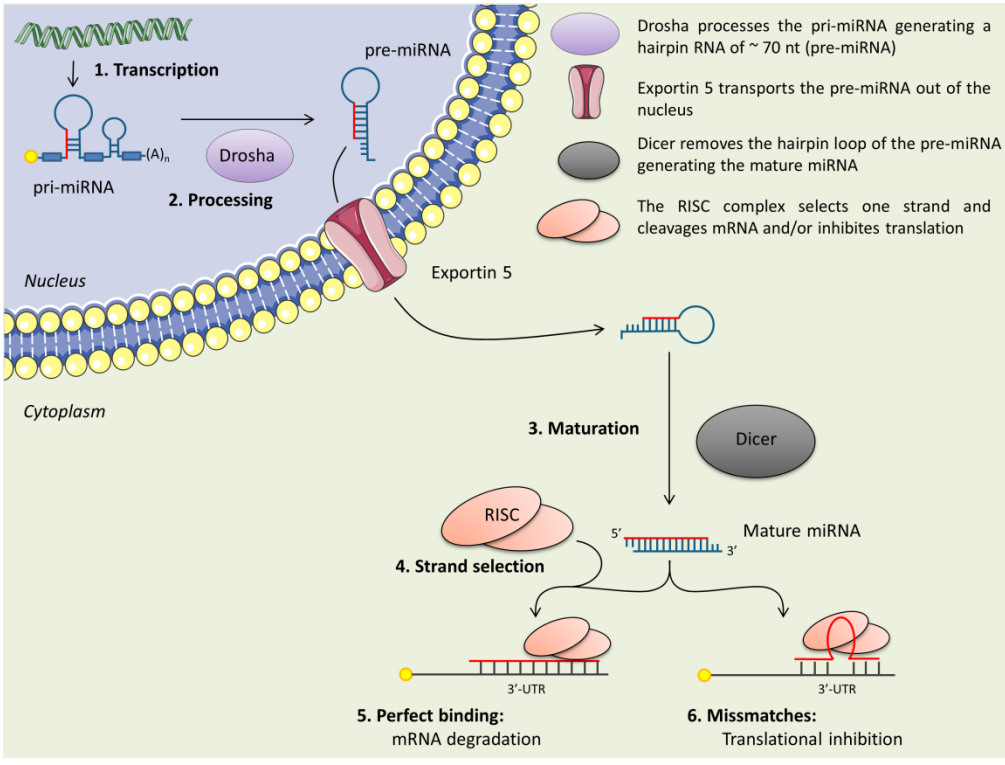

Fig 6. The biogenesis of miRNAs involves several steps. First at all, miRNAs are transcribed by RNA pol II creating the pri-miRNA. Drosha enzyme processes the pri-miRNA generating a hairpin RNA of approximately 70 nt (pre-miRNA). This pre-miRNA is translocated to the cytoplasm by the exporting 5 and is then cleavage by the Dicer endonuclease which eliminates the loop. The mature miRNA can now be incorporated into the RISC complex that selects one of the strands. miRNA, together with the RISC complex, is now ready to bind to its target sites and regulate gene expression (adapted from Flipowicz *et al.* 2008).

Mechanisms of Regulation of Gene Expression by miRNAs

There are diverse post-transcriptional regulations mediated by miRNAs depending on the degree of complementarity between the miRNA and the binding site. Usually, miRNAs bind to their target transcripts at the 3'-untranslated region (UTR). The most common binding between miRNAs and target sites involves nucleotides 2 and 7 at the 5' end of the miRNA, named as "seed" sequence (Melo & Melo, 2014).

1) One mechanisms in where miRNAs control post-transcriptional gene expression is by endonuclease cleavage of target mRNA, referred to as "Slicer" activity. This endonucleolytic cleavage is performed as a result of perfect base-pairing between miRNA-mRNA, although some mismatches are allowed (Valencia-Sanchez et al., 2006). In mammals, only Ago2 is capable of directing cleavage mRNA. The sliced product appears to be degraded by the same enzymes that degrade the greater part of cellular mRNA. This process involved the exosome, a exonuclease complex that degrades mRNA cleavage triggered by miRNAs binding (Valencia-Sanchez et al., 2006).

2) It is now proposed that miRNAs also target mRNAs by slicer-independent mechanisms. This process requires the action of processing bodies (P-bodies), which are recruiter sites for mRNA decapping and degradation (Sheth & Parker, 2003).

3) The third way by which miRNAs silence gene expression is by interfering in their translation. It has been suggested that some argonaute proteins, miRNAs, and miRNA-mRNA targets accumulate in P-bodies, which affect translation initiation by a competition between assembly of the translation initiation factors and P-bodies- RISC complexes (Valencia-Sanchez et al., 2006).

Although the most common miRNA regulatory mechanism is the repression of targets genes by binding to the 3'-UTR of the mRNA, there are also other mechanisms by which miRNAs can interact and regulate their target transcripts. It has been demonstrated that miRNAs can also bind at the 5'-UTRs or coding regions. The 5'-UTR region of mRNA is the sequence immediately upstream of the translation start codon and has important regulatory roles, since it contains sites for RNA binding proteins, open reading frames and start codons, which have dramatic

importance in the ignition of translation (Araujo et al., 2012). First, translation repression was observed when the 3'-UTR target site for let-7a was moved to the 5'-UTR (Lytle et al., 2007). Lee *et al.* reported combinatory interactions between a single miRNA and both end regions (5'-UTR and 3'-UTR), demonstrating that miR-34a binds to both 3'-UTR and 5'-UTR (Lee et al., 2009). Moreover, interaction between miRNA and mRNA in the coding region of the transcript has been also evidenced. Indeed, Forman *et al.* demonstrated that let-7 miRNA directly targeted Dicer within its coding sequence, establishing that miRNAs act not only bind to -UTRs regions, but also in the coding body of the target mRNA (Forman et al., 2008).

On the other hand, while the majority of miRNAs are found in the cytoplasm, there are evidences that Ago protein and miRNAs can navigate into the nucleus to lead miRNA gene regulation (Nishi et al., 2013), underpinning de idea that miRNAs could have important regulatory roles in the nucleus.

When a group of purines of one strand of dsDNA are together, Hoogsteen pairing could occur, allowing a third strand to assemble into the duplex in a Watson-Crick pattern, resulting in a triple-stranded helices (triplexes) (Goni et al., 2004). In this context, it has been postulated that miRNAs of sufficient length may form triplexes with dsDNA and thereby directly interact with target DNA sequences in regulatory regions and gene promoters. Indeed, an accurate study using a fluorescence resonance energy transfer-based method demonstrated that miR-483-5p forms triplexes with dsDNA and could directly alter gene expression (Paugh et al., 2016).

It is important to mention that under certain conditions, miRNAs have been shown to activate gene expression in a process known as RNA activation (RNAa). One example is *TNF* gene. Upon cell cycle arrest, the AU-rich elements (ARE) of its mRNA are transformed into a translation activation signal, recruiting AGO and fragile X mental retardation-related protein 1 (FXR1) factors resulting in an increased expression. It has been observed that miR-369-3 recruits AGO and FXR1 and associates the complex to the ARE region, inducing *TNF* expression. Furthermore, in the same study, it was demonstrated that *let-7* and synthetic miRcxcr4-likewise induce translation up-regulation of target mRNAs on cell cycle arrest (Vasudevan et al., 2007). Moreover, Tsai and colleagues found that miR-346 elevates RIP140

protein levels, binding to the 5'-UTR of *RIP140* mRNA in an AGO2-independent way (Tsai et al., 2009), demonstrating that miRNAs binding to the 5'-UTR of target genes may also activate gene expression.

Epigenetic Regulation of miRNAs

In recent years, the number of studies documenting the epigenetic regulation of miRNAs and their implication in human health has significantly increased (Kunej et al., 2011). Around 50% of the miRNAs are enclosed by CpGs islands, being therefore susceptible for hyper- or hypo- DNA methylation and thus, target in multiple cancers [for a comprehensive review of miRNA-methylation related diseases, see (Silahtaroglu & Stenvang, 2010, Tuna et al., 2016, Moutinho & Esteller, 2017)]. miRNAs can also be dysregulated as a consequence of altered levels of histone modifiers (Scott et al., 2006), or through abnormal activation of transcription factors.

It is important to highlight that miRNAs could also regulate epigenetic machinery and thus could be involved in an indirect epigenetic regulation. Epi-miRNAs are defined as those miRNAs whose targets are, in a direct or indirect manner, effectors of the epigenetic machinery. For instance, miRNAs can affect histone methyltransferases, which disturb histones or DNA methylation (Moutinho & Esteller, 2017).

miRNAs Involved in Obesity and Related Comorbidities

The contribution of miRNAs in gene regulation emphasizes their impact in the onset or evolution of disease. Indeed, miRNAs play key roles in vital cellular processes such as division and death, metabolism, intracellular signaling, immunity and cell movement that can cause malignant outcomes (Li & Kowdley, 2012). The aberrant expression of these miRNAs can lead to the onset of pathologies like several cancers (breast, lung, gastric and liver cancer), viral diseases (HCV, HIV-1 or influenza virus), immune-related diseases (multiple sclerosis, systemic lupus erythematosus, T2D, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis) or neurodegenerative disorders (Parkinson's and Alzheimer's disease) (Li & Kowdley, 2012).

In this context, it has been suggested that miRNAs could be implicated also in the development and progression of MetS and related comorbidities (Pomatto & Gai, 2018). By applying deep sequencing technologies, several miRNAs have been identified to be differentially expressed in human adipose tissue in obese vs. lean subjects (Ortega et al., 2010, Casado-Díaz et al., 2016, Armenise et al., 2017, Kristensen et al., 2017). Several studies have shown that during pre-adipocyte fat cell development miRNAs expression is altered, and that they can act as both negative regulators or capable of accelerating adipocyte differentiation (McGregor & Choi, 2011). Indeed, miRNAs are involved in many obesity-associated processes, such as angiogenesis, adipogenesis, inflammation and oxidative stress (Pomatto & Gai, 2018), in part due to their capacity to modulate glucose and lipid metabolism in the liver (Liu et al., 2014), insulin production in pancreas (Plaisance et al., 2014), or leptin signaling in the hypothalamus (Derghal et al., 2015). Interestingly, 13 miRNAs appeared differentially expressed in plasma when comparing diabetic and controls subjects (Zampetaki et al., 2010), and 107 miRNAs changed their expression during mouse pancreas development (Lynn et al., 2007). A summary of some relevant miRNAs associated with obesity and related diseases is shown (Table 1).

miRNAs as Biomarkers and Diagnostics

Circulating miRNAs are very stable in blood due to their resistance to RNAse digestion. This feature, together with their implication in many diseases, makes miRNAs suitable prognostic indicators of disease (Chen et al., 2008). For instance, several studies have demonstrated that miRNAs could be used as biomarkers of cancer (Lu et al., 2005, Pimentel et al., 2015). Indeed, Lu et al. used a bead-based flow cytometric miRNA expression profiling method to analyze 217 mammalian miRNAs from 334 samples of multiple human cancers. They observed a down-regulation of miRNAs in tumor samples compared with normal tissues, and were able to classify poorly differentiated tumors by using miRNA expression profiles (Lu et al., 2005). Additionally, Pimentel and collaborators identified and quantified miRNAs as potential breast cancer biomarkers (Pimentel et al., 2015).

Table 1: Most relevant miRNAs involved in obesity-associated events and their target genes

Event	miRNA	Target Genes	Ref.
Adipogenesis	miR-26a/b	*UCP1, ADAM17*	(Karbiener et al., 2014)
	miR-27	*Ppara, Ppargc1a, Prdm16*	(Sun & Trajkovski, 2014)
	miR-106b-93	*Ucp1, Prdm16, Ppara, Ppargc1a*	(Wu et al., 2013)
	miR-133	*Prdm16*	(Trajkovski et al., 2012, Yin et al., 2013)
	miR-143	*Mapk7*	(Zhang et al., 2018)
	miR-155	*Cebpb, Ppargc1a*	(Chen et al., 2013)
	miR-193b/365	*Runx1t1, Ppara, Cebpa*	(Sun et al., 2011)
	miR-196a	*Hoxc8*	(Mori et al., 2012)
	miR-377-3p	*LIFR*	(Li et al., 2018)
	miR-378	*Msc, Cebpb, Cebpa*	(Gerin et al., 2010, Gagan et al., 2011)
	miR-455	*Ucp1*	(Cai et al., 2016)
	Let-7	*Hmga2*	(Wei et al., 2014)
	miR-15a	*Dlk1*	(Andersen et al., 2010)
	miR-223	*GPAM*	(Li et al., 2019)
	miR-519d	*PPARA*	(Martinelli et al., 2010)
Cardiovascular Disease	miR-133	*RHOA, CDC42, NELFA*	(Carè et al., 2007)
	miR-21	*PDCD4, PTEN, SPRY1, SPRY2; Map2k3*	(Cheng & Zhang, 2010, Canfran-Duque et al., 2017)
	miR-143	*KLF4*	(Zhao et al., 2015)
	miR-145	*KLF4*	(Zhao et al., 2015)
	miR-146	*Sort*	(Cheng et al., 2017)
	miR-155	*Atr1*	(Yang et al., 2014)
	miR-181	*Notch1*	(An et al., 2017)
	miR-223	*TNNI3K, TNNI3*	(Chen et al., 2017)
Diabetes	miR-191	*PCSK9*	(Naeli et al., 2017)
	miR-375	*Mtpn, PDK1*	(Atkinson & Eisenbarth, 2001, Achenbach et al., 2005)

Table 1: *(Continued)*

Event	miRNA	Target Genes	Ref.
	miR-124a	*Foxa2*	(Baroukh et al., 2007)
	miR-657	*IL-37*	(Wang et al., 2018)
	miR-144	*Irs1*	(Karolina et al., 2011)
	miR-146a/b	*IRAK1*	(Kim et al., 2012)
	miR-221	*SIRT1*	(Liu et al., 2018)
	miR-24	*P3*	(Huang et al., 2009)
Inflammation	miR-125b	*Tnf*	(Tili et al., 2007)
	miR-126	*VCAM1*	(Sturgeon et al., 2012)
	miR-132	*SIRT1*	(Strum et al., 2009)
	miR-155	*Nfkb*	(O'Connell et al., 2007)
	miR-187	*TNF, IL6, IL12*	(Rossato et al., 2012)
	miR-222	*ICAM1*	(Ueda et al., 2009)
	Let-7	*IL6*	(Sung et al., 2013)

Moreover, miRNAs have been related to obesity-related complications. Serum miR-23a and miR-126 levels can be used for early detection of T2D (Liu et al., 2014, Yang et al., 2014). Similarly, three serum miRNAs (miR-132, miR-29a and miR-222) are predictive markers of gestational diabetes mellitus (Zhao et al., 2011). In CVD, circulating miRNAs have great potential as markers for myocardial infarction, and miR-423, among others, has been proposed as biomarker for this cardiac complication (Tijsen et al., 2010).

In obesity, several anthropometric and biochemical parameters correlate with different miRNA expression. Certainly, Ortega et al. found that 17 miRNAs were altered in obesity and correlated with BMI and glycemia (Ortega et al., 2010). Parallelly, Arner et al. also correlated disrupted miRNA expression profiles with BMI, weight, insulin resistance, and adipocyte function parameters (Arner et al., 2012). Finally, serum levels of miR-122 and miR-519d are strongly correlated with obesity and are BMI-associated (Adams et al., 2018).

Regarding weight loss prediction, there are also several miRNAs that have been proposed as biomarkers of prediction. In this context, previous studies have identified several miRNAs that are differentially expressed at baseline between responders and non-responders to a weight loss intervention. Indeed, miR-935 and miR-4772 were up-regulated in non-responders while miR-223, miR-224 and

miR-376b are down-regulated. Additionally, miR-935 and miR-4772 were strongly associated with the magnitude of weight loss (Milagro et al., 2013).

miRNAs as Therapeutic Targets

In the last years, the idea of inhibiting or overexpressing miRNAs for therapeutic purposes has become a new frontier in modern medicine. miRNA mimics or miR-inhibitors have been widely studied as possible therapeutic agents. miRNA mimics are synthetic miRNAs that mimic natural miRNAs, and can be used to reconstitute miRNAs that are down-regulated during disease or to down-regulate pathways involved in disease development (Kreth et al., 2018). The first use of a miRNA mimic as clinical tool was MRW34, which mimics a tumor suppressor (miR-34) underexpresssed in several cancers (Beg et al., 2017). This mimic was tested in a phase I clinical trial in 2013 but was stopped in 2016 due to multiple immune-related severe adverse events.

Recently a mimic of miR-29b started a phase 1 study. The molecule (MRG-201) decreased the expression of collagen and other proteins involved in fibrous scar formation. As well as for mimicking miRNAs action, pharmaceutical approaches to inhibit miRNAs regulation have been developed. Anti-miR molecules block natural miRNAs and thus silence miRNAs that are overexpressed during disease. Anti-miRs can be administered dissolved in saline solution and are easily taken by different tissues and organs. Hepatitis C virus infection has been effectively silenced using miravirsen (Gebert et al., 2014). Miravirsen targets miR-122 in liver, which is hijacked by the hepatitis C virus to bind to RNA viral 5'-UTR sequences, enhancing virus replication. Another anit-miR is MRG-106, that inhibits miR-155. miR-155 plays a crucial role in differentiation, function, and proliferation of blood and lymphoid cells. Therefore, inhibitors of miR-155 could be useful in patients experiencing cutaneous T-cell lymphoma (Querfeld et al., 2016). Nevertheless, a major challenge in miRNA-based therapies is the development of delivery systems enabling cell-specific uptake and the design of therapeutic molecules without toxic side effects (Kreth et al., 2018).

Finally, much effort is being made in nutrition in order to find dietary patterns, specific foods, nutrients and bioactive compounds that are able to specifically

increase or decrease the expression of miRNAs involved in the treatment or onset of metabolic diseases. For example, the protective roles of PUFAs in various human diseases, including cancer, could be attributed at least in part to miRNA-regulating mechanisms. A study that evaluated the chemopreventive effects of PUFAs on colon cancer in rats demonstrated the role of fish oil in protecting the colon from carcinogen-induced miRNA dysregulation (Davidson et al., 2009). Interestingly, dietary fisetin (a flavonol present in fruits and vegetables) can protect against hepatic fat accumulation in mice fed a high-fat diet via inhibition of miR-328 expression (Jeon et al., 2013), suggesting the use of fisetin to target miRNAs as an effective intervention against metabolic diseases.

In parallel, resveratrol and quercetin in combination have anticancer activity in colon cancer cells through the regulation of miRNA-27a levels (Del Follo-Martinez et al., 2013). Moreover, a Mediterranean-based nutritional intervention was able to induce changes in the expression of important miRNAs such as let-7b and miR-155-3p, that are known to be associated with MetS, cancer, and atherogenic or adipogenic processes (Marques-Rocha et al., 2016). Lastly, rats fed a vitamin E deficient diet for 6 months displayed reduced concentrations of miR-122a and miR-125b, which may play an important role in lipid metabolism, carcinogenesis, and inflammation, suggesting that a dietary regimen with appropriate vitamin E levels could prevent cancer progression via miRNA regulation (Gaedicke et al., 2008).

Altogether, these studies support the use of dietary compounds or specific dietary patterns to modify miRNA levels to use them as therapeutic targets against metabolic diseases.

Although further studies are needed to better understand the molecular mechanisms involving miRNA regulation, it is clear that miRNAs are involved in the pathophysiology of obesity and its related comorbidities. Moreover, several studies reveal that miRNAs regulate important obesogenes and that they could be used as biomarkers of response to diet interventions, and also as therapeutic targets for many diseases.

HYPOTHESIS AND OBJECTIVES

Hypothesis

Epigenetic phenomena mediated by miRNAs have been implicated in gene expression regulation and could also be involved in the onset and progression of several metabolic alterations, including obesity and associated manifestations.

In this context it was hypothesized that miRNAs can be used as biomarkers to define and diagnose obesity predisposition at early stages of the disorder. On the other hand, miRNAs can be predictive factors of the response to an energy restriction strategy in obese subjects via target gene regulation.

Objectives

General Objective

The general objective of the present project was to identify specific miRNAs whose expression patterns are associated with obesity and that can be predictive of the response to specific weight loss treatments.

Specific Objectives

1. To demonstrate a potential role of the epigenetic miRNA regulation in obesity by describing specific DNA miRNA-coding sequences whose methylation levels in white blood cells (WBC) are associated with adiposity in children (Chapter 1).
2. To identify through -omics technologies, specific miRNAs that can be used as biomarkers in the personalization of the dietary treatment of obesity in adults (Chapters 2 and 3).
3. To verify the involvement of the selected miRNAs in the regulation of predicted target genes expression implicated in obesity-related inflammatory and metabolic pathways (Chapters 2 and 3).

METHODOLOGY

The design followed in the present thesis project is described in detail in the materials and methods section of each article, which in turn corresponds to each chapter. This section describes the general methodology applied to identify miRNAs and to validate their putative regulatory roles over target genes.

Study Population

GENOI (Chapter 1)

The GENOI (Grupo Navarro de Obesidad Infantil) study consisted in a cross-sectional trial of Spanish Children that started in 2001 and finished in 2003. Participants were recruited from Virgen del Camino Hospital, Clínica Universidad de Navarra and primary care centers when they were attended for routine medical examinations or vaccinations. At the end of the recruitment period, the Cases group (n= 185) were Spanish children of Caucasian ethnicity with BMI above the age- and sex-specific 97th percentile according to the Spanish BMI reference charts (B. Sobradillo et al., 2004); and Controls group (n= 185) were Caucasian healthy subjects with the BMI below the 97th percentile for the same references charts. Exclusion criteria were exposure to hormonal treatment or development of secondary obesity due to endocrinopathies or serious intercurrent illness. The study was approved by the Ethics Committee of the University of Navarra. Consequently, parents and adolescents gave written informed consent for participation in agreement with the Declaration of Helsinki.

In the current project, an identification sample (IS) of 24 subjects (12 Cases and 12 Controls) matched for age and gender was used to perform the methylation array-based approach.

RESMENA (Chapter 2, 3)

The RESMENA study (Reducción del Síndrome Metabólico en Navarra) was a randomized, longitudinal and controlled trial conducted in 96 adults with MetS in Navarra. These patients were allocated to one of two different energy-restricted dietary patterns (AHA diet as reference diet, and RESMENA diet as intervention diet) during 8 weeks, both with an energy restriction of -30% of the studied

requirements of energy intake. As no differences were found either in anthropometric or biochemical variables between groups after the intervention, both dietary groups were merged to increase the statistical power of the study. Aiming to identify miRNA-type biomarkers implicated in the response to the weight loss treatments, participants were categorized into "High Responders" (HR), when weight loss was ≥ 8%, and "Low Responders" (LR) when weight loss was < 8%.
The study was performed following the CONSORT 2010 guidelines and properly approved by the Ethics Committee of the University of Navarra (065/2009) and registered at www.clinicaltrials.gov (NTC01087086). All participants provided written informed consent for participation.

miRNAs and Target Genes Identification

In order to identify candidate miRNAs potentially implicated in obesity, three different -omics approaches were applied. In a second step assembling results, putative target genes of the selected miRNAs were predicted by using bioinformatics algorithms (Fig 7).

Methylation Microarray (Chapters 1, 2)

Genomic DNA was isolated from WBC of the IS (12 Case and 12 Controls) of the GENOI study (Chapter 1), and from a subpopulation (31 LR vs 16 HR) of the RESMENA cohort (Chapter 2) using the MasterPureTM DNA Purification Kit (Epicentre Biotechnologies). Sodium bisulfite modification of 500 ng of genomic DNA was carried out using the EZ DNA methylation kit (Zymo Research). Bisulfite-treated DNA was subsequently hybridized in an Infinium HumanMethylation450 BeadChip array (Illumina HM450K), which assessed the CpGs methylation levels of many miRNAs coding regions.

Expression Microarray (Chapters 2, 3)

Total RNA from the WBC (14 LR vs 10 HR) of the RESMENA study was reverse transcribed using High Capacity Complementary DNA reverse transcription kit (Life Technologies), and cDNA was hybridized in an Illumina Human HT-12 v4 gene

expression BeadChip array; analyzing both gene transcripts and miRNA expression levels.

miRNA-Seq (Chapter 3)

miRNAs from WBC of 6 LR and 5 HR from the RESMENA study were deep sequenced by Illumina's miRNA-Seq Technology, which is a technique to isolate and sequence small RNA species, such as miRNAs. RNA quality was evaluating before sequencing using the RNA Agilent Small RNA Bioanalyzer.

Bioinformatic Prediction (Chapters 1, 2, 3)

To predict putative target genes of the selected miRNAs, publically available miRBase (www.mirbase.org) or miRWalk 2.0 (mirwalk.umm.uni-heidelberg.de) algorithms were used. For each miRNA, these databases provides references in literature, genomic coordinates and links to databases of predicted and validated miRNA target sites such as DIANA-microT, microRNA.org, miRDB, RNA22, TargetMiner and TargetScan. Predictive target genes were filtered and selected if appeared at least in 6/11 databases and by focusing on obesity-related genes.

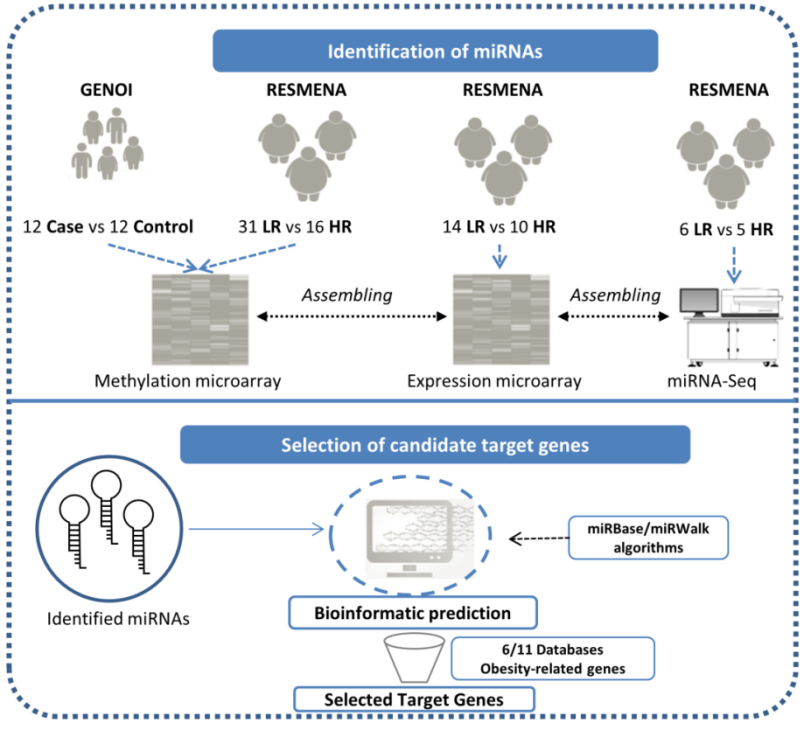

Fig 7. Procedure to identify candidate miRNAs and target genes.

Validation of miRNA Methylation and Expression Data

Results from microarrays and miRNA-Seq were further confirmed by different techniques to obtain robust miRNAs candidates for complete validation (Fig 8).

Validation of Methylation Microarray (Chapters 1, 2)

miRNA methylation results from the methylation microarray were validated by using MassArray EpITYPER technology. This method uses matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE), which allows, after designing specific primers, to quantify specific DNA sequence methylation.

Validation of Expression Microarray and miRNA-Seq (Chapters 2, 3)

In Chapter 2, expression levels of miRNAs differently expressed between HR and LR in the RESMENA study were validated by measuring expression levels of selected miRNAs in WBCs from RESMENA individuals. Quantitative PCR (qPCR) was performed with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using commercial Taqman probes (Applied Biosystems).

In Chapter 3, when miRNAs appeared significantly differential in both expression microarray and miRNA-Seq approaches, it was considered unnecessary to apply a third technique in order to validate their expression values.

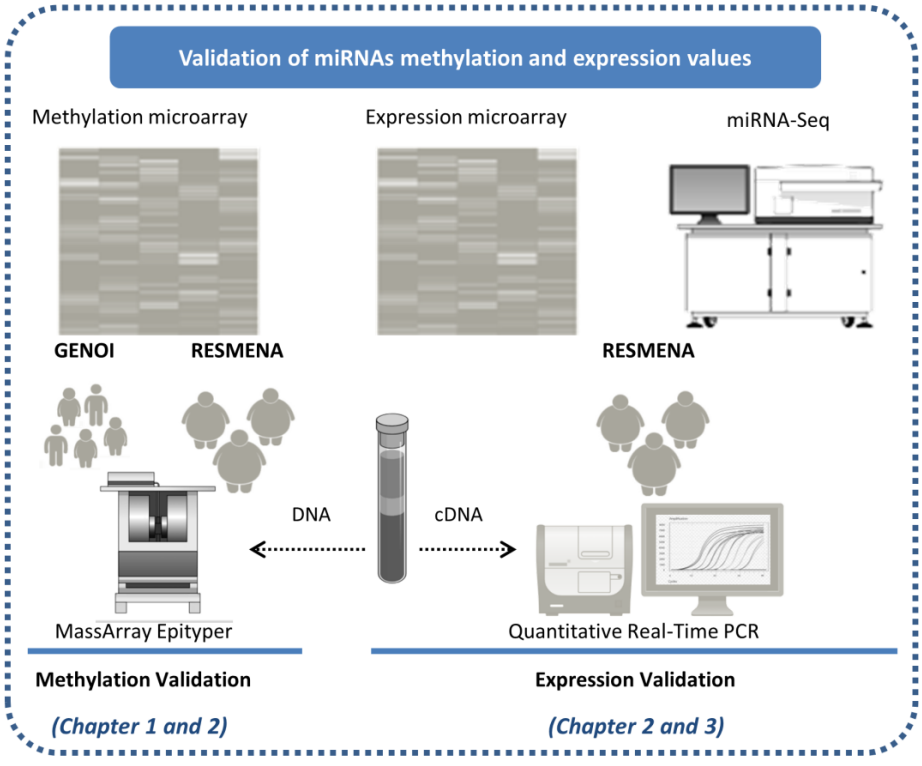

Fig 8. Experimental design for the validation of miRNAs methylation and expression results.

Validation of miRNA-Predicted Targets

After the identification of putative target genes by bioinformatic algorithms, different experimental approaches were used to elucidate if selected miRNAs might have regulatory roles over their predicted target genes. We analyzed their mRNA expression levels in WBCs by qPCR, performed miRNA overexpression experiments in human primary adipocytes and THP-1 cells, and created expression vectors containing the specific 3'-UTR region of predicted genes to accomplished Dual-Luciferase Reporter assays. (Fig 9)

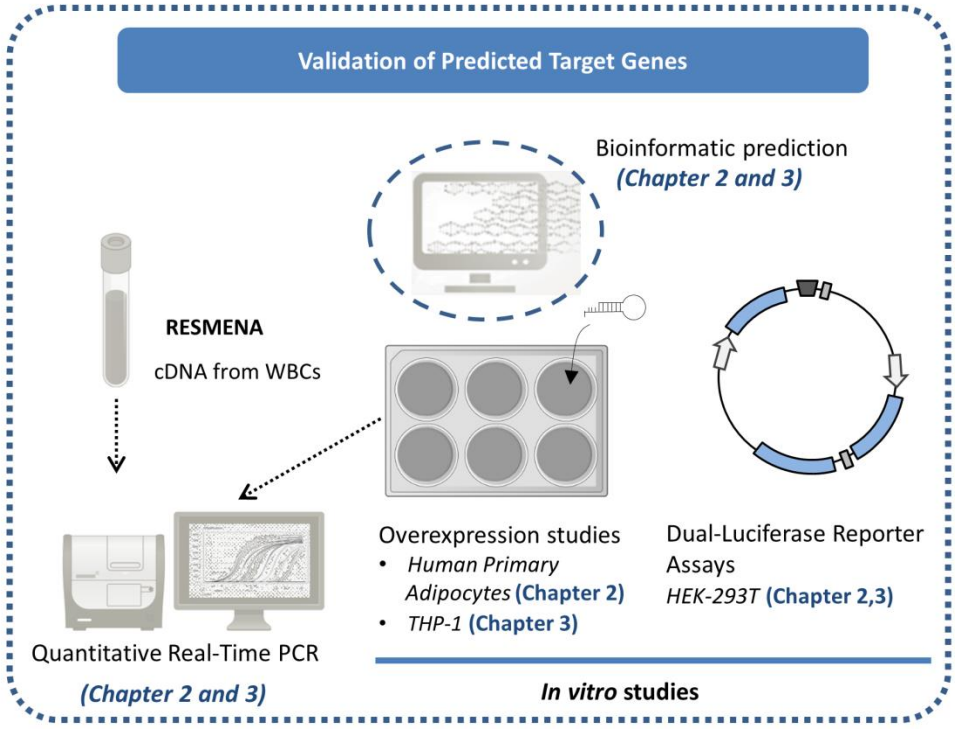

Fig 9. Experimental design for the validation of predicted target genes

mRNA expression Levels in WBCs (Chapters 2, 3)

Expression levels of predicted genes were evaluated in WBCs from RESMENA subjects by performing qPCRs with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad) or with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), using commercial miScript Primer Assays (QIAGEN) or Taqman probes (Applied Biosystems).

miRNA Overexpression in *in vitro* Experiments (Chapters 2, 3)

To ascertain if miRNAs might affect endogenous mRNA levels of predicted target genes, miRNA overexpression experiments were performed by transfecting synthetic miRNA mimics into different cellular models. Primary adipocyte cultures (Chapter 2) were obtained from subcutaneous white adipose tissue (WAT) from lean and overweight subjects undergoing cosmetic liposuction at the Karolinska University Hospital. Stromal vascular fraction was isolated and differentiated into mature adipocytes for 12 days as described elsewhere (Pettersson et al., 2013). On

the other hand, human monocytes from the leukemia cell line THP-1 (Chapter 3) were differentiated and activated to M1-phenotype macrophages as follows: 50 ng/mL of Phorbol 12-myristate 13-acetate (TPA) (Sigma-Aldrich) were added for 48 h for differentiating monocytes into macrophage-like cells, and 100 ng/mL of lipopolysaccharide (LPS) (Invitrogen) were added afterwards for 24 h to activate macrophages to a M1-phenotype. Adipocytes and macrophages were maintained at 37°C in a 5% carbon dioxide humidified atmosphere. Hiperfect Reagent (QIAGEN) was used to transfect miRNA mimics (at doses of 20/40 nM) in adipocytes, and Lipofectamine 2000 (Applied Biosystems) was used to transfect activated macrophages. 48h (in the case of adipocytes) and 24h (in the case of macrophages) after transfection, RNA was extracted and reverse transcribed. mRNA expression levels of predicted target genes were evaluated by qPCR and by using commercial miScript Primer Assays (QIAGEN) or Taqman probes (Applied Biosystems).

Dual Luciferase Reporter Vectors (Chapters 2, 3)

To determine if miRNAs regulate their target genes by binding to its 3'-UTR, specific expression vectors were constructed cloning the particular 3'-UTR of each gene into the pmiR-GLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) immediately downstream of the firefly luciferase (*luc*) gene. This process is explained in detail in Fig 10.

1) 3'-UTR binding sites of each miRNA were examined using TargetScan software (www.targetscan.org). After selection of regions of interest (sites where miRNAs bound to their predictive target-genes), primers amplifying particular sequences were designed using FastPCR software. Restriction sites for the enzymes *NheI* and *XbaI* (New England Biolabs) were added at the 5' end of forward and reverse primers to facilitate digestion and ligation into the vector.

2) Human genomic DNA was amplified from a pool using designed primers to obtain the desired sequences with restriction enzymes sites in 5' and 3' terminus. PCRs were carried out using *Taq* DNA polymerase (New England BioLabs) and were set up by testing different annealing temperatures and elongation times (Table 2).

Table 2: Conditions used to amplify the desired 3'UTR products

PCR Product	Annealing T°	Elongation Time
CD40	63° C	1'
TP53	63° C	1'
***GSK3B*-1185-1**	61.9° C	1'
***GSK3B*-548q**	61.9° C	1'

3) To analyze the PCR products, agarose gel electrophoresis was used. If nonspecific products were not detected, PCR products were purified using QIAquick PCR Purification Kit (QIAGEN).

4) *XbaI* and *NheI* restriction enzymes (New England BioLabs) were used to digest the PCR products (containing the specific miRNA 3'-UTR binding site) and to open the pmiR-GLO Dual-Luciferase miRNA Target Expression Vector, generating in both cases cohesive endings. Digestions were performed at 37°C over-night (O/N) and enzymes were inactivated at 65°C during 20 minutes. Digested products were purified using QIAquick PCR Purification Kit (QIAGEN). Afterwards the vector was treated with the alkaline phosphatase calf intestinal (CIP) (New Englands BioLabs) at 37°C during 1 hour to avoid plasmid recircularization. Plasmid was purified again with QIAquick PCR Purification Kit after CIP treatment. Ligations of digested PCR products and open plasmids were carried out at 16°C O/N at different ratios (2:1, 3:1 and 5:1) using T4 DNA ligase (New England BioLabs).

5) XL1-Blue supercompetent bacteria (Agilent Technologies) were used in the transformation step. 30 µL of the supercompetent bacteria were incubated with 10 uL of the ligation product on ice during 30 minutes. A heat shock was then applied for 45 seconds at 42°C and bacteria were put again on ice during 5 additional minutes. Transformation reactions were performed in 950 µL of lysogeni broth (LB) at 37°C for 1 hour with shaking at 240 rpm. Once incubation period was over, bacterial cultures were centrifuged during 10 minutes at 1000 gs and resuspended in 150 µL of LB. Then, each transformation reaction was plated on

LB-agar plates supplemented with ampicillin to a final concentration of 50 μg/mL and incubated O/N at 37°C.

6) To check ligation and transformation efficiency, colonies were selected and subsequently cultured in 500 μL of LB medium supplemented with ampicillin at 37°C for 1 hour with shaking at 240 rpm. After incubation, 100 μL of the culture were lysed by applying a heat pulse of 100°C during 5 minutes. Next, 1 μL of the lysate was used to check correct ligation and orientation of products by PCR using a combination of primers hybridizing outside of the MCS of the plasmid and designed primers.

7) If colonies were positive, 200 μL of the incubation suspension were used to inoculate 5 mL of LB supplemented with ampicillin and cultured O/N at 37°C shaking at 240 rpm. Afterwards, a Miniprep (QIAGEN) was performed to obtain higher number of plasmid copies and to sequence the vector to avoid insert mutations.

8) Finally, HEK-293T cells were seeded at a density of 15,000 cells/well in 96-well plates for Dual Luciferase Assays. After 24 h, cells were co-transfected using 1.5 μL/well of Lipofectamine 2000 (Applied Biosystems) with either 0.25 μg of empty pmiR-GLO, pmiR-GLO containing the 3'-UTR of each target gene, or a combination of the later and 7.5 pmol of specific miRNA mimics. 24 h after incubation, firefly luciferase activity was then evaluated with a Dual-Luciferase Reporter Assay System (Promega). The bindings of miRNAs over their predictive target sites were considered validated when cells co-transfected with both the vector encoding the 3'-UTR of predicted site and the miRNA mimic, showed lower levels of firefly/Renilla activity.

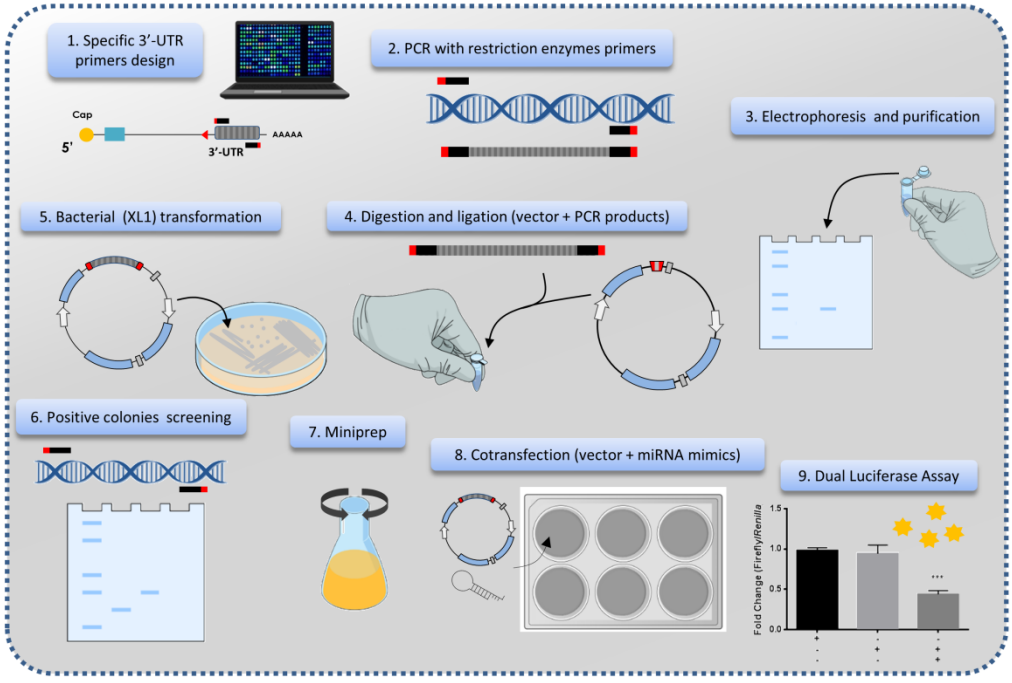

Fig 10. Explanation of expression vectors construction

Statistical Analyses

Data of both microarrays (methylation and expression) and miRNA-Seq were analyzed using Limma package in R. Corrections for multiple comparisons were carried out in all platforms by using the Benjamini-Hochberg procedure.

In Chapter 1, data are expressed as means ± SD, except as otherwise stated. Multiple linear regression models were performed to analyze the prediction of BMI-SDS (outcome) for selected CpG sites of miRNAs. A lineal regression analysis was performed to explain the variation of related variables. The associations were calculated with Pearson's correlation coefficients. For stringency, a p-value < 0.05 was considered significant and took into account for further analyses.

In Chapters 2 and 3, human data are presented as mean ± SD and data from cells are presented as mean ± SEM. Differences between groups were calculated using the Student's t- or ANOVA tests when indicated. Volcano plots were created by plotting the negative log10 of the p-value (y axis) and the mean differences between groups

for each variable (x axis). Statistical analyses and graphics were performed using SPSS Statistics 19 software (IBM Corporation, Somers, NY, USA) and GraphPad Prism version 6.0C (La Jolla, CA, USA).

Summary of the Experimental Approach

A summary of the experimental approach of the present thesis is shown in Fig 11. It is separated by chapters, indicating the specific cohort used (GENOI or RESMENA), the –omic technologies used for identified candidate miRNAs potentially implicated in obesity (methylation/expression microarrays or miRNAs-Seq Technology), as well as the procedure to validate microarrays and miRNA-Seq data (MassArray EpiTYPER or qPCR) and target genes predictions (qPCR, overexpression studies, or Dual Luciferase Reporter Assays).

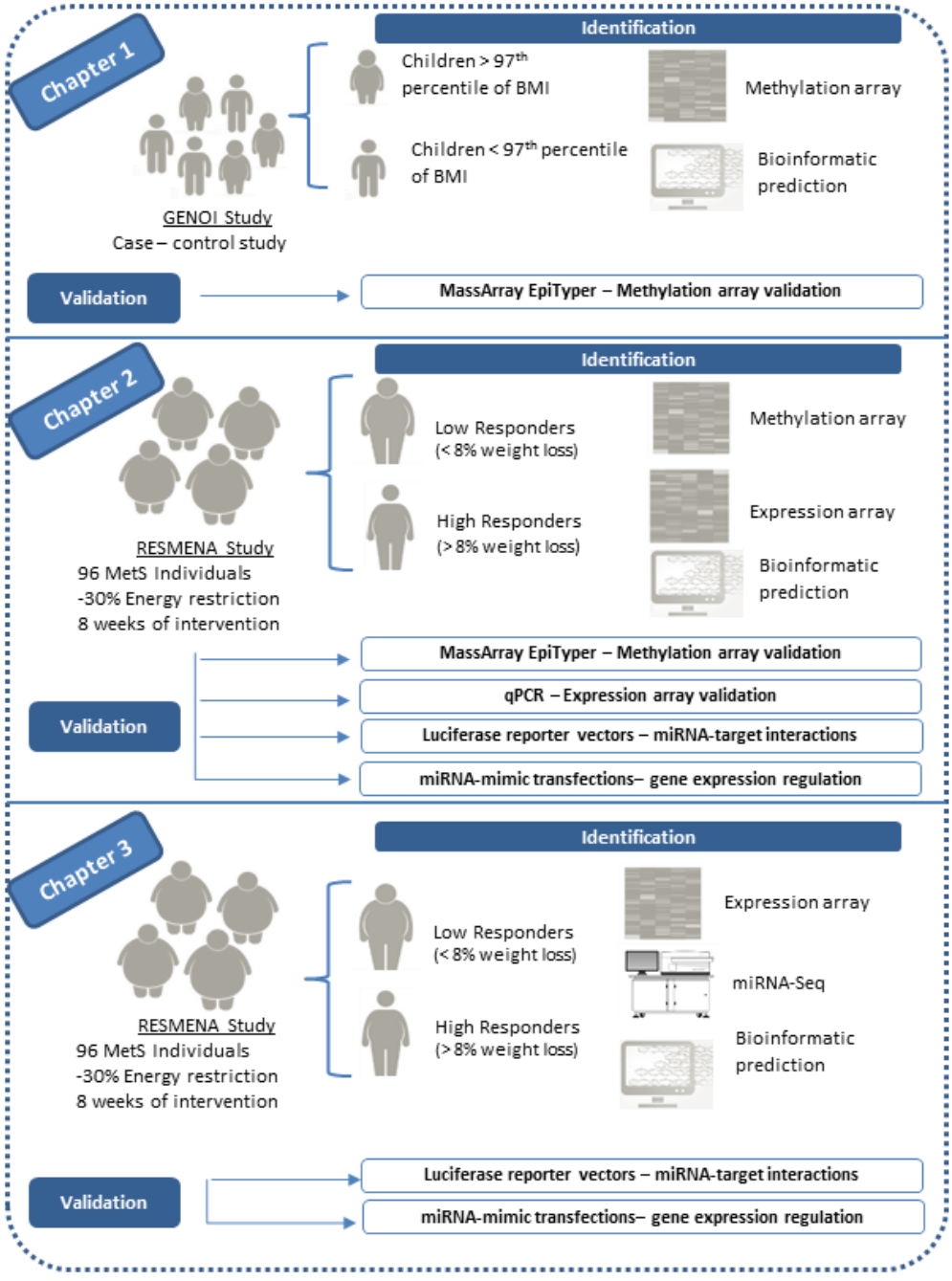

Fig 11. General experimental approaches used in each chapter

RESULTS

Chapter 1

DNA methylation of miRNA coding sequences putatively associated with childhood obesity

M.L. Mansego [1,2,3†], M. Garcia-Lacarte [1,2 †], F.I. Milagro [1,2,3], A. Marti [1,3,4],
J. A. Martinez [1,2,3,4] and GENOI members [‡]

[1] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.

[2] Nutrition Research Center, University of Navarra, Pamplona, Spain.

[3] CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Institute of Health, Madrid, Spain.

[4] IdiSNA, Navarra's, Health Research Institute, Pamplona, Spain.

[†] The authors contributed equally to this work.

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Supplementary Data Chapter 1

Supplementary table 1. Selected 2783 CpG sites of miRNAs included in the HumanMethylation450 BeadChips.

IlmnID	Symbol	CHR	MAPINFO	SourceSeq	Strand
cg00002531	miR593	7	127721794	CGGCAGCACCTCCATGCCTGAGCCCAAGCCAGGGCTCATGTGAAGGCTCC	R
cg00009001	miR1178	12	120151890	CAGGTGGAGGTGATTTTTTTCTAACTCATCATATAGTGCATTCATTTTTCG	F
cg00014998	miR10B	2	177014962	CGACGCAGCCACCCGCACTTTCTGAGACAGTTCAGGTAGCCTAATGGGCT	R
cg00030523	miR1251	12	97885353	CACAATTTTAAAATGCAATACCTCTTCCCAACTGGTCCAGGTTAAGCTCG	F
cg00034556	miR1977	1	566731	CGCCATATCGGGGGGCACCGATTATTAGGGCAACTAGTCAGTTGCCAAAGC	R
cg00047185	miR526B	19	54196849	CGGAGTATTCTCTGTGTTTAGAAGAGTATTTTGTGTGTACATAATATATA	R
cg00054301	miR328	16	67237548	CGGCTCTGTGGCCACCCCCAGTTCTACCTGTGGACAGATGGGGTCAGTGCC	F
cg00057966	miR142	17	56408694	GGGCTCTTTGGAGCAGGAGTCAGGAGGCCTGGGCAGCCTGAACAGTACACG	F
cg00066270	miR548F1	1	186265270	CGTCTGGGCCCTTTTCCAATAAATGAGGAAAGTTTGATTTCATAGGTTGT	F
cg00074313	miR548N	2	179371342	CGCCCTTGTGATATGTTAATACCAGATTGCTGTTTACACTAATTCCATAT	F
cg00089864	miR1272	15	65055305	TTTCCTATGGGAGGCGTAAGAGCAACCTGCTGGTGCCAAGCCTAAGCGGG	F
cg00094751	miR106B;miR93	7	99692902	GGAATTTTCCACTTAAAAGCTGATCTGAGGCTCTGGACATGTCTGTAGCG	R
cg00096742	miR770	14	101318357	CTCTCAATTCCTATTTGGGACATATAAAAACACTGGATTCTTTAACAAGCG	R
cg00101104	miR521-2	19	54219607	TGCTCTTCTCCAGAAATCTTACCAATTCTCCTGCTTAAATATAAGCTACG	R
cg00104570	miR548G	3	99536649	TGCTTCTGCCTGTCGGCGGCCTGTGTGGAAACTGCGACGAGTCTGGGAGCG	R
cg00109776	miR518E	19	54232332	CGCACAAACAACCTCCAGGATAGACCACACAGGTTAGGCCATAAAAGCTCCT	R
cg00111333	miR181B1;miR181A1	1	198828249	CGCTGTCGGTGAGTTTGGAATTAAAATCAAAACCATCGACCGTTGATTGT	R
cg00124993	miR886	5	135416412	GATGCGGGCGGGAGGAACTGAGAGTCCCTCCAGGACACGTTAGCAGGACG	F
cg00128812	miR661	8	145019377	AGGCGGCAGGCCTGAGCCCTGGTTTCGGGCTGCCTGGGTCTCTGGCCTGCG	F
cg00135888	miR130A	11	57407543	GGACAGAATTCCAGTTGGGAGGCTCTTCCTCTGGGCTTCCCTCTAGGCTCG	F
cg00138641	miR193B	16	14397074	CCTTTTTCTTTTTTTTTCTAACTTCTTTTTCCTAACTTGGTGTTGTCTCG	F
cg00152708	miR429	1	1104316	TCTGCAGACCCAGGAGGGATCAGGGCAGCTGCAGGAGGTGGCAGGAGCCG	R
cg00158530	miR140	16	69966973	CGTGGTGACCTCCTCTCCAGGCTCTGCTTGGTGGGCTTCTGGTGTGGCCG	R
cg00175487	miR380;miR299	14	101490186	CGCTTCTTGGTATCCAGCCCAGCCATCCAAACCTCCCTGGAGAAAGCCTG	F
cg00176888	miR142	17	56408804	GGAGGCAAGTCTGGCGCCATGCTGAGTCACGCCCCACAAGGCCCAGGGCG	F
cg00182416	miR516A2	19	54263917	CGGTACTTGTGATTTAGGTTCAAATTTTGATGAGTTTTTTATGACCAAGCC	F
cg00190642	miR1256	X	103268431	CGGGGCAAAAGCGCATCCGCCCTTCTCAGCTGATCCATGCCACACTGTGA	F
cg00210994	miR548G	3	99594893	CGCTCCACCAAGGGGCACTGCTGTCAGACAGCCAGGTAGCAAGTCTGTGG	R
cg00213281	miR1978	2	149639822	AGCTCTGCCACTTCTTATCTGTGAGAACTTGGGCAAATTCCTAAACCACG	R
cg00213334	miR548G	3	99341589	CGGTCAGAGAGATTCCGCCTTGCTGACTTTAAAGATGGAGAAAAGGTTCC	R

Supplementary table 2. Primers, amplified regions and amplicon's information for the Sequenom MassArray® EpiTYPER™

Assay name	Primer sequences	Screened CpGs/ Amplicon length
miR412 SME	F- TTAGTGTTTATGGTTTTTGAAGGGA	8/ 275 bp
cg21230021	R- AAAAACACCCAAAAAATACCTCCTA	
miR216 SME	F- TAGAGATTATTGTGAGGGATTGTATG	4/248 bp
cg24343835	R- CATATACCAAAAATCTATAAATTCAACAAA	
miR1306 SME	F- TTTTGGTTTATTGTTAGGGTTTTTTT	4/209 bp
cg21285564	R- AAAACCTAAATACCACCTACAACCC	
miR1203 SME	F- TTGTTGTGGTTTGAGTTGTATTTTG	3/381 bp
cg11210138	R- TCCACCATACCTTTCCTAATCTAAA	

SME: Sequenom MassArray® EpiTYPER™ MS-HRM: Methylation-sensitive high-resolution melting.

Supplementary table 3. Anthropometric and biochemical measures in children from the GENOI population (VS) according to obesity status (BMI above or below the 97 percentile adjusted for age and sex).

Variables	All (n=95)	Obese (n=48)	Non-obese (n=47)	p-value
Age (years)	10.6 (0.8)	10.5 (0.7)	10.6 (1.0)	0.481
Sex (M/F)	48/47	24/24	24/23	0.540
Body weight (kg)	49.3 (14.4)	60.2 (11.3)	38.3 (6.8)	<0.001
BMI (kg/m^2)	22.8 (5.2)	27.2 (3.4)	18.4 (1.9)	<0.001
BMI-SDS (kg/m^2)	2.2 (2.3)	4.0 (1.6)	0.3 (0.8)	<0.001
Fat mass (%)	27.4 (11.5)	36.1 (7.0)	17.9 (7.3)	<0.001
Glucose (mg/dL)	83.4 (15.0)	87.4 (14.9)	79.1 (14.1)	0.007
TC (mg/dL)	165.8 (32.7)	164.9 (33.9)	166.7 (31.9)	0.796
HDL-c (mg/dL)	57.0 (14.5)	49.9 (12.3)	64.5 (12.8)	<0.001
Triglycerides (mg/dL)	67.3 (28.7)	73.4 (34.2)	60.8 (19.5)	0.035

Data are expressed as mean (SD). Abbreviations: BMI, body mass index; SDS, standard deviation score; TC, total cholesterol; HDL-c, high density lipoprotein–cholesterol. p-value from ANCOVA test.

Supplementary table 4. Obesity-related biological networks and pathways according to the miRNA coding regions differentially methylated (by microarray) between obese and non-obese children.

KEGG pathway[1]	p-value	#miRNAs
TGF-beta signaling pathway (hsa04350)	2.75E-24	11
MAPK signaling pathway (hsa04010)	8.02E-18	13
Wnt signaling pathway (hsa04310)	1.24E-16	13
Neurotrophin signaling pathway (hsa04722)	3.92E-11	14
Insulin signaling pathway (hsa04910)	1.46E-10	14
Notch signaling pathway (hsa04330)	3.38E-09	10
Focal adhesion (hsa04510)	5.26E-08	14
Jak-STAT signaling pathway (hsa04630)	3.98E-07	11
Aldosterone-regulated sodium reabsorption (hsa04960)	4.85E-07	8
Circadian rhythm (hsa04710)	1.70E-06	7
mTOR signaling pathway (hsa04150)	3.52E-05	9
Gap junction (hsa04540)	6.44E-05	12
HIF-1 signaling pathway (hsa04066)	7.76E-05	10
Leukocyte transendothelial migration (hsa04670)	0.0001243	10
Adipocytokine signaling pathway (hsa04920)	0.0001896	10
Retrograde endocannabinoid signaling(hsa04723)	0.0017943	12
Type II diabetes mellitus (hsa04930)	0.0027992	10
GnRH signaling pathway (hsa04912)	0.0051178	14
Salivary secretion (hsa04970)	0.0126147	11

[1] Kyoto Encyclopedia of Genes and Genomes. Highlighted in bold, pathways where the selected miRNAs for further validation by Sequenom MassArray® EpiTYPER™ (miR-1203, miR-412, miR-216A and miR-1306) were involved.

Supplementary table 5. Multiple regression analyses showing the independent contributions of variables of studied domains[a] to the variation of BMI-SDS in the Identification sample (IS). Data are ranked by B coefficient.

BMI-SDS as dependent	B coefficient	95% CI	R^2	p-val
cg08316488_miR2114	0.882	(-0.016; 0.585)	0.302	*0.027*
cg11210138_miR1203	**0.593**	**(0.066; 0.343)**	**0.439**	**0.00**
cg21285564_miR1306	**0.417**	**(0.003; 0.469)**	**0.318**	**0.02**
cg27069588_miR892A	0.413	(0.031; 0.491)	0.351	0.015
cg07059402_miR659	0.337	(-0.023; 0.346)	0.284	0.033
cg11755251_miR557	0.315	(-0.034; 0.274)	0.260	0.044
cg00944580_miR548D2	-0.308	(-0.389; 0.046)	0.263	0.042
cg06606539_miR572	-0.326	(-0.322; 0.028)	0.275	0.037
cg02206323_miR1180	-0.341	(-0.333; 0.033)	0.270	0.039
cg03918703_miR208B	-0.356	(-0.285; 0.012)	0.295	0.029
cg15554966_miR519E	-0.374	(-0.143; 0.002)	0.307	0.025
cg04735660_miR488	-0.394	(-0.166; 0.006)	0.243	0.067
cg15787712_miR24_others	-0.468	(-0.535; -0.072)	0.397	0.008
cg27337176_miR1	-0.510	(-0.376; -0.040)	0.377	0.010
cg21230021_miR412_others	**-0.517**	**(-0.382; -0.059)**	**0.410**	**0.00**
cg24343835_miR216A	**-0.538**	**(-0.581; -0.082)**	**0.401**	**0.00**

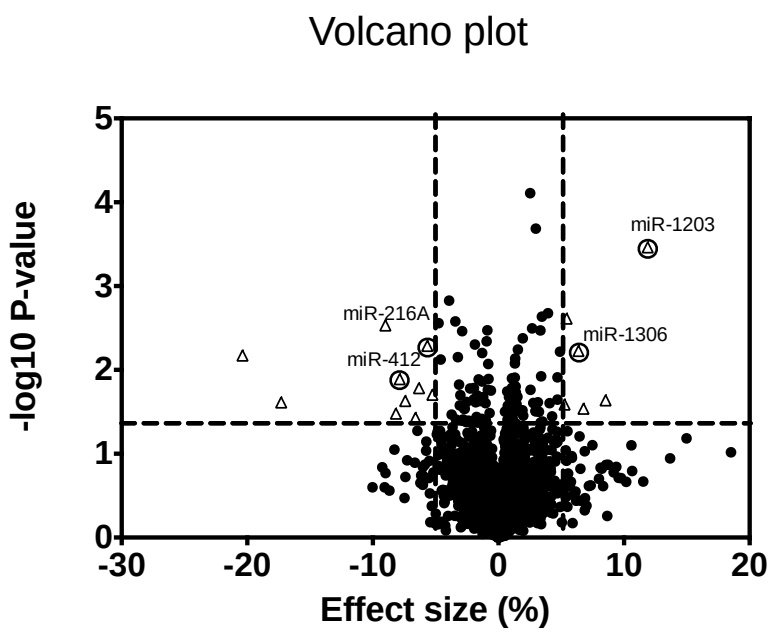
Adjusted R^2 and all independent variables included in each model are shown in the table. Bold and underlined style highlights those miRNAs selected for further validation. In bold the 4 CpGs finally selected for validation. In underlined style those for which we were not able to design primers. Domains are DNA methylation (%) in miRNA coding region, age, gender and batch effect. CI: Confidence interval.

[a] DNA methylation, age, gender and batch effect

Supplementary table 6. Association and comparison between the CpGs methylation levels measured by Sequenom MassArray® EpiTyper™ or microarray in the VS (n=95).

	Sequenom MassArray® EpiTyper™			**Microarray**		
CpG sites by Sequenom	**Obese (n=48)**	**Non-Obese (n=47)**	**p-value**	**Obese (n=12)**	**Non-Obese (n=12)**	**p-value**
MIR412_CG1	67.3±4.8	71.1±5.9	0.001	--	--	--
MIR412_CG2	**84.0±1.9**	**85.6±3.1**	**0.004**	73.9±1.9	81.7±1.9	0.013
MIR412_CG3	95.8±1.5	96.4±2.1	0.125	--	--	--
MIR412_CG4	77.1±9.5	80.3±10.5	0.127	--	--	--
MIR412_CG5	97.0±2.1	96.3±1.8	0.095	--	--	--
MIR412_CG6.7	87.7±1.6	88.6±2.1	0.014	--	--	--
MIR412_CG8	91.1±1.1	91.4±1.8	0.287	--	--	--
MIR216A_CG1	70.8±5.2	68.8±3.7	0.038	--	--	--
MIR216A_CG2	**81.5±3.5**	**85.6±5.9**	**<0.0001**	78.1±1.2	83.8±1.2	0.0052
MIR216A_CG4	96.6±3.3	97.4±4.6	0.296	--	--	--
MIR1306_CG1	59.8±10.9	49.2±12.9	<0.0001	--	--	--
MIR1306_CG2	86.7±1.7	87.3±1.7	0.073	--	--	--
MIR1306_CG3	**96.4±0.7**	**96.1±0.9**	**0.058**	75.3±1.4	68.9±1.4	0.0059
MIR1306_CG4	90.5±2.1	90.2±2.4	0.516	--	--	--
MIR1203_CG1	14.3±5.0	10.6±6.5	0.003	--	--	--
MIR1203_CG2	**55.5±10.8**	**48.2±11.9**	**0.002**	50.9±1.9	39.0±1.9	0.00034
MIR1203_CG3	39.4±10.9	32.9±11.7	0.006	--	--	--

Data are expressed as mean (SD). Bold style highlights those miRNAs selected for further validation by Sequenom MassArray® EpiTyper™. p-value from ANCOVA



Supplementary Figure 1. Identification of genes differentially methylated between obese and non-obese children in the IS. Volcano plot of log of raw p-values versus mean methylation differences (size effect) between groups. Sixteen differentially methylated CpG sites are shown in triangles. Six miRNA probes (6 miRNAs) hypermethylated in obese children are highlighted in the upper right corner. Ten miRNA probes (10 miRNAs) hypomethylated in obese samples are highlighted in the upper left corner. Probes not differentially methylated (raw p>0.05) are shown in black circles. Selected miRNAs for further validation by Sequenom MassArray® EpiTYPER™ are rounded.

Chapter 2

Implication of miR-612 and miR-1976 in the regulation of *TP53* and *CD40* and their relationship in the response to specific weight-loss diets

Marcos Garcia-Lacarte [1,2], J. Alfredo Martinez [1,2,3,4], M. Angeles Zulet [1,2,,3,4], Fermin I. Milagro [1,2,3,4],

[1] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.

[2] Center for Nutrition Research, University of Navarra, Pamplona, Spain.

[3] CIBERobn, Physiopathology of Obesity and Nutrition, Center of Biomedical Research Network, ISCIII Madrid, Spain.

[4] IdiSNA, Navarra's, Health Research Institute, Pamplona, Spain.

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RESEARCH ARTICLE

Implication of miR-612 and miR-1976 in the regulation of *TP53* and *CD40* and their relationship in the response to specific weight-loss diets

Marcos Garcia-Lacarte[1,2], J. Alfredo Martinez[1,2,3,4]*, M. Angeles Zulet[1,2,3,4], Fermin I. Milagro[1,2,3,4]

1 Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain, **2** Center for Nutrition Research, University of Navarra, Pamplona, Spain, **3** CIBERobn, Physiopathology of Obesity and Nutrition, Center of Biomedical Research Network, ISCIII Madrid, Spain, **4** IdiSNA, Navarra's Health Research Institute, Pamplona, Spain

* jalfmtz@unav.es

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Abstract

Background

Non-coding RNAs (i.e., miRNAs) play a role in the development of obesity and related comorbidities and the regulation of body weight.

Objective

To identify candidate miRNA biomarkers throughout omics approaches in order to predict the response to specific weight-loss dietary treatments.

Design

Genomic DNA and cDNA isolated from white blood cells of a subset from the RESMENA nutritional intervention study (Low-responders (LR) vs High-responders (HR)) was hybridized in Infinium Human Methylation450 BeadChip and in Illumina Human HT-12 v4 gene expression BeadChips arrays respectively. A bioinformatic prediction of putative target sites of selected miRNAs was performed by applying miRBase algorithms. HEK-293T cells were co-transfected with expression vectors containing the 3'-UTR of candidate genes to validate the binding of miRNAs to its target sites.

Results

134 miRNAs were differentially methylated between HR and LR in the methylation array, whereas 44 miRNAs were differentially expressed between both groups in the expression array. Specifically, miR-1237, miR-1976, miR-642, miR-636, miR-612 and miR-193B were simultaneously hypomethylated and overexpressed in HR. miR-612 and miR-1976 showed greatest differences in methylation and expression levels, respectively. The bioinformatic prediction revealed that *TP53* was a putative target gene of miR-612 and *CD40* of miR-

analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

1976. Moreover, *TP53* was downregulated in the expression array when comparing HR vs LR expression levels adjusted by sex, diet, age and baseline weight, and *CD40* showed a statistical trend. Furthermore, gene expression levels of *TP53* and *CD40* in white blood cells, when measured by qPCR, were also downregulated in HR. Finally, miR-612 and miR-1976 potently repressed *TP53* and *CD40* respectively by targeting its 3'-UTR regions.

Conclusion

miR-612 and miR-1976 levels could be prospective biomarkers of response to specific weight-loss diets and might regulate the gene expression of *TP53* and *CD40*.

Introduction

The rates of obesity incidence have doubled in many countries since 80's [1]. This growing prevalence and the related disease burdens highlight the need to understand not only the involved process but also to identify and evaluate biomarkers to address this problem. In the last decades, several types of biomarkers have been investigated in relation to their potential application in cardiovascular disease, infections, immunological and genetic disorders, and cancer diagnosis and management [2]. Concerning obesity, different biomarkers of response to different dietary approaches have been identified [3–5].

MicroRNAs (miRNAs) are non-coding RNAs (ncRNAs) of 18–25 nucleotides in length, that bind to a 3'-UTR target mRNA resulting usually in post-transcriptional regulation of gene expression [6] via transcript degradation (when the complementarity is nearly perfect) and/or inhibition of translation or deadenylation (when there are some mismatches in the binding).

On the other hand, miRNAs isolated from white blood cells [7] or directly from the circulation [8] are a good source of biomarkers, and their implication in obesity-related diseases has been well documented [9]. The identification of reliable biomarkers of response to anti-obesity treatments is of crucial importance in order to boost motivation, increase weight loss and maintenance success, and save time and money [10]. In this context, the use of biomarkers that predict the efficacy of weight loss treatments is considered a milestone in the design of precision nutrition strategies against obesity and related comorbidities [11].

In this study, we aimed to identify miRNAs from blood white cells that could be predictive of the outcome of a specific weight-loss intervention. For this purpose, in order to evaluate the interaction between dietary patterns targeting obesity and related transcriptomic biomarkers (miRNAs), we used a miRNAomic approach including methylation and expression microarrays.

Materials and methods

Subjects and study protocol

The current study was conducted in a subsample of the RESMENA (Metabolic Syndrome Reduction in Navarra) nutritional intervention trial. In this study, 96 adults with metabolic syndrome underwent two energy-restricted dietary patterns (AHA diet as reference diet, and RESMENA diet as intervention diet) during 8 weeks, both with an energy restriction of -30% of the studied requirements [12]. As no differences were found neither in anthropometric or biochemical variables between groups after the intervention, both dietary groups were merged for further analyses to increase the statistical power of the study, classifying subjects in "high-

responders" (HR) when weight loss was ≥8%, and "low responders" (LR) when weight loss was ≤8%, as previously published [4].

The study was performed following the CONSORT 2010 guidelines and properly approved by the Ethics Committee of the University of Navarra (065/2009) and registered at www.clinicaltrials.gov (NTC01087086). All participants provided written informed consent for participation.

Microarray analyses

Genomic DNA isolated from white blood cells of a subpopulation (31 LR vs 16 HR) of the RESMENA cohort was hybridized in an Infinium HumanMethylation450 BeadChip array (Illumina HM450K). Also, RNA from the same cells (14 LR vs 10 HR) was reverse transcribed and hybridized in an Illumina Human HT-12 v4 gene expression BeadChip array. Microarray data were analysed using Limma package in R [13]. Corrections for multiple comparisons were carried out in both microarrays (expression and methylation) by using the Benjamini-Hochberg procedure.

Bioinformatic study

A bioinformatic study of putative target sites of selected miRNAs was performed by applying miRBase algorithms (www.mirbase.org). For each miRNA, miRBase provides references in literature, genomic coordinates and links to databases of predicted and validated miRNA target sites such as DIANA-microT, microRNA.org, miRDB, RNA22, TargetMiner and TargetScan [14].

MassArray Epityper validation

In order to validate the results of the methylation microarray, miR-612 methylation levels of 47 subjects selected from the subpopulation sample were analyzed by MassArray EpiTyp0065r (Sequenom, San Diego, CA, USA) after designing specific primers encompassing 6 CpGs sites (F: GTTTTATGGTAGTGGGAAGGGATTT; R: AATAAAACCCAAACAACAAACAATC). This method has been previously applied to validate methylation microarray data [15].

Luciferase reporter constructs

To verify if selected miRNAs regulate the 3'-UTR of the two predicted target genes, expression vectors containing the 3'-UTR region of each gene provided by the bioinformatic prediction were designed. To amplify the 3'-UTR region of *TP53* and *CD40* genes, specific primers incorporating *NheI* and *XbaI* restriction enzymes sites were designed. The PCR products were subsequently cloned downstream of the firefly luciferase (*luc*) gene in the pmiR-GLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) (S1 Fig). Primer sequences are shown in Table 1.

Table 1. Primer sequences used to amplify the 3'-UTR region of target genes and the amplicon length.

CD40-F	5′-TTAGCTAGCACTTTACATGGATGCCAACC-3′	766 bp
CD40-R	5′-TTATCTAGACACCACTCTTCGAGCTGT-3′	
TP53-F	5′-TTAGCTAGCGCCAAACCCTGTCTGACAA-3′	1010 bp
TP53-R	5′-TTATCTAGAAACCCAGGTATCCTGCCA-3′	

F: Forward. R: Reverse. Underlined: *NheI* and *XbaI* target sites

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Cell culture

Human HEK-293T cells were purchased from the ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin at 37ºC in a 5% carbon dioxide humidified atmosphere.

Dual-luciferase reporter assays

To assess miRNA-target interactions, HEK-293T cells were seeded in 96-well plates at a density of 20000 cells per well. After 8 h, cells were transiently co-transfected with either 0.25 μg of empty pmiR-GLO, pmiR-GLO-*TP53*-3'-UTR, or pmiR-GLO-*CD40*-3'-UTR, and 7.5 pmol of miR-612 and miR-1976 mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Firefly and *Renilla* luciferase activities were evaluated 24 h after co-transfection using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized using *Renilla* luciferase activity. Determinations were carried out in three independent experiments, each assayed in triplicate.

RNA isolation and quantitative real-time PCR

RNA from white blood cells was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol. cDNA was synthesized from 0.5 ug of total RNA using random primers and MultiScribe^{TM} MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). For mature miR-1976, 20 ng of RNA were reverse transcribed by using a Taqman MicroRNA RT kit (Applied Biosystems) and miRNA-specific primer sets supplied by the manufacturer. Quantitative real time PCR (qPCR) was performed with the ABI prism 7900HT Sequence Detection System (Applied Biosystems) using Taqman probes. Both mRNAs and miRNAs relative expression was calculated with the $2^{-\Delta\Delta CT}$ method and normalized using *GAPDH* and U48 mature miRNA, respectively.

Statistical analysis

Differences between groups were calculated using the Student's t-test or an ANCOVA test when indicated. Data are presented as mean ± SEM. p-values less than 0.05 were defined as statistically significant. Volcano plots were created by plotting the negative $\log_{10}$ of the p-value (y axis) and the mean differences between groups for each variable (x axis). An effect size of ±1.5% in the methylation differences and an effect size of ±1% in the expression differences were considered of interest. Statistical analyses and graphics were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 6.0C (La Jolla, CA, USA).

Results

miR-612 and miR-1976 are hypomethylated and overexpressed in HR

When analyzing array data, 134 miRNAs differentially methylated (87 hypomethylated and 47 hypermethylated), and 44 miRNAs differentially expressed (10 downregulated and 34 upregulated) were identified when comparing HR and LR (S1 and S2 Tables). From them, miR-1237, miR-1976, miR-642, miR-636, miR-612 and miR-193B were simultaneously hypomethylated and overexpressed in HR (Fig 1A and 1B). miR-612 showed significant difference in expression levels (1.43% effect size; p = 0.019) and the greatest difference in methylation levels (10% effect size; p = 0.003). Likewise, miR-1976 showed also significant difference in methylation levels (3.24% effect size; p = 0.041) and greatest differences in expression levels (12% effect size; p = 0.043). Although nominal statistically differences in miR-612 and miR-1976 between HR and LR were found, they disappeared after correction for multiple comparisons

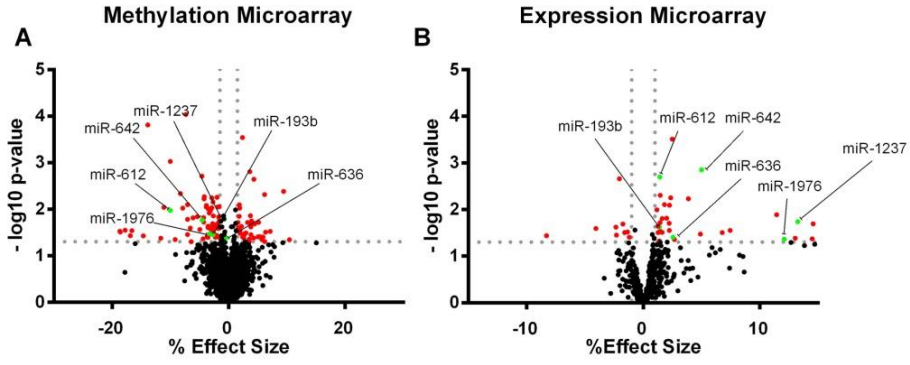

Fig 1. Identification of miR-612 and miR-1976 in the methylation and expression microarrays. A) Volcano plot of miRNAs differentially methylated between HR and LR. B) Volcano plot of miRNAs differentially expressed between HR and LR.

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(Benjamini-Hochberg). Nevertheless, as these miRNAs showed notable differences in both methylation and expression microarrays, they were selected for further evaluation.

TP53 is a putative target gene of miR-612

To select putative miRNAs implicated in the response to the diet, a bioinformatic study using miRBase algorithms assigning p-values to putative target binding sites of those miRNAs simultaneously hypomethylated and overexpressed in arrays was carried out. We then focused on obesity-related genes and filtered the target sites of each miRNA. We noted that *TP53* was predicted to be regulated by miR-612. EpiTyper analysis of the DNA methylation levels of miR-612 in white blood cells showed a positive correlation ($p < 0.001$) with respect to the DNA methylation levels measured by microarray (Fig 2A). Moreover, *TP53* was downregulated in the expression array ($p = 0.024$) when comparing HR vs LR expression levels adjusted by sex, diet, age and baseline weight (Fig 2B). Furthermore, *TP53* levels were also significantly lower in HR than in LR when measured by qPCR ($p = 0.04$), supporting the idea that miR-612 might affect *TP53* gene expression (Fig 2C). Finally, cells co-transfected with the pmiR-GLO-*TP53*-3'-UTR vector and miR-612 showed significantly lower firefly/*Renilla* activity ($p < 0.001$) than controls transfected only with the pmiR-GLO-*TP53*-3'-UTR vector (Fig 2D), confirming that *TP53* is a target gene of miR-612.

CD40 is a putative target gene of miR-1976

Similarly to miR-612, we carried out the same approach to identify putative obesity-related target genes of miR-1976. According to miRBase, *CD40* could be regulated by miR-1976. First, we found that miR-1976 expression profile in white blood cells was statistically different between HR and LR ($p = 0.019$) and also showed a positive correlation ($p = 0.012$) with miR-1976 expression levels measured by microarray (Fig 3A–3B). *CD40* expression level showed a trend toward significance ($p = 0.069$) when comparing HR vs LR adjusted by sex, diet, age and baseline weight (Fig 3C). Interestingly, gene expression levels of *CD40* were also significant negatively correlated ($p = 0.023$; $R = -0.505$) with miR-1976 expression profile (Fig 3D).

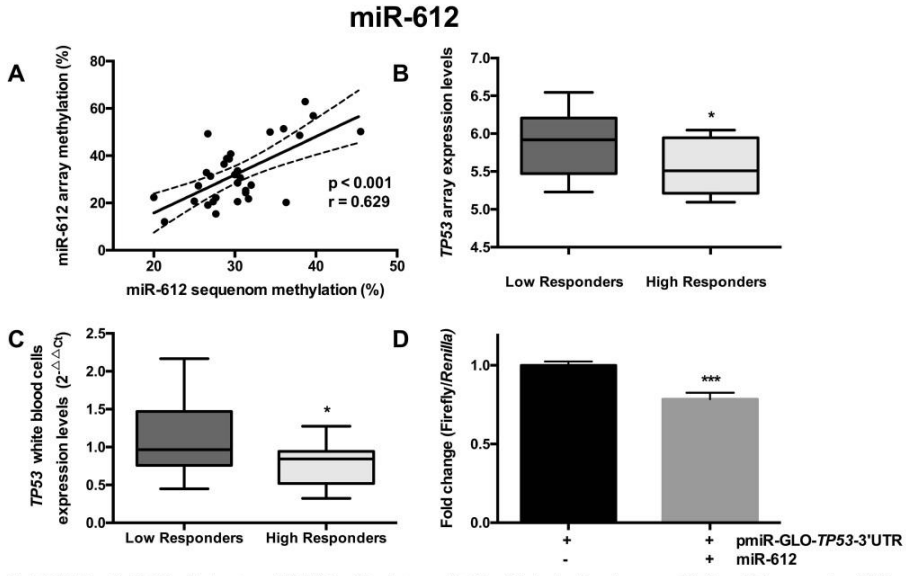

Fig 2. Validation of miR-612 and its target gene *TP53*. A) Correlation between miR-612 methylation levels by microarray and EpiTyper. B) Gene expression of *TP53* in HR and LR in the microarray. C) Validation of *TP53* expression profile in HR and LR white blood cells by qPCR, adjusted for age, sex, diet and baseline body weight. * $p < 0.05$ from ANCOVA test. D) Luciferase activity assay of pmiR-GLO-*TP53*-3'-UTR after co-transfection with miR-612. Normalized luciferase activity is presented as the mean ± SEM of three separate triplicate experiments. *** $p < 0.001$ from Student t-test.

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Furthermore, *CD40* expression in white blood cells, when measured by qPCR, were significantly lower in HR than in LR (p = 0.02) (Fig 3E), suggesting an interaction between miR-1976 and *CD40*. Similarly, cells co-transfected with the pmiR-GLO-*CD40*-3'-UTR construct and miR-1976 showed also a significantly reduction in firefly/*Renilla* activity (p = 0.014) than controls transfected only with the expression vector (Fig 3F), confirming that *CD40* is a target gene of miR-1976.

Discussion

In this study, a miRNAomic approach was performed in order to find transcriptomic biomarkers (especially miRNAs) associated to the response to specific weight loss diets. It is well established that miRNAs can regulate the expression of genes by binding to its target sites, usually resulting in degradation or translation inhibition [6]. miRNAs have been implicated in many development and diseases processes [16], including obesity and associated comorbidities [17]. For example, in type 2 diabetes, a well-known obesity-related disease, several miRNAs are down or upregulated [18]. miR-103 and miR-107 have been reported to contribute to adipose growth by accelerating adipocyte differentiation, and both are upregulated in obese individuals [19]. Moreover, several miRNAs have been defined as important modulators in human

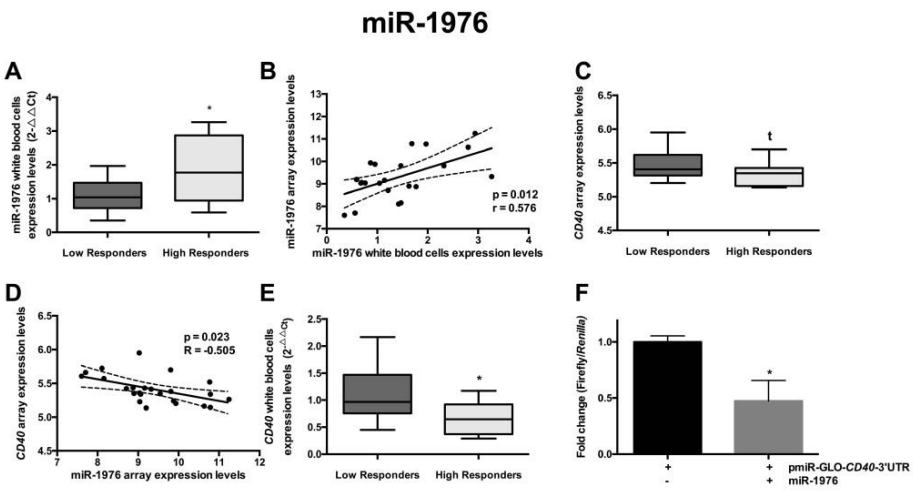

Fig 3. Validation of miR-1976 and its target gene *CD40*. A) miR-612 expression levels by qPCR in HR and LR, adjusted for age, sex, diet and baseline body weight. * $p < 0.05$ from ANCOVA test. B) Pearson's correlation between miR-1976 expression levels in the microarray and by qPCR, adjusted by sex, age, baseline body weight and diet. C) Gene expression of *CD40* in HR and LR in the microarray. t p = 0.069. D) Pearson's correlations between miR-1976 expression levels and its target gene (*CD40*) in the microarray, adjusted by sex, age, baseline body weight and diet. E) Validation of *CD40* expression profile in HR and LR white blood cells by qPCR adjusted for age, sex, diet and baseline body weight. * $p < 0.05$ from ANCOVA test. F) Luciferase activity assays of pmiR-GLO-*CD40*-3'-UTR after co-transfection with miR-1976. Normalized luciferase activity is presented as the mean ± SEM of three separate triplicate experiments. * $p < 0.05$ from Student t-test.

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obesity-related inflammation [20] or white adipose tissue inflammation [21], adipogenesis and adipose tissue signaling [22]. Additionally, changes in miRNA levels in plasma, serum, urine and other fluids have been associated with different diseases such as prostate cancer [23], bladder cancer [24] or cell carcinoma [25]. Thus, the identification of circulating miRNAs could serve as useful clinical biomarkers of diagnosis and prognosis of several diseases [26].

The present study has demonstrated that miR-612 and miR-1976 bind to *TP53* and *CD40* respectively and regulate their expression. *TP53* gene encodes p53 protein, a tumor suppressor whose deficiency enhances the initiation and/or progression of cancer [27]. Noticeably, Minamino et al. found that the expression of proinflammatory cytokines in mice decreased and insulin resistance improved after inhibition of p53 in adipose tissue, suggesting an important role of p53 in the regulation of obesity-related inflammation and insulin resistance [28]. Furthermore, they also evidenced that adipose tissue from subjects with diabetes showed higher levels of p53 protein compared with tissue from nondiabetic subjects, and that the expression of inflammatory cytokines was also significantly increased in adipose tissue.

Moreover, *ob/ob* mice show higher levels of p53 than wild type mice, and the disruption of p53 in *ob/ob* mice restores the expression of lipogenic enzymes [29]. Conversely, Molchadsky *et al*. showed that p53 may exert either a positive or negative effect according to the adipogenic differentiation program [30]. In our study, those subjects who responded better to the diet had lower expression of *TP53* than LR. It can be speculated that LR had higher inflammatory state than HR, and that this inflammation could trigger an activation of *TP53*.

In white blood cells, *TP53* is downregulated in obese subjects with type 2 diabetes after bariatric surgery, suggesting that *TP53* is upregulated in white blood cells of obese subjects [31]. Taking all these data together, our findings of decrease whole blood *TP53* mRNA in HR are consistent with these studies.

Several *TP53*-directed miRNAs have been experimentally and *in silico* identified. Some of these miRNAs that target *TP53* are miR-125b, miR-504, miR-1285, miR-92, miR-141, miR-380-5p, miR-15a, miR-16, miR-25, miR-30d, miR-200a, miR-453, miR-98, miR-19b, miR-518c and miR-638 [32]. Interestingly, miR-1285 has the same seed sequence as miR-612. In a previous study, Tian *et al.* tried to validate the binding of miR-612 to *TP53* and found that the luciferase activity of the p-LUC-p53-3'-UTR reporter did not change when transiently transfected with miR-612 [33]. However, in the present study, our data show that miR-612 binds to the 3'-UTR of *TP53* and that there exists a negative relationship between miR-612 levels and *TP53* expression (in blood and in the microarray), indicating that miR-612 could regulate *TP53* expression.

On the other hand, CD40 is a surface glycoprotein expressed in hematopoietic and nonhematopoietic cells, and has an important role in the ability to stimulate adaptive immunity [34]. Concerning obesity, *CD40* is highly expressed in leukocytes, adipocytes and the stromal cells of adipose tissue [35], and is involved in the regulation of adipose tissue metabolism [36]. Furthermore, soluble CD40L levels have been positively correlated with obesity and metabolic syndrome [37,38], and studies in rodents have shown that vascular inflammation and atherosclerosis could be prevented by CD40 deficiency [39,40]. There exists evidence that *CD40* could be regulated by miRNAs [41,42], even though to date there are no articles showing a miRNA-regulation of *CD40* in obese subjects.

To our knowledge, this is the first study to explore the relationships between miR-612 and miR-1976 and *TP53* and *CD40*, respectively, and was able to connect the expression of these miRNAs and genes with the response to a dietary intervention in obese subjects. However, further studies are needed to better understand the complex regulation of these miRNAs on their target genes. Transcriptomic biomarkers of the response to specific dietary strategies are a first step towards the personalization of weight-loss treatment, being miRNAs particularly relevant for this purpose.

Supporting information

S1 Fig. miR-612 and miR-1976 regulate the 3'-UTR region of *TP53* and *CD40*, respectively. A) Location of putative target sites for miR-612 and miR-1976 in the 3'-UTR of *TP53* and *CD40* predicted by TargetScan. B) miR-GLO Dual-Luciferase miRNA Target Expression Vector used to create the 3'-UTR expression vectors cloning the PCR product into the MCS. MCS: Multiple Cloning Site.
(PDF)

S1 Table. Significantly differentiated methylated miRNAs between HR and LR. In bold style, those miRNAs that were above the selected threshold of ±1.5%.
(DOCX)

S2 Table. Significantly differentiated expressed miRNAs between HR and LR. In bold style, those miRNAs that were above the selected threshold of ±1%.
(DOCX)

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Author Contributions

Data curation: Fermin I. Milagro.

Investigation: Marcos Garcia-Lacarte, Fermin I. Milagro.

Methodology: Marcos Garcia-Lacarte, M. Angeles Zulet.

Supervision: J. Alfredo Martinez, Fermin I. Milagro.

Writing – original draft: Marcos Garcia-Lacarte, J. Alfredo Martinez, Fermin I. Milagro.

Writing – review & editing: Marcos Garcia-Lacarte, J. Alfredo Martinez, M. Angeles Zulet, Fermin I. Milagro.

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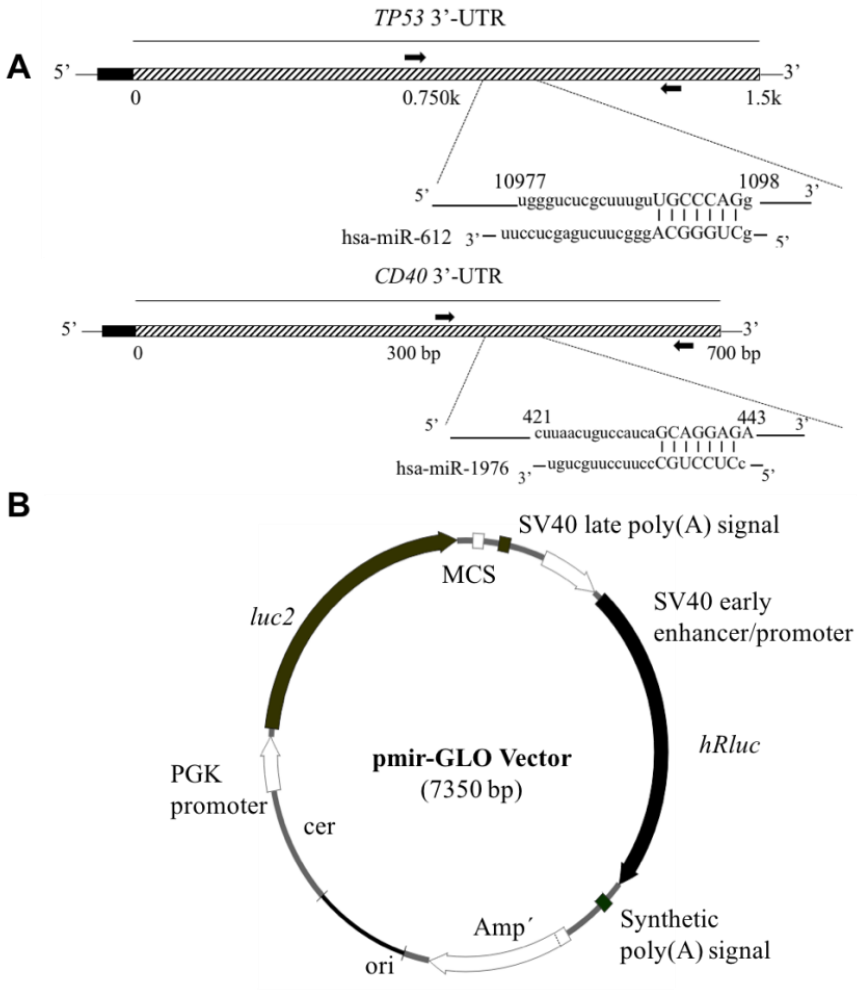
Supplementary Data Chapter 2



S1 Fig. miR-612 and miR-1976 regulate the 3'-UTR region of *TP53* and *CD40*, respectively. A) Location of putative target sites for miR-612 and miR-1976 in the 3'-UTR of *TP53* and *CD40* predicted by TargetScan. B) pmiR-GLO Dual-Luciferase miRNA Target Expression Vector used to create the 3'-UTR expression vectors cloning the PCR product into the MCS. MCS: Multiple Cloning Site.

S1 Table. Significantly differentiated methylated miRNAs between HR and LR. In bold style, those miRNAs that were above the selected threshold of ±1.5 %.

Name	% Effect Size	"-logP-value"	p-value
MIR505	-7.356	4.033	0.0001
MIR1284	-13.876	3.808	0.0002
MIR2116	2.371	3.540	0.0003
MIR548N	-10.024	3.026	0.0009
MIRLET7G	3.666	2.807	0.0016
MIR141	-4.585	2.710	0.0020
MIR548H4	4.360	2.644	0.0023
MIR548N	9.442	2.380	0.0042
MIR487B;MIR539	-8.283	2.333	0.0046
MIR1256	6.190	2.315	0.0048
MIR657;MIR338	-4.195	2.267	0.0054
MIR130A	-1.980	2.254	0.0056
MIRLET7A3;MIRLET7B	-2.880	2.242	0.0057
MIRLET7B	-4.224	2.221	0.0060
MIR525;MIR523	-2.688	2.157	0.0070
MIR647	-7.063	2.097	0.0080
MIR220B	1.865	2.094	0.0081
MIR130A	-3.808	2.082	0.0083
MIR375	-1.633	2.052	0.0089
MIR935	-3.842	2.050	0.0089
MIR106B	-1.893	2.050	0.0089
MIR487B;MIR539	-11.121	2.039	0.0091
MIR182	-2.899	2.032	0.0093
MIR301A	1.913	2.023	0.0095
MIR1208	-7.880	2.021	0.0095
MIR130B;MIR301B	-3.497	2.005	0.0099
MIR199A1	1.175	1.987	0.0103
MIR200A;MIR429	-3.108	1.974	0.0106
MIR612	-10.032	1.971	0.0107
MIR671	-3.322	1.919	0.0120
MIR654;MIR376B;MIR376A2;MIR300;MIR376A1	3.755	1.861	0.0138
MIR548J	-0.845	1.858	0.0139
MIR1537	-2.602	1.856	0.0139
MIR512-1;MIR512-2	1.826	1.846	0.0142
MIR662	-5.388	1.841	0.0144
MIR628	-1.587	1.841	0.0144
MIRLET7C	-1.717	1.832	0.0147
MIR637	-4.269	1.819	0.0152
MIR339	-6.132	1.816	0.0153

Note: and 3 pages more.

S2 Table. Significantly differentiated expressed miRNAs between HR and LR.

In bold style, those miRNAs that were above the selected threshold of ±1%.

Name	% Effect Size	"-logP-value"	p-value
MIR623	2.491	3.510	0.0003
MIR642	5.007	2.853	0.0014
MIR612	1.427	2.699	0.0020
MIRLET7C	-2.045	2.660	0.0022
MIR668	1.459	2.305	0.0050
MIR548K	2.351	2.253	0.0056
MIR542	3.872	2.232	0.0059
MIR497	1.819	2.106	0.0078
MIR1200	2.261	2.094	0.0081
MIR539	1.181	1.996	0.0101
MIR2116	11.447	1.891	0.0129
MIR890	2.009	1.816	0.0153
MIR1257	1.626	1.805	0.0157
MIR1237	13.262	1.739	0.0182
MIR449B	1.448	1.719	0.0191
MIR892A	2.232	1.707	0.0196
MIR324	14.582	1.692	0.0203
MIR576	-1.745	1.689	0.0205
MIR196B	1.264	1.645	0.0227
MIR193B	1.391	1.623	0.0238
MIR128-1	-2.345	1.622	0.0239
MIR342	-4.060	1.596	0.0254
MIR152	-0.716	1.561	0.0275
MIR1185-1	7.457	1.553	0.0280
MIR548B	2.249	1.552	0.0280
MIR761	1.452	1.535	0.0292
MIR543	-1.344	1.517	0.0304
MIR590	1.313	1.510	0.0309
MIR495	1.621	1.509	0.0310
MIR548I2	-1.586	1.508	0.0310
MIR943	6.773	1.507	0.0311
MIR509-2	1.280	1.505	0.0313
MIR1238	4.905	1.474	0.0336
MIR765	0.779	1.465	0.0343
MIR571	-2.291	1.451	0.0354
MIR155HG	-8.295	1.438	0.0365
MIR636	2.557	1.407	0.0392
MIR147	-1.191	1.405	0.0393
MIR939	13.048	1.386	0.0411
MIR520H	0.936	1.377	0.0420
MIR877	14.486	1.373	0.0424
MIR1976	12.089	1.367	0.0430
MIR1236	2.670	1.355	0.0442

APPENDIX TO CHAPTER 2: CONGRESS COMMUNICATION

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Barcelona, Spain 30 May – 1 June 2018

miR-1976 regulates *CD40* expression in adipocytes by binding to its 3'-UTR region.

Garcia-Lacarte M[1], Åström G[4], Rydén M[4], Martinez JA[1,2,3], Milagro FI[1,2,3].

[1] Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, University of Navarra. Pamplona, Spain.

[2] CIBERobn, Physiopathology of Obesity and Nutrition, Center of Biomedical Research Network, ISCII Madrid, Spain.

[3] IdiSNA, Navarra's Health Research Institute, Pamplona, Spain.

[4] Department of Medicine, Karolinska Institutet, c2-94, Karolinska University Hospital Huddinge, Stockholm, Sweden

Background: miRNAs play a key role in regulating WAT inflammation and obesity. We have previously evidenced that miR-1976 could be a prospective biomarker of response to specific weight loss diets and might regulate the expression of its predicted target gene *CD40*. *CD40* attenuates obesity-induced insulin resistance whose deficiency is associated with adipose tissue inflammation and insulin resistance. The aim of this study was to elucidate the relationship between miR-1976 and *CD40* in adipocytes. **Material and methods**: A time course analysis in human preadipocytes was performed to assay the expression of both miR-1976 and *CD40* during adipocyte differentiation. For downregulation experiments, preadipocytes isolated from stromal vascular fraction were differentiated into mature adipocytes for 12 days and transfected with either 20/40 nM of miR-1976 mimic, or Negative Control using Hiperfect Reagent. RNA expression was analyzed 48 h post transfection. To assess miRNA-target interactions, the 3'UTR region of CD40 was cloned downstream of the firefly luciferase gene in the pmiR-GLO Dual-Luciferase miRNA Target Expression Vector. Then, HEK-293T cells were transiently co-transfected with either 0.25 μg of empty pmiR-GLO or pmiR-GLO-*CD40*-3'UTR, and 7.5 pmol of miR-1976 mimics using Lipofectamine

2000. Firefly and Renilla luciferase activities were evaluated 24 h after co-transfection using a Dual-Luciferase Reporter Assay. **Results**: miR-1976 expression increased during adipocyte differentiation reaching a maximum at day 12. However, *CD40* mRNA expression remained unchanged. Transfection with miR-1976 mimic inhibited *CD40* expression. Lastly, HEK-293T cells co-transfected with the pmiR-GLO-*CD40*-3'-UTR construction and miR-1976 showed a significantly reduction in firefly/Renilla activity than controls transfected only with the expression vector, demonstrating that miR-1976 binds to the 3'UTR of *CD40*. **Conclusion**: *CD40* is a target gene of miR-1976 whose expression is regulated by the binding of the miRNA to the 3'UTR region. These results suggest that miR-1976 could be implicated in adipose tissue inflammation and insulin resistance processes.

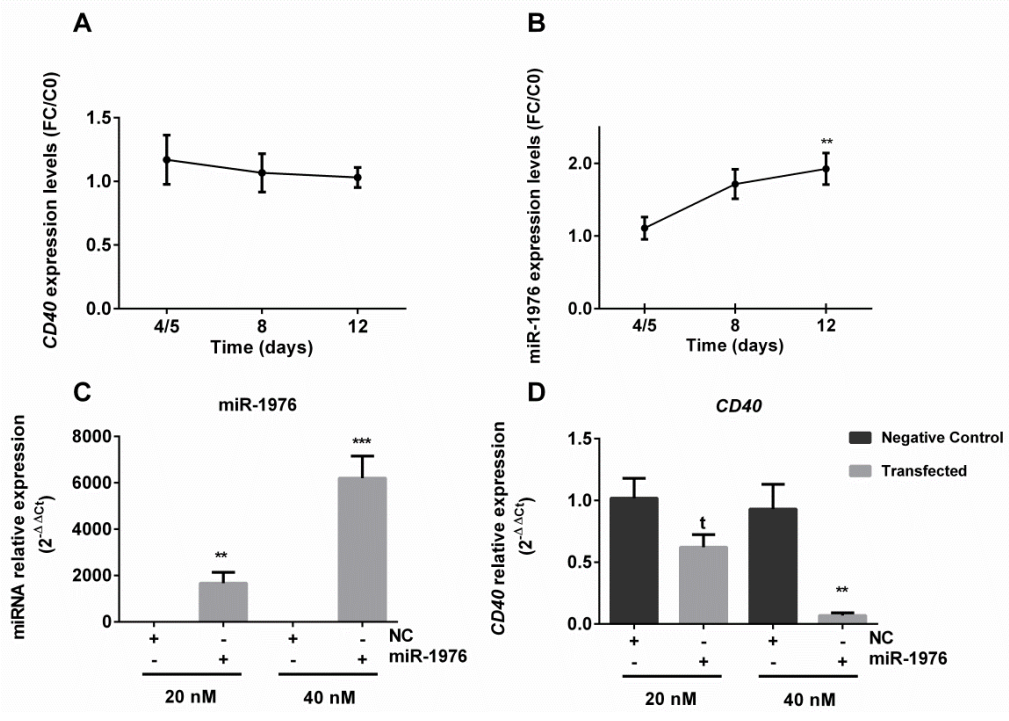

Fig 1. miR-1976 transfection of adipocytes at day 12 of differentiation. A) *CD40* expression during adipocyte differentiation. B) miR-1976 expression during adipocyte differentiation. C) miR-1976 mimic transfection verification. D) Downregulation of *CD40* mRNA levels 48h after miR-1976 mimic transfection. *p< 0.05; **p< 0.01; ***p< 0.001; t< 0.1 from a Student's t-test.

Chapter 3

miR-1185-1 and miR-548q regulate *GSK3B* expression and may mediate the response to weight loss

Marcos Garcia-Lacarte [a,b], J. Alfredo Martinez [a,b,c,d,e], Fermin I. Milagro [a,b,c,d]

[a] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.

[b] Center for Nutrition Research, University of Navarra, Pamplona, Spain.

[c] CIBERobn, Physiopathology of Obesity and Nutrition, Center of Biomedical Research Network, ISCIII Madrid, Spain.

[d] IdiSNA, Navarra's, Health Research Institute, Pamplona, Spain

[e] Madrid Institute of Advance Studies (IMDEA), IMDEA Food, Madrid, Spain.

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Abstract

Background. miRNAs play crucial roles in the inflammatory response and the regulation of body weight. The aim of the present investigation was to identify putative miRNAs involved in the response to weight loss in order to eventually understand obesity management. **Material and Methods.** Reverse-transcripted mRNA isolated from white blood cells (WBCs) of a subpopulation from the RESMENA study (14 low-responders (LR) and 10 high-responders (HR)) was hybridized in a gene expression microarray. miRNAs from WBC from 6 LR and 5 HR were analyzed by miRNA-Seq. A bioinformatic prediction of putative target genes of the selected miRNAs was performed. For confirmatory experiments, THP-1 cells were transfected with either 20/40 nM of miR-548q/miR-1185-1 mimics. To assess miRNA-target interactions, the particular 3'-UTR binding regions of *GSK3B* were cloned downstream of the firefly luciferase gene. Then, HEK-293T cells were transiently co-transfected with either 0.25 μg of empty pmiR-GLO or pmiR-GLO-548q-3'-UTR/pmiR-GLO-1185-1-3'-UTR and 7.5 pmol of miR-548q/ miR-1185-1 mimics. **Results**. miR-548q and miR-1185-1 were overexpressed in HR, both in the expression microarray and in the miRNA-Seq. Interestingly, *GSK3B* was a putative target gene for miR-548q and miR-1185-1 and was down-regulated in HR. miR-1185-1 bound to the 3'-UTR region of *GSK3B* and inhibited the associated target mRNA expression when transfected in THP-1 cells at doses of 20/40 nM. miR-548q also repressed the expression of *GSK3B* at a dose of 40 nM. **Conclusion**. miR-548q and miR-1185-1 levels in WBCs are associated with the response to specific weight loss diets and could be involved in the regulation of the proinflammatory gene *GSK3B*.

Keywords: inflammation; obesity; miRNA; weight loss; biomarkers.

1. Introduction

Obesity and overweight prevalence is increasing worldwide, as well as their accompanying comorbidities, such as cardiovascular diseases, T2D, chronic kidney disease, nonalcoholic fatty liver disease, and musculoskeletal disorders (Afshin *et al.* 2017). Obesity, insulin resistance and T2D are closely related to a chronic inflammatory state. White adipose tissue (WAT) in obese individuals presents

higher macrophages infiltration and an increased secretion of proinflammatory cytokines than non-obese WAT, leading to a greater macrophage recruitment that aggravates the inflammatory response (Hotamisligil, 2006). In any case, obesity status is complex and multifactorial, and lifestyle modifications including dietary-induced weight loss and physical activity are not equally effective for every person. Therefore, continuous efforts are being made to develop personalized approaches based on nutrigenetic and nutrigenomic data (Goni, Cuervo, Milagro, & Martinez, 2016). Consequently, novel biomarkers of prognosis or even of response to dietary treatments to overcome obesity and its inflammatory state are imperatively required.

In this context, microRNAs (miRNAs) are small single stranded noncoding RNA molecules, 18-25 nucleotides approximately in length, that bind to the 3'-UTR region of target genes regulating its transcription and resulting usually in degradation of mRNA or inhibition of the translation (Filipowicz, Bhattacharyya, & Sonenberg, 2008). A single miRNA has different target genes, and one single gene transcript is regulated by several miRNAs, generating an enormous cluster of miRNAs-target gene regulatory pathways (Ross & Davis, 2014).

The enrolment of miRNAs in gene regulation highlights their impact in the control of metabolic homeostasis and their implication in the development of obesity- and metabolic syndrome-related comorbidities (Pomatto & Gai, 2018). For example, miRNAs are involved in the control of angiogenesis, adipogenesis, inflammation, and oxidative stress processes (Goody & Pfeifer, 2018; Pomatto & Gai, 2018; Price & Fernandez-Hernando, 2016), and have capacity to modulate glucose and lipid metabolism in the liver (Liu *et al.* 2014), insulin production in the pancreas (Plaisance, Waeber, & Regazzi, 2014), or leptin signaling in the hypothalamus (Derghal *et al.* 2015). On the other hand, there are several examples in which miRNAs are used as biomarkers or clinical tools for diagnosis and prognosis of several diseases, including obesity and diabetes (Jones *et al.* 2017; Pescador *et al.* 2013; Toiyama, Okugawa, Fleshman, Richard Boland, & Goel, 2018; Vijayan & Reddy, 2016).

One of the areas where miRNA biomarkers could be helpful is in the prediction of the response to a weight loss intervention. Many miRNAs have been proposed as

predictors of the response to dietary interventions. For example, circulating miR-935 and miR-140 levels were described as biomarkers of the magnitude of weight loss to an exercise and nutritional intervention (Parr *et al.* 2016). Moreover, our group has previously demonstrated that miRNAs expression in blood cells could be also used as prognostic biomarker of weight loss (Garcia-Lacarte, Martinez, Zulet, & Milagro, 2018; Milagro *et al.* 2013). In this study, we aimed to search miRNA-type biomarkers to predict the response to a specific dietary treatment, trying to elucidate their impact over the expression of target genes and their mechanism of action.

2. Materials and methods

2.1. Subjects

A subsample from the RESMENA (Metabolic Syndrome Reduction in Navarra) nutritional intervention trial was used in the present study. 96 metabolic syndrome adults underwent two hypocaloric diets (-30% of energy restriction). As no differences were found neither in anthropometric nor in biochemical variables between groups after the intervention, both dietary groups were merged to increase the statistical power of the study. After the dietary intervention, aiming to identify miRNA-type biomarkers implicated in the response to the weight-loss intervention, participants were categorized into "High Responders" (HR), when weight loss was ≥8%, and "Low Responders" (LR) when weight loss was <8%, as previously published (Garcia-Lacarte *et al.* 2018).

The study was approved by the Ethics Committee of the University of Navarra (065/2009) and was registered at www.clinicaltrials.gov (NTC01087086). All participants provided written informed consent for participation.

2.2. RNA isolation and reverse transcription

Plasma, erythrocytes and white blood cells (WBCs) were separated from whole blood by centrifugation at 3,500 rpm at 4°C for 15 min (Model 5804R, Eppendorf AG, Hamburg, Germany), and were stored at -80 °C until analyses. Total RNA from WBCs was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using 0.5 μg of

total RNA from WBCs and the miScript HiFlex Buffer of miScript II RT Kit (QIAGEN, Hilden, Germany), enabling detection of several miRNAs and mRNAs from the same cDNA preparation.

2.3. Microarray analyses and miRNA-Seq

Total RNA was extracted from WBC of 14 matched LR and 10 HR by using TRizol Reagent. 1 μg of RNA from each sample was reverse-transcribed using High Capacity Complementary DNA reverse transcription kit (Life Technologies, Carlsbad, CA) and subsequently hybridized to a HumanHT-12 v4 Expression BeadChip kit (Illumina Inc., San Diego, CA, USA) containing 31,000 annotated genes with more than 47,000 probes and scanned using the Illumina HiScan SQ platform.
Also, miRNAs from WBCs of 6 LR and 5 HR were sequenced by using Illumina's miRNA-Seq following the standardized protocol. Limma package in R was used to analyse microarray and miRNA-Seq data as published (Ritchie *et al.* 2015). Multiple comparison corrections were carried out by using Benjamini-Hochberg procedure in both techniques.

2.4. Bioinformatic study

To predict target genes of the selected miRNAs, miRWalk 2.0 algorithms were applied. miRWalk 2.0 is a database which links to databases such as DIANA-microT, microRNA.org, miRDB, RNA22, TargetMiner, and TargetScan, giving information of predicted and validated miRNA target sites.

2.5. Luciferase reporter constructs

Expression vectors for each miRNA were constructed by cloning the particular 3'-UTR binding region of the *GSK3B* gene provided by the bioinformatic prediction into the pmiR-GLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). Primers containing *NheI* and *XbaI* restriction enzymes sites were used to amplify each specific *GSK3B* 3'-UTR binding region. PCR products were purified and subsequently digested and cloned downstream of the firefly luciferase (*luc*) gene after vector linearization. Primers sequences are shown in **Table 1.**

Table 1. Primer sequences used to amplify the 3'-UTR regions of *GSK3B* and amplicon lengths.

GSK3B-miR-548q-F	5'- TTAGCTAGCACAGTAGGTACCGGCCTGTA -3'	668 bp
GSK3B-miR-548q -R	5'- TTATCTAGAGGTGGCACTCCGTGCAGT -3'	
GSK3B-miR-1185-1-F	5'- TTTGCTAGCCCGATGGATCACTTGGGCCT -3'	856 bp
GSK3B-miR-1185-1-R	5'- TTATCTAGAGGAGGTACAGCCCCACTGTT -3'	

F: Forward. R: Reverse. Underlined: *NheI* and *XbaI* target sites

2.6. Cell culture

Human monocytes from the leukemia cell line THP-1 (for overexpression experiments) were purchased from the ATCC (Manassas, VA, USA) and maintained in GIBCO™ RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin at 37°C in a 5% CO_2 humidified atmosphere. Phorbol 12-myristate 13-atectate (TPA) (Sigma-Aldrich, San Louis, MO, USA) was applied for 48 h at a final concentration of 50 ng/ml for differentiating monocytes into macrophage-like cells, and 100 ng/ml of lipopolysaccharide (LPS) (Invitrogen, Carlsbad, CA, USA) was applied afterwards for 24 h to activate macrophages.

HEK-293T cells (for luciferase-reporter assays) were purchased from the ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100 U/ml penicillin-streptomycin at 37°C in a 5% CO_2 humidified atmosphere.

2.7. mirVana miRNA mimic transfections

For downregulation experiments, THP-1 cells were seeded at 250,000 cells/well in 24-well plates, and differentiated into macrophage-like cells by activation as explained in 2.6 subsection. Activated macrophages were transiently transfected with either 20 nM or 40 nM of *mir*Vana® miR-548q mimic, *mir*Vana® miR-1185-1 mimic, or *mir*Vana® miRNA mimic negative control miR-1 (Applied Biosystems, Foster City, CA, USA) using 3 µl/well of Lipofectamine 2000 Transfection Reagent (Applied Biosystems). To optimize transfection efficacy, the BLOCK-iT Alexa Fluor Red Fluorescent Oligo control (Invitrogen) was transfected and fluorescence was measured 24 h post transfection (excitation 540 nm, emission 590 nm).

2.8. Dual-luciferase reporter assays

miRNA-target interactions were carried out in HEK-293T cells seeded at a density of 15,000 cells per well in 96-well plates. After 24 h, cells were transiently co-transfected with either 0.25 μg of empty pmiR-GLO, pmiR-GLO-548q-3'-UTR, or pmiR-GLO-1185-1-3'-UTR, and 7.5 pmol of miR-548q and miR-1185-1 mimics using 1.5 μl/well Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was normalized using *Renilla* luciferase activity 24 h after co-transfection with a Dual-Luciferase Reporter Assay System (Promega). Determinations were carried out in three independent experiments, each assayed in triplicate.

2.9. Quantitative real-time PCR

Total RNA from THP-1 cells was extracted 24 h after transfection following the TRIzol protocol. A total of 50 ng of RNA were reverse-transcripted using miScript HiFlex Buffer of miScript II RT Kit (QIAGEN). Quantitative PCR (qPCR) was performed with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using commercial Taqman probes for *GSK3B* (Applied Biosystems, Foster City, CA, USA) or miScript Primer Assays for miRNAs (QIAGEN, Hilden, Germany).

mRNA and miRNA expressions were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized using glyceraldehyde-3-phosphate dehydrogenase *GAPDH* and small nucleolar RNA, C/D box 68 (SNORD68) mature miRNA as housekeeping genes, respectively.

2.10. Statistical analysis

Data from humans are presented as mean ± SD and data from cells are presented as mean ± SEM. Differences between groups were calculated using the Student's t- or ANOVA tests when indicated. In this context, p-values less than 0.05 were defined as statistically significant. In the case of expression microarray and Illumina's miRNA-Seq, in order to select the best candidates to act as predictive biomarkers, a p-value< 0.1 was considered relevant if specific miRNAs were found in both platforms. Volcano figures were created by plotting the negative log of the p-value (y axis) and the mean differences between groups for each variable (x axis). An

effect size (ES) of ±1% in the expression differences and a 10xlog Fold Change (FC) of ±1 in the miRNA-Seq were considered relevant. Statistical analyses and graphics were performed using GraphPad Prism version 6.0C (La Jolla, CA, USA).

3. Results

3.1. miR-548q and miR-1185-1 are overexpressed in high responders to the weight loss intervention

When combining the results from the same subjects in both platforms (expression microarray and miRNA-Seq), only two miRNAs (miR-548q and miR-1185-1) showed statistically significant differences between HR and LR subjects, being both miRNAs overexpressed in HR **(Fig 1).**

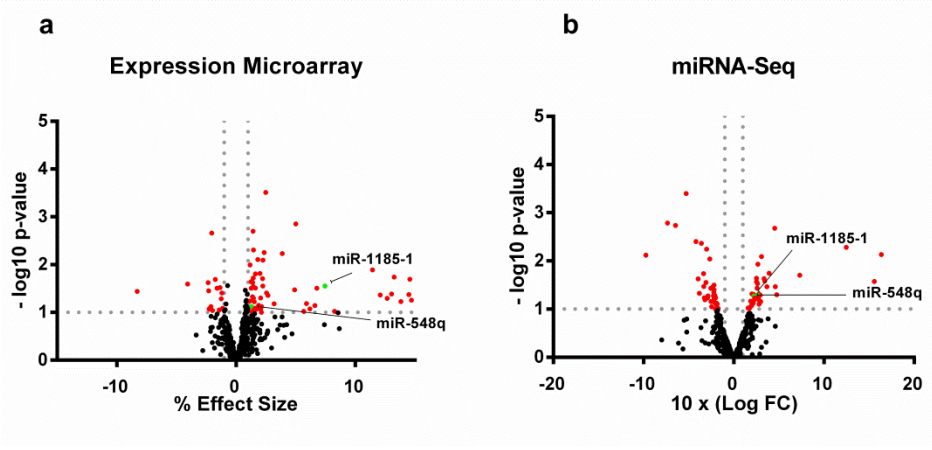

Fig 1. Identification of miR-548q and miR-1185-1 as differentially expressed in the expression array and in the miRNA-Seq. Volcano plots of miRNAs differentially expressed between HR and LR in both the expression microarray a) and in the miRNA-Seq b), respectively. p< 0.1 and ES >1% or 10xlog FC >1.

In particular, miR-548q showed an ES= 1.29% in the expression array (p = 0.075), and a 10xlog FC= 2.22 in the miRNA-Seq (p= 0.053). Meanwhile, miR-1185-1 showed an ES= 7.46% in the expression array (p= 0.028), and a 10xlog FC= 2.60 in the miRNA-Seq (p= 0.052) as illustrated **(Fig 2B-C).**

These differences were not significant after multiple comparison corrections. Nonetheless, as these miRNAs appeared in both the expression array and in the miRNA-Seq, they were selected for target gene predictions with the aim of finding

feasible candidates for further evaluation, considering that these experimental approaches were performed with small sample.

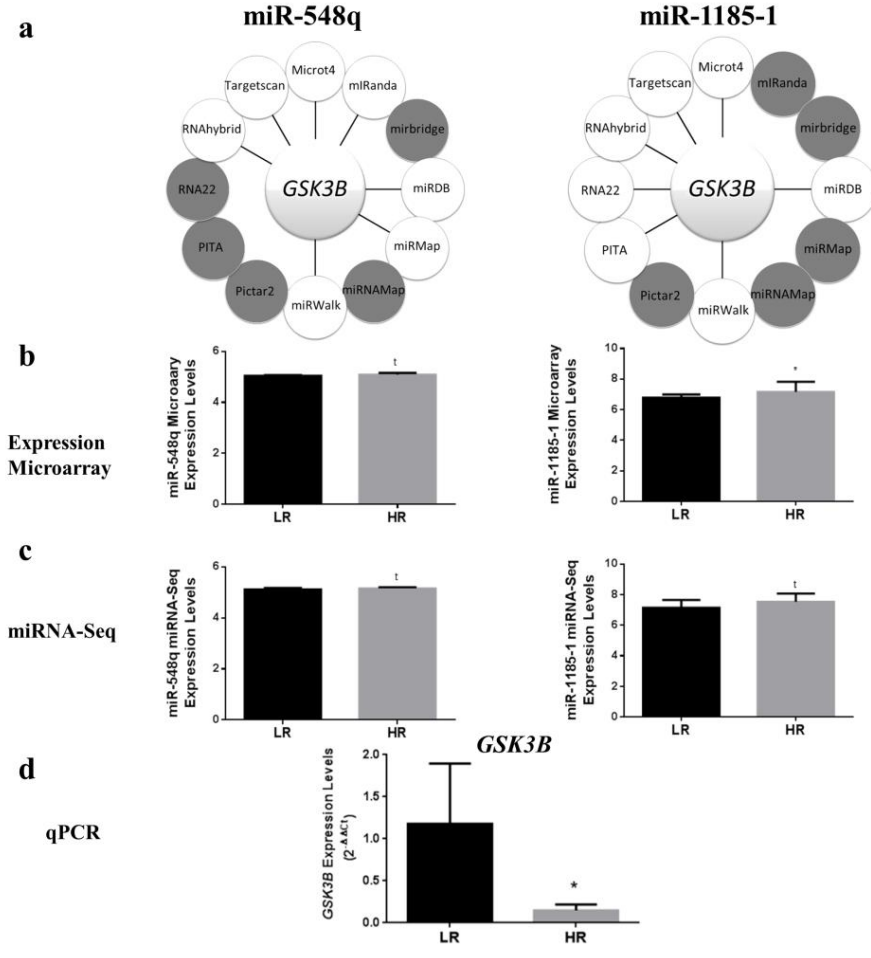

Fig 2. *GSK3B* is a putative target gene of miR-548q and miR-1185-1. a) Bioinformatic predictions of miRWalk 2.0 for selected miRNAs. *GSK3B* appeared to be a potent regulated gene in 7 data bases from a total of 11. ○ Databases which predicted *GSK3B* as a putative target gene of selected miRNAs. ● Databases which did not predict *GSK3B* as a putative target gene of selected miRNAs b) Microarray expression levels of miR-548q and miR-1185-1 in HR and LR to the weight loss intervention. c) miRNA-Seq expression levels of miR-548q and miR-1185-1 in HR and LR to the weight loss intervention. d) Validation of *GSK3B* expression profile in HR and LR WBCs by qPCR in the microarray subjects. *p< 0.05; t< 0.1 from a Student's t-test.

3.2 *GSK3B* is a putative target gene for miR-548q and miR-1185-1

We applied bioinformatic algorithms to screen for genes that are putative targets of the selected miRNAs. We encompassed results from all databases linked by miRWalk 2.0 and selected only those genes that appeared at least in 6/11 databases. Then, results were filtered by focusing on obesity-related genes. Finally, we noticed that *GSK3B* could be a target gene for both miR-548q and miR-1185-1 **(Fig 2A).** According to miRWalk 2.0, the *GSK3B* mRNA 3'-UTR contains one sequence motif complementary to miR-548q and two binding sites complementary to the miR-1185-1 sequence **(Fig 3A).**

Interestingly, *GSK3B* mRNA levels in WBC were significantly lower ($p= 0.014$) in HR than in LR when measured the microarray subjects by qPCR **(Fig 2D),** suggesting a possible regulation of these miRNAs over *GSK3B* expression.

3.2 miR-1185-1 binds to the 3'-UTR of *GSK3B*

To determine if *GSK3B* is regulated by the binding of miR-548q and miR-1185-1 to their specific binding sites, each predicted sequence was cloned immediately downstream of the luciferase reporter gene in two different expression vectors (pmiR-GLO-548q-3'-UTR and pmiR-GLO-1185-1-3'-UTR) **(Fig 3A-B)**. Cells co-transfected with pmiR-GLO-1185-1-3'-UTR vector and miR-1185 mimic showed lower levels of firefly/*Renilla* activity ($p < 0.001$) than controls transfected only with the pmiR-GLO-1185-1-3'-UTR vector (Fig 3C), suggesting that *GSK3B* is a target gene of miR-1185-1. Regrettably, firely/*Renilla* activity was not detected as affected in cells co-transfected with the pmiR-GLO-548q-3'-UTR vector and miR-548q mimic when comparing with cells transfected only with the pmiR-GLO-548q-3'-UTR vector **(Fig 3C).**

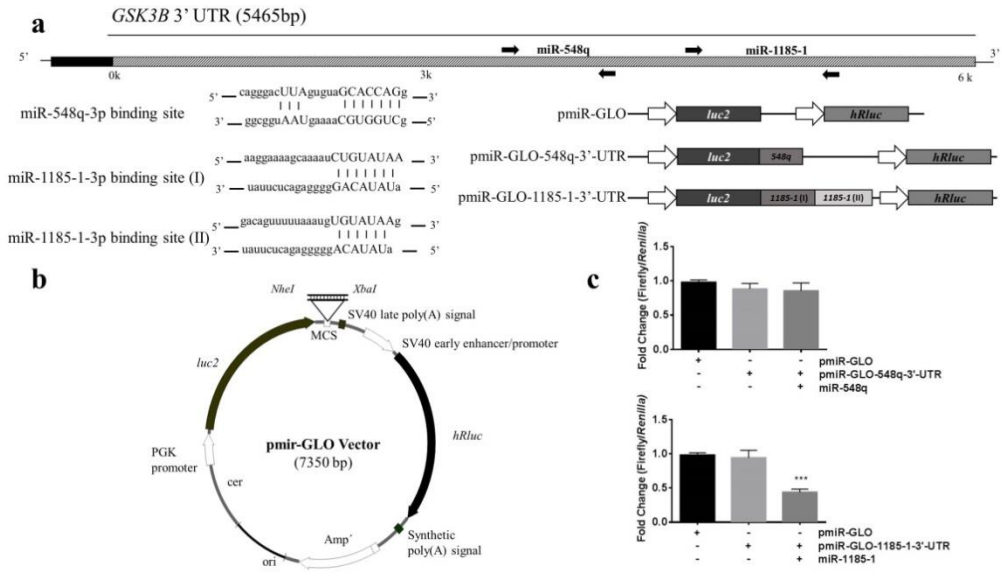

Fig 3. miR-1185-1 binds to the 3'-UTR of *GSK3B*. a) Location of the predicted target sites for miR-548q and miR-1185-1 in the 3'-UTR of *GSK3B*. b) Dual-Luciferase miRNA Target Expression Vector used to create the 3'-UTR expression vectors cloning the PCR product into the multiple cloning site (MCS). c) Luciferase activity assay of pmiR-GLO-548q-3'-UTR and pmiR-GLO-1185-3'-UTR after co-transfection with either miR-548q or miR-1185-1 mimics. Normalized luciferase activity is presented as the mean ± SEM of three separate triplicate experiments. *** p< 0.001 from an ANOVA test.

3.3 miR-548q and miR-1185-1 decrease the endogenous *GSK3B* mRNA levels

To ascertain if miR-548q and miR-1185 regulate GSK3B by affecting its endogenous mRNA levels, miR-548q and miR-1185-1 mimics or control were transfected into THP-1 macrophage-like cells at two different doses (20 nM and 40 nM).. GSK3B mRNA levels were significantly reduced in miR-548q and miR-1185-1 transfected cells at the higher dose of 40 nM and in cells transfected with miR-1185-1 at a dose of 20 nM **(Fig 4).**

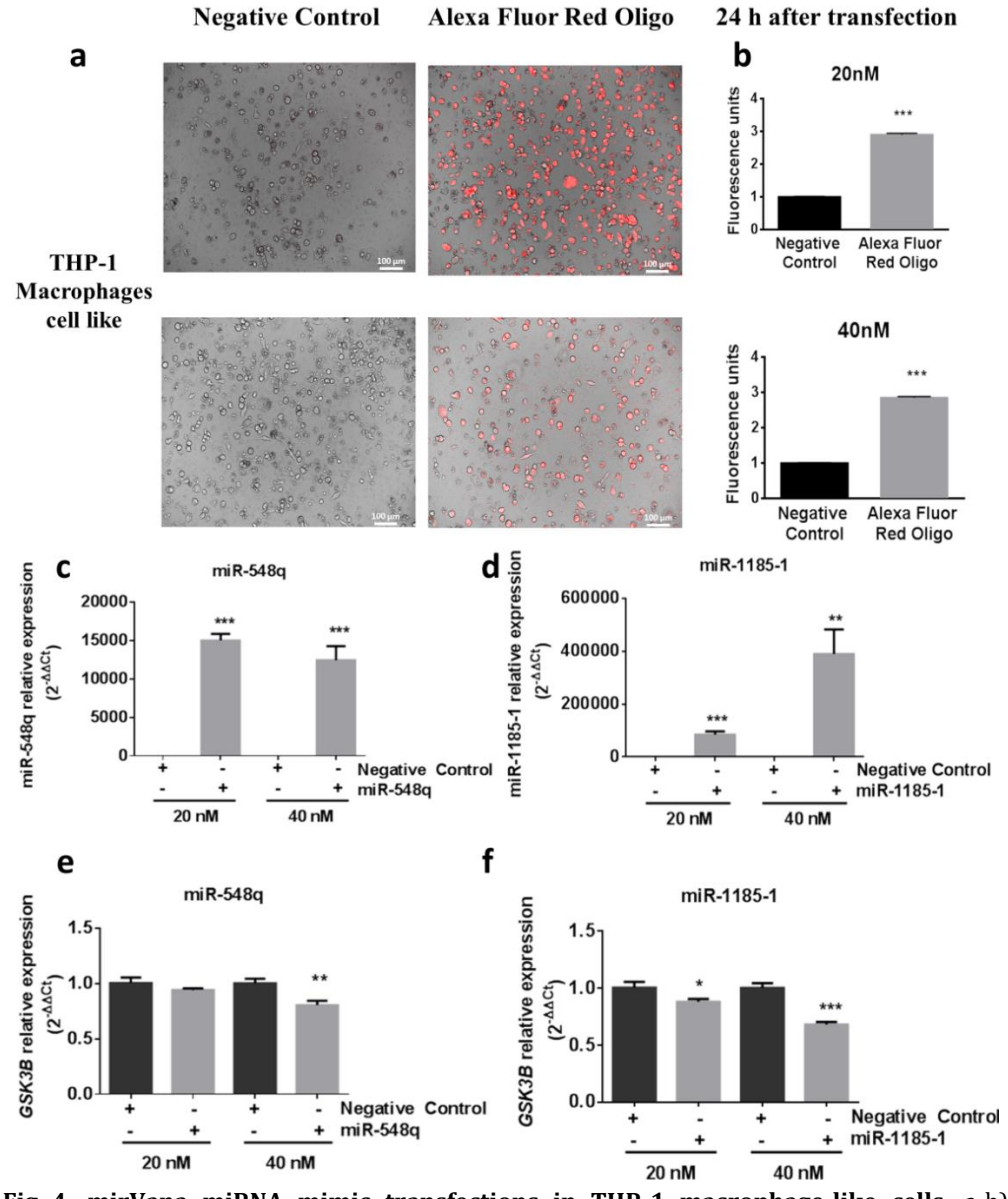

Fig 4. mirVana miRNA mimic transfections in THP-1 macrophage-like cells. a-b) Transfection optimization using the BLOCK-iT Alexa Fluor Red Fluorescent Oligo. 20 nM or 40 nM of the fluorescent oligo were transfected into THP-1 macrophage-like cells using Lipofectamine 2000 to confirm the positive transfection of small nucleotides in the cells, 24 h after transfection. c-d) Efficiency of miRNA mimic transfections measuring miR-548q and miR-1185-1 levels by qPCR. e-f) Downregulation of *GSK3B* mRNA 24h after miR-548q and miR-1185-1 mimic transfection into TPH-1 cells at different doses (20 nM and 40 nM). *p< 0.05; **p< 0.01; ***p< 0.001 from a Student's t-test.

4 Discussion

In the present study, we performed different miRNAomic approaches and we identified two presumed miRNAs that could distinguish the level of response to a specific weight loss dietary treatment. Considering that about 70% of the human genome is transcribed but only up to 2% is translated to proteins, transcriptomic has become an emerging alternative in the search for biomarkers for personalizing diagnosis, prognosis and treatment of diseases. Recent advances in the RNA-seq workflow have enabled to elucidate new biomarkers without a prior known association with different physiological and pathological conditions (Kukurba & Montgomery, 2015) and so that, next-generation sequencing (miRNA-seq) is the platform of choice for the discovery of new potential biomarkers of disease diagnosis, prognosis and therapy (Gong *et al.* 2014).

In this context, circulating and exosomal miRNAs have been proposed as useful in diagnostics of diverse diseases including cancer, nervous system disorders, cardiovascular disease, diabetes and other metabolic conditions (J. Wang, Chen, & Sen, 2016).

One of the best known manifestations of obesity is the alteration of adipose tissue metabolism that leads to a chronic inflammatory state characterized by the infiltration of macrophages and the secretion of proinflammatory cytokines that aggravate the process (Weisberg *et al.* 2003). These inflammatory mediators secreted by the adipose tissue can trigger different metabolic alterations, impairing insulin signaling and inducing oxidative stress, leading to systemic insulin resistance and cardiovascular disease (Maury & Brichard, 2010). Similarly, dysregulation of macrophage signaling can impair insulin sensitivity (Varma *et al.* 2009). Likewise, hypothalamic inflammation may provoke hyperphagia (X. Wang *et al.* 2012), enhancing chronic excess of nutrient intake and thus, metabolic dysfunction.

Altogether, these results indicate that miR-548q and miR-1185-1 are able to reduce and eventually modulate the mRNA levels of *GSK3B.*

Several miRNAs have been implicated in different inflammatory processes. For example, miR-21 has an essential role as a negative modulator of inflammation. In

vitro overexpression studies in macrophages showed that miR-21 reduced the secretion of IL-6 and IL-10, implying an anti-inflammatory effect (Feng *et al.* 2014). Another miRNA with anti-inflammatory properties is miR-24, which inhibits the production of pro-inflammatory cytokines in LPS-stimulated macrophages (Fordham, Naqvi, & Nares, 2015). miR-124, miR-145, miR-146, miR-149, miR-155, or miR-181 family are other miRNAs that may act as negative regulators in inflammation (Tahamtan, Teymoori-Rad, Nakstad, & Salimi, 2018). However, this is the first time that miR-548q and miR-1185-1 have been related to inflammation or body weight regulation.

Regarding inflammation, glycogen synthase kinase-3 (GSK3) has emerged as an important regulator of the process. GSK3 is a multitasking serine/threonine kinase that have over 50 substrates (Jope, Yuskaitis, & Beurel, 2007). Initially GSK3 was thought to be only related to the metabolism of glycogen. However, interest began growing when it was described that GSK3 is a key member of the insulin and Wnt signaling pathways, and is involved in inflammatory responses (Jope *et al.* 2007). GSK3 enhances the expression of genes activated by NF-κB (Steinbrecher, Wilson, Cogswell, & Baldwin, 2005). For example, IL-6 and MCP-1 require GSK3B (an isoform of GSK3) for efficient expression (Steinbrecher *et al.* 2005). The inflammatory role of GSK3 and the importance of its inhibitors is clear since GSK3 promotes the expression of pro-inflammatory cytokines such as IL-1β, IL-6 or TNF-α among others (Beurel, 2011). Interestingly, we found a negative correlation between microarray expression levels of miR-1185-1 and serum levels of IL-6 ($r = -0.44$; $p = 0.033$), suggesting that the regulation of GSK3B by miR-1185-1 may imply a reduction in the inflammatory status.

On the other hand, mice receiving LPS showed decreased levels of proinflammatory cytokines, such as TNF-α, IL6, IL-1β or MCP-1, after the administration of a GSK3 inhibitor (Martin, Rehani, Jope, & Michalek, 2005). Consequently, inhibitors of GSK3 seem to provide strong therapeutic protection against inflammation and many prevalent associated diseases, such as diabetes or obesity. In obese mice, Gsk3 activity is increased in adipose tissue, and its inhibition prevents adipocyte differentiation (Bennett *et al.* 2002; Eldar-Finkelman, Schreyer, Shinohara, LeBoeuf, & Krebs, 1999), supporting the notion that *GSK3* is involved in obesity. Regarding

body weight, a double knockdown of both Gsk3a and Gsk3b featured a decrease in body weight (Patricia, Wolfgang, & Ralf, 2008), whereas an overexpression of human *GSK3B* in mice skeletal muscle resulted in impaired glucose tolerance, hyperlipidemia and an increase in fat mass and body weight gain (Pearce *et al.* 2004). Moreover, when inhibiting Gsk3b activity, diet-induced obese mice significantly ameliorated obesity symptoms, such as body weight gain, increased adiposity, dyslipidemia, and hepatic steatosis, due to the marked reduction of whole-body lipid content (Lee *et al.* 2013).

Applying different high-throughput technologies, here we found two miRNAs that interact with *GSK3B*. Our results showed that both miR-548q and miR-1185-1 regulate *GSK3B* mRNA levels in a dose-dependent manner. At least for miR-1185-1, it occurred by directly binding to the associated 3'-UTR region. The regulation of miR-548q over *GSK3B* could happen in an indirect manner. Thus, it is important to highlight that miRNAs could also regulate epigenetic machinery and may be involved in an indirect epigenetic regulation. In this context, epi-miRNAs are defined as those miRNAs whose targets are, in a direct or indirect way, effectors of the epigenetic machinery. For instance, miRNAs can affect histone methylatransferases that disturb methylation of histones or DNA (Moutinho & Esteller, 2017). On the other hand, miR-548q could target the expression of some *GSK3B* enhancers or transcription factors, resulting in an ultimate reduction of *GSK3B* mRNA levels.

Although not in inflammation, GSK3B has been reported to interact with miRNAs in neoplasia and several types of cancer. In gastric tumors, miR-92, miR-182 and miR-183 expressions were increased in *Gsk3b* knockout mice (Tang *et al.* 2014). In hepatocellular carcinoma, *GSK3B* increases miR-122 levels (Zeng *et al.* 2010) and activates miR-181 expression (Ji, Yamashita, & Wang, 2011).

To date, this is the first study to identify miR-1185-1 and miR-548q as biomarkers of weight loss that could predict the level of response to a dietary intervention, being involved in energy homeostasis. Moreover, we have demonstrated that these miRNAs are involved in the regulation of *GKS3B*, suggesting a role of these miRNAs in inflammation and body weight control. On the other hand, this result suggests that GKS3B could be an important mediator of weight control, and that its

expression could also early differentiate the patients that are going to respond well to the dietary treatment of obesity. In summary, those patients with low expression of this gene in WBCs respond poorly to the hypocaloric dietary intervention.

This article was not devoid of limitations. First, the sample size used in both microarray and miRNA-seq was relatively low. Second, differences in miRNA expression between HR and LR were not significant after multiple comparisons, although we selected miRNAs that were differentially expressed in both techniques. Third, we cannot conclude if the changes in the expression of miR-548q and miR-1185-1 are a cause or a consequence of the obese state of the participants.

On the other hand, the study presents strengths that are important to remark. Two different miRNAomic technologies were applied to search for miRNAs differentially expressed. Moreover, *in vitro* experiments complemented results from WBCs. Co-transfection experiments were performed using different expression target vectors encoding the 3'-UTR of *GSK3B* gene, being this strategy one of the most accurate to validate the binding of miRNAs over target genes. These experiments were verified by overexpression studies with specific miRNA mimics.

5 Conclusion

The present article identified two miRNAs that are implicated in the regulation of one important inflammatory gene. miR-548q and miR-1185-1 were overexpressed in individuals that responded better to a weight loss intervention, and the mRNA expression in blood of their target gene *GSK3B* was down-regulated. This happened through a direct binding of miR-1185-1 over the 3'-UTR of *GSK3B* and by an indirect regulation of miR-548q. The fact that *GSK3B* is implicated in many inflammatory events, and considering that IL-6 levels in blood were negatively correlated with miR-1185-1, indicates that these miRNAs could have a key role in the progression of obesity-induced inflammation; and that this gene could be an important target for struggle against obesity related complications.

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GENERAL DISCUSSION

Genetic and environmental factors can alter the expression pattern in an individual, and interact with traditional risk factors that may lead to obesity-related manifestations (Musaad & Haynes, 2007). In this context, a number of studies in the last years have evidenced that epigenetic mechanisms play an important role in the susceptibility and development of several disease, including obesity (Herrera et al., 2011). The three mayor epigenetic mechanisms that regulate gene expression are: 1) DNA methylation (incorporation of a methyl group at the C-5 position of cytosine adjacent to a guanine resulting in 5-methylcytosine); 2) Histone modifications, such as methylation or demethylation of arginine, lysine and histidine amino acid residues; and 3) ncRNAs, that include miRNAs that post-transcriptionally regulate gene expression (Zhang & Pradhan, 2014).

Depending on the degree of complementarity between miRNAs and their binding sites, there are different post-transcriptional regulations mediated by miRNAs. Usually, miRNAs bind to their target transcripts at the 3'-UTR of the mRNA, although there are also evidences of gene expression activation mediated by miRNAs (Vasudevan et al., 2007). Additionally, miRNAs are also reported to bind into the 5'-UTR of their target genes (Lee et al., 2009), or even within the coding gene body (Forman et al., 2008). Lastly, it has been demonstrated that miRNAs form triplexes with dsDNA and could directly alter gene expression (Paugh et al., 2016).

Therefore, the general objective of the present dissertation was the identification of obesity-related miRNA-type biomarkers associated with obesity and the response to specific weight loss treatments. We performed different miRNA-omic approaches to identify and further validate miRNAs that could distinguish between obese and non-obese children (Chapter 1), and to predict the level of response to specific weight loss dietary treatments (Chapters 2 and 3).

Childhood Obesity miRNA Biomarkers

As obesity incidence in childhood is disturbingly increasing, and considering the dramatic incoming consequences that childhood obesity has in the quality of life of children, methods to combat obesity in the early steps of the life are imperatively required. In this sense, validated biomarkers serve as valuable tools to diagnose and

understand the etiology and progression of a disease (Mayeux, 2004). Regarding obesity, one of the most extended ways to use biomarkers to diagnose diseases or predict future risks in children is directly applying validated methods in adults, using as reference their values and standards ranges. However, this approach is imprecise because some diseases are unique to children (Goldman et al., 2011). Nonetheless, studying obesity in its early onset in infants provides an important opportunity to overcome the disease because obesity-associated comorbidities are not present at those steps in childhood, so changes in the levels of some biomarkers could only be attributable to the pathology *per se*, and thus the reliance of the markers would be enlarged.

Trying to shed more light on this issue, the first objective of this dissertation was to investigate the associations between epigenetic biomarkers, specifically DNA methylation profiles (focusing on miRNAs coding regions), and childhood obesity (Chapter 1).

In relation to this, several studies have focused on identifying miRNAs in infant populations as biomarkers in different diseases. Yani et al. found that down-regulated miR-203 in urine may be indicative of severe inflammation in children with atopic dermatitis (Lv et al., 2014). In another study examining the expression levels of miRNAs in kidney biopsies from nephrotic children, Meiling et al. reported that miR-191 and miR-151-3p were up- and down-regulated in all subtypes of kidney diseases compared to controls (Lu et al., 2015).

Regarding obesity and related comorbidities, several studies have analyzed the changes in the expression of miRNAs in childhood. In a study involving 45 obese children, it was described that a significant association was present between circulating levels of miR-370, miR-33, miR-378, miR-27, miR-335, miR-143 and miR-758 and childhood obesity. In the same study, low circulating levels of miR-335, miR-143 and miR-758, and high levels of miR-27, miR-378, miR-33 and miR-370 were associated with triglyceridemia (Can et al., 2016). A cross-sectional validation study from Prats-Puig et al. disclosed that 15 miRNAs were not only significantly de-regulated in prepubertal obesity, but also associated with BMI, percent fat mass, waist circumference, regional fat distribution, and biochemical parameters. Moreover, plasma concentrations of 10 of these circulating miRNAs

changed significantly and differently during a 3 year follow-up in those children who increased or decreased their normalized weight (Prats-Puig et al., 2013). Interestingly, in a recent study in obese children, three miRNAs (miR-486, miR-146b and miR-15b) were found up-regulated in obese children and adult T2D patients. miR-486 was implicated in accelerating preadipocyte proliferation whereas miR-146b and miR-15b were involved in the suppression of high concentration glucose-induced pancreatic insulin secretion, being all of them implicated in the pathological processes of obesity and T2D, indicating that they could be used as predictors of risk of T2D in obese children (Cui et al., 2018).

As previous studies have demonstrated (Samblas et al., 2018), the present study revealed that epigenetic modifications at early-life may be linked to the onset of obesity.

Advances in high-throughput technologies are allowing large-scale epigenome-wide association studies (EWAS) that investigate the impact of nutrition and lifestyle factors on the epigenome and the role of epigenetics in develop obesity (van Dijk et al., 2015). Recent researches have focused on the associations between obesity/metabolic diseases in children and DNA methylation across the genome. For example, Clarke-Harris et al. demonstrated that *PPARGC1A* promoter methylation in blood at 5-7 years predicts adiposity from 9 to 14 years (Clarke-Harris et al., 2014). In a European children cohort (CHOP-Study), 212 methylated sites were associated with BMI, 230 with fat mass, 120 with fat free mass, 24 with fat mass index and 15 with fat free mass index (Rzehak et al., 2017). In a genome-wide methylation analysis, 129 CpGs were differentially methylated in white blood cells between obese and control children, in which immune system regulation, regulation of cell signaling, and small GPT-ase mediated signal transduction pathways were enriched (Huang et al., 2015). Not only in blood, but also in saliva, methylation of DNA has been associated with obesity. In an analysis in saliva samples from 50 lean and 50 heavy adolescent girls, 100 CpG sites and 5 CpG islands differed between the two groups (Rounge et al., 2016).

From these studies it is clear that altered methylation patterns have a role in the development of metabolic diseases, and have emerged as biomarkers associated with, or perhaps predictive of, metabolic health outcomes, especially in children.

As ordinary DNA sequences, expression of miRNAs might be epigenetically regulated and thus miRNAs coding regions are susceptible of DNA hyper- o hypomethylation. Interestingly, these epigenetic marks could be use of putative biomarkers since significant correlations with several cancers have been found [reviewed in (Silahtaroglu & Stenvang, 2010, Tuna et al., 2016, Moutinho & Esteller, 2017)].

According to obesity or related comorbidities, few cases of DNA methylation regulation of miRNAs are available. Kameswaran et al. found a cluster of miRNAs on chromosome 14q32 that was highly and specifically expressed in human β-cells and dramatically down-regulated in islets from T2D patients. Remarkably, the down-regulation of this locus was strongly correlated with hypermethylation of its promoter, suggesting a possible epigenetic regulation (Kameswaran et al., 2014). Another miRNA that is regulated by epigenetic mechanisms in diabetes is miR-375. This miRNA is encoded in an intergenic region and has a promoter containing CpG islands. Intriguingly, in a study involving 100 patients suffering from T2D and 100 healthy controls, it was found that miR-375 promoter was hypomethylated in T2D subjects regulating the expression of miR-375 and contributing to the pathogenesis of T2D (Sun et al., 2014).

In the present thesis, after comparing 2783 miRNAs CpG sites in obese children compared to lean ones, we identified 16 differentially methylated CpGs in miRNAs coding regions (6 hypermethylated and 10 hypomethylated). Among them, the methylation levels of 3 CpG sites (corresponding to miR-1203, miR-412 and miR-216A) correlated with BMI-SDS.

Thus, miR-216A has been implicated in obesity-related pathways, as for example the regulation of human adipose-derived mesenchymal stem cells (Li et al., 2015). Specifically, miR-216A is up-regulated during osteogenic differentiation in human adipose-derived mesenchymal stem cells (Li et al., 2015) and is also implicated in diabetic complications, such as nephropathy (El-Samahy et al., 2018) or diabetic neuropathy (Li et al., 2016). In particular, in pediatric type I diabetes, miR-216A is down-regulated in urine samples and can be considered as early biomarker of nephropathy (El-Samahy et al., 2018). Moreover, miR-216A, which expression is augmented in activated macrophages (Yang et al., 2018), promotes M1

macrophages polarization and atherosclerosis progression via Smad3/NF-κB pathway (Yang et al., 2018), suggesting a regulatory role of miR-216A in inflammation processes.

Little is known about the other two miRNAs whose coding regions methylation levels were altered in obese children. In any case, miR-1203 has been related to several cancers. For example miR-1203 is more than threefold de-regulated in serum samples from prostate cancer and could be used as diagnosis biomarker (Haldrup et al., 2014). Additionally, miR-1203 expression levels are also correlated with esophageal (Okumura et al., 2016) and renal cell carcinomas (Zhu et al., 2016). Regarding miR-412, it has been reported to be up-regulated in squamous cell lung carcinoma tissues when compared to normal tissues (Gao et al., 2011). Moreover, it has been recently found to be up-regulated in salivary extracellular vesicles from oral squamous cell carcinoma patients compared to controls (Gai et al., 2018).

To our knowledge, this is the first time that epigenetic (methylation) regulatory processes have been associated to these miRNAs, relating their methylation levels to childhood obesity.

Although not many evidences are present in the literature connecting the expression of these miRNAs with obesity-related diseases, the predicted and validated genes of these miRNAs suggest a role in the development of obesity and its comorbidities. Interestingly, miR-1203 has two important validated genes related to obesity. One of them is DNA fragmentation factor-alpha-like effector A (*CIDEA*), that is known to play a role in adipose tissue energy expenditure (Gummesson et al., 2007) and serves as a brown-like adipocyte marker in children (Rockstroh et al., 2015). Its expression in human adipose tissue is low in obese subjects and normalizes after weight reduction (Dahlman et al., 2005), while *Cidea*-null mice present a lean phenotype and are resistant to diet-induce obesity and diabetes (Zhou et al., 2003). Moreover, *CIDEA* V115F polymorphism has been associated with phenotypes of MetS (Zhang et al., 2008), and with abdominal obesity, enlarged fat cells, insulin resistance and increased basal lipolysis (Nordstrom et al., 2005).

The other validated miR-1203 target gene is *CD44*, which is implicated in inflammation and insulin resistance and whose neutralization in mice prevents

macrophage infiltration and activation in white adipose tissue (Kodama et al., 2015). Recently, it was demonstrated that individuals that responded better to an energy-restricted program showed higher mRNA and lower methylation *CD44* levels (Samblas et al., 2018). In obese patients, hepatic *CD44* is strongly up-regulated in non-alcoholic steatohepatitis and its soluble form is increased in severe steatosis (Patouraux et al., 2017). In addition, Liu et al. found that serum CD44 concentrations were significantly increased in insulin resistant participants and that its expression in subcutaneous adipose tissue was threefold higher in the same individuals. Lastly, they observed that CD44 density was associated with pro-inflammatory M1 macrophage polarization (Liu et al., 2015). Moreover, miR-1203 has two putative predicted obesogenes: perilipin 4 (*PLIN4*), a kind of perilipin that regulates lipid storage and lipolysis (Smith & Ordovás, 2012); and tumor suppressor protein p53 (*TP53*), whose protein levels are increased in the adipose tissue of *ob/ob* mice and plays a key role in the regulation of insulin resistance (Minamino et al., 2009).

Similarly, miR-412 targets perilipin 2 (*PLIN2*), which, when abrogated in mice, protects against diet-induced obesity, inflammation and hepatic steatosis (McManaman et al., 2013). miR-412 has several predicted genes related to obesity, such as leptin receptor (*LEPR*), *IL6*, Fas cell surface death receptor (*FAS*) and leptin (*LEP*).

Finally, miR-216A targets phosphatase and tensin homolog (*PTEN*) (Kato et al., 2009), a gene that has been implicated in T2D and plays a central role in both insulin signaling/resistance and apoptosis (Bulger et al., 2015). In addition, *PTEN* could regulate brown adipose function and energy expenditure and exerts an important role in lipid metabolism (Ortega-Molina et al., 2012). Moreover, it is well established that *PTEN* is a main regulator of the PI3K signaling pathway, which is involved in lipid metabolism and glucose transport in 3T3-L1 adipocyte (Nakashima et al., 2000). Additionally, miR-216A has several obesity-related predicted genes, including *CD44*, *LEPR*, peroxisome proliferator-activated receptor gamma (*PPARG*), adiponectin receptor 1 (*ADIPOR1*), caveolin 1 (*CAV1*) and caveolin 2 (*CAV2*). In this context, our investigation demonstrated that miR-216A methylation levels correlated significantly with HOMA-IR and insulin levels.

Although the circulating levels of several miRNAs have been associated with childhood obesity in previous studies (Prats-Puig et al., 2013, Cui et al., 2018), this is the first work describing that methylation levels in CpG sites of miRNA coding regions are associated with childhood obesity; and none of the miRNAs identified in the current project were described by Cui et al. or Puig et al. (Prats-Puig et al., 2013, Cui et al., 2018).

Moreover, our work described a cluster of 16 miRNAs with differential methylation levels between obese and non-obese children that are putatively implicated in obesity-relevant biological pathways. These pathways are well known to have relevance in obesity comorbidities and include transforming growth factor-beta (TGF-β) (Yadav et al., 2011), insulin signaling pathway (Qatanani & Lazar, 2007), as well as mitogen-activated protein kinases (MAPK) (Bost et al., 2005) and Wnt signaling pathway (Christodoulides et al., 2009), which share 13 miRNAs of the cluster.

Studies in cell and animal models have characterized the key role of TGF-β superfamily signaling members in many biological processes related to obesity and its comorbidities, such as adipocyte development, adiposity, and energy expenditure (Zamani & Brown, 2011). Polymorphisms in insulin signaling-related genes have been implicated in the development of insulin resistance (George et al., 2004). Moreover, processes such as insulin secretion, insulin recruitment or beta cell development are known to be influenced by miRNAs (Chakraborty et al., 2014).

MAPKs are intracellular signaling proteins that play a key role in essential cellular processes, such as proliferation and differentiation. Indeed, *in vitro* studies in preadipocyte cell lines have analyzed the role of MAPKs in adipocyte differentiation (Bost et al., 2005). In fact, Engelman et al. were the first to describe that a treatment with a p38 inhibitor blocked adipogenesis in 3T3-L1 adipocytes during the early stages of differentiation (Engelman et al., 1998).

Lastly, the Wnt family regulates the maintenance and remodeling of adult adipose tissue, resulting in adipose cell communication. Certainly, several reports implicate Wnt signaling pathways in adipogenesis regulation (Christodoulides et al., 2009). Altogether, the miRNAs identified in Chapter 1 add to others whose methylation is altered in obese children and emerge as important transcriptional regulators of

obesity and related comorbidities in the early steps of the life. These data open the door to deepen into the mechanisms by which these miRNAs regulate specific obesity-related genes and molecular pathways.

miRNA Biomarkers of Response to Weight Loss Interventions

Although it is critical to identify biomarkers to prevent obesity in the first steps of this disease in the childhood, it is a reality that nowadays there is an important prevalence of obesity worldwide, both in young and adult population. Thus, it is necessary to investigate new personalized strategies to treat the disease. As previously mentioned, one of the most classical obesity treatments is the use of reduced-energy diets. However, the response to body weight loss varies widely between individuals, and one of the main causes is the difference in the epigenetic marks (Bouchard et al., 2010). In order to achieve personalization of weight loss treatments, another aim of the present thesis was to identify predictive miRNA-type biomarkers of response to dietary interventions (Chapters 2 and 3). In this sense, many studies have aimed to identify novel predictors of these variations that may bias the goal of weight loss interventions (Rudkowska et al., 2015, Tremblay et al., 2015, Vallee Marcotte et al., 2016). Importantly, our group demonstrated that DNA methylation levels of several CpGs at the *WT1* promoter were statistically more methylated in individuals that responded better to a weight loss diet than in low responders (Milagro et al., 2011). Moreover, Samblas et al. established that individuals that responded better to an energy-restricted program showed higher mRNA and lower methylation CD44 levels (Samblas et al., 2018). *LEP* and *TNFA* promoter methylation levels could also be used as predictive biomarkers for weight loss response (Cordero et al., 2011). [For a systematic review of studies of DNA methylation in the context of weight loss interventions, see Aronica et al. (Aronica et al., 2017) and Milagro et al. (Milagro et al., 2015)].

As in the case of the children study in Chapter 1, we also performed epigenetic and transcriptomic deep-sequencing studies to identify, in this case, predictive biomarkers of response to weight loss interventions in adults. Indeed, we not only

used data from a methylation microarray similar to that of Chapter 1, but also performed an expression microarray (Chapters 2 and 3) and sequenced miRNAs in WBCs by using miRNA-Seq technology (Chapter 3). This approach has been previously applied by several authors to identify candidate genes for explaining the inter-individual differences in the response to weight loss strategies (Wahl et al., 2016). Although we focused on miRNAs, our results are consistent with previous studies in blood cells where expression levels of several genes at baseline could predict changes after a nutritional intervention in BMI and body weight (Rendo-Urteaga et al., 2015, Armenise et al., 2017). Similarly, after performing a transcriptome microarray assay, Bolton and colleagues found circulating transcriptomic biomarkers associated with weight control that could be useful in obesity management and genes that may be prognostic for successful weight maintenance (Bolton et al., 2017).

Several studies have proposed miRNAs as predictors of weight loss. For example, in a prospective study, 10 Caucasian obese women were selected among the participants in a weight loss trial. Differences in baseline expression of several miRNAs were found between responders (>5% of weight loss) and non-responders (<5% of body weight loss). Specifically, miR-935 and miR-4772 were up-regulated and miR-223, miR-224 and miR-376b were down-regulated in the non-responder group (Milagro et al., 2013). Evelyn and colleagues found that circmiR-935 and circmiR-140 were differentially expressed between high and low responders before and after a chronic weight loss intervention, revealing the potential for circmiRNAs to act as biomarkers of the magnitude of weight loss to a dietary intervention (Parr et al., 2016).

In the current study, we have identified 134 miRNAs that were differentially methylated between HR and LR, whereas 44 miRNAs appeared differentially expressed between groups in the expression array. Moreover when combining both microarrays (Chapter 2), 6 miRNAs (miR-1237, miR-1976, miR-642, miR-636, miR-612 and miR-193B) were simultaneously hypomethylated and overexpressed in HR. Additionally, when combining results from expression microarray and miRNA-Seq (Chapter 3), two miRNAs (miR-548q and miR-1185-1) were overexpressed in HR. In *in vitro* studies we have demonstrated solid relationships

between the following clusters of miRNAs-target genes: miR-612 – *TP53*, miR-1976 – *CD40*, miR-1181-1 – *GSK3β*, and miR-548q – *GSK3β*. Although almost nothing is known about these four miRNAs, the target genes have been repeatedly associated with obesity-related traits.

TP53 encodes p53, a tumor suppressor protein whose deficiency can enhance the initiation or progression of cancer (Bieging et al., 2014). Regarding inflammation, p53 knockdown adipose tissue mice presented lower expression of pro-inflammatory cytokines, suggesting a role of p53 in the regulation of obesity-related inflammation (Minamino et al., 2009). Moreover, in the same study it was evidenced that adipose tissue from subjects suffering diabetes showed higher levels of p53 compared with tissue from nondiabetic subjects, and that the expression of inflammatory cytokines was also significantly increased in adipose tissue. In a cohort of 230 obese subjects, omental adipose tissue p53 levels were positively associated with BMI and percent fat mass, and were also correlated with TNF-α expression (Ortega et al., 2013). In this sense, Yahagi and colleagues demonstrated that p53 and its target genes are highly induced in adipocytes of ob/ob mice, and that the disruption of p53 in this mice restores the expression of lipogenic enzymes (Yahagi et al., 2003). Additionally, there are considerable evidence pointing to a crucial role for p53 in CVD (Sano et al., 2007). In our study, those subjects who responded better to the diet had lower expression levels of *TP53* than LR. It can be speculated that LR had higher inflammatory state than HR, and that this inflammation could trigger an activation of *TP53*. Our findings of decreased whole blood *TP53* mRNA levels in HR are consistent with other studies, since it has been observed that *TP53* is down-regulated in WBC from obese subjects with T2D after bariatric surgery, suggesting an up-regulation of *TP53* in WBC of obese subjects (Berisha et al., 2011).

Regarding miRNA regulation, *TP53* is a target gene of several miRNAs such as miR-125b, miR-504, miR-1285, miR-92, miR-141, miR-380-5p, miR-15a, miR-16, miR-25, miR-30d, miR-25, miR-30d, miR-200a, miR-453, miR-98, miR-19b, miR-518c and miR-638, whose interactions have been experimentally validated (Vijayakumaran et al., 2015).

Remarkably, miR-1285 has the same seed sequence as miR-612. However, in previous studies Tian et al. found that the luciferase activity of the p-LUC-p53-3'-UTR reporter did not change when was transiently transfected with miR-612 (Tian et al., 2010). Nevertheless, our data showed that miR-612 binds to the 3'-UTR of *TP53* and that there exists a negative relationship between miR-612 levels and *TP53* expression (in blood and in the microarray), suggesting that miR-612 could regulate *TP53* expression.

Otherwise, *CD40* gene encodes CD40, a surface glycoprotein that is expressed in both hematopoietic and nonhematopoietic cells, and that participates in the stimulation of adaptive immunity (Fujii et al., 2004). *CD40* is expressed in different cells involved in adipose tissue inflammation, as for example leukocytes, adipocytes and stromal cells of adipose tissue (Poggi et al., 2009); and has relevant roles in the regulation of adipose tissue metabolism (Chatzigeorgiou et al., 2013). In inflammation, the interaction of the soluble ligand of CD40 (sCD40) with CD40 is known to contribute to inflammatory status inducing pro-inflammatory cytokines and chemokines secretion (Suttles & Stout, 2009). Moreover, adipocyte stimulation with CD40L increases the expression of *CD40* as well as of different adipocytokines, as several authors have reported (Poggi et al., 2009, Chatzigeorgiou et al., 2013). In obesity-induced adipose tissue, it has been found that loss of macrophage CD40 is not sufficient to induce obesity metabolic dysregulation and that *CD40*-deficiency on other cell-types than macrophages (as for example adipocytes) is responsible for the metabolic dysregulation and adipose tissue inflammation associated to obesity (Aarts et al., 2018). Concerning obesity, the soluble ligand of sCD40L is increased in obese adults (Desideri & Ferri, 2003, Santilli et al., 2007) and children (Luciardi et al., 2018), as well as in T2D individuals (Cipollone et al., 2005), and subjects with MetS (Angelico et al., 2006). Notably, studies in rodents have shown that Cd40 deficiency could prevent vascular inflammation, atherosclerosis and obesity-associated insulin resistance (Lutgens et al., 2010, Chatzigeorgiou et al., 2014), presenting regulation of *CD40* as a therapeutic option in obesity-associated comorbidities. There exists evidence that *CD40* expression could be regulated by miRNAs (Luo et al., 2015, Guo et al., 2016); however to the date there is no available information showing a miRNA-regulation of *CD40* in obese subjects. Here we

present solid evidences that *CD40* is highly expressed in LR compared to HR WBCs, and that its levels correlate negatively with the levels of miR-1976. Moreover, we demonstrated that miR-1976 binds to the 3'-UTR of *CD40* and that, when transfected into mature human adipocytes, mRNA levels of *CD40* decrease, suggesting that miR-1976 could be implicated in adipose tissue inflammation and insulin resistance processes.

In the present dissertation we also identified two additional miRNAs that could distinguish the level of response to a specific weight loss dietary treatment and that regulate the expression of an important gene involved in the regulation of inflammation. Indeed, we found that miR-1185-1 and miR-548q regulate *GSK3B* gene expression.

There are several studies were miRNAs have been implicated in different inflammatory processes. One of these studies was performed by Feng et al. in macrophages when studying miR-21. They showed that overexpression studies in macrophages reduced the secretion of IL-6 and IL-10, implying an anti-inflammatory effect of miR-21 (Feng et al., 2014). miR-24 is another miRNA with anti-inflammatory properties, as studies in LPS-stimulated macrophages have shown (Fordham et al., 2015). Here, transfection of miR-24 inhibits the production of pro-inflammatory cytokines affecting the polarization and plasticity of macrophages. miR-124, miR-145, miR-146, miR-149, miR-155, or miR-181 family are other miRNAs that may act as negative regulators in inflammatory processes (Tahamtan et al., 2018). In this sense, the role of miRNAs in obesity-induced metabolic disorders and immune response has been recently reviewed by Zhong et al. (Zhong et al., 2018).

Regarding inflammation, glycogen synthase kinase-3 (*GSK3*) is one important regulatory gene. GSK3 is a multitasking serine/threonine kinase that acts over 50 substrates (Jope et al., 2007). Initially GSK3 was thought to be only related to the metabolism of glycogen. However, when it was found that GSK3 is a key member of the insulin and Wnt signaling pathways (Jope et al., 2007), the interest in investigating the role of *GSK3* in metabolic complications began growing. It is known that GSK3 enhances the transcription of genes that are activated by NF-κB (Steinbrecher et al., 2005). For example, the presence of an isoform of GSK3

(GSK3B) is necessary to the efficient expression of cytokines such as IL-6 and MCP-1 (Steinbrecher et al., 2005). The inflammatory role of *GSK3* and the importance of its inhibitors is clear since IL-6, IL-1β or TNF-α expressions are promoted by GSK3 (Beurel, 2011). In this sense, we found an important negative correlation between microarray expression levels of miR-1185-1 and serum levels of IL-6, which suggests that the regulation of *GSK3B* by miR-1185-1 may imply a down-regulation of pro-inflammatory cytokines and an attenuation of the inflammatory status.

Several studies have shown the importance of GSK3B inhibitors in reducing inflammation. Certainly, the administration of a GSK3 inhibitor to LPS-induced mice decreased the levels of pro-inflammatory cytokines such as TNF-α, IL6 or MCP-1 (Martin et al., 2005). Consequently, inhibitors of GSK3 seem to provide strong therapeutic protection not only against inflammation, but also against many metabolic-related diseases, such as diabetes or obesity. Gsk3 is increased in obese mice adipose tissue, and its inhibition prevents adipocyte differentiation (Eldar-Finkelman et al., 1999, Bennett et al., 2002). Recently, it has been demonstrated that GSK3 is essential for adipocyte differentiation and that Sfrp1 expression (an obesity-induced gene) can be reversed by GSK3 inhibitors (Wang et al., 2018). Moreover, GSK3 inhibition results in transcriptional suppression of inflammation-related genes (Beurel, 2011) and reverses obesity-induced white adipose tissue inflammation (Wang et al., 2018), suggesting a key role of *GSK3* in obesity progression.

On the other hand, a double knockdown of both isoforms of *Gsk3* (*Gsk3a* and *Gsk3b*) implied a decrease in body weight (Steuber-Buchberger et al., 2008), whereas an overexpression of human *GSK3B* in mice skeletal muscle featured an impaired glucose tolerance, hyperlipidemia and an increase in fat mass and body weight (Pearce et al., 2004). Additionally, Gsk3b activity inhibition in mice ameliorated diet-induced obesity, decreased body weight gain, adiposity, dyslipidemia, and hepatic steatosis (Lee et al., 2013). Altogether, these results suggest that *GSK3* is a key effector of obesity-induced inflammation, and that inhibitors of its activity may be useful in obesity treatment.

Using different –omics technologies, we identified 2 miRNAs that interact with *GSK3B* affecting its mRNA levels. Our results showed that miR-548q and

miR-1185-1 regulate *GSK3B* expression levels in a dose-dependent manner. At least for miR-1185-1, it occurred by the binding of the miRNA over the specific 3'-UTR *GSK3B* region. As commented in the introduction, (Epigenetic Regulation of miRNAs section), miRNAs (epi-miRNAs) could regulate the epigenetic machinery and may be involved in an indirect epigenetic regulation. Thus, epi-miRNAs could affect histone methyltransferases disturbing DNA or histones methylation (Moutinho & Esteller, 2017). In this context, the regulation of miR-548q over *GSK3B* could happen in an indirect manner. On the other hand, it is possible that miR-548q could target some *GSK3B* enhancers or transcription factors, resulting in an ultimate reduction of *GSK3B* mRNA levels.

Although not in inflammation, there are several studies reporting interactions between *GSK3B* and miRNAs in neoplasia and cancer. Regarding gastric tumors, *Gsk3b* knockout mice showed higher levels of miR-92, miR-182 and miR-183 (Tang et al., 2014). On the other hand, in hepatocellular carcinoma, GSK3B increased miR-122 levels (Zeng et al., 2010) and activated the expression of miR-181 (Ji et al., 2011).

To our knowledge, this is the first time that miR-1185-1 and miR-548q have been proposed as biomarkers of weight loss that could predict the level of response to dietary treatments. Moreover, we showed evidences about the involvement of these miRNAs in *GSK3B* gene expression regulation, suggesting a role of miR-1185-1 and miR-548q in energy homeostasis, inflammation and body weight control. Furthermore, our results propose that *GSK3B* could be an important mediator of weight control, and that its expression could also early differentiate patients that are going to respond well to the dietary treatment of obesity.

Strengths and Limitations

The present project uses –omic approaches to find predictive miRNA-type biomarkers of obesity and weight loss in child and adult populations.

One of the main strengths of the present research is the robust designs of the GENOI and RESMENA studies. The design of the studies allowed to obtain a general perspective of the obesity pathology between obese and non-obese individuals, and

then to separate the higher responders to a weight loss intervention from the lower responders. The GENOI study has an important advantage: children in that stage of life are not affected by pernicious lifestyle practices and there are not obesity-associated comorbidities. Moreover, the RESMENA study, as a prospective study, allowed to analyze different variables at two different stages.

The –omic approaches used in the present dissertation have been demonstrated to be useful for identifying transcriptomic patterns related to different phenotypes and populations, categorizing subjects into groups of response to a given dietary strategy. Indeed, combining methylome and transcriptome –omic analyses improves the understanding of biological mechanisms related to obesity and associated comorbidities. In this sense, we used three different high-throughput technologies (methylation and expression microarrays and miRNA-Seq) in parallel with validation studies in larger populations (MassArray EpITYPER, qPCR or *in vitro* models) that has been proved to be a useful strategy to achieve our aims of discovering new potential biomarkers of disease diagnosis, prognosis and therapy (Gong et al., 2014).

Moreover, we used *in vitro* experiments to confirm the biological effects of identified miRNAs over their target genes. Thus, we designed expression target vectors that encoded the 3'-UTR of each target gene. This approach is one of the strongest points of the current work, since it is the most accurate strategy to validate the binding of a miRNA on target genes (Elton & Yalowich, 2015). With this strategy we demonstrated that miR-612, miR-1976 and miR-1185-1 bound to those target genes that were previously selected by bioinformatics predictions (*TP53*, *CD40* and *GSK3B*, respectively).

Additionally, for overexpression experiments, we used a THP-1 cell model that has been widely used to study immune responses due to the possibility to differentiate cells into macrophage-cells like, and because they present a relative equal response patterns than WBCs. However, some differences have been reported concerning gene expression and cytokine secretion (Schildberger et al., 2013). Furthermore, we also used primary adipocyte cultures from subcutaneous WAT.

This work was not avoided of limitations. Since our main objective was to find predictive biomarkers of obesity or weight loss, we selected WBCs as sample type

for further studies. Although blood is not a metabolically relevant tissue for the study of obesity or comorbidities, blood sampling is one of the easiest and non-invasive procedures to obtain RNA and DNA, and it has been demonstrated that blood-based biomarkers reflect the DNA methylation changes in key metabolic tissues as adipose tissue (Crujeiras et al., 2017), which supports the use of these type of sample type for studying the importance of epigenetic in metabolic disorders.

Regarding the pediatric cohort, factors that are involving in the perinatal period (smoke exposure, preterm newborns, gestational diabetes, maternal diet, etc.) are able to impact in the DNA methylation levels and future health outcomes. Thus, the mentioned factors should be considered in future studies. Another shortcoming in chapter 1 was sample availability. Given that there were no RNA samples from the individuals that participated in the GENOI study, we could not establish a relationship between miRNA methylation levels and expression. Therefore we only could hypothesize about the underlying biological processes. Indeed, we used bioinformatics software to predict putative target genes of the identified miRNAs. However, because of the lack of RNA and protein samples, we could not confirm in the GENOI samples the regulatory mechanisms of miRNAs over their predicted target genes.

Another limitation is the relative small sample size, especially in the high-throughput studies, which might limit the statistical power to detect differences between methylation and expression levels of miRNAs. This issue could lead to an increase in the risk of type II errors (failing in the detection of real differences). Likewise, differences in miRNA expression between HR and LR in Chapters 2 and between HR and LR were not significant after multiple comparisons (Benjamini-Hochberg method), implying in type I errors (accepting as valid results that are not real). Nonetheless, since the objective of the project was to select candidates to act as biomarkers, we used raw microarray and miRNA-Seq data, and selected miRNAs that were found in at least two platforms. Then, we further confirmed the results by qPCR, MassArray EpiTYPER, as well as by *in vitro* cell co-transfections.

Lastly, in some cases we were not able to validate expression microarray data due to the low expression levels of selected miRNAs in blood; and we failed when trying to validate methylation microarray data because we were not able to design primers for MassArray EpiTYPER validation.

For these reasons, further studies in larger and more diverse populations are needed for validating the impact of the observed miRNAs over obesity and inflammation, and to deepen into the molecular mechanisms involved in the regulation of these miRNAs.

Concluding Remarks

Summing up, the results of the current research have demonstrated that miRNAs are implicated in obesity and accompanying comorbidities through the regulation of the expression of important genes involved in inflammation and metabolism. Moreover, we have shown that miRNA DNA coding sequences could be epigenetically regulated and that methylation levels may distinguish between obese and non-obese subjects in a young population. The application of different epigenetic and transcriptomic high-throughput technologies enabled to find plausible miRNA biomarkers to predict obese susceptibility at early stages or the response to diet-intervention. Indeed, 16 differentially methylated CpGs in miRNA DNA coding regions when comparing obese and normal-weight children were found. Furthermore, miR-1203, miR-412 and miR-216A differed in their methylation levels between obese and non-obese children, and showed significantly correlations whit BMI-SDS, explaining up to 40% of the variation of BMI-SDS. In addition, the pathways and target genes of the identified miRNAs highlighted the relevance of epigenetic miRNA-coding region regulation in childhood obesity development.

On the other hand, in a cohort of adult obese subjects underwent a weight loss trial, 6 miRNAs were simultaneously hypomethylated and overexpressed in the group that responded well to the diet (HR). Certainly, miR-612 and miR-1976 were hypomethylated in HR, their expression levels were up-regulated, and their target genes were down-regulated. Specifically, miR-612 and miR-1976 regulate *TP53* and

CD40 mRNA levels respectively, which are genes with important obese-related effects. This regulation is mediated by the binding of the miRNAs to the specific 3'-UTR of each gene.

The integration of two deep-sequencing transcriptomic technologies identified two miRNAs that regulated *GSK3B*, a gene that has been associated with obesity-induced inflammation. miR-548q and miR-1185-1 appeared overexpressed in HR, and both miRNAs down-regulated *GSK3B* in *in vitro* studies. Moreover, the regulation of miR-1185-1 over *GSK3B* was mediated by the direct binding over its 3'-UTR site.

As a corollary, the findings of the present investigation confirmed that miRNAs are involved in the pathophysiology of obesity and mediate the individual response to weight loss treatments, being implicated in the interindividual susceptibility to obesity and the response to dietary interventions. -Omics technologies are powerful tools for identifying new epigenetic/transcriptomics biomarkers related to metabolic alterations such as obesity, insulin resistance and inflammation.

Although further studies are needed to understand the molecular mechanisms involving miRNA regulation, the results presented in this dissertation add new insights into the therapeutic uses of miRNAs in the management of human diseases, especially of obesity and inflammation-related diseases.

CONCLUSIONS

Conclusions

1. The methylation study in a children population revealed that sixteen CpGs regulating miRNAs expression were found differentially methylated between obese and normal-weight children, suggesting an epigenetic regulating role of specific miRNAs in childhood obesity.
2. DNA methylation levels in miR-1203, miR-412 and miR-216A coding regions significantly correlated with BMI-SDS and explained up to 40% of the variation of BMI-SDS between obese and normal-weight children.
3. Epigenetic and transcriptomic deep-sequencing studies evidenced that miR-612 and miR-1976 were hypomethylated and overexpressed in obese adults that responded better to the weight loss intervention.
4. miR-612 targeted *TP53* and miR-1976 targeted *CD40* by directly binding over the specific 3'-UTR site of each gene. Moreover, miR-1976 was able to down-regulate *CD40* expression when transfected into mature human adipocytes.
5. The combination of an expression array and miRNA-Seq technology showed that miR-548q and miR-1185-1 were up-regulated in those individuals that responded better to a weight loss intervention, while the mRNA expression of their target gene *GSK3B* was down-regulated. This feature happened through a direct binding of miR-1185-1 over the 3'-UTR of *GSK3B* and by an indirect regulation of miR-548q.
6. Both miR-548q and miR-1185-1 may play a role in the progression of obesity-induced inflammation, and could be new biomarkers and targets against obesity-related complications, given that their target gene *GSK3B* is implicated in diverse inflammatory and metabolic pathways.

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APPENDICES

Appendix 1

Global DNA Methylation in Obesity, Diabetes and Cardiovascular Diseases and the Influence of Environmental Factors

M. Garcia-Lacarte 1, Fermin I. Milagro 1,2 and Alfredo J. Martinez1,2

[1] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.

[2] CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Institute of Health, Madrid, Spain.

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Chapter 14

GLOBAL DNA METHYLATION IN OBESITY, DIABETES AND CARDIOVASCULAR DISEASES AND THE INFLUENCE OF ENVIRONMENTAL FACTORS

M. Garcia-Lacarte[1], *Fermín I. Milagro*[1,2] *and Alfredo J. Martinez*[1,2]

[1]Department of Nutrition, Food Science and Physiology and the Centre for Nutrition Research, University of Navarra, Pamplona, Spain
[2]Instituto de Salud Carlos III, CIBER Fisiología Obesidad y Nutrición (CIBERobn), Madrid, Spain

ABSTRACT

As the prevalence of obesity and related diseases such as cardiovascular disease and type 2 diabetes is increasing worldwide, different strategies and novel approaches are being investigated to manage these pathological conditions. In this context, dynamic changes in DNA as a consequence of environmental interactions have gained interest in understanding these diseases. Indeed, several studies have demonstrated that the epigenome is affected by external factors, such as diet, physical activity, stress, or exposure to chemical carcinogens. Global methylation status is commonly used as a surrogate measure of overall methylation changes. Actually, aberrations in DNA methylation are often related to disease. In this sense, global DNA methylation pattern changes, measured in *LINE-1* and *Alu* sequences, have been associated to body weight regulation and the onset of obesity, cardiovascular diseases and type 2 diabetes, and could be used as possible diagnostic and prognostic biomarkers. In relation to cardiovascular diseases, *LINE-1* is the principal sequence that trends to hyper or hypomethylation depending on the tissue analysed. In type 2 diabetes, changes in *LINE-1* methylation levels have been reported in peripheral blood mononuclear cells (PBMC). Focusing on obesity, several studies have reported changes in the methylation status of both, *LINE-1* and *Alu* sequences. For the near future, the principal challenges in nutriepigenetics are the large number of variables, intermediate markers and measurements to be considered, as well as the dynamic nature of nutrients and the limited outcome information.

Appendix 2

Regulatory roles of miR-155 and let-7b on the expression of inflammation-related genes in THP-1 cells: effects of fatty acids.

J.L. Marques-Rocha [1] & M.Garcia Lacarte [2] & M. Samblas [2] & J. Bressan [3] & J. A. Martínez [4,5,6] & F. I. Milagro [2,4]

[1] Department of Integrated Education of Health, Federal University of Espirito Santo, Vitória, Brazil

[2] Department of Nutrition, Food Science and Physiology; Centre for Nutrition Research, University of Navarra, c/Irunlarrea 1, 31008 Pamplona, Navarra, Spain

[3] Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Brazil

[4] CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Health Institute, Madrid, Spain

[5] IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

[6] IMDEA Food, Madrid, Spain

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ORIGINAL ARTICLE

Regulatory roles of miR-155 and let-7b on the expression of inflammation-related genes in THP-1 cells: effects of fatty acids

J.L. Marques-Rocha[1] · M. Garcia-Lacarte[2] · M. Samblas[2] · J. Bressan[3] · J.A. Martínez[2,4,5,6] · F.I. Milagro[2,4]

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Abstract
The main aim of this investigation was to study the regulatory roles of let-7b and miR-155-3p on the expression of inflammation-associated genes in monocytes, macrophages, and lipopolysaccharide (LPS)-activated macrophages (AcM). A second goal was to analyze the potential modulatory roles of different fatty acids, including oleic, palmitic, eicosapentaenoic (EPA), and docosahexaenoic (DHA), on the expression of these miRNAs in the three cell types. This hypothesis was tested in human acute monocytic leukemia cells (THP-1), which were differentiated into macrophages with 2-O-tetradecanoylphorbol-13-acetate (TPA) and further activated with LPS for 24 h. Monocytes, macrophages, and AcM were transfected with a negative control, or mimics for miR-155-3p and miR-let-7b-5p. The expression of both miRNAs and some proinflammatory genes was analyzed by qRT-PCR. Interestingly, let-7b mimic reduced the expression of *IL6* and *TNF* in monocytes, and *SERPINE1* expression in LPS-activated macrophages. However, *IL6*, *TNF*, and *SERPINE1* were upregulated in macrophages by let-7b mimic. *IL6* expression was higher in the three types of cells after transfecting with miR-155-3p mimic. Similarly, expression of *SERPINE1* was increased by miR-155-3p mimic in monocytes and macrophages. However, *TLR4* was downregulated by miR-155-3p in monocytes and macrophages. Regarding the effects of the different fatty acids, oleic acid increased the expression of let-7b in macrophages and AcM and also increased the expression of miR-155 in monocytes when compared with DHA but not when compared with non-treated cells. Overall, these results suggest anti- and proinflammatory roles of let-7b and miR-155-3p in THP-1 cells, respectively, although these outcomes are strongly dependent on the cell type. Noteworthy, oleic acid might exert beneficial anti-inflammatory effects in immune cells (i.e., non-activated and LPS-activated macrophages) by upregulating the expression of let-7b.

Keywords SERPINE1 · Macrophages · Monocytes · Oleic acid · DHA · EPA

Introduction

Non-coding RNAs are emerging as a critical group of modulators on gene expression. Indeed, some of these regulatory RNAs are termed microRNAs (miRNAs), which control biological and physiopathological phenomena by blocking target genes through the inhibition of protein translation or by promoting mRNA degradation [21]. Recent findings have evidenced that miRNAs play important roles in cell proliferation, differentiation, and development, as well as in the regulation of genes implicated in many inflammation-related diseases. A pioneer research linking miRNA with immunocompetence arrived from miRNA expression analyses in a monocytic cell line treated with lipopolysaccharide (LPS), a toll-like receptor (TLR)-4 ligand [35]. Currently, cells of the immune

✉ F.I. Milagro
fmilagro@unav.es

1 Department of Integrated Education of Health, Federal University of Espirito Santo, Vitória, Brazil

2 Department of Nutrition, Food Science and Physiology; Centre for Nutrition Research, University of Navarra, c / Irunlarrea 1, 31008 Pamplona, Navarra, Spain

3 Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Brazil

4 CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Health Institute, Madrid, Spain

5 IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

6 IMDEA Food, Madrid, Spain

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Appendix 3

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

Ana Gracia [a], Jonatan Miranda [a,c], Alfredo Fernández-Quintela [a,c], Itziar Eseberri [a,c], Marcos Garcia-Lacarte [b], Fermin I. Milagro [b,c], J. Alfredo Martinez [b,c], Leixuri Aguirre [a,c] and María P. Portillo* [a,c].

[a] Nutrition and Obesity Group, Department of Nutrition and Food Science, University of the Basque Country (UPV/EHU) and Lucio Lacaray Research Institute, Vitoria, Spain. E-mail: mariapuy.portillo@ehu.es; Fax: +34-945-013014; Tel: +34-945-013067

[b] Department of Nutrition, Food Sciences and Physiology, Centre for Nutrition Research, University of Navarra, Pamplona, Spain

[c] CIBERobn Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Spain

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

Ana Gracia,[a] Jonatan Miranda,[a,c] Alfredo Fernández-Quintela,[a,c] Itziar Eseberri,[a,c] Marcos Garcia-Lacarte,[b] Fermín I. Milagro,[b,c] J. Alfredo Martínez,[b,c] Leixuri Aguirre[a,c] and María P. Portillo*[a,c]

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 the 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^{-1} d^{-1}). A miRNA microarray was carried out in perirenal adipose tissue. The 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 (*ppary*: peroxisome proliferator-activated receptor gamma, *hsl*: hormone sensitive lipase and *sp1*: SP1 transcription factor) in the 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.

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1 Introduction

Resveratrol has been shown to elicit anti-obesity properties in animal models such as mice,[1–3] rats[4–6] and primates.[7] 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.[8,9]

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.[10,11] 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.[12] Epigenetic mechanisms include non-coding RNAs, such as microRNAs (miRNAs).[13]

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.[14,15] MiRNAs are essential regulators of diverse biological processes, comprising lipid metabolism and pre-adipocyte differentiation.[16–18]

[a]Nutrition and Obesity Group, Department of Nutrition and Food Science, University of the Basque Country (UPV/EHU) and Lucio Lacaray Research Institute, Vitoria, Spain. E-mail: mariapuy.portillo@ehu.es; Fax: +34-945-013014; Tel: +34-945-013067

[b]Department of Nutrition, Food Sciences and Physiology, Centre for Nutrition Research, University of Navarra, Pamplona, Spain

[c]CIBERobn Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Spain

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Appendix 4

LINE-1 methylation levels, a biomarker of weight loss in obese subjects, are influenced by dietary antioxidant capacity

Marcos Garcia-Lacarte [1], Fermin I. Milagro [1,2], Maria A. Zulet [1,2], J. Alfredo Martinez [1,2] & Maria L. Mansego [1,2]

[1] Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, University of Navarra, Pamplona, Spain

[2] Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (CIBERobn), Carlos II Health Insitute, Madrid, Spain

Redox Report

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LINE-1 methylation levels, a biomarker of weight loss in obese subjects, are influenced by dietary antioxidant capacity

Marcos Garcia-Lacarte[1], Fermin I. Milagro[1,2], Maria A. Zulet[1,2], J. Alfredo Martinez[1,2], Maria L. Mansego[1,2]

[1]Department of Nutrition, Food Science and Physiology, Centre for Nutrition Research, University of Navarra, Pamplona, Spain, [2]Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición (CIBERobn), Carlos II Health Insitute, Madrid, Spain

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Objectives: Epigenetic markers, and in particular DNA methylation, have come to the fore as new tools in the personalization of the treatment of obesity and its comorbidities. The objectives of the current investigation were to identify epigenetic biomarkers that might be predictive of response to a weight-loss intervention, and to better understand the influence of certain nutrients (particularly antioxidants) on the epigenome.
Methods: Global DNA (*LINE-1*) methylation levels were assessed in peripheral blood mononuclear cells (PBMCs) from 96 obese volunteers of the Metabolic Syndrome Reduction in Navarra study, using a methylation-sensitive high resolution melting approach after bisulfite modification.
Results: Baseline *LINE-1* DNA methylation levels were significantly higher (5.41%) in high responders (>8% of weight loss) as compared to low responders (<8%) to the energy-restricted treatment. Indeed, a *LINE-1* methylation higher than 84.15% may be predictive of a high response to the hypocaloric diet. Statistically significant correlations were found between *LINE-1* baseline DNA methylation levels and the response to the treatment involving total fat mass and body weight. Furthermore, *LINE-1* baseline methylation levels positively correlated with baseline dietary total antioxidant capacity (TAC).
Discussion: *LINE-1* methylation levels in PBMCs might be used to predict response to a dietary weight-loss intervention, and seem to be related to the dietary TAC.
Trial Registration: www.clinicaltrials.gov: NCT01087086.

Keywords: Antioxidants, Biomarkers, Epigenetic, Energy-restriction, *LINE-1*, Methylation, Obesity

Background

Obesity, which is defined as the excessive accumulation of adiposity in relation to body mass, occurs as a result of an imbalance in energy homeostasis between energy intake and energy expenditure.[1] Given the steady increase in the prevalence of obesity,[2] there is an urgent need to find new robust biomarkers that could lead to better prevention of the disorder and a more personalized form of treatment.[3] In recent years, different epigenetic biomarkers have been identified in relation to the personalized response to different dietary approaches to treat obesity.[4,5]

Epigenetics is defined as the heritable changes in gene activity and expression that occur without abnormalities in DNA sequences, and is involved in the onset and progression of obesity and obesity-related diseases.[6] At a general level, DNA methylation is usually assessed in peripheral blood mononuclear cells (PBMCs) by measuring repeat interspersed regions, such as long interspersed nucleotide element 1 (*LINE-1*) or *Alu* regions, a kind of short interspersed nucleotide element (*SINE*). PBMCs, which have the longest life span of all blood cells, are considered to best represent the biological changes related to environmental exposures or modifications to lifestyle and behavior.[7] On the other hand, *LINE-1* and *SINE* sequences are retrotransposons, mobile sequences that use a 'copy-and-paste' mechanism to change their location and their copy number using an RNA intermediate.[8] In the case of obesity, one trial reported that a high or low birth weight, as well as premature birth, was associated with significantly lower *LINE-1* methylation levels.[9] Similarly, Perng *et al.* found that *LINE-1* hypomethylation was related to the development of adiposity in boys.[10]

Although nutrition plays a role in disease prevention, the interactions between the epigenome

Correspondence to: J. Alfredo Martinez, C/Irunlarrea 1, University of Navarra, 31008 Pamplona, Spain.
Email: jalfmtz@unav.es

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