Universidad de Navarra

Facultad de Farmacia y Nutrición

Epigenetic biomarkers in obesity, weight loss and inflammation: a role for circadian rhythm and methyl donors

Mirian Samblas García

Pamplona, 2018

Universidad de Navarra

Facultad de Farmacia y Nutrición

Memoria presentada por Dña. Mirian Samblas García para aspirar al grado de Doctor por la Universidad de Navarra.

Mirian Samblas García

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación y Fisiología de la Facultad de Farmacia y Nutrición de la Universidad de Navarra y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 26 de Febrero de 2018

VºBº Director

Dr. Fermín I. Milagro Yoldi

VºBº Co-Director

Prof. J. Alfredo Martínez Hernández

Este trabajo ha sido posible gracias a la financiación de diversas entidades: Ministerio de Economía y Competitividad (AGL2013-45554-R), Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBERObn), Instituo de Salud Carlos III (ISCIII), Centro de Investigación en Nutrición (Universidad de Navarra). La investigación que ha dado lugar a estos resultados ha sido impulsada por la beca predoctoral 2014-2015 del Centro de Investigación en Nutrición y las becas predoctoral 2015-2018 y de movilidad del Ministerio de Educación, Cultura y Deporte.

“Necesitamos especialmente de la imaginación en las ciencias. No todo es matemáticas y no todo es simple lógica, también se trata de un poco de belleza y poesía”

Maria Montessori

Dedicado a las dos personas que me lo han dado todo,

Mi aita Ray,

Mi ama Ana

Con especial cariño a,

Mi hermana, Bea

Mi sobrina, Aroa

Mi abu, Esther

Agradecimientos/Acknowledgements

En estas líneas quisiera mostrar mi más sincero agradecimiento a todas aquellas personas e instituciones que han hecho posible la actual tesis. No hay palabras ni espacio suficiente para plasmar todo el apoyo, ayuda, fuerza, ánimo y cariño que se me ha dado, y solo me queda daros las gracias, MUCHAS GRACIAS a cada uno de vosotros.

Mis primeras palabras de agradecimiento me gustaría dedicarlas a los directores de la presente tesis el Dr. Fermín Milagro y el Prof. Alfredo Martínez. En primer lugar quisiera expresaros mi agradecimiento por la confianza depositada en mí desde el primer momento y haberme dado la oportunidad de realizar este trabajo. Gracias por haber guiado y dirigido esta tesis. Juntos habéis formado el equilibrio perfecto para guiarme y dirigirme a lo largo de esto años de trabajo. Sé que no siempre ha sido fácil, sin embargo siempre he contado con vuestro tiempo, implicación, paciencia y apoyo. Ha sido un placer poder crecer profesionalmente y personalmente junto a dos grandes investigadores y personas. Os agradezco todo el esfuerzo invertido en mí y sinceramente espero haber cumplido, aunque sea mínimamente, vuestras expectativas. Muchísimas gracias.

Quisiera agradecer al Centro de Investigación en Nutrición de la Universidad de Navarra (beca predoctoral) y al Ministerio de Educación, Cultura y Deporte (beca predoctoral y de movilidad) por la financiación recibida durante estos años de tesis doctoral, sin la cual todo esto no hubiera sido posible. Muchísimas gracias a la Universidad de Navarra, en especial a la Facultad de Ciencias, en la que comencé mi formación ya hace 11 años, y a la Facultad de Farmacia y Nutrición por haberme acogido y haberme ofrecido la oportunidad de comenzar mi formación como investigadora.

No quisiera dejar de agradecer a todo el personal del Departamento de Ciencias de la Alimentación y Fisiología que han hecho más fácil este camino. A las super técnicos Ana, Verónica, Neira y María que son fundamentales en el departamento. A nuestra gran secretaría Alba, gracias por tener siempre una sonrisa y haberme ayudado en todo lo posible. A los grandes profesores e investigadores, que siempre me han ayudado cada vez que lo necesitaba. A Paula por estar siempre dispuesta a ayudarme. A Jose Ignacio por su humor y su buen rollismo que nos alegra cada día. A Marian y Amelia por hacer más divertidos los ratos del café. A Pedro y Silvia por sus consejos. A Jaione por haberme ayudado a desarrollarme como profesora de prácticas. A Eva, Carlos, Santiago y Xavi gracias por vuestras conversaciones tan provechosas.

Además, no quisiera olvidarme de todos aquellos doctorandos (ya doctores) que formaron parte del desarrollo de las intervenciones, ya que sin ser conscientes han hecho posible que yo pueda realizar esta tesis. Y como no, gracias a los voluntarios que han participado en cada uno de los estudios aplicados en el presente trabajo, ya que sin ellos esto no hubiera sido posible.

Así mismo, me gustaría agradecer de todo corazón a todos los compañeros y amigos que han hecho el camino junto a mí todos estos años de tesis. Gracias a todos los compañeros con los que compartí mis primeros momentos y ya no están, por haber tenido la oportunidad de haber conocido grandes investigadores y mejores personas. Kike, bioinformático y amigo, gracias por toda tu ayuda y por todos los ratos que pasamos juntos. Amaya y Leire, comenzamos juntas esta aventura y me enorgullece decir que juntas la estamos acabando pero como amigas. Amaya, Leire, Irene y Marcos habéis sido un pedazo de apoyo en todos estos años profesionalmente y personalmente. Sé que os esperan grandes cosas y espero vivirlas junto a vosotros. No puedo expresar con palabras todo el agradecimiento que siento, solo os puedo decir que parte de esta tesis os pertenece. Gracias a Elisa y Ana, mis andaluzas favoritas, por todos los buenos momentos que hemos vivido juntas y porque ¡sois increíbles! Gracias a Jorge, Cris, Ana A., Lidia, Lucía, Amanda y Angy por todas las risas y los buenos momentos que hemos tenido. De verdad, muchas gracias a todos, sois una parte importante de este trabajo.

No me puedo olvidar de mis amigas de Brasil. Muchas gracias de coraÇão a Carol y Julia por haberme dado la oportunidad de conoceros no solo profesionalmente si no personalmente. Hemos disfrutado de muchos momentos juntas, aquí en Pamplona y allá en Brasil, pero sé que son aún muchos los que nos quedan por vivir. ¡Muchas gracias! Obrigada!

I wish to express my sincere gratitude to Dr. Miguel Constanza for giving me the opportunity to stay and work with your team at the Cambridge University. Thank you to all team for the warm welcome. Many thanks for the kind help to Niamh.

Gracias a mis bioamigas por todos estos (11) años, porque aunque cada una estemos casi en una punta del mundo, cuando nos juntamos es cómo si no hubiera pasado el tiempo. En especial a Teresa, Bea y Miriam, sé que os esperan grandes éxitos en la vida y estoy muy orgullosa de vosotras.

Muchísimas gracias a mis "Neskatxis" por estar siempre conmigo y preguntarme sobre mi trabajo, sé que os parece confuso pero siempre me escucháis con total atención. No sabéis la suerte que tengo de contar con vosotras y vuestra amistad. Muy en especial a mi amiga Irantzu, por compartir tantos momentos juntas, apoyarme en mis "tesis bajones" y saber sacarme siempre una sonrisa. ¡Maite zaituztet!

Maitia, has sido mi mejor apoyo en estos años. Eres parte de esta tesis, no solo porque hayas sido el ilustrador de este trabajo, sino también por haber caminado junto a mí en todo este proceso. Gracias por tu cariño, paciencia y sobre todo compresión, gracias por sacarme miles de carcajadas, gracias por hacerme tan feliz.

Y por último, gracias a mi familia. A ti abuela porque siempre has sido la luz de mi vida. Gracias Bea por estar siempre ahí y haberme ayudado toda mi vida. ¡Eres la mejor hermana del mundo! Gracias a

Oskar y a ti por todos esos ratos, cenas, risas y conversaciones que me ayudan a desconectar. Pero sobre todo gracias por Aroa, la única que consigue que con solo mirarla se me olviden todas las preocupaciones. Mis últimas palabras de agradecimiento pertenecen a mis padres. Muchísimas gracias ama y aita por todo lo que habéis hecho por mí. Sois lo mejor y más importante de mi vida, sin vuestro apoyo, cariño, consejos, ánimo y amor no hubiera llegado a este momento. Ni en mil vidas os podría agradecer y devolver todo lo que me habéis dado. Os quiero con toda mi alma y corazón, gracias.

List of abbreviations

5mC	5-methylcytosine
AA	Arachidonic acid
ABCG1	ATP binding cassette subfamily G member 1
AHA	American heart association
Alu	Transposable element characterized by the action of the *Arthrobacter luteus* restriction endonuclease
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMAL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
BMI	Body mass index
BMI z-score	Conversion of BMI values into standard deviation scores
BP	Blood pressure
CAMKK2	Calcium/calmodulin-dependent protein kinase kinase 2
CCL	C-C motif chemokine ligand
CD40	Cluster of differentiation 40
CD44	CD44 molecule (Indian blood group)
cDNA	Complementary DNA
CGI	Cpg islands
CLOCK	Circadian locomoter output cycles kaput
CpG	Cytosine linked by a phosphate to guanine
CPT1A	Carnitine palmitoyltransferase 1A
CRY1	Cryptochrome 1
CVD	Cardiovascular disease
DHA	Docosahexanoic acid
DHF	Dihydrofolate
DMR	Differentially methylated region
DNMT	DNA methyltransferase
EPA	Eicosapentaenoic acid
ESRA	Estrogen receptor 1
EWAS	Epigenome-wide association study
FA	Fatty acid

FHL2	Four and a half LIM domains 2
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GENOI	Grupo de estudio Navarro de la obesidad infantil
hcy	Homocysteine
HDL-C	High-density lipoprotein cholesterol
HFD	High fat diet
HIF3A	Hypoxia-inducible factor 3 alpha
HOMA	Homeostatic model assessment
HR	High responders
IBD	Inflammatory bowel disease
IFN	Interferon
IGF2	Insulin-like growth factor 2
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IPA	Ingenuity pathway analysis
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
KLF14	Kruppel like factor 14
LEP	Leptin
LINE	Long interspersed nuclear element
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
LR	Low responders
MBD	Methylated DNA-binding
MECP2	Methyl-cpg binding protein 2
MEG3	Maternally expressed 3
MetS	Metabolic syndrome
miRNA	MicroRNA
mRNA	Messenger RNA
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase

n-3	Omega 3
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ncRNA	Non-coding RNA
NF-κB	Nuclear factor-kappa B
NR3C1	Nuclear receptor subfamily 3 group C member 1
PA	Palmitic acid
PAI-1	Plasminogen activator inhibitor-1
PBMC	Peripheral blood mononuclear cells
PDGFA	Platelet derived growth factor subunit A
PDK4	Pyruvate dehydrogenase kinase 4
PER2	Period circadian regulator 2
PER3	Period circadian regulator 3
PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase 1
POMC	Pro-opiomelanocortin
PPARD	Peroxisome proliferator-activated receptor delta
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pparγ	Peroxisome proliferator-activated receptor gamma
PTPRS	Receptor-type tyrosine-protein phosphatase S
PUFA	Polyunsaturated fatty acid
RESMENA	Reducción del síndrome metabólico en Navarra
SAM	S-adenosyl methionine
SERPINE1	Serpin family E member
SFA	Saturated fatty acid
SHMT	Serine hydroxymethyltransferase
SREBF1	Sterol regulatory element-binding transcription factor 1
STAT	Signal transducer and activator of transcription
T2D	Type 2 diabetes
TC	Total cholesterol
TET	Tet methylcytosine dioxygenase
TFA	Trans fatty acid
TG	Triglyceride
THF	Tetrahydrofolate

TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphobol-13-acetate
TXNIP	Thioredoxin interacting protein
VLDL	Very-low density lipoprotein
WAT	White adipose tissue
WC	Waist circumference

Abstract

Epigenetics refers to all the modifications that alter gene activity without nucleotide sequence modification, but including the chromatin structure alteration as a direct consequence. Indeed, the most widely studied epigenetic mechanism is DNA methylation, which involves the addition of a methyl group onto cytosine nucleotide. DNA methylation may be modified by environmental stimuli including dietary patterns and nutrients. The DNA methylation pattern alteration has been associated with the development of obesity, inflammation and metabolic disturbances (type 2 diabetes, hypercholesterolemia, hypertension, cardiovascular disease). In this context, obesity is considered a contributing factor to the onset and aggravation of the conditions that lead to metabolic syndrome. In the last years it has been reported that low-grade inflammation underlies the pathological processes that are tied to obesity and metabolic syndrome, meanwhile the disruption of the circadian system has also been associated with higher risk to develop obesity-related comorbidities. Furthermore, in the era of "personalized nutrition", the DNA methylation pattern of each individual has emerged as a promising tool for the prediction, screening, diagnosis and prognosis of obesity and related pathologies. Likewise, the modulation of DNA methylation marks by different dietary compounds may be a target for newer therapeutic strategies concerning the prevention and treatment of these diseases.

In this context, this research work has taken advantage of "omics" and high-throughput screening technologies in order to address the following aims: 1) to analyse the association between DNA methylation in white blood cells and the development of obesity in a pediatric population; 2) to investigate the influence of a weight loss intervention in the DNA methylation levels of genes involved in the circadian system, and the association between DNA methylation and changes in the lipid profile; 3) to identify potential epigenetic biomarkers for weight loss within a weight-loss program by integrating transcriptome and methylome microarray data; 4) to evaluate whether a low intake of folic acid is related to adverse metabolic features in obese subjects through changes in gene-specific DNA methylation pattern, and 5) to study whether folic acid and other dietary methyl donors can prevent the inflammatory response in an in vitro model through epigenetic mechanisms.

In relation to the first objective, the results of the first chapter of this thesis suggest a role for DNA methylation, particularly in *PTPRS* and *PER3* genes, in childhood obesity development. Concerning the second objective, we observe that DNA methylation in circadian genes, particularly in *BMAL1*, is dependent on dietary factors such as energy and carbohydrate intake, and could be used as a biomarker of the lipid profile response to the diet. The third chapter demonstrates that *CD44* may have a role in body weight regulation, and its methylation levels can be used as a predictor of the success to a weight-loss intervention. The fourth chapter evidences that subjects with lower folate intake showed more adiposity and higher circulating levels of insulin, glucose, PAI-1, and cortisol, but lower *CAMKK2* methylation levels. Moreover, *CAMKK2* methylation was negatively associated with HOMA-IR index whereas *CAMKK2* expression positively correlated with insulin resistance, suggesting that the methylation of this gene could be an epigenetic mechanism underlying low folic acid intake-mediated insulin resistance. Finally, in relation to the fifth objective, an *in vitro* study conducted in THP-1 monocytes and macrophages confirms that methyl donors, particularly folic acid, are able to decrease the expression and secretion of several pro-inflammatory mediators like IL-1β and TNF-α, which was accompanied by epigenetic modifications such as increased methylation of *IL1B*, *SERPINE1* and *IL18*.

Resumen

Epigenética hace referencia a todas las modificaciones que alteran la actividad de los genes sin modificar la secuencia de nucleótidos, y sin embargo son capaces de alterar la estructura de la cromatina como consecuencia directa. El mecanismo epigenético más ampliamente estudiado es la metilación del ADN, que normalmente implica la adición de un grupo metilo en el nucleótido citosina. La metilación del ADN puede ser modificada por estímulos ambientales como son los patrones dietéticos y determinados nutrientes. La alteración del patrón de metilación del ADN se ha asociado con el desarrollo de obesidad, inflamación y alteraciones metabólicas (diabetes tipo 2, hipercolesterolemia, hipertensión, enfermedad cardiovascular). En este contexto, la obesidad se considera un factor común que contribuye a la aparición y agravamiento de las condiciones que conducen al síndrome metabólico. En los últimos años se ha observado que la inflamación de bajo grado subyace a los procesos patológicos que están vinculados a la obesidad y al síndrome metabólico. También la alteración del sistema circadiano se ha asociado con un mayor riesgo de desarrollar comorbilidades relacionadas con la obesidad. En la era de la "nutrición personalizada", el patrón de metilación del ADN de cada individuo se ha convertido en una herramienta prometedora para la predicción, el cribado, el diagnóstico y el pronóstico de la obesidad y las patologías relacionadas. Del mismo modo, la modulación de las marcas de metilación del ADN por diferentes compuestos de la dieta puede ser un objetivo para nuevas estrategias terapéuticas relacionadas con la prevención y el tratamiento de dichas enfermedades.

En este contexto, este trabajo de investigación ha aprovechado las tecnologías "ómicas" y de cribado de alto rendimiento para abordar los siguientes objetivos: 1) analizar la asociación entre la metilación del ADN en los células blancas de la sangre y el desarrollo de obesidad en una población pediátrica; 2) investigar la influencia de una intervención dietética de pérdida de peso en los niveles de metilación del ADN de los genes implicados en el sistema circadiano y la asociación entre la metilación de estos genes y los cambios en el perfil lipídico; 3) identificar posibles biomarcadores epigenéticos de pérdida de peso mediante la integración de datos de microarrays de transcriptoma y metiloma; 4) evaluar si una baja ingesta de ácido fólico está relacionada con anomalías metabólicas en sujetos obesos a través de cambios en el patrón de metilación del ADN, y 5) estudiar si el ácido fólico y otros compuestos donantes de metilo pueden disminuir la respuesta inflamatoria en un modelo *in vitro* a través de mecanismos epigenéticos.

En relación con el primer objetivo, los resultados del primer capítulo de esta tesis sugieren un papel de la metilación del ADN, particularmente en los genes *PTPRS* y *PER3*, en el desarrollo de obesidad infantil. Con respecto al segundo objetivo, observamos que la metilación del ADN en genes circadianos, particularmente en *BMAL1*, depende de factores dietéticos tales como la ingesta de energía y carbohidratos, y podría usarse como un biomarcador de la respuesta del perfil lipídico a la dieta. El tercer capítulo muestra que *CD44* puede tener un papel en la regulación del peso corporal, y sus niveles de metilación pueden usarse como un predictor del éxito de las intervenciones de pérdida de peso. El cuarto capítulo demuestra que los sujetos con una menor ingesta de ácido fólico muestran más adiposidad y niveles circulantes más altos de insulina, glucosa, PAI-1 y cortisol, pero menores niveles de metilación del gen *CAMKK2*. Además, la metilación de *CAMKK2* se asoció negativamente con el índice HOMA-IR mientras que la expresión de *CAMKK2* se correlacionó positivamente con la resistencia a la insulina, sugiriendo que la metilación de este gen podría ser un mecanismo epigenético subyacente a la baja resistencia a la insulina mediada por la ingesta de ácido fólico. Finalmente, en relación con el quinto objetivo, un

estudio *in vitro* realizado en monocitos y macrófagos THP-1 confirma que los compuestos donantes de metilo, particularmente el ácido fólico, son capaces de disminuir la expresión y secreción de varios mediadores proinflamatorios como IL-1β y TNF -α, al mismo tiempo que induce modificaciones epigenéticas como el aumento de metilación de *IL1B, SERPINE1* e *IL18*.

Table of contents

INTRODUCTION **1**

1. EPIGENETICS 3

1.1 Definition 3

1.2 Epigenetic mechanisms in gene expression regulation 4

1.3 Factors associated with DNA methylation 11

2. MODULATION OF DNA METHYLATION BY SPECIFIC NUTRIENTS 18

2.1 Methyl donors 19

2.2. Fatty acids 27

2.3 Other dietary bioactive compounds 31

2.4 Future perspectives 31

3. DNA METHYLATION MARKERS IN OBESITY AND RELATED COMORBIDITIES 33

3.1 Obesity, related comorbidities and DNA methylation 33

3.2 DNA methylation and chronobiology in the context of obesity 55

3.3 DNA methylation markers in weight loss 57

4. DNA MARKERS IN INFLAMMATION 60

4.1 DNA methylation and immune cells 61

4.2 DNA methylation and mediators of inflammation 62

4.3 DNA methylation role in obesity and other chronic inflammation-related diseases 64

HYPOTHESIS AND OBJECTIVES **67**

1. HYPOTHESIS 69

2. GENERAL OBJECTIVE 69

3. SPECIFIC OBJECTIVES 69

SUBJECTS AND METHODS **71**

1. STUDY POPULATIONS 74

2. CLINICAL AND BIOCHEMICAL ASSESSMENT (Chapter 1, 2, 3 and 4) 79

3. CELL MODEL AND EXPERIMENTAL DESIGN (Chapter 5) 80

4. DNA METHYLATION ANALYSIS 81

5. mRNA EXPRESSION ANALYSIS 82

6. OTHER TECHNIQUES 83

RESULTS **85**

CHAPTER 1: *PTPRS* and *PER3* methylation levels are associated with childhood obesity: results from a genome-wide methylation analysis 87

Supplementary data CHAPTER 1 89

CHAPTER 2: Methylation on the circadian gene *BMAL1* is associated with the effects of a weight loss intervention on serum lipid levels 99

Supplementary data CHAPTER 2 101

CHAPTER 3: An integrated transcriptomic and epigenomic analysis identifies *CD44* gene as a potential biomarker for weight loss within an energy-restricted program 105

Supplementary data CHAPTER 3 107

CHAPTER 4: Association of low dietary folate intake with lower *CAMKK2* gene methylation, adiposity, and insulin resistance in obese subjects 113

CHAPTER 5: Folic acid improves the inflammatory response in LPS-activated THP-1 macrophages 115

Supplementary data CHAPTER 5 117

GENERAL DISCUSSION **121**

1. EPIGENETIC MARKERS IN CHILDREN OBESITY (CASE-CONTROL STUDY) 123
2. EPIGENETIC MARKERS IN WEIGHT LOSS (INTERVENTION STUDIES) 125
3. EFFECT OF METHYL DONORS ON EPIGENETIC MARKS AND INFLAMMATION 129
4. STRENGTHS AND LIMITATIONS 133
5. COROLLARY 136

CONCLUSIONS **139**

Conclusions 141

REFERENCES **143**

References 145

APPENDICES **175**

Appendix 1: Noncoding RNAs, cytokines, and inflammation-related diseases 177

Appendix 2: Congress communications 179

INTRODUCTION

1. EPIGENETICS

1.1 Definition

Epigenetics concepts were introduced in the early 1940s by the scientist Conrad Waddington, who defined epigenetics such as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being"(Waddington, 1968). Epigenetics term is born from the sum Greek prefix "epi" that means "over" and the word "genetics". So literally, epigenetics refers all the mechanisms of regulation at a level above those of genetic mechanism. Specifically, epigenetics involve all the modifications that alter gene activity without nucleotide sequence modification, but including the chromatin structure modifications as a direct consequence (Dupont et al., 2009).

Epigenetic modifications may be stable and passed on to next generations or may be dynamic (plasticity) and alter as a consequence of environmental stimuli (Abdul et al., 2017). Several studies observed that exposure to environmental factors in one generation caused phenotypical effects in unexposed future generations and considered epigenetic mechanism in origin, calling this phenomenon transgenerational epigenetics inheritance. This transfer is caused because epigenetic modifications during gametogenesis and early embryogenesis are not completely erased; many of them persist and are transferred to the offspring (Campion et al., 2009). Transgenerational epigenetic inheritance has been described in embryonic development, ageing, health and disease (Sharma, 2017). On the other hand, one of the main advantages of epigenetics is the plasticity that allow modifying the epigenetic pattern by the environmental factors, such as dietary intake (Zhang, 2015). Numerous studies have shown that some nutrients affect the pathways involved in the epigenetic mechanism and may prevent the onset or ameliorate the outcome of some diseases (Cianciulli et al., 2016; Tian et al., 2017). These investigations demonstrate that epigenetic mechanisms are involved in the development of a variety of diseases and, through the modifications of environmental factors, specifically the diet, would alter epigenetic marks for the benefit of the individuals.

1.2 Epigenetic mechanisms in gene expression regulation

The epigenetic mechanisms play an elementary role in the regulation of gene activity (Wolffe & Matzke, 1999). From unicellular to eukaryotic organisms, epigenetics is involved in the mechanisms of gene expression (Adhikari & Curtis, 2016; Labbé et al., 2017; Pikaard & Scheid, 2014; Zhang & Pradhan, 2014). Although the epigenetic mechanisms have been considered gene expression repressors, recent studies have described that these mechanisms are also involved in transcriptional activation (Zhang & Pradhan, 2014).

The epigenetic control of gene expression can be considered a normal regulation of cellular activities, which requires the switch on and off of several genes. For example, during embryonic development a large number of different and specialized cell types came from a single totipotent stem cell. The epigenetic mechanisms allow totipotent stem cell to activate and repress needed genes for differentiating into various pluripotent cells. Pluripotent cells in turn also use the epigenetic mechanisms for developing the specialized cell types (Cheedipudi et al., 2014). Sometimes, the epigenome (global epigenetic information of one organism) of a cell type becomes abnormal resulting in a dysregulation of the gene transcription that can trigger the development of diseases. This condition is the case of multiple types of cancer, where the cells gain and lose their epigenomic marks (Baylin & Jones, 2011). However, in recent years there are more and more pathogenic situations, such as obesity, inflammation or metabolic diseases, in which an abnormal epigenetic pattern has been observed (Holmes et al., 2017; Marques-Rocha et al., 2015; Robertson, 2005).

The most relevant epigenetic mechanisms involved in gene activity regulation are histone modifications, non-coding RNAs (ncRNA) and DNA methylation (**Figure 1**).

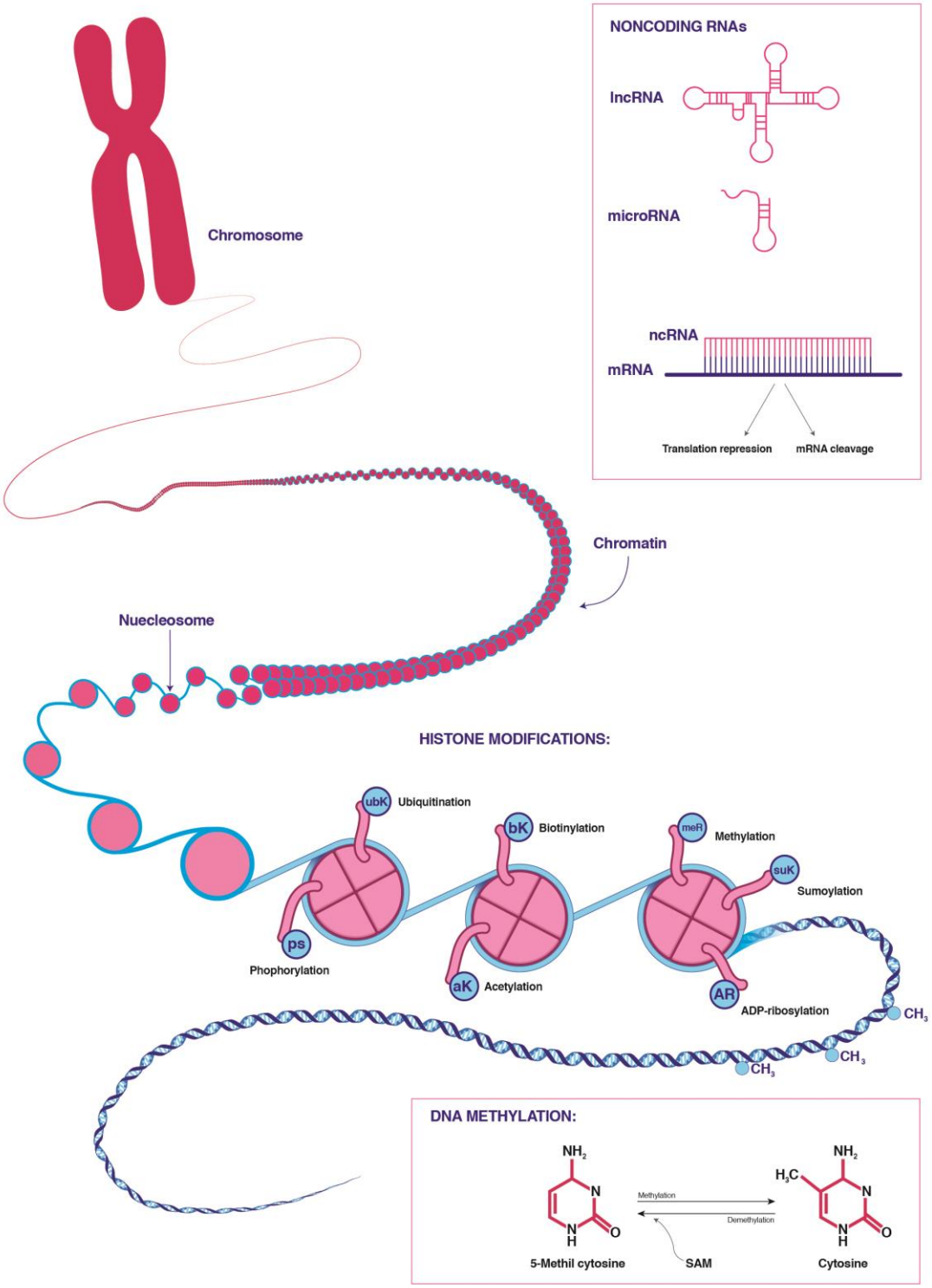


Figure 1. **The most relevant epigenetic mechanisms involved in gene activity regulation**. Adapted from Zaidi et al., (2010). Abbreviations: lncRNA, long non-coding RNA; ncRNA, non-coding RNA; mRNA, messenger RNA; SAM, S-adenosyl methionine.

1.2.1 Histone modifications

Histones are proteins found in the cell nuclei and are involved in the packaging of DNA into structural units named nucleosomes (Luger et al., 1997). There exist five major families of histones (Hs) in the chromatin: H1/H5, H2A, H2B, H3, and H4 (Bhasin et al., 2006). The aminoacids-terminal tail residues of the histones, specifically serine, lysine and arginine residues, are susceptible of being modified by methylation or demethylation, acetylation, phosporilation, ADP-ribosylation, biotinylation, *O*-GlcNAcylation, propionylation, sumoylation, and ubiquitination. These modifications play a central role in the epigenetic regulation of gene expression (Zhang & Pradhan, 2014). The trimethylation of H3 lysine 4 (H3K4), H3K79 and H3K36 have been particularly associated with gene expression activation, whereas the trymethylation of H4 lysine 27 (H4K27), di- and trimethylation of H3K9, and trimethylation of H3K20 have been associated with transcriptional repression (Abdul et al., 2017).

1.2.2 ncRNA (microRNAs and long noncoding RNAs)

ncRNA are RNA molecules that are not translated into proteins. Among others, ncRNA include microRNAs (miRNA) and long noncoding RNAs (lncRNA). miRNA is the most studied ncRNA in relation to gene regulation. miRNA are small ncRNA, comprised of 18-25 nucleotides, which play a role in the post-transcriptional modulation of gene expression. RNA polymerase II transcribes miRNA genes into primary miRNAs. Primary miRNAs are processed in the nucleus by ribonuclease III Drosha and DiGeorge syndrome chromosomal region 8 (DGCR8) into precursor miRNA, which are then translocated to the cytoplasm, where ribonuclease III Dicer modifies precursor miRNA and results in mature miRNA products (Abdul et al., 2017). miRNA regulates gene function through complementarity to messenger RNA (mRNA) sequences. miRNA binds to complementary mRNA sequence. miRNA-mRNA interactions with high degree of match result in mRNA cleavage and degradation, whereas low degree of match causes translational repression (Mohr & Mott, 2015). The repression of mRNA by a miRNA is not complete. Generally, the union of miRNA-mRNA reduces gene expression (Bartel, 2009). Similarly to other epigenetic mechanisms, miRNA expression profiles diverge between normal and diseased tissues. miRNA have been also identified in exosomes and

extracellular vesicles, which can be taken up by neighboring or distant cells and be involved in cell-to-cell communication (Théry, 2011). The principal advantage of secreted miRNAs is their stability in body fluids (blood, saliva, urine, faeces, bile and other fluids), providing the potential for a non-invasive biormarker of prognosis and diagnosis in several diseases (Chen et al., 2008; Wald et al., 2017).

lncRNA are long transcripts from 200 nucleotides to 100 kilobase (kb) that are not translated into protein and are usually spliced, capped and polyadenylated similarly to mRNA molecules (Quinn & Chang, 2015). lncRNA are involved in numerous biological processes including gene expression. Transcription of lncRNA regulated in close genomic proximity or in distant through targeting transcriptional activators or repressors by a variety of mechanisms (Ponting et al., 2009). In the same way to miRNA, lncRNA are also being used as biomarkers of diagnosis in a variety of diseases (Bolha et al., 2017).

Epigenetic ncRNA also include small interfering RNA (siRNA) and piwi-interacting RNA (piRNA) (Kaikkonen et al., 2011). Both of them are short ncRNA similar to miRNA. siRNA mediates post-transcriptional gene silencing as a result of mRNA degradation and induces heterochromatin formation via RNA-induced transcriptional silencing (RITS) complex, which promotes H3K9 methylation and chromatin condensation (Carthew & Sontheimer, 2009). On the other hand, piRNA interacts with piwi family of proteins, regulating chromatin and suppressing transposon activity in germline and somatic cells (Kaikkonen et al., 2011).

1.2.3 DNA methylation

The most extensively studied epigenetic mark in the mammalian genome in relation to gene expression regulation has been DNA methylation (Robertson, 2005). DNA methylation is an epigenetic mechanism involving the covalent addition of a methyl group (-CH3) onto the 5 position of cytosine, resulting in 5-methylcytosine (5mC). Although in mammals methylation generally is restricted to cytosine linked by a phosphate to guanine (CpG site), small percentage of methylation may occurs in non-CpG sites (CHG and CHH, where H = A, C or T) (Lister et al., 2009; Smith & Meissner, 2013).

Dietary folate is reduced to dihydrofolate (DHF) in the intestine and/or liver and subsequently to tetrahydrofolate (THF) via the methionine synthase reaction. Vitamin B_6 provides the enzymatic support to serine hydroxymethyltransferase (SHMT) enzyme necessary for the reversible and simultaneous conversion of L-serine to glycine and THF to 5,10-methyleneTHF (Perry et al., 2007). Furthermore, vitamin B_2 acts as a co-factor of methylenetetrahydrofolate reductase (MTHFR) for the transformation of 5,10-methyleneTHF to 5-methylTHF (Goyette et al., 1994). Cofactor vitamin B_{12} provides the enzymatic support to methionine synthase (MTR) enzyme necessary for the transformation into THF. This form of folate donates the methyl group to homocysteine (hcy) converting it to methionine. In addition, choline by a choline oxidase enzyme is transformed into betaine, which donates the methyl group to hcy for the conversion into methionine. Methyl group of the methionine becomes activated by adenosine triphosphate (ATP) with the addition of adenosine to the sulfur of methionine to transform into S-Adenosyl methionine (SAM). With the help of DNA methyltransferases (DNMTs), methyl group from SAM is finally transferred to cytosine residues of the DNA (Anderson et al., 2012) (**Figure 2**).

Three principal enzymes of the DNMT family are responsible for establishing and maintaining DNA methylation: DNMT1, DNMT3A and DNMT3B. These DNMTs catalyze the transfer of the methyl group from methyl donor SAM onto cytosine. DNMT1 plays the role of maintaining DNA methylation pattern after cell DNA replication cycle, whereas DNMT3A and DNMT3B mediate *de novo* CpG methylation principally during embryogenesis (Rondelet & Wouters, 2017). The activity of these enzymes is connected with a complex regulatory network, in which DNMTs interact with each other and with histones and methylcytosine-binding proteins (Gagnidze & Pfaff, 2013).

Mammalian genomes present approximately 28 million CpG sites, among which 60-80 % is generally methylated. Very CpG-dense regions in the genome are known as CpG islands (CGIs). The majority of the CGIs are located in gene promoter region, but they also appear in gene bodies, often acting as alternative promoters (Saxonov et al., 2006). Contrary to CpG sites, most of the CGIs in promoter regions usually are unmethylated to maintain transcription of the active gene.

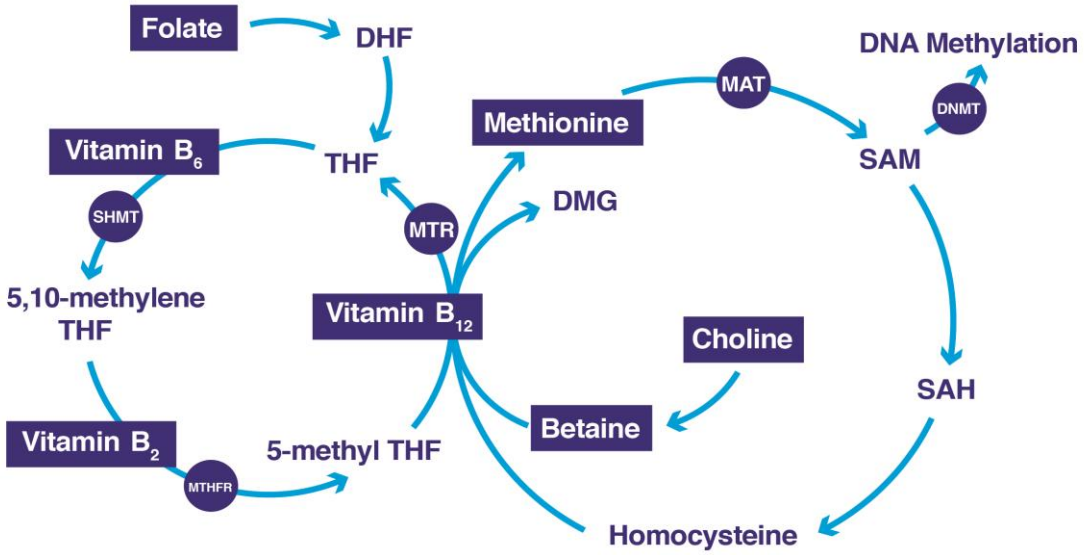


Figure 2. Involvement of dietary micronutrients in one-carbon metabolism. Substrates obtained via diet are highlighted in blue rectangle. Enzymes involved in the metabolism are highlighted in blue circle. Adapted from Anderson (2012). Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; SHMT, serine hydroxymethyltransferase; DMG, dimethyl glycine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MTR, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; MAT, methionine adenosyltransferase; DNMT, DNA methyltransferase.

In this sense, DNA methylation of CGIs of promoter regions has been associated with transcriptional repression, whereas methylation of CGIs in gene body is enriched in highly transcribed genes (Deaton & Bird, 2011). DNA methylation could affect gene expression through three ways: i) by modifying transcription factor-gene promoter binding affinity; ii) affecting the binding between promoter or gene body to methylation-specific recognition factors; and iii) repressing the spatial accessibility of transcription factors or DNA binding proteins to promoters by altering chromatin structure (Zhang & Pradhan, 2014).

DNA methylation is a normal and essential gene regulatory mechanism of the cell and is associated with a number of key processes such as gametogenesis, embryogenesis and aging. It contributes to genomic imprinting, X-chromosome inactivation and repression of transposable elements, but is also involved in several diseases (Reik, 2001; Jones, 2012; Smith & Meissner, 2013b; Nilsson & Ling, 2017). Numerous studies have shown that different stages and types of diseases produce a different epigenetic pattern (Belzeaux et al., 2017; Goel et al., 2017; Leygo et al., 2017; Nicoletti et al., 2017).

In this sense, DNA methylation is a promising biomarker not only for diagnosis, but also for prognosis of different malignancies.

Differentially methylated region (DMR) is an area of the genome where multiple adjacent CpGs show different methylation status between phenotypes; they are regarded as possible functional regions involved in gene transcriptional regulation (Shen et al., 2017). Differential methylation measured regionally is more biologically interpretable and statistically powerful than CpGs measured individually (Zhang et al., 2011). These characteristics allow DMR to be powerful biomarker of biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. DNA methylation profiles might also be modified by environmental factors. Several studies have investigated the CpG methylation dynamics changes as a consequence of the restriction or supplementation with different nutrients. Moreover, physical activity, lifestyle, metals, chemicals, pesticides, and air pollution also alter DNA methylation signature (reviewed in (Abdul et al., 2017). Noteworthy, DNA methylation is being applied as a therapeutic tool in a variety of pathologies through the correct changes of these environmental factors.

Another important epigenetic modification in DNA of mammalian cells has been described: cytosine hydroxymethylation. Hydroxymethylation replaces the hydrogen atom at the C5-position in cytosine with a hydroxymethyl group (**Figure 3**). Wanunu et al., (2011) suggested that hydroxymethylation may affect the flexibility and stability of DNA duplexes differently than common methylation. In addition, it has been demonstrated that hydroxymethylation is involved in gene expression regulation (Severin et al., 2013).

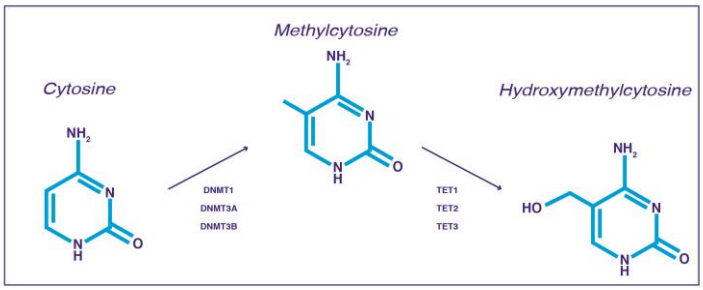


Figure 3. Schematic view of the cytosine hydroxymethylation process. (Kinde, 2015).

A series of current publications demonstrated the existence of non-CpG methylation, including methylation at cytosines followed by adenine (CpA), thymine (CpT) or another cytosine (CpC) in a variety of multi-cellular organism (*Chlamydomonas, Caenorhabditis elegans* and *Drosophila*) and, in a very low levels, in mammalian cells (Koziol et al., 2016; Kigar et al., 2017). Non-CpG methylation could be the result of the hyperactivity of non-specific *de novo* methylation of CpGs by DNMT3A and DNMT3B (Jang et al., 2017). Different reports have indicated that non-CpGs methylation levels in promoter regions are correlated with gene repression (Jang et al., 2017) by affecting chromatin availability to transcription factors and other regulatory protein binding (Pinney, 2014).

New investigations reported the methylation of adenine nucleotide. Adenine is methylated on the exocyclic NH_2 group at the 6-position of the purine ring (Iyer et al., 2016). Increased N6-methyladenine (6mA) has been associated with gene repression and described as an epigenetic biomarker of the development of stress-induced neuropathology (Kigar et al., 2017).

1.2.4 Other epigenetic modifications

The packaging of DNA into nucleosomes may affect all stages of transcription, thereby regulation gene expression (Portela & Esteller, 2010). Indeed, nucleosomes might block the access of activators and transcription factors to their sites on DNA. Thus, the nucleosome positioning determines accessibility of the transcription factors. Several groups of large molecular complexes are known to modify, destabilize or restructure nucleosomes in an ATP hydrolysis-dependent manner. The chromatin remodelling complexes are classified into four families: switch/sucrose non fermentable (SWI/SNF), chromodomain helicase DNA binding (CHD), and INO80 (Portela & Esteller, 2010). Nucleosome positioning has been reported to be involved in several diseases such as cancer, neurodevelopmental disorders, neurodegenerative and neurological diseases, and autoimmune diseases (Portela & Esteller, 2010).

1.3 Factors associated with DNA methylation

1.3.1 Genetic factors

DNA methylation is involved in a variety of biological processes. The DNA methylation regulation depends on a complex machinery interaction that includes DNMTs, methylated DNA-binding proteins

(MBDs) and methyl group metabolism pathway. Mutations in DNMTs, MBDs and methyl group metabolism genes result in an aberrant DNA methylation pattern that may cause numerous human diseases.

Mutations in genes encoding methyltransferases

DNA methylation, as explained before, is mediated principally by DNMT1, DNMT3A and DNMT3B enzymes. Several studies have found numerous mutations in these genes that cause different diseases. For example, there have been described 10 missense mutations (point mutation in a nucleotide results in an aminoacid changing) in *DNMT1* that result in global hypomethylation but site-specific hypermethylation. These mutations are involved in the development of dementia, hearing loss type IE, autosomal dominant cerebellar ataxia, deafness, or Alzheimer's disease (reviewed in (Hamidi et al., 2015). However, the homozygous deletion of *Dnmt1* in mice results in embryonic lethality (Chen & Li, 2006). The missense, nonsense (point mutation in a nucleotide results in a stop codon) and insertion mutations in *DNMT3A* result in hematologic abnormalities and overgrowth syndrome by the focal hypomethylation of specific genes such as Hox genes. Similarly, different mutations in *DNMT3B* have been hypothesized that produce mislocalization of the gene, leading to aberrant *de novo* methylation by affecting SAM binding, methyl group transfer or DNA association. Immunodeficiency and centromeric instability with hypogammaglobulineamia or agammaglobulinemia are some of the diseases produced by mutations in *DNMT3B*. (reviewed in (Hamidi et al., 2015) **(Table 1).**

Mutations in the gene encoding methyl-CpG binding protein 2

The genetic variants of methyl-CpG binding protein 2 (*MECP2*) gene have been extensively studied. More than 600 missense as well as numerous intronic and untranslated region (UTR) mutations have been described. The loss of function of MECP2 protein inhibits the union of different molecules to 5mC. These mutation are associated for example with the Rett syndrome (Hamidi et al., 2015).

Table 1. Different diseases-related phenotypes associated with mutations in *DNMT1, DNMT3A* and *DNMT3B.*

Molecule	Mutation	Phenotype
DNMT1	D506E P507Y Y511C Y511H K521def H569R A570V C596R G605A V606F	Dementia and hearing loss type IE Alzheimer's disease Autoinmune attack of some neurons and mitochondrial dysfunction Autosomal dominant cerebellar ataxia, deafness and narcolepsy
DNMT3A	> 50 % R882 to histidine or other residues 86 more mutations: frameshift deletion, frameshift substitution, deletion, missense and nonsense	Acute myeloid leukemia Myeloproliferative neoplasms Myelodysplastic syndrome T-cell acute lymphoblastic leukemia Angioimmunoblastic T-cell lymphoma Overgrowth disorders Intellectual disability Distintive facial appearance
DNMT3B	S270P	Immunodeficiency, centromeric instability and facial anomalies Defects in B cell maturation and immunoglobulin production

Data from Hamidi et al., (2015).

Mutations in genes of the methyl group metabolism pathway

Several single nucleotide polymorphisms (SNPs) in *MTHFR* are associated with the development of obesity-related non-alcoholic steatohepatitis (NASH) (Mehta et al., 2014). The patients present low levels of methylation and aberrant gene expression (Sazci et al., 2008; Mehta et al., 2014). Moreover, different SNPs in the genes *MTR*, methionine synthase reductase (*MTRR*), cystationine betha-syntase (*CBS*), transcobalamin-II (*TCN2*) and paraoxonase-1 (*PON1*) have been associated with obesity (Bokor et al., 2011). In addition, the missense mutation with the substitution of an arginine for a glutamine (R239Q) in betaine-homocysteine methyltransferase (*BHMT*) gene has been associated with coronary artery disease (Weisberg et al., 2003). Although these genes affect directly the methyl group availability, the effect in genome DNA methylation is still unknown.

1.3.2 Environmental factors

A number of environmental factors have been related to the modification of DNA methylation profile (**Figure 4**).

1.3.2.1 Diet

Prenatal maternal diet

Methylation marks are critical for cellular differentiation of individual's tissues (Bird, 2002). During gametogenesis and embryogenesis, demethylation and remethylation is occurring each time. In this period of high dynamic flux of methylation, any endogenous or exogenous factor could be able to induce changes in methylation. A number of studies have demonstrated existing links between prenatal nutrition and future individual health in early and later life, and the risk of disease (Barker, 2007; Wadhwa et al., 2009). Several researchers have proposed an "epigenetic programming hypothesis", which explains that suboptimal maternal diet induces epimutations in offspring during early embryonic development, and this is maintained during adulthood, sometimes producing diseases (Li et al., 2010). Epimutations may occur in somatic cells and germ cells of the embryo, so the effects of the DNA methylation changes could be seen in the adulthood.

Numerous studies in animals have described a genome-wide alteration in hepatic DNA methylation and gene expression of the offspring after the maternal exposure to high-fat diet (HFD) during pregnancy, and the consequent development of metabolic syndrome (MetS) in early postnatal life (Altmann et al., 2013; Ge et al., 2014; Marco et al., 2014; Seki et al., 2017). In addition, female mice fed a high fat-sucrose diet (HFSD) during pregnancy resulted in DNA methylation changes of genes involved in renin-angiotensin system (RAS) leading to autonomic dysfunction in the male offspring (Mukerjee et al., 2017).

In humans the classical example of the association between maternal diet and DNA methylation was the analysis of individuals born during the Dutch Hunger Winter of 1944-1945. The study showed that the people who were conceived during the famine presented lower methylation of the insulin-like growth factor (IGF) 2 even 6 decades later (Heijmans et al., 2008). On the other hand, the seasonal fluctuation in nutrient intake and the maternal nutritional status of Gambian women modified the DNA methylation pattern of several metastable epialleles in hair follicles and lymphocytes in the offspring

(Waterland et al., 2010; Dominguez-Salas et al., 2014). A recent study has demonstrated the association between circulating cytokine concentrations during pregnancy as a consequence of maternal pro-inflammatory diet, and maternally expressed 3 (*MEG3*) gene methylation in the offspring (McCullough et al., 2017). In addition, the supplementation or the restriction of a range of dietary factors such as folate, methionine, betaine or choline, in maternal diet, has been shown to modify the establishment of DNA methylation profile in the offspring (Pauwels et al., 2017b).

Early postnatal diet

Nutritional programming in infant development may explain the predisposition of some individuals to suffer some chronic diseases such as obesity, type 2 diabetes (T2D), MetS, cognitive and behavioural disorders (Oken & Gillman, 2003; Martínez et al., 2012; Vaag et al., 2012). There are increasing evidences that abnormal DNA methylation is one of the mechanisms by which the early postnatal and infancy nutrition may cause these health problems. For example, a study about the effect of childhood malnutrition demonstrated that the individuals who had moderate to severe protein-energy malnutrition at the first year of life, presented epigenetic dyresgulation associated with attentional and cognitive deficits in adults (Peter et al., 2016). Moreover, the suboptimal diet in early infancy is associated with DNA methylation profile changes and increased risk of obesity in later life (van Dijk et al., 2015). Finally, longer breastfeeding duration is associated with lower levels of infant leptin (*LEP*) methylation and, as a consequence, with lower risk of deficient appetite regulation and obesity in adulthood (Obermann-Borst et al., 2013).

Diet in adulthood

Numerous studies have evidenced that nutrition in adulthood is associated with changes in CpG methylation profile that can translate in beneficial or pernicious outcome. For example, DMRs have been reported in adult adipose tissue in response to calorie restriction (Bouchard et al., 2010). In addition, healthy young men presented widespread DNA methylation modifications in skeletal muscle after a high-fat overfeeding (HFO) diet and, interestingly, changes partially reversed 6-8 weeks after returning to control diet (Jacobsen et al., 2012). A dietary pattern characterized by a high intake of vegetables and fruits prevented against the global DNA hypomethylation caused by high intake of meats, refined products, sweets, deserts, oils, and potatoes (Bouchard-Mercier et al., 2013). Moreover,

seven weeks of diets rich in either polyunsaturated or saturated fat increased mean levels of methylation in human adipose tissue, evidencing different DNA methylation modifications between both diets (Perfilyev et al., 2017). An energy restriction diet also modified pro-inflammatory gene interleukin-6 (*IL6*) methylation after a 6 month treatment (Nicoletti et al., 2016). The change of dietary pattern to a hypocaloric diet altered the DNA methylation of ATPase phospholipid transporting 10A (*ATP10A*) and Wilms tumor 1 (*WT1*) genes in human peripheral blood mononuclear cells (PBMCs) (Milagro et al., 2011). Numerous studies about anorexia, an eating disorder characterized by intense restriction of energy intake and nutrients deficiency, have associated this disease with methylation modifications of norepinephrine transporter (*SLC6A2*), dopamine transporter (*SLC6A3*), dopamine receptor D2 (*DRD2*), *LEP*, brain-derived neurotrophic factor (*BDNF*), oxytocin receptor (*OXTR*) and proopiomelanocortin (*POMC*) genes, and global DNA hypomethylation (reviewed in (Thaler & Steiger, 2017).

1.3.2.2 Physical activity

The benefits of physical activity on health are well known. Exercise prevents or reduces the risk factors for the development of cardiovascular diseases (CVDs), inflammation, obesity, insulin resistance (IR) and other diseases (reviewed in (Ntanasis-Stathopoulos et al., 2013). Recent studies described how the regular physical activity modulated the DNA methylation profile of several genes related with muscle development and metabolism. For example, a study analysing skeletal muscle biopsies of healthy sedentary subjects after an aerobic exercise showed a general hypomethylation of DNA (Barrès et al., 2012). Moreover, acute exercise in healthy men and women produced an intensity-dependent promoter hypomethylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*), pyruvate dehydrogenase kinase 4 (*PDK4*) and peroxisome proliferator-activated receptor delta (*PPARD*) linked with increasing in mRNA expression of these genes (Barrès & Zierath, 2016). Notably, these are genes involved in glucose and lipid metabolism. But not only DNA methylation is altered in skeletal muscle; other organs also response to physical activity. For example, some studies found that adipose tissue also remodelled the CpG methylation pattern after an exercise training program (Rönn et al., 2013; Kanzleiter et al., 2015).

1.3.2.3 Others influencing factors

Stress, drugs or air pollution are examples of factors that may also alter global epigenome signature. Environmental stress studies have described modifications in DNA methylation markers that alter gene transcription in the brain and neuroendocrine systems (Cadet, 2016). A recent review summarized the evidence association between traumatic stress and stress-related disorders (anxiety and depression), and DNA methylation in subjects during prenatal period, early life environment and adolescence/adulthood. A high number of gene methylation and mRNA expression differences were found between health individuals and subjects that suffered a stress episode in the different periods across the life span (Vinkers et al., 2015; Bartlett et al., 2017).

Several studies have described potential DNA methylation modifications that are secondary to drug consumption. For example, subjects that consume alcohol usually presented higher methylation in the genes histone cluster 2 H2A family member c (*HIST2H2AC*) and histone cluster 1 H4 family member e (*HIST1H4E*), altering histone regulation, as compared with controls. Moreover, cocaine administration also increased the expression of *MECP2* and upregulated DNMT3A and DNMT3B in mouse brain resulting in *de novo* DNA methylation. In addition, methamphetamine increased DNA methylation by enhancing DNMT1 expression in the brain. Nicotine also modified the DNA methylation pattern of numerous genes (reviewed in (Brown & Feng, 2017). The exposure to air pollution may be the origin of inflammation, oxidative damage or mitochondrial dysfunction. Some genome-wide DNA methylation studies found an inverse association between long-term exposure to air pollution and global methylation of long interspersed nuclear element (LINE) and Alu transposable elements and other functional regions on the genome including CGI shores and shelves, and gene bodies (De Prins et al., 2013; Janssen et al., 2013; Sanchez-Guerra et al., 2015; Plusquin et al., 2017).

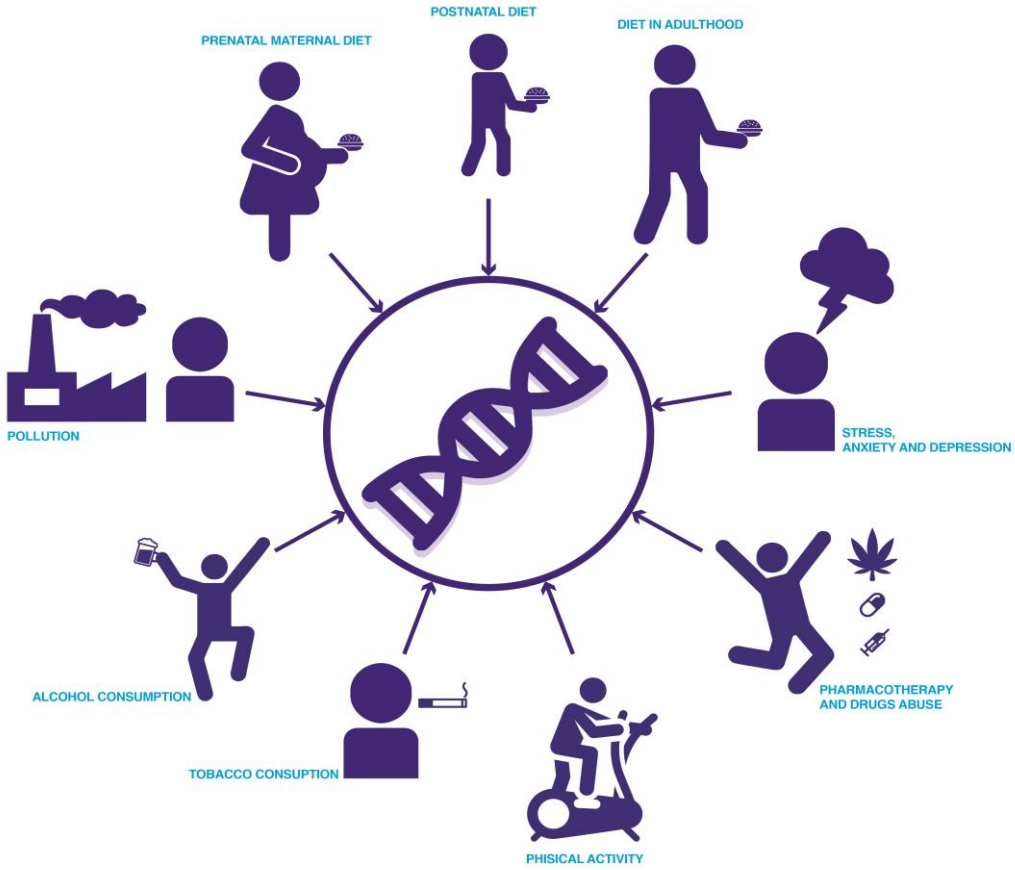


Figure 4. Different environmental factors that modify DNA methylation profile in humans.

2. MODULATION OF DNA METHYLATION BY SPECIFIC NUTRIENTS

The role of nutrition as the base of individual health is widely recognized. In the 19th century the philosopher and anthropologist Ludwig Feuerbach stated that "You are what you eat", without being conscious that his affirmation would literally be true in the area of health. Nowadays, our comprehension is deeper, and the science has studied the associations that exist between the diet intake and its influence in the onset of diseases (*National academy of Sciences*, 1989).

The relationship with the diet might be beneficial or detrimental for the individual health. A numerous research has been investigated the role of the food and food components in the activation of pathways and mechanisms related to cardiovascular and metabolic health, inflammation, cancer or behavioural disorders (Baker et al., 2017; Manning, 2017; Yusufov et al., 2017). However, scientific research has demonstrated how the adherence to specific diets and the consumption of specific foods have positive

health outcomes and improve several diseases (Alissa & Ferns, 2015; Martín-Peláez et al., 2017; Vanamala, 2017).

The discovery of the DNA methylation mechanism and its linker role between food and health enlarged the knowledge in the nutrition study area. More and more research evidence how nutrients act activating pathways that modify the methyl profile. This results in gene expression alterations and in the last instance in beneficial or harmful effects on health (Shimizu, 2017).

2.1 Methyl donors

Methyl donors are substances involved in the DNA methylation process. By definition, methyl donors donate a methyl group to an acceptor molecule. The methyl group can be directly delivered by diet from methionine, choline, betaine, and folate (**Figure 2**). The metabolic pathways of these sources of methyl groups interact and participate in the DNA methylation process. For this reason, folate, choline and betaine supplementation have usually been associated with increased DNA methylation, whereas low intake of these compounds is linked to hypomethylation.

2.1.1 Folate

Folate is a water-soluble member of the B vitamin family with a group of heterocyclic compounds based on the 4-[(pteridin-6-ylmethyl)amino]benzoic acid base conjugated with L-glutamate units (**Figure 5**). The "folate" name includes the many forms of the vitamin, such as THF (activated form of the vitamin), 5-methyl THF, methenyl THF, folic acid (artificial folate), and folinic acid (Cornish-Bowden, 1987).

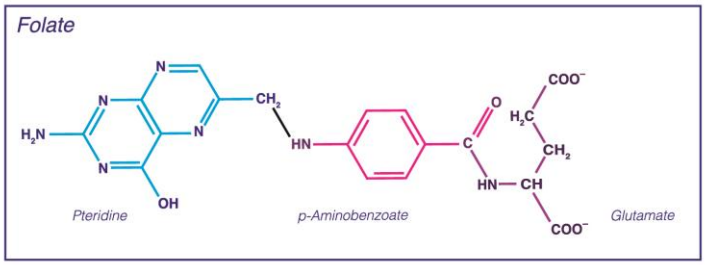


Figure 5. Folate chemical structure.

Folate is present naturally in a wide variety of foods, including green leafy vegetables, fruits, nuts, beans, peas, dairy products, liver and meat, eggs, seafood, and grains (database from Spanish Food Composition Database). Folic acid is the oxidized monoglutamate form that rarely is present in nature

but is contained in fortified foods and vitamin supplements. The bioavailability of food folates depends on the intestinal environment, food matrix, chemical stability, and the presence of other food components (McNulty & Pentieva, 2004).

Folate deficiency is caused by several factors, such as dietary insufficiency. Nowadays, the increasing consumption of HFD and the lower accessibility of folate-rich foods in undeveloped countries result in a reducing intake of folate. This is the case of Mediterranean countries that, although are characterized by a high consumption of vegetables and fish, in the last years the consumption of these products is decreasing, which may explain the current folate deficiency in Mediterranean population (Samaniego-Vaesken et al., 2017).

Folate deficiency has been associated with an increased risk pregnancy complications and birth defects, anemia, depression cancer, CVDs, obesity, and IR (McKay & Mathers, 2016; Li et al., 2017; Thomas-Valdés et al., 2017). The folate deficiency may contribute to an increased susceptibility to develop diseases through the modification of specific gene or genome-wide DNA methylation pattern. Some examples of diseases attributed to folate deficiency acting by DNA methylation mechanism are summarized in **Table 2.**

In the same way, the use of folic acid supplementation for preventing or reducing numerous diseases by DNA methylation mechanisms has been studied (Remely et al., 2015; Fu et al., 2017; Tian et al., 2017). Different studies in animals evidenced the beneficial effect of folic acid. For example, folic acid supplementation reduced the liver lipid accumulation after a HFS diet in rats (Cordero et al., 2011a). Moreover, folic acid normalized DNMTs and methylation levels resulting in an amelioration of brain damage in neurovascular diseases in mice (Kalani et al., 2014). In addition, new studies suggest that folic acid supplementation might be useful in the management of Alzheimer-like disease (Kalani et al., 2014). In humans, folic acid supplementation in Nepalese women during pregnancy resulted in a reduction of MetS prevalence in school-age children (Stewart et al., 2009). Furthermore, folic acid supplementation in overweight and obese men with T2D improved biochemical indices (Gargari et al., 2011), and was effective for stroke prevention in patients with CVDs (Tian et al., 2017). Nonetheless, few studies in humans have analysed the effect of folic acid supplementation on DNA methylation for the prevention and treatment of diseases.

Table 2. Studies analyzing the association between DNA methylation and folate deficiency.

Sample type	Target gene	Outcome	Methylation alteration	Reference
Blood (human)	*IGF-2*	Growth alteration	hypomethylation	(Heijmans et al., 2008)
Blood (human)	*INSIGF, IL10, LEP, ABCA1, GNASAS*	Metabolic disorders	hypomethylation	(Tobi et al., 2009)
Blood (mouse)	genome-wide	Atherogenesis	hypomethylation	(McNeil, 2011)
WBC (human)	*TFAP2A, STX11, CYS1, OTX*	Potential tumorigenic effects in offspring	hypomethylation	(Gonseth et al., 2015)
serum (human)	telomeres	Shorter telomeres in newborn	hypomethylation	(Entringer et al., 2015)
WIL2-NS cells (human)	telomeres	Increase chromosome fusions and terminal deletion	hypomethylation	(Bull et al., 2014)
Sputum (human)	*p16, MGMT, DAPK, RASSF1A, GATA4, GATA5, PAX5a, PAX5b*	Higher risk of lung cancer	abnormal hypomethylation	(Stidley et al., 2010)
Tumor tissue (human / *in vitro*)	genome-wide	Gliomagenesis	hypomethylation	(Hervouet et al., 2009)
Tumor tissue (human)	*APC-1A, P14ARF, P16INK4A, Hmlh1, O6-MGMT, RASSF1A*	Sporadic colorectal cancer	abnormal hypomethylation	(Van Den Donk et al., 2007)
Neonatal immune cells (human)	*ZFP57*	Higher risk of cancer in the offspring	hypomethylation	(Amarasekera et al., 2014)
Liver (mouse)	*ApoE, Foxa1, Foxa2*	Higher severity of NAFLD	hypermethylation	(Tryndyak et al., 2011)

Notes: WBC, white blood cells; WIL2-NS, non secreting B lymphocyte variant of WIL-2 cell line; NAFLD: non-alcoholic fatty liver disease.

2.1.2 Choline and Betaine

Choline and betaine are metabolically-related quaternary ammonium compounds (**Figure 6**). Choline is a water soluble molecule that is considered an essential nutrient since 1998 by the "Food and Nutrition Board" of the American Institute of Medicine (Subcommittee on Upper Reference Levels of Nutrients., 1998)**.** Choline can be acquired from the diet and via *de novo* biosynthesis which occurs in liver. However, *de novo* synthesis is not enough for the human requirements. Choline is present in higher concentrations in chicken, milk, salmon, eggs, wheat germ, and quinoa (Unites States Department of Agriculture Database). However, numerous studies in different populations have found that choline consumption is usually below the adequate intake (Shaw et al., 2004; Bidulescu et al., 2009). Choline is the major dietary source of methyl groups for DNA methylation via SAM (Stead et al., 2006). In addition, choline is involved in the production of phosphatidylcholine,

lysophosphatidylcholine, choline plasmalogen, and sphigomyelin, which are essential components of the cell membranes (Zeisel et al., 1991).

Betaine is specially obtained from wheat bran, wheat germ and spinach but is also synthesized by oxidation of choline (**Figure 5**) (Sakamoto et al., 2002). This is a two-step enzymic reaction in which choline is transformed by choline oxidase into betaine aldehyde, and then betaine aldehyde is converted into betaine by means of betaine aldehyde dehydrogenase (Lin & Wu, 1986). The synthesis of betaine links choline to folate-mediated one-carbon metabolism given that betaine donates one methyl group to hcy for the formation of methionine. Several studies have shown that the diminished folate availability increases the demand of choline/betaine as a methyl group donor, and on the contrary, decreased choline availability increases folate demand, demonstrating the interchangeable sources of methyl donors for the methionine formation (Holm et al., 2007; Fox & Stover, 2008; Ganz et al., 2016).

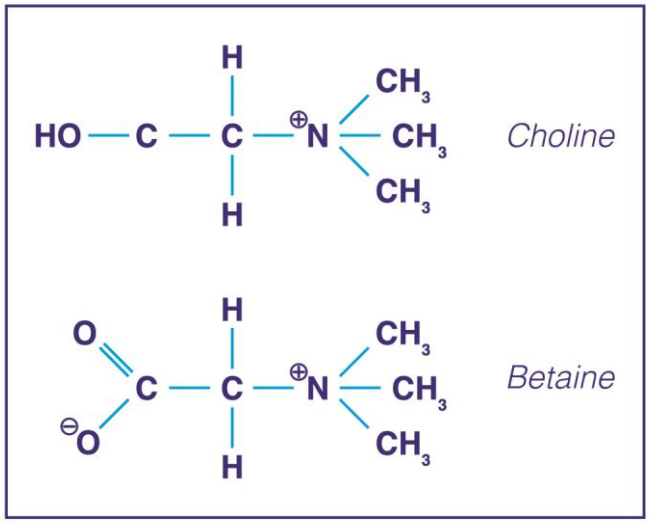


Figure 6. Choline and betaine chemical structures.

Choline and betaine deficiency is involved in the increase of plasma hcy, fatty liver, liver and muscle damage, neural tube defects in offspring, anxiety, CVD, and cancer (reviewed in (Ueland, 2011)). On the contrary, choline/betaine supplementation has been related with amelioration of the adverse effects of alcohol consumption on liver, may prevent liver steatosis, normalizes very-low density lipoprotein (VLDL) secretion, has dose-dependent antiatherogenic effect, improves memory and learning tasks in Alzheimer's disease patients, reduces inflammatory markers in plasma, and might protect against

progression of certain cancers and obesity (reviewed in (Ueland, 2011)) (Gao et al., 2016; Kritis et al., 2016).

The role of betaine and choline in all these functions appears to be beyond their effect on gene methylation. Several studies have looked into the effect of the deficiency and supplementation of choline/betaine on health **(Table 3).**

Studies have demonstrated that the maternal choline and betaine status specially affects DNA methylation pattern in the fetus (Pauwels et al., 2017a). Studies in animals and humans have shown that choline and betaine deficient diets alter the methylation status of *Igf2*, corticotropin-releasing hormone (*Crh*), glucocorticoid receptor (*Nr3c1*), cyclin-dependent kinase inhibitor 3 (*Cdkn3*), vascular endothelial growth factor C (*Vefgc*), angiopoietin 2 (*Angpt2)*, ephrin type-B receptor 2 (*EphB2*), and von Willebrand factor (*VWF*) resulting in growth abnormalities, blood vessel malformation and enhanced predisposition to develop steatohepatitis in offspring (Kovacheva et al., 2007; Medici et al., 2014; Pauwels et al., 2017a; Wankhade et al., 2017; Zeisel, 2017). In addition, adult deficiency is related to risk of cancer (Tsujiuchi et al., 1999; Tryndyak et al., 2011). However, choline and betaine supplementations are related to the correct regulation of lipid, glucose and cholesterol metabolism (Chen et al., 2015; Idriss et al., 2016; Jiang et al., 2016; Zeisel, 2017), reducing lipogenesis (Xing et al., 2011) and regulating offspring body weight (Medici et al., 2014). In humans, maternal choline/betaine supplementation has been associated with offspring genome-wide hypermethylation, *IGF2* DNA methylation and prenatal growth (Pauwels et al., 2017b). Meanwhile, paternal methyl-group donor intake on offspring global and *IGF2* DMR DNA methylation, and prenatal growth (Pauwels et al., 2017b).

2.1.3 Vitamin B_{12}

Vitamin B_{12} (also called cobalamin) is a water-soluble vitamin that contains cobalt element positioned in the centre of a corrin ring (**Figure 7**). Humans are not capable to synthesize B_{12}, only some bacteria and archaea have the needed enzymes for its production. We naturally obtain B_{12} from animal products consumption, such as liver, meat, eggs, milk and shellfish (data from National Institutes of Health). Some gut bacteria are able to produce B_{12} but humans cannot absorb it since the location of the production (colon) is too far from the location of absorption (small intestine) (Gille & Schmid, 2015).

Vegetarians and vegans, who restrict or eliminate consumption of animal-source foods, need to consume B_{12} supplements for avoiding the development of B_{12} deficiency (Pawlak et al., 2013).

The results of B_{12} deficiency are a pernicious anemia with megaloblastic anemia and neuropathy. The neuropathy symptoms include degeneration of the spinal cord, decrease of proprioceptive sensation, spastic in the lower limbs, and sometimes, depression and loss of memory (Truswell, 2007).

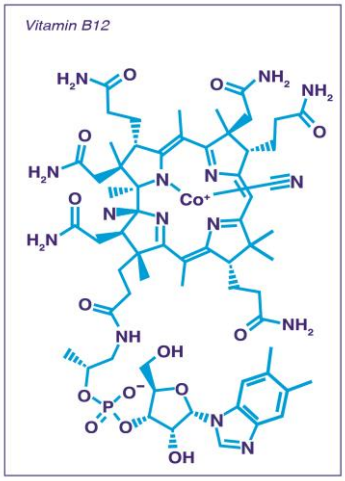


Figure 7. Vitamin B_{12} chemical structure.

Vitamin B_{12} is involved in DNA methylation as it acts as a co-factor of the enzyme methionine synthase, for the transformation of hcy into methionine (Toohey, 2006). In this sense, B_{12} is involved in several health questions derived from this function **(Table 4)**.

Numerous studies have demonstrated that B_{12} is associated with obesity. For example, B_{12} supplementation was associated with mouse body weight and liver DNA methylation (Sabet et al., 2016), in agreement with other human study in which hypoxia-inducible factor 3 alpha (*HIF3A*) gene hypermethylation modified body mass index (BMI) (Huang et al., 2015). In addition, B_{12} deficiency has also been associated with metabolism, affecting intestinal absorption and function through hypermethylation of peptide transporter 1 (*Pept1*) in pigs (Liu et al., 2017), modifying hepatic triglycerides (TGs) and cholesterol levels via global DNA methyaltion in mice (Sabet et al., 2016), and also by altering lipid profile after hypomethylation of sterol regulatory element-binding transcription factor 1 (*SREBF1*) and low density lipoprotein receptor (*LDLR*) genes in human adipocytes (Adaikalakoteswari et al., 2015). Moreover, B_{12}-related DNA methylation has been associated with cancer (Ba et al., 2011; Piyathilake et al., 2014), cognitive and behavioural functions (Caramaschi et

al., 2017; McKee et al., 2017), telomere length (Pusceddu et al., 2016), oxidative stress and inflammation (Zhong et al., 2017), and offspring outcomes (McKay et al., 2012; Khot et al., 2017).

Table 3. Studies analyzing the association between DNA methylation and choline/betaine deficiency and supplementation.

Sample type	Target gene	Outcome	Methylation alteration	Reference
Choline and betaine deficiency				
Placenta (human)	*CRH, NR3C1*	Higher cord blood, leukocyte promoter methylation, higer cord plasma cortisol	Hypomethylation	(Jiang et al., 2012)
Fetal brain (mouse)	*Cdkn3*	Cell cycling inhibition	Hypomethylation	(Niculescu, 2006)
Fetal brain (mouse)	*Vefgc, Angpt2*	Reduction of blood vessel formation	Hypomethylation	(Mehedint et al., 2010)
Liver (mouse)	*Igf2*	Epigenome modification	Hypermethylation	(Kovacheva et al., 2007)
Liver (mouse)	*c-myc*	Risk for hepatocarcinoma	Hypomethylation	(Tsujiuchi et al., 1999)
Liver (mouse)	*p53, p16$^{INK4\alpha}$, PtprO, Cdh1, Cx26*	Decrease of tumor-suppresors expression	Hypermethylation	(Tryndyak et al., 2011)
Liver (mouse)	*EphB2, VWF*	Enhance of offspring predisposition to steatohepatitis	Hypermethylation	(Wankhade et al., 2017)
Choline and betaine supplementation				
HepG2 human cell line	*ACOX1*	Modification of the transcription of fatty acid and glucose metabolism	Hypermethylation	(Jiang et al., 2016)
Liver (rat)	*Srebf2, Agpat3, Esr1*	Protective effect on liver fat accumulation	Hypomethylation	(Cordero et al., 2013a)
Liver (rat)	FASN	NAFLD improvement	Hypermethylation	(Corderoet al., 2013b)
Liver (tx mouse)	genome-wide	Rescue the lower body weight	Hypermethylation	(Medici et al., 2014)
Fat mass (chicken)	LPL	Decreases of lipogenesis	Hypomethylation	(Xing et al., 2011)
Fat (mouse)	FTO	Diminishes of hepatic fat accumulation	Hypomethylation	(Chen et al., 2015)
Neurons (rat)	POMC	Prevents the adverse effects of etanol in neuron	Hypermethylation	(Bekdash et al., 2013)
Hhypothalamus (cockerel)	HMGCR, ABCA1, ACAT1	Regulate cholesterol metabolism in brain	Hypomethylation	(Idriss et al., 2016)
Blood (human)	Genome-wide	Regulation of birth weight in offspring	Hypermethylation	(Pauwels et al., 2017b)
Buccal cells (human)	*RXRA*	Metabolism regulation	Hypermethylation	(Pauwels et al., 2017c)

Abbreviations: NAFLD, non alcoholic fatty liver disease.

2.1.4 Vitamin B_6 and B_2

Vitamin B_6 consists of a 2-methyl-3-hydroxypyridine and exists in different isoforms. Pyridoxal 5'-phosphate (PLP) is the metabolically active form of vitamin B_6 **(Figure 8)** and is involved in macronutrient metabolism, one-carbon metabolism, hemoglobin synthesis, regulation of immune system, and also has anti-oxidative and anti-inflammation properties, and effects on carcinogenesis (Wit, 2011). Vitamin B_6 is present in vegetables, whole grain cereals, nuts, and muscle meats (Wit, 2011).

Vitamin B_2 or riboflavin is an essential heat-stable and water-soluble nutrient belonging to the vitamin B family. Vitamin B_2 is involved in fat, carbohydrate and protein metabolism, cell respiration, and erythrocyte integrity. Vitamin B_2 is found in small amounts in many foods, including organ meats, milk, eggs, whole grains and leafy vegetables (Powers, 2003).

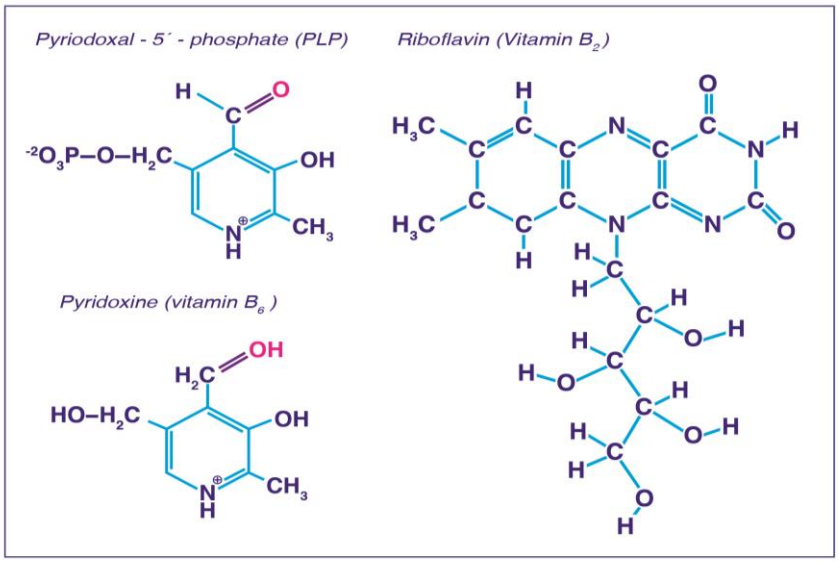


Figure 8. Vitamin B_6 and its metabolically active isoform (pyridoxal 5'-phosphate), and vitamin B_2.

Regarding the role of these vitamins in one-carbon metabolisms, only a few human studies have demonstrated modulating effects on DNA methylation. For example, maternal PLP was positively associated with *MEG3* DMR methylation in a cohort of 496 mother-infant pairs (McCullough et al., 2016). In addition, a prospective cohort study of 254 mother-infant pairs demonstrated that maternal vitamin B_2 intake was positively associated with DMR methylation of the gene encoding the zinc finger protein pleomorphic adenoma gene 1 (*PLAG1*) (Azzi et al., 2014).

Table 4. Studies analyzing the association between DNA methylation and B_{12} levels, deficiency and supplementation.

Sample type	Target gene	Phenotypic effect	Methylation alteration	Reference
Vitamin B_{12} plasma levels				
Liver (mouse)	Genome-wide	Body weight regulation, modification of hepatic triglycerides and cholesterol levels	Hyper and hypomethylation	(Sabet et al., 2016)
Cord blood (human)	Genome-wide	Gestational length	Hyper and hypomethylation	(McKay et al., 2012)
Cord blood (human)	*APOL2*	Childhood intelligence	Hypermethylation	(Caramaschi et al., 2017)
Vitamin B_{12} deficiency				
Cord blood (human)	*IGF2*	Weight gain during pregnancy	Hypermethylation	(Ba et al., 2011)
Placenta (human)	*MTHFR, MTR*	Preterm birth	Hypermethylation	(Khot et al., 2017)
Human adipocyte cells (*in vitro*)	*SREBF1, LDLR*	Lipid profile alteration	Hypomethylation	(Adaikalakoteswari et al., 2015)
Vitamin B_{12} supplementation				
Prefrontal cortex (mouse)	Genome-wide	Improve cognitive and motivational behaviour	Hypermethylation	(McKee et al., 2017)
Blood (human)	Genome-wide	Attenuated acute exposure to fine particles-related inflammation and oxidative stress	Hyper and hypomethylation	(Zhong et al., 2017)
Blood (human)	LINE-1	Increase in telomere length	Hypomethylation	(Pusceddu et al., 2016)
Blood (human)	*HIF3A*	Greater increase in BMI	Hypermethylation	(Huang et al., 2015)
Cervical cells (human)	E6 enhancer	Lower risk of cervical intraepithelial neoplasia	Hypomethylation	(Piyathilake et al., 2014)

Abbreviations: BMI, body mass index.

2.2. Fatty acids

A fatty acid (FA) is a carboxylic acid with an aliphatic chain, which can be saturated (no double bond) or unsaturated (minimum one double bond). Natural FAs commonly have a chain from 4 to 28 carbons (IUPAC, 2012). FAs are present in a variety of foods and oils from animal and vegetal sources. Some examples of FAs, their length and sources are shown in **Table 5**.

There are some FAs that cannot be synthesized *de novo* in sufficient quantities for normal physiological function. Linoleic acid (LA) and linolenic acid (ALA) are essential for humans and are able to convert to subsequent polyunsaturated FAs (PUFAs). However, there are other PUFAs whose requirements are sometimes increased and are known as conditionally essential FAs (Cunnane, 2003).

For example, newborn infants or breast-fed children need higher levels of docosahexanoic acid (DHA) or arachidonic acid (AA) for normal development (Birch et al., 1998; Carlson & Neuringer, 1999; Cunnane et al., 2000).

Table 5. Examples of fatty acids, characteristics, lenght and sources.

Name	Length	Nº of double bonds	Position of the double bond	Food source
Monounsaturated				
Myristoleic acid	14	1	ω-5	Butter, beef, cheese, cream
Palmitoleic acid	16	1	ω-7	Chicken, beef, turkey, buffer
Oleic acid	18	1	ω-9	Olive oil, rape oil, hazelnut, corn margarine
Polyunsaturated				
Linoleic acid	18	2	ω-6	Grape seed oil, walnut oil, sunflower oil, soy oil
α-Linolenic acid	18	3	ω-3	Flaxseed oil, rape oil, soy oil, corn margarine
Arachidonic acid	20	4	ω-6	Pork, pork brain, veal brain, lamb brain
EPA	20	5	ω-3	Salmon, mussel, trout, herring
DHA	22	6	ω-3	Herring, trout, salmon, albacore
Saturated				
Lauric acid	12	0	-	Coconut oil, commercial biscuits, butter, cheese
Myristic acid	14	0	-	Coconut oil, butter, biscuit, cheese
Palmitic acid	16	0	-	Oil palm, meats, cotton oil, butter
Stearic acid	18	0	-	Chocolate, butter, sesame oil, palm oil
Arachidic acid	20	0	-	Nuts, peanut butter, vegetable oil, corn oil

Notes: Length, number of carbon atoms; ω, omega symbol denotes the carbon atom furthest from the carboxyl group of a fatty acid; Food source, food with higher fatty acid (grams) content per 100 g.

Saturated FAs (SFAs), especially shorter-chain SFAs, such as lauric, myristic and palmitic acids (PA), have been associated with damages in intestinal permeability, inflammatory processes, carcinogenesis and cardiovascular damage (Fattore & Fanelli, 2013; Wang & Hu, 2017). However, many monounsaturated FAs (MUFAs) and PUFAs may have beneficial effects on obesity-related comorbidities, dyslipemias, inflammation, non-alcoholic fatty liver disease (NAFLD), and IR (Ma et al., 2016; Silva Figueiredo et al., 2017). In addition, industrially produced *trans* FAs (TFAs) (unsaturated fatty acids with the double bond in the *trans* configuration) promote inflammation and increase the risk of CVD (Wang & Hu, 2017). Numerous studies have evidenced a role of SFAs and unsaturated FAs on DNA methylation, maybe by affecting the enzymes involved in the processes or by modifying the availability of the substrates necessary for the processes (Milagro et al., 2013).

Recent studies on the effect of FAs on DNA methylation are summarized in **Table 6**. Some FAs, generally SFAs and TFAs, through DNA methylation, are involved in the outcome of several diseases. For example, PA supplementation in animals models induced global hypermethylation in pancreatic tissue, impaired insulin secretion and higher risk of T2D (Hall et al., 2014). In addition, supplementation with AA increased global DNA methylation resulting in a methylation profile similar to the initial phase of atherosclerosis (Silva-Martínez et al., 2016) and decreased the methylation of angiogenesis and carcinogenesis-related genes, promoting carcinogenesis (Kiec-Wilk et al., 2011). SFAs supplemented-diet induces tumor necrosis factor (TNF) hypomethylation and overexpression in mice, resulting in adipose tissue inflammatory response and adipogenic profile, in agreement with a similar study in which elaidic acid (*trans* modification) supplementation resulted in a pro-inflammatory and adipogenic transcriptional profile in human monocytes and mouse fat tissue (Flores-Sierra et al., 2016).

Different studies have demonstrated that the supplementation with omega 3 (n-3) PUFAs may improve the symptoms caused by several diseases, such as cancer, obesity, T2D, inflammation, and neurodegenerative disease. The supplementation with these FAs in overweight and obese patients resulted in changes in the global methylation profile of obesity-related genes, and also decreased plasma TGs, total cholesterol (TC) and the ratio TC/high-density lipoprotein cholesterol (HDL-C). Similarly, an inverse association has been detected between BMI and global DNA methylation after the supplementation (de la Rocha et al., 2016; Tremblay et al., 2017). Moreover, studies in animals and humans have observed that n-3 PUFAs change the methylation pattern in brain and blood and improve long-term memory and learning, decrease cognition deterioration in AD, and increase neurogenesis (Fan et al., 2016; Chakraborty et al., 2017; Karimi et al., 2017).

Some studies have mentioned possible mechanism attributed to maternal FAs blood levels and the effect on the DNA methylation profile of the offspring. n-3 PUFA supplementation during pregnancy might modify the availability of the key metabolites of one carbon cycle in the fetus, which may have a direct effect on the fetus methylation profile (Kulkarni et al., 2011). The n-3 PUFAs imprint long-term changes that may persist until 5 years of age of the offspring (Fan et al., 2016; Dijk et al., 2016).

To identify the causal mechanism of association between FAs, DNA methylation and outcome phenotype, gene expression has been evaluated. The supplementation with eicosapentaenoic acid (EPA) was accompanied by demethylation and consequently higher expression of the tumour suppressor gene CCAAT/enhancer-binding protein delta (*C/EBPδ*) in human promonocytes U937, showing the importance of FAs in cancer, and in agreement with studies in human umbilical vein endothelial cells (HUVECs) that found demethylation and higher expression of the carcinogenic genes kinase insert domain receptor (*KDR*) and neurogenic locus notch homolog 4 (*Notch4*) following the supplementation with AA (Ceccarelli et al., 2011; Kiec-Wilk et al., 2011). The gene *Bdnf* was found hypomethylated and its expression increased in mouse pups after maternal n-3 PUFAs supplementation during pregnancy. *BDNF* gene is important for the long-term potentiation, maintaining neuronal populations and connections, concluding that n-3 FAs during pregnancy are important for the correct brain development in the offspring (Fan et al., 2016). Additional studies have found associations between FAs and inflammation-related genes in several diseases. For instance, n-3 FAs were inversely associated with *IL6* methylation in human blood, reducing pro-inflammatory gene expression. In animals, SFAs reduce *Tnfa* and increase peroxisome proliferator-activated receptor gamma (*Pparγ1*) methylation, increasing and decreasing the expression of these genes and leading to an inflammatory environment in adipose tissue. These findings provide the possible mechanistic basis for how obesity increases inflammation and leads to the development of obesity-associated metabolic diseases, including IR (García-Escobar et al., 2017; Wang & Hu, 2017). Moreover, rats fed a mostly saturated fat diet showed lowest methylation and highest expression on *Vegfb* gene. In contrast, PUFAs supplementation was associated with higher methylation and lower expression of this gene. *Vegfb* is involved in angiogenesis and these results provide the evidence of the role of FAs via DNA methylation in the formation of new blood vessels in adipose tissue, which is very important for preadipocyte differentiation and adipose tissue expansion (Monastero et al., 2017). In mice, HFD diet supplementation with EPA and DHA decreased global DNA methylation in the adipose tissue and increased *Pparγ2* promoter methylation in adipose tissue and muscle, attenuating the obesogenic effects of the HFD (Amaral et al., 2015).

2.3 Other dietary bioactive compounds

There are evidences that other dietary bioactive compounds are also involved in changes in the epigenetic profile. For example, during the last decade much attention has been paid to the potential epigenetic regulatory role of polyphenols. These phenolic compounds are widely distributed in fruits, vegetables, red wine, and plants, and are converted to secondary metabolites by microbial metabolism in gut (Bhat, 2017). Emerging evidence suggests that dietary polyphenols from soy, green tea, coffee or apples modify the epigenetic state, leading to gene activation or repression (Ayissi et al., 2014). Many polyphenols such as curcumin, resveratrol, and catechins have been reported to prevent the development of cancer, neurodegenerative diseases and metabolic disorders via epigenetic modifications (Ayissi et al., 2014). The beneficial effects of dietary polyphenols can be linked to their ability to inhibit DNMT activity by increasing SAH levels or inhibiting the catalytic site of DNMT (Lim, 2012). Understanding the role and mechanism of action of polyphenols in epigenetic regulation may allow the identification of new therapeutic options against obesity-related diseases (Ayissi et al., 2014).

2.4 Future perspectives

While these epigenetic studies demonstrated the effects of nutrients on genome-wide and gene-specific DNA methylation status, it is still needed to clarify how each specific nutrient targets specific genes to be methylated and demethylated. In addition, although the role of FAs has been more extensively evaluated, until now, few studies have investigated the folate and choline direct effects on gene-specific DNA methylation in humans. It is also necessary to investigate how DNA methylation affects the pathophysiology of specific diseases, and the real effect on health and disease outcomes. Finally, these studies could also help guide the use of DNA methylation regulation as a new therapeutic target for the prevention and treatment of obesity and other metabolic diseases.

Table 6. Studies analyzing the association between DNA methylation and fatty acids.

Name	Sample type	Target gene	Main results	Reference
SFA and PUFA	Adipose tissue (human)	Metabolism-related genes such as *NFAM1, ACO1, SLC37A2, MC2R*	Distinct DNA methylation profile between SFA and PUFA The baseline DNA methylation can predict weight increase in response to diet	(Perfilyev et al., 2017)
SFA and PUFA	Adipose tissue (rat)	*Vegfb*	SFA associated with hypomethylaton of *Vegfb* and with angiogenic effects PUFA associated with hypermethylation of *Vegfb*	(Monastero et al., 2017)
SFA	Mouse 3T3-L1 cells (*in vitro*)	*Tnfα*	Hypomethylation. Increase the adipose tissue inflammatory response.	(García-Escobar et al., 2017)
SFA	Mouse MD1KO cells (*in vitro*)	*Dnmt1* and *Pparγ1*	Hypomethylation of *Dnmt1* and hypermethylation of *Ppar γ1* Macrophage phenotypic switch to a more proinflammatory M1 phenotype Development of insulin resistance	(Wang et al., 2016)
OA	Human THP-1 cells (*in vitro*)	Genome-wide and specific genes	Global hypomethylation Participates in shaping metabolic disease-specific DNA methylomes through b-oxidation, PPAR-a, and sirtuin 1 signaling, has potential implications for diet-oriented therapy and prevention	(Silva-Martínez et al., 2016)
EA (Trans)	Human THP-1 cells (*in vitro*) and epididymal fat (mouse)	Genome-wide	Causes global DNA hypermetilation Induces a pro-inflammatory and adipogenic transcriptional profile	(Flores-Sierra et al., 2016)
ω-3 fatty acids	Blood (human)	Genome-wide	Changes of DNA methylation profile of genes involved in obesity-related pathways ω-3 fatty acids supplementation decrease plasma TG, TC and the ratio TC/HDL-c in overweight and obese adults	(Tremblay et al., 2015)
ω-3 fatty acids	Left hemibrain (mouse)	Genome-wide	Hypermethylation of genes related to long-term potentiation, memory, cognition and learning	(Chakraborty et al., 2017)
ω-3 fatty acids	Brain (mouse)	*Bdnf*	Hypomethylation and upregulation of *Bdnf* gene Increases neurogenesis.	(Fan et al., 2016)
ω-3 fatty acids	Blood (human)	*IL-6*	Hypomethylation and downregulation of *IL-6* Ameliorate systematic inflammation.	(Ma et al., 2016)
ω-3 + ω-6 fatty acids	Left hemibrain (mouse)	Genome-wide	Hypermethylation of genes related to the formation of neuronal precursors	(Kulkarni et al., 2011)
AA	Human THP-1 cells (*in vitro*)	Genome-wide and specific genes	Hypermethylation profile similar to atherosclerosis DNA methylation profile in the initial phases	(Silva-Martínez et al., 2016)
AA	Human HUVECs cells (*in vitro*)	*KDR, Notch4*	Hypomethylation of these genes Control angiogenesis and carcinogenesis	(Kiec-Wilk et al., 2011)
EPA and AA	Blood (human)	Genome-wide	Global hypermethylation. Inverse association between BMI and global DNA methylation	(de la Rocha et al., 2016)
EPA	Human U937 cells (*in vitro*)	*C/EBPδ*	Hypomethylation and upregulation of the tumor suppressor *C/EBPδ*	(Ceccarelli et al., 2011)
DHA	Blood (human)	Genome-wide	DNA methylation profile alteration during pregnancy that persist until 5 years of age, supporting the role of epigenetics in developmental programming	(van Dijk et al., 2016)
DHA	Placenta (rat)	Genome-wide	Changes in maternal fatty acids levels could alter one carbon metabolism in the fetus Reverse the DNA methylation alteration by a folic acid and vitamin B12 deficiency	(Kulkarni et al., 2011)
EPA and DHA	Liver and muscle (mouse)	*Pparγ2* and global methylation	Decrease global DNA methylation and increase *Pparγ2* promoter methylation. Attenuate body weight gain after a HFD	(Amaral et al., 2015)
EPA, DPA and DHA	Blood (human)	Genome-wide	Global hypomethylation that reduces cognitive deterioration in AD	(Karimi et al., 2017)
PA	Pancreatic islets (human)	Genome-wide	Impaires insulin secretion and contributes to the development of T2D	(Hall et al., 2014)

Abbreviations: SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; OA, oleic acid; EA, elaidic acid; ω, position of double bond; TG, triglyceride; TC, total cholesterol; HDL-c, high density lipoprotein-cholesterol; AA, arachidonic acid; EPA, eicosapentaenoic acid; BMI, body mass index; DHA, docosahexanoic acid; HFD, high fat diet; DPA, docosapentaenoic acid; AD, Alzheimer's disease; PA, palmitic acid; T2D, type 2 diabetes.

3. DNA METHYLATION MARKERS IN OBESITY AND RELATED COMORBIDITIES

Biomarkers can help to predict the outcome and may be a useful tool for the development of new therapeutic targets in the fight against diseases (Neidhart, 2016). In this context, and as it has been evidenced during this introduction, important links between DNA methylation deregulation and several metabolic-related diseases have been reported. In the era of "personalized medicine", DNA methylation markers have emerged as a useful tool for the risk prediction, screening, diagnosis and prognosis of obesity and metabolic diseases. Deepening into the knowledge about the molecular mechanisms involved in disease development is essential for finding biomarkers involved in them and more personalized therapeutic strategies.

The next paragraphs provide a concise review of the role of DNA methylation in obesity, metabolic syndrome and weight regulation.

3.1 Obesity, related comorbidities and DNA methylation

Obesity has been defined as an abnormal or excessive fat accumulation that contributes to increase morbidity and mortality (WHO, 2017). Obesity is attributed to a chronic positive energy imbalance between calories intake and calories expenditure (Chatzigeorgiou et al., 2014), although, several factors could affect the energy equation, such as lifestyle behaviours (dietary habits, exercise, sleep behaviour) (Forouzanfar et al., 2015; Biddle et al., 2017; St-Onge, 2017), social factors (educational level, economic status) (Kim et al., 2017), endocrine disorders (hypothyroidism) (Weaver, 2007) or prescription of certain medication (corticosteroids) (Martínez de Morentin et al., 2013). However, not all individuals under the same environmental factors develop obesity, so it has been suggested a role of genetics (Singh et al., 2017), epigenetics (Campion et al., 2009) and gut microbiota (Khan et al, 2016).

Obesity may represent a risk that contributes to the development of many comorbidities such as T2D, CVD, dyslipidemia, hypertension, liver steatosis, respiratory problems and certain types of cancer (reviewed in (Guh et al., 2009)).

Obesity is considered the pandemic of XXI century. The prevalence of obesity closely tripled between 1975 and 2016. In 2016, more than 1.9 billion adults were overweight and 650 million were obese (WHO, 2017). Also among children and adolescents the obesity prevalence has hugely increased in the last decades. A recent meta-analysis of 2416 population-based studies in 128.9 million children and adolescents described an increase of prevalence of worldwide obesity from 0.7 % in 1975 to 5.6 % in 2016 in girls, and from 0.9 % in 1975 to 7.8 % in 2016 in boys, postulating that in 2022 child and adolescent obesity would surpass moderate and severe underweight (Abdeen et al., 2017).

Despite extensive efforts, obesity prevalence continues increasing in every country. At the same time, new evidence of novel biomarkers has risen to understanding the pathophysiology of obesity and associated health problems. In this sense, a number of studies have discovered associations between genome-wide or gene-specific DNA methylation levels and individual's response to the development of obesity and other related disorders (Campion et al., 2009; Wang et al., 2010; Milagro et al., 2011, 2013; Remely et al., 2015; van Dijk et al., 2015; Wahl et al., 2016; Abdul et al., 2017; Castellano-Castillo et al., 2017; Nilsson & Ling, 2017). These studies allow understanding, at least in part, the variability in obesity-related phenotypes and the interactions between genetics and environmental factors, and might be the base for the development of novel biomarkers and therapies.

3.1.1 DNA methylation markers in obesity

Cross-sectional studies

Numerous cross-sectional studies reported a significant association between obesity or adiposity status, and DNA methylation **(Table 7)**.

In the analysis performed in 2861 children who participated in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort study, 28 CpG sites were differentially methylated in cord blood from offspring of obese mothers in comparison with offspring of normal weight mothers (Sharp et al., 2015). Other case-control study in 12 children also described 19 differentially methylated regions (involving 18 genes) in obese individuals' blood cells. These regions were mainly hypomethylated in obese and located in the gene body region and revealed a unique cluster of obese individuals that was differentiated from the normoweight children (Rhee et al., 2017). Similarly, a recent study involving 374 children found 212 differentially methylated CpGs in blood cells associated with adiposity and

230 associated with fat mass, but finally only 13 genes were significantly associated after Bonferroni correction. These genes are involved in lipid and glucose metabolism, differential body size and body composition in children (Rzehak et al., 2017). However, any of the genes matched with the genes found in the case-control study of 12 children.

The majority of cross-sectional studies have investigated DNA methylation in blood cells from adult population. An interesting study in 547 subsaharians described 15 differentially methylated positions associated with BMI and 7 with obesity (Meeks et al., 2017). Similar to other cross-sectional study involving 991 individuals, that found 8 differentially methylated positions associated with BMI and waist circumference (WC), although finally, only carnitine palmitoyltransferase 1A (*CPT1A*), cluster of differentiation (*CD38*) and phosphoglycerate dehydrogenase (*PHGDH*) genes were validated in blood samples taken from two additional independent studies (Aslibekyan et al., 2015). Interestingly, *CPT1A* gene also was found in the EWAS analysis of Arab population and monozygotic and dizygotic twins' cohort blood samples, where *CPT1A* gene was associated with T2D and obesity. *CPT1A* is implicated in several metabolic processes, including fasting TG and VLDL levels (Irvin et al., 2014). These studies suggest an important role of this gene in obesity.

Several studies also associated gene-specific and global methylation with obesity and obesity-related parameters. In this sense, melanin concentrating hormone receptor 1 (*MCHR1*) gene, involved in the control of energy metabolism, was associated with BMI in humans and rodents (Stepanow et al., 2011). *POMC* gene, which is involved in the regulation of appetite, showed hypermethylation in obese subjects (Kuehnen et al., 2012). Global DNA (*LINE-1)* hypermethylation and adiposity genes association was described in 40 healthy subjects. Global DNA hypermethylation was associated with higher circulating levels of glucose, insulin and homeostatic model assessment (HOMA)-IR, and lower adiponectin concentration. These results suggested global methylation as an early epigenetic marker of metabolic outcomes and obesity (Carraro et al., 2016).

Not only blood cell methylation profile has been associated with obesity, but also methylation of other tissues and body fluids. An epigenome-wide association study (EWAS) identified predominantly DNA hypermethylation in white adipose tissue (WAT) from obese subjects related with gene expression of pro-inflammatory pathways. This suggests that DNA methylation mechanisms may link dysfunctional

adipocytes to WAT inflammation and IR in obesity (Petrus et al., 2017). Hypermethylation of beta-3 adrenergic receptor (*ADRB3*) gene was also observed in WAT of obese subjects, and increased the susceptibility to visceral obesity and altered body fat distribution (Guay et al., 2014). This gene methylation was previously described in blood cells (Guay et al., 2014), suggesting that the methylation changes of *ADRB3* gene in blood reflect obesity-related DNA methylation changes of this gene in WAT. In addition, a case-control study demonstrated an association between hypomethylation of *LEP* gene in obese individuals, measured in saliva, and obesity-related parameters (Dunstan et al., 2017). Likewise, an EWAS performed in 92 children's saliva samples described 17 CpGs associated with maternal BMI (Oelsner et al., 2017). The saliva sample analysis in 50 girls with and without obesity found two interesting genes neuron navigator 3 (*NAV3*) and melanocortin 2 receptor (*MC2R*) whose methylation levels were associated with BMI (Rounge et al., 2016). These studies revealed that saliva is a probable viable medium for epigenetic testing in obesity. However, further testing would have to include both saliva and blood samples for demonstrating that saliva is consistent to whole blood findings. With this purpose, a recent study provided a novel application of non-invasive buccal samples for the identification of DNA methylation markers in relation to overweight management and insulin sensibility (San-Cristobal et al., 2016).

All these studies evidenced that DNA methylation is linked to obesity and obesity-related measures. However, the causal direction of phenotypic outcomes and DNA methylation profile remains unclear and longitudinal analyses in other populations is required.

Table 7. Cross-sectional studies analyzing the association between DNA methylation and obesity.

Study population	Sample type	Target gene	Major finding	Reference
n = 2861 children	Cord blood	Genome-wide	28 CpG differentially methylated between offspring of obese and normal weigh mothers. Maternal weight modifies offspring epigenome	(Sharp et al., 2015)
n = 6 obese and n = 6 non obese children	PBC	*21orf56, ZNF154, SDK1, KIAA0146, SKIV2L, GPR125, SORBS2, C14orf70, POLR3E,CTBP2,DLGAP2,CAPS2,GIMAP1,RNF 213,MND1,SRM,TGM6,WDR27*	Different DNA methylation profile between obese/non obese children Markers for early diagnosis of obesity	(Rhee et al., 2017)
n = 374 children	PBC	*SNED1(IRE-BP1), KLHL6, WDR51A(POC1A), CYTH4-ELFN2, CFLAR, PRDM14, SOS1, ZNF643(ZFP6 9B), ST6GAL1, C3orf70, CILP2, MLLT4*	Association between DNA methylation and BMI, fat mass, lipid and glucose metabolism	(Rzehak et al., 2017)
n = 24 obese and n = 23 non obese	PBC	*CERCAM, DPYD, IL12A, ZNF35, ZNF362, TSC22D2, CBX6, FOXF1, PSMD7, H1FX, PRRC2C, MSI1, COL4A1, NBPF3, USP5, PLOD2, TLE3, RPS24, DVL3, POLD3*	20 genes differentially methylated between obese and non obese adults Biomarkers for the understanding of obesity	(Almén et al., 2012)
n = 547 adults	Blood	15 DMP and *CPT1A*	Association between DNA methylation and BMI, obesity, and abdominal adiposity	(Meeks et al., 2017)
3 cohort: GOLDN (n = 991), ARIC (n = 2,106) and FHS (n = 442)	CD4+ T-cells	*CPT1A, CD38, PHGDH*	Association between DNA methylation and BMI and waist circumference	(Aslibekyan et al., 2015)
n = 49 from Popgen biobank	PBC	*MCHR1*	DNA methylation associated with BMI DNA methylation contributes to the age-related specific effects	(Stepanow et al., 2011)
Non obese n = 90 and obese n = 171 children	PBC	*POMC*	Hypermethylaton associated with obesity Appetite regulation	(Kuehnen et al., 2012)
Obese n = 7 and non-obese n = 7 adults	PBL	*UBASH3A, TRIM3*	Hypermethylation of *UBASH3A* and hypomethylation of *TRIM3* genes in obese subjects compared with lean controls	(Wang et al., 2010)
n = 40 healthy subjects	PBMC	*SERPINE1, LINE-1*	Association between hypermethylation of *LINE-1* and *SERPINE1*, and WC and BMI	(Carraro et al., 2016)
Two cohort: RESMENA n = 48 and OBEPALIP n = 25	WBC	*GPR13, ITGB5*	Association between DNA methylation and BMI	(Mansego et al., 2015)
Two cohort: Qatari descent from 15 families n = 30 and TwinsUK cohort n = 32	Blood	*CPT1A, TXNIP, ABCG1*	Asocciation between hypomethylation of *CPT1A* and *TXNIP* and obesity Association between hypermethylation of *ABCG1* and BMI	(Al Muftah et al., 2016)

Table 7. Cross-sectional studies analyzing the association between DNA methylation and obesity (continuation).

Study population	Sample type	Target gene	Major finding	Reference
Two cohort: n = 61 men with hypercholesterolemia and n = 30 morbid obese men	VAT and Blood	*ADRB3*	Association between hypermethylation of *ADRB3* gene and visceral obesity and fat distribution in VAT Association between hypermethylation of *ADRB3* and LDL-c and higher waist-to-hip ratio in blood	(Guay et al., 2014)
Obese n = 50 and non obese girls n = 50	Saliva	*NAV3, MC2R*	Association between hypermethylation and BMI	(Rounge et al., 2016)
n = 431 adults	Saliva	*LEP*	Association between DNA methylation and BMI	(Dunstan et al., 2017)
n = 92 children	Saliva	Genome-wide	Association between 17 CpGs methylation and BMI	(Oelsner et al., 2017)
n = 43 adults	Blood and oral mucosa	*GAP43, ATP2A3, ADARB2*	Association between genes methylation and overweight	(San-Cristobal et al., 2016)
NEST cohort n = 92	Blood	*PLAGL1, NEG3*	Hypermethylation of *PLAGL1* and hypomethylation of *NEG3* in obese mothers	(Soubry et al., 2015)
n = 88 children	Cord blood	*MMP7, KCNK4, TRPM5, NFKB1*	Association between GWG and hypermethylation of genes	(Morales et al, 2014)
BBC cohort n = 309	Blood leukocytes	Genome-wide	Association between maternal BMI and DNA methylation of 20 CpGs	(Liu et al., 2014)
Four cohorts: Cardiogenic Consortium cohort n = 479, MARTHA cohort n = 339, KORA F4 cohort n = 1789 and MuTHER cohort n = 635	Blood and adipose tissue	*HIF3A*	Association between DNA hypermethylation of gene and BMI	(Dick et al., 2014)
n = 84 MZ twin pairs	PBC	*SLC6A4*	Association between DNA hypermethylation of gene and BMI, body weight and WC	(Zhao et al., 2013)
ARIC cohort n = 2097	WBC	Genome-wide	Association between 8 CpG methylation and BMI changes	(Demerath et al., 2015)

Abbreviations: CpG, cytosine linked by a phosphate to guanine; PBC, peripheral blood cells; BMI, body mass index; DMP, differentially methylated positions; GOLDN, Genetics of Lipid Lowering Drugs and Diet Network; ARIC, Atherosclerosis Risk in Communities; FHS, Framingham Heart Study; PBMC, peripheral blood mononuclear cells; WC, waist circumference; RESMENA, Metabolic Syndrome Reduction in Navarra; OBEPALIP, Effects of Lipoic Acid and Eicosapentaenoic Acid in Human Obesity; WBC, white blood cells; VAT, visceral adipose tissue; LDL-c, low-density lipoprotein cholesterol; NEST, The Newborn Epigenetic Study; GWG, gestational weight gain; BBC, Boston Birth Cohort; MARTHA, MARseille THrombosis Association; KORA, Cooperative Health Research in the Region of Augsburg; MuTHER, Multiple Tissue Human Expression Resource; MZ, monozygotic.

Longitudinal studies

Few longitudinal studies have tried to establish the causal effect of DNA methylation in the development of obesity. In addition, the majority of longitudinal and prospective studies have been focused on pediatric population (**Table 8**).

A prospective study in 483 children, who were monitored for 5 years, identified 65 DMRs at birth that were associated with obesity parameters in childhood (van Dijk et al., 2017). This was in agreement with other prospective study that described DNA methylation of several weight-linked loci in newborns that continued to show a longitudinal association with adiposity in early children (Lin et al., 2017). In addition, the site-specific methylation of cyclin dependent kinase inhibitor 1C (*CDKN1C*) at birth in 178 newborns was associated with body size at childhood (Relton et al., 2012). Similarly, other longitudinal and prospective studies, identified the effect of methylation of retinoid X receptor alpha (*RXRA*) and tumor associated calcium signal transducer 2 (*TACSTD2*) genes at birth with later adiposity and fat mass (Godfrey et al., 2011; Groom et al., 2012). Global DNA methylation also was associated with development of adiposity in boys who participated in a longitudinal study (Perng et al., 2013).

To identify the causal nature of association between DNA methylation and obesity in later life, a prospective study was performed in 258 individuals. DNA methylation in ATP binding cassette G1 (*ABCG1*) and phosphoethanolamine/phosphocholine phosphatase (*PHOSPHO1*) genes in blood were correlated positively with BMI, glycated haemoglobin (HbA1c), fasting insulin, and TGs levels after a mean of 8 years in adult population (Dayeh et al., 2016).

In summary, the advances in the study of DNA methylation and the establishment of longitudinal relevant models in the study of obesity, may now allow to detect novel DNA methylation markers for obesity. Mainly, the first potential DNA methylation markers at birth have been detected. This might help to predict obesity risk, adiposity and body size at a young age and gives the opportunity for the development of prevention strategies.

Table 8. Longitudinal studies analyzing the association between DNA methylation and obesity.

Study population	Sample type	Target gene	Major finding	Reference
Botnia prospective study n = 129 who developed T2D; n = 129 controls, 8 years	Blood	*PHOSPHO1, ABCG1*	Associated with BMI, insulin, TG.	(Dayeh et al., 2016)
438 children, 5 years	Blood	Genome-wide	Associated with obesity, BMI z-score, HOMA-IR, insulin and glucose	(van Dijk et al., 2017)
GUSTO cohort n = 987, 48 months	Blood	*CDKN2B/P4HA 3*	Associated with BMI	(Lin et al., 2017)
CHAMACOS study n= 373, 9 years	Blood	*PPARγ*	Associated with body size	(Volberg et al., 2017)
Two birth cohorts: ALSPAC n = 178, 7 years, and PTBGS n=24, 11-13 years	Blood	*CDKN1C, EPHA1, CASP10, HLA, NID1*	2.08% and 0.80% increase in BMI per 1% increase in methylation at *CDKN1C* and *EPHA1* respectively Increase of 5.16% and 1.84% in fat mass per 1% increase in methylation respectively. Associated with BMI and fat mass	(Relton et al., 2012)
Two prospective cohorts: PAH, n = 78, 9 years and SWS n = 239 6 years	Blood	*RXRA*	Higher methylation of *RXRA*, was associated with lower maternal carbohydrate intake and neonatal adiposity and fat mass	(Godfrey et al., 2011)
Two cohort: NPBGS n = 94, 11 years and ALSPAC n = 161, 7 years	Blood	*TACSTD2*	Hypomethylation associated with higher fat mass	(Groom et al., 2012)
BSCC study, n = 600 children, 30 months	Blood	LINE-1	Hypomethylation associated with adiposity	(Perng et al., 2013)

Abbreviations: T2D, type 2 diabetes; BMI, body mass index; TG, triglycerides; HOMA-IR, homeostasis model assessment - insulin resistance; GUSTO, Growing Up in Singapore Towards healthy Outcomes; CHAMACOS, Center for the Health Assessment of Mothers and Children of Salinas; ALSPAC, Avon Longitudinal Study of Parents and Children; PTBGS, Preterm Birth Growth Study; PAH, Princess Anne Hospital; SWS, Southampton Women's Survey; NPBGS, Newcastle Preterm Birth Growth Study; BSCC, Bogota School Children Cohort.

3.1.2 DNA methylation markers in pathologies associated with obesity

As it has been previously noted, obesity amplifies the risk of developing various diseases. The risk depends especially on excess body weight and accumulation of adiposity, mainly in the visceral area, and the inflammation associated with the condition. The most frequent pathologies associated with adiposity and body weight are metabolic disorders (T2D, hypertension, and dyslipemia), CVDs and liver disorders (hepatic steatosis, NASH and cirrhosis) (Salas-Salvadó et al., 2007).

The characteristic accumulation of visceral adiposity in obesity is associated with metabolic dysregulation including IR, hyperglycaemia, dyslipidaemia and hypertension and increased risk of developing T2D, contributing thus to the mortality and morbidity of obesity (Salas-Salvadó et al., 2007). The epigenetic mechanism underlying metabolic disorders include DNA methylation

modification (Barrès & Zierath, 2016) **(Table 9)**. DNA methylation profile alteration has been observed in individuals with metabolic disorders, CVD and digestive disorders, suggesting that DNA methylation could be used as a biomarker in obesity comorbidities. Moreover, this contributes to a better insight into the pathophysiology of these illnesses.

3.1.2.1 DNA methylation and hypertension

Two meta-analyses including 17010 individuals from European, African-American and Hispanic ancestry identified three CpGs whose methylation alteration explained 1.4 % and 2 % of the interindividual variation in systolic and diastolic blood pressure (BP), respectively. In addition, the study showed that the methylation of TATA box-binding protein-associated factor RNA polymerase I subunit B (*TAF1B)*-Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide (*YWHAQ)* gene influenced BP, while BP altered the methylation status of zinc finger MIZ-type containing 1 (*ZMIZ1)*, *CPT1A* and solute carrier family 1 member 5 (*SLC1A5*) genes (Richard et al., 2017). These findings suggest that DNA methylation plays a role in hypertension.

3.1.2.2 DNA methylation and T2D

DNA methylation changes are described in the tissues that undergo metabolic alteration in obese and diabetic patients, including adipose tissue, liver, skeletal muscle, and pancreas (Cheng et al., 2018). A recent article reviewed all these DNA methylation alterations associated with metabolic disorders in the different metabolic tissues **(Table 9)** (Cheng et al., 2018).

Concerning adipose tissue, 15627 CpGs were differentially methylated between monozygotic twin pairs discordant for T2D. Interestingly, these CpGs were involved in insulin signaling, adipogenesis and metabolism (Nilsson et al., 2014). Another EWAS analysis in 190 healthy subjects' adipose tissue discovered several genes including nicotinamide adenine dinucleotide phosphate oxidase 4 (*NOX4*), plasminogen (*PLG*), ELOVL fatty acid elongase 2 (*ELOVL2*), kruppel like factor 14 (*KLF14*), glycine receptor alpha 1 (*GLRA1*), alpha-ketoglutarate dependent dioxygenase (*FTO*), inter-alpha-trypsin inhibitor heavy chain family member 5 (*ITIH5*), C-C motif chemokine ligand 18 (*CCL18*), mitochondrial carrier 2 (*MTCH2*), insulin receptor substrate 1 (*IRS1*), four and a half LIM domains 2 (*FHL2*), *HIF3A* and secreted phosphoprotein 1 (*SPP1*) associated with T2D. Some of these genes have

a function in critical pathways, such as *IRS1* and *FHL2* in insulin signaling and secretion, and *HIF3A* in adipocyte differentiation and function (Rönn et al., 2015).

When analysing the possible associations between DNA methylation changes in liver and metabolic disorders, two studies have come to the same conclusion. A case-control study including 35 T2D and 60 non-diabetic subjects presented 251 CpGs representing 162 different genes that had discordant DNA methylation profile. The 94 % of the significant CpGs were hypomethylated in liver from T2D subjects. Growth factor receptor bound protein 10 (*GRB10*), *ABCC3*, monoacylglycerol O-acyltransferase 1 (*MOGAT1*) and PR/SET domain 16 (*PRDM16*) were validated as important genes involved in T2D development (Nilsson et al., 2015). Likewise, Kirchner et al., (2016) identified hypomethylation of genes involved in hepatic glycolysis and IR in liver of obese diabetic individuals in comparison with non-diabetics (Kirchner et al., 2016).

Three DNA methylation studies in pancreas, the most relevant tissue for diabetes, associated epigenetic alterations with T2D. In a T2D case-control study, Dayeh et al., (2016) identified methylation alterations of the genes *ABCG1*, *PHOSPHO1*, *SREBF1* and thioredoxin interacting protein (*TXNIP*) in pancreatic islets. Noteworthy, these genes' methylation differences also were identified in blood and skeletal muscle, and were associated with future risk of the development of T2D (Dayeh et al., 2016). Another EWAS study in blood and pancreatic islets from non-diabetic and individuals with risk to develop T2D described differently methylated CpGs between groups. Identified CpGs were involved in mitochondrial function, insulin secretion and glucose homeostasis pathways, and were located in the genes cyclin D2 (*CCND2*), cartilage intermediate layer protein 2 (*CILP2*), *FHL2*, glucosamine-phosphate N-acetyltransferase 1 (*GNPNAT1*), helicase like transcription factor (*HLTF*), *KLF14*, PBX homeobox 4 (*PBX4*), SH2B adaptor protein 3 (*SH2B3*), serotonin transporter (*SLC6A4*), transcription factor 7 (*TCF7*) and zinc finger protein 518B (*ZNF518B*). Notably, the DNA methylation changes observed in the human pancreatic islets were further validated in blood samples (Bacos et al., 2016). Finally, a case-control study with 34 non-diabetic and 15 diabetic subjects found 457 genes differently methylated in T2D individuals' pancreatic islets, including insulin promoter factor 1 (*PDX1*), adenylate cyclase 5 (*ADCY5*), glucose transporter 2 (*SLC2A2*) and the nuclear receptor subfamily 4 group A member 3 (*NR4A3*) tumor suppressor.

Table 9. Studies analyzing the association between DNA methylation and metabolic disorders.

Study population	Tissue sample	Methylation strategy	Target gene	Major results	Reference
Two meta-analysis: n = 17010 individuals	Blood	EWAS	*TAF1B-YWHAQ, ZMIZ1, CPT1A, SLC1A5*	*TAF1B-YWHAQ* gene methylation influences BP BP modifies DNA methylation status of *ZMIZ1, CPT1A, SLC1A5*	(Richard et al., 2017)
N = 14 pairs of MZ twins diabetic-healthy subjects, n = 176 diabetic-healthy subjects	Adipose tissue	EWAS	15627 CpGs	15627 CpGs differentially methylated in diabetic subjects Association between 15627 CpGs methylation and insulin signalling, adipogenesis and metabolism	(Nilsson et al., 2014)
Cross-sectional study n = 294 individuals	Adipose tissue	EWAS	*FHL2, NOX4, PLG, ELOVL2, KLF14, GLRA1, FTO, ITIH5, CCL18, MTCH2, IRS1 and SPP1, HIF3A*	Association between genes methylation and glucose and fatty acid metabolism, mitochondrial function, oxidative stress, insulin signalling and adipocyte differentiation	(Rönn et al., 2015)
Cross-sectional study n = 133 individuals	Adipose tissue	Gene-specific	*PPARGC1A*	Association between gene methylation and mitochondrial biogenesis, energy expenditure and balance, browning of adipose tissue	(Gillberg & Ling, 2015)
n = 129 who developed T2D and n = 129 who did not develop T2D	Blood	Gene-specific	*ABCG1, PHOSPHO1*	Association between genes methylation and lipogenesis, dyslipemia, cytokine signaling, redox and insulin resistance	(Dayeh et al., 2016)
n = 7 non obese, n = 7 obese non diabetic and n = 8 obese T2D subjects	Liver	EWAS	*PRKCE, ABR, PDGFA, ARHGEF16, ADCY6 and RPS6KA1*	Hypomethylation of the genes associated with insulin signaling, hepatic glycolysis and *de novo* lipogenesis in obese T2D subjects	(Kirchner et al., 2016)
n = 60 healthy controls and n =5 T2D subjects	Liver	EWAS	251 CpG representing 162 genes, *GRB10, ABCC3, MOGAT1, PRDM16*	Association between DNA methylation and insulin sensitivity, hepatic glycogen synthesis and glucose homeostasis	(Nilsson et al., 2015)
n = 12 healthy control and n = 12 T2D subjects	Skeletal muscle	EWAS	*DAPK3*	Association between DNA hypomethylation with high glucose levels	(Mudry et al., 2017)
n = 87 healthy controls, n = 112 subjects with risk of T2D, n = 105 converters to T2D and n = 194 non-converters to T2D	Blood and pancreatic islets	EWAS	*CCND2, CILP2, FHL2, GNPNAT1, HLTF, KLF14, PBX4, SH2B3, SLC6A4, TCF7, ZNF518B*	Association between gene methylation and mitochondrial function, insulin secretion and glucose homeostasis	(Bacos et al., 2016)
n = 34 healthy controls and n = 15 T2D subjects	Pancreatic islets	EWAS	*PDX1, TCF7L2, ADCY5, NR4A3, PARK2, PID1, SLC2A2, SOCS2* and more	Association between DNA methylation and β-cell proliferation, mitochondrial function and insulin secretion	(Volkov et al., 2017)

Abbreviations: EWAS, epigenome-wide association studies; BP, blood pressure; MZ, monozygotic; CpG, cytosine linked by a phosphate to guanine; T2D, type 2 diabetes. Data from Cheng et al., (2018).

These genes participate in β-cell proliferation, mitochondrial function and insulin secretion (Volkov et al., 2017). These studies highlight the importance of DNA methylation alterations in metabolic tissues and blood and the pathogenesis of metabolic disorders.

3.1.2.3 DNA methylation and cardiovascular disease

CVDs are the first cause of mortality and morbidity worldwide, being responsible of 31 % of deaths. These deaths are mainly due to coronary heart disease (CoHD) and stroke ("WHO | CVDs," 2017). Of 17.7 million people who die due to CVDs, an estimated 7.4 million were because of CoHD and 6.7 million of stroke. Atherosclerosis is the main underlying mechanism for the development of CoHD. Increasing evidence suggests an important role for DNA methylation in the development of atherosclerosis. A recent review summarized all available evidence related to the association between DNA methylation and CoHD (Fernández-Sanlés et al., 2017). The studies were classified depending on the region of DNA methylation analysis: (a) global methylation studies, (b) specific-gene methylation studies, and (c) EWAS. For assessing global methylation, the majority of the studies used repetitive elements such as LINE-1 or Alu. The results of these studies were inconsistent. Thus, six studies described an association between hypomethylation and CoHD or atherosclerosis, whereas other studies associated the global hypermethylation with same traits **(Table 10).** The studies of specific-gene methylation were performed in blood and vascular tissues. The review described that there were some genes that appear in more than one study such as estrogen receptor 1 (*ESRA*), *ABCG1*, apolipoprotein E (*APOE*), forkhead box *P3* (*FOXP3*) and *IL6*. The hypermethylation of *ESRA*, *ABCG1* and *FOXP3* were associated with CoHD, whereas the hypomethylation of *IL6* was associated with CoHD. However, in other study the association between *ABCA1* methylation and CoHD and atherosclerosis was not significant.

Table 10. Studies analyzing the association between DNA methylation (measured by global and genome-wide approaches) and CVD.

Study population	Sample type	Conclusion	Reference
Atherosclerotic vascular patients (n = 17) and healthy controls (n = 15)	Blood	Association between global DNA hypomethylation and CHD	(Castro et al., 2003)
Ischemic stroke cases (n = 280) and healthy controls (n = 280)	Blood	Association between global DNA hypomethylation by *LINE-1* and ischemic stroke	(Lin et al., 2014)
CHD subjects (n = 334) and healthy controls (n = 788)	Blood	Association between global DNA hypomethylation by *LINE-1* and CHD	(Wei et al., 2014)
Prevalent CHD subjects (n = 292), healthy controls (n = 247) incident CHD subjects (n = 317) and healthy controls (n = 262)	Blood	Association between global DNA hypomethylation by *LINE-1* and CHD and MI	(Guarrera et al., 2015)
Prevalent CVD subjects (n = 242), CVD free at baseline subjects (n = 470), incident CVD subjects (n = 44) and n = 86 deaths	Blood	Association between global DNA hypomethylation by *LINE-1* and CHD and stroke	(Baccarelli et al., 2010)
Postmenopausal women (n = 90)	Blood	Association between global DNA hypomethylation and higher risk of development of CVD	(Ramos et al., 2016)
CHD patients (n = 137) and healthy controls (n = 150)	Blood	Association between global DNA hypermethylation and CHD, especially in elderly	(Sharma et al., 2008)
Prevalent CHD subjects (n = 101) and incident CHD subjects (n = 52)	Blood	Association between global DNA hypermethylation with prevalence and incidence of CHD, MI, stroke and hypertension	(Kim et al., 2010)
15 atherosclerotic and 15 normal donor-paired samples, 19 carotid samples	Aorta and carotid	Association between genome-wide DNA hypermethylation and atherosclerosis	(Zaina et al., 2014)
6 individuals with 4 donor-paired samples for 3 tissues	Atherosclerotic right coronary artery, internal mammary arteries and great saphenous veins	Association between genome-wide DNA hypermethylation and coronary athrosclerosis	(Nazarenko et al., 2015)
CHD subjects with high hypoxia (n = 13) and with low hypoxia (n = 13)	Right atrial appendages	Association between global DNA hypermethylation in hypoxic subjects and fibrotic burden	(Watson et al., 2014)

Abbreviations: CoHD, coronary heart disease; MI, myocardial infarction; CVD, cardiovascular disease. Adapted from Fernández-Sanlés et al., (2017).

The list of candidate genes was summarized in **Table 11**. All EWAS that examine the association between DNA methylation and CoHD or atherosclerosis, identified 2625 CpGs and 111 CpG islands showing differential methylation in CoHD or atherosclerosis. Only 60 genes were identified in the same methylation direction in more than one EWAS (**Table 12**). In addition, recent EWAS analyzing the link between DNA methylation, obesity and coronary artery disease (CAD) identified 83 CpG sites that presented differential methylation depending on the BMI in CAD. Only *SREBF1* gene was found with differential methylation and expression that could be implicated in BMI, adiposity-related traits and CAD **(Table 12)** (Mendelson et al., 2017).

Table 11. Studies analyzing the association between specific-gene methylation levels and CVD.

Gene	Methylation status	Outcomes
ESRβ	Hypermethylated	Associated with atherosclerosis
AMT, CBS, EC-SOD, NPC1, p15INK4b, TCN2	Hypermethylated	Associated with CoHD
ABCA, PLA2G7	Hypermethylated	Associated with CoHD and aging
DDAH2	Hypermethylated	Associated with CoHD and dysfunction of endothelial progenitor cells in CoHD
MCT3, MT-CO1, MT-CO2, MT-CO3, MT-TL1	Hypermethylated	Associated with CVD
TIMP1	Hypermethylated	Associated with CVD and aging
GNASAS, INS	Hypermethylated	Associated with incidence of MI in women
ANRIL	Hypermethylated	Associated with increased arterial stiffness and higher CVD risk
FOXP3, GALNT2, ABCG1	Hypermethylated	Associated with increased risk of CoHD
MTHFR	Hypermethylated	Associated with ischemic stroke
ESRα	Hypermethylated	Associated with atherosclerosis, aging and CVD
COL15A1	Hypomethylated	Associated with atherosclerosis
BNIP3	Hypomethylated	Associated with CoHD
COMT	Hypomethylated	Associated with CoHD and aging
F7	Hypomethylated	Associated with CoHD in wild-type A1A1 genotypes
F2RL3	Hypomethylated	Associated with higher CVD mortality
GCK, IL-6	Hypomethylated	Associated with increased risk of CoHD
TNF-α	Hypomethylated	Associated with stroke development
ANGPTL2	Hypomethylated	Associated with the pro-inflammatory environment in post-acute coronary patients

Abbreviations: CoHD, coronary heart disease; CVD, cardiovascular disease; MI, myocardial infarction. Data from Fernández-Sanlés et al., (2017).

Table 12. Epigenome-wide association studies (EWAS) analyzing the association between DNA methylation and CVD.

Gene	Reference
Hypermethylated	
GRIP1, KCNJ14, PKD2, HRH2, NGEF, TNS1, ABCB4, KCNJ14, ACOT2, DLG2, SH2D4B, NGEF, SLC6A6, C1QTNF7, OLFML3, PART1, CALD1, THSD4, TNS1, PKD2, CORT, GRIP1, HRH2	(Nazarenko et al., 2015; Zaina et al., 2014)
SFRP4, MECOM, DOCK5, SMOC2, DYSF, SYTL3, PDZD2, PHACTR2, TRANK1, VWC2, RASGRF1, HMCN1, DFNA5	(Rask-Andersen et al., 2016; Zaina et al., 2014)
FYN, RNF216, SEPT9, CAMTA1	(Yamada et al., 2014; Zaina et al., 2014)
DSCAML1, PKNOX2, CTNNA3, DFNA5, DIP2C	(Sharma et al., 2008; Zaina et al., 2014)
FOXJ3, ZBTB16	(Ek et al., 2016; Zaina et al., 2014)
DLC1	(Oudejans et al., 2016; Rask-Andersen et al., 2016)
GATA3-AS1, AR	(Nazarenko et al., 2015; Sharma et al., 2008)
HAND2	(Rask-Andersen et al., 2016; Sharma et al., 2008)
C4orf48	(Oudejans et al., 2016; Rask-Andersen et al., 2016)
CBFA2T3	(Guarrera et al., 2015; Zaina et al., 2014)
Hypomethylated	
AIM2, F2RL3	(Ek et al., 2016; Nazarenko et al., 2015)
CRELD2	(Ek et al., 2016; Yamada et al., 2014)
GPR143	(Castillo-Díaz et al., 2010; Nazarenko et al., 2015)
HOXC5	(Nazarenko et al., 2015; Zaina et al., 2014)
MLC1	(Ek et al., 2016; Nazarenko et al., 2015; Zaina et al., 2014)
SREBF1	(Mendelson et al., 2017)

3.1.2.4 DNA methylation and liver diseases

NAFLD involves a variety of liver disorders associated with obesity (Chalasani et al., 2012). The steatosis is the benign condition that can be complicated to NASH, which can progress to cirrhosis and finally liver failure (Chalasani et al., 2012). The relevance of DNA methylation in NAFLD has been reported **(Table 13).**

Two EWAS analyses in the literature studied the DNA methylation profile in the different stages of NAFLD. In the first one, Ahrens et al., (2013) identified 74 CpGs to be differentially methylated in liver between healthy individuals, healthy obese, subjects with steatosis and NASH. NAFLD-specific metylation differences were found in 9 genes that participate in glucose metabolism, FA synthesis and

liver metabolism (pyruvate carboxylase (*PC*), ATP citrate lyase (*ACLY*) and phospholipase C gamma 1 (*PLCG1*)), and insulin signaling pathway (*IGF1*, insulin-like growth factor binding protein 2 (*IGFBP*) and protein kinase C epsilon (*PRKCE*)) (Ahrens et al., 2013).

The second study used liver biopsies from 33 mild NAFLD and 28 advanced NAFLD subjects and found 52,830 CpGs hypomethylated and 16,417 hypomethylated in advanced NAFLD comparing with mild NAFLD individuals. The hypomethylated genes in NAFLD were associated with matrix remodeling functions including those associated with fibrosis and cirrhosis (collagen type I alpha 1 chain (*COL1A1*), collagen type I alpha 2 chain (*COL1A2*), collagen type IV alpha 1 chain (*COL4A1*), collagen type IV alpha 2 chain (*COL4A2*), laminin subunit alpha 4 (*LAMA4*), laminin subunit beta 1 (*LAMB1*), connective tissue growth factor (*CTGF*) and platelet derived growth factor subunit A (*PDGFA*)), chemokines (C-C motif chemokine receptor 7 (*CCR7*) and *CCL5*, and associated with inflammation (signal transducer and activator of transcription 1 (*STAT1*), TNF alpha induced protein 8 (*TNFAIP8*) and caspase 1 (*CASP1*)). However, the hypermethylated genes in NAFLD were *APOC4*, cytochrome P450 family 2 subfamily C member 19 (*CYP2C19*), sodium/bile acid cotransporter (*SLC10A*), and acyl-CoA oxidase 1 (*ACOX*) among others (Murphy et al., 2013).

Moreover, a global methylation analysis using LINE showed 59 NASH-associated CpG sites correlated with fasting insulin levels, but not in subjects with simple steatosis (de Mello et al., 2017).

Furthermore, gene-specific DNA methylation studies also identified several genes with an important role in the development and progression of NAFLD. The demethylation of dipeptidyl peptidase 4 (*DPP4*) gene in obese subjects might contribute to deteriorations in hepatic functions and the development of hepatosteatosis (Baumeier et al., 2017). In addition, the DNA methylation status of tet methylcytosine dioxygenase (*TET*), *PPARGC1A* and transcription factor A mitochondrial (*TFAM*) genes were different in NAFLD subjects comparing with healthy subjects (Sookoian et al., 2010; Pirola et al., 2015).

Mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 6 (*MT-ND6*) gene methylation was different between steatosis and NASH (Pirola et al., 2015). In addition, a study that included 14 mild NAFLD and 12 advanced NAFLD blood and liver samples, described hypermethylation of peroxisome proliferator-activated receptor gamma (*PPARG*) and

hypomethylation of *PDGF* genes in severe NAFLD in comparison to mild NAFLD (Hardy et al., 2017). Zeybel et al., studied the DNA methylation differences of transforming growth factor beta 1 (*TGFβ1*), *PDGFA*, peroxisome proliferator-activated receptor alpha (*PPARA*) and *PPARD* between mild and advanced liver fibrosis. The investigation concluded that the combination of higher methylation at *TGFβ1* and *PDGFA* with lower methylation at *PPARA* and *PPARD* could be a useful tool for determining progression to liver fibrosis (Zeybel et al., 2015). These data suggested that DNA methylation play an important role in the development of liver diseases and may be used as putative biomarkers for the progression and severity of NAFLD.

3.1.2.5 DNA methylation and MetS

MetS is characterized by a cluster of risk factors associated with CVD, diabetes, obesity and stroke, which include visceral adiposity, IR, hypertension, hypertriglyceridemia and low HDL-C ("What Is Metabolic Syndrome?, NIH," 2016). There are several criteria for diagnosis of MetS. According to World Health Organization (WHO) criteria, the diagnosis of MetS requires hyperinsulinemia plus two or more other parameters. However, the most used definition is the revised Adult Treatment Panel-III (ATP-III), which requires at least three or more alterations **(Table 14).**

The prevalence of MetS is rapidly increasing in most countries, affecting more than 20% of the global adult population (Onat, 2011). Spanish adult population presented higher prevalence of MetS, specifically, 38.37 % in men and 29.62 % in women, and these percentages increased in the elderly (Marcuello et al., 2013). The high prevalence of MetS makes necessary to implement new strategies for its prevention.

Table 13. Studies analyzing the associations between DNA methylation and liver diseases.

Study population	Tissue sample	Methylation strategy	Target gene	Major results	Reference
Cross sectional n= 48 obese	Liver	Gene-specific	*DPP4*	Association between gene hypomethylation and early deterioration in hepatic function.	(Baumeier et al., 2017)
Cross sectional n = 141 subjects	Liver	EWAS	DNAm age	Association between DNAm age and insulin resistance and liver cancer	(Horvath et al., 2014)
n = 23 subjects with SS and n = 23 with NASH	Liver	Gene-specific	*MT-ND6*	Association between gene hypermethylation and histological severity of NAFLD	(Pirola et al., 2013)
n = 11 healthy controls and n = 63 NAFLD	Liver	Gene-specific	*PPARGC1A, TFAM*	Association between genes hypermethylation and peripheral IR	(Sookoian et al., 2010)
n = 23 healthy control and n = 67 NAFLD	Liver	Gene-specific	*TET*	Association between gene methylation and pathogenesis of NAFLD	(Pirola et al., 2015)
n = 12 healthy control, n = 12 NASH subject and n = 12 with SS	Liver	Global-methylation	*LINE-1*	Association between lower DNA methylation and NASH, but not SS	(de Mello et al., 2017)
n = 14 mild NAFLD fibrosis and n = 12 advanced NAFLD fibrosis	Liver and blood	Gene-specific	*PPARγ, PDGFα*	Association between *PPARγ* hypermethylation and disease progression and development of fibrosis. *PDGFα* becomes hypomethylated with increasing fibrosis severity.	(Hardy et al., 2017)
n = 18 healthy controls, n = 18 obese, n = 12 SS and n = 15 NASH	Liver	EWAS	*GALNTL4, ACLY, GRID1, IGFBP2, PLCG1, PRKCE, IGF1, IP6K3, PC*	Genes differentially methylated between phenotypic groups. *IGFBP2* hypermethylation associated with liver metabolism	(Ahrens et al., 2013)
n = 8 NAFLD with minimal fibrosis and n = 9 NAFLD with advanced fibrosis	Liver	Gene-specific	*PPARα, PPARδ, TGFβ1, PDGFα*	Association between *TGFβ1, PDGFα* hypermethylation and *PPARα, PPARδ* hypomethylaton with minimal fibrosis in NAFLD	(Zeybel et al., 2015)
n = 33 mild NAFLD and n = 23 advanced NAFLD	Liver	EWAS	69,247 CpGs	Association between DNA hypomethylation with matrix remodeling factors, fibrosis and cirrhosis, several chemokines and pro-inflammatory immune response in advanced NAFLD	(Murphy et al., 2013)

Abbreviations: NASH, non alcoholic steatohepatitis; NAFLD, non alcoholic fatty liver disease; IR, insulin resistance; SS, simple steatosis; CpGs, cytosine linked by a phosphate to guanine.

Numerous studies in human populations have looked into the possible association between DNA methylation and MetS features, including visceral adiposity, IR, hypertension, HDL-C and hypertriglyceridemia. Most of them observed that the DNA methylation pattern of different genes were associated with MetS parameters, resulting in a worse prognosis or a higher risk of presenting other several diseases **(Table 15)**. Moreover, several studies have evidenced that MetS risk factors are likely to play a role in the DNA methylation pattern and this modification of gene methylation suggests an initial phase of the development of MetS.

Table 14. WHO and ATPIII criteria for diagnosis of metabolic syndrome.

Parameter	WHO	ATPIII
Obesity	BMI ≥ 30 Kg/m^2 or	WC ≥ 100 cm in males
	WC ≥ 100 cm in males	WC ≥ 88 cm in females
	WC ≥ 88 cm in females	
BP	Hypertension or	Hypertension or
	SBP ≥ 130 mm Hg	SBP ≥ 130 mm Hg
	DBP ≥ 85 mm Hg	DBP ≥ 85 mm Hg
Cholesterol	HDL ≤ 35 mg/dL in males	HDL ≤ 40 mg/dL in males
	HDL ≤ 39 mg/dL in females	HDL ≤ 50 mg/dL in females
TG	TG ≥ 150 mg/dL	TG ≥ 150 mg/dL
Glucose	Diabetes or fasting glucose ≥ 110 mg/dL	Diabetes or fasting glucose ≥ 100 mg/dL
Insulin*	Hyperinsulinemia	

Abbreviations: BMI, body mass index; WC, waist circumference; SPB, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; TG, trygliceride.
* WHO requires hyperinsulinemia plus two or more other parameters. ATPII requires three or more parameters

Two prospective case-control studies in newborns from mothers who suffered a hyperglicemia during pregnancy, presented changes in guanine nucleotide binding protein alpha stimulating (*GNAS*), mesoderm specific transcript (*MEST*) and *NR3C1* genes methylation pattern supporting the hypothesis that these alterations contribute to the life-long risk of development of obesity and other metabolic disorders (El Hajj et al., 2013; Chen et al., 2014). In addition, an EWAS involving 8,165 participants integrating data from six independent cohorts, 2 case-control and 8 retrospective studies,

Table 15. Studies analyzing the association between DNA methylation and MetS features.

Study population	Sample type	Methylation strategy	Target gene	Major results	Reference
n = 87 with GDM women and n = 81 healthy pregnant women	Umbilical cord blood	Gene-specific	*GNAS*	GDM increases methylation level at fetal *GNAS* Hypermethylation of *GNAS* associated with high risk for diseases in offspring	(D. Chen et al., 2014)
n = 88 GDM dietetically treated, n = 98 GDM insulin-dependent, n = 65 healthy pregnant	Cord blood and chorionic villi	Gene-specific	*MEST*	GDM decreases *MEST* methylation in newborn *MEST* hypomethylation contributes to risk of development MetS	(El Hajj et al., 2013)
n = 70 healthy controls and n = 64 MetS subjects	VAT	Gene-specific	*LPL*	Association betwen *LPL* hypermethylation and etiology of MetS	(Castellano-Castillo et al., 2017)
n = 82 GDM and n = 67 T1D in pregnancy	Skeletal muscle, SAT and blood	Gene-specific	*TXNIP*	Association between intrauterine hyperglycemia and changes in *TXNIP* methylation	(Houshmand-Oeregaard et al., 2017)
n = 48 healthy controls and n = 12 T2D	Pancreatic islets	Gene-specific	*PPARGC1A*	*PPARGC1A* hypermethylation in T2D subjects Association between *PPARGC1A* hypermethylation and lower insulin secretion	(Ling et al., 2008)
n =61 subjects with FH and n = 30 severely obese non-FH	Blood and VAT	Gene-specific	*ADRB3*	Association between *ADRB3* hypomethylation and higher LDL-c, WC and apoB levels	(Guay et al., 2014)
n =13 healthy middle-age sedentary men	Muscle biopsies	Gene-specific	*ANGPTL4*	Association between *ANGPTL4* hypomethylation and low blood glucose and insulin sensitivity	(Laker et al., 2017)
Three cohort: n = 96, n = 94 and n = 104	SAT and blood	EWAS	2825 genes, 711 CpGs	Association between 2825 gene methylation and BMI Association between 711 CpGs methylation and HbA1c	(Rönn et al., 2015)
8 retrospective studies n = 8,061, 2 case-control study n = 687 and 3 cohorts n = 1,490	Blood	EWAS	187 CpGs	Association between 187 CpGs methylation and BMI Future risk for the development of T2D	(Wahl et al., 2016)
n = 185 adults	Blood	EWAS	*CPT1A, TXNIP, ABCG1*	Association between *CPT1A* and *TXNIP* hypomethylation and T2D and BMI	(Al Muftah et al., 2016)
LOLIPOP study n = 25,372	Blood	EWAS	*ABCG1, PHOSPHO1, SOCS3, SREBF1*	Association between gene methylation and BMI, WC, insulin concentration and HOMA-IR	(Chambers et al., 2015)
n = 438 children	Neonatal blood	EWAS	69 CpGs	Association between 69 CpGs methylation and HOMA-IR, insulin and glucose levels	(van Dijk et al., 2017)
Three prospective cohorts: n = 355, n = 167 and n = 645	Blood	EWAS	*TXNIP*	Association between DM and hypomethylation of *TXNIP* gene. *TXNIP* hypomethylation is a consequence of hyperglycemia levels	(Soriano-Tárraga et al., 2016)

Table 15. Studies analyzing the association between DNA methylation and MetS features (continuation).

Study population	Sample type	Methylation strategy	Target gene	Major results	Reference
Newborns categorized in LBW n = 3, NBW n = 3 and HBW n = 3	Cord blood and placenta	EWAS	360 DMR in LBW and 773 DMR in HBW	Genes encoding p38 MAPK signaling components are hypermethylated in LBW and HBW PI3K/AKT pathway components hypomethylated in HBW Association between DNA hypomethylation and glucose transport in HBW	(Mao et al., 2017)
ESTHER study n =527	Blood	EWAS	*TXNIP*	Association between *TXNIP* hypomethylation and increasing fasting glucose and HbA1c	(Florath et al., 2016)
n = 276 subjects with FH and n = 26 healthy subjects	Blood	EWAS	*TNNT1*	*TNNT1* methylation is related to 10 % of the interindividual variation in HDL-c levels	(Guay et al., 2012)
Three independent cohorts n = 99,994	WBC	EWAS	*IGFBP3, KCNK3, PDE3A, PRDM6,ARHGAP24,OSR 1,SLC22A7,TBX2*	Association between gene methylation and BP and hypertension	(Kato et al., 2015)
n = 850 subjects	Blood	EWAS	*CPT1A, ABCG1*	Association between gene methylation and TG storage	(Mamtani et al., 2016)
n = 59 healthy subjects and n = 58 obese	SAT	EWAS	Genome-wide	Association between global DNA hypermethylation and WAT inflammation and IR	(Petrus et al., 2017)
Botnia study: n = 129 subjects who developed T2D and n = 129 who did not develop T2D	Blood	Gene-specific	*PHOSPHO1, ABCG1*	Association between *PHOSPHO1* methylation and HDL-c levels. Association between *ABCG1* methylation and BMI, HbA1c, fasting insulin and TG levels	(Dayeh et al., 2016)
Human SW62 cell line (*in vitro*)	Cell	Gene-specific	*HSD11B2*	Association between *HSD11B2* methylation and blood pressure	(Alikhani-Koopaei et al., 2004)
Human NCI H295R cell line (*in vitro*)	Cell	Gene-specific	*AGT*	Association between *AGT* hypomethylation and angiotensin II enzyme activity Hypomethylation of *AGT* may result in hypertension and kidney injury.	(Wang et al., 2014)
C57BL/6J mice with IH	Endothelial cells	Gene-specific	*Ace1, Agt*	Association between gene methylation and systolic hypertension in mice exposed to IH	(Chu et al., 2015)

Abbreviations: GDM, gestational diabetes mellitus; MetS, metabolic syndrome; VAT, visceral adipose tissue; T2D, type 2 diabetes; SAT, subcutaneous adipose tissue; FH, familiar hypercholesterolemia; LDL-c, low density lipoprotein cholesterol; WC, waist circumference; apoB, apolipoprotein B; EWAS, epigenome-wide association study; CpG, cytosine linked by a phosphate to guanine; BMI, body mass index; HbA1c, hemoglobin A1C; LOLIPOP, London Life Sciences Prospective Population Study; HOMA-IR, homeostasis model assessment-insulin resistance; WAT, white adipose tissue; IR, insulin resistance; LBW, low birth weight; HBW, high birth weight; DMR, differentially methylation region; ESTHER, Epidemiologische Studie zu Chancen der Verhütung, Früherkennung und optimierten THerapie chronischer ERkrankungen in der älteren Bevölkerung; HDL-c, high density lipoprotien cholesterol; TG, triglycerides; BMI, body mass index, SW62, colon carcinoma cell line; NCIH295R, adrenocortical cell line; IH, intermitent hipoxia.

showed a causal relationship between adiposity and DNA methylation alteration in blood and adipose tissue. These data support an important function of altered DNA methylation mediated by visceral adiposity, in the development of several diseases, such as T2D, obesity, CVD and cancer (Rönn et al., 2015; Wahl et al., 2016). Moreover, a case-control study with 64 subjects with hypertriglyceridemia found an association between lipoprotein lipase (*LPL*) gene promoter methylation, which was higher in these individuals, with poor metabolic profile and the development of MetS (Castellano-Castillo et al., 2017).

An EWAS performed in 483 children identified a number of DMRs at birth that were associated with insulin sensitivity in childhood. Many of these changes in DNA methylation were causally related to the health outcomes (van Dijk et al., 2017). In addition, methylation pattern of different genes have been associated with the risk of development of MetS. The DNA methylation alteration of *PPARGC1A*, *CPT1A, TXNIP*, *ABCG1*, *PHOSPHO1*, suppressor of cytokine signaling *3* (*SOCS3*) and *SREBF1* were associated with increased IR and the future development of T2D (Ling et al., 2008; Chambers et al., 2015; Al Muftah et al., 2016). Some of these genes, such as *CPT1A*, *TXNIP*, *ABCG1* and *PHOSPHO1* were also involved in the dysregulation of glucose metabolism, hypertriglyceridemia and decreased HDL-C levels, suggesting a role in the development of T2D and CVDs (Florath et al., 2016; Dayeh et al., 2016; Mamtani et al., 2016). Furthermore, the methylation patterns of *IGFBP3*, potassium two pore domain channel subfamily K member 3 (*KCNK3*), phosphodiesterase 3 A (*PDE3A*), PR/SET domain 6 (*PRDM6*), Rho GTPase activating protein 24 *(ARHGAP24*), odd-skipped related transciption factor 1 (*OSR1*), solute carrier family 22 member 7a (*SLC22A7*), t-box 2 (*TBX2*) and hydroxysteroid 11-beta dehydrogenase 2 (*HSD11B2*) were associated with BP and alteration in endothelial vascular function resulting in hypertriglyceridemia (Alikhani-Koopaei et al., 2004; Kato et al., 2015).

As a summary, it has been shown that DNA methylation is altered in subjects with one or various risk factors of MetS. The assessment of DNA methylation could be a good predictor of MetS and related diseases. Conversely, the majority of genes that were found are not validated among different populations, suggesting that may be weak biomarkers of MetS.

3.2 DNA methylation and chronobiology in the context of obesity

In mammals, the synchronization between circadian rhythms and environmental stimuli, including daily rhythms of natural light, external temperature and food intake, is driven by the circadian system, a hierarchical multilevel organization (Tarquini & Mazzoccoli, 2017). The most frequent biological rhythms are hallmarked by a 24-hour period with the solar illumination. The light/darkness is perceived by the retina via melanopsin-containing ganglion cells and transferred to the suprachiasmatic nuclei (SCN) of the hypothalamus (Tarquini & Mazzoccoli, 2017). Although photic cue is the main signal, alternative signals such as feeding time can also disengage central and peripheral oscillators present in most tissues and cells (Tarquini & Mazzoccoli, 2017).

Circadian oscillation is regulated by the molecular circadian clockwork that consists on a positive and negative transcription-translation feedback loop **(Figure 9)**. It is initiated by the transcription factor aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1), that dimerizes with circadian locomoter output cycles kaput (CLOCK) or neuronal PAS domain protein 2 (NPAS2) to activate the expression of the negative regulators of period circadian regulator (*PER*) and cryptochrome 1 (*CRY1*) genes through E-box elements (5'-CACGTG-3') (Kim et al., 2015). PER/CRY proteins in a negative feedback suppress BMAL1/CLOCK activity (Garaulet & Gómez-Abellán, 2013). Additional components of the core clock stimulated by BMAL1/CLOCK include receptor tyrosine kinase-like orphan receptor (ROR) and Rev-erbα, which bind ROR DNA elements (ROREs) for stimulating or repressing transcription. The BMAL1/CLOCK heterodimer also can stimulate the transcription of DP beta (DPB) and E4 promoter–binding protein 4 (E4BP4), which bind to D-box elements contributing to the circadian output of the molecular clock (Papazyan et al., 2016).

Recent studies have reported the interaction between the circadian clock and energy regulation and metabolism. Indeed, disruption of circadian rhythms compromises metabolic homeostasis and contributes to the development of obesity and obesity-related pathologies (Antunes et al., 2010; Bass & Takahashi, 2010; Garaulet et al., 2010). Many metabolic functions such as glucose homeostasis, lipid metabolism and fasting/feeding cycles, are regulated by the internal clock system (Tarquini & Mazzoccoli, 2017). Furthermore, an online database (CircaDB) of circadian transcriptional profiles in

mammalian tissues disclosed that numerous epigenetic genes such as histone deacetylase 4 (*Hdac4*), *Dnmt3a*, *Dnmt3b*, and *Tet2*, present rhythmic tissue-specific expression patterns in male Siberian hamsters (Stevenson, 2017). In addition, a comprehensive review discussed the evidence demonstrating that chromatin remodeling and other epigenetic mechanisms, including histone modifications and DNA methylation, regulate the expression of the main circadian clock genes by (Sahar & Sassone-Corsi, 2013).

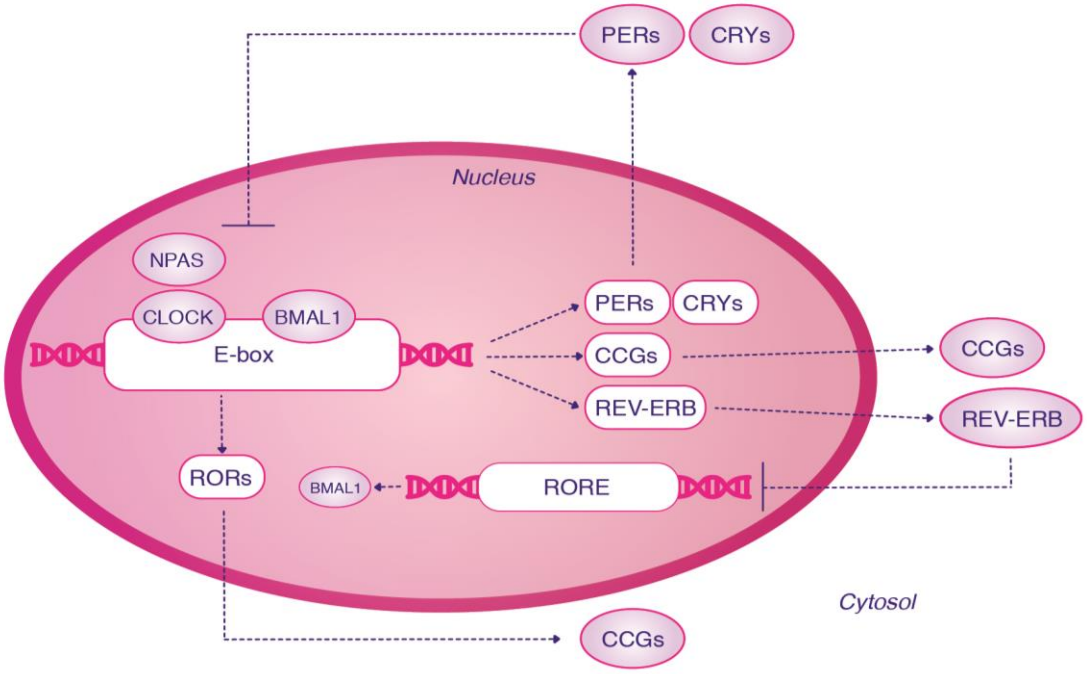


Figure 9. Molecular circadian clock mechanisms in a cell. Adapted from Albrecht, (2012).

A crossover clinical study in 15 healthy men who followed acute total sleep deprivation presented hypermethylation of *CRY1* and period circadian regulator 1 (*PER1*) genes in adipose tissue and were associated with metabolic disruption (Cedernaes et al., 2015). In addition, children with obstructive sleep apnea exhibited *FOXP3* gene hypermethylation and higher systemic inflammatory response (Kim et al., 2012). In relation to obesity, a study performed in 60 normoweight, overweight and obese women described hypermethylation of three CpGs in *CLOCK* and two CpGs in *BMAL1* genes in overweight/obese women in comparison to normal-weight. Moreover, the methylation of these genes were associated with BMI, body fat, WC, HOMA, and MetS score (Milagro et al., 2012). These

findings suggest that the circadian system might be implicated in the onset of obesity through a DNA methylation mechanism.

3.3 DNA methylation markers in weight loss

As obesity has been attributed to a positive energy balance (Chatzigeorgiou et al., 2014), reduction of energy intake, changes in macronutrient distribution, increase of physical activity, behavioural approaches, and pharmacological or surgical treatments are the strategies followed in order to induce a negative energy balance (Salas-Salvadó et al., 2007; Biddle et al., 2017). Although many strategies have been investigated for inducing a weight reduction, individual response vary widely.

As it has been described previously, DNA methylation has been described to be associated with body weight regulation, since it is involved in appetite, adiposity, adipogenesis and glucose and lipid metabolism (Cheng et al., 2018). In addition, dietary factors, such as FAs, polyphenols or methyl donors, exercise and pharmacological and surgical treatments can induce changes in the DNA methylation pattern (Milagro et al., 2012; Barres et al., 2013; Milagro et al., 2013; Fiorito et al., 2014; Remely et al., 2015; Tremblay et al., 2017). In this context, several studies have identified DNA methylation as a regulatory mechanism of inter-individual metabolic response to weight loss **(Table 16).**

Several studies based on energy-restricted treatments for weight loss reported many CpGs whose methylation was associated with the response to the intervention. Thus, 35 different loci were differentially methylated between high and low responders to an energy-restricted diet in adipose tissue from overweight and obese women. Moreover, the methylation status of potassium voltage-gated channel subfamily A member 3 (*KCNA3*), GLI similar 3 (*GLIS3*), E26 transformation-specific (*ETS*), nuclear factor 1 X-type (*NFIX),* insulinoma-associated protein 1 (*INSM1*), corticotropin releasing hormone receptor 2 (*CHRH2*), enoyl coenzyme A hydratase short chain 1 (*ECHS1*) and cholecystokinin B receptor (*CCKBR*) genes were associated with weight control and insulin secretion (Bouchard et al., 2010).

Table 16. Studies aznalyzing the association between DNA methylation and weight loss.

Weight loss strategy	Sample study	Sample type	Target gene	Main results	Reference
Genotypic information	n = 95 obese and overweight	Blood	Global DNA	Association between global DNA hypomethylation and increased weight loss	(Pirini et al., 2018)
Physical activity	n = 20 healthy women	Blood	*NAMPT, RUNX3, BR, SLCO4C, WNT7A, RASGRP3, CYP2E, CA13, KANK4, SMOC2, SLIT3, GABRG3*	Baseline DNA methylation was able to predict the percent body weight change over the six-month period	(McEwen et al., 2017)
Behavioural and ER	n = 20 NW, n = 20 overweight/obese and n = 20 morbid obese women	Blood	*CLOCK, BMAL1, PER2*	Association between the baseline methylation of genes and the magnitude of weight loss.	(Milagro et al., 2012)
No treatment	n = 51 subjects	Blood	*POMC*	Association between *POMC* hypermethylation and individuals body weight.	(Kühnen et al., 2016)
RYGB	n = 5 obese and n = 6 non-obese women	Skeletal muscle	409 DMR	409 DMR after weight loss.	(Barres et al., 2013)
ER	n = 14 overweight and obese women	Adipose tissue	35 CpGs	Association between 35 loci methylation and weight control.	(Bouchard et al., 2010)
ER	n = 27 obese women	Adipose tissue	*LEP, TNF*	Association between hypomethylation of *LEPTIN* and *TNF-α* at baseline with better response to the dietary intervention.	(Cordero et al., 2011b)
ER	n = 24 overweight and obese adolescents	Blood	*AQP9, DUSP22, HIPK3, TNNT1, TNNI3*	Association between basal DNA methylation in *QP9, DUSP22, HIPK3, TNNI3,* and *TNNT1* with changes in body weight, BMI-SDS, WC, and body fat mass after the weight loss intervention.	(Moleres et al., 2013)
ER	n = 18 obese men	Blood	*POMC, NPY*	Association between baseline *NPY* hypomethylation with weight-loss regain Association between *POMC* hypomethylation with success in weight-loss maintenance	(Crujeiras et al., 2013)
BS	n = 9 healthy control, n = 22 obese + ER, n = 14 obese + BS	Blood	*SERPINE1*	Baseline *SERPINE1* methylation may be a predictor of weight loss after BS	(Nicoletti et al., 2016)
ER	n = 12 obese and overweight	Blood	*ATP10A, CD44, WT1*	*ATP10A* and *CD44* genes showed baseline methylation differences depending on the weight-loss outcome. DNA methylation levels of on the *WT1* gene were hypermethylated in the high than in the low responders.	(Milagro et al., 2011)

Abbreviations: ER, energy-restriction; NW, normal weight; RYGB, Roux-en- Y gastric bypass; DMR, differentially methylated region; CpG, cytosine linked by a phosphate to guanine; BMI-SD, body mass index-standard deviation; WC, waist circumference; BS, bariatric surgery.

In addition, the methylation status in adipose tissue and blood of several genes that participate in the regulation of BP, inflammation, lipid metabolism, appetite and energy homeostasis, such as *TNF*, *POMC,* neuropeptide Y (*NPY*), *ATP10*, CD44 molecule (Indian blood group) (*CD44*) and *LEP* have been related to significant differences in the weight loss response after a low calorie diet (30 % of energy-restriction) (Cordero et al., 2011b; Milagro et al., 2011; Crujeiras et al., 2013). Moreover, clock genes, such as *CLOCK*, *BMAL1* and period circadian regulator 2 (*PER2*), are implicated in the regulation of circadian clockwork and one study associated baseline methylation of these genes with body-weight loss in a women population within a behavioural and energy-restricted intervention (Milagro et al., 2012).

Some studies have analyzed other factors, such as physical activity or gastric surgery, in relation to the interaction between weight loss and DNA methylation. DNA methylation analysis in blood samples from 20 healthy women within a physical activity program identified significant associations between the methylation profile of 12 CpGs and weight loss (McEwen et al., 2017). A lifestyle and nutritional educational weight loss program in 24 overweight and obese adolescents associated the methylation status of genes related to glucose metabolism, IR, inflammation and CVD, such as aquaporin-9 (*AQP9*), dual specificity protein phosphatase 22 (*DUSP22*), homeodomain-interacting protein kinase 3 (*HIPK3*), slow skeletal muscle troponin T 1 (*TNNT1*) and *TNNI3*, with weight loss modifications (Moleres et al., 2013). Moreover, two investigations with bariatric surgery intervention identified 409 differentially methylated regions (DMR) after weight loss, and specifically, methylation of *PPARGC1A*, serpin family E member 1 (*SERPINE1*) and *PDK4* were involved in weight control and glucose and lipid metabolism (Barres et al., 2013; Nicoletti et al., 2016).

Although genetic factors are involved in the regulation of body weight, genetic variants only partially explain the individual variation observed in the response to weight loss treatment. Thus, DNA methylation could play a role in the environment-gene interaction in weight regulation. Pirini et al., (2018) studied the genetic and epigenetic alterations associated with weight loss within a personalized weight reduction program designed on the basis of genotypic information. The investigation identified an inverse association between global DNA methylation and weight loss depending on individual genetic variants for insulin induced gene 2 (*INSIG2*), melanocortin 4 receptor (*MC4R*), *ADRB2*,

APOA4 and guanine nucleotide-binding protein 3 (*GNB3*) (Pirini et al., 2018). In summary, all these studies confirm that DNA methylation, in combination with genetic variants and other biomarkers, could be useful in the personalization of the clinical management of obesity.

4. DNA MARKERS IN INFLAMMATION

Inflammation is a normal physiological response of a body to harmful stimuli, such as pathogens, damaged cells and chemical irritants (Larsen & Henson, 1983). Acute inflammation is a crucial component of the immune response, but when inflammation persists for a long period, it becomes chronic. Although the symptoms in chronic inflammation are not as severe as in acute inflammation, the persistent condition is involved in the development of numerous diseases including inflammatory bowel disease (IBD), T2D, atherosclerosis, obesity, and/or MetS (Solas et al., 2017).

Inflammation is a complex response in which a variety of interacting pathways, molecules and different cells types are implicated (Larsen & Henson, 1983). The response involves three major steps: i) An increased blood supply to the damage tissue; ii) Migration and recruitment of cells from bloodstream to inflamed regions by the signaling of chemoattractants from the site of inflammation and by the adhesion molecules on the endothelium; iii) Secretion of mediators from cells at the site of inflammation **(figure 10)** (Calder et al., 2009). The inflammatory response requires a sophisticated regulatory mechanism to carry out functions at signal- and gene-specific levels. In addition, cells must be able to phenotypically adapt continuously their response by the expression, production and secretion of the different inflammatory mediators (Shi & Pamer, 2011). In this context, transcription factors such as nuclear factor-kappa B (NF-κB), FOXP3, interferon regulatory factor (IRF) and STAT families, in combination with epigenetics and, specifically, DNA methylation, have been suggested as the mechanisms involved in the regulation of inflammatory response.

Several groups have shown that DNA methylation modulate mediators of inflammation including immune cells and inflammatory molecules. In addition, it is well documented that DNA methylation may also be involved in the development of chronic inflammation-related diseases (Stenvinkel et al., 2007), including obesity and MetS

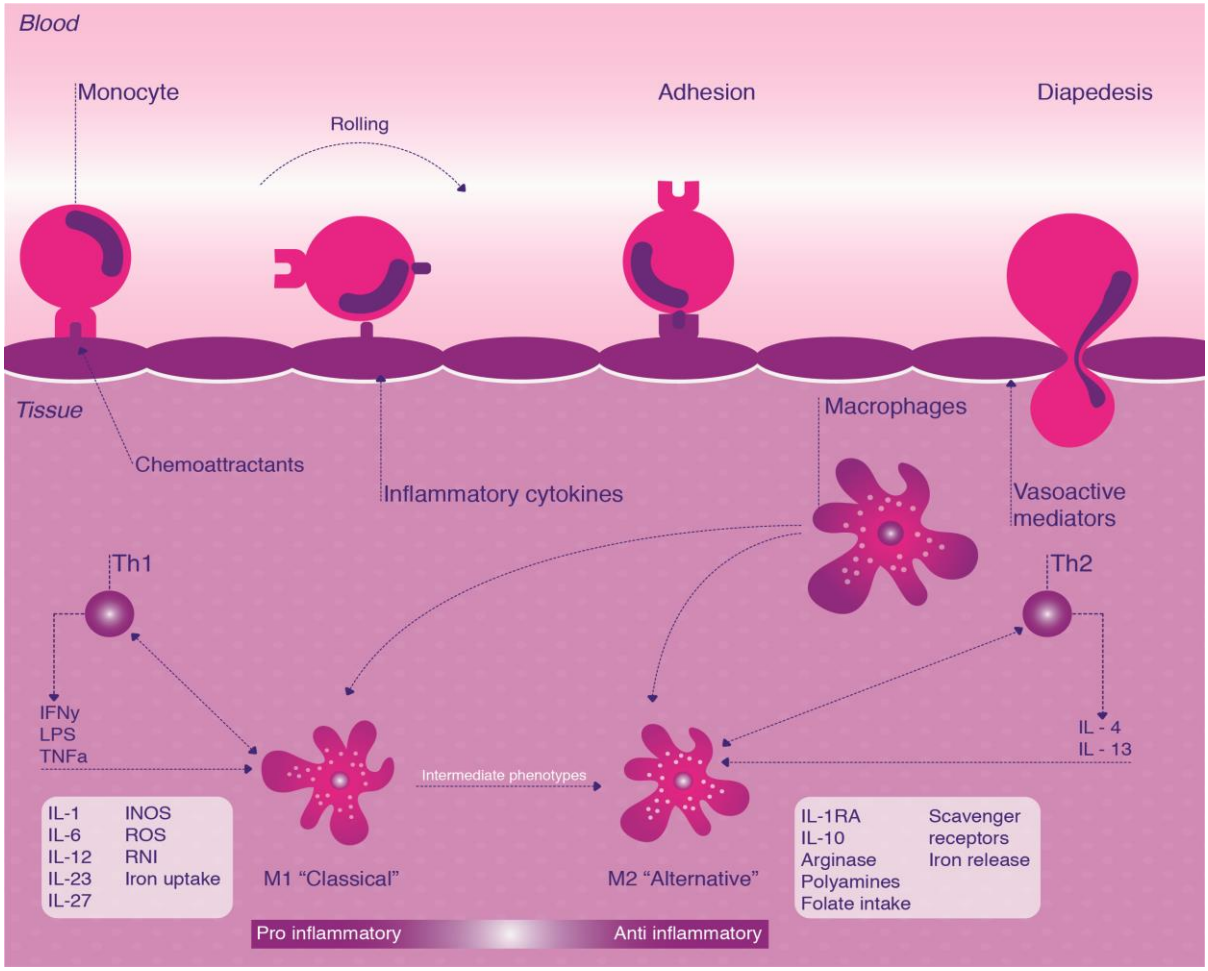


Figure 10. Generalised view of the inflammatory process including interaction between T cells and macrophages, and M1 and M2 macrophage polarization profiles. Adapted from Calder et al., (2009) and Sima et al., (2013)

4.1 DNA methylation and immune cells

Monocytes and macrophages are the main cells involved in the inflammatory response. Monocytes are involved in the homeostasis during infection and tissue repair (Saeed et al., 2014). Commonly, circulating monocytes migrate to inflamed tissues and transform into monocyte-derived macrophages (Mantovani et al., 2013). Macrophages have been classified as classic M1 and alternative M2 phenotypes. Activated M1 macrophages are proinflammatory cells whose activation is induced by interferon (IFN) γ or biological pathogens. The continuous activity of M1 macrophages results in chronic inflammatory diseases and tissue damage (Hoeksema et al., 2012). On the other hand, M2 phenotype is induced by IL-4 and IL-10 and is involved in the decrease of inflammatory response and the promotion of tissue repair. In this context, numerous studies have shown that DNA methylation

has a functional role in the monocyte-to-macrophage differentiation and macrophage polarization (Saeed et al., 2014; Fogel et al., 2017).

On the other hand, T cells have been identified as key players in inflammation-related diseases; specifically, T helper (Th) 1 and Th17 participate in cell-mediated immunity (Fogel et al., 2017). Similarly to monocyte/macrophage cells, dynamic DNA methylation and demethylation are necessary to develop and differentiate T lineage (Ansel et al., 2003). The alteration of the DNA methylation landscape during these processes may produce several diseases. For example, the inhibition of the methylation of T cells results in a lupus-like disease (Teitell & Richardson, 2003). In contrast to monocyte/macrophages and T cells, B cells have been scarcely studied in inflammatory diseases. However, these cells are involved in the physiopathology of IBD, Crohn's disease or ulcerative colitis (Fogel et al., 2017). In this context, DNA methylation regulates Toll-like receptor (TLR) 2 and *IL-8* expression, which are necessary for the function of B cells (Noronha et al., 2009).

4.2 DNA methylation and mediators of inflammation

It is known that inflammation is controlled by cellular and extracellular mediators that are produced by immune cells. These molecules are cytokines, growth factors, eicosanoids, and several peptides (Turner et al., 2014). TLRs are also inflammatory regulators that are expressed in the membranes of a great variety of cell types including immune cells. The TLR family includes 11 proteins, from TLR1 to TLR11. One of the major functions of these molecules is to induce signalling pathways which ultimately lead to the expression of inflammatory cytokines, chemokines, and IFNs (Chen et al., 2007).

Cytokines are essential modulators of inflammation via complex networks of interactions. Cytokines are classified based on the nature of immune response, cell type or location. Key inflammatory cytokines include IL-1, IL-6 and TNF-α. The IL-1 family includes 11 protein members, expressed by numerous cells types and comprise pro- and anti-inflammatory proteins such as IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-6, and IL-10. Except for IL-1Ra, all the anti-inflammatory cytokines have also some proinflammatory properties (Opal & DePalo, 2000). TNF-α is a potent inflammatory mediator, involved in cytokine production, adhesion molecule activation and growth factor stimulation

(Turner et al., 2014). Under the stimulation of IL-1, TNF-α or lipopolysaccharide (LPS), cells produce chemokines, which are small cytokines with a chemotactic function. These molecules participate in the inflammatory response attracting leukocytes, monocytes and other effector cells to the site of inflammation. Some examples are C-X-C motif ligand (CXCL) 8, CCL2 and CCL4, among others. In addition, IFNs are another group of signaling proteins that contribute to the inflammatory cytokine production (Chen, 2017).

Although it is widely known that DNA methylation is involved in the regulation of inflammation, little is known about the role of DNA methylation in the expression control of inflammatory genes during the inflammatory response. In this context, **Table 17** summarizes the studies that evidence the regulation of inflammatory mediators by DNA methylation.

Table 17. Studies analyzing association between DNA methylation and inflammatory mediators.

Molecule	Main sources	Major function	DNA methylation modulation	Reference
TLR2	Leukocytes	Stimulation of NF-κB	Association between promoter methylation and higher pro-inflammatory response in cystic fibrosis	(Shuto et al., 2006)
TLR4	Macrophages	Stimulation of cytokines	DNA methylation regulates *TLR4* expression in intestinal epithelial cells	(Takahashi et al., 2009)
IL1β	Macrophages	Pyrogenic, pro-inflammatory, proliferation and differentiation	IL1β acts as demethylating agent and changes DNA methylation of *IL6* and *IL8*	(Caradonna et al., 2017)
IL6	Th cells, macrophages, fibroblasts	Differentiation into plasma cells	Promoter hypomethylation decreases *IL6* expression	(Ma et al., 2016)
TNFα	Monocytes and macrophages	Phagocyte cell activation, endotoxic shock	Association between higher DNA methylation and *TNFα* low expression in monocytes Association between *TNFα* demethylation and pluripotent stem cells differentiation to hematopoietic stem cells	(Sullivan et al., 2007)
CCL2	Endothelium, monocytes, macrophages	Recruits monocytes, memory T cells and DC	Association between global DNA hypermethylation and higher expression of *CCL2*	(Petrus et al., 2017)
IFN	Leukocytes, fibroblast, T cells	Anti-viral, macrophage activation, increases monocyte function	Aberration of *IFN* methylation is associated with autoimmune diseases	(Chen et al., 2017)

Abbreviations: NF-κB, nuclear factor kappa B; DC, dentritic cells.

4.3 DNA methylation role in obesity and other chronic inflammation-related diseases

The excess adiposity characteristic of obesity is an established risk factor for the development of IR, T2D, hypertension, NAFLD, polycystic ovarian diseases, and some cancers (Hotamisligil & Erbay, 2008). Hypertrophied adipocytes and adipose tissue-resident immune cells (i.e lymphocytes and macrophages) contribute to the inflammatory condition by increasing the secretion of proinflammatory cytokines, which enhance tissular inflammation (Makki et al., 2013). A number of studies have evidenced that the inflammatory trigger in obesity is mainly caused by an excess of nutrient consumption (Gregor & Hotamisligil, 2011). For example, several animal studies have suggested that changes in body weight, adiposity and inflammatory response occur very early after a short-term HFD feeding. When the obesogenic diet lasts in time, animals become glucose intolerant and IR in all metabolic tissues including liver and muscle (Sun et al., 2012).

Adipose tissue macrophages (ATMs) are categorized into M1 or M2 phenotypes based on their differential expression of surface markers and cytokines. Obesity is associated with a positive deregulation of ATM polarization into M1 macrophages, which are characterized by a more proinflammatory phenotype and contribute to obesity-induced inflammation (Wang et al., 2016). In this context, several investigations have associated DNA methylation with the inflammatory state in obesity. A study in animals demonstrated that DNMT1-mediated *Pparg1* promoter DNA methylation in macrophages was significantly enhanced in obesity and switched to a more pro-inflammatory M1 phenotype (Xianfeng Wang et al., 2016). Another study in mice described the association between the hypomethylation of *Tnf* and higher adipose tissue inflammatory response (García-Escobar et al., 2017). A study in the human monocyte/macrophage THP-1 cell line associated global DNA hypermethylation with the induction of pro-inflammatory and adipogenic transcriptional profile (Flores-Sierra et al., 2016). In addition, the hypermethylation of *IL-6* was correlated with increased childhood adiposity in cord blood of Mexican children (Wu et al., 2018) and with overweight and obesity in blood cells of Korean women (Na et al., 2015).

On the other hand, MetS is characterized by a chronic low-grade inflammatory condition that is associated with the pathophysiological consequences of the syndrome (Monteiro & Azevedo, 2010). For example, high circulating levels of IL-6 and TNF-α have been associated with MetS in women and men cohorts. Interestingly, low circulating levels of the anti-inflammatory cytokine IL-10 have been found to be associated with higher inflammation and risk for MetS (Esposito & Giugliano, 2004). And elevated levels of C-reactive protein (CRP), an inflammatory biomarker, are a strong predictors of diabetes and CVD (Esposito & Giugliano, 2004).

DNA methylation and inflammation may together affect the susceptibility to MetS. For example, an EWAS identified an association between global DNA hypermethylation and WAT inflammation and IR in obese subjects in comparison with healthy subjects (Petrus et al., 2017). DNA methylation influences inflammatory-related gene transcription and pathways, and subsequently different organ function. An extensive review has described that DNA methylation of inflammatory genes regulates circulating cholesterol and TG levels, and is associated with obesity (Khyzha et al., 2017). Specifically, Lopez-Legarrea et al., (2013) described the association between high methylation levels of *SERPINE1* at baseline in peripheral white blood cells and higher changes in body weight, fat mass, TC and TG after a dietary program (Lopez-Legarrea et al., 2013). In addition, other studies have found that *IL-6* methylation in peripheral blood presented a negative association with BP (Mao et al., 2017), meanwhile *IL-10* hypomethylation in blood was associated with the development of MetS (Tobi et al., 2009).

HYPOTHESIS AND OBJECTIVES

1. Hypothesis

This work is based on the hypothesis that the DNA methylation patterns of some genes are related to life style factors including the diet (e.e., calorie intake, nutrients and other bioactive compounds), and can have an effect on the onset of obesity and its comorbidities, as well as associated with the individualized response to different weight loss programs. As suggested by previous investigations, the identification of novel epigenetic biomarkers may help to determine the risk of obesity and related pathologies, and to design more personalized weight loss strategies in the framework of precision nutrition. Some of these diseases are characterized by a low-grade chronic inflammatory state. In this study, we also hypothesize that dietary methyl donors are able to modify the inflammatory response through the epigenetic regulation of inflammation mediators.

2. General objective

The general aim of this work was to identify DNA methylation patterns that are associated with obesity and the response to specific weight-loss treatments, deepening into the interactions between epigenetic marks and dietary patterns and nutrients in order to link them with the development of obesity and inflammation.

3. Specific objectives

1. To analyse the association between the DNA methylation pattern in white blood cells and the development of obesity in a pediatric population (*chapter 1*).

2. To investigate the influence of a weight loss intervention in the DNA methylation levels of genes involved in the circadian system, and the association between DNA methylation and changes in the lipid profile (*chapter 2*).

3. To identify potential epigenetic biomarkers for weight loss within a weight-loss program by integrating transcriptome and methylome microarray data (*chapter 3*).

4. To evaluate whether a low intake of folic acid is related to adverse metabolic features in obese subjects through changes in gene-specific DNA methylation patterns (*chapter 4*).

5. To study whether folate and other dietary methyl donors can prevent the inflammatory response in a human monocyte/macrophage in vitro model through epigenetic mechanisms (*chapter 5*).

SUBJECTS AND METHODS

In order to study the possible DNA methylation marks underlying obesity and related disorders as well as potential interactions between DNA methylation patterns with dietary patterns and nutrients different populations were used in the present work (**Figure 11**): the GENOI ("Grupo de Estudio Navarro de la Obesidad Infantil") study, the Women cohort and the RESMENA ("REducción del Síndrome Metabólico en Navarra") study. In addition, THP-1 cells were used for the *in vitro* study.

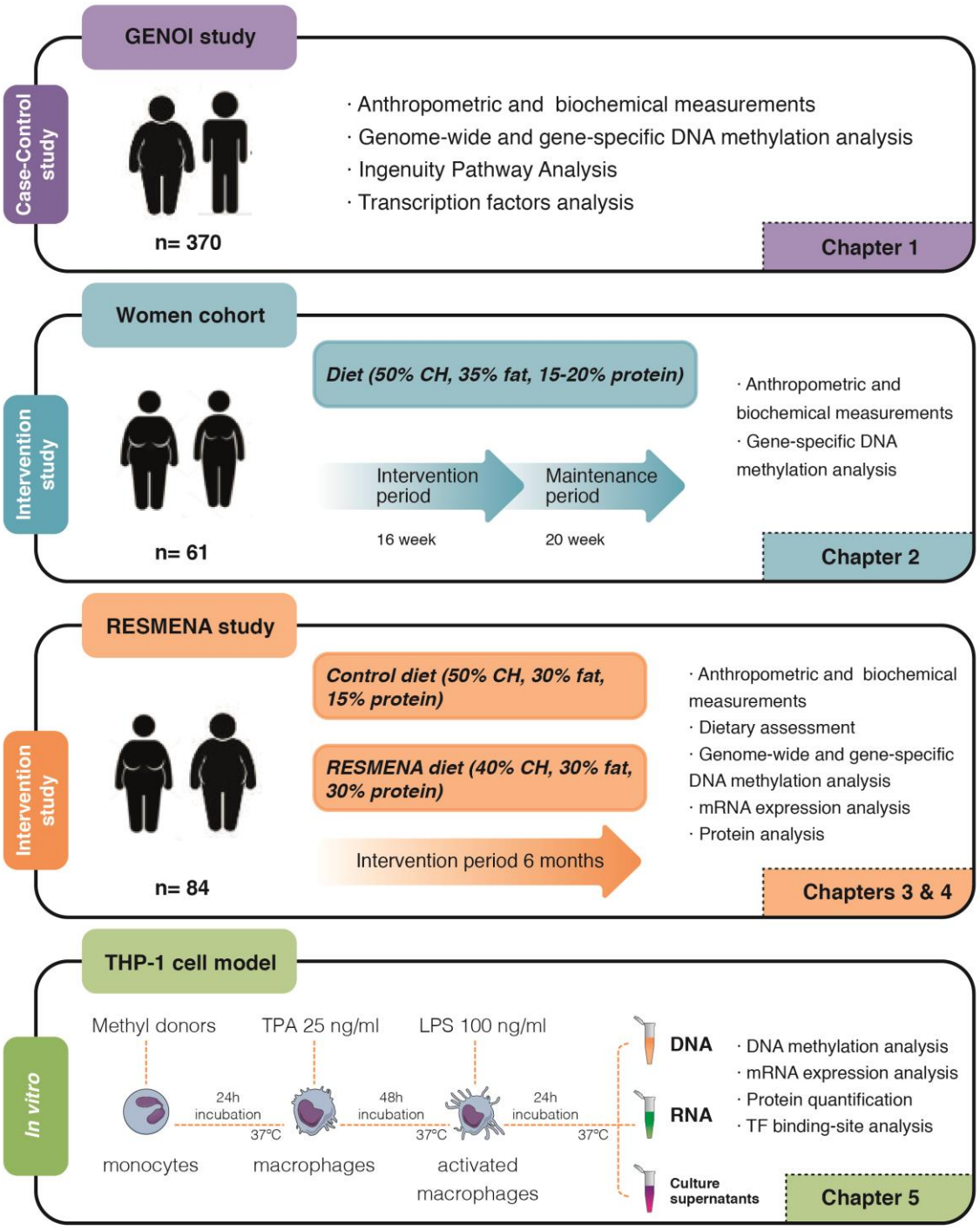


Figure 11. Overview of the experimental designs conducted from Chapter 1 to Chapter 5.

The objectives proposed for the present work have been addressed in different chapters (Chapters 1 to Chapter 5). In this section a brief explanation of each experimental design and the most relevant laboratory techniques and procedures used will be given.

1. STUDY POPULATIONS

❖ GENOI study (Chapter 1)

The GENOI study is a cross-sectional study of cases and controls started in 2001 and finished in 2003. The study was designed to understand the role of lifestyle and genetics factors on obesity development in children and adolescents in the region of Navarra, Spain.

The obese (cases) and non-obese (controls) participants were recruited from Virgen del Camino Hospital, Clinica Universidad de Navarra and other primary care centers when they were attended for routine medical examinations or vaccinations. The study was approved by the Ethics Committee of the University of Navarra. Consequently, the parents and adolescents aged above 12 years gave written informed consent for participation in agreement with the Declaration of Helsinki.

The GENOI project recruited 370 children and adolescents between 5 to 19 years old. Cases (n = 185) were Spanish children of Caucasian ethnicity with BMI above the age- and sex-specific 97^{th} percentile according to the Spanish BMI reference charts (Sobradillo, Aguirre, & Aresti, 2000). Controls (n = 185) were healthy subjects with the BMI below the 97^{th} percentile for the same references charts.

Exclusion criteria were exposure to hormonal treatment or development of secondary obesity diseases due to endocrinopathies or serious intercurrent illness. Further aspects of the design of the GENOI study have previously been detailed elsewhere (Ochoa et al., 2007).

❖ Women cohort (Chapter 2)

The study was designed as a randomized, longitudinal and controlled trial to study the influence of a weight loss nutritional intervention in the methylation status of clock system. The participants followed a weight reduction program named Garaulet method (Corbalán et al., 2009).

The clinical investigations were conducted in accordance with the guidelines of the Declaration of Helsinki and were approved by the Ethics Committee of the Virgen de la Arrixaca Hospital (2011-01-26). The experimental protocol was conformed to international ethical standards (Portaluppi et al.,

2010). The study was explained to the participants before starting, and the volunteers provided signed informed consent prior to participating in the study. Patient data were codified to guarantee anonymity.

The study sample was composed by 61 overweight/obese women between 16 and 77 years old, with an average BMI of 28.6 ± 3.4 kg/m^2 who voluntarily attended 5 nutrition clinics during 2009-2010 in the city of Murcia, southeastern Spain. Those women who were out of this range of age, under treatment with thermogenic or lipogenic drugs, following a special diet at the beginning of the study, or pre-diagnosed with diabetes mellitus, chronic renal failure, hepatic diseases such as NAFLD, steatohepatitis or cirrhosis, or cancer were excluded from the study**.**

A total of 68 potential participants were asked to attend a screening session. Finally, 9 % of the volunteers were dropped out and 61 women were included in the study.

- **Characteristics of the intervention**

The characteristics of the weight reduction program (Garaulet method) have been described completely elsewhere (Corbalan et al., 2009) and are represented in the **Figure 12**.

The study was divided into two phases: initial period for the first 4 months, followed by a maintenance period for 5 months. In the initial period, subjects attended a weekly 60-min therapy session in support groups (n = 10). During the maintenance phase, the group therapy was fortnightly initially and monthly subsequently. Sessions were conducted by a nutritionist. Treatment was based on the following issues:

- Dietary treatment: At the beginning of the dietary intervention, total energy intake was 2079 ± 701 kcal/day (43% of energy was supplied by carbohydrate (CH) and 41% by fat). During weight loss management, the total energy intake was reduced to 1408±297 kcal/day (50% of energy was supplied by CH and 35% by fat). Total energy intake and macronutrient composition were determined using Grunumur software (version 2.0; University of Murcia, Murcia, Spain**)** (Perez-Llamas et al., 2012), a nutritional evaluation program, in conjunction with Spanish food composition tables. Dietary individual energy requirements were assessed by calculating 1) resting energy expenditure according to the Harris-Benedict formula and 2) total energy expenditure (TEE) according to the type and duration of physical activity. Next, about 2.6 MJ/day was subtracted from the TEE. The final dietary

energy content ranged from 1200 to 1800 kcal/day for women to induce an approximate loss of 0.5 to 1 kg/week. The recommendations were based on the Mediterranean dietary pattern (Corbalan et al., 2009), in which the distribution of macronutrients followed the recommendations of the Spanish Society of Community Nutrition.

- Nutritional education: Nutritionist provided guidelines during group therapy for helping subjects plan their own menu and adopt appropriate lifetime eating habits.
- Physical activity: Individualized long-term goals were accentuated with 15 to 30 min or more of moderate-intensity physical activity, and preferably, 2 or 3 times a week, unless medically contraindicated. Patients were encouraged to use a pedometer to reach at least 10,000 steps per day.
- Behavioral techniques: Different behavioral techniques were included in the group therapy, such as stimulus control, self-monitoring, positive reinforcement, and cognitive behavioral therapy.

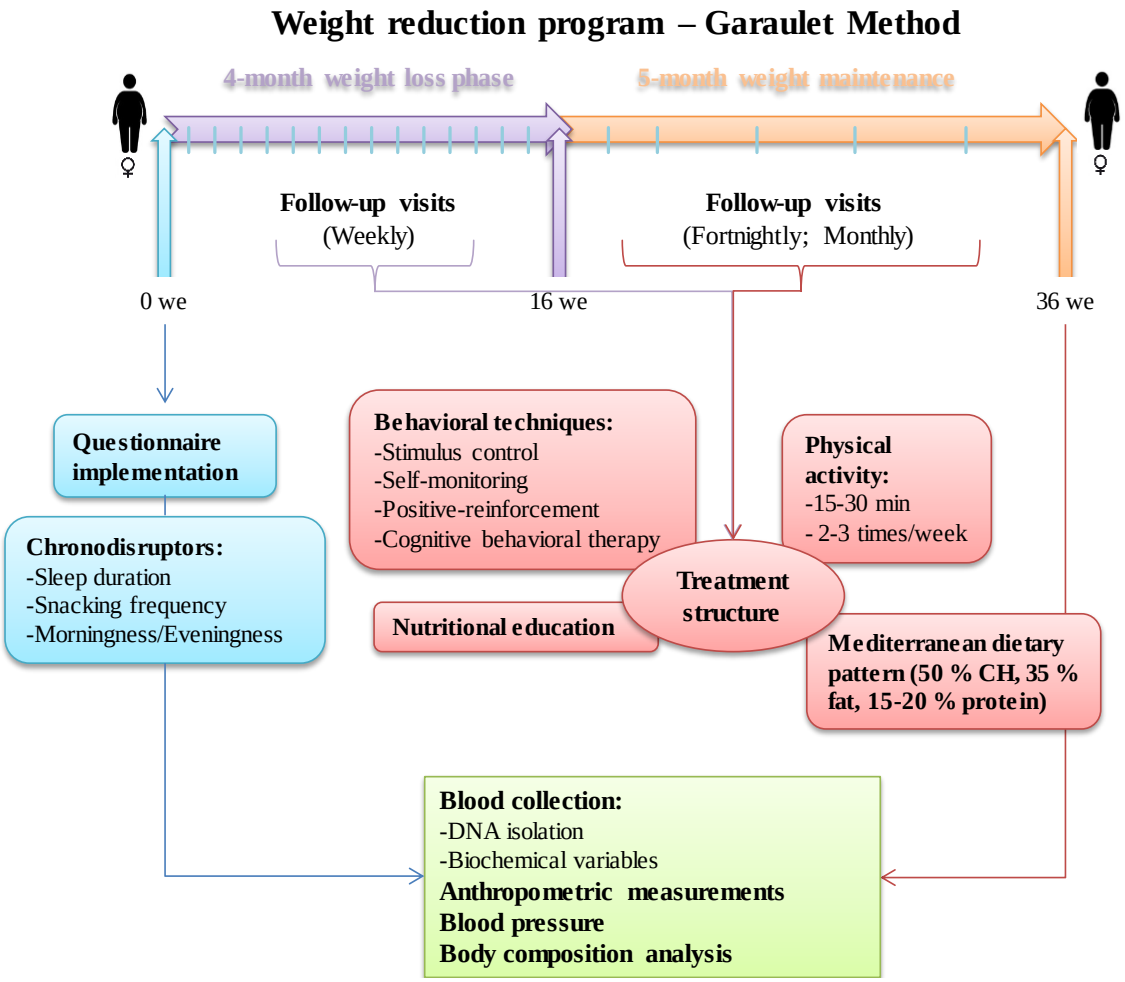


Figure 12. Main characteristics of the Garaulet Method for weight management. Abbreviations: we, week; CH, carbohydrate.

- **Morningness-Eveningness Score**

The score was developed using the Morning-Evening Questionnaire (MEQ) elaborated by Horne and Ostberg (1976) (Horne & Ostberg, 1976). The final score is divided into five behavioural categories: definitively morning times, moderately morning types, neither types, moderately evening times and definitively evening types. The score is useful to characterize subjects depending on individual preferences of wake/sleep patterns and the time of day people feel best. The complete questionnaire methodology was completely described by Garaulet et al., (2011).

❖ RESMENA study (Chapter 3 and 4)

The RESMENA study is a randomized, longitudinal and controlled trial that was designed to compare the effects of two dietary strategies on improving the comorbidities of MetS such as body composition, biochemical, hormonal, oxidative stress or epigenetics parameters over a six-month period (Zulet et al., 2011).

The CONSORT (CONsolidated Standards Of Reporting Trials) 2010 guidelines (Moher et al., 2012) were followed to design this intervention. The study was approved by the Research Ethics Committee of the University of Navarra (ref.065/2009). All the volunteers gave written informed consent before starting the intervention trial (www.clinicaltrials.gov; NCT01087086). The exclusion criteria (Perez-Cornago et al., 2014) are detailed in **Table 19**.

Table 19. Exclusion criteria.

Exclusion criteria of the RESMENA study
• **Subjects with difficulty for changing dietary habits.**
• **Having psychiatric or psychological disorders.**
• **Eating disorders.**
• **Subjects with weight instability for 3 months before the study.**
• **Pharmacological treatment (except drugs included in the IDF diagnostic criteria for the MetS).**
• **Pre-existing chronic diseases related to the metabolism of energy and nutrients (gastric ulcer, disorders of the digestive system, hyperthyroidism or hypothyroidism).**
• **Following special diets.**
• **Food allergies or intolerances.**

The RESMENA project recruited 109 volunteers with MetS symptoms, but only 93 subjects were diagnosed with the MetS according to the International Diabetes Federation criteria (Alberti et al., 2006). Finally, 84 participants completed whole intervention. 22 % of the volunteers were dropped out. Participants were divided randomly in two groups to follow one of the two energy-restricted diets, the Control diet or the RESMENA diet. The Control diet followed the recommendations of the American Heart Association (AHA). In addition, the main characteristics if the RESMENA diet were: high intake of dietary antioxidants from natural sources, low glycemic index and a macronutrient distribution if 40 % of CH, 30 % of fat and 30 % of protein distributed with 7 meals *per* day that compete to conventional AHA-recommendations (Zulet et al., 2011).

The study lasted for 6 months divided in two sequential stages: during the first period of 2 months the subjects received nutritional assessment every two weeks (Lopez-Legarrea et al., 2013), followed by a 4 months self-control period in which the volunteers applied the first period acquired dietary habits (de la Iglesia et al., 2014). During the whole study, the professionals asked the participants to maintain their habitual physical activity (Lopez-Legarrea et al., 2014) (**Figure 13**).

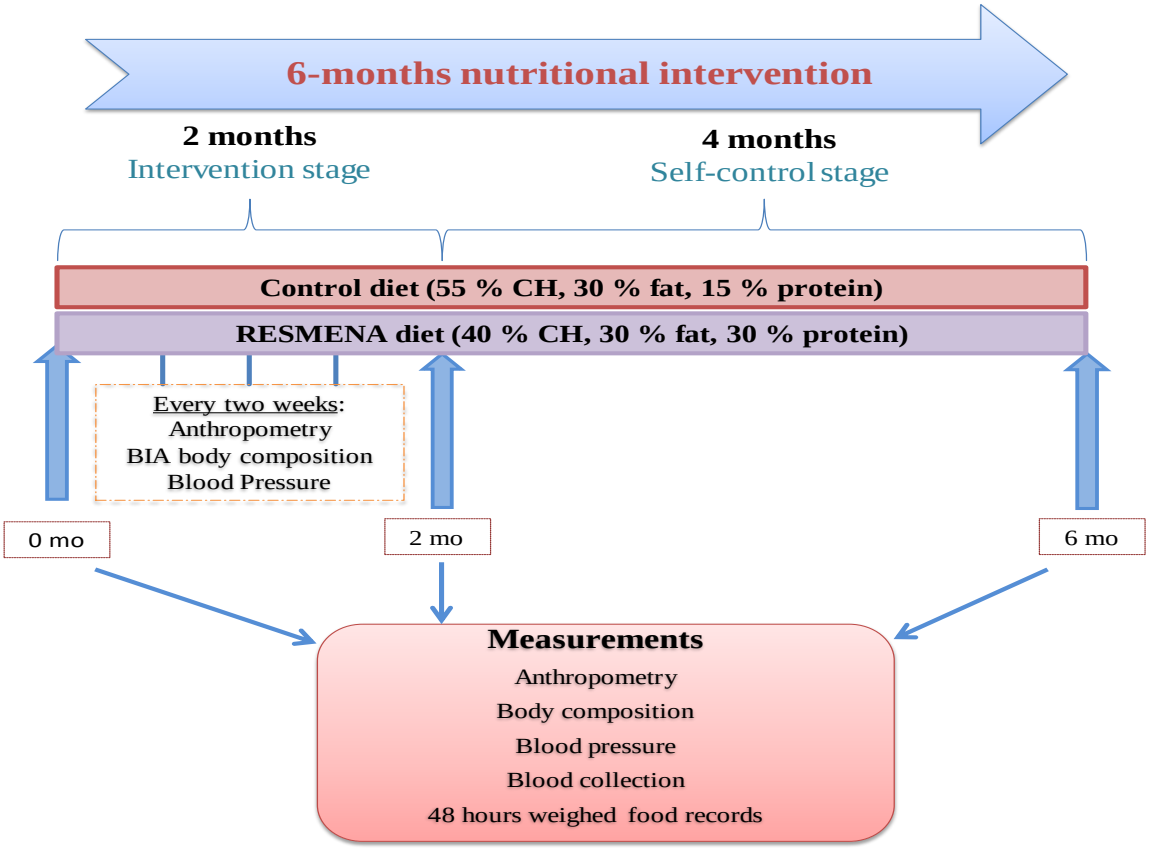


Figure 13. Design of the RESMENA study. Abbreviations: mo, month; CH, carbohydrate; BIA, bioelectrical impedance analysis.

- **Dietary assessment**

The habitual dietary intake of the volunteers was assessed using a validated food frequency questionnaire that includes the daily servings of cereals, vegetables, fruits, dairy products and meat and it also considers the percentage of energy provided by total and SFAs, the amount of cholesterol and the variety of the diet expressed by the number of different foods consumed daily. The energy, nutrient content and variety of the diet were determined using DIAL software (Alce Ingeniería, Madrid, Spain)(de la Iglesia et al., 2013).

2. CLINICAL AND BIOCHEMICAL ASSESSMENT (Chapter 1, 2, 3 and 4)

❖ Anthropometric measurements

Subjects were weighed to the nearest 100 g with a digital balance and height was measured using a stadiometer. BMI was calculated as the body weight divided by height squared (kg/m^2). In GENOI study, BMI z-score was computed by the conversion of BMI values into standard deviation scores using age and sex according to the criteria by Cole (2000) (Cole, 2000). Total body fat mass was determined by bioelectrical impedance (TANITA, Tanita corporation, Japan) and DEXA (DEXA Lunar Prodigy, GE Medical Systems, WI, USA).

❖ Biochemical measures

Biochemical parameters were determined in blood samples. Plasma and serum were separated by centrifugation at 1,400 xg, 5 ºC, 15 min. VLDL-C was separated by ultracentrifugation (Havel et al., 1955), whereas HDL-C was determined after precipitation of apoB-containing lipoproteins with dextran sulfate and magnesium (Warnick et al., 1982). LDL-C was calculated using Friedewald equation (Friedewald et al., 1972): LDL-C = TC – HDL – TG/5. Plasma glucose, TC and TG values were obtained by automated chemical analysis (Roche Diagnostics GmbH, Mannheim, Germany) and in a Pentra C-200 autoanalyser (HORIBA ABX, Madrid, Spain). Serum fasting insulin was measured with three different radioimmunoassay kits: DPC (LA, CA, USA), TKIN1 (Diagnostic Products, Madrid, Spain) and Mercodia (Sweeden). HOMA was stimated as [fasting insulin (µU/mL) x fasting glucose (mM)]/22.5.

3. CELL MODEL AND EXPERIMENTAL DESIGN (Chapter 5)

Human monocyte THP-1 cells were treated with folic acid (11.3 µM) dissolved in NaOH 1 M, choline chloride (105 µM), vitamin B_{12} (18.5 nM) and a mix of methyl donors that consisted in a combination of folic acid, choline chloride and vitamin B_{12} in the concentrations previously indicated. The concentrations of the treatments were chosen multiplying ten times the basal concentration present in RPMI-1640 medium for each compound. After 24 h, cells were differentiated into macrophages by incubation with 25 ng/ml 12-O-tetradecanoylphobol-13-acetate (TPA) for 48 h, and then were activated by incubation with 100 ng/ml LPS for 24 hours. Finally, RNA and DNA were extracted, and supernatants were collected for enzyme-linked immunosorbent assay (ELISA) analysis (**Figure 14**).

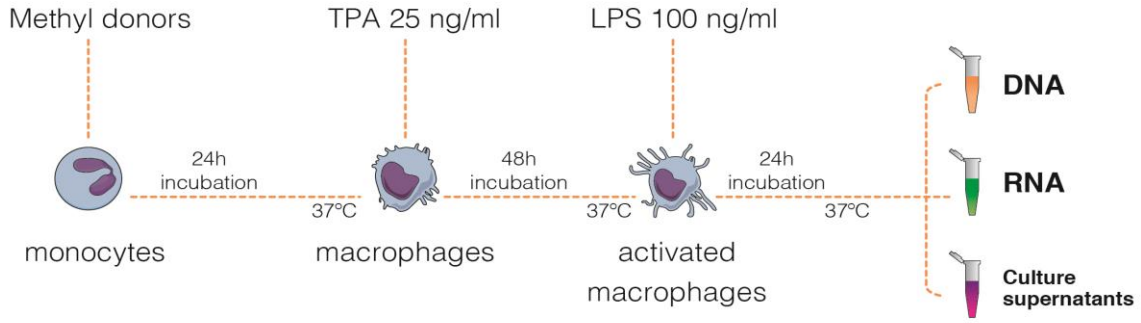


Figure 14. ***In vitro*** **experimental design for chapter 5.**

❖ Evaluation of cell viability

For the study of cell viability after treatments, 20 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well and plates were incubated for 2 h at 37 ºC. Formazan crystal formation was solubilised in 100 µL/well dimethylformamide (DMF)-glacial acetic acid-SDS solution consisting in 40 % DMF, 2 % glacial acetic acid and 16 % w/v sodium dodecyl sulfate (SDS). Formazan production was quantified by absorbance at 570 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland). The results were expressed as relative cell viability (%).

❖ Chromatin Immunoprecipitation (ChIP) Assay

ChiP assay was performed with the ChIP-IT™ Express Enzymatic Kit (Active Motif, CA, USA) following the manufacturer's instruction. Chromatin was immunoprecipitated using rabbit polyclonal

antibody for NF-κB (ab7970, Abcam, MA, USA). Real time quantitative PCR was performed using primers for *IL1B*: sense 5'-agcaacaaagctgccactta-3' and antisense 5'-tgacgtgctgtgtgaatttg-3', and *TNF*: sense 5'-ggagaatgtccagggctatg-3' and antisense 5'-tcctggaggctctttcactc-3'.

4. DNA METHYLATION ANALYSIS

❖ Genome-wide methylation (Chapter 1, 3 and 4)

Array-based specific DNA methylation analysis was performed with the Infinium HumanMethylation450 BeadChip kit (Ilumina, CA) and scanned using Illumina iScanSQ platform (Mansego et al., 2013). The GenomeStudio methylation module software (v1.9.0, Illumina) was used to extract the intensity of the images. Pre-processing and analysis of the Infinium 450k data was performed using the R package. Methylation values were represented as β-values calculated using the formula β-value = M/(U+M), where M and U are the raw "methylated" and "unmethylated" signals, respectively. Methylation array data were normalized using Subset Quantile Normalization in R program. The algorithm of Houseman et al. (2012) incorporated in R program was used to estimate cell type composition (Houseman et al., 2012).

❖ Gene-specific methylation (Chapter 1, 2, 3 and 5)

Specific sequence methylation was quantified using MassARRAY® EpITYPER™ (Bruker-Sequenom, CA, USA). This method uses matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). Polymerase chain reaction (PCR) primers for the regions of interest were designed using EpiDesigner software (Bruker-Sequenom, CA, USA) (**Figure 20)**. PCR products were purified from 2% agarose gels by Qiagen Gel Extraction Kit (Qiagen), and eluted with 1X Roche FastStart high-fidelity reaction buffer (Roche). Unincorporated dNTPs in the PCR products were dephosphorylated by adding 2 µl shrimp alkaline phosphatase (SAP) (37ºC, 20 min), and SAP was then heat-inactivated for 5 min at 85ºC. Subsequently, samples were incubated for 3h at 37ºC with 5 µl of Transcleave reaction mix (Sequenom) for simultaneous *in vitro* transcription and base-specific cleavage. The cleaved fragments were deionized with 20 µl of deionized water and then were spotted onto silicon matrix-preloaded chips (Spectro-CHIP, Sequenom) by nanodispensation. The fragments were analyzed using MALDI-

TOF MS Compact Unit. Matched peak data were exported using EpiTyper software. Methylation percentages were calculated using the ratio of the unmethylated versus methylated peaks. In addition, DNA methylation standards were used to control for amplification bias.

Table 20. Primer sequences of the MassARRAY EpiTYPER analyses in chapters 1, 2, 3

Gene symbol	Primer sequence	bp	Genebank number
PTPRS	5'-TTTTTTTTGGTTTTTGTGTTTTTGT-3' 3'-AAACCACACCAACCTTAATCCTC-5'	272	NC_000019.9
PER3	5'-TGATTTTTTTTAATTGGATGTTAGA-3' 3'-CAAAAACTCACCAAAACATTCATAA-5'	365	NC_000001.11
BMAL1	5'-TGAGATTTTGGTAAATTAGGGATTTT-3' 3'-ACTACTTTCCTACCACCAATCATTTAAC-5'	376	NC_000011.9
CLOCK	5'-TTTTTTTAGGAGATGGGAGAAGATG-3' 3'-CCTAAAAACTCTTTAACTTTCCCCC-5'	271	NC_000004.11
NR1D1	5'-AGAGTTTTTTGTTTTAGGGAAAGGT-3' 5'-TTACCCCCTAAACACTAACTAAAAA-3'	477	NC_000017.11
CD44	5'-GGGGTGAAGTAATGGATAGTAATTAGG-3' 3'-AATTATATATTCCAAAAAATCCCA-5'	312	NC_000011.10
IL-1β	5'-GTGTTTGTAGTTTTAGTTGTTGGG-3' 3'-TCTCTTAATAATACCAACCAAAAATTATCA-5'	331	NC_000002.12
TNF	5'-TTTGGTTTTTAAAAGAAATGGAGGT-3' 3'-TCCTTAATAAAAAAACCCATAAACTCA-5'	273	NC_000006.12
SERPINE1	5'-TTTGGTATAAAAGGAGGTAGTGGTT-3' 3'-ACTCTCCTACAATCACCCCTAAAAC-5'	343	NC_000007.14
IL-18	5'-TTTGTTGAGTTTTTTGTTTTTTTGG-3' 3'-CCTCTAATTACCATAACTAACTTTCCA-5'	251	NC_000011.10

5. mRNA EXPRESSION ANALYSIS

❖ Genome-wide mRNA expression analysis (Chapter 3 and 4)

Total RNA was extracted from WBC by using TRIzol Reagent (Life Technologies, Carlsbad, CA). A total of 1 μg of RNA from each sample was reverse-transcribed using High Capacity Complementary DNA reverse transcription kit (Life Technologies, Carlsbad, CA) and hybridized to a HumanHT-12 v4 Expression BeadChip kit (Illumina Inc., San Diego, CA, USA) containing annotated 31,000 genes with more than 47,000 probes and scanned using the Illumina HiScan SQ platform. Illumina GenomeStudio v2011.1 software was used to extract the data (v1.9.0, Illumina Inc., San Diego, CA, USA).

❖ Gene-specific expression analysis (Chapter 3 and 5)

For the gene individual expression study, 2 µg of RNA was reverse-transcribed using MultiScribeTM Reverse Transcriptase kit following the manufacturer's instruction (Thermo Fisher Scientific Inc., Waltham, MA, USA). cDNA was amplified with the predesigned Taqman primers for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Hs02758991_g1), *CD44* (Hs01075861_m1), *IL1B* (Hs01555410_m1), *TNF* (Hs00174128_m1), *IL18* (Hs01038788_m1), *SERPINE1* (Hs01126606_m1), cluster of differentiation 40 (*CD40)* (Hs01002913_g1) and *TLR4* (Hs00152939_m1). Gene mRNA levels were normalized to the endogenous control *GAPDH*. The comparative 2-ΔΔCT method was used for quantification of relative expression. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

6. OTHER TECHNIQUES

❖ Protein analysis (Chapter 3 and 5)

For the protein analysis plasma samples (**Chapter 3**) and culture supernatants (**Chapter 5**) were collected and stored at – 80 ºC. IL-1β, TNF-α, plasminogen activator inhibitor-1 (PAI1), CD40 and CD44 protein concentrations were measured with standard ELISA kits (R&D Systems Europe, UK) according to manufacturer's instructions **(Table 21)**. Absorbance was measured at 450 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland).

Table 21. List of ELISA kits used in Chapters 3 and 5.

Antibody	Manufacturer	Reference	Sample type
CD44	R&D	DY7045-05	Human plasma
IL-1β	R&D	DLB50	Culture supernatant
TNF-α	R&D	DTA00C	Culture supernatant
PAI1	R&D	DTSE100	Culture supernatant
CD40	R&D	DCCD40	Culture supernatant

❖ Bioinformatic analyses of transcription factors (Chapter 1, 2 and 5)

The bioinformatic analysis for the putative transcription factor binding site in the sequence of genes of interest was performed using LASAGNA Search 2.0 TRANSFAC (Lee & Huang, 2014).

❖ Pathway analysis (Chapter 1)

Potential pathways in which the genes receptor-type tyrosine-protein phosphatase S (*PTPRS*) and period circadian regulator 3 (*PER3*) participate were detected using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA).

RESULTS

CHAPTER 1

PTPRS and *PER3* methylation levels are associated with childhood obesity: results from a genome-wide methylation analysis

Mirian Samblas[1], Fermin Ignacio Milagro[1,2], Maria Luisa Mansego[1], Amelia Marti[1,2,3], Jose Alfredo Martinez[1,2,3] and GENOI members

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

[2] CIBERobn, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain

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

Pediatric Obesity, 2018

DOI: 10.1111/ijpo.12224

Impact factor (2016): 3.4

10/121 in Pediatrics, Q1

Samblas M, Milagro FI, Mansego ML, et al, PTPRS and PER3 methylation levels are associated with childhood obesity: results from a genome-wide methylation analysis. Pediatric Obesity, 2018, 13(3):149-158. https://doi.org/10.1111/ijpo.12224

Supplementary data CHAPTER 1

Supplementary Table S1. CpG sites differently methylated between cases and controls (p<0.001) in the methylation array (n=24).

Probe ID	Gene	p value	p adjusted	Control methylation (%)	Case methylation (%)	Mean diff.	Chr	Gene region	CpG island
cg06842518	*FYTTD1;KIAA0226*	2.473E-06	0.309	6.3	5.3	-1.0	3	TSS200; 1stExon; 5'UTR; TSS1500; TSS1500	Island
cg04757389	*PTPRS*	2.641E-06	0.309	91.6	88.6	-3.0	19	Body; Body; Body; Body	Island
cg24927646	*CHSY1*	3.337E-06	0.309	88.6	92.0	3.5	15	Body	Open sea
cg12513481	*SKAP1*	3.470E-06	0.309	13.3	10.8	-2.5	17	TSS1500; TSS1500	S_Shore
cg04902474	*KIAA1522*	3.841E-06	0.309	21.2	25.4	4.2	1	Body	N_Shelf
cg12943082	*CCL26*	4.097E-06	0.309	28.2	42.8	14.6	7	TSS1500	Open sea
cg26433494	*PLXNA4*	6.049E-06	0.309	75.1	71.0	-4.1	7	Body; 3'UTR	Open sea
cg01878214	*TBRG4;SNORA5C;SNORA5A*	6.947E-06	0.309	79.5	85.5	6.0	7	Body; TSS1500; TSS1500; Body; Body	Open sea
cg11210138	*MIR1203;SKAP1*	7.899E-06	0.309	38.1	51.8	13.6	17	TSS200; Body; Body	Open sea
cg01109012	*DYNLT3*	8.413E-06	0.309	33.8	25.2	-8.6	X	TSS1500	S_Shore
cg18797923	*AMT;NICN1*	1.074E-05	0.309	88.5	85.4	-3.1	3	TSS1500; TSS1500; TSS1500; 3'UTR; TSS1500; TSS1500	Open sea
cg00252701	*CDC73*	1.230E-05	0.309	6.2	5.3	-0.8	1	TSS200	Island
cg23912763	*PWP1*	1.359E-05	0.309	2.9	3.8	0.9	12	Body	Island
cg03448938	*PIKFYVE*	1.418E-05	0.309	6.1	5.1	-1.0	2	TSS1500; TSS1500	Island
cg19558848	*MYH3*	1.425E-05	0.309	78.8	87.0	8.2	17	5'UTR	Open sea
cg04531202	*MTHFSD;FLJ30679*	1.459E-05	0.309	4.6	3.8	-0.8	16	TSS200; TSS200; TSS200; TSS200; TSS200; TSS200; TSS200; Body	Island
cg27104173	*PTPRN2*	1.495E-05	0.309	95.6	94.6	-1.0	7	Body; Body; Body	Open sea
cg14925137	*IQCB1;EAF2*	1.519E-05	0.309	6.2	5.3	-0.9	3	TSS200; TSS200; TSS200	Island
cg00565090	*SNORD23;GLTSCR2*	2.029E-05	0.396	34.7	40.1	5.3	19	TSS1500; Body	Open sea

Note: the rest of the table can be accessed in link ijpo12224-sup-0001-SI.pdf.

Supplementary Table S2. Linear Regression Models of Association between BMI z-score and gene methylation levels.

BMI z-score as dependent	**Standardized B coefficient**	**95 % CI**	**adj R^2**	**p value**
cg_04757389 *(PTPRS)*	- 0.804	(-1.660; -0.940)	0.747	< **0.001**
age	- 0.309	(- 4.118; - 0.557)		**0.013**
gender	0.021	(-1.305; 1.553)		0,858
cg_10059324 *(PER3)*	-0.751	(-0.469; - 0.201	0.582	< **0.001**
age	- 0.159	(-3.178; 1.059)		**0.308**
gender	- 0.059	(- 1.985; 1.358)		**0.698**

Note: Adjusted by age and gender.

Supplementary Table S3. Associations between the methylation levels of the different CpG sites of *PTPRS* and *PER3* genes.

CpG site *PTPRS*	CpG_6		
	n	R coefficient	p value
cg_04757389 (array)	24	**0.637**	**0.001**
CpG_2	91	**0,297**	**0.004**
CpG_3	91	**0,602**	**< 0.001**
CpG_5	91	**0,567**	**< 0.001**
CpG_7	91	**0,712**	**< 0.001**
CpG_8.9	91	0,105	0,322
CpG_10	91	**0,342**	**< 0.001**
CpG_11	91	**0,476**	**< 0.001**
CpG_13	91	0,088	0,409

R = Pearson's correlation coefficient; p = probability value

CpG site *PTPRS*	CpG_3		
	n	R coefficient	p value
cg_04757389 (array)	24	**0.641**	**0.001**

R = Pearson's correlation coefficient; p = probability value

CpG site *PER3*	CpG_1		
	n	R coefficient	p value
cg_10059324 (array)	24	0.256	0.238
CpG_2	91	-0,054	0.614
CpG_3	91	-0,057	0.594
CpG_4	91	-0,107	0.312
CpG_15	91	0,020	0.852

R = Pearson's correlation coefficient; p = probability value

Supplementary Table S4. Transcription factors that putatively bind to the selected *PTPRS* and *PER3* sequences.

PTPRS

Name (TRANSFAC ID)	Sequence	Strand	Score	p-value	E-value	CpG binding site
SRF(M00215)	CCTTTTTTGGTTTT	+	13.38	0.000775	0.222	-
Sp1(M00008)	CAGGCAGGAA	-	6.72	0.000875	0.255	CpG_1
AP-2(M00189)	CTCCCCCAGGCA	-	7.93	0.000575	0.166	CpG_1
Zic1(M00448)	GGGGTCGTC	+	6.4	0.000975	0.285	CpG_1
MEIS1B:HOXA9(M00421)	TGACGGTTTTTGAG	+	7.67	0.0008	0.230	CpG_2
Brn-2(M00145)	CACATGGAAAAACAAC	-	8.13	0.000775	0.221	-
N-Myc(M00055)	TTCCATGTGAAT	+	8.11	0.000575	0.166	-
Oct-1(M00195)	TTCCATGTGAATCGG	+	7.99	0.000875	0.250	CpG_3
USF(M00217)	TCACATG	-	10.9	0.00065	0.190	-
GATA-6(M00462)	CCCGATTCAC	-	7.97	0.00065	0.189	CpG_3
GATA-1(M00075)	CCCGATTC	-	12.53	0.000475	0.138	CpG_3
AREB6(M00415)	GGGTTTGAA	+	8.44	0.000275	0.080	-
Zic1(M00448)	GGGGTGGTC	-	6.81	0.0006	0.175	CpG_4
Zic2(M00449)	GGGGTGGTC	-	7.19	0.000325	0.095	CpG_4
MZF1(M00084)	GGGCGAGGGGGTC	-	7.25	0.0008	0.230	CpG_6, CpG_7
Ik-2(M00087)	CGCTGGGACCCC	+	9.75	0.000275	0.079	CpG_9
NF-kappaB(M00194)	CTGGGACCCCCCGG	+	7.18	0.00095	0.273	CpG_10
CREB(M00113)	TCGGTGACGAGA	+	9.08	0.0007	0.202	CpG_11, CpG_12
ATF(M00017)	CGGTGACGAGAAGA	+	9.52	0.00035	0.100	CpG_11, CpG_12
STATx(M00223)	TTCTCGTCA	-	7.39	0.001	0.292	CpG_12
GR(M00205)	GTTTCCAGCTCTTCT	-	11.3	0.000825	0.235	-
NF-AT(M00302)	AGCTGGAAACCA	+	7.85	0.0009	0.260	-

ZID(M00085)	TGGCTCTGGTTTC	-	7.17	0.0008	0.230	-
Tal-1beta:E47(M00065)	CGCTCCAGCTGGCTCT	-	6.1	0.00095	0.271	CpG_13
Tal-1beta:ITF-2(M00070)	CGCTCCAGCTGGCTCT	-	8.36	0.000125	0.036	CpG_13
AP-4(M00005)	AGAGCCAGCTGGAGCGTG	+	12.63	0.0001	0.0283	CpG_13
AP-4(M00005)	GAGCCAGCTGGAGCGTG	+	8.77	0.000875	0.248	CpG_13
MyoD(M00184)	TCCAGCTGGC	-	9.61	0.000325	0.095	-
Lmo2 complex(M00277)	GCCAGCTGGAG	+	12.39	0.000325	0.094	-
E2(M00181)	ACACCAGCCTTGGTCC	-	9.47	0.000225	0.064	-
E2(M00107)	GGACCAAGGCTGGTGT	+	10.39	0.000225	0.064	-
Poly A downstream element(M00211)	TGTGGTCCC	+	9.1	0.000175	0.051	-
Sp1(M00008)	CAGGCAGGTT	-	6.83	0.000825	0.240	-

Note: Transcription factors that bind to CpG sites of interest are highlited in bold. CpG analysed by sequenom are underlined.

PER3

Name (TRANSFAC ID)	**Sequence**	**Strand**	**Score**	**p-value**	**E-value**	**CpG binding site**
FOXO4(M00472)	AAAAACAGGC	-	10.62	0.0009	0.34	-
NF-AT(M00302)	CAGAGGAAAAAC	-	9.93	0.0001	0.037	-
v-ErbA(M00239)	CAGGAAGGTCTCGCCT	-	9.06	0.0005	0.185	CpG_1
Zic2(M00449)	AGGAAGGTC	-	6.23	0.001	0.38	-
NF-kappaB(M00208)	GAGACTCTCCT	-	9.52	0.000475	0.178	-
Lyf-1(M00141)	TCTGGCGGA	+	7.79	0.001	0.38	CpG_2
MRF-2(M00454)	CGGAATAGTGC	-	16.38	0.00075	0.279	CpG_4
AhR(M00139)	TCCGCAGCTTGCGTGAG	+	13.64	0	0	CpG_4, CpG_5
AhR(M00139)	CCGCAGCTTGCGTGAGC	+	11.11	0	0	CpG_4, CpG_5
AhR(M00139)	CGCAGCTTGCGTGAGC	+	6.7	0.000975	0.36	CpG_4, CpG_5
CREB(M00177)	GCTCACGCAAGC	-	8.51	0.000675	0.252	CpG_5

CP2(M00072)	GCTCACGCAAG	-	9.1	0.00035	0.131	CpG_5
v-ErbA(M00239)	GCTGAAGCTCACGCAA	-	7.9	0.000775	0.287	CpG_5
AP-4(M00175)	TTCAGCTCTT	+	8.44	0.0009	0.34	-
AP-4(M00175)	TCAGCTCTT	+	8.44	0.0009	0.34	-
COMP1(M00057)	TGAGAGGATTAGCAGGAACA	-	19.78	0.00085	0.308	-

Note: Transcription factors that bind to CpG sites of interest are highlited in bold. CpG analysed by sequenom are underlined.

Supplementary Figure S1.

A) Genomic DNA position of targeted CpG and sequences of the amplicons. Number of the left of the sequence represents the position with respect to the first nucleotide of the mRNA (start of transcription). Underlined and consecutively numbered CpGs were reliably quantified by EpiTYPER. The CpG that was present in the methylation array is framed in a box. **B)** Polymerase chain reaction (PCR) primers covering 13 CpG sites of the *PTPRS* gene and 5 CpG sites of the *PER3* gene were designed using Epidesigner software (Bruker-Sequenom).

A) ***PTPRS*** *(NC_000019.9) chr19: 5,250,515-5,250,814*

```
                                                                  1
+ 89975 CCCTTTTTTGGTTTTTGTGTTTCTGCCTCTTCCTGCCTGGGGGAGGGGTCGTCTCCTGGT
                  2                                   3            4
+ 90035 TGCTTTGACGGTTTTTGAGTTGTTTTTCCATGTGAATCGGGGTTTGAAGCGACCACCCCC
                                          5                6       7       8
+ 90095 CACAACCTGCCACCCCCCCACCCCACCGCCCACCCTCCCGACCCCCTCGCCCAAGCGGGT
                9                10          11      12
+ 90155 CCCCCCGCTGGGACCCCCCGGCTACTCGGTGACGAGAAGAGCTGGAAACCAGAGCCAGCT
               13
+ 90215 GGAGCGTGCTGAGGACCAAGGCTGGTGTGGTCCCCTTATAACCTGCCTGGACCCTCCATT
```

PER3 **(***NC_000001.11) chr1: 7,884,624-7,885,010*

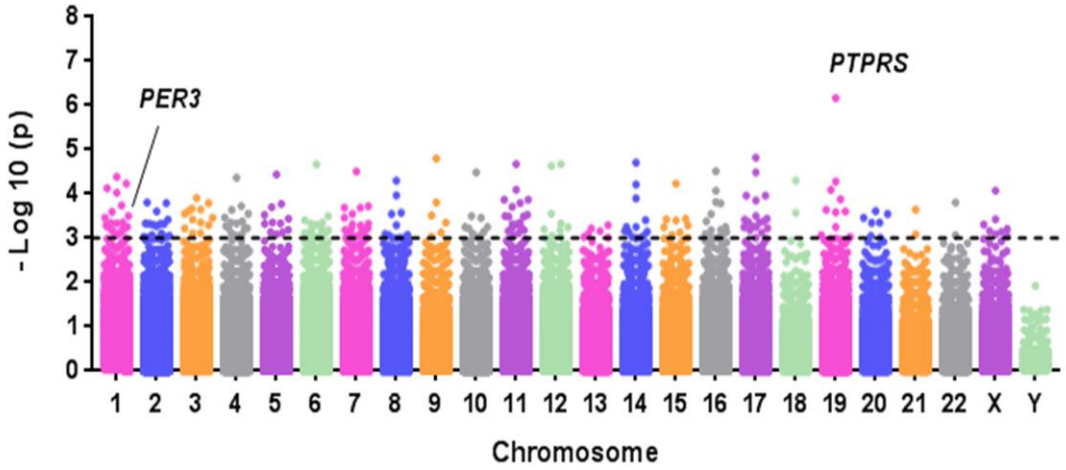
```
+ 40212 GTGATTCTTTCTAATTGGATGCCAGATACTGTGAATGGGTACCAAATATATTTGTATTCC

+ 40272 TATAAATATTCTTGATCTTTATTCTGGGTCCCAGTGAAGTTACCTGTAAACAGATTGATC

+ 40332 CTTTCAGGCTTTGCTTTTTAAATTTGCTAGGCAGGACAAATAGTGTGTAGTCTAGGCCTG
                                  1
+ 40392 TTTTTCCTCTGCTACAAAGGCGAGACCTTCCTGAGTGCTCCTTGTGATGCCTCAGGAGTT
                                  2           3       4       5
+ 40452 AGGAGAGTCTCTAGTCTGGCGGATGGGAACCGGCACTATTCCGCAGCTTGCGTGAGCTTC

+ 40512 AGCTCTTGTTCCTGCTAATCCTCTCAGGTGGTTTTTTCCTCCCATGAATGCCCTGGTGAG

+ 40572 TCCTTGGAAGCCCTCCAGGCTGCAC
```

B) For *PTPRS* (272 bp length)

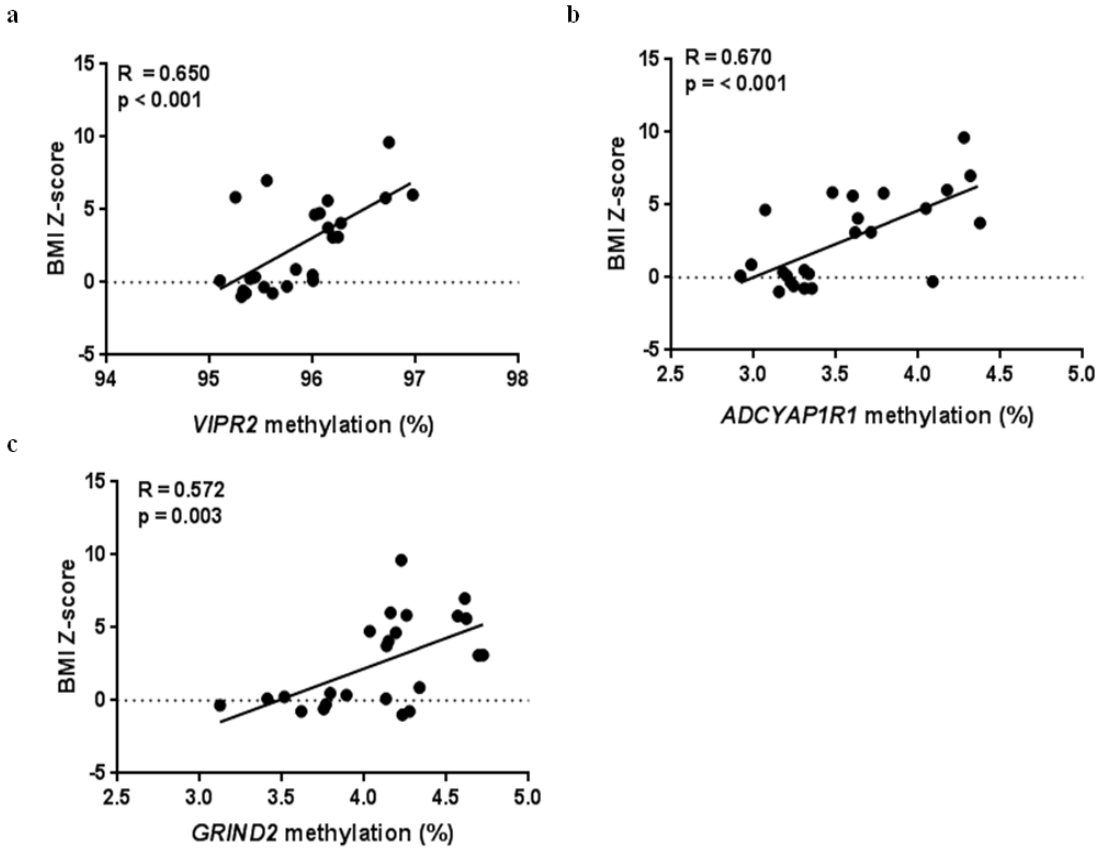
Left, TTTTTTTTGGTTTTTGTGTTTTTGT
Right, AAACCACACCAACCTTAATCCTC

For *PER3* (365 bp length)

Left, TGATTTTTTTTAATTGGATGTTAGA
Right, CAAAAACTCACCAAAACATTCATAA



Supplementary Figure S2. Manhattan plot showing genome-wide P values of association between methylation levels and BMI Z-score. The y axis shows the −log10 P values of 468,317 CpG sites, and the x axis shows their chromosomal positions. Horizontal dashed line represents the thresholds of p=1x10-3 for selecting CpG sites for biological pathway analysis.



Supplementary Figure S3. Methylation levels of genes involved in circadian rhythm regulation are associated with obesity. Correlations between BMI z-score and microarray methylation levels of (a) *VIPR2* (b) *ADCYAP1R1* and (c) *GRIND2* genes in the methylation array population (n=24). R=Pearson's coefficient; p<0.05.

CHAPTER 2

Methylation on the circadian gene *BMAL1* is associated with the effects of a weight loss intervention on serum lipid levels

Mirian Samblas[1], Fermin I. Milagro[1,2], Purificación Gómez-Abellán[3], J. Alfredo Martínez[1,2,4] and Marta Garaulet[3]

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

[2] CIBERobn, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain

[3] Department of Physiology, Faculty of Biology, University of Murcia; IMIB, Murcia, Spain

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

Journal of Biological Rhythms, 2016

DOI: 10.1177/0748730416629247

Impact Factor (2016): 3.5

13/85 in Biology, Q1

19/84 in Physiology, Q1

Samblas M, Milagro FI, Gomez-Abellan P, et al, Methylation on the Circadian Gene BMAL1 Is Associated with the Effects of a Weight Loss Intervention on Serum Lipid Levels. Journal of Biological Rhythms, 20(10):1-10.
https://doi.org/10.1177/0748730416629247

Supplementary data CHAPTER 2

Figure S1. Genomic DNA position of targeted CpGs and sequence of the amplicons. Number on the left of each sequence represents the positions with respect to the first nucleotide of the mRNA (transcription start site). CpGs are highlighted in bold and consecutively numbered in the sequences. Underlined CpGs are the sites that were reliably quantified by EpiTYPER. Boxes in the sequences indicate that these CpGs could not be discriminated by the EpiTYPER technique.

BMAL1 (NC_000011.9) chr11: 13,298,705-13,299,004

NR 1D1 (NC_000017.11) chr17:40,100,188-40,100,615

CHAPTER 3

An integrated transcriptomic and epigenomic analysis identifies *CD44* gene as a potential biomarker for weight loss within an energy-restricted program

Mirian Samblas[1], Maria Luisa Mansego[1,2], Maria Angeles Zulet[1,2,3], Fermín I. Milagro[1,2], J. Alfredo Martínez[1,2,3]

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

[2] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III. Madrid, Spain.

[3] Navarra Institute for Health Research (IdiSNA), Pamplona, Spain

European Journal of Nutrition

Under review (EJON-D-18-00027)

Impact Factor (2016): 4.37

14/81 in Nutrition & Dietetics (Q1)

An integrated transcriptomic and epigenomic analysis identifies *CD44* gene as a potential biomarker for weight loss within an energy-restricted program

Mirian Samblas [1], Maria Luisa Mansego [1,2], Maria Angeles Zulet [1,2,3], Fermín I. Milagro [1,2*], J. Alfredo Martínez [1,2,3]

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

[2] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III. Madrid, Spain.

[3] Navarra Institute for Health Research (IdiSNA), Pamplona, Spain

Address of the authors: Irunlarrea 1, 31008 Pamplona, Navarra, Spain

[*]**Corresponding author:**
Fermín I. Milagro. Department of Nutrition, Food Science and Physiology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Navarra, Spain. E-mail: fmilagro@unav.es. Phone: +34-948-425-665. Fax: +34-948-425-740.

ORCID:

Mirian Samblas: 0000-0003-0866-2300

Maria Luisa Mansego: 0000-0001-8914-7890

Maria Angeles Zulet: 0000-0002-3926-0892

Fermin I. Milagro: 0000-0002-3228-9916

J. Alfredo Martinez: 0000-0001-5218-6941

Conflict of interest

The authors have nothing to declare.

Acknowledgments

We thank the participants of the RESMENA project and technical assistance of Enrique Buso (UCIM, University of Valencia) for the MassARRAY measurements. The technical assistance of Veronica Ciaurriz and Ana Lorente is gratefully acknowledged. We credit the financial support of Ministry of Economy, Industry and Competitiveness (Nutrigenio Project reference AGL2013-45554-R) and Spanish Biomedical Research Centre CIBER de Fisiopatología de la Obesidad y Nutrición (CIBERobn). Mirian Samblas holds a FPI grant from the Ministry of Education, Culture and Sport (BES-2014-068409).

Abstract

Purpose: The interindividual variable response to weight-loss treatments requires the search for new predictive biomarkers for improving weight-loss programs success. The aim of this study is to identify novel genes that distinguish individual responses to a weight loss dietary treatment by using the integrative analysis of mRNA expression and DNA methylation arrays.

Methods: Subjects from Metabolic Syndrome Reduction in Navarra (RESMENA) project were classified as Low (LR) or High (HR) responders depending on their weight loss. Transcriptomic and epigenomic patterns were determined by array-based genome-wide technologies in human white blood cells at the baseline of the treatment period. *CD44* expression was validated by qRT-PCR and methylation degree of CpGs of the gene was validated by MassARRAY® EpiTYPER™ CD44 protein levels were measured by ELISA in human plasma.

Results: Different expression and DNA methylation profiles were identified in LR in comparison to HR. The integrative analysis of both array data identified four genes: *CD44*, *ITPR1*, *MTSS1* and *FBXW5* that were differently methylated and expressed between groups. *CD44* showed higher expression and lower DNA methylation levels in LR than in HR. Although differences in CD44 protein levels between LR and HR were not statistically significant, a positive association was observed between *CD44* mRNA expression and protein levels.

Conclusions: In summary, the combination of a genome-wide methylation and expression array dataset can be a useful strategy to identify novel genes that might be considered as predictors of the dietary response. *CD44* gene transcription and methylation may be a possible candidate biomarker for weight loss prediction.

Keywords: mRNA, methylation, weight loss, obesity, metabolic syndrome

Introduction

Metabolic syndrome (MetS) encompasses a group of manifestations, such as obesity, insulin resistance and abnormal lipid profile, that are associated to quality of life and longevity [1]. Indeed, MetS is the consequence of a combination of genetic, epigenetic, environmental, and lifestyle factors including inactivity or unbalanced diets. One of the main strategies for managing MetS features are dietary and lifestyle programs [2]. Given that most MetS subjects are overweight or obese, dietary strategies are focused on weight reduction and include energy restriction and changes in macronutrient distribution, which subsequently may improve lipid profile and serum glucose concentrations [3]. Nevertheless, many factors influence the effectiveness of low-calorie diets for body weight loss, and patients could be more or less sensitive to these treatments. Therefore, it is mandatory to improve the success of weight loss strategies, being necessary to deepen into the knowledge of all the factors

involved in the metabolic processes in order to implement more personalized health care programs. In this context, genetic and epigenetic markers have been suggested as promising tools for diagnosis, prognosis, monitoring, and management of metabolic diseases [4].

In order to explain the inter-individual variability of the metabolic response to specific diets, several association studies have been carried out to identify those genetic variants that may be implicated in the process (reviewed in [5]). In this sense, a number of genes have been identified as important players in the heterogeneous response to diet [6–8]. For example, the metabolic response to different weight loss diets has been associated to specific genetic variants located in obesity (*FTO* and *NPY*) or MetS (*IRS1*)-related genes [9, 10]. On the other hand, several dietary factors, such as methyl donors or polyphenols, can induce changes in the epigenetic marks [11, 12]. The epigenetic mechanisms, including DNA methylation, contribute to regulate gene expression [13]. Thus, epigenetics could represent a mechanistic link in the diet-gene interaction, since specific nutrients or bioactive compounds may modify gene expression via epigenetic mechanisms [14–16]. In this context, research concerning personal genome, epigenome and transcriptome may be useful to identify new potential biomarkers that predict the inter-individual response to specific dietary treatment. For example, the study of the transcriptomic profile from adipose tissue of obese and overweight individuals during a low-calorie diet identified predictors of body weight and glycemic evolution [17]. In the last years, genome-wide array technologies (GWAS and EWAS) have facilitated the discovery of gene alterations influencing the success of weight loss dietary treatments, and are describing novel candidates for explaining successful weight reduction [18]. In this context, the present study aimed to evaluate white blood cell transcriptome and methylome before an energy-restricted diet, and to identify novel genes that are able to distinguish individual's responses by using the integrative analysis of mRNA expression and DNA methylation arrays.

Methods

Study design

The RESMENA study ("REducción del Síndrome Metabólico en Navarra") is a Spanish, randomized controlled and longitudinal trial (NCT01087086) over a six-month period that aimed to improve the parameters related with the MetS, including body fat composition, biochemistry, inflammation, or oxidative stress. The volunteers were recruited from Navarra Hospital, "Clínica Universidad de Navarra" and other Navarrese primary care centres. The volunteers who presented difficulties for changing dietary habits, psychological or eating disorders, weight instability the last 3 months before the study, pharmacological treatment, metabolism of energy- or nutrients-related chronic diseases, and food allergies or intolerances were excluded from the study. The volunteers were

randomly ascribed to one of the two following groups: the control diet was based on the American Heart Association (AHA) criteria and the RESMENA diet was characterized by a higher meal frequency and a different macronutrient distribution (40% carbohydrates, 30% lipids and 30% proteins). The prescribed diets had the same energy restriction (-30% of the studied requirements). The study design has been explained in detail previously [19].

Participants

For all the analyses performed in this research, both control and RESMENA group were mixed together as a unique observational cohort group, as both diets were equally successful for weight loss. Subjects were classified in two groups depending on their weight loss at the end of the treatment: low responders (LR) when the weight loss was < 8 % of the initial weight, and high responders (HR) when the volunteers lost >8% of initial weight. The investigation was carried out in three subsamples from RESMENA project. (i) Discovery Population for Expression (DPE): the expression array approach of the study was performed in a subsample of 24 subjects (LR, n = 14; HR, n = 10); (ii) Discovery Population for Methylation (DPM): for the methylation array a subsample of 47 subjects (LR, n = 31; HR, n= 16) was selected; and (iii) Validation Population (VP): the subsequent validation of selected gene from both arrays was conducted in a subsample of 47 volunteers, which contained 26 LR and 21 HR.

Ethics

The study was approved by the Research Ethics Committee of the University of Navarra (ref.065/2009). All the participants gave written informed consent for participation in agreement with the Declaration of Helsinki (www.clinicaltrials.gov; NTC01087086). The design of this study followed the CONSORT (CONsolidated Standards Of Reporting Trials) 2010 guidelines [20].

Anthropometric and Biochemical measurements

Anthropometric measurements were taken in fasting conditions. Body weight was measured to the nearest 0.1 kg with bioelectric impedance (TANITA SC-330, Tanita Corporation, Tokyo, Japan). Height, waist and hip circumferences were performed by trained researchers following validated protocols. Body mass index (BMI) was calculated as the body weight divided by height squared (kg/m^2). Total body fat mass was measured using DXA (Lunar iDXAe, software version 6.0; GE Healthcare, Madison, WI, USA).

Blood samples were collected at baseline and at the end of the trial after fasting overnight. The plasma, serum and WBCs were separated from whole blood by centrifugation at 3,500 rpm, 5ºC, 15 min (Model 5804R, Eppendorf AG, Hamburg, Germany), and were stored at -80ºC. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and glucose concentrations were measured by a Pentra C-200 autoanalyser (Horiba ABX, Madrid, Spain) with specific kits. Low-density lipoprotein cholesterol (LDL-c) concentration was calculated using the Friedewald equation: LDL-c = TC- HDL-c – TG/5 [21]. Serum insulin was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweeden) in a Triturus autoanalyser (Grifols SA, Barcelona, Spain). Insulin resistance was estimated with the homeostasis model assessment (HOMA-IR) as [fasting insulin (µIU/mL) x fasting glucose (mM)] / 22.5.

Genome-wide mRNA expression analysis

Total RNA was extracted from WBC by using TRIzol Reagent (Life Technologies, Carlsbad, CA). A total of 1 µg of RNA from each sample was amplified and hybridized to a HumanHT-12 v4 Expression BeadChip kit (Illumina Inc., San Diego, CA, USA) containing annotated 31,000 genes with more than 47,000 probes. Illumina GenomeStudio v2011.1 software was used to extract the data.

Genome-wide DNA methylation analysis

Genomic DNA was isolated from WBC with the MasterPureTM DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA). Array-based DNA methylation analyses were performed with the Infinium Human Methylation 450K BeadChip kit (Illumina). Bisulfite-treated DNA was amplified, hybridized to HumanMethylation450 BeadChips (Ilumina) and scanned using the Illumina iScanSQ platform. Illumina GenomeStudio methylation module software (v 1.9.0) was used to extract the intensity of the images. β-values (β-value = M/(U + M, where M is the raw 'methylated' and U is 'unmethylated' signal) were computed. Subsequently, β-values were corrected for type I and II bias using peak-based correction. Data was normalized using categorical Subset Quantile Normalization method through the pipeline described by Toulemain and Tost [22]. All the process was explained in detail previously [23].

mRNA expression analysis by quantitative real-time PCR

For the individual gene expression study, 2 µg of RNA from a total of 47 samples was reverse-transcribed using MultiScribeTM Reverse Transcriptase kit following the manufacturer's instruction (Thermo Fisher Scientific Inc., Waltham, MA, USA). *CD44* gene was selected for confirmation of the mRNA expression array data. Complementary DNA (cDNA) was amplified with the predesigned Taqman primers for *GAPDH*

(Hs02758991_g1) and *CD44* (Hs01075861_m1). *CD44* mRNA levels were normalized to the endogenous control *GAPDH*. The comparative $2^{-\Delta\Delta CT}$ method was used for quantification of relative expression. Real-time PCR was performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA)

DNA methylation analysis by MALDI-TOF mass spectrometry

Specific gene methylation was quantified using Ma MassARRAY® EpiTYPER™ ssARRAY EpiTYPER (Sequenom, San Diego, CA, USA). Genomic DNA was sodium bisulfite transformed by EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) primers covering 5 CpG sites (CpGs) of the *CD44* gene (including one from the microarray study) were designed by Epidesigner software (Sequenom). The primers used were: 5'-ggggtgaagtaatggatagtaattagg-3' and 5'-accctaaaaaaccttatatattaa-3'. The complete methodology was previously described by Milagro et al., 2011 [24].

Enzyme-linked immunosorbent assay (ELISA)

Plasma samples were collected at the beginning of the treatment and stored at -80 ºC for protein analysis. Protein concentration of CD44 was measured with standard ELISA kits (R&D Systems Europe, Ltd., Abingdon, UK) according to manufacturer's protocols. Absorbance was measured at 450 nm using a Multiskan microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistical analysis

Quantitative variables were expressed as means and confidence intervals and qualitative variables as numbers. The LIMMA package [25] for the R statistical software was used to compute a one-way ANOVA test for the statistical differences between DNA methylation values and diet response categories (HR vs LR), adjusted by age, sex, baseline weight and array chips [26]. For the methylation analysis, a significant threshold of $p < 0.05$ and 5 % of methylation variation between groups was used, whereas for the expression study false discovery rate (FDR) cut-off of 0.05, B-statistic ≥ 0 and a logarithmic fold change (logFC) $> \pm 0.58$ were accepted. Pearson's correlation coefficient was calculated to analyse the associations between the methylation levels of candidate CpGs measured by methylation array and MALDI-TOF mass spectrometry, association between expression level of *CD44* gene identified in microarray and qRT-PCR and the association between expression and protein levels. The associations are always two-tailed expect when noted otherwise. Student t-test was used to compare anthropometric, biochemical, expression, methylation and protein differences between HR and LR. A p-value

less than 0.05 was considered significant if not otherwise specified. Statistics were performed using SPSS v.15 (SPSS Inc., Chicago, IL, USA) and Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Anthropometric characteristics of the study populations

Clinical characteristics of the subjects of DPE, DPM and VP at the beginning of the intervention are shown in **Table 1**. HR group of the DPE lost 10.5 ± 1.1 % of initial body weight while LR lost 5.9 ± 0.9 %. As designed, the difference of weight loss between the two groups was very significant ($p < 0.001$). In the DPM subsample, the HR lost 10 ± 1.7 % of body weight and the LR lost 5.2 ± 1.6 % ($p < 0.001$). In the VP, the HR lost 9.5 ± 0.9 % whereas LR lost only 5.7 ± 0.8 % of body weight ($p < 0.001$).

Changes in WBC gene expression and methylation patterns between LR and HR subjects and integrated analysis of the data

A subset of 24 subjects (14 LR and 10 HR subjects) with available RNA and DNA of high quality and clinically representative of both groups was selected for array studies. The applied workflow is described in **Fig. 1.** For the microarray analysis, a total of 908 genes were differentially expressed between both groups with a FDR < 0.05. Among these, 156 transcripts presented a logFC ± 0.58 and a $B \geq 0$, twenty of which were down-regulated in LR and up-regulated in HR and 136 up-regulated in LR and down-regulated in HR **(Online Resource 1)**.

From the genome-wide DNA methylation study, a total of 2,102 CpGs in 1,785 genes differentially methylated between LR and HR, after the adjustment for age, gender and baseline weight, with an absolute methylation variation above 5 % and raw p-value < 0.05 were identify. Nine hundred and fifty two CpGs were hypomethylated and 1,150 hypermethylated in the LR group compared to the HR group **(Online Resource 2)**. However, none of these CpGs remained statistically significant after a Benjamini-Hochberg correction for multiple comparisons.

Since DNA methylation may regulate gene expression, it was tested whether the identified diet response-associated CpGs methylation changes correlated with expression of respective annotated genes. According to the data obtained from the mRNA expression and DNA methylation array, there were 4 genes differentially methylated and expressed between LR and HR **(Fig. 2)**. Among them, 3 genes were up-regulated and hypomethylated in LR (*CD44*, *ITPR1* and *MTSS1*) and one gene (*FBXW5*) was down-regulated and hypomethylated in LR **(Table 2)**.

Functions of genes in obesity, weight loss and inflammation

To further understand the biological relevance of the identified genes, a literature search was performed to investigate the potential involvement of *CD44*, *ITPR1*, *MTSS1* and *FBXW5* in obesity, MetS, and inflammation and also as biomarker. The literature search was carried out using each gene name and the following terms; obesity, insulin resistance, inflammation, weight loss or biomarker (neither cancer nor tumour). The results showed that *CD44* had been described in 2,998 studies associated with obesity, MetS or inflammation, and also as biomarker **(Fig. 3a)**. In contrast, *MTSS1* and *ITPR1* only appeared in 3 and 33 studies, respectively (data not shown). No results were found concerning the *FBXW5* gene (data not shown).

Validation of expression and methylation changes of *CD44* in human WBC

Due to the biological relevance of *CD44* gene in obesity, weight loss and inflammation, this gene was selected to validate the expression and methylation changes found between LR and HR in arrays analysis. qRT-PCR and MassARRAY® EpiTYPER™ were used to technically validate and biologically replicate the results obtained in *CD44* gene. The expression validation was performed on WBC from 24 of the subjects in the original microarray analysis, and a significant correlation was obtained between qRT-PCR and microarray expression data ($R=0.479$, $p = 0.020$) **(Fig. 3b)**. For the DNA methylation 32 subjects from the methylation array analysis were selected. The region analysed of *CD44* contained the same CpG selected in the methylation array (CpG5) and two additional CpGs (CpG1 and CpG4). DNA methylation levels of CpG5 measured by MassARRAY® EpiTYPER™ showed a significant correlation ($R=0.382$, $p = 0.031$) with the DNA methylation levels quantified by microarray **(Fig. 3e)**. In addition, CpG5 methylation levels in the microarray also significantly correlated with those involving CpG1 ($R = 0.562$, $p < 0.001$) and CpG4 ($R = 0.412$, $p = 0.018$) **(Fig. 3c-d)**. The biological replication was performed on WBC from VP (n=47, 31 LR and 16 HR). *CD44* gene expression was significantly higher in LR than in HR ($p < 0.05$) **(Fig. 4a)**. Moreover, lower methylation levels were observed in LR in comparison to HR for the three CpGs, although only CpG4 and CpG5 reached statistical significance ($p < 0.05$) as graphically depicted **(Fig. 4b)**. Gene expression and methylation in VP followed opposite direction, in accordance to the results of the arrays, showing that LR subjects presented higher expression and lower methylation of *CD44* comparing to HR individuals. Finally, although CD44 protein levels in plasma were not significantly different between LR and HR (Data not shown), a significant association was found between protein and expression data ($p < 0.05$) **(Fig. 4c)**.

Discussion

Obesity features by an excessive fat accumulation and may contribute to the development of the other characteristics of MetS including dyslipemia, insulin resistance or hypertension [27]. Thus, weight loss might be a powerful strategy to prevent MetS risk factors from progressing to disease status. Nevertheless, because of interindividual variability in the response to body weight loss interventions, recent research using genome-wide array technologies are trying to understand the variability in individual's responses to specific dietary treatments. The present research demonstrated differential transcription and methylation profiles between subjects who respond successfully or were resistant to an energy restriction-based weight loss program. Moreover, the integrated analysis of mRNA expression and DNA methylation arrays identified *CD44* gene as a novel important regulator of the personalized response to the diet.

From the 156 differentially expressed genes between HR and LR identified with the Illumina HumanHT-12 v4 array, most of them were down-regulated in HR. These results suggested that gene expression levels may be important to understand the response to specific diet, and is in agreement with a previous study performed in obese young boys, where expression levels of several genes at baseline helped to predict the changes in BMI after a nutritional intervention [28]. However, few studies have associated baseline genome-wide expression profile with weight loss outcome in adult obese subjects. Interestingly, Mutch et al. (2007) reported that adipose gene expression profiling prior to the consumption of a low-fat diet was able to differentiate responders from non-responders to the treatment, while Arnemise et al (2017) demonstrated that adipose gene expression combined with clinical variables allowed to distinguish weight and glycemic responders from non-responders to a low-calorie diet. Other studies have found no differences in the baseline transcriptomic profiling of subcutaneous adipose tissue between LR and HR [29]. However, several studies using methylation array-based technologies have been successful in the identification of novel markers of age-related diseases or obesity [30, 31]. In the present study, we described 2,102 CpGs that were differentially methylated between LR and HR before the energy-restriction diet. Similarly, other authors have reported that methylation levels of different genomic regions in different cell types are associated with individual response to a weight loss nutritional intervention [24, 29, 32].

DNA methylation is considered as a gene expression regulatory mechanism [33]. In this sense, methylation changes have been associated with mRNA expression alteration in age, diabetes, embryonic development, or cancer [34, 35]. Regarding the main objective of the current investigation, the identification of novel genes for distinguishing personal response by the integrated analysis of expression and DNA methylation data, the

combination of both high-throughput technologies pointed out four genes that presented differential expression and methylation profiles between LR and HR at baseline.

DNA methylation has been associated with transcriptional repression, but emerging data showed that the effect of DNA methylation depends of the genomic location [33]. Our results revealed that hypomethylated CpG regions corresponding to *CD44*, *ITPR1* and *MTSS1* exhibited up-regulation of gene expression, and in contrast, hypomethylated CpGs of *FBXW5* exhibited down-regulation of gene expression in LR comparing HR. Of these four genes, a literature survey indicated that only *CD44* and *ITPR1* were implicated in obesity or weight loss. In addition, a previous study of our group identified an association between methylation of *CD44* promoter and changes in waist circumference, BMI and fat mass after a weight loss treatment [36]. These findings suggest that this gene could be implicated in weight loss regulation, and we selected *CD44* as a candidate gene to further validation. qRT-PCR results in the VP showed higher *CD44* expression in LR versus HR. Moreover, the MassARRAY® EpiTYPER™ technique allowed us to study neighbouring CpGs to the CpG selected by the array, covering more than 300 bp. Interestingly, CpG4 and CpG5 presented lower methylation levels in LR than in HR. Finally, gene expression is often directly associated with protein levels [37] and our results demonstrated a positive association between *CD44* mRNA expression in WBC and protein levels of CD44 shed from cell surfaces in plasma samples. The role of LR in this trend seems stronger than HR. Although *CD44* gene encodes an immune-cell surface receptor [38], few studies have evidenced that serum CD44 is associated with a protein release from human cells [38, 39]. Nevertheless, the population size could have been a limitation to find significant changes in protein levels between groups.

The expression of *CD44* has been related to adipose tissue macrophage accumulation and liver steatosis in morbid obesity, with a dramatic expression decrease as a result of massive weight loss [40]. In addition, *CD44* is implicated in the development of adipose tissue inflammation and insulin resistance [39], having been suggested as a biomarker for insulin resistance and a possible therapeutic target for T2D [38]. In contrast to our research, these studies were performed in adipose tissue biopsies. The choice of using WBC instead of other metabolic relevant tissues was based because blood is relatively easily to obtain in humans and is a non-invasive source of mRNA and DNA. In addition, recent studies have demonstrated that DNA methylation changes in blood reflect DNA methylation changes in pancreatic islets, and that DNA methylation levels in leukocytes mirror subcutaneous adipose tissue methylation pattern, which support the use of circulating cells to study epigenetic alterations in primary tissues [30]. In this context, the *CD44* methylation and expression profile found in the present study might reflect the profile in other metabolism-related tissue. Based on these evidences and our

current data, we hypothesise that *CD44* is an important gene involved in the amplification of the inflammatory process in obese subjects, and the higher expression of the gene before an energy-restricted diet may impair the effectiveness of weight loss dietary interventions.

As a limitation, despite that relevant statistical outcomes were found, type 1 and type 2 errors cannot be discarded. On the other hand, the use of false discovery rate (FDR) tests in gene expression analysis minimizes the risk to highlight genes that appear differentially expressed between LR and HR by chance. However, in the methylation array results, no CpG remained statistically significant after applying FDR; in order to minimize type 1 error, the lowest p values were selected for the analysis.

One of the strengths of the present work is the use of a two steps strategy, validating the results obtained in the DPs in a second, different one called VP for both measurements (DNA methylation and gene expression). Another remarkable point is the concordance observed between DNA methylation and mRNA levels, which suggests a putative mechanistic role of the epigenetic mechanism on the regulation of gene expression.

In summary, DNA methylation has been suggested as a powerful tool for diagnosis and prognosis. It is noteworthy that the integration of genome-wide array data can be a useful strategy to identify novel genes that might be considered as predictors of the response to specific nutritional interventions. In addition, we demonstrated for the first time that the expression and DNA methylation of the immune-cell receptor gene, *CD44*, has a different profile between LR and HR at baseline, suggesting a putative role of *CD44* in body weight regulation.

References

1. Kaur J, Jaspinder (2014) A comprehensive review on metabolic syndrome. Cardiol Res Pract 2014:943162 . doi: 10.1155/2014/943162
2. Goni L, Cuervo M, Milagro FI, Martínez JA (2015) A genetic risk tool for obesity predisposition assessment and personalized nutrition implementation based on macronutrient intake. Genes Nutr 10:445 . doi: 10.1007/s12263-014-0445-z
3. de la Iglesia R, Lopez-Legarrea P, Abete I, Bondia-Pons I, Navas-Carretero S, Forga L, Martinez J. A, Zulet MA (2014) A new dietary strategy for long-term treatment of the metabolic syndrome is compared with the American Heart Association (AHA) guidelines: the MEtabolic Syndrome REduction in NAvarra (RESMENA) project. Br J Nutr 111:643–652 . doi: 10.1017/S0007114513002778
4. Guo S, Yan F, Xu J, Bao Y, Zhu J, Wang X, Wu J, Li Y, Pu W, Liu Y, Jiang Z, Ma Y, Chen X, Xiong M, Jin L, Wang J (2015) Identification and validation of the methylation biomarkers of non-small cell lung cancer (NSCLC). Clin Epigenetics 7:3 . doi: 10.1186/s13148-014-0035-3
5. de Toro-Martín J, Arsenault B, Després J-P, Vohl M-C (2017) Precision Nutrition: A Review of

Personalized Nutritional Approaches for the Prevention and Management of Metabolic Syndrome. Nutrients 9:913 . doi: 10.3390/nu9080913

6. Tremblay BL, Cormier H, Rudkowska I, Lemieux S, Couture P, Vohl MC (2015) Association between polymorphisms in phospholipase A2 genes and the plasma triglyceride response to an n-3 PUFA supplementation: a clinical trial. Lipids Health Dis 14:12 . doi: 10.1186/s12944-015-0009-2

7. Rudkowska I, Pérusse L, Bellis C, Blangero J, Després JP, Bouchard C, Vohl MC (2015) Interaction between Common Genetic Variants and Total Fat Intake on Low-Density Lipoprotein Peak Particle Diameter: A Genome-Wide Association Study. J Nutrigenet Nutrigenomics 8:44–53 . doi: 10.1159/000431151

8. Vallée Marcotte B, Cormier H, Guénard F, Rudkowska I, Lemieux S, Couture P, Vohl MC (2016) Novel Genetic Loci Associated with the Plasma Triglyceride Response to an Omega-3 Fatty Acid Supplementation. J Nutrigenet Nutrigenomics 9:1–11 . doi: 10.1159/000446024

9. González-Muniesa P, Mártinez-González M-A, Hu FB, Després JP, Matsuzawa Y, Loos R, Moreno LA, Bray GA, Martinez JA (2017) Obesity. Nat Rev Dis Prim 3:17034 . doi: 10.1038/nrdp.2017.34

10. Heianza Y, Qi L (2017) Gene-Diet Interaction and Precision Nutrition in Obesity. Int J Mol Sci 18:787 . doi: 10.3390/ijms18040787

11. Cordero P, Campion J, Milagro FI, Martinez JA (2013) Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: Effect of dietary methyl donor supplementation. Mol Genet Metab 110:388–395 . doi: 10.1016/j.ymgme.2013.08.022

12. Martínez JA, Etxeberría U, Galar A, Milagro FI (2013) Role of Dietary Polyphenols and Inflammatory Processes on Disease Progression Mediated by the Gut Microbiota. Rejuvenation Res 16:435–437 . doi: 10.1089/rej.2013.1481

13. Choi S-W, Claycombe KJ, Martinez JA, Friso S, Schalinske KL (2013) Nutritional epigenomics: a portal to disease prevention. Adv Nutr 4:530–2 . doi: 10.3945/an.113.004168

14. Remely M, Lovrecic L, De La Garza AL, Migliore L, Peterlin B, Milagro FI, Martinez AJ, Haslberger AG (2015) Therapeutic perspectives of epigenetically active nutrients. Br. J. Pharmacol. 172:2756–2768

15. Karimi M, Vedin I, Freund Levi Y, Basun H, Faxén-Irving G, Eriksdotter M, Wahlund L-O, Schultzberg M, Hjorth E, Cederholm T, Palmblad J (2017) DHA-rich n-3 fatty acid supplementation decreases DNA methylation in blood leukocytes: the OmegAD study. Am J Clin Nutr 106:1157–1165 . doi: 10.3945/ajcn.117.155648

16. Perfilyev A, Dahlman I, Gillberg L, Rosqvist F, Iggman D, Volkov P, Nilsson E, Risérus U, Ling C (2017) Impact of polyunsaturated and saturated fat overfeeding on the DNA-methylation pattern in human adipose tissue: A randomized controlled trial. Am J Clin Nutr 105:991–1000 . doi: 10.3945/ajcn.116.143164

17. Armenise C, Lefebvre G, Carayol J, Bonnel S, Bolton J, Di Cara A, Gheldof N, Descombes P, Langin D, Saris WH, Astrup A, Hager J, Viguerie N, Valsesia A (2017) Transcriptome profiling from adipose tissue during a low-calorie diet reveals predictors of weight and glycemic outcomes in obese, nondiabetic subjects. Am J Clin Nutr 106:736–746 . doi: 10.3945/ajcn.117.156216

18. Goni L, Milagro FI, Cuervo M, Martínez JA (2014) Single-nucleotide polymorphisms and DNA methylation markers associated with central obesity and regulation of body weight. Nutr Rev 72:673–

690 . doi: 10.1111/nure.12143

19. Zulet MA, Bondia-Pons I, Abete I, De La Iglesia R, Lopez-Legarrea P, Forga L, Navas-Carretero S, Martinez JA (2011) The reduction of the metabolyc syndrome in Navarra-Spain (RESMENA-S) study; A multidisciplinary strategy based on chrononutrition and nutritional education, together with dietetic and psychological control. Nutr Hosp 26:16–26 . doi: 10.3305/nh.2011.26.1.5050

20. Moher D, Hopewell S, Schulz KF, Montori V, Gøtzsche PC, Devereaux PJ, Elbourne D, Egger M, Altman DG (2012) CONSORT 2010 explanation and elaboration: Updated guidelines for reporting parallel group randomised trials. Int J Surg 10:28–55 . doi: 10.1016/j.ijsu.2011.10.001

21. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18:499–502

22. Touleimat N, Tost J (2012) Complete pipeline for Infinium ® Human Methylation 450K BeadChip data processing using subset quantile normalization for accurate DNA methylation estimation. Epigenomics 4:325–341 . doi: 10.2217/epi.12.21

23. Mansego ML, Milagro FI, Campión J, Martínez JA (2013) Techniques of DNA Methylation Analysis with Nutritional Applications. J Nutrigenet Nutrigenomics 6:83–96 . doi: 10.1159/000350749

24. Milagro FI, Campion J, Cordero P, Goyenechea E, Gomez-Uriz AM, Abete I, Zulet MA, Martinez JA (2011) A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J 25:1378–1389 . doi: 10.1096/fj.10-170365 [doi]

25. Diboun I, Wernisch L, Orengo CA, Koltzenburg M (2006) Microarray analysis after RNA amplification can detect pronounced differences in gene expression using limma. BMC Genomics 7:252 . doi: 10.1186/1471-2164-7-252

26. Smyth GK (2005) limma: Linear Models for Microarray Data. In: Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer-Verlag, New York, pp 397–420

27. Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH (2009) The incidence of co-morbidities related to obesity and overweight: A systematic review and meta-analysis. BMC Public Health 9:88 . doi: 10.1186/1471-2458-9-88

28. Rendo-Urteaga T, García-Calzón S, González-Muniesa P, Milagro FI, Chueca M, Oyarzabal M, Azcona-Sanjulián MC, Martínez JA, Marti A (2015) Peripheral blood mononuclear cell gene expression profile in obese boys who followed a moderate energy-restricted diet: Differences between high and low responders at baseline and after the intervention. Br J Nutr 113:331–342 . doi: 10.1017/S0007114514003584

29. Bouchard L, Rabasa-Lhoret R, Faraj M, Lavoie ME, Mill J, Pérusse L, Vohl MC (2010) Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction. Am J Clin Nutr 91:309–320 . doi: 10.3945/ajcn.2009.28085 [doi]

30. Bacos K, Gillberg L, Volkov P, Olsson AH, Hansen T, Pedersen O, Gjesing AP, Eiberg H, Tuomi T, Almgren P, Groop L, Eliasson L, Vaag A, Dayeh T, Ling C (2016) Blood-based biomarkers of age-associated epigenetic changes in human islets associate with insulin secretion and diabetes. Nat Commun 7:11089 . doi: 10.1038/ncomms11089

31. Chaplin A, Palou A, Serra F (2017) Methylation analysis in fatty-acid-related genes reveals their plasticity associated with conjugated linoleic acid and calcium supplementation in adult mice. Eur J Nutr

56:879–891 . doi: 10.1007/s00394-015-1135-3

32. Moleres A, Campion J, Milagro FI, Marcos A, Campoy C, Garagorri JM, Gomez-Martinez S, Martinez JA, Azcona-Sanjulian MC, Marti A (2013) Differential DNA methylation patterns between high and low responders to a weight loss intervention in overweight or obese adolescents: the EVASYON study. FASEB J 27:2504–2512 . doi: 10.1096/fj.12-215566 [doi]

33. Zhang G, Pradhan S (2014) Mammalian epigenetic mechanisms. IUBMB Life 66:240–256

34. Zhuang J, Peng W, Li H, Wang W, Wei Y, Li W, Xu Y (2012) Methylation of p15INK4b and Expression of ANRIL on Chromosome 9p21 Are Associated with Coronary Artery Disease. PLoS One 7:e47193 . doi: 10.1371/journal.pone.0047193

35. Wadhwa PD, Buss C, Entringer S, Swanson JM (2009) Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. Semin. Reprod. Med. 27:358–368

36. Milagro FI, Campion J, Cordero P, Goyenechea E, Gomez-Uriz AM, Abete I, Zulet MA, Martinez JA (2011) A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J 25:1378–1389 . doi: 10.1096/fj.10-170365

37. Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13:227–232 . doi: 10.1038/nrg3185

38. Kodama K, Horikoshi M, Toda K, Yamada S, Hara K, Irie J, Sirota M, Morgan AA, Chen R, Ohtsu H, Maeda S, Kadowaki T, Butte AJ (2012) Expression-based genome-wide association study links the receptor CD44 in adipose tissue with type 2 diabetes. Proc Natl Acad Sci U S A 109:7049–54 . doi: 10.1073/pnas.1114513109

39. Liu LF, Kodama K, Wei K, Tolentino LL, Choi O, Engleman EG, Butte AJ, McLaughlin T (2015) The receptor CD44 is associated with systemic insulin resistance and proinflammatory macrophages in human adipose tissue. Diabetologia 58:1579–1586 . doi: 10.1007/s00125-015-3603-y

40. Bertola A, Deveaux V, Bonnafous S, Rousseau D, Anty R, Wakkach A, Dahman M, Tordjman J, Clément K, McQuaid SE, Frayn KN, Huet PM, Gugenheim J, Lotersztajn S, Marchand-Brustel YL, Tran A, Gual P (2009) Elevated expression of osteopontin may be related to adipose tissue macrophage accumulation and liver steatosis in morbid obesity. Diabetes 58:125–133 . doi: 10.2337/db08-0400

Figure captions

Fig. 1 Schematic diagram of the study design and integrative analysis of methylation and expression arrays.

Fig. 2 Venn diagram of statistically significant genes differentially expressed and methylated according to the diet response. A total of 4 genes were statistically significant in both transcription and methylation analyses. Four genes were significant in both arrays, 3 of them presented methylation and expression changes in opposite direction and only one of them presented changes in the same direction.

Fig. 3 Graphic illustration of the literature search results of the association between *CD44* and obesity, weight loss, inflammation, or biomarker. PubMed was searched for papers containing the gene name and the terms (a). Positive association between *CD44* expression levels by qRT-PCR and ILMN_1803429 (*CD44*) expression by microarray (n = 24) (b), and between DNA methylation data of several *CD44* CpG sites located in *CD44* measured by MassARRAY® EpiTYPER™ (CpG1 (c) CpG4 (d) and CpG5 (e))with the CpG site (cg08688659, corresponding to CpG5) from the methylation array (n = 32) (c-e). Data was analysed by Pearson's test, $p < 0.05$.

Fig. 4 Differences of *CD44* mRNA expression levels between LR and HR subjects **(a).** Differences of DNA methylation levels of three CpG sites located in *CD44* between LR and HR **(b).** The *CD44* mRNA expression was found to be correlated with protein expression **(c)**. Results are expressed as means ± SD (LR, n = 31; HR, n = 16). Unpaired t Student's test was used to compare LR with HR. * $p < 0.05$. Linear relationship between protein and mRNA expression was tested using Pearson's correlation coefficient (R), $p < 0.05$, one-tailed test.

Table 1. Characteristics of study subjects of the discovery populations and validation population, and divided according to weight loss (LR < 8 % of initial weight; HR > 8 % of initial weight) at the beginning of the study.

Characteristics	Discovery Population for Expression		Discovery Population for Methylation		Validation Population	
	LR	HR	LR	HR	LR	HR
No. of subjects	14	10	31	16	26	21
Age (y)	48.4 ± 3.5	49.6 ± 4.0	46.5 ± 9.7	52.1 ± 9.2	48.3 ± 2.9	51.7 ± 3.8
Sex (M/F)	8/6	5/5	18/13	7/9	14/12	12/9
Weight (kg)	105.3 ± 12.2	98.4 ± 9.6	106.5 ± 18.5	94.3 ± 14.1*	102.4 ± 8.2	94.8 ± 6.0
Weight loss (%)	5,9 ± 0.9	10.5 ± 1.1***	5.2 ± 1.6	10.0 ± 1.7***	5.7 ± 0.8	9.5 ± 0.9***
BMI (kg/m^2)	37.0 ± 2.1	34.9 ± 3.0	36.9 ± 3.4	34.8 ± 4.3	36.1 ± 1.9	33.8 ± 1.5
Body fat mass (%)	40.5 ± 5.5	38.8 ± 8.6	39.6 ± 6.2	37.8 ± 8.6	39.9 ± 6.3	37.0 ± 7.0
Glucose (mg/dL)	135.6 ± 23.4	113.1 ± 14.6	126.9 ± 38.2	112.1 ± 19.7	124.2 ± 15.2	118.0 ± 12.8
Insulin (mUI)	17.3 ± 4.2	12.7 ± 4.3	17.2 ± 10.5	11.6 ± 6.3	16.9 ± 3.9	12.4 ± 2.7
HOMA-IR	5.6 ± 1.3	3.6 ± 1.3	5.6 ± 3.7	3.2 ± 1.9*	5.3 ± 1.4	3.8 ± 1.1
Total cholesterol (mg/dL)	214.9 ± 16.5	221.2 ± 16.5	211.7 ± 47.8	232.2 ± 52.8	222.5 ± 15.8	227.7 ± 12
HDL (mg/dL)	40.8 ± 4.7	43.2 ± 3.2	41.7 ± 8.5	46.1 ± 12.4	43.7 ± 4.5	43.7 ± 2.9
LDL (mg/dL)	129.9 ± 15.6	139.0 ± 12.2	129.9 ± 38.2	149.4 ± 50.8	135.4 ± 14.1	143.4 ± 11.4
Triglycerides (mg/dL)	221.4 ± 60.5	195.2 ± 72.9	200.7 ± 119.5	183.9 ± 106.0	216.8 ± 47.8	202.7 ± 52.1

Data presented as mean ± SEM. Differences between variables were compared using Student's t-test;*$p < 0.05$; *** $p < 0.001$. Abreviations: DPE, discovery population for expression; DPM, discovery population for methylation; VP, validation population; LR, low responder; HR, high responder; BMI, body mass index; HOMA-IR, homeostasis model assessment-insulir resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 2. Significant differentially expressed and methylated loci between LR and HR.

Gene name	Probe set	logFC[a]	p value[b]	FDR[c]	LR vs HR
CD44	cg08688659	0.105	0.008	0.999	LR < HR
	ILMN_1803429	0.640	< 0.001	0.028	LR > HR
FBXW5	cg14357259	0.078	0.047	0.999	LR > HR
	ILMN_1701375	-0.740	< 0.001	0.029	LR < HR
ITPR1	cg18689402	0.159	0.042	0.999	LR < HR
	ILMN_1789505	0.912	<0.001	0.022	LR > HR
MTSS1	cg03102442	0.055	0.009	0.999	LR < HR
	ILMN_2073289	0.702	<0.001	0.022	LR > HR

ANOVA test was applied for the differences analysis. FC, fold change; FDR, false discovery rate; LR, low responder; HR, high responder.

[a] the LR was the reference group for FC calculation.

[b] adjusted by gender, age and baseline weight

[c] after Benjamini-Hochberg correction

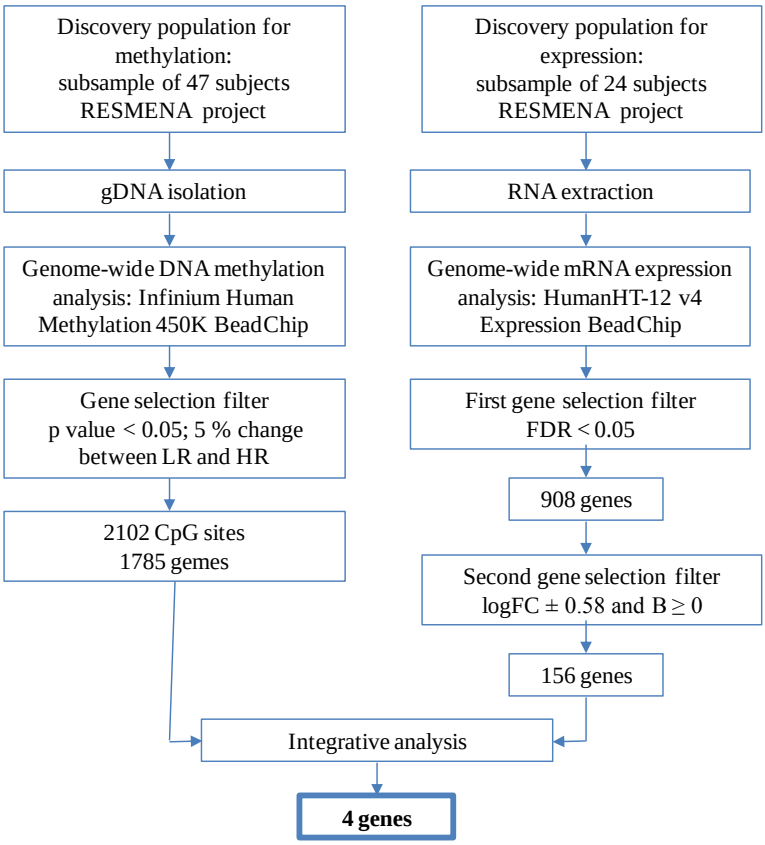


Fig. 1 Schematic diagram of the study design and integrative analysis of methylation and expression arrays.

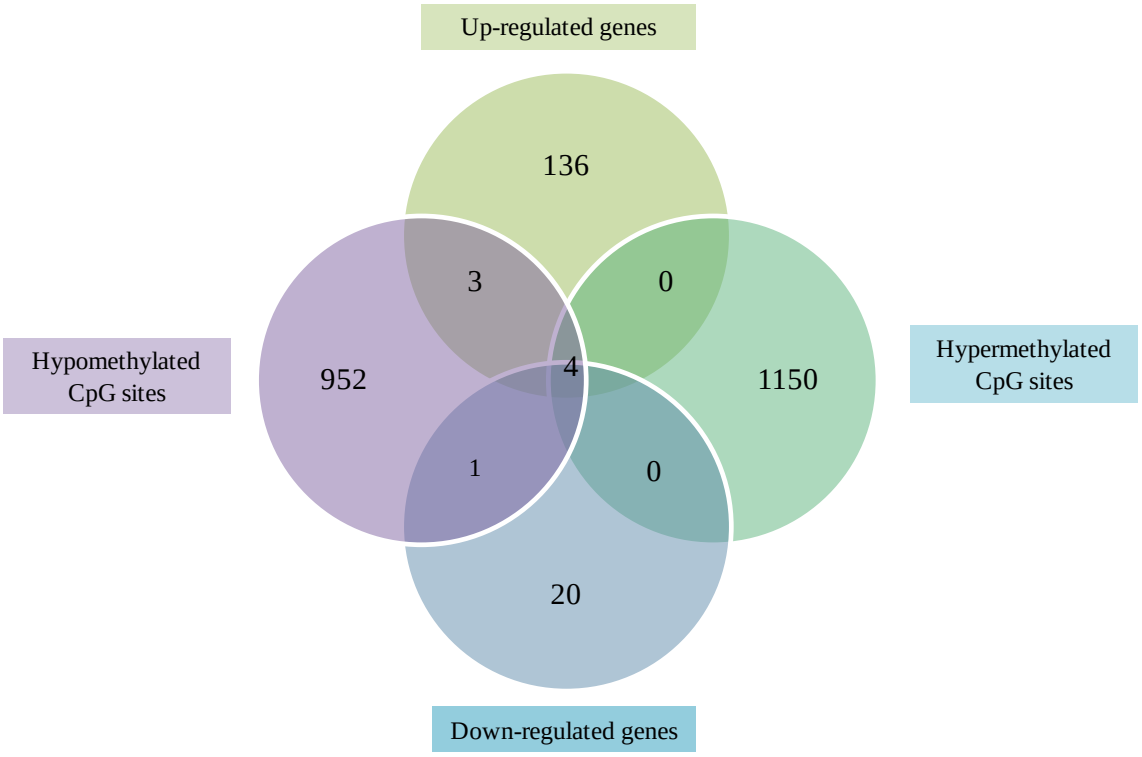


Fig. 2 Venn diagram of statistically significant genes differentially expressed and methylated according to the diet response. A total of 4 genes were statistically significant in both transcription and methylation analyses. Four genes were significant in both arrays, 3 of them presented methylation and expression changes in opposite direction and only one of them presented changes in the same direction.

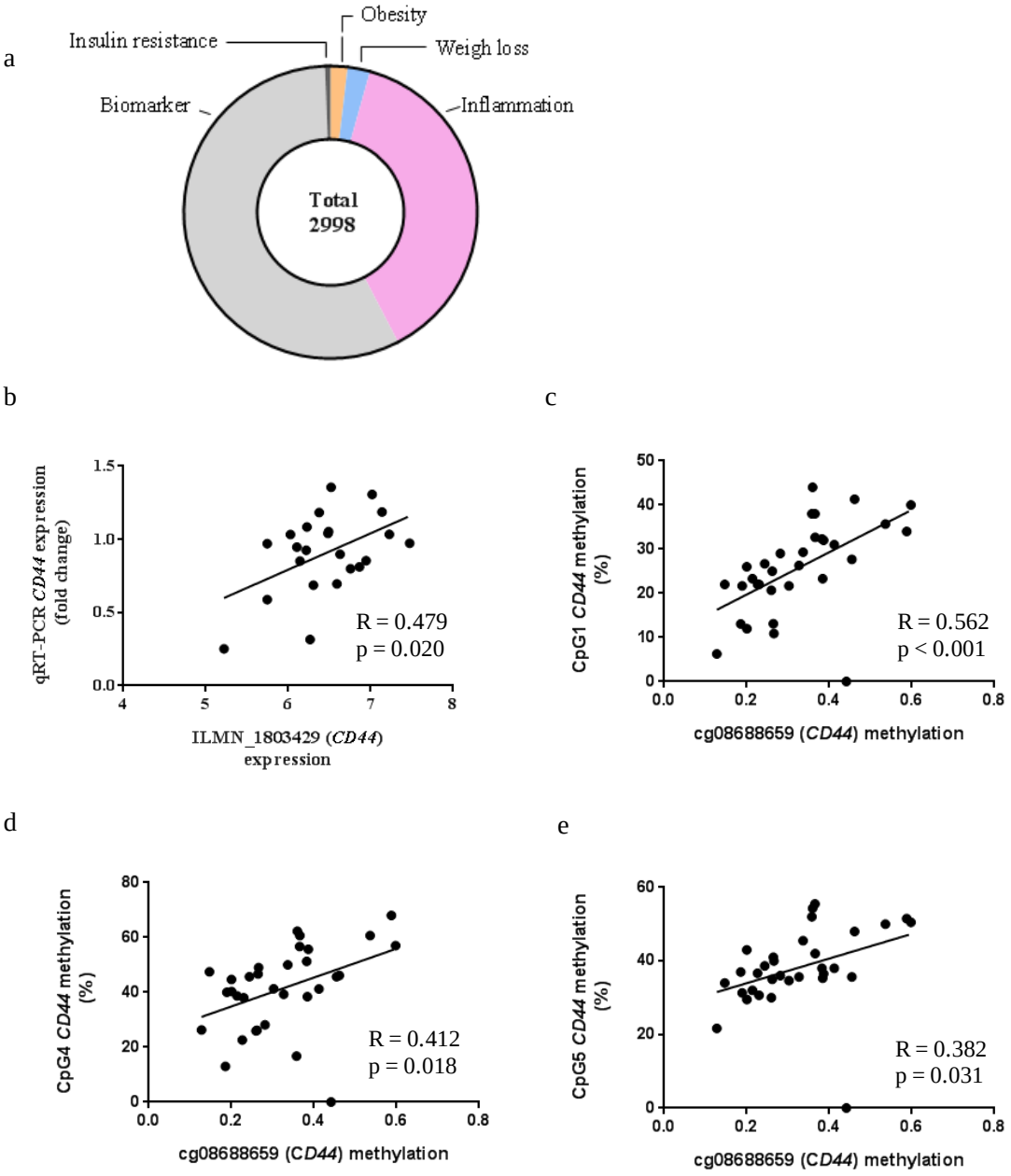


Fig. 3 Graphic illustration of the literature search results of the association between *CD44* and obesity, weight loss, inflammation, or biomarker. PubMed was searched for papers containing the gene name and the terms (a). Positive association between *CD44* expression levels by qRT-PCR and ILMN_1803429 (*CD44*) expression by microarray (n = 24) (b), and between DNA methylation data of several *CD44* CpG sites located in *CD44* measured by MassARRAY® EpiTYPER™ (CpG1 (c) CpG4 (d) and CpG5 (e))with the CpG site (cg08688659, corresponding to CpG5) from the methylation array (n = 32) (c-e). Data was analysed by Pearson's test, $p < 0.05$.

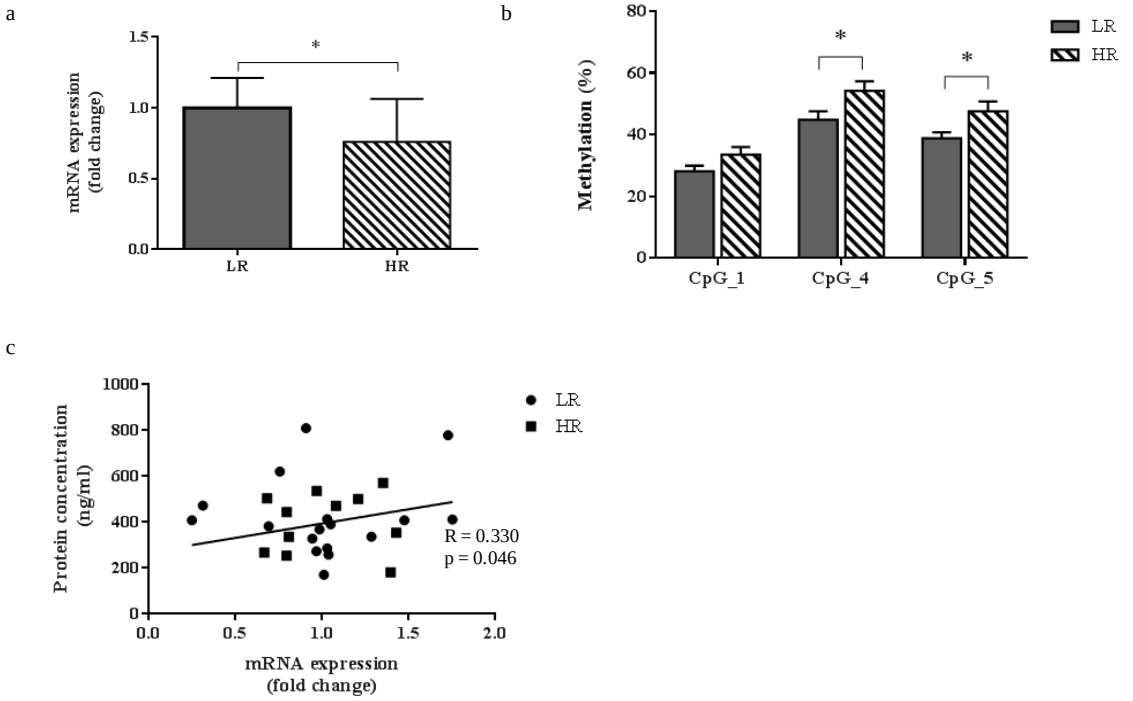


Fig. 4 Differences of *CD44* mRNA expression levels between LR and HR subjects **(a).** Differences of DNA methylation levels of three CpG sites located in *CD44* between LR and HR **(b).** The *CD44* mRNA expression was found to be correlated with protein expression **(c)**. Results are expressed as means ± SD (LR, n = 31; HR, n = 16). Unpaired t Student's test was used to compare LR with HR. * $p < 0.05$. Linear relationship between protein and mRNA expression was tested using Pearson's correlation coefficient (R), $p < 0.05$, one-tailed test.

Supplementary data CHAPTER 3

Supplementary data Table 1.

Identify genes differently expressed between low responders and high responders (n=24) with FDR < 0.05. Illumina ID: Probe name from the Illumina database; LogFC: logarithm of fold change; Average Expression Average expression value for that gene; t: Moderated t-statistic; Unadjusted P Value: P-value of moderated t-statistic; FDR: False discovery rate, adjusted p-value for multiple testing; B value: The log odds that the mRNA is differentially expressed; LR vs HR: low responders > or < expression than high responders.

Supplementary data Table 2.

CpG sites differently methylated between low responders and high responders (n=47) with methylation variation above 5 % and raw p value < 0.05. Probe set: Unique CpG locus identifier from the Illumina CG database; UCSC RefGene: Gene accession number (UCSC); LogFC: logarithm of fold change; Average Methylation: Average methylation value for that CpG site; t: Moderated t-statistic; P.Value: P-value of moderated t-statistic; adjusted P.Value: Adjusted p-value for multiple testing; B value: The log odds that the CpG site is differentially methylated; Chr: number of chromosome where is located the CpG; Localization: coordinates - genome build 37; LR vs HR: low responders > or < methylation than high responders.

Supplementary Table 1.

Illumina ID	Gene Name	logFC	Average Expression	t	Unadjusted P value	FDR	B value	LR vs HR
ILMN_1780368	GPR18	0,78	7,33	6,52	1,80E-06	0,0175	5,04	LR>HR
ILMN_1677824	RAB4A	0,91	6,05	6,07	4,90E-06	0,0175	4,15	LR>HR
ILMN_2297997	LIPT1	0,61	6,14	6,02	5,50E-06	0,0175	4,05	LR>HR
ILMN_1704472	EID2	0,93	6,37	5,90	7,20E-06	0,0195	3,80	LR>HR
ILMN_1809850	RCN3	-0,87	6,37	-5,85	8,10E-06	0,0195	3,70	LR<HR
ILMN_1676423	CCNC	0,82	6,16	5,68	1,21E-05	0,0223	3,34	LR>HR
ILMN_1685260	DNM1L	0,64	6,46	5,59	1,47E-05	0,0223	3,16	LR>HR
ILMN_1681628	ZNF277	0,78	7,85	5,53	1,69E-05	0,0223	3,04	LR>HR
ILMN_1857915	LOC401397	0,92	8,12	5,29	2,97E-05	0,0223	2,53	LR>HR
ILMN_1718907	TSHZ1	0,81	6,67	5,28	3,04E-05	0,0223	2,51	LR>HR
ILMN_2073289	MTSS1	0,70	8,13	5,26	3,20E-05	0,0223	2,46	LR>HR
ILMN_3235065	ZNHIT6	0,70	6,89	5,25	3,30E-05	0,0223	2,44	LR>HR
ILMN_1736940	HPRT1	0,77	7,98	5,22	3,50E-05	0,0223	2,38	LR>HR
ILMN_1813685	RAB7L1	0,80	8,02	5,21	3,58E-05	0,0223	2,36	LR>HR
ILMN_1691570	METTL5	0,84	7,92	5,21	3,62E-05	0,0223	2,35	LR>HR
ILMN_1662129	RCN2	0,67	7,71	5,20	3,68E-05	0,0223	2,34	LR>HR
ILMN_2088410	PSMG2	0,64	9,39	5,20	3,70E-05	0,0223	2,33	LR>HR
ILMN_2373831	BTN3A3	0,62	7,74	5,16	4,01E-05	0,0223	2,26	LR>HR
ILMN_1657632	ZMYM6	0,72	9,01	5,15	4,11E-05	0,0223	2,24	LR>HR
ILMN_1671554	LPIN1	1,05	8,48	5,10	4,70E-05	0,0223	2,11	LR>HR
ILMN_1737988	PRNP	0,73	9,27	5,09	4,74E-05	0,0223	2,11	LR>HR
ILMN_1747078	HYLS1	0,71	6,18	5,04	5,35E-05	0,0223	2,00	LR>HR
ILMN_1757336	LRCH3	0,71	6,03	5,03	5,44E-05	0,0223	1,98	LR>HR
ILMN_2059294	RTCD1	0,87	6,77	5,03	5,46E-05	0,0223	1,98	LR>HR
ILMN_1772743	PIGK	0,60	6,70	5,00	5,95E-05	0,0223	1,90	LR>HR

Note: and 5 pages more.

Supplementary Table 2.

Probe set	Gene name	UCSC RefGene	Average methylation	logFC	t	P value	Adjusted P value	B value	Chr	Localization	LR vs HR
cg19846991	MYO9A	NM_006901;NM_145204;NM_001166340	37,47	0,06	-5,60	1,81E-06	0,68279	4,12	15	72411513	LR < HR
cg26572973	GPT	NM_005309	76,79	0,12	-5,35	4,14E-06	0,68279	3,30	8	145728501	LR < HR
cg13372635	RNASEL	NM_021133	24,61	-0,10	5,29	4,99E-06	0,68279	3,11	1	182557217	LR > HR
cg13780303	TOMM22	NM_020243	68,66	0,17	-5,01	1,21E-05	0,92577	2,24	22	39078941	LR < HR
cg08608086	POLS	NM_006999	91,70	0,07	-4,92	1,61E-05	0,92577	1,96	5	6755235	LR < HR
cg16719099	MIR505	NR_030230	84,36	0,09	-4,65	3,75E-05	0,99998	1,13	X	139007085	LR < HR
cg20218571	PQLC1	NM_001146345;NM_001146343;NM_025078	75,00	0,48	-4,34	9,83E-05	0,99998	0,18	18	77678514	LR < HR
cg02960500	NTM	NM_001048209	85,93	0,07	-4,30	1,11E-04	0,99998	0,06	11	131469924	LR < HR
cg14739500	EHMT2	NM_006709;NM_025256	75,56	-0,08	4,25	1,29E-04	0,99998	-0,08	6	31855598	LR > HR
cg16280667	CXCR5	NM_001716;NM_001716	83,91	-0,07	4,24	1,33E-04	0,99998	-0,12	11	118754593	LR > HR
cg13179549	KRT16	NM_005557	59,40	0,07	-4,17	1,64E-04	0,99998	-0,32	17	39769175	LR < HR
cg14370752	DNHD1	NM_144666;NM_173589	78,67	0,06	-4,15	1,73E-04	0,99998	-0,37	11	6518438	LR < HR
cg05483199	LOC100132963	NM_001162936	20,80	-0,13	4,10	2,00E-04	0,99998	-0,51	X	154056033	LR > HR
cg18107006	HNRPLL	NM_001142650;NM_138394	20,22	0,07	-4,08	2,17E-04	0,99998	-0,59	2	38831166	LR < HR
cg15565576	IL17REL	NM_001001694	76,83	0,09	-4,03	2,46E-04	0,99998	-0,71	22	50451108	LR < HR
cg22198044	SH3BP2	NM_003023;NM_001145855;NM_001145856;NM_001122681	34,40	0,16	-3,99	2,85E-04	0,99998	-0,86	4	2819614	LR < HR
cg20318662	FAM110C	NM_001077710	24,98	-0,07	3,98	2,87E-04	0,99998	-0,86	2	47452	LR > HR

Note: and 102 pages more.

CHAPTER 4

Association of low dietary folate intake with lower *CAMKK2* gene methylation, adiposity, and insulin resistance in obese subjects

Omar Ramos-Lopez [a,1], Mirian Samblas [a,1], Fermín I. Milagro [1,2], Maria Angeles Zulet [1,2,3], Maria L. Mansego [1], Jose I. Riezu-Boj [1,3], J. Alfredo Martínez [1,2,3,4]

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

[2] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III. Madrid, Spain.

[3] Navarra Institute for Health Research (IdiSNA), Pamplona, Spain

[4] Madrid Institute of Advances Studies (IMDEA Food), Madrid, Spain

Nutrition Research, 2018

DOI: 10.1016/j.nutres.2017.11.007

Impact Factor (2016): 2.737

36/81 in Nutrition & Dietetics (Q2)

Ramos-Lopez O, Samblas M, Milagro FI, et al, Association of low dietary folate intake with lower CAMKK2 gene methylation, adiposity, and insulin resistance in obese subjects. Nutrition Research, 2018, 50:53-62.
http://doi.org/10.1016/j.nutres.2017.11.007

CHAPTER 5

Folic acid improves the inflammatory response in LPS-activated THP-1 macrophages

Mirian Samblas[1,2], Alfredo Martínez[1,2,3] and Fermín Milagro[1,2]

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

[2] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III. Madrid, Spain

[3] Navarra Institute for Health Research (IdiSNA), Pamplona, Spain

Mediators of inflammation

Under review (1312626.v1)

Impact Factor (2016): 3.232

95/190 in Cell Biology, Q2

69/151 in Immunology, Q2

Folic acid improves the inflammatory response in LPS-activated THP-1 macrophages

Mirian Samblas[1,2], Alfredo Martínez[1,2,3] and Fermín Milagro[1,2*]

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

[2] CIBERobn, CIBER Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III. Madrid, Spain.

[3] IdiSNA, Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain

Email adresses:

Mirian Samblas: msamblas@alumni.unav.es

Alfredo Martínez: jalfmtz@unav.es

* Corresponding author:

Fermín Milagro: fmilagro@unav.es

ABSTRACT

DNA methylation has been suggested as one regulatory mechanism behind some inflammatory processes. The physiological actions of methyl donors, such as folic acid, choline, and vitamin B_{12} on inflammatory-related diseases have been associated to an involvement in the synthesis of the universal methyl-donor S-adenosyl methionine (SAM). The aim of this study was to evaluate the effects of folic acid, choline, and vitamin B_{12} on preventing the lipopolysaccharide (LPS)-induced inflammatory response in human THP-1 monocyte/macrophage cells. Folic acid and a mix of methyl donors reduced interleukin 1 beta (*IL1B*) and tumor necrosis factor (*TNF*) expression and protein secretion by such cells. Moreover, the methyl donor mix also reduced Cluster of differentiation 40 (*CD40*) expression, but increased serpin family E member 1 (*SERPINE1)* gene expression. All the methyl donors increased methylation levels in the CpGs located in *IL1B*, *SERPINE1*, and interleukin 18 (*IL18)* genes. However, they did not modify *TNF* methylation. ChIP analysis showed no changes in the binding affinity of NF-κB to *IL1B* and *TNF* promoters region after the treatment with folic acid and the methyl donors' mix. The findings of this study suggest that folic acid might be a factor for controlling chronic inflammation in inflammatory-related diseases.

INTRODUCTION

Inflammation has been traditionally defined as the short-term adaptive response of the body to fight against injuries on cells and tissues caused by pathogens or biological and chemical stimuli [1]. Although acute inflammation is a crucial component for maintaining body homeostasis, when persists longer (chronic inflammation) these processes are involved in the development of several clinical manifestations and diseases. For example, chronic inflammation is linked with osteoarthritis, autoimmune and degenerative diseases, type 2 diabetes, atherosclerosis and/or obesity [2].

A variety of interacting mechanisms, molecules, mediators and cells participate in the inflammatory outcome [3]. An important feature of the inflammatory process is the migration and recruitment of monocytes from bloodstream to inflamed regions [4]. Chemokines produced in the inflamed region orchestrate the recruitment of monocytes into these sites, where they differentiate into macrophages and secrete cytokines to mediate the inflammatory response [5]. This is the case of macrophage infiltration and activation in liver and adipose tissue in non-alcoholic fatty liver disease and visceral obesity [6, 7]. Monocytes must be able to phenotypically continuously adapt their response to both external and internal environmental signals [8]. In this sense, epigenetics, defined as heritable changes in gene expression without changes in genomic DNA, has been suggested as the group of mechanisms that may alter transcriptomic patterns of these cells in function of the requirements [9]. DNA methylation is the most studied epigenetic mechanism and consists in the addition of a methyl group in the 5' position of cytosine next to a guanine nucleotide [10]. Several studies have described that epigenetic mechanisms are involved in the pathogenesis of most chronic inflammatory diseases by regulating important steps such as macrophage infiltration or cytokine secretion [11][12]. For instance, typically inflammatory molecules like interleukin (IL)-6, *IL-4*, *IL-8*, *IL-1β* or *INF-γ* have been described to be differently methylated in several chronic inflammatory diseases [9][11].

One of the advantages of epigenetics phenomena is the plasticity that enables modifying the epigenome pattern by the environment factors, including the diet [13]. Actually, that a variety of nutrients, such as fatty acids, polyphenols and methyl-donors, may modify DNA methylation pattern,

either at the global scale or at specific sites [14][15–17]. Methionine, folate, betaine, choline, vitamin B_2, B_6 and B_{12} are considered methyl-donor precursor compounds present in the diet [18]. These compounds participate in the methionine pathway for the synthesis of S-adenosyl methionine (SAM), which is the universal methyl-donor for DNA methylation reactions. In this context, low availability of these compounds is usually accompanied by a reduction of global DNA methylation [13]. A variety of inflammation-related pathologies are linked to methyl-donor deficiency, and several studies have described the anti-inflammatory effect of these compounds. For example, folic acid deficiency has been related to hyperhomocysteinemia, hypertension, diabetes, and stroke [19], and the supplementation with this compound might improve disease outcome by reducing the inflammatory response [20, 21]. Furthermore, choline deficiency has been associated with the development of fatty liver and with worse liver fibrosis outcomes in patients with non-alcoholic steatohepatitis (NASH) as described elsewhere [22]. Nevertheless, NASH patients that were choline deficient exhibited amelioration of steatohepatitis after choline supplementation [23]. Finally, vitamin B_{12} deficiency has been associated with pro-inflammatory cytokines and low-grade systemic inflammation [24], and the development of hyperhomocysteinamemia, obesity, hypertension, and insulin resistance (Li, Gueant-Rodriguez, & Quilliot, 2017). However, the effects of these compounds in the inflammatory response of macrophages and the role of DNA methylation in this process are seldom studied. For this reason, the aim of this study was to investigate the effects of methyl donors, both individually and in combination, on the prevention of LPS-induced inflammatory response in human THP-1 monocyte/macrophage cells by assessing methylation pattern modifications. For this purpose, monocytes were incubated with folic acid, choline, vitamin B_{12} or a methyl donors' mix that consisted in a combination of folic acid, choline and vitamin B_{12} and, then, the monocytes were differentiated into macrophages and an inflammatory response was induced with LPS.

MATERIAL AND METHODS

Reagents

Folic acid, vitamin B_{12} and choline chloride were supplied from Sigma-Aldrich (MO, USA). Phorbol 12-myristate 13-acetate (TPA) from Sigma-Aldrich was used for differentiating THP-1 monocytes into macrophage-like cells, whereas lipopolysaccharide (LPS) from E. coli K12 strain (Invitrogen, CA, USA) was applied for activating macrophages. Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma-Aldrich) was required in order to investigate the toxic effects of methyl donors on THP-1 cells.

Cell culture and treatments

Human monocyte THP-1 cells were purchased from American Type Cell Culture (ATCC® TIB-202™, VA, USA). Cells were maintained at 37ºC and 5 % CO_2 in RMPI-1640 medium (GIBCO) modified to contain 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g/l glucose and 1.5 g/l sodium bicarbonate, and supplemented with 10 % fetal bovine serum (GIBCO), 100 U/ml penicillin, and 100 µg/ml streptomycin.

THP-1 cells were treated with folic acid (11.3 µM) dissolved in NaOH 1 M, choline chloride (105 µM), vitamin B_{12} (18.5 nM) and a mix of methyl donors that consisted in a combination of folic acid, choline chloride and vitamin B_{12} in the concentrations previously indicated. The concentrations of the treatments were chosen multiplying ten times the basal concentration present in RPMI-1640 medium for each compound. After 24 h, cells were differentiated into macrophages by incubation with 25 ng/ml TPA for 48 h, and then were activated by incubation with 100 ng/ml LPS for 24 hours. Finally, RNA and DNA were extracted, and supernatants were collected for ELISA analysis.

Cell viability analysis

For viability assay, THP-1 cells were pretreated with the compounds at the selected concentrations during 24 h as described above in a 96-well plate. After the treatments, 20 µl MTT (5 mg/ml) was added to each well and plates were incubated for 2 h at 37 ºC. Formazan crystal formation was solubilised in 100 µL/well DMF-glacial acetic acid-SDS solution consisting in 40 % DMF, 2 % glacial

acetic acid and 16 % w/v sodium SDS. Formazan production was quantified by absorbance at 570 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland). The results were expressed as relative cell viability (%).

Analysis of mRNA expression by quantitative real-time PCR

Total RNA was extracted from cells with TRizol® reagent (Invitrogen). RNA quality and concentrations were measured with Nanodrop Spectrophotometer ND1000 (Thermo Fisher Scientific, MA, USA). About 1 µg of total RNA was reverse-transcribed into cDNA by MultiScribe™ Reverse Transcriptase kit following the manufacturer's instruction (Thermo Fisher Scientific, MA, USA). Real-time PCR was performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, CA, USA). Predesigned TaqMan primers and probes for *IL1B* (Hs01555410_m1), *TNF* (Hs00174128_m1), *IL18* (Hs01038788_m1), *SERPINE1* (Hs01126606_m1), *CD40* (Hs01002913_g1) and *TLR4* (Hs00152939_m1) genes, and Taqman Universal Master Mix (Applied Biosystem). The levels of these mRNAs were normalized to the level of *GAPDH* (Hs02758991_g1) mRNA expression. Relative expression was determined by using the comparative $2^{-\Delta\Delta Ct}$ method.

Cytokine secretion analysis by Enzyme-linked immunosorbent assay (ELISA)

Culture supernatants were collected after the treatments and stored at -80 ºC for cytokine analysis. Protein concentrations of IL-1β, TNF-α, PAI1 and CD40 were measured with standard ELISA kits (R&D Systems Europe, UK) according to manufacturer's protocols. Absorbance was measured at 450 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Finland).

DNA methylation analysis by MALDI-TOF mass spectrometry

DNA was isolated from cells using MasterPure™ DNA Purification Kit (Illumina, WI, USA) according to manufacturer's guidelines. Genomic DNA was sodium bisulfite-converted using the EpitTect Bisulfite Kit (Qiagen, CA, USA). DNA methylation quantification was performed by MassARRAY EpiTYPER technology (Sequenom Inc., CA, USA). This method uses matrix-assisted

laser desorption ionization time-of-flight (MALDITOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE). Four amplicons covering 32 CpG sites were selected. EpiDesigner software (Sequenom; http://www.epidesigner.com/start3.html) was used for designing PCR primers for the amplicons of interest concerning *IL1B* (chr2: 112,837,566-112,837,895), *TNF* (chr6: 31,575,209-31,575,481), *SERPINE1* (chr7: 101,127,068-101,127,411) and *IL18* (chr11: 112,163,853-112,164,105). The designed primers are shown in **supplementary table 1** and the complete amplicon sequences are shown in **supplementary figure 1**. The complete methodology was previously explained [26].

Chromatin Immunoprecipitation (ChIP) Assay

ChiP assay was performed with the ChIP-IT^{TM} Express Enzymatic Kit (Active Motif, CA, USA) following the manufacturer's instruction. Briefly, THP-1 cells were cultured for 24 hours with methyl donors, and then were differentiated with TPA (25 ng/µl) for 48 hours and activated with LPS (100 ng/µl) during 24 hours. The cell medium was discarded and 36.5 % formaldehyde was added directly to the cell surface for 10 min for the crosslink between proteins and DNA. Cross-linking was stopped by the addition of glycine for 5 min at room temperature and cells were scraped to collect them. Then, cells were incubated with lysis buffer for 30 min at 4 ºC and the DNA was fragmented via enzyme-based digestion for 10 min at 37 ºC. Chromatin was immunoprecipitated using rabbit polyclonal antibody to NF-κB (ab7970, Abcam, MA, USA). After immunoprecipitation, crosslinking of protein-DNA complexes was reversed and DNA was ready for analysis. Real time quantitative PCR was performed using primers for *IL1B*: sense 5'-agcaacaaagctgccactta-3' and antisense 5'-tgacgtgctgtgtgaatttg-3', and *TNF*: sense 5'-ggagaatgtccagggctatg-3' and antisense 5'-tcctggaggctctttcactc-3'.

Transcription factor-binding site analysis

In order to identify the putative transcription factor binding site in the CpG sites of *IL-1β* gene, a bioinformatic analysis was performed through LASAGNA-Search 2.0 by using TRANSFAC matrices and aligned models [27].

Statistical analysis

Normality was assessed by Kolmogorov-Smirnov and Shapiro-Wilk tests. For the statistical analysis of the results, one-way ANOVA followed by Dunnett's test for multiple comparisons between groups, and unpaired Student's t test for the direct comparisons between two groups, were used. Differences were considered significant at p value < 0.05. Statistics were performed using Prism 5.0 (GraphPad Software, CA, USA).

RESULTS

Methyl donors' treatment did not affect cell viability

To exclude the possibility that changes of the levels of the inflammatory genes were due to toxicity of methyl donors, cell viability was measured by MTT assay after the incubation with folic acid at 11.3 µM, choline at 105 µM, vitamin B12 at 18.5 nM, and methyl donor's mix. The selected concentrations were within the range proposed by previously studies (Cianciulli, Salvatore, Porro, Trotta, & Panaro, 2016b; Feng, Zhou, Xia, & Ma, 2011b; Jiang et al., 2016). Cell viability was not significantly affected by methyl donors at these concentrations (**Supplementary Figure 2**).

Comparative effects of methyl donors on genes associated with the inflammatory response in THP-1 macrophages activated with LPS

The treatment of THP-1 cells with the different compounds before the differentiation with TPA and activation with LPS altered the expression of most of the inflammation-related genes compared to the control treatment **(Figure 1)**. Folic acid and methyl donors' mix reduced *IL1B* ($P<0.05$ for folic acid; $P < 0.01$ for methyl donors' mix) and *TNF* ($P<0.05$ for folic acid; $P<0.001$ for methyl donors' mix) mRNA expression. Folic acid treatment also reduced *TLR4* ($P<0.05$), but increased *SERPINE1* ($P<0.05$) gene expression. Moreover, methyl donors' mix incubation reduced the levels of *CD40* ($P<0.05$) but increased *SERPINE1* ($P<0.05$). However, no statistically significant changes were observed after choline and vitamin B_{12} incubation.

The pretreatment with folic acid and methyl donors' mix reduced IL-1β and TNF-α secretion of LPS-activated macrophages

We next evaluated the effects of folic acid, choline, vitamin B12 and methyl donors' mix on the secretion of proinflammatory cytokines in the macrophages activated with LPS. The incubation with folic acid and methyl donors' mix reduced the secretion of IL-1β ($P<0.01$) and TNF-α ($P<0.01$ for folic acid, and $P<0.05$ for methyl donors' mix), but not CD40 and PAI-1. On the other hand, no changes were observed with the other methyl donors **(Figure 2)**.

Incubation with methyl donors increased DNA methylation in the inflammatory genes

The regions under study of *IL1B*, *SERPINE1* and *IL18* displayed an overall gain of methylation when LPS-activated macrophages were treated with the different methyl donors. This hypermethylation was significant after the incubation with folic acid. As shown in **table 1**, folic acid increased significantly ($P<0.05$) the methylation levels of CpG_1 (190 %), CpG_5 (680 %) and CpG_6 (200 %) of *IL1B*, CpG_1 (750 %), CpG_2 (88 %), CpG_3.4 (136 %), CpG_7 (1003 %) and CpG_9 (88 %) of *SERPINE1*, and CpG_4 (53 %) and CpG_5 (27 %) of *IL18* compared with the methylation percentage of the non-treated LPS-activated macrophages. As an exception, no methylation changes were noted in the analysed region of *TNF* after the treatment. In the case of choline chloride, vitamin B_{12} and methyl donors' mix incubation, the incubation with these compounds also significantly increased ($P<0.05$) the methylation levels of some CpG sites of the genes (**Table 1**).

NF-κB binding site to *IL-1β* and *TNF-α* is not affect after the incubation with folic acid and mix

IL1B and *TNF* gene expression and secretion decreased after the incubation with folic acid and methyl donor's mix **(Figure 1-2)**. In the case of *IL1B,* DNA methylation levels were increased, but not in the case of *TNF*. To determine the effect of DNA methylation in the sequence of proinflammatory genes in the NF-κB binding to *IL1B* and *TNF* promoters a ChIP assay was performed. The analysis showed no significant changes in NF-κB binding to specific *IL1B* and *TNF* promoters region in chromatin from THP-1 cells treated with folic acid and methyl donors' mix (**Figure 3**).

DISCUSSION

In the present study, we demonstrated that some methyl donors, particularly folic acid alone or in combination with other methyl donors, reduced the inflammatory response in THP-1 activated-macrophages by evidencing the decrease in the expression of proinflammatory genes and the secretion of cytokines (e.g., IL-1β and TNF-α).

Previous studies in humans have analysed the association between folic acid and inflammation. For example, a case-control study showed a reduction of cytokines levels after 12 weeks treatment with folic acid [31]. In addition, folic acid supplementation in patients with high risk of coronary artery disease also was associated with a reduction in pro-inflammatory cytokines (e.g., MCP-1) in human monocytes [32]. In the present study, we demonstrated that folic acid and a mix of methyl donors reduced the expression of proinflammatory genes (e.g *TNF-a*, *IL-1B*, *CD40*, *TLR4*) in THP-1 monocytes when the monocytes were differentiated into macrophages and activated with LPS. In agreement with our results, incubation of murine monocyte RAW 264.7 cells with folic acid was found to reduce the expression of pro-inflammatory genes during LPS activation [33]. Contrariwise, folic acid deficiency in the same cell line enhanced the pro-inflammatory gene expression [34].

Current data revealed that the folic acid and methyl donor`s mix treatment of THP-1 monocytes not only reduce pro-inflammatory gene expression, but also decrease the secretion of TNF-α and IL-1β cytokines from cells when are differentiated to macrophages and activated by LPS. During the inflammatory response, the monocytes migrated from blood into the surrounding tissue, promoted by release of chemoattractants from the site of inflammation [4, 35]. During chronic inflammation there exists an overproduction of TNF-α and IL-1β by macrophages in the inflamed tissue, which amplifies the inflammatory process and attracts more monocytes to the inflammation area, contributing to tissue damage and disease [36]. In this context, our results suggest that folic acid and a mix of methyl donors might contribute to prevent this undesirable effect. Indeed, these methyl compounds could reduce the inflammatory response of the monocytes that are recruited from blood to surrounding tissues, and the

macrophages derived from these monocytes would produce lower cytokines levels, contributing thus to reduce inflammation at the final instance.

The intimate mechanisms for the beneficial effect of folic acid or methyl donors on inflammation have not been elucidated. One of the possible explanations that has been suggested is epigenetics, via DNA and histone methylation [34]. Folate, choline and vitamin B_{12} directly participate in the formation of S-adenosyl methionine (SAM) molecule, which is the major donor of methyl groups for DNA methylation [37]. Several research groups have reported an association between methyl donors' consumption, DNA methylation and inflammation [38] [34]. Specifically, an association between methyl donors' supplementation and DNA methylation changes in early liver steatosis in rats has been reported (Cordero, Campion, Milagro, & Martinez, 2013), evidencing the potential use of methyl donor's in the amelioration of inflammation. In the current trial, folic acid and methyl donors' mix increased the methylation levels of *IL1B*, *SERPINE1* and *IL18* in comparison with the non-treated LPS-stimulated THP-1 cells, and reduces the inflammatory response.

Also, DNA methylation has been associated with transcriptional repression by altering transcription factor-gene promoter binding affinity or the spatial accessibility of transcription machinery due to chromatin structure changes (Zhang & Pradhan, 2014)[41]. In the present study, only *IL1B* presented lower gene expression and protein secretion, and hypermethylation after folic acid supplementation. This result suggests that *IL1B* gene expression may be modulated by DNA methylation changes induced by folic acid. However, the methylation changes of *SERPINE1* and *IL18* were not correlated with changes in gene expression. The methyl donors' incubation was for 24 hours and then, the monocytes were differentiated for 48 hours and activated by LPS for other 24 hours. A recent study of *IL18* expression in after LPS-stimulated murine macrophages showed that the maximum level of expression of this interleukin was 3-6 hours after the induction, and not changes were showed at 24 hours, suggesting by an earlier enzymatic activation of PARP-1 that induces *IL18* expression (Liu et al., 2012). Moreover, other studies found that *IL18* and *SERPINE1* expression levels were modified by DNA methylation [43, 44]. In this context, the time or the concentration of supplements might have been insufficient to evaluate subtle changes in *IL18* and *SERPINE1* expression. Surprisingly, no

changes in DNA methylation levels in the *TNF* gene after the treatment with methyl donors were featured. In line with these results, Kolb *et al.* [34] found that, although folate deficiency in murine macrophages reduced DNA methyltransferase expression, DNA methylation did not change. In addition, in murine macrophages, incubation with exogenous SAM attenuated the LPS-stimulated expression of *TNF* [45].

Nuclear factor-κB (NF-κB) regulates the expression of many genes involved in the inflammatory response and the pro-inflammatory function of NF-κB has been studied widely in macrophages (Liu, Zhang, Joo, & Sun, 2017). For instance, lead-induced inflammatory response exhibited an increase in NF-κB expression that was associated with more inflammatory cell infiltration and IL-1B production [47]. In addition, it is known that NF-κB transcription factor is essential in the processes of LPS-mediated inflammatory response [48] and regulates the expression of cytokines by direct binding to promoter sequences [49–51]. Interestingly, after a folic acid supplementation the inflammatory response was reduced with a decrease of cytokine and cell infiltration [47]. In accordance with these findings, Feng *et al.* [33] reported that folic acid inhibited TNF-α and IL-1β production by inhibiting NF-κB pathway, although the direct effects of DNA methylation on NF-κB binding affinity to gene sequences had not been apparently reported. Furthermore, LPS stimulates the NF-κB pathway and induces the expression of a number of inflammatory genes, including *TNF* and *IL1B*, by binding to specific DNA elements or κB enhancers [52]. Available data in this research reveals that the DNA methylation induction of folic acid and methyl donors' mix in *IL1B* sequence did not affected the binding affinity of NF-κB to *IL1B* promoter. The binding levels of the transcription factor of *IL1B* promoter were similar to *TNF* promoter and to macrophages without methyl donors' supplementation.

Results of this investigation suggest a direct effect of methyl donors in the methylation of pro-inflammatory genes in a human-derived monocyte/macrophage cell line and the reduction of the expression and the production of pro-inflammatory cytokines. In addition, although the methyl donors' supplementation did not modify *TNF* promoter methylation, the results evidenced that it reduced TNF-α production induced by LPS. However, binding affinity of NF-κB to pro-inflammatory genes was unaffected, suggesting a minor role of the transcription factor binding in the transcriptional regulation

of these genes. Taking into account these results, it can be speculated about the molecular mechanisms under the regulation of these molecules. Thus, the bioinformatic analysis of the selected sequence of *IL1B* identified a putative PU.1 (Spi-1) transcription factor binding site, which could be involved in the regulation of the expression of this gene. It is known that PU.1 is a transcription factor that binds to GC-regions of genes to activate transcription, hence DNA methylation might impair the binding of PU.1 to the analysed sequence and downregulate gene transcription. Interestingly, PU.1 transcription factor is involved in macrophage differentiation and also in the transcriptional control of genes in mature macrophages [53, 54].

CONCLUSION

The findings of this study suggest that folic acid supplementation could contribute to ameliorate chronic inflammation, which could be in part mediated by increased DNA methylation in CpG for inflammatory genes. Nevertheless, the mechanisms where folic acid improves the inflammatory response are not totally understood concerning the potential anti-inflammatory effect of this molecule in human macrophages. In any case, folic acid might be a factor for controlling chronic inflammation in inflammatory-related diseases.

CONFLICT OF INTEREST

The authors have nothing to declare.

DATA AVAILABILITY

Access to these data will be considered by the author upon request.

ACKNOWLEDGEMENTS

We thank the technical assistance of Enrique Buso (UCIM, University of Valencia) for the MassArray measurements. We acknowledge the financial support of MINECO (Nutrigenio Project reference AGL2013-45554-R) and Spanish Biomedical Research Centre in Physiopathology of Obesity and Nutrition (CIBERobn). Mirian Samblas holds a FPI grant from the MECD (BES-2014-068409).

TABLE LEGENDS

Table 1. Percentages of CpG methylation in *IL-1β, TNF-α, IL-18* and *SERPINE1* after the incubation with treatments, measured by MassARRAY®. Unpaired t Student's test was used to compare each CpG with its control. * p value < 0.05; ** p value < 0.01; *** p value < 0.001

FIGURE LEGENDS

Figure 1. Comparative effects of folic acid, choline, vitamin B12 and methyl donors' mix on inflammatory genes expression. Results are expressed as means ± SD (n=8). Differences between groups and control were analyzed by one-way ANOVA test for each concentration * p value < 0.05, ** p value < 0.01, *** p value < 0.001 vs control (THP-1 treated with TPA and LPS).

Figure 2. The effect of methyl donors' treatment on cytokine secretion. **A)** IL-1B, **B)** TNF-α, **C)** CD40 and **D)** PAI-1 secretion of THP-1-derived activated macrophages treated with methyl donors' mix for 24 hours. Results are expressed as means ± SD (n=7-8). Differences between groups in relation to control were compared using one-way ANOVA test. * p value < 0.05; ** p value < 0.01.

Figure 3. The relative binding of NF-κB and *IL-1β* and *TNF-α* promoter was compared between folic acid and mehtyl donors' mix with control (THP-1 differentiated with TPA and LPS activated). Results are expressed as means ± SD (n=8). Differences between groups in relation to control were compared using one-way ANOVA test, p value < 0.05..

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primer sequences used for MassArray Epityper assay.

Supplementary Figure 1. Genomic localization and nucleotide sequence of CpGs sites covered by the MassArray Epityper probe for the study of DNA methylation levels of IL-1β, TNF-α, SERPINE1 and IL-18 genes regions. Number of the left of each sequences represents the positions with respect the first nucleotide of the mRNA (start of transcription or TSS). Nucleotides in the box are the sequences selected for each gene. Underlined and highlighted in bold CpGs are the sites that quantified by EpiTYPER. Transcription Start Site (TSS). Coding DNA Sequence (CDS).

Supplementary Figure 2. Relative cell viability after the treatments was measured by MTT assay. THP-1 cells were incubated with folic acid (11.3 µM), choline (105 µM), vitamin B12 (18.5 nM) and methyl donors' mix. Data are shown as the means ± SD (n=6). Differences between groups in relation to control were compared using one-way ANOVA test, p value < 0.05.

338 **Table 1.** Percentages of CpG methylation in IL-1β, TNF-α, IL-18 and SERPINE1 after the
339 incubation with treatments, measured by MassARRAY®. Unpaired t Student's test was used to
340 compare each CpG with its control. * p value < 0.05; ** p value < 0.01; *** p value < 0.001

	Control	Folic acid	Choline	Vitamin B12	Methyl donors' mix
IL-1β					
CpG_1	6.88 ± 3.97	15.5 ± 6.14*	11.4 ± 1.55*	9.38 ± 0.95	14.0 ± 3.11*
CpG_2	94.9 ± 3.14	96.2 ± 1.66	94.8 ± 2.06	9.76 ± 0.85	96.9 ± 0.85
CpG_3	2.88 ± 2.09	1.75 ± 1.26	5.75 ± 1.77	3.75 ± 3.89	3.12 ± 3.75
CpG_4	2.75 ± 2.06	1.88 ± 1.79	1.25 ± 1.19	1.75 ± 0.96	3.75 ± 1.55
CpG_5	2.12 ± 2.49	6.50 ± 4.06*	1.25 ± 0.64	3.25 ± 1.55	10.0 ± 1.13
CpG_6	0.75 ± 0.50	7.12 ± 6.14*	9.50 ± 0.58***	9.25 ± 2.33***	0.88 ± 0.48
TNF-α					
CpG_1	98.2 ± 1.32	96.1 ± 2.78	98.0 ± 1.47	95.2 ± 2.72	95.8 ± 3.07
CpG_2	67.8 ± 3.95	64.8 ± 7.59	65.8 ± 4.48	69.9 ± 1.93	63.4 ± 6.46
CpG_3	48.1 ± 12.6	48.6 ± 8.53	51.2 ± 10.3	47.9 ± 4.71	45.0 ± 4.65
CpG_4.5.6	19.9 ± 6.76	15.2 ± 4.41	17.4 ± 3.09	19.8 ± 0.87	17.8 ± 1.79
CpG_8	33.9 ± 3.49	29.6 ± 4.37	31.8 ± 3.93	34.2 ± 2.53	34.0 ± 3.24
IL-18					
CpG_1	12.5 ± 17.4	2.0 ± 0.5	2.12 ± 0.85*	1.25 ± 1.32	2.5 ± 2.0
CpG_2	6.88 ± 1.55	6.0 ± 1.22	7.38 ± 2.06*	9.50 ± 1.0	10.1 ± 2.25
CpG_3	0.67 ± 0.29	2.0 ± 0.82	0.62 ± 0.25	1.38 ± 0.75	0.88 ± 0.75
CpG_4	11.0 ± 2.04	8.38 ± 4.09*	7.75 ± 2.22	8.0 ± 1.91	7.25 ± 1.85
CpG_5	19.0 ± 3.19	13.2 ± 2.10*	15.8 ± 2.90	16.6 ± 1.11	15.0 ± 2.16
SERPINE1					
CpG_1	2.62 ± 0.63	22.0 ± 13.7**	10.8 ± 14.2	17.0 ± 16.1*	56.2 ± 5.14
CpG_2	33.5 ± 3.39	63.0 ± 12.5***	50.8 ± 7.09**	65.0 ± 5.40***	45.4 ± 8.68*
CpG_3.4	38.5 ± 9.81	91.1 ± 8.23**	79.5 ± 7.99***	85.2 ± 10.9***	62.6 ± 24.0*
CpG_6	100 ± 0.00	89.4 ± 4.09	90.9 ± 11.3	87.1 ± 10.2	96.6 ± 5.49
CpG_7	2.88 ± 1.60	32.1 ± 15.4**	13.9 ± 7.97*	28.2 ± 23.1*	15.8 ± 15.8*
CpG_8	95.1 ± 2.62	91.5 ± 6.77	92.6 ± 6.74	93.1 ± 2.66	94.9 ± 2.06
CpG_9	33.5 ± 3.39	63.0 ± 12.5**	50.8 ± 7.09**	65.0 ± 5.40***	45.4 ± 8.68
CpG_10	97.2 ± 2.59	95.0 ± 3.03	96.6 ± 1.60	88.5 ± 19.7	96.9 ± 2.46
CpG_11	97.5 ± 2.91	98.2 ± 2.36	97.1 ± 2.69	98.1 ± 0.75	98.5 ± 2.68
CpG_12	94.0 ± 7.22	95.0 ± 2.42	94.2. ± 5.52	96.0 ± 3.58	92.4 ± 6.26

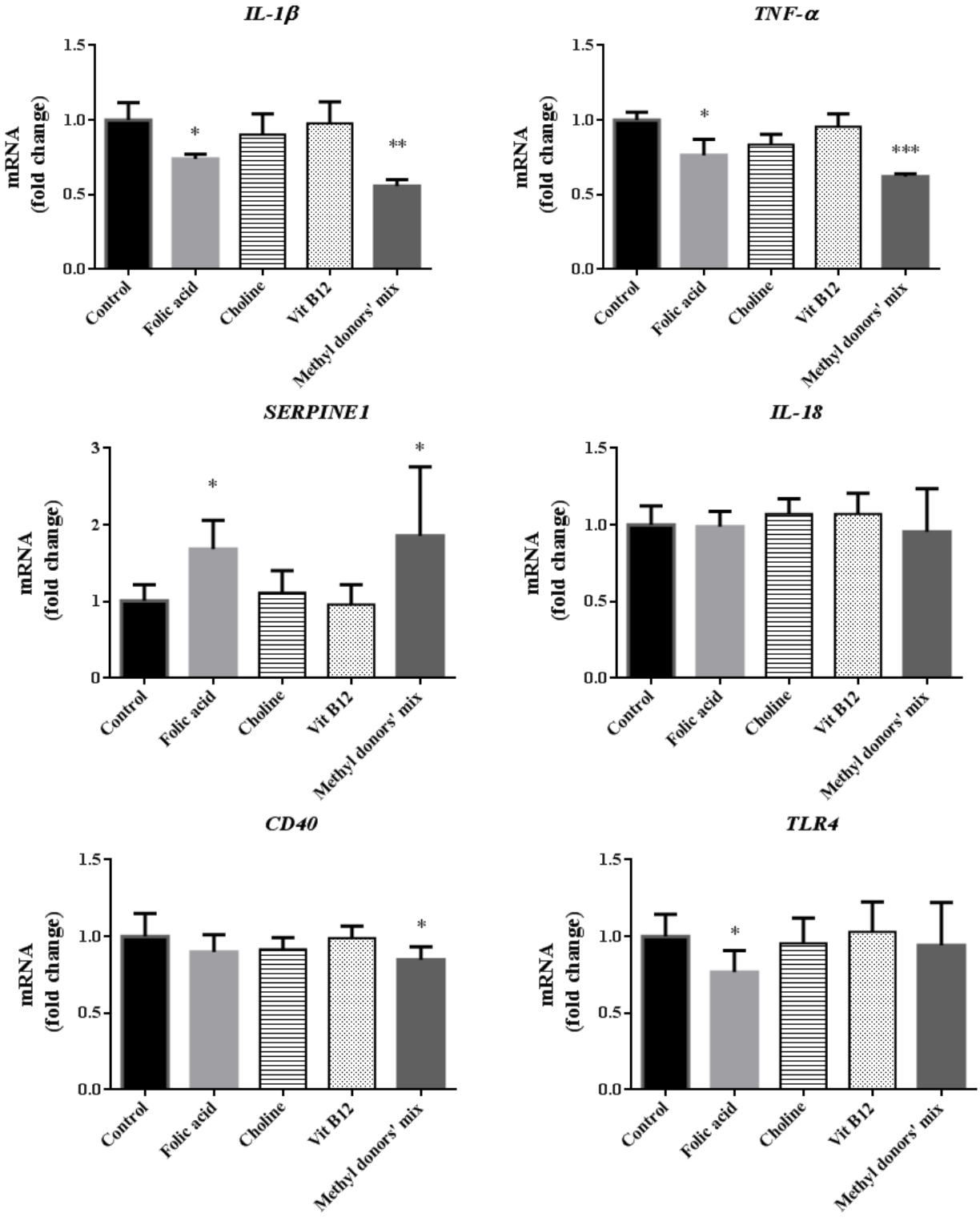


Figure 1. Comparative effects of folic acid, choline, vitamin B12 and methyl donors' mix on inflammatory genes expression. Results are expressed as means ± SD (n=8). Differences between groups and control were analyzed by one-way ANOVA test for each concentration * p value < 0.05, ** p value < 0.01, *** p value < 0.001 vs control (THP-1 treated with TPA and LPS).

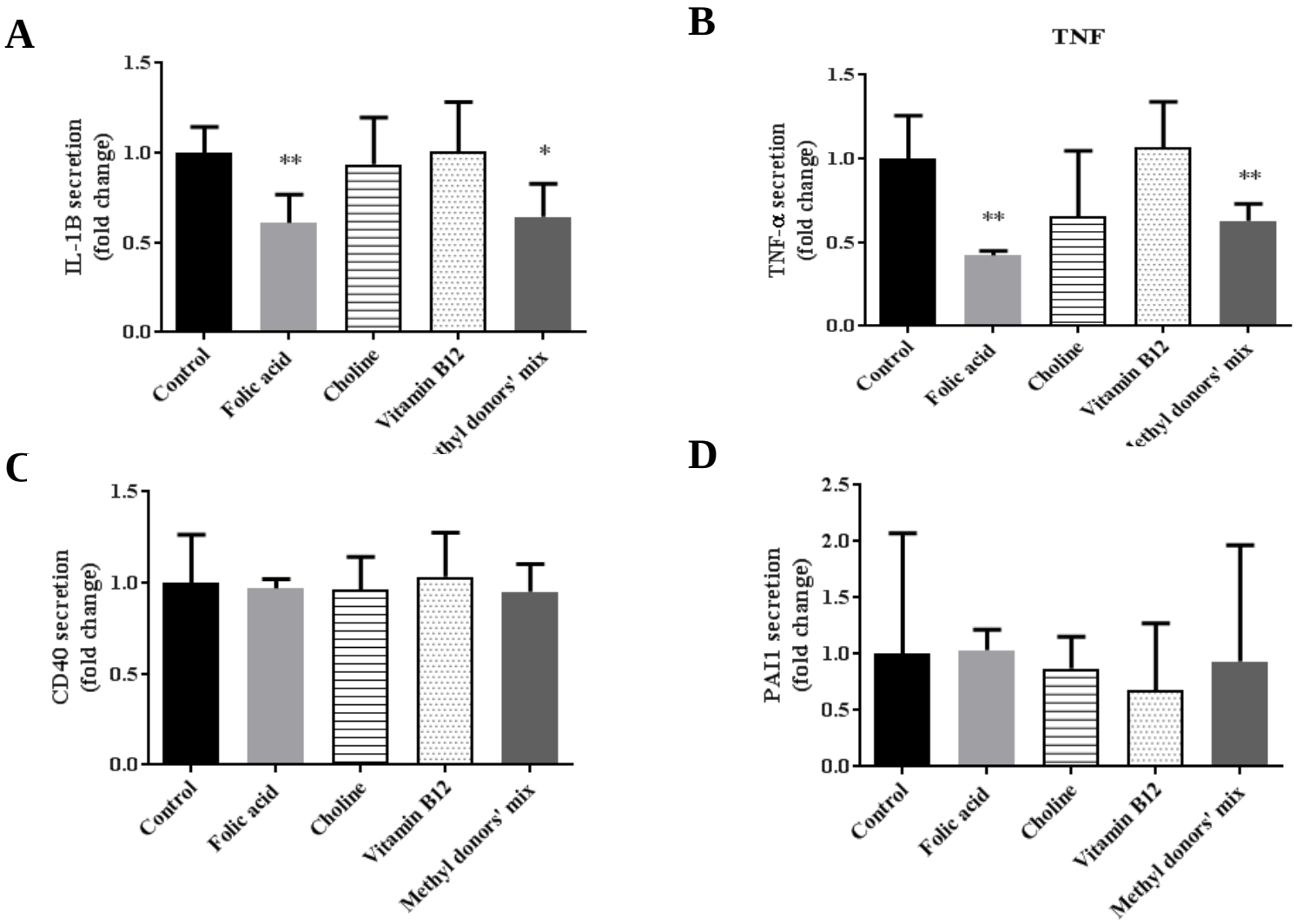


Figure 2. The effect of methyl donors' treatment on cytokine secretion. **A)** IL-1B, **B)** TNF-α, **C)** CD40 and **D)** PAI-1 secretion of THP-1-derived activated macrophages treated with methyl donors' mix for 24 hours. Results are expressed as means ± SD (n=7-8). Differences between groups in relation to control were compared using one-way ANOVA test. * p value < 0.05; ** p value < 0.01.

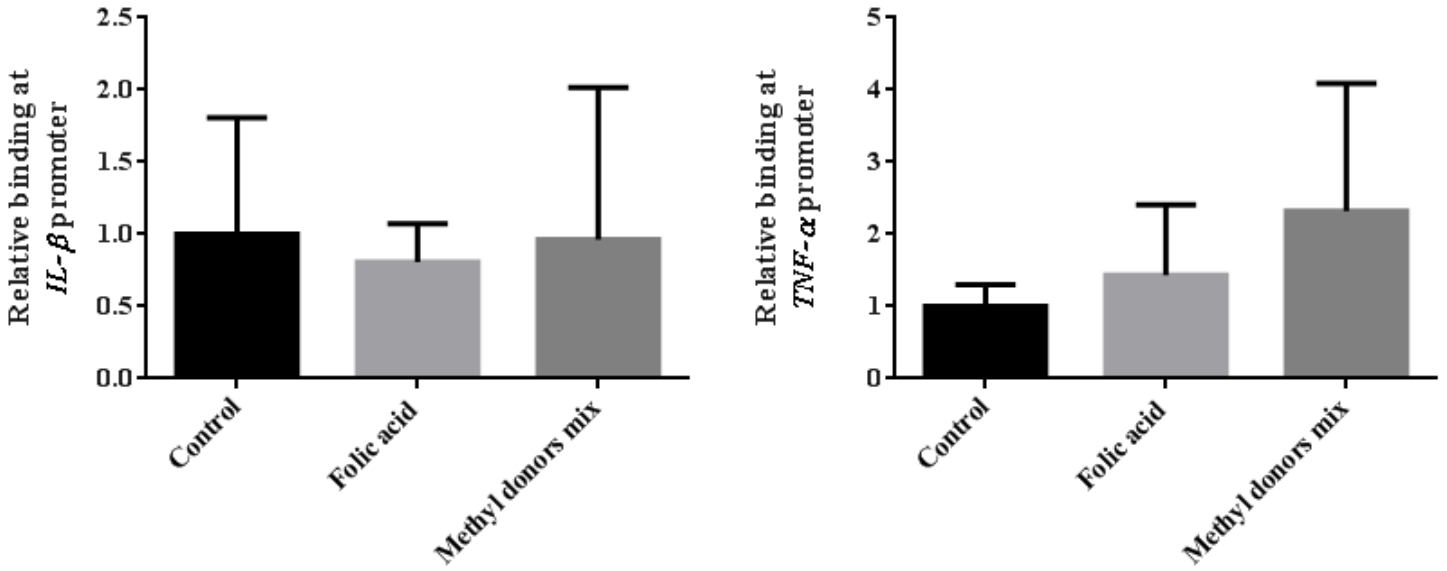


Figure 3. The relative binding of NF-κB and *IL-1β* and *TNF-α* promoter was compared between folic acid and mehtyl donors' mix with control (THP-1 differentiated with TPA and LPS activated). Results are expressed as means ± SD (n=8). Differences between groups in relation to control were compared using one-way ANOVA test, p value < 0.05..

REFERENCES

1. Larsen GL, Henson PM (1983) Mediators of inflammation. Annu Rev Immunol 1:335–59 . doi: 10.1146/annurev.iy.01.040183.002003
2. Solas M, Milagro FI, Ramírez MJ, Martínez JA (2017) Inflammation and gut-brain axis link obesity to cognitive dysfunction: plausible pharmacological interventions. Curr Opin Pharmacol 37:87–92 . doi: 10.1016/j.coph.2017.10.005
3. Massey HD, McPherson RA (2011) Mediators of Inflammation
4. Shi C, Pamer EG (2014) Monocyte Recruitment Suring Infection and Inflammation. Nat Rev Immunol 11:762–774 . doi: 10.1038/nri3070.Monocyte
5. Shi C, Pamer EG (2014) Monocyte Recruitment Suring Infection and Inflammation. Nat Rev Immunol 11:762–774 . doi: 10.1038/nri3070.Monocyte
6. Westerbacka J, Kolak M, Kiviluoto T, et al (2007) Genes involved in fatty acid partitioning and binding, lipolysis, monocyte/macrophage recruitment, and inflammation are overexpressed in the human fatty liver of insulin-resistant subjects. Diabetes 56:2759–2765 . doi: 10.2337/db07-0156
7. Engin AB (2017) Adipocyte-macrophage cross-talk in obesity. In: Advances in Experimental Medicine and Biology. Springer, Cham, pp 327–343
8. Seo JW, Yang EJ, Yoo KH, Choi IH (2015) Macrophage differentiation from monocytes is influenced by the lipid oxidation degree of low density lipoprotein. Mediators Inflamm 2015:1–10 . doi: 10.1155/2015/235797
9. Simon TC, Jeffries MA (2017) The Epigenomic Landscape in Osteoarthritis. Curr Rheumatol Rep 19:30 . doi: 10.1007/s11926-017-0661-9
10. Tost J (2010) DNA Methylation: An Introduction to the Biology and the Disease-Associated Changes of a Promising Biomarker. Mol Biotechnol 44:71–81 . doi: 10.1007/s12033-009-9216-2
11. Fogel O, Richard-Miceli C, Tost J (2017) Epigenetic Changes in Chronic Inflammatory Diseases. In: Advances in Protein Chemistry and Structural Biology. Academic Press, pp 139–189
12. Marques-Rocha JL, Samblas M, Milagro FI, et al (2015) Noncoding RNAs, cytokines, and inflammation-related diseases. FASEB J 29:3595–611 . doi: 10.1096/fj.14-260323
13. Zhang N (2015) Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. Anim. Nutr. 1:144–151
14. Amaral CL do, Milagro FI, Curi R, Martínez JA (2014) DNA Methylation Pattern in Overweight Women under an Energy-Restricted Diet Supplemented with Fish Oil. Biomed Res Int 2014:1–10 . doi: 10.1155/2014/675021
15. Cordero P, Milagro FI, Campion J, Martinez JA (2013) Maternal methyl donors supplementation during lactation prevents the hyperhomocysteinemia induced by a high-fat-sucrose intake by dams. Int J Mol Sci 14:24422–24437 . doi: 10.3390/ijms141224422 [doi]
16. Burdge GC, Lillycrop KA (2014) Fatty acids and epigenetics. Curr Opin Clin Nutr Metab Care 17:156–161 . doi: 10.1097/MCO.0000000000000023 [doi]
17. Hoile SP, Clarke-Harris R, Huang RC, et al (2014) Supplementation with N-3 long-chain polyunsaturated fatty acids or olive oil in men and women with renal disease induces differential changes in the DNA methylation of FADS2 and ELOVL5 in peripheral blood mononuclear cells.

PLoS One 9:e109896 . doi: 10.1371/journal.pone.0109896 [doi]
18. Shorter KR, Felder MR, Vrana PB (2015) Consequences of dietary methyl donor supplements: Is more always better? Prog. Biophys. Mol. Biol. 118:14–20
19. Liew S-C, Liew S-C (2016) Folic acid and diseases - supplement it or not? Rev Assoc Med Bras 62:90–100 . doi: 10.1590/1806-9282.62.01.90
20. Tian H, Tian D, Zhang C, et al (2017) Efficacy of Folic Acid Therapy in Patients with Hyperhomocysteinemia. J Am Coll Nutr 36:528–532 . doi: 10.1080/07315724.2017.1330162
21. Cianciulli A, Salvatore R, Porro C, et al (2016) Folic Acid Is Able to Polarize the Inflammatory Response in LPS Activated Microglia by Regulating Multiple Signaling Pathways. Mediators Inflamm 2016:1–10 . doi: 10.1155/2016/5240127
22. Guerrerio AL, Colvin RM, Schwartz AK, et al (2012) Choline intake in a large cohort of patients with nonalcoholic fatty liver disease. Am J Clin Nutr 95:892–900 . doi: 10.3945/ajcn.111.020156
23. Buchman AL, Dubin MD, Moukarzel AA, et al (1995) Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. Hepatology 22:1399–403
24. Al-Daghri NM, Rahman S, Sabico S, et al (2016) Association of vitamin B12 with pro-inflammatory cytokines and biochemical markers related to cardiometabolic risk in Saudi subjects. Nutrients 8: . doi: 10.3390/nu8090460
25. Li Z, Gueant-rodriguez R, Quilliot D (2017) Folate and vitamin B12 status is associated with insulin resistance and metabolic syndrome in morbid obesity. Clin Nutr 1–7 . doi: 10.1016/j.clnu.2017.07.008
26. Milagro FI, Campion J, Cordero P, et al (2011) A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J 25:1378–1389 . doi: 10.1096/fj.10-170365 [doi]
27. Lee C, Huang C-H (2014) LASAGNA-Search 2.0: integrated transcription factor binding site search and visualization in a browser. Bioinformatics 30:1923–1925 . doi: 10.1093/bioinformatics/btu115
28. JIANG Y, MA S, ZHANG H, et al (2016) FABP4-mediated homocysteine-induced cholesterol accumulation in THP-1 monocyte-derived macrophages and the potential epigenetic mechanism. Mol Med Rep 14:969–976 . doi: 10.3892/mmr.2016.5315
29. Feng D, Zhou Y, Xia M, Ma J (2011) Folic acid inhibits lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and NF-κB activation. Inflamm Res 60:817–822 . doi: 10.1007/s00011-011-0337-2
30. Cianciulli A, Salvatore R, Porro C, et al (2016) Folic Acid Is Able to Polarize the Inflammatory Response in LPS Activated Microglia by Regulating Multiple Signaling Pathways. Mediators Inflamm 2016:5240127 . doi: 10.1155/2016/5240127
31. Solini A, Santini E, Ferrannini E (2006) Effect of short-term folic acid supplementation on insulin sensitivity and inflammatory markers in overweight subjects. Int J Obes (Lond) 30:1197–202 . doi: 10.1038/sj.ijo.0803265
32. Wang G, Dai J, Mao J, et al (2005) Folic acid reverses hyper-responsiveness of LPS-induced

chemokine secretion from monocytes in patients with hyperhomocysteinemia. Atherosclerosis 179:395–402 . doi: 10.1016/j.atherosclerosis.2004.10.033

33. Feng D, Zhou Y, Xia M, Ma J (2011) Folic acid inhibits lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and NF-??B activation. Inflamm Res 60:817–822 . doi: 10.1007/s00011-011-0337-2

34. Kolb AF, Petrie L (2013) Folate deficiency enhances the inflammatory response of macrophages. Mol Immunol 54:164–172 . doi: 10.1016/j.molimm.2012.11.012

35. Yang J, Zhang L, Yu C, et al (2014) Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res 2:1 . doi: 10.1186/2050-7771-2-1

36. Calder PC, Albers R, Antoine J-M, et al (2009) Inflammatory Disease Processes and Interactions with Nutrition. Br J Nutr 101:1 . doi: 10.1017/S0007114509377867

37. Anderson OS, Sant KE, Dolinoy DC (2012) Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. J. Nutr. Biochem. 23:853–859

38. Martínez JA, Cordero P, Campión J, Milagro FI (2012) Interplay of early-life nutritional programming on obesity, inflammation and epigenetic outcomes. Proc Nutr Soc 71:276–283 . doi: 10.1017/S0029665112000055

39. Cordero P, Campion J, Milagro FI, Martinez JA (2013) Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: Effect of dietary methyl donor supplementation. Mol Genet Metab 110:388–395 . doi: 10.1016/j.ymgme.2013.08.022

40. Zhang G, Pradhan S (2014) Mammalian epigenetic mechanisms. IUBMB Life 66:240–256

41. Deaton AM, Bird A (2011) CpG islands and the regulation of transcription. Genes Dev 25:1010–1022 . doi: 10.1101/gad.2037511

42. Liu L, Ke Y, Jiang X, et al (2012) Lipopolysaccharide activates ERK-PARP-1-RelA pathway and promotes nuclear factor-kB transcription in murine macrophages. Hum Immunol 73:439–447 . doi: 10.1016/j.humimm.2012.02.002

43. Pan J-X, Qu F, Wang F-F, et al (2017) Aberrant SERPINE1 DNA methylation is involved in carboplatin induced epithelial-mesenchymal transition in epithelial ovarian cancer. Arch Gynecol Obstet 296:1145–1152 . doi: 10.1007/s00404-017-4547-x

44. Rusiecki JA, Byrne C, Galdzicki Z, et al (2013) PTSD and DNA methylation in select immune function gene promoter regions: A repeated measures case-control study of U.S. military service members. Front Psychiatry 4:56 . doi: 10.3389/fpsyt.2013.00056

45. Ara AI, Xia M, Ramani K, et al (2008) S-adenosylmethionine inhibits lipopolysaccharide-induced gene expression via modulation of histone methylation. Hepatology 47:1655–1666 . doi: 10.1002/hep.22231

46. Liu T, Zhang L, Joo D, Sun S-C (2017) NF-κB signaling in inflammation. Signal Transduct Target Ther 2:17023 . doi: 10.1038/sigtrans.2017.23

47. Abd allah ESH, Badary DM (2017) Folic acid protects against lead acetate-induced hepatotoxicity by decreasing NF-κB, IL-1β production and lipid peroxidation mediataed cell injury. Pathophysiology 24:39–44 . doi: 10.1016/j.pathophys.2017.02.002

48. Sultana F, Rasool M (2015) A novel therapeutic approach targeting rheumatoid arthritis by combined administration of morin, a dietary flavanol and non-steroidal anti-inflammatory drug indomethacin with reference to pro-inflammatory cytokines, inflammatory enzymes, RANKL and transcr. Chem Biol Interact 230:58–70 . doi: 10.1016/j.cbi.2015.02.007

49. Collart MA, Baeuerle P, Vassalli P (1990) Regulation of tumor necrosis factor alpha transcription in macrophages: involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. Mol Cell Biol 10:1498–506 . doi: 10.1128/MCB.10.4.1498

50. Shakhov AN, Collart MA, Vassalli P, et al (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. J Exp Med 171:35–47 . doi: 10.1084/jem.171.1.35

51. Hiscott J, Marois J, Garoufalis J, et al (1993) Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. Mol Cell Biol 13:6231–6240 . doi: 10.1128/MCB.13.10.6231

52. Sun S-C, Chang J-H, Jin J (2013) Regulation of nuclear factor-κB in autoimmunity. Trends Immunol 34:282–289 . doi: 10.1016/j.it.2013.01.004

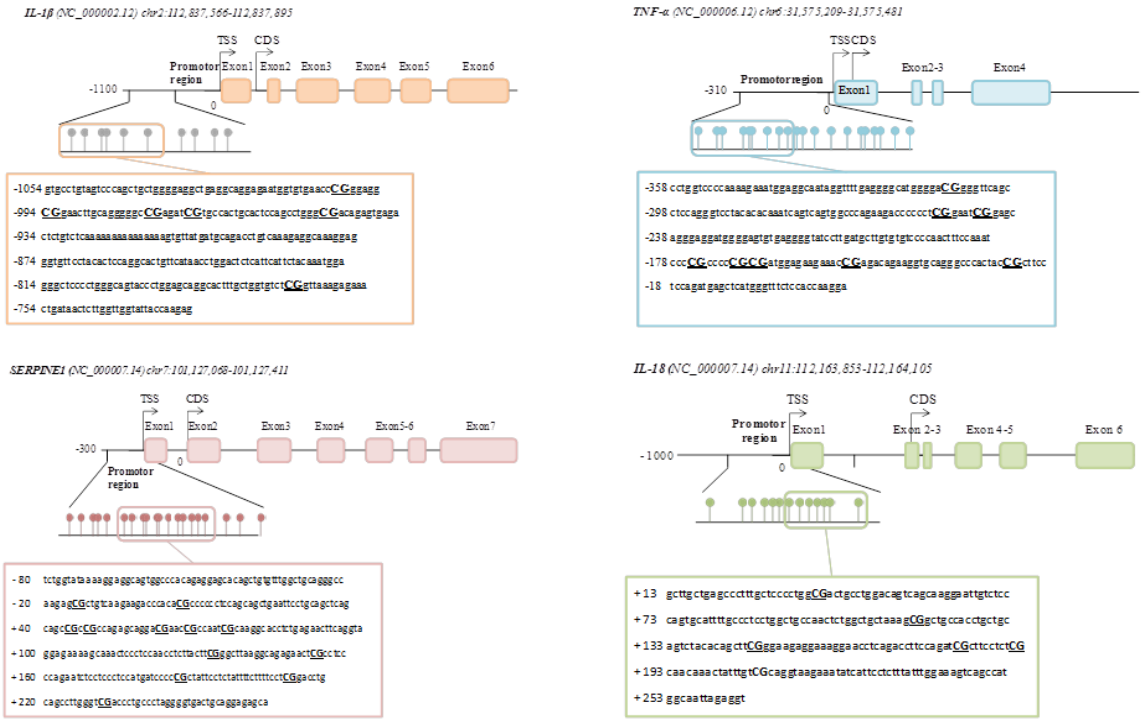
53. Ghisletti S, Barozzi I, Mietton F, et al (2010) Identification and Characterization of Enhancers Controlling the Inflammatory Gene Expression Program in Macrophages. Immunity 32:317–328 . doi: 10.1016/j.immuni.2010.02.008

54. Yuan Z, Syed MA, Panchal D, et al (2012) Curcumin mediated epigenetic modulation inhibits TREM-1 expression in response to lipopolysaccharide. Int J Biochem Cell Biol 44:2032–2043 . doi: 10.1016/j.biocel.2012.08.001

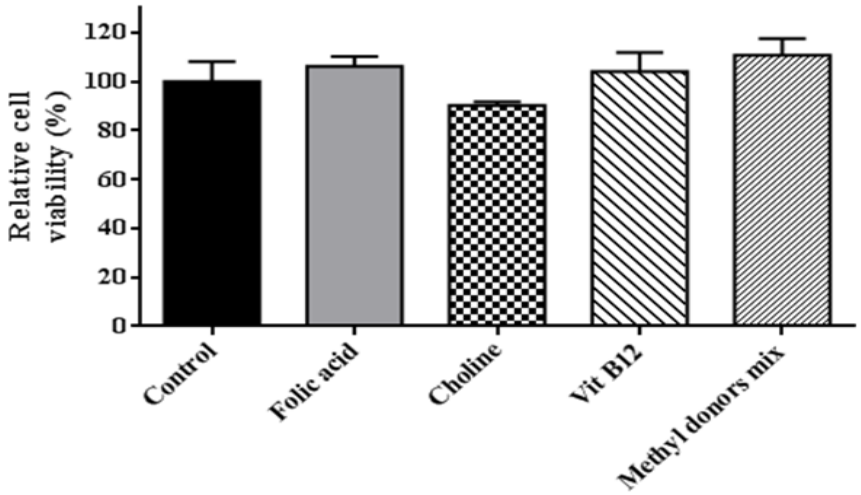
Supplementary data CHAPTER 5

Supplementary table 1. Primer sequences used for MassArray Epityper assay.

Gene	Primer	Sequence	Length (pb)	CpG covered
IL-1β	left	5'-gtgtttgtagttttagttgttggg-3'	331	6
	right	3'-tctcttaataataccaaccaaaaattatca-5'		
TNF-α	left	5'-tttggtttttaaaagaaatggaggt-3'	273	8
	right	3'-tccttaataaaaaaacccataaactca-5'		
SERPINE1	left	5'-tttggtataaaaggaggtagtggtt-3'	343	12
	right	3'-actctcctacaatcacccctaaaac-5'		
IL18	left	5'-tttgttgagtttttgttttttgg-3'	251	6
	right	5'-cctctaattaccataactaactttcca-3'		



Supplementary figure 1. Genomic localization and nucleotide sequence of CpGs sites covered by the MassArray Epityper probe for the study of DNA methylation levels of *IL-1β*, *TNF-α*, *SERPINE1* and *IL-18* genes regions. Number of the left of each sequences represents the positions with respect the first nucleotide of the mRNA (start of transcription or TSS). Nucleotides in the box are the sequences selected for each gene. Underlined and highlighted in bold CpGs are the sites that quantified by EpiTYPER. Transcription Start Site (TSS). Coding DNA Sequence (CDS).



Supplementary Figure 2. Relative cell viability after the treatments was measured by MTT assay. THP-1 cells were incubated with folic acid (11.3 µM), choline (105 µM), vitamin B12 (18.5 nM) and methyl donors' mix. Data are shown as the means ± SD (n=6). Differences between groups in relation to control were compared using one-way ANOVA test, p value < 0.05.

GENERAL DISCUSSION

1. Epigenetic markers in pediatric obesity (case-control study)

Obesity is defined as an excessive fat accumulation resulting from a dysregulation between energy intake and expenditure (Chatzigeorgiou et al., 2014). Obesity is highly prevalent worldwide and is considered the pandemic of the current century, where genetics and lifestyle factors are involved (González-Muniesa et al., 2017). Most importantly, the prevalence has dramatically increased among children in the last years (Abdeen et al., 2017). Therefore, treating obesity in childhood is critical to prevent adult obesity and obesity-related complications. In this context, epigenetics is one of the factors that can be involved in the increased prevalence of obesity and accompanying comorbidities, being influenced by some of the dietary and lifestyle factors that are commonly associated with obesity risk (Milagro et al., 2013). In addition, obesity and its comorbidities (hyperglycemia, hyperlipidemia, hypertension, inflammation…) can also modify the DNA methylation levels (Demerath et al., 2015).

In this sense, the research in children populations enables us to study the development of obesity at the first stages of the process, in a population who does not usually present obesity-related complications. Although some candidate gene-specific methylation studies have been carried out in children, very few EWAS analyses in pediatric cohorts have been published (reviewed by Rzehak et al., 2017). Trying to shed more light on this issue, the first objective of this work investigates the putative associations between DNA methylation profiles and childhood obesity. Thus, a genome-wide DNA methylation analysis identified 734 sites differently methylated in obese children when compared to lean ones. As other authors have previously reported (Faienza et al, 2016), the present study revealed that epigenetic modifications at different stages of early-life may be linked to the onset of obesity. In addition, most of the CpGs were located in the gene body region, and children with obesity presented higher methylation levels in these sites but lower methylation in promoter loci. In contrast with these results, lower methylation in the gene body regions in children with obese phenotype (Rhee et al., 2017) and higher methylation in gene promoters (de Mello et al., 2014) have been previously described. Interestingly, DNA methylation fluctuations in significant sites occurred in CpG-rich regions, and increased with obesity. One of the strongest associations was found between *PTPRS* gene

methylation and obesity. A higher BMI z-score correlated with lower methylation levels of *PTPRS*. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family and regulates a variety of cellular processes such as cell division, growth, differentiation, and oncogenic transformation (Denu & Dixon, 1998). Studies in animals have evidenced that this gene is highly expressed during mammalian embryonic development and regulates hematopoietic stem cell (Elchebly et al., 1999; Quarmyne et al., 2015). The methylation machinery is very dynamic during the embryogenesis period and numerous studies have described that some epigenetic alterations that occur during this stage remain in adulthood (Li et al., 2010). A recent investigation in mice described that exposure to HFD during pregnancy was associated with genome-wide DNA methylation alterations and long-term gene expression changes in the liver, and the development of MetS in the offspring (Seki et al., 2017).

Pathway analysis results indicated that the genes whose CpGs presented significant methylation differences between obese and non-obese children were involved in important biological processes such as oxidative stress, which has been associated with obesity, diabetes and other metabolic disorders (Gharib et al., 2016), and circadian rhythm signalling. Specifically, the methylation status of the circadian genes vasoactive intestinal peptide receptor 2 (*VIPR2*), glutamate ionotropic receptor NMDA type subunit 2D (*GRIN2D*), pituitary adenylate cyclase-activating polypeptide type I receptor (*ADCYAP1R1*) and *PER3* was associated with BMI z-score. Moreover, the current research confirmed a negative correlation between *PER3* methylation and childhood obesity, concluding that children with higher methylation levels of this gene exhibited lower degree of obesity. *PER3* is one of the three *PER* family genes in humans, which encodes important components of the circadian rhythm machinery that have been associated with locomotor activity, behaviour, and metabolism. Interestingly, *PER3* expression levels in PBMCs have been previously related to visceral adiposity (Kim et al., 2017). These findings suggest a relationship between obesity in children and the methylation pattern of *PTPRS* and *PER3* genes. It is noteworthy that obese children are predisposed to suffer obesity in adulthood and to develop severe comorbidities (Lipsky et al., 2017). There is also increasing evidence that DNA methylation alterations during childhood are determinant in the development of obesity in adulthood (Wahl et al., 2016). However, the results of the current study are not able to demonstrate if

the differences in DNA methylation between obese and non-obese children are a cause or a consequence of obesity. To correctly answer this question, it would be necessary to study the methylation pattern of the same individuals before and after developing obesity. For example, a recent genome-wide DNA methylation analysis from 478 children in cord blood identified novel CpG loci associated with adiposity outcomes at 3 and 8 years old (Kresovich et al., 2017). In this context, it may be particularly interesting to measure the methylation levels in DNA from cord blood or placenta. In addition, studies in growth-restricted neonates (Hillman et al., 2015; Côté et al., 2016) and children of women suffering from gestational diabetes (Finer et al., 2015), have been able to identify genome-wide DNA methylation variations that may have a mechanistic role in metabolic disease programming through interaction of the pregnancy environment with gene function.

As pointed out by previous results, epigenetic mechanisms affecting genes of the circadian clock system, which participates in the regulation of energy homeostasis and metabolism, seem to play a relevant role in the pathogenesis of obesity and MetS (Lopez-Minguez et al., 2016). These data support previous investigations, which reported that the methylation of the circadian-related genes *CLOCK*, *PER2* and *BMAL1* was associated with BMI, body fat, WC, HOMA, and MetS features in adult overweight and obese subjects (Milagro et al., 2012). In a similar way, *CRY1* hypermethylation has been found in adipose tissue of healthy subjects after an acute total sleep deprivation and was associated with metabolic dysregulation (Cedernaes et al., 2015), confirming that disruptions in the circadian rhythm can alter the epigenetic and transcriptional profile of core clock genes in key metabolic tissues. These findings underline the importance of epigenetic mechanisms, particularly DNA methylation, in circadian clock regulation in relation to the control of metabolic processes in peripheral tissues, body weight and adiposity.

2. Epigenetic markers in weight loss (intervention studies)

Obese children are predisposed to become obese in adulthood. Moreover, the subjects who maintain obese phenotype over time present higher risk for developing severe comorbidities such as MetS, inflammation or T2D in the future. Thus, a number of strategies have been investigated not only to induce a negative energy balance and weight loss (Biddle et al., 2017), but also to improve the health

status of the subject. Nevertheless, individual responses to body weight loss interventions may vary widely (Bouchard et al., 2010). For this reason, numerous studies have aimed to identify novel predictors of this variability that may be the basis for a precision management of obesity and its comorbidities (Rudkowska et al., 2015; Tremblay et al., 2015; Marcotte et al., 2016). In this context, previous publications from our group have demonstrated that epigenetic markers are associated with adiposity, metabolism, inflammation, appetite and weight loss (Milagro et al., 2011; Cordero et al., 2013a; Goni et al., 2014; Carraro et al., 2016; Ramos-Lopez et al., 2018). With the final objective of identifying new biomarkers that could be useful in the personalization of the weight loss strategies, an aim of the present research has been to deepen into the association of DNA methylation marks in white blood cells, analyzed by different techniques, and body weight loss after different dietary interventions.

Given that circadian clock system is involved in energy metabolism and weight control at different periods of life , the study of circadian system genes is of great interest (Garaulet et al., 2010). In this sense, we investigated the association between the methylation levels of *BMAL1*, *CLOCK* and *NR1D1* genes and the outcomes of a weight-loss nutritional intervention. The epigenome may be altered by environmental factors, including dietary intake (Milagro et al., 2013). Indeed, different dietary compounds, such as methyl donors (Cordero et al., 2013b), FAs (Karimi et al., 2017), and polyphenols (Boqué et al., 2013; Remely et al., 2015), may modify epigenetic marks. For this reason, this work also focused on the association between diet composition (nutrients and calorie intake) and the DNA methylation levels of the core clock genes, specifically *BMAL1* and *NR1D1*. The weight loss intervention was based on a restricted Mediterranean dietary pattern, and previous studies have found that Mediterranean diet-based interventions were able to modify epigenetic marks in blood cells (Lopez-Legarrea et al., 2013; Arpón et al., 2018). In the present study, higher energy and carbohydrate intakes were associated with higher methylation levels in the CpG 5 to 9 region of *BMAL1*. Interestingly, these findings suggest that energy content and the type of macronutrients might modulate the synchronization between individual clock and biological functions. Interestingly, previous investigations have demonstrated the association between several nutrients and the circadian clock rhythms by altering the expression of clock genes (Froy, 2007; Garaulet et al., 2009). Also,

several polymorphisms in clock genes have been reported to be associated with differential effectiveness of weight-loss interventions depending on nutrient composition (Garaulet et al., 2011; Garcia-Rios et al., 2014; Dashti et al., 2015).

On the other hand, the genome methylation levels have been associated with weight loss outcomes after a low calorie diet in different populations (Cordero et al., 2011b; Crujeiras et al., 2013; Milagro et al., 2011). In relation to methylation of clock genes, our group has previously demonstrated an association between the methylation status of some clock genes (*CLOCK* and *PER2*) and some parameters related to MetS (Milagro et al., 2012). An important result of the current research was to feature an association between the baseline methylation profile of *BMAL1* and the changes in the serum lipid profile induced by the weight loss treatment. Moreover, positive correlations were found between changes in methylation levels in the CpG 5 to 9 region of *BMAL1* due to the intervention and changes in serum lipids. These results allow to hypothesize about a possible causality between DNA methylation and lipid metabolism. *BMAL1* encodes a transcription factor that creates a complex with CLOCK (the CLOCK-BMAL1 heterodimer) and plays a key role as one of the positive elements in the mammalian transcription and translation regulation (Froy, 2007). *BMAL1* has been identified as a candidate gene for the development of hypertension, infertility, diabetes, and other glucose metabolism alterations (Pappa et al., 2013; Richards et al., 2014). In addition, *BMAL1* gene activity has been related to impairments in adipogenesis, lipogenesis and other lipid metabolism processes (Froy, 2012). Moreover, the mRNA levels of this gene are increased during hyperlipidemic and hyperglycaemic periods in obesity and may regulate other genes involved in metabolic processes (Tahira et al., 2011). These results support our finding that the methylation changes of *BMAL1* were associated with the habitual energy and carbohydrate intake, and blood lipid levels. It is noteworthy that these results provide evidence that an energy-restricted intervention based on the Mediterranean diet is able to modify the methylation status of *BMAL1* gene in white blood cells. It should be interesting to carry out studies in other tissues (i.e., liver, adipose tissue, skeletal muscle, pancreas...) to confirm if these epigenetic changes induced by the diet could affect the expression of genes and the function of these important metabolic organs. Unfortunately, we did not have samples from these tissues.

In the last years, microarray technology and EWAS have allowed the discovery of candidate genes for explaining the inter-individual differences in the response to weight loss strategies (Wahl et al., 2016). Accordingly to the previous association reported on DNA methylation changes within weight loss intervention, and using the "omics" technologies, we also analysed the differential transcription and DNA methylation profiles of the RESMENA cohort in relation to weight loss response. The transcriptomic analysis pointed out 156 differentially expressed transcripts between subjects who responded successfully to the diet (high responders, HR) and subjects who responded poorly (low responders, LR). These results are consistent with previous studies in blood cells where expression levels of several genes at baseline helped to predict changes in BMI and weight after a nutritional intervention (Rendo-Urteaga et al., 2015; Armenise et al., 2017). Interestingly, in the RESMENA cohort, the methylation array analysis found 2,102 CpGs that were differentially methylated before the energy-restriction diet between LR and HR. Although neither the treatments nor the characteristics of the populations were similar to the RESMENA study, we did not find genes coincident when comparing these different studies.

On the other hand, this is not the first study using methylation-array based tools for the identification of novel markers of age-related diseases or obesity (Gómez-Úriz et al., 2015) Other studies have used alternative technologies, such as pyrosequencing (Aumueller et al., 2015) or global DNA methylation and hydroxymethylation (Nicoletti et al., 2016) for the same purpose. Similar to our study, the methylation levels of different genomic regions have been assessed in relation to the individual response to different weight loss nutritional interventions (Bouchard et al., 2010; Milagro et al., 2011; Moleres et al., 2013). DNA methylation has been associated with transcriptional regulation; while DNA methylation in gene promoters has been usually associated with transcriptional repression, body gene methylation is more often associated with gene expression activation (Zhang & Pradhan, 2014). LR subjects exhibited DNA hypomethylation and over-expression of *CD44*, *ITPR1* and *MTSS1* genes, and, in contrast, *FBXW5* was hypomethylated and under-expressed. Specifically, two CpGs located in *CD44* presented lower methylation and higher expression levels in LR comparing with HR. This result could be related with a previous study that described an association between methylation of *CD44*,

which encodes an immune-cell surface receptor (Kodama et al., 2012), and changes in WC, BMI and fat mass after a weight loss treatment (Milagro et al., 2011).

The excessive fat accumulation and chronic low-grade inflammation characteristic of obesity are an underlying principle in the development of numerous obesity comorbidities and MetS, including dyslipidemia, IR, and hypertension (Guh et al., 2009). Physiologically, adipose tissue hosts resident leukocytes (mostly macrophages) that participate in the response to nutritional signals. The macrophage recruitment in visceral adipose tissue is necessary for controlling the flow of lipids and inhibiting lipolysis (Dali-Youcef et al., 2013). During obesity, excess fat accumulation and cholesterol dysregulation may induce adipocytes to produce pro-inflammatory molecules and cytokines (such as NOS2, TNF-α, MCP-1, VCAM-1, ICAM-1, and interleukins IL-12, IL-6 and IL-1β) which further propagate inflammation (Kirwan et al., 2017). In this sense, the expression of *CD44* has been associated with macrophage accumulation in morbid obesity and liver steatosis, whereas its expression decreases after a massive weight loss (Bertola et al., 2009). In addition, *CD44* is implicated in the development of inflammation in adipose tissue and IR (Liu et al., 2015), having been suggested as a biomarker for IR and putative therapeutic target for T2D (Kodama et al., 2012). Regarding these results and our current data available, it can be speculated that *CD44* could have an important role in the inflammatory process in obese subjects, and the DNA hypermethylation and therefore lower expression of *CD44* at baseline may improve the response to an energy-restricted diet.

3. Effect of methyl donors on epigenetic marks and inflammation

The dietary strategy followed by the RESMENA study was based on the Mediterranean diet. Previous studies of our group have associated Mediterranean dietary patterns with lower prevalence and incidence of MetS and amelioration of obesity-related inflammation, and can be a useful tool for obesity management (de la Iglesia et al., 2014). The term "Mediterranean diet" is applied to a spectrum of diverse dietary patterns that contains extra virgin olive oil as a major source of fat, but also includes high consumption of vegetables, fruits, legumes, cereals, and fish (Galland, 2010). Mediterranean diet represents an important source of methyl donors, like folate and vitamin B_{12}, which are essential cofactors in the methionine/homocysteine cycle. However, the increasing consumption of

HFDs and western dietary patterns explains a reduction in the methyl donors intake in Mediterranean countries (Samaniego-Vaesken et al., 2017). In this context, the prevalence of micronutrient deficiency caused by unbalanced diets, including folate deprivation, constitutes an important aspect in the high prevalence of overweight and obesity in the population (Hwalla et al., 2017). For example, different observational studies have previously associated low folate intake with overweight and obesity (Tungtrongchitr et al., 2003; Mahabir et al., 2008; J. K. Bird et al., 2015). In the current trial, half of the studied RESMENA sample presented low consumption of folate according to the Spanish reference intake tables ("Ingestas Dietéticas de Referencia (IDR) para la Población Española" 2010). Interestingly, as in previous studies, individuals with lower folate intake presented higher body adiposity compared with those who consumed high folate.

In agreement with these results concerning folate intake, negative associations between serum folate concentrations and BMI and fat mass have been previously reported in both pediatric (Gunanti et al., 2014) and adult (Mahabir et al., 2008; Bradbury et al., 2014; Bird et al., 2015) populations. Moreover, the subjects with low folate levels exhibited metabolic alterations characterized by higher levels of glucose, insulin, HOMA-IR, and TG. These outcomes suggest that folate deficiency is also associated with metabolic disturbances. Indeed, previous studies in humans revealed that IR in the offspring was associated with maternal folate intake (Krishnaveni et al., 2014). However, a study based on NHANES data paradoxically found a positive association between red blood cell folate and BMI that was opposed to the negative association between serum folate and BMI (Bird et al., 2015). Thus, future research is needed to understand how obesity differentially alters serum and red blood cell folate status.

Since folate participates as a substrate in one-carbon cycle, where SAM donates a methyl group for DNA methylation, it has been suggested that folate acts in the pathophysiology of obesity and MetS through the regulation of the expression of specific genes by altering DNA methylation patterns (Fu et al., 2017). In agreement with this hypothesis, we have found an association between inadequate folate intake and lower methylation of calcium/calmodulin-dependent protein kinase kinase 2 (*CAMKK2*) gene. Furthermore, *CAMKK2* hypomethylation was associated with HOMA-IR index, whereas higher *CAMKK2* gene expression correlated with IR. These findings suggest that epigenetic modifications in

CAMKK2 due to methyl donor intake may have a metabolic impact by modifying *CAMKK2* expression. *CAMKK2* encodes for a kinase that is involved in appetite and weight loss (Anderson et al., 2008). Moreover, previous experimental studies have reported a relationship between CAMKK2 and folate status in the prevention of cardiac dysfunction in a model of IR and adiposity (Anderson et al., 2008, 2012; Racioppi & Means, 2012; Roe et al., 2013). Taken together, our data show that low folate intake is associated with IR in obese subjects through *CAMKK2* hypomethylation.

On the other hand, inflammation has emerged as an important factor in the pathophysiology of obesity (Engin, 2017). The characteristic chronic low-grade inflammation within the metabolic tissues is known as metabolic inflammation, or "meta-inflammation" (Lyons et al., 2016). This meta-inflammation is linked with the development of IR in obese subjects (Solas et al., 2017). An increasing number of investigations have evidenced that some nutrients and bioactive compounds, including carbohydrates, flavonoids, FAs, and vitamins, may be implicated in the regulation of inflammation, increasing or reducing the inflammatory response (Galland, 2010). In this context, deficiency of folate and other methyl-donors (i.e choline, methionine, betaine, vitamin B_{12}) has been related to a variety of inflammation-related manifestations. For example, low methyl-donor intake has been associated with hypertension, NASH, IR, and MetS (Liew, 2016; Li et al., 2017). In rats fed an obesogenic diet, it is well known that methyl donor deficiency produces steatohepatitis (Bison et al., 2016). On the other hand, a study showed that the supplementation of choline, in subjects with deficient choline levels and hepatic steatosis, ameliorated liver disease (Buchman et al., 1995). For example, folic acid attenuated the hypoxia-induced inflammatory response of THP-1 cells through inhibition of the PI3K/Akt/HIF-1α pathway (Huang et al., 2016). Also, folic acid supplementation has been associated with a decrease of cytokine secretion in a model of lead acetate-induced hepatotoxicity in rats (Abd allah & Badary, 2017). Finally, dietary supplementation with methyl donors prevents NAFLD in rats (Cordero et al., 2013b).

According to the previous findings and our own results associating lower intake of folate with higher adiposity in the RESMENA study, we have tested the possible anti-inflammatory effects of methyl donors, especially folic acid alone or in combination with other methyl donors, in an *in vitro* model of LPS-activated THP-1 macrophages, and analysed whether epigenetic mechanisms were involved in

the effect. THP-1 is a human leukemia monocytic cell line that has been widely used to study monocyte and macrophage functions, mechanisms, signaling pathways, and nutrient effects (Chanput et al., 2014).

Previous studies have described that folic acid supplementation reduced cytokine levels in overweight subjects (Solini, Santini, & Ferrannini, 2006) and also decreased the risk of coronary artery disease by decreasing cytokines secretion (Wang et al., 2005). In the present study, we found that the incubation of LPS-activated macrophages with folic acid and a mix of methyl donors was associated with a reduction in the expression of several pro-inflammatory genes such as *TNF*, *IL1B*, *CD40*, and *TLR4*. Similar to our results, the incubation of murine monocytes with folic acid was found to reduce the expression of pro-inflammatory genes (Feng et al., 2011), whereas folic acid deficiency increased the expression of inflammatory mediators like IL-1β, IL-6, TNF-α, and MCP-1 at the RNA and protein level in the mouse monocyte-macrophage lineage RAW 264.7 (Kolb & Petrie, 2013). During chronic inflammation, macrophages overproduce TNF-α and IL-1β in the inflamed tissue for attracting more monocytes to damaged tissue and amplify inflammatory response (Calder et al., 2009). Current data revealed that folic acid and a mix of methyl donors also decrease the secretion of TNF-α and IL-1β by LPS-activated THP-1 macrophages. In this context, our results suggest that these methyl compounds could reduce the inflammatory response of the monocytes that are recruited from blood to surrounding inflamed tissues, and the macrophages derived from these monocytes would secrete less cytokines, contributing thus to reduce inflammation at the final instance and to improve the metabolic profile and delay the development of metabolic complications.

Furthermore, choline and vitamin B_{12} are involved in the conversion of homocysteine to methionine and the synthesis of SAM, which is the major donor of methyl groups for many substrates such as DNA, RNA, histones and co-regulators of nuclear receptors that play a key role in epigenetic mechanisms (Anderson et al., 2012). In the present research, folic acid and a mix of methyl donors increased the methylation levels of the proinflammatory genes *IL1B*, *SERPINE1* and *IL18* in LPS-activated macrophages in comparison with the non-activated ones. Several research groups have associated the intake of methyl donors with DNA methylation changes in inflammation-related diseases. For example, research in rats have reported that methyl donor consumption modified DNA

methylation in early liver steatosis, envisaging a potential use of these compounds in the improvement of inflammation (Cordero et al., 2011b; Cordero et al., 2013a; 2013b).

Since there is scientific evidence that transcriptional regulation by DNA methylation is the result of the alteration of transcription factor-gene promoter binding affinity or the spatial accessibility of transcriptional machinery (Zhang, 2015), a focus to investigate the effect of DNA methylation on the binding affinity of the NF-κB transcription factor to *IL1B* and *TNF* binding sites was aimed. NF-κB has been extensively studied in macrophages due to its regulatory function on the expression of many inflammatory genes (Liu et al., 2017). For instance, NF-κB transcription factor is essential in the LPS-mediated inflammatory response by increasing monocyte infiltration and IL-1β production (Abd allah & Badary, 2017), and modulates cytokine expression by direct binding to the promoters of their genes (Hiscott et al., 1993). Data of the present research reveals that the increase of DNA methylation by folic acid and a mix of methyl donors in *IL1B* gene did not affect the binding affinity of NF-κB to *IL1B* binding site. In accordance with this finding, a previous study reported that folic acid inhibited TNF-α and IL-1β production by inhibiting NF-κB pathway, whereas the direct effect of DNA methylation on binding affinity to gene promoters has not been elucidated (Feng et al., 2011).

In our model, LPS-induced inflammation ameliorated by the supplementation of folic acid and a mix of methyl donors. Although binding affinity of NF-κB to *IL1B* gene remained unaffected, it can be speculated about the molecular mechanisms regulated by this molecule that can be involved in the control of the expression of *IL1B*. The bioinformatic analysis of the selected sequence of the gene identified a putative PU.1 (Spi-1) transcription factor binding site. Interestingly, the PU.1 transcription factor is involved in macrophage differentiation and in the control of the expression of genes in mature macrophages (Yuan et al., 2012).

4. Strengths and limitations

This research has successfully identified novel DNA methylation signatures for obesity and metabolic disorders and for the response to an energy-restricted diet. In addition, it has found interesting association between the diet (particularly energy and carbohydrate intake) and the methylation levels of core clock genes. Finally, this research provides a descriptive picture of the impact of methyl

donors on the inflammatory response.

One of the main strengths of the present research is the robust design of the Women cohort and the RESMENA study. Given their prospective nature, they allowed us to analyze DNA methylation and other variables at two points. Moreover, the randomized design of the clinical trial is other strength of the RESMENA study as this design is the best to assess the effectiveness of the interventions. However, the studies carried out in pediatric population, such as the GENOI study, have the advantage that they are not affected by pernicious lifestyle practices. The use of "omics" approach has demonstrated to be useful for identifying DNA methylation patterns related to different phenotypes and populations, and to categorize individuals into different clusters depending on the nutrient pattern or success of a given dietary strategy. Integrative "omics" analyses that involve the integration of methylome and transcriptome data greatly improve our understanding of the biological mechanisms. Indeed, the combination of high-throughput technologies (arrays) and further validation in larger populations (by MassARRAY® EpiTYPER or pyrosequencing, and qRT-PCR) has proven to be a useful strategy to achieve the goal. The major limitation of this research is that no clear causality could be given between DNA methylation patterns and obesity and related phenotypes due to the studies design. For this reason, this project opens new questions that need to be addressed in further studies, and some limitations must be declared. For example, although we have performed some *in vitro* studies trying to deepen into the molecular mechanisms implicated in the onset of the diet-induced epigenetic changes, more mechanistic studies must be performed in order to explain some of the findings.

One shortcoming of the current research is the sample type of some analyses. On the one hand, DNA methylation and expression analyses were performed in white blood cells, rather than in metabolically relevant tissues, since blood is relatively easy to obtain and is a non-invasive source for RNA and DNA. Although blood is not a metabolically relevant tissue for the study of obesity and metabolic abnormalities, recent investigations have demonstrated that blood-based biomarkers reflect the DNA methylation changes in key metabolic tissues, as is the case of pancreatic islets (Bacos et al., 2016) and adipose tissue (Crujeiras et al., 2017), which supports the use of blood cells to study epigenetic alterations related to metabolic disorders. On the other hand, in the *in vitro* study, THP-1 cell model

was chosen to study the effects of methyl donor supplementation on the inflammatory response. THP-1 monocytic cell line was isolated from peripheral blood and has been widely used to study immune responses due to the possibility to differentiate into macrophage-like cells. However, a number of investigations have compared the similarity between THP-1 cells and human PBMCs and, although in most cases both types presented relative equal response patterns, some differences have been reported concerning gene expression and cytokine secretion.

We are aware that epigenetics is not the only factor contributing to natural human variation. Apart from DNA methylation, many other factors are implicated in differential response to dietary and metabolic environments, including genetics, gut microbiota, perinatal factors, family socio-economic status, and other life style characteristics. Particularly, factors that are involved in the perinatal period have been shown to alter the DNA methylation levels and future health outcomes (prenatal smoke exposure, preterm newborns, gestational diabetes, birth length and weight, maternal diet and physical exercise, maternal weight gain and adiposity,...). In this regard, DNA methylation has been reported to be a sensitive biomarker and potential therapeutic target (Crujeiras et al., 2017; Day et al., 2017). However, the mentioned factors should be considered in further studies.

Sample availability is another limitation of the present research. Given that we have no RNA samples from the individuals that participated in the GENOI and Women cohorts, we could not establish any direct relation between DNA methylation and gene expression. Therefore, in the present trial we could only hypothesize about the underlying biological processes. For example, bioinformatics analysis identified several transcription factor binding sites as putatively involved in the regulation of the expression of *BMAL1*, *PER3* and *PTPRS*. Interestingly, the identified transcription factors, such as glucocorticoid receptors, GATA-1/-6 and MZF1, are involved in adipogenesis (Tong et al., 2000), lipid metabolism (Watts et al., 2005) and inflammation (Powell et al., 2013). However, because of the lack of protein samples, we could not confirm the involved mechanisms behind the observed associations between DNA methylation profiles and phenotypes. In addition, *CD44* methylation levels were not measured at the end of the intervention, being not possible to analyse whether the dietary treatment had an effect on *CD44* gene methylation levels. Moreover, the lack of measures of blood/plasma folate concentrations in the subjects from the RESMENA study did not allow studying

the associations between folate intake and blood folate concentrations, and between folate levels and DNA methylation and expression.

Another limitation is related to the relative small samples size of some of the cohorts, which might limit the statistical power to detect differences in DNA methylation and expression of genes across the obesity and related disorders phenotypes and gene-diet interactions. The lack of large sample sizes could lead to increase the risk of type II errors (no detect real differences). Moreover, comparable experimental trials (focused on epigenetic biomarkers) with a balance in number of subjects and reasonable statistical power (> 80 %) have been performed to diminish type II error (Hermsdorff et al., 2013; Carraro et al., 2016). In addition, the array data were controlled by multiple comparisons correction by applying the Benjamini-Hochberg method, to avoid type I errors (accepting something that is not real). In order to minimize type I error, the lowest p values were selected for the analyses.

Lastly, another limitation is related with the use of questionnaires to collect weighed food records in the RESMENA study. Although it is true that dietary recalls and food composition tables may have some inconsistencies (Shim et al., 2014), food frequency questionnaires are relatively simple, cost-effective, and time-efficient (Shim et al., 2014). For this reason, they are widely used in similar nutritional studies.

The use of one cross-sectional study and two intervention trials enable us to identify putative epigenetics biomarkers for pediatric obesity and the success of different weight-loss interventions. Due to the limitation of each study we could not deepen into the molecular mechanisms involved in these epigenetic outcomes. For these reasons, further studies in larger and more diverse populations are needed for validating the observed associations between DNA methylation and phenotypes, before implementing epigenetic biomarkers for clinical diagnosis and personalized weight loss treatment decisions.

5. Corollary

The results of the current research have demonstrated that epigenetic factors could modulate susceptibility to obesity and related metabolic disorders. Specifically, hypomethylation of *PTPRS* and *PER3* genes were associated with higher adiposity in children. In addition, the pathways analysis

highlighted the relevance of circadian rhythm signalling in childhood obesity, but also suggested that some circadian genes (*VIPR2*, *GRIN2D*, *ADCYAP1R1*, and *PER3*) could play an important role via methylation-dependent mechanisms. In this context, a higher intake of energy and carbohydrates was associated with higher methylation levels of *BMAL1* clock gene in an adult population. This association supports an interaction between circadian and food intake systems through an epigenetic mechanism. It is noteworthy to highlight that changes in the methylation pattern of *BMAL1* as a consequence of the dietary intervention were associated with blood lipid markers. Indeed, the current data support the hypothesis that methylation of clock genes could be involved in the control of adiposity and lipid metabolism, but also that dietary interventions are able to modify the methylation pattern of genes intervening in important metabolic pathways such as the circadian clock and inflammation.

The integration of genome-wide array data has been shown to be a powerful tool for identifying novel biomarkers that predict the response to specific nutritional interventions. This is the case of the methylation and expression of *CD44*, which has been identified as a potential diagnostic tool to personalize the dietary treatment of obesity. Furthermore, our research also contributes to better understand not only the role of DNA methylation in obesity and body weight loss, but also how some micronutrients (i.e., methyl donors) can influence mechanisms associated with obesity and metabolic disorders via epigenetic modifications. For example, low folate intake was apparently associated with lower *CAMKK2* gene methylation and IR in obese individuals. In addition, *in vitro* studies in human monocyte/macrophage cells suggested potential anti-inflammatory effects of folic acid, alone and in combination with choline and vitamin B_{12} that could be in part mediated by DNA methylation mechanisms.

In summary, the findings of the present work confirm that the genes regulating circadian rhythm and inflammation are involved in the pathophysiology of obesity and in the individual response to weight loss treatments. “Omics” technologies are powerful tools for identifying new biomarkers related to metabolic alterations such as obesity, insulin resistance and inflammation. In the current work, we have demonstrated that epigenetics is implicated in the interindividual susceptibility to obesity and the response to dietary interventions. Moreover, these epigenetic mechanisms are dependent on dietary

factors, including energy and macronutrient intake, but also micronutrients and bioactive compounds such as folate and other methyl donors. In this context, our data confirm that the study of the epigenetic profile is a good strategy to find biomarkers that help to personalize the prevention and treatment of obesity and its related pathologies. The results of the present research work add new insights into the application of epigenome-based precision nutrition in the management of obesity and inflammatory related diseases. However, further studies must consider other factors that are also implicated in differential response to dietary and metabolic environments, including genetics, gut microbiota, and other life style characteristics.

CONCLUSIONS

Conclusions

1. Several CpGs in the *PTPRS* and *PER3* genes were identified as higher methylated in obese children in comparison with non-obese, suggesting a role for DNA methylation in these genes in childhood obesity development.
2. The methylation levels of *BMAL1* and other core clock genes were modified by a weight loss intervention and were associated with changes in serum lipid levels. These results evidenced that DNA methylation of clock genes is dependent on the diet composition and can be used as a biomarker of the lipid profile response to the diet.
3. The expression and DNA methylation of the immune-cell receptor gene *CD44* can be used as a predictor of the success to specific weight-loss interventions, revealing that this gene may have a putative role in body weight regulation.
4. Subjects within the RESMENA study with lower folate intake showed more fat mass, higher serum levels of glucose, insulin, cortisol, and PAI-1, and lower *CAMKK2* methylation than those with higher folate intake. Interestingly, *CAMKK2* methylation was negatively associated with HOMA-IR index whereas *CAMKK2* expression positively correlated with HOMA-IR, which suggests that *CAMKK2* methylation could be an epigenetic mechanism underlying low folic acid consumption-mediated insulin resistance in obese subjects.
5. Folic acid alone or mixed with other methyl donors (choline and vitamin B_{12}), when administered before the differentiation of THP-1 monocytes, reduced the inflammatory response when further differentiated into macrophages and activated by LPS. Folate addition reduced the expression of pro-inflammatory genes and the secretion of IL-1β and TNF-α in comparison with the non-treated LPS-stimulated THP-1 cells, which was accompanied by an increased methylation on CpGs concerning *IL1B*, *SERPINE1* and *IL18*.

REFERENCES

References

Abd allah, E. S. H., & Badary, D. M. (2017). Folic acid protects against lead acetate-induced hepatotoxicity by decreasing NF-κB, IL-1β production and lipid peroxidation mediataed cell injury. *Pathophysiology*, *24*(1), 39–44. doi: 10.1016/j.pathophys.2017.02.002

Abdeen, Z. A., Hamid, Z. A., Abu-Rmeileh, N. M., et al., (2017). Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *Lancet*. doi: 10.1016/S0140-6736(17)32129-3

Abdul, Q. A., Yu, B. P., Chung, H. Y., et al., (2017). Epigenetic modifications of gene expression by lifestyle and environment. *Archives of Pharmacal Research*, pp. 1–19. Pharmaceutical Society of Korea. doi: 10.1007/s12272-017-0973-3

Adaikalakoteswari, A., Finer, S., Voyias, P. D., et al., (2015). Vitamin B12 insufficiency induces cholesterol biosynthesis by limiting s-adenosylmethionine and modulating the methylation of SREBF1 and LDLR genes. *Clinical Epigenetics*, *7*(1), 14. doi: 10.1186/s13148-015-0046-8

Adhikari, S., & Curtis, P. D. (2016). DNA methyltransferases and epigenetic regulation in bacteria. *FEMS Microbiology Reviews*, *23*(40), 575–591. doi: 10.1093/femsre/fuw023

Ahrens, M., Ammerpohl, O., von Schönfels, W., et al., (2013). DNA Methylation Analysis in Nonalcoholic Fatty Liver Disease Suggests Distinct Disease-Specific and Remodeling Signatures after Bariatric Surgery. *Cell Metabolism*, *18*(2), 296–302. doi: 10.1016/j.cmet.2013.07.004

Al Muftah, W. A., Al-Shafai, M., Zaghlool, S. B., et al., (2016). Epigenetic associations of type 2 diabetes and BMI in an Arab population. *Clinical Epigenetics*, *8*(1), 13. doi: 10.1186/s13148-016-0177-6

Alberti, K. G., Zimmet, P., & Shaw, J. (2006). Metabolic syndrome-a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabetic Medicine*, *23*(5), 469–480. doi: 10.1111/j.1464-5491.2006.01858.x

Albrecht, U. (2012). Timing to Perfection: The Biology of Central and Peripheral Circadian Clocks. *Neuron*, *74*(2), 246–260. doi: 10.1016/J.NEURON.2012.04.006

Alikhani-Koopaei, R., Fouladkou, F., Frey, F. J., et al., (2004). Epigenetic regulation of 11β-hydroxysteroid dehydrogenase type 2 expression. *The Journal of Clinical Investigation*, *114*(8), 1146–1157. doi: 10.1172/JCI200421647

Alissa, E. M., & Ferns, G. A. (2015). Dietary Fruits and Vegetables and Cardiovascular Diseases Risk. *Critical Reviews in Food Science and Nutrition*, doi: 10.1080/10408398.2015.1040487

Almén, M. S., Jacobsson, J. A., Moschonis, G., et al., (2012). Genome wide analysis reveals association of a FTO gene variant with epigenetic changes. *Genomics*, *99*(3), 132–137. doi: 10.1016/j.ygeno.2011.12.007

Altmann, S., Murani, E., Schwerin, M., et al., (2013). Dietary protein restriction and excess of pregnant German Landrace sows induce changes in hepatic gene expression and promoter methylation of key metabolic genes in the offspring. *Journal of Nutritional Biochemistry*, *24*(2), 484–495. doi: 10.1016/j.jnutbio.2012.01.011

Amaral, C. L., Crisma, A. R., Masi, L. N., et al., (2015). DNA methylation changes induced by a high-fat diet and fish oil supplementation in the skeletal muscle of mice. *Journal of Nutrigenetics and Nutrigenomics*, *7*(4–6), 314–326. doi: 10.1159/000381777

Anderson, K. A., Lin, F., Ribar, T. J., et al., (2012). Deletion of CaMKK2 from the Liver Lowers Blood Glucose and Improves Whole-Body Glucose Tolerance in the Mouse. *Molecular Endocrinology*, *26*(2), 281–291. doi: 10.1210/me.2011-1299

Anderson, K. A., Ribar, T. J., Lin, F., et al., (2008). Hypothalamic CaMKK2 Contributes to the Regulation of Energy Balance. *Cell Metabolism*, *7*(5), 377–388. doi: 10.1016/j.cmet.2008.02.011

Anderson, O. S., Sant, K. E., & Dolinoy, D. C. (2012). Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism and DNA methylation. *Journal of Nutritional Biochemistry*. doi: 10.1016/j.jnutbio.2012.03.003

Ansel, K. M., Lee, D. U., & Rao, A. (2003). An epigenetic view of helper T cell differentiation. *Nature Immunology*. doi: 10.1038/ni0703-616

Antunes, L. C., Levandovski, R., Dantas, G., et al., (2010). Obesity and shift work: Chronobiological aspects. *Nutrition Research Reviews*, *23*(1), 155–168. doi: 10.1017/S0954422410000016

Armenise, C., Lefebvre, G., Carayol, J., et al., (2017). Transcriptome profiling from adipose tissue during a low-calorie diet reveals predictors of weight and glycemic outcomes in obese, nondiabetic subjects. *The American Journal of Clinical Nutrition*, *106*(3), 736–746. doi: 10.3945/ajcn.117.156216

Arpón, A., Milagro, F. I., Razquin, C., et al., (2018). Impact of consuming extra-virgin olive oil or nuts within a mediterranean diet on DNA methylation in peripheral white blood cells within the PREDIMED-navarra randomized controlled trial: A role for dietary lipids. *Nutrients*, *10*(1), 15. doi: 10.3390/nu10010015

Aslibekyan, S., Demerath, E. W., Mendelson, M., et al., (2015). Epigenome-wide study identifies novel methylation loci associated with body mass index and waist circumference. *Obesity (Silver Spring, Md.)*, *23*(7), 1493–1501. doi: 10.1002/oby.21111

Aumueller, E., Remely, M., Baeck, H., et al., (2015). CpG Methylation and Body Weight Correlate Differently in Type 2 Diabetes Patients Compared to Obese and Lean Controls. *Journal of Nutrigenetics and Nutrigenomics*, *8*(1), 26–35. doi: 10.1159/000381714

Ayissi, V. B. O., Ebrahimi, A., & Schluesenner, H. (2014). Epigenetic effects of natural polyphenols: A focus on SIRT1-mediated mechanisms. *Molecular Nutrition and Food Research*. doi: 10.1002/mnfr.201300195

Azzi, S., Sas, T. C. J., Koudou, Y., et al., (2014). Degree of methylation of ZAC1 (PLAGL1) is associated with prenatal and post-natal growth in healthy infants of the EDEN mother child cohort. *Epigenetics*, *9*(3), 338–45. doi: 10.4161/epi.27387

Ba, Y., Yu, H., Liu, F., et al., (2011). Relationship of folate, vitamin B12 and methylation of insulin-like growth factor-II in maternal and cord blood. *European Journal of Clinical Nutrition*, *65*(4), 480–5. doi: 10.1038/ejcn.2010.294

Baccarelli, A., Wright, R. O., Bollati, V., et al., (2009). Rapid DNA methylation changes after exposure to traffic particles. *American Journal of Respiratory and Critical Care Medicine*, *179*(7), 572–578. doi: 10.1164/rccm.200807-1097OC

Bacos, K., Gillberg, L., Volkov, P., et al., (2016). Blood-based biomarkers of age-associated epigenetic changes in human islets associate with insulin secretion and diabetes. *Nature Communications*, *7*, 11089. doi: 10.1038/ncomms11089

Baker, K. D., Loughman, A., Spencer, S. J., et al., (2017). The impact of obesity and hypercaloric diet consumption on anxiety and emotional behavior across the lifespan. *Neuroscience & Biobehavioral*

Reviews, *83*, 173–182. doi: 10.1016/j.neubiorev.2017.10.014

Barker, D. J. P. (2007). The origins of the developmental origins theory. In *Journal of Internal Medicine 261*, 412–417. doi: 10.1111/j.1365-2796.2007.01809.x

Barres, R., Kirchner, H., Rasmussen, M., et al., (2013). Weight Loss after Gastric Bypass Surgery in Human Obesity Remodels Promoter Methylation. *Cell Reports*, *3*(4), 1020–1027. doi: 10.1016/j.celrep.2013.03.018

Barrès, R., Yan, J., Egan, B., et al., (2012). Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metabolism*, *15*(3), 405–411. doi: 10.1016/j.cmet.2012.01.001

Barrès, R., & Zierath, J. R. (2016). The role of diet and exercise in the transgenerational epigenetic landscape of T2DM. *Nature Reviews Endocrinology*, *12*(8), 441–451. doi: 10.1038/nrendo.2016.87

Bartel, D. P. (2009). MicroRNAs: Target Recognition and Regulatory Functions. *Cell*. doi: 10.1016/j.cell.2009.01.002

Bartlett, A. A., Singh, R., & Hunter, R. G. (2017). Anxiety and epigenetics. *Advances in Experimental Medicine and Biology 978*, 145–166. doi: 10.1007/978-3-319-53889-1_8

Bass, J., & Takahashi, J. S. (2010). Circadian integration of metabolism and energetics. *Science*. doi: 10.1126/science.1195027

Baumeier, C., Saussenthaler, S., Kammel, A., et al., (2017). Hepatic DPP4 DNA methylation associates with fatty liver. *Diabetes*, *66*(1), 25–35. doi: 10.2337/db15-1716

Baylin, S. B., & Jones, P. A. (2011). A decade of exploring the cancer epigenome - biological and translational implications. *Nature Reviews. Cancer*, *11*(10), 726–34. doi: 10.1038/nrc3130

Belzeaux, R., Lin, R., Ju, C., et al., (2017). Transcriptomic and epigenomic biomarkers of antidepressant response. *Journal of Affective Disorders*. doi: 10.1016/j.jad.2017.08.087

Bertola, A., Deveaux, V., Bonnafous, S., et al., (2009). Elevated expression of osteopontin may be related to adipose tissue macrophage accumulation and liver steatosis in morbid obesity. *Diabetes*, *58*(1), 125–133. doi: 10.2337/db08-0400

Bhasin, M., Reinherz, E. L., & Reche, P. A. (2006). Recognition and Classification of Histones Using Support Vector Machine. *Journal of Computational Biology*, *13*(1), 102–112. doi: 10.1089/cmb.2006.13.102

Bhat, M. I., & Kapila, R. (2017). Dietary metabolites derived from gut microbiota: Critical modulators of epigenetic changes in mammals. *Nutrition Reviews*, *75*(5), 374–389. doi: 10.1093/nutrit/nux001

Biddle, S. J. H., Bengoechea Garcia, E., Pedisic, Z., et al., (2017). Screen Time, Other Sedentary Behaviours, and Obesity Risk in Adults: A Review of Reviews. *Current Obesity Reports*, *6*(2), 134–147. doi: 10.1007/s13679-017-0256-9

Bidulescu, A., Chambless, L. E., Siega-Riz, A. M., et al., (2009). Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) study. *Nutrition Journal*, *8*, 14. doi: 10.1186/1475-2891-8-14

Birch, E. E., Hoffman, D. R., Uauy, R., et al., (1998). Visual Acuity and the Essentiality of Docosahexaenoic Acid and Arachidonic Acid in the Diet of Term Infants. *Pediatric Research*, *44*(2), 201–209. doi: 10.1203/00006450-199808000-00011

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes and Development*. doi: 10.1101/gad.947102

Bird, J. K., Ronnenberg, A. G., Choi, S.W., et al., (2015). Obesity Is Associated with Increased Red Blood Cell Folate Despite Lower Dietary Intakes and Serum Concentrations. *Journal of Nutrition*, *145*(1), 79–86. doi: 10.3945/jn.114.199117

Bison, A., Marchal-Bressenot, A., Li, Z., et al., (2016). Foetal programming by methyl donor deficiency produces steato-hepatitis in rats exposed to high fat diet. *Scientific Reports*, *6*(1), 37207. doi: 10.1038/srep37207

Bokor, S., Meirhaeghe, A., Ruiz, J. R., et al., (2011). Common polymorphisms in six genes of the methyl group metabolism pathway and obesity in European adolescents. *International Journal of Pediatric Obesity*, *6*(2–2), e336–e344. doi: 10.3109/17477166.2010.500386

Bolha, L., Ravnik-Glavač, M., & Glavač, D. (2017). Long Noncoding RNAs as Biomarkers in Cancer. *Disease Markers*. doi: 10.1155/2017/7243968

Boqué, N., de la Iglesia, R., de la Garza, A. L., et al., (2013). Prevention of diet-induced obesity by apple polyphenols in Wistar rats through regulation of adipocyte gene expression and DNA methylation patterns. *Molecular Nutrition and Food Research*, *57*(8), 1473–1478. doi: 10.1002/mnfr.201200686

Bouchard-Mercier, A., Paradis, A.M., Rudkowska, I., et al., (2013). Associations between dietary patterns and gene expression profiles of healthy men and women: a cross-sectional study. *Nutrition Journal*, *12*(1), 24. doi: 10.1186/1475-2891-12-24

Bouchard, L., Rabasa-Lhoret, R., Faraj, M., et al., (2010). Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction. *American Journal of Clinical Nutrition*, *91*(2), 309–320. doi: 10.3945/ajcn.2009.28085

Bradbury, K. E., Williams, S. M., Mann, J. I., et al., (2014). Estimation of Serum and Erythrocyte Folate Concentrations in the New Zealand Adult Population within a Background of Voluntary Folic Acid Fortification. *Journal of Nutrition*, *144*(1), 68–74. doi: 10.3945/jn.113.182105

Brown, A. N., & Feng, J. (2017). Drug addiction and DNA modifications. In *Advances in Experimental Medicine and Biology*, *978*, 105–125. doi: 10.1007/978-3-319-53889-1_6

Buchman, A. L., Dubin, M. D., Moukarzel, A. A., et al., (1995). Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology*, *22*(5), 1399–403. doi: 10.1002/hep.1840220510

Cadet, J. L. (2016). Epigenetics of Stress, Addiction, and Resilience: Therapeutic Implications. *Molecular Neurobiology*. doi: 10.1007/s12035-014-9040-y

Calder, P. C., Albers, R., Antoine, J.M., et al., (2009). Inflammatory Disease Processes and Interactions with Nutrition. *British Journal of Nutrition*, *101*(S1), 1. doi: 10.1017/S0007114509377867

Campion, J., Milagro, F. I., & Martinez, J. A. (2009). Individuality and epigenetics in obesity. *Obesity Reviews: An Official Journal of the International Association for the Study of Obesity*, *10*(4), 383–392. doi: 10.1111/j.1467-789X.2009.00595.x

Caramaschi, D., Sharp, G. C., Nohr, E. A., et al., (2017). Exploring a causal role of DNA methylation in the relationship between maternal vitamin B12 during pregnancy and child's IQ at age 8, cognitive performance and educational attainment: a two-step Mendelian randomization study. *Human Molecular Genetics*, *26*(15), 3001–3013. doi: 10.1093/hmg/ddx164

Carlson, S. E., & Neuringer, M. (1999). Polyunsaturated fatty acid status and neurodevelopment: a summary and

critical analysis of the literature. *Lipids*, *34*(2), 171–178. doi: 10.1007/s11745-999-0351-2

Carraro, J. C. C., Hermsdorff, H. H. M., Mansego, M. L., et al., (2016). Higher fruit intake is related to TNF-α hypomethylation and better glucose tolerance in healthy subjects. *Journal of Nutrigenetics and Nutrigenomics*, *9*(2–4), 95–105. doi: 10.1159/000448101

Carraro, J. C. C., Mansego, M. L., Milagro, F. I., et al., (2016). LINE-1 and inflammatory gene methylation levels are early biomarkers of metabolic changes: association with adiposity. *Biomarkers*, *21*(7), 625–632. doi: 10.3109/1354750X.2016.1171904

Carthew, R. W., & Sontheimer, E. J. (2009). Origins and Mechanisms of miRNAs and siRNAs. *Cell*. doi: 10.1016/j.cell.2009.01.035

Castellano-Castillo, D., Moreno-Indias, I., Fernández-García, J. C., et al., (2017). Adipose Tissue LPL Methylation is Associated with Triglyceride Concentrations in the Metabolic Syndrome. *Clinical Chemistry*, doi: 10.1373/clinchem.2017.277921

Ceccarelli, V., Racanicchi, S., Martelli, M. P., et al., (2011). Eicosapentaenoic acid demethylates a single CpG that mediates expression of tumor suppressor CCAAT/enhancer-binding protein delta in U937 leukemia cells. *The Journal of Biological Chemistry*, *286*(31), 27092–102. doi: 10.1074/jbc.M111.253609

Cedernaes, J., Osler, M. E., Voisin, S., et al., (2015). Acute sleep loss induces tissue-specific epigenetic and transcriptional alterations to circadian clock genes in men. *Journal of Clinical Endocrinology and Metabolism*, *100*(9), E1255–E1261. doi: 10.1210/JC.2015-2284

Chakraborty, N., Muhie, S., Kumar, R., et al., (2017). Contributions of polyunsaturated fatty acids (PUFA) on cerebral neurobiology: an integrated omics approach with epigenomic focus. *Journal of Nutritional Biochemistry*, *42*, 84–94. doi: 10.1016/j.jnutbio.2016.12.006

Chalasani, N., Younossi, Z., Lavine, J. E., et al., (2012). The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology*, *142*(7), 1592–1609. doi: 10.1053/j.gastro.2012.04.001

Chambers, J. C., Loh, M., Lehne, B., et al., (2015). Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: A nested case-control study. *The Lancet Diabetes and Endocrinology*, *3*(7), 526–534. doi: 10.1016/S2213-8587(15)00127-8

Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: An in vitro cell model for immune modulation approach. *International Immunopharmacology*. doi: 10.1016/j.intimp.2014.08.002

Chatzigeorgiou, A., Kandaraki, E., Papavassiliou, A. G., et al., (2014). Peripheral targets in obesity treatment: A comprehensive update. *Obesity Reviews*, *15*(6), 487–503. doi: 10.1111/obr.12163

Cheedipudi, S., Genolet, O., & Dobreva, G. (2014). Epigenetic inheritance of cell fates during embryonic development. *Front Genet*, *5*, 19. doi: 10.3389/fgene.2014.00019

Chen, D., Zhang, A., Fang, M., et al., (2014). Increased methylation at differentially methylated region of GNAS in infants born to gestational diabetes. *BMC Medical Genetics*, *15*, 108.doi: 10.1186/s12881-014-0108-3

Chen, J., Zhou, X., Wu, W., et al., (2015). FTO-dependent function of N6-methyladenosine is involved in the hepatoprotective effects of betaine on adolescent mice. *Journal of Physiology and Biochemistry*, *71*(3), 405–413. doi: 10.1007/s13105-015-0420-1

Chen, K., Huang, J., Gong, W., et al., (2007). Toll-like receptors in inflammation, infection and cancer.

International Immunopharmacology. doi: 10.1016/j.intimp.2007.05.016

Chen, K., Liu, J., & Cao, X. (2017). Regulation of type I interferon signaling in immunity and inflammation: A comprehensive review. *Journal of Autoimmunity*. doi: 10.1016/j.jaut.2017.03.008

Chen, T., & Li, E. (2006). Establishment and maintenance of DNA methylation patterns in mammals. *Current Topics in Microbiology and Immunology*, *301*, 179–201. doi: 10.1007/3-540-31390-7_6

Chen, X., Ba, Y., Ma, L., et al., (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Research*, *18*(10), 997–1006. doi: 10.1038/cr.2008.282

Cheng, Z., Zheng, L., & Almeida, F. A. (2018). Epigenetic reprogramming in metabolic disorders: nutritional factors and beyond. *The Journal of Nutritional Biochemistry*, *54*, 1–10. doi: 10.1016/J.JNUTBIO.2017.10.004

Chu, A., Gozal, D., Cortese, R., et al., (2015). Cardiovascular dysfunction in adult mice following postnatal intermittent hypoxia. *Pediatric Research*, *77*(3), 425–433. doi: 10.1038/pr.2014.197

Cianciulli, A., Salvatore, R., Porro, C., et al., (2016). Folic Acid Is Able to Polarize the Inflammatory Response in LPS Activated Microglia by Regulating Multiple Signaling Pathways. *Mediators of Inflammation*, *2016*, 1–10. doi: 10.1155/2016/5240127

Cole, T. J. (2000). Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ*, *320*(7244), 1240–1240. doi: 10.1136/bmj.320.7244.1240

Corbalan, M. D., Morales, E. M., Canteras, M., et al., (2009). Effectiveness of cognitive-behavioral therapy based on the Mediterranean diet for the treatment of obesity. *Nutrition*, *25*(7–8), 861–869. doi: 10.1016/j.nut.2009.02.013

Cordero, P., Campion, J., Milagro, F. I., et al., (2011b). Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. *Journal of Physiology and Biochemistry*, *67*(3), 463–470. doi: 10.1007/s13105-011-0084-4

Cordero, P., Campion, J., Milagro, F. I., et al., (2013a). Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: Effect of dietary methyl donor supplementation. *Molecular Genetics and Metabolism*, *110*(3), 388–395. doi: 10.1016/j.ymgme.2013.08.022

Cordero, P., Campion, J., Milagro, F. I., & Martínez, J. A. (2011a). Dietary supplementation with methyl donor groups could prevent nonalcoholic fatty liver. *Hepatology*. doi: 10.1002/hep.24164

Cordero, P., Gomez-Uriz, A. M., Campion, J., et al., (2013b). Dietary supplementation with methyl donors reduces fatty liver and modifies the fatty acid synthase DNA methylation profile in rats fed an obesogenic diet. *Genes and Nutrition*, *8*(1), 105–113. doi: 10.1007/s12263-012-0300-z

Cornish-Bowden, A. (1987). Nomenclature and symbols for folic acid and related compounds (Recommendations 1986). *Pure and Applied Chemistry*, *59*(6). doi: 10.1351/pac198759060833

Commite of Diet and Health (1989) *Diet and Health*. doi: 10.17226/1222

Côté, S., Gagné-Ouellet, V., Guay, S.-P., et al., (2016). PPARGC1α gene DNA methylation variations in human placenta mediate the link between maternal hyperglycemia and leptin levels in newborns. *Clinical Epigenetics*, *8*(1), 72. doi: 10.1186/s13148-016-0239-9

Crujeiras, A. B., Campion, J., Díaz-Lagares, A., et al., (2013). Association of weight regain with specific methylation levels in the NPY and POMC promoters in leukocytes of obese men: A translational study. *Regulatory Peptides*, *186*, 1–6. doi: 10.1016/j.regpep.2013.06.012

Crujeiras, A. B., Diaz-Lagares, A., Sandoval, J., et al., (2017). DNA methylation map in circulating leukocytes mirrors subcutaneous adipose tissue methylation pattern: a genome-wide analysis from non-obese and obese patients. *Scientific Reports*, *7*, 41903. doi: 10.1038/srep41903

Cunnane, S. C. (2003). Problems with essential fatty acids: Time for a new paradigm? *Progress in Lipid Research*. doi: 10.1016/S0163-7827(03)00038-9

Cunnane, S. C., Francescutti, V., Brenna, J. T., et al., (2000). Breast-fed infants achieve a higher rate of brain and whole body docosahexaenoate accumulation than formula-fed infants not consuming dietary docosahexaenoate. *Lipids*, *35*(1), 105–111. doi: 10.1007/s11745-000-0501-6

Dali-Youcef, N., Mecili, M., Ricci, R., et al., (2013). Metabolic inflammation: Connecting obesity and insulin resistance. *Annals of Medicine*, *45*(3), 242–253. doi: 10.3109/07853890.2012.705015

Daly, L. E., Kirke, P. N., Molloy, A., et al., (1995). Folate levels and neural tube defects. Implications for prevention. *JAMA : The Journal of the American Medical Association*, *274*(21), 1698–1702. doi: 10.1001/jama.274.21.1698

Dashti, H. S., Follis, J. L., Smith, C. E., et al., (2015). Gene-Environment Interactions of Circadian-Related Genes for Cardiometabolic Traits. *Diabetes Care*, *38*(8), 1456–1466. doi: 10.2337/dc14-2709

Day, S. E., Coletta, R. L., Kim, J. Y., et al., (2017). Potential epigenetic biomarkers of obesity-related insulin resistance in human whole-blood. *Epigenetics*, *12*(4), 254–263. doi: 10.1080/15592294.2017.1281501

Dayeh, T., Tuomi, T., Almgren, P., et al., (2016). DNA methylation of loci within ABCG1 and PHOSPHO1 in blood DNA is associated with future type 2 diabetes risk. *Epigenetics*, *11*(7), 482–488. doi: 10.1080/15592294.2016.1178418

de la Iglesia, R., Lopez-Legarrea, P., Abete, I., et al., (2014). A new dietary strategy for long-term treatment of the metabolic syndrome is compared with the American Heart Association (AHA) guidelines: the MEtabolic Syndrome REduction in NAvarra (RESMENA) project. *British Journal of Nutrition*, *111*(4), 643–652. doi: 10.1017/S0007114513002778

de la Iglesia, R., Lopez-Legarrea, P., Celada, P., et al., (2013). Beneficial effects of the RESMENA dietary pattern on oxidative stress in patients suffering from metabolic syndrome with hyperglycemia are associated to dietary TAC and fruit consumption. *International Journal of Molecular Sciences*, *14*(4), 6903–6919. doi: 10.3390/ijms14046903

de la Rocha, C., Pérez-Mojica, J. E., León, S. Z.D., et al., (2016). Associations between whole peripheral blood fatty acids and DNA methylation in humans. *Scientific Reports*, *6*, 25867. doi: 10.1038/srep25867

de Mello, V. D. F., Pulkkinen, L., Lalli, M., et al., (2014). DNA methylation in obesity and type 2 diabetes. *Annals of Medicine*, *46*(3), 103–113. doi: 10.3109/07853890.2013.857259

de Mello, V. D., Matte, A., Perfilyev, A., et al., (2017). Human liver epigenetic alterations in non-alcoholic steatohepatitis are related to insulin action. *Epigenetics*, *12*(4), 287–295. doi: 10.1080/15592294.2017.1294305

De Prins, S., Koppen, G., Jacobs, G., et al., (2013). Influence of ambient air pollution on global DNA methylation in healthy adults: A seasonal follow-up. *Environment International*, *59*, 418–424. doi: 10.1016/j.envint.2013.07.007

Deaton, A. M., & Bird, A. (2011). CpG islands and the regulation of transcription. *Genes and Development*, *25*(10), 1010–1022. doi: /10.1101/gad.2037511

Demerath, E. W., Guan, W., Grove, M. L., et al., (2015). Epigenome-wide association study (EWAS) of BMI, BMI change and waist circumference in African American adults identifies multiple replicated loci. *Human Molecular Genetics*, *24*(15), 4464–4479. doi: 10.1093/hmg/ddv161

Denu, J. M., & Dixon, J. E. (1998). Protein tyrosine phosphatases: Mechanisms of catalysis and regulation. *Current Opinion in Chemical Biology*, *2*(5), 633–641. doi: 10.1016/S1367-5931(98)80095-1

Dick, K. J., Nelson, C. P., Tsaprouni, L., et al., (2014). DNA methylation and body-mass index: a genome-wide analysis. *The Lancet*, *383*(9933), 1990–1998. doi: 10.1016/S0140-6736(13)62674-4Dominguez-Salas, P., Moore, S. E., Baker, M. S., et al., (2014). Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nature Communications*, *5*, 3746. doi: 10.1038/ncomms4746

Dunstan, J., Bressler, J. P., Moran, T. H., et al., (2017). Associations of LEP, CRH, ICAM-1, and LINE-1 methylation, measured in saliva, with waist circumference, body mass index, and percent body fat in mid-childhood. *Clinical Epigenetics*, *9*, 29. doi: 10.1186/s13148-017-0327-5

Dupont, C., Armant, D. R., & Brenner, C. A. (2009). Epigenetics: Definition, mechanisms and clinical perspective. *Seminars in Reproductive Medicine*. doi: 10.1055/s-0029-1237423

El Hajj, N., Pliushch, G., Schneider, E., et al., (2013). Metabolic programming of MEST DNA methylation by intrauterine exposure to gestational diabetes mellitus. *Diabetes*, *62*(4), 1320–1328. doi: 10.2337/db12-0289

Elchebly, M., Wagner, J., Kennedy, T. E., et al., (1999). Neuroendocrine dysplasia in mice lacking protein tyrosine phosphatase σ. *Nature Genetics*, *21*(3), 330–333. doi: 10.1038/6859

Engin, A. (2017). The Pathogenesis of Obesity-Associated Adipose Tissue Inflammation. *Advances in Experimental Medicine and Biology*, 221–245. doi: 10.1007/978-3-319-48382-5_9

Esposito, K., & Giugliano, D. (2004). The metabolic syndrome and inflammation: Association or causation? *Nutrition, Metabolism and Cardiovascular Diseases*. doi: 10.1016/S0939-4753(04)80048-6

Faienza, M. F., Wang, D. Q., Fruhbeck, G., et al., (2016). The dangerous link between childhood and adulthood predictors of obesity and metabolic syndrome. *Internal and Emergency Medicine*, *11*(2), 175–182. doi: 10.1007/s11739-015-1382-6 [doi]

Fan, C., Fu, H., Dong, H., et al., (2016). Maternal n-3 polyunsaturated fatty acid deprivation during pregnancy and lactation affects neurogenesis and apoptosis in adult offspring: associated with DNA methylation of brain-derived neurotrophic factor transcripts. *Nutrition Research*, *36*(9), 1013–1021. doi: 10.1016/j.nutres.2016.06.005

Fattore, E., & Fanelli, R. (2013). Palm oil and palmitic acid: a review on cardiovascular effects and carcinogenicity. *International Journal of Food Sciences and Nutrition*, *64*(5), 648–659. doi: 10.3109/09637486.2013.768213

Feng, D., Zhou, Y., Xia, M., et al., (2011). Folic acid inhibits lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages by suppressing MAPKs and NF-kB activation. *Inflammation Research*, *60*(9), 817–822. doi: 10.1007/s00011-011-0337-2

Fernández-Sanlés, A., Sayols-Baixeras, S., Subirana, I., et al., (2017). Association between DNA methylation and coronary heart disease or other atherosclerotic events: A systematic review. *Atherosclerosis*. doi: 10.1016/j.atherosclerosis.2017.05.022

Finer, S., Mathews, C., Lowe, R., et al., (2015). Maternal gestational diabetes is associated with genome-wide

DNA methylation variation in placenta and cord blood of exposed offspring. *Human Molecular Genetics*, *24*(11), 3021–3029. doi: 10.1093/hmg/ddv013

Fiorito, G., Guarrera, S., Valle, C., et al., (2014). B-vitamins intake, DNA-methylation of One Carbon Metabolism and homocysteine pathway genes and myocardial infarction risk: The EPICOR study. doi: 10.1016/j.numecd.2013.10.026

Florath, I., Butterbach, K., Heiss, J., et al., (2016). Type 2 diabetes and leucocyte DNA methylation: an epigenome-wide association study in over 1,500 older adults. *Diabetologia*, *59*(1), 130–138. doi: 10.1007/s00125-015-3773-7

Flores-Sierra, J., Arredondo-Guerrero, M., Cervantes-Paz, B., et al., (2016). The trans fatty acid elaidate affects the global DNA methylation profile of cultured cells and in vivo. *Lipids in Health and Disease*, *15*, 75. doi: 10.1186/s12944-016-0243-2

Fogel, O., Richard-Miceli, C., & Tost, J. (2017). Epigenetic Changes in Chronic Inflammatory Diseases. In *Advances in Protein Chemistry and Structural Biology*, *106*, 139–189. doi: 10.1016/bs.apcsb.2016.09.003

Forouzanfar, M. H., Alexander, L., Anderson, H. R., et al., (2015). Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, *386*(10010), 2287–2323. doi: 10.1016/S0140-6736(15)00128-2

Fox, J. T., & Stover, P. J. (2008). Chapter 1 Folate-Mediated One-Carbon Metabolism. *Vitamins and Hormones*. doi: 10.1016/S0083-6729(08)00401-9

Friedewald, W. T., Levy, R. I., & Fredrickson, D. S. (1972). Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical Chemistry*, *18*(6), 499–502.

Froy, O. (2007). The relationship between nutrition and circadian rhythms in mammals. *Frontiers in Neuroendocrinology*, *28*(2–3), 61–71. doi: 10.1016/j.yfrne.2007.03.001

Froy, O. (2012). Circadian rhythms and obesity in mammals. *ISRN Obesity*, *2012*, 437198. doi: 10.5402/2012/437198

Fu, Y., Wang, X., & Kong, W. (2017). Hyperhomocysteinaemia and vascular injury: Advances in mechanisms and drug targets. *British Journal of Pharmacology*. doi: 10.1111/bph.13988

Gagnidze, K., & Pfaff, D. W. (2013). Epigenetic Mechanisms: DNA Methylation and Histone Protein Modification. *Neuroscience in the 21st Century*, 1939–1978. doi: 10.1007/978-1-4614-1997-6_69

Galland, L. (2010). Diet and inflammation. *Nutrition in Clinical Practice*. doi: 10.1177/0884533610385703

Ganz, A. B., Shields, K., Fomin, V. G., et al., (2016). Genetic impairments in folate enzymes increase dependence on dietary choline for phosphatidylcholine production at the expense of betaine synthesis. *FASEB Journal*, *30*(10), 3321–3333. doi: 10.1096/fj.201500138RR

Gao, X., Wang, Y., Randell, E., et al., (2016). Higher Dietary Choline and Betaine Intakes are Associated with Better Body Composition in the Adult Population of Newfoundland, Canada. *PLoS ONE*, *11*(5), e0155403. doi: 10.1371/journal.pone.0155403

Garaulet, M., & Gómez-Abellán, P. (2013). Chronobiology and obesity. *Nutr Hosp*, *28 Suppl 5*(28), 114–120. doi: 10.3305/nh.2013.28.sup5.6926

Garaulet, M., Lee, Y. C., Shen, J., et al., (2009). CLOCK genetic variation and metabolic syndrome risk:

modulation by monounsaturated fatty acids. *The American Journal of Clinical Nutrition*, *90*(6), 1466–1475. doi: 10.3945/ajcn.2009.27536

Garaulet, M., Ordovás, J. M., & Madrid, J. A. (2010). The chronobiology, etiology and pathophysiology of obesity. *International Journal of Obesity*, *34*(12), 1667–1683. doi: 10.1038/ijo.2010.118

Garaulet, M., Sanchez-Moreno, C., Smith, C. E., et al., (2011). Ghrelin, sleep reduction and evening preference: relationships to CLOCK 3111 T/C SNP and weight loss. *PloS One*, *6*(2), e17435. doi: 10.1371/journal.pone.0017435

García-Escobar, E., Monastero, R., García-Serrano, S., et al., (2017). Dietary fatty acids modulate adipocyte TNFa production via regulation of its DNA promoter methylation levels. *Journal of Nutritional Biochemistry*, *47*, 106–112. doi: 10.1016/j.jnutbio.2017.05.006

Garcia-Rios, A., Gomez-Delgado, F. J., Garaulet, M., et al., (2014). Beneficial effect of *CLOCK* gene polymorphism rs1801260 in combination with low-fat diet on insulin metabolism in the patients with metabolic syndrome. *Chronobiology International*, *31*(3), 401–408. doi: 10.3109/07420528.2013.864300

Gargari, B. P., Aghamohammadi, V., & Aliasgharzadeh, A. (2011). Effect of folic acid supplementation on biochemical indices in overweight and obese men with type 2 diabetes. *Diabetes Research and Clinical Practice*, *94*(1), 33–38. doi: 10.1016/j.diabres.2011.07.003

Ge, Z. J., Luo, S. M., Lin, F., et al., (2014). DNA methylation in oocytes and liver of female mice and their offspring: Effects of high-fat-diet-induced obesity. *Environmental Health Perspectives*, *122*(2), 159–164. doi: 10.1289/ehp.1307047

Gharib, M., Tao, H., Fungwe, T. V, et al., (2016). Cluster Differentiating 36 (CD36) Deficiency Attenuates Obesity-Associated Oxidative Stress in the Heart. *PloS One*, *11*(5), e0155611. doi: 10.1371/journal.pone.0155611

Gille, D., & Schmid, A. (2015). Vitamin B12 in meat and dairy products. *Nutrition Reviews*, *73*(2), 106–115. doi: 10.1093/nutrit/nuu011

Godfrey, K. M., Sheppard, A., Gluckman, P. D., et al., (2011). Epigenetic gene promoter methylation at birth is associated with child's later adiposity. *Diabetes*, *60*(5), 1528–34. doi: 10.2337/db10-0979

Goel, N., Karir, P., & Garg, V. K. (2017). Role of DNA methylation in human age prediction. *Mechanisms of Ageing and Development*, *166*, 33–41. doi: 10.1016/j.mad.2017.08.012

Gómez-Úriz, A. M., Milagro, F. I., Mansego, M. L., et al., (2015). Obesity and ischemic stroke modulate the methylation levels of KCNQ1 in white blood cells. *Human Molecular Genetics*, *24*(5), 1432–1440. doi: 10.1093/hmg/ddu559

Goni, L., Milagro, F. I., Cuervo, M., et al., (2014). Single-nucleotide polymorphisms and DNA methylation markers associated with central obesity and regulation of body weight. *Nutrition Reviews*, *72*(11), 673–690. doi: 10.1111/nure.12143

González-Muniesa, P., Mártinez-González, M. A., Hu, F. B., et al., (2017). Obesity. *Nature Reviews Disease Primers*, *3*, 17034. doi: 10.1038/nrdp.2017.34

Goyette, P., Sumner, J. S., Milos, R., et al., (1994). Human methylenetetrahydrofolate reductase: Isolation of cDNA, mapping and mutation identification. *Nature Genetics*, *7*(2), 195–200. doi: 10.1038/ng0694-195

Gregor, M. F., & Hotamisligil, G. S. (2011). Inflammatory Mechanisms in Obesity. *Annual Review of Immunology*, *29*(1), 415–445. doi: 10.1146/annurev-immunol-031210-101322

Groom, A., Potter, C., Swan, D. C., et al., (2012). Postnatal growth and DNA methylation are associated with differential gene expression of the TACSTD2 gene and childhood fat mass. *Diabetes*, *61*(2), 391–400. doi: 10.2337/db11-1039

Guay, S. P., Brisson, D., Lamarche, B., et al., (2014). ADRB3 gene promoter DNA methylation in blood and visceral adipose tissue is associated with metabolic disturbances in men. *Epigenomics*, *6*(1), 33–43. doi: 10.2217/epi.13.82

Guay, S.-P., Voisin, G., Brisson, D., et al., (2012). Epigenome-wide analysis in familial hypercholesterolemia identified new loci associated with high-density lipoprotein cholesterol concentration. *Epigenomics*, *4*(6), 623–639. https://doi.org/10.2217/epi.12.62

Guh, D. P., Zhang, W., Bansback, N., et al., (2009). The incidence of co-morbidities related to obesity and overweight: A systematic review and meta-analysis. *BMC Public Health*, *9*(1), 88. doi: 10.1186/1471-2458-9-88

Gunanti, I. R., Marks, G. C., Al-Mamun, A., et al., (2014). Low Serum Vitamin B-12 and Folate Concentrations and Low Thiamin and Riboflavin Intakes Are Inversely Associated with Greater Adiposity in Mexican American Children. *Journal of Nutrition*, *144*(12), 2027–2033. doi: 10.3945/jn.114.201202

Hall, E., Volkov, P., Dayeh, T., et al., (2014). Effects of palmitate on genome-wide mRNA expression and DNA methylation patterns in human pancreatic islets. *BMC Medicine*, *12*, 103. doi: 10.1186/1741-7015-12-103

Hamidi, T., Singh, A. K., & Chen, T. (2015). Genetic alterations of DNA methylation machinery in human diseases. *Epigenomics*, *7*(2), 247–265. doi: 10.2217/epi.14.80

Hardy, T., Zeybel, M., Day, C. P., et al., (2017). Plasma DNA methylation: A potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut*, *66*(7), 1321–1328. doi: 10.1136/gutjnl-2016-311526

Havel, R. J., Eder, H. A., & Bragdon, J. H. (1955). The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *The Journal of Clinical Investigation*, *34*(9), 1345–1353. doi: 10.1172/JCI103182

Heijmans, B. T., Tobi, E. W., Stein, A. D., et al., (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences*, *105*(44), 17046–17049. doi: 10.1073/pnas.0806560105

Hermsdorff, H. H., Mansego, M. L., Campión, J., et al., (2013). TNF-alpha promoter methylation in peripheral white blood cells: Relationship with circulating TNFα, truncal fat and n-6 PUFA intake in young women. *Cytokine*, *64*(1), 265–271. doi: 10.1016/j.cyto.2013.05.028

Hillman, S. L., Finer, S., Smart, M. C., et al., (2015). Novel DNA methylation profiles associated with key gene regulation and transcription pathways in blood and placenta of growth-restricted neonates. *Epigenetics*, *10*(1), 50–61. doi: 10.4161/15592294.2014.989741

Hiscott, J., Marois, J., Garoufalis, J., et al., (1993). Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Molecular and Cellular Biology*, *13*(10), 6231–6240. doi: 10.1128/MCB.13.10.6231

Hoeksema, M. A., Stöger, J. L., & de Winther, M. P. J. (2012). Molecular Pathways Regulating Macrophage Polarization: Implications for Atherosclerosis. *Current Atherosclerosis Reports*, *14*(3), 254–263. doi: 10.1007/s11883-012-0240-5

Holm, P. I., Hustad, S., Ueland, P. M., et al., (2007). Modulation of the homocysteine-betaine relationship by methylenetetrahydrofolate reductase 677 C->T genotypes and B-vitamin status in a large-scale epidemiological study. *Journal of Clinical Endocrinology and Metabolism*, *92*(4), 1535–1541. doi: 10.1210/jc.2006-1471

Holmes, M. V, Pulit, S. L., & Lindgren, C. M. (2017). Genetic and epigenetic studies of adiposity and cardiometabolic disease. *Genome Medicine*, *9*(1), 82. doi: 10.1186/s13073-017-0474-5

Horne, J. A., & Ostberg, O. (1976). A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *International Journal of Chronobiology*, *4*(2), 97–110.

Horvath, S., Erhart, W., Brosch, M., et al., (2014). Obesity accelerates epigenetic aging of human liver. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(43), 15538–43. doi: 10.1073/pnas.1412759111

Hotamisligil, G. S., & Erbay, E. (2008). Nutrient sensing and inflammation in metabolic diseases. *Nature Reviews Immunology*. doi: 10.1038/nri2449

Houseman, E. A., Accomando, W. P., Koestler, D. C., et al., (2012). DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*, *13*(1), 86. doi: 10.1186/1471-2105-13-86

Houshmand-Oeregaard, A., Hjort, L., Kelstrup, L., et al., (2017). DNA methylation and gene expression of TXNIP in adult offspring of women with diabetes in pregnancy. *PloS One*, *12*(10), e0187038. doi: 10.1371/journal.pone.0187038

Huang, T., Zheng, Y., Qi, Q., et al., (2015). DNA methylation variants at HIF3A locus, B-vitamin intake, and long-term weight change: Gene-diet interactions in two U.S. Cohorts. *Diabetes*, *64*(9), 3146–3154. doi: 10.2337/db15-0264

Huang, X., He, Z., Jiang, X., et al., (2016). Folic Acid Represses Hypoxia-Induced Inflammation in THP-1 Cells through Inhibition of the PI3K/Akt/HIF-1α Pathway. *PLOS ONE*, *11*(3), e0151553. doi: 10.1371/journal.pone.0151553

Hwalla, N., Al Dhaheri, A. S., Radwan, H., et al., (2017). The prevalence of micronutrient deficiencies and inadequacies in the middle east and approaches to interventions. *Nutrients*. doi: 10.3390/nu9030229

Idriss, A. A., Hu, Y., Sun, Q., et al., (2016). Prenatal betaine exposure modulates hypothalamic expression of cholesterol metabolic genes in cockerels through modifications of DNA methylation. *Poultry Science*, 1715–1724. doi: 10.3382/ps/pew437

Ingestas Dietéticas de Referencia (IDR) para la Población Española, 2010. (2010). *Actividad Dietetica*, *14*(4), 196–197. doi: 10.1016/S1138-0322(10)70039-0

Institute of Medicine (U.S.). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes., Institute of Medicine (U.S.). Subcommittee on Upper Reference Levels of Nutrients. (1998). Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B_6, folate, vitamin B_{12}, pantothenic acid, biotin, and choline. doi: 10.17226/6015

Irvin, M. R., Zhi, D., Joehanes, R., et al., (2014). Epigenome-wide association study of fasting blood lipids in the genetics of lipid-lowering drugs and diet network study. *Circulation*, *130*(7), 565–572. doi: 10.1161/CIRCULATIONAHA.114.009158

IUPAC. (2012). Fatty acids. *PAC, (Glossary of Class Names of Organic Compounds and Reactivity Intermediates Based on Structure (IUPAC Recommendations 1995)) 1335*, *1307*, 2330. doi:

10.1351/goldbook.F02330

Iyer, L. M., Zhang, D., & Aravind, L. (2016). Adenine methylation in eukaryotes: Apprehending the complex evolutionary history and functional potential of an epigenetic modification. *BioEssays*, *38*(1), 27–40. doi: 10.1002/bies.201500104

Jacobsen, S. C., Brøns, C., Bork-Jensen, J., et al., (2012). Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. *Diabetologia*, *55*(12), 3341–3349. doi: 10.1007/s00125-012-2717-8

Jang, H. S., Shin, W. J., Lee, J. E., et al., (2017). CpG and non-CpG methylation in epigenetic gene regulation and brain function. *Genes*. doi: 10.3390/genes8060148

Janssen, B. G., Godderis, L., Pieters, et al., (2013). Placental DNA hypomethylation in association with particulate air pollution in early life. *Particle and Fibre Toxicology*, *10*(1), 22. doi: 10.1186/1743-8977-10-22

Jiang, X., Greenwald, E., & Jack-Roberts, C. (2016). Effects of Choline on DNA Methylation and Macronutrient Metabolic Gene Expression in In Vitro Models of Hyperglycemia. *Nutrition and Metabolic Insights*, *9*, 11–17. doi: 10.4137/NMI.S29465

Jones, P. A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492. doi: 10.1038/nrg3230

Kaikkonen, M. U., Lam, M. T. Y., & Glass, C. K. (2011). Non-coding RNAs as regulators of gene expression and epigenetics. *Cardiovascular Research*. doi: 10.1093/cvr/cvr097

Kalani, A., Kamat, P. K., Givvimani, S., et al., (2014). Nutri-epigenetics Ameliorates Blood-Brain Barrier Damage and Neurodegeneration in Hyperhomocysteinemia: Role of Folic Acid. *Journal of Molecular Neuroscience*, *52*(2), 202–215. doi: 10.1007/s12031-013-0122-5

Kanzleiter, T., Jähnert, M., Schulze, G., et al., (2015). Exercise training alters DNA-methylation patterns in genes related to muscle growth and differentiation in mice. *American Journal of Physiology. Endocrinology and Metabolism*, *308*(10). doi: 10.1152/ajpendo.00289.2014.

Karimi, M., Vedin, I., Freund Levi, Y., et al., (2017). DHA-rich n-3 fatty acid supplementation decreases DNA methylation in blood leukocytes: the OmegAD study. *The American Journal of Clinical Nutrition*, *106*(4), 1157–1165. doi: 10.3945/ajcn.117.155648

Kato, N., Loh, M., Takeuchi, F., et al., (2015). Trans-ancestry genome-wide association study identifies 12 genetic loci influencing blood pressure and implicates a role for DNA methylation. *Nature Genetics*, *47*(11), 1282–1293. doi: 10.1038/ng.3405

Khan, M. J., Gerasimidis, K., Edwards, C. A., et al., (2016). Role of Gut Microbiota in the Aetiology of Obesity: Proposed Mechanisms and Review of the Literature. *Journal of Obesity*. doi: 10.1155/2016/7353642

Khot, V. V, Yadav, D. K., Shrestha, S., et al., (2017). Hypermethylated CpG sites in the MTR gene promoter in preterm placenta. *Epigenomics*, *9*(7), 985–996. doi: 10.2217/epi-2016-0173

Khyzha, N., Alizada, A., Wilson, M. D., et al., (2017). Epigenetics of Atherosclerosis: Emerging Mechanisms and Methods. *Trends in Molecular Medicine*. doi: 10.1016/j.molmed.2017.02.004

Kiec-Wilk, B., Sliwa, A., Mikolajczyk, M., et al., (2011). The CpG island methylation regulated expression of endothelial proangiogenic genes in response to beta-carotene and arachidonic acid. *Nutrition and Cancer*, *63*(7), 1053–1063. doi: 10.1080/01635581.2011.596644

Kigar, S. L., Chang, L., Guerrero, C. R., et al., (2017). N6-methyladenine is an epigenetic marker of mammalian early life stress. *Scientific Reports*, *7*(1), 18078. doi: 10.1038/s41598-017-18414-7

Kim, J., Bhattacharjee, R., Khalyfa, A., et al., (2012). DNA methylation in inflammatory genes among children with obstructive sleep apnea. *American Journal of Respiratory and Critical Care Medicine*, *185*(3), 330–338. doi: 10.1164/rccm.201106-1026OC

Kim, J. Y., Kwak, P. B., Gebert, M., et al., (2015). Purification and analysis of PERIOD protein complexes of the mammalian circadian clock. *Methods in Enzymology*, *551*, 197–210. doi: 10.1016/bs.mie.2014.10.013

Kim, S., Lee, H. S., Park, H. K., et al., (2017). Visceral adiposity and expression of clock genes in peripheral blood mononuclear cells: A pilot study. *Chronobiology International*, *34*(8), 1–10. doi: 10.1080/07420528.2017.1337780

Kim, T. J., Roesler, N. M., & von dem Knesebeck, O. (2017). Causation or selection – examining the relation between education and overweight/obesity in prospective observational studies: a meta-analysis. *Obesity Reviews*. doi: 10.1111/obr.12537

Kinde, B., Gabel, H. W., Gilbert, C. S., et al., (2015). Reading the unique DNA methylation landscape of the brain: Non-CpG methylation, hydroxymethylation, and MeCP2. *Proceedings of the National Academy of Sciences*, *112*(22), 6800–6806. doi: 10.1073/pnas.1411269112

Kirchner, H., Sinha, I., Gao, H., et al., (2016). Altered DNA methylation of glycolytic and lipogenic genes in liver from obese and type 2 diabetic patients. *Molecular Metabolism*, *5*(3), 171–183. doi: 10.1016/j.molmet.2015.12.004

Kirwan, A. M., Lenighan, Y. M., O'Reilly, et al., (2017). Nutritional modulation of metabolic inflammation. *Biochemical Society Transactions*, *45*(4), BST20160465. doi: 10.1042/BST20160465

Kodama, K., Horikoshi, M., Toda, K., et al., (2012). Expression-based genome-wide association study links the receptor CD44 in adipose tissue with type 2 diabetes. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(18), 7049–54. doi: 10.1073/pnas.1114513109

Kolb, A. F., & Petrie, L. (2013). Folate deficiency enhances the inflammatory response of macrophages. *Molecular Immunology*, *54*(2), 164–172. doi: 10.1016/j.molimm.2012.11.012

Kovacheva, V. P., Mellott, T. J., Davison, J. M., et al., (2007). Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. *Journal of Biological Chemistry*, *282*(43), 31777–31788. doi: 10.1074/jbc.M705539200

Koziol, M. J., Bradshaw, C. R., Allen, G. E., et al., (2016). Identification of methylated deoxyadenosines in vertebrates reveals diversity in DNA modifications. *Nature Structural and Molecular Biology*, *23*(1), 24–30. doi: 10.1038/nsmb.3145

Kresovich, J. K., Zheng, Y., Cardenas, A., et al., (2017). Cord blood DNA methylation and adiposity measures in early and mid-childhood. *Clinical Epigenetics*, *9*(1), 86. doi: 10.1186/s13148-017-0384-9

Krishnaveni, G. V., Veena, S. R., Karat, S. C., et al., (2014). Association between maternal folate concentrations during pregnancy and insulin resistance in Indian children. *Diabetologia*, *57*(1), 110–121. doi: 10.1007/s00125-013-3086-7

Kritis, A. A., Gouta, C. P., Liaretidou, E. I., et al., (2016). Latest aspects of aldosterone actions on the heart muscle. *Journal of Physiology and Pharmacology*. American Society for Nutrition. doi: 10.1525/jlin.2007.17.1.130.130

Kuehnen, P., Mischke, M., Wiegand, S., et al., (2012). An alu element-associated hypermethylation variant of the POMC gene is associated with childhood obesity. *PLoS Genetics*, *8*(3), e1002543. doi: 10.1371/journal.pgen.1002543

Kulkarni, A., Dangat, K., Kale, A., et al., (2011). Effects of Altered Maternal Folic Acid, Vitamin B12 and Docosahexaenoic Acid on Placental Global DNA Methylation Patterns in Wistar Rats. *PLoS ONE*, *6*(3), e17706. doi: 10.1371/journal.pone.0017706

Labbé, C., Robles, V., & Herraez, M. P. (2017). Epigenetics in fish gametes and early embryo. *Aquaculture*, *472*, 93–106. doi: 10.1016/j.aquaculture.2016.07.026

Laker, R. C., Garde, C., Camera, D. M., et al., (2017). Transcriptomic and epigenetic responses to short-term nutrient-exercise stress in humans. *Scientific Reports*, *7*(1), 15134. doi: 10.1038/s41598-017-15420-7

Larsen, G. L., & Henson, P. M. (1983). Mediators of inflammation. *Annual Review of Immunology*, *1*(85), 335–59. doi: 10.1146/annurev.iy.01.040183.002003

Lee, C., & Huang, C. H. (2014). LASAGNA-Search 2.0: Integrated transcription factor binding site search and visualization in a browser. *Bioinformatics*, *30*(13), 1923–1925. doi: 10.1093/bioinformatics/btu115

Leygo, C., Williams, M., Jin, H. C., et al., (2017). DNA Methylation as a Noninvasive Epigenetic Biomarker for the Detection of Cancer. *Disease Markers*, *2017*, 1–13. doi: 10.1155/2017/3726595

Li, C. C. Y., Maloney, C. A., Cropley, J. E., et al., (2010). Epigenetic programming by maternal nutrition : shaping future generations Review. *Epigenomics*, *2*(4), 539–549. doi: 10.2217/epi.10.33

Li, J., Goh, C. E., Demmer, R. T., et al., (2017). Association between Serum Folate and Insulin Resistance among U.S. Nondiabetic Adults. *Scientific Reports*, *7*(1), 9187. doi: 10.1038/s41598-017-09522-5

Li, Z., Gueant-rodriguez, R., & Quilliot, D. (2017). Folate and vitamin B12 status is associated with insulin resistance and metabolic syndrome in morbid obesity. *Clinical Nutrition*, 1–7. doi: 10.1016/j.clnu.2017.07.008

Liew, S. C. (2016). Folic acid and diseases - supplement it or not? *Revista Da Associação Médica Brasileira*, *62*(1), 90–100. doi: 10.1590/1806-9282.62.01.90

Lim, U., & Song, M. A. (2012). Dietary and lifestyle factors of DNA methylation. *Methods in Molecular Biology*. doi: 10.1007/978-1-61779-612-8_23

Lin, C. S., & Wu, R. D. (1986). Choline oxidation and choline dehydrogenase. *Journal of Protein Chemistry*, *5*(3), 193–200. doi: 10.1007/BF01025488

Lin, X., Lim, I. Y., Wu, Y., et al., (2017). Developmental pathways to adiposity begin before birth and are influenced by genotype, prenatal environment and epigenome. *BMC Medicine*, *15*(1), 50. doi: 10.1186/s12916-017-0800-1

Ling, C., Del Guerra, S., Lupi, R., et al., (2008). Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia*, *51*(4), 615–622. doi: 10.1007/s00125-007-0916-5

Lipsky, L. M., Gee, B., Liu, A., et al., (2017). Body mass index and adiposity indicators associated with cardiovascular biomarkers in youth with type 1 diabetes followed prospectively. *Pediatric Obesity*, *12*(6), 468–476. doi: 10.1111/ijpo.12167

Lister, R., Pelizzola, M., Dowen, R. H., et al., (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, *462*(7271), 315–322. doi: 10.1038/nature08514

Liu, H., Wang, J., Mou, D., et al., (2017). Maternal methyl donor supplementation during gestation counteracts

the bisphenol a-induced impairment of intestinal morphology, disaccharidase activity, and nutrient transporters gene expression in newborn and weaning pigs. *Nutrients*, *9*(5). doi: 10.3390/nu9050423

Liu, L. F., Kodama, K., Wei, K., et al., (2015). The receptor CD44 is associated with systemic insulin resistance and proinflammatory macrophages in human adipose tissue. *Diabetologia*, *58*(7), 1579–1586. doi: 10.1007/s00125-015-3603-y

Liu, T., Zhang, L., Joo, D., et al., (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*, 17023. doi: 10.1038/sigtrans.2017.23

Liu, X., Chen, Q., Tsai, H. J., et al., (2014). Maternal preconception body mass index and offspring cord blood DNA methylation: Exploration of early life origins of disease. *Environmental and Molecular Mutagenesis*, *55*(3), 223–230. doi: 10.1002/em.21827

Lopez-Legarrea, P., de la Iglesia, R., Abete, I., et al., (2014). The protein type within a hypocaloric diet affects obesity-related inflammation: The RESMENA project. *Nutrition*, *30*(4), 424–429. doi: 10.1016/j.nut.2013.09.009

Lopez-Legarrea, P., Mansego, M. L., Zulet, M. A., et al., (2013). SERPINE1, PAI-1 protein coding gene, methylation levels and epigenetic relationships with adiposity changes in obese subjects with metabolic syndrome features under dietary restriction. *Journal of Clinical Biochemistry and Nutrition*, *53*(3), 139–144. doi: 10.3164/jcbn.13-54

Lopez-Minguez, J., Gómez-Abellán, P., & Garaulet, M. (2016). Circadian rhythms, food timing and obesity. *Proceedings of the Nutrition Society*, *75*(4), 501–511. doi: 10.1017/S0029665116000628

Luger, K., Mäder, A. W., Richmond, R. K., et al., (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, *389*(6648), 251–260. doi: 10.1038/38444

Lyons, C. L., Kennedy, E. B., & Roche, H. M. (2016). Metabolic inflammation-differential modulation by dietary constituents. *Nutrients*. Multidisciplinary Digital Publishing Institute. doi: 10.3390/nu8050247

Ma, Y., Smith, C. E., Lai, C.-Q., et al., (2016). The effects of omega-3 polyunsaturated fatty acids and genetic variants on methylation levels of the interleukin-6 gene promoter. *Molecular Nutrition & Food Research*, *60*(2), 410–9. doi: 10.1002/mnfr.201500436

Mahabir, S., Ettinger, S., Johnson, L., et al., (2008). Measures of adiposity and body fat distribution in relation to serum folate levels in postmenopausal women in a feeding study. *European Journal of Clinical Nutrition*, *62*(5), 644–650. doi: 10.1038/sj.ejcn.1602771

Makki, K., Froguel, P., & Wolowczuk, I. (2013). Adipose Tissue in Obesity-Related Inflammation and Insulin Resistance: Cells, Cytokines, and Chemokines. *ISRN Inflammation*, *2013*, 1–12. doi: 10.1155/2013/139239

Mamtani, M., Kulkarni, H., Dyer, T. D., et al., (2016). Genome- and epigenome-wide association study of hypertriglyceridemic waist in Mexican American families. *Clinical Epigenetics*, *8*(1), 6. doi: 10.1186/s13148-016-0173-x

Manning, L. (2017). Categorizing food-related illness: Have we got it right? *Critical Reviews in Food Science and Nutrition*, *57*(9), 1938–1949. doi: 10.1080/10408398.2015.1038776

Mansego, M. L., Milagro, F. I., Campión, J., et al., (2013). Techniques of DNA Methylation Analysis with Nutritional Applications. *Journal of Nutrigenetics and Nutrigenomics*, *6*(2), 83–96. doi: 10.1159/000350749

Mansego, M. L., Milagro, F. I., Zulet, M. A., et al., (2015). Differential DNA Methylation in Relation to Age and Health Risks of Obesity. *International Journal of Molecular Sciences*, *16*(8), 16816–16832. doi: 10.3390/ijms160816816

Mantovani, A., Biswas, S. K., Galdiero, M. R., et al., (2013). Macrophage plasticity and polarization in tissue repair and remodelling. *Journal of Pathology*. doi: 10.1002/path.4133

Mao, L. L., Xiao, X. H., Zhang, Q., et al., (2017). DNA Methylation and Birth Weight: a Genome-wide Analysis. *Biomedical and Environmental Sciences*, *30*(9), 667–670. doi: 10.3967/bes2017.088

Mao, S., Sun, J., Gu, T., et al., (2017). Hypomethylation of interleukin-6 (IL-6) gene increases the risk of essential hypertension: a matched case–control study. *Journal of Human Hypertension*, *31*(8), 530–536. doi: 10.1038/jhh.2017.7

Marco, A., Kisliouk, T., Tabachnik, T., et al., (2014). Overweight and CpG methylation of the Pomc promoter in offspring of high-fat-diet-fed dams are not "reprogrammed" by regular chow diet in rats. *FASEB Journal*, *28*(9), 4148–4157. doi: 10.1096/fj.14-255620

Marcuello, C., Calle-Pascual, A. L., Fuentes, M., et al., (2013). Prevalence of the metabolic syndrome in Spain using regional cutoff points for waist circumference: The di@bet.es study. *Acta Diabetologica*, *50*(4), 615–623. doi: 10.1007/s00592-013-0468-8

Marques-Rocha, J. L., Samblas, M., Milagro, F. I., et al., (2015). Noncoding RNAs, cytokines, and inflammation-related diseases. *FASEB Journal*, *29*(9), 3595–611. doi: 10.1096/fj.14-260323

Martín-Peláez, S., Mosele, J. I., Pizarro, N., et al., (2017). Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota. *European Journal of Nutrition*, *56*(1), 119–131. doi: 10.1007/s00394-015-1063-2

Martínez, J. A., Cordero, P., Campión, J., et al., (2012). Interplay of early-life nutritional programming on obesity, inflammation and epigenetic outcomes. *Proceedings of the Nutrition Society*, *71*(2), 276–283. doi: 10.1017/S0029665112000055

Martínez de Morentin Aldabe, B. E., Hernández Ruiz de Eguilaz, M., Pérez Díez, S., et al., (2013). Influencia de la administración de psicofármacos en el aumento del peso corporal. *Revista Española de Nutrición Humana Y Dietética*. doi: 10.14306/renhyd.17.1.4

McCullough, L. E., Miller, E. E., Calderwood, L. E., et al., (2017). Maternal inflammatory diet and adverse pregnancy outcomes: Circulating cytokines and genomic imprinting as potential regulators? *Epigenetics*, doi: 10.1080/15592294.2017.1347241

McCullough, L. E., Miller, E. E., Mendez, M. A., et al., (2016). Maternal B vitamins: effects on offspring weight and DNA methylation at genomically imprinted domains. *Clinical Epigenetics*, *8*, 8. doi: 10.1186/s13148-016-0174-9

McEwen, L. M., Gatev, E. G., Jones, M. J., et al., (2017). DNA methylation signatures in peripheral blood mononuclear cells from a lifestyle intervention for women at midlife: A pilot RCT. *Applied Physiology, Nutrition, and Metabolism*, doi: 10.1139/apnm-2017-0436

McKay, J. A., Groom, A., Potter, C., et al., (2012). Genetic and non-genetic influences during pregnancy on infant global and site specific DNA methylation: role for folate gene variants and vitamin B12. *PloS One*, *7*(3), e33290. doi: 10.1371/journal.pone.0033290

McKay, J. A., & Mathers, J. C. (2016). Maternal folate deficiency and metabolic dysfunction in offspring.

Proceedings of the Nutrition Society, *75*(1), 90–95. https://doi.org/10.1017/S0029665115004280

McKee, S. E., Grissom, N. M., Herdt, C. T., et al., (2017). Methyl donor supplementation alters cognitive performance and motivation in female offspring from high-fat diet-fed dams. *FASEB Journal*, *31*(6), 2352–2363. doi: 10.1096/fj.201601172R

McNulty, H., & Pentieva, K. (2004). Folate bioavailability. *Proceedings of the Nutrition Society*, *63*(4), 529–536. doi: 10.1079/PNS2004383

Medici, V., Shibata, N. M., Kharbanda, K. K., et al., (2014). Maternal choline modifies fetal liver copper, gene expression, DNA methylation, and neonatal growth in the tx-j mouse model of Wilson disease. *Epigenetics*, *9*(2), 286–296. doi: 10.4161/epi.27110

Meeks, K. A. C., Henneman, P., Venema, A., et al., (2017). An epigenome-wide association study in whole blood of measures of adiposity among Ghanaians: the RODAM study. *Clinical Epigenetics*, *9*, 103. doi: 10.1186/s13148-017-0403-x

Mehta, R., Birerdinc, A., & Younossi, Z. M. (2014). Host genetic variants in obesity-related nonalcoholic fatty liver disease. *Clinics in Liver Disease*. doi: 10.1016/j.cld.2013.09.017

Mendelson, M. M., Marioni, R. E., Joehanes, R., et al., (2017). Association of Body Mass Index with DNA Methylation and Gene Expression in Blood Cells and Relations to Cardiometabolic Disease: A Mendelian Randomization Approach. *PLoS Medicine*, *14*(1), e1002215. doi: 10.1371/journal.pmed.1002215

Milagro, F. I., Campion, J., Cordero, P., et al., (2011). A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. *The FASEB Journal*, *25*(4), 1378–1389. doi: 10.1096/fj.10-170365

Milagro, F. I., Gomez-Abellan, P., Campion, J., et al., (2012). CLOCK, PER2 and BMAL1 DNA methylation: association with obesity and metabolic syndrome characteristics and monounsaturated fat intake. *Chronobiology International*, *29*(9), 1180–1194. doi: 10.3109/07420528.2012.719967

Milagro, F. I., Mansego, M. L., De Miguel, C., et al., (2013). Dietary factors, epigenetic modifications and obesity outcomes: Progresses and perspectives. *Molecular Aspects of Medicine*. doi: 10.1016/j.mam.2012.06.010

Moher, D., Hopewell, S., Schulz, K. F., et al., (2012). CONSORT 2010 explanation and elaboration: Updated guidelines for reporting parallel group randomised trials. *International Journal of Surgery*, *10*(1), 28–55. doi: 10.1016/j.ijsu.2011.10.001

Mohr, A. M., & Mott, J. L. (2015). Overview of microRNA biology. *Seminars in Liver Disease*. doi: 10.1055/s-0034-1397344

Moleres, A., Campion, J., Milagro, F. I., et al., (2013). Differential DNA methylation patterns between high and low responders to a weight loss intervention in overweight or obese adolescents: the EVASYON study. *The FASEB Journal*, *27*(6), 2504–2512. doi: 10.1096/fj.12-215566

Monastero, R., García-Serrano, S., Lago-Sampedro, A., et al., (2017). Methylation patterns of Vegfb promoter are associated with gene and protein expression levels: the effects of dietary fatty acids. *European Journal of Nutrition*, *56*(2), 715–726. doi: 10.1007/s00394-015-1115-7

Monteiro, R., & Azevedo, I. (2010). Chronic inflammation in obesity and the metabolic syndrome. *Mediators of Inflammation*. doi: 10.1155/2010/289645

Morales, E., Groom, A., Lawlor, D. A., et al., (2014). DNA methylation signatures in cord blood associated with

maternal gestational weight gain: results from the ALSPAC cohort. *BMC Research Notes*, *7*(1), 278. doi: 10.1186/1756-0500-7-278

Mukerjee, S., Zhu, Y., Zsombok, A., et al., (2017). Perinatal Exposure to Western Diet Programs Autonomic Dysfunction in the Male Offspring. *Cellular and Molecular Neurobiology*. doi: 10.1007/s10571-017-0502-4

Murphy, S. K., Yang, H., Moylan, C. A., et al., (2013). Relationship between methylome and transcriptome in patients with nonalcoholic fatty liver disease. *Gastroenterology*, *145*(5), 1076–1087. doi: 10.1053/j.gastro.2013.07.047

Na, Y. K., Hong, H. S., Lee, W. K., et al., (2015). Increased Methylation of Interleukin 6 Gene Is Associated with Obesity in Korean Women. *Molecules and Cells*, *38*(5), 452–456. doi: 10.14348/molcells.2015.0005Neidhart, M. (2016). *DNA methylation and complex human disease*. doi: 10.1016/B978-0-12-420194-1

Nicoletti, C., Cortes-Oliveira, C., Pinhel, M., et al., (2017). Bariatric Surgery and Precision Nutrition. *Nutrients*, *9*(9), 974. doi: 10.3390/nu9090974

Nicoletti, C. F., Nonino, C. B., de Oliveira, B. A. P., et al., (2016). DNA Methylation and Hydroxymethylation Levels in Relation to Two Weight Loss Strategies: Energy-Restricted Diet or Bariatric Surgery. *Obesity Surgery*, *26*(3), 603–611. doi: 10.1007/s11695-015-1802-8

Nilsson, E., Jansson, P. A., Perfilyev, A., et al., (2014). Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. *Diabetes*, *63*(9), 2962–2976. doi: 10.2337/db13-1459

Nilsson, E., & Ling, C. (2017). DNA methylation links genetics, fetal environment, and an unhealthy lifestyle to the development of type 2 diabetes. *Clinical Epigenetics*, *9*(1), 105. doi: 10.1186/s13148-017-0399-2

Nilsson, E., Matte, A., Perfilyev, A., et al., (2015). Epigenetic Alterations in Human Liver From Subjects With Type 2 Diabetes in Parallel With Reduced Folate Levels. *The Journal of Clinical Endocrinology & Metabolism*, *100*(11), E1491–E1501. doi: 10.1210/jc.2015-3204

Noronha, A. M., Liang, Y., Hetzel, J. T., et al., (2009). Hyperactivated B cells in human inflammatory bowel disease. *Journal of Leukocyte Biology*, *86*(4), 1007–1016. doi: 10.1189/jlb.0309203

Ntanasis-Stathopoulos, J., Tzanninis, J. G., Philippou, A., et al., (2013). Epigenetic regulation on gene expression induced by physical exercise. *Journal of Musculoskeletal Neuronal Interactions*. doi: 10.17756/jrds.2015-011

Obermann-Borst, S. A., Eilers, P. H. C., Tobi, E. W., et al., (2013). Duration of breastfeeding and gender are associated with methylation of the LEPTIN gene in very young children. *Pediatric Research*, *74*(3), 344–349. doi: 10.1038/pr.2013.95

Ochoa, M. C., Moreno-Aliaga, M. J., Martinez-Gonzalez, M. A., et al., (2007). Predictor factors for childhood obesity in a Spanish case-control study. *Nutrition (Burbank, Los Angeles County, Calif.)*, *23*(5), 379–384. doi: 10.1016/j.nut.2007.02.004

Oelsner, K. T., Guo, Y., To, S. B. C., et al., (2017). Maternal BMI as a predictor of methylation of obesity-related genes in saliva samples from preschool-age Hispanic children at-risk for obesity. *BMC Genomics*, *18*(1), 57. doi: 10.1186/s12864-016-3473-9

Oken, E., & Gillman, M. W. (2003). Fetal Origins of Obesity. *Obesity Research*, *11*(4), 496–506. doi:

10.1038/oby.2003.69

Onat, A. (2011). Metabolic syndrome: nature, therapeutic solutions and options. *Expert Opinion on Pharmacotherapy*, *12*(12), 1887–1900. doi: 10.1517/14656566.2011.585462

Opal, S. M., & DePalo, V. A. (2000). Anti-inflammatory cytokines. *Chest*, *117*(4), 1162–1172. doi: 10.1378/chest.117.4.1162

Papazyan, R., Zhang, Y., & Lazar, M. A. (2016). Genetic and epigenomic mechanisms of mammalian circadian transcription. *Nature Structural and Molecular Biology*. doi: 10.1038/nsmb.3324

Pappa, K. I., Gazouli, M., Anastasiou, E., et al., (2013). The major circadian pacemaker ARNT-like protein-1 (BMAL1) is associated with susceptibility to gestational diabetes mellitus. *Diabetes Research and Clinical Practice*, *99*(2), 151–157. doi: 10.1016/j.diabres.2012.10.015

Pauwels, S., Ghosh, M., Duca, R. C., et al., (2017a). Dietary and supplemental maternal methyl-group donor intake and cord blood DNA methylation. *Epigenetics*, *12*(1), 1–10. doi: 10.1080/15592294.2016.1257450

Pauwels, S., Ghosh, M., Duca, R. C., et al., (2017c). Maternal intake of methyl-group donors affects DNA methylation of metabolic genes in infants. *Clinical Epigenetics*, *9*(1), 16. doi: 10.1186/s13148-017-0321-y

Pauwels, S., Truijen, I., Ghosh, M., et al., (2017b). The effect of paternal methyl-group donor intake on offspring DNA methylation and birth weight. *Journal of Developmental Origins of Health and Disease*, *8*(3), 311–321. doi: 10.1017/S2040174417000046

Pawlak, R., Parrott, S. J., Raj, S., et al., (2013). How prevalent is vitamin B12 deficiency among vegetarians? *Nutrition Reviews*. doi: 10.1111/nure.12001

Perez-Cornago, A., Lopez-Legarrea, P., de la Iglesia, R., et al., (2014). Longitudinal relationship of diet and oxidative stress with depressive symptoms in patients with metabolic syndrome after following a weight loss treatment: The RESMENA project. *Clinical Nutrition*, *33*(6), 1061–1067. doi: 10.1016/j.clnu.2013.11.011

Perez-Llamas, F., Garaulet, M., Torralba, C., et al., (2012). Development of a current version of a software application for research and practice in human nutrition (GRUNUMUR 2.0). *Nutricion Hospitalaria*, *27*(5), 1576–1582. doi: 10.3305/nh.2012.27.5.5940

Perfilyev, A., Dahlman, I., Gillberg, L., et al., (2017). Impact of polyunsaturated and saturated fat overfeeding on the DNA-methylation pattern in human adipose tissue: A randomized controlled trial. *American Journal of Clinical Nutrition*, *105*(4), 991–1000. doi: 10.3945/ajcn.116.143164

Perng, W., Mora-Plazas, M., Marín, C., et al., (2013). A prospective study of LINE-1DNA methylation and development of adiposity in school-age children. *PloS One*, *8*(4), e62587. doi: 10.1371/journal.pone.0062587

Perry, C., Yu, S., Chen, J., et al., (2007). Effect of Vitamin B6 Availability of Serine Hydroxymethyltransferase in MCF-7 cells. *Arch. Biochem. Biophys.*, *462*(1), 21–27. doi: 10.1016/j.biotechadv.2011.08.021.Secreted

Peter, C. J., Fischer, L. K., Kundakovic, M., et al., (2016). DNA Methylation Signatures of Early Childhood Malnutrition Associated With Impairments in Attention and Cognition. *Biological Psychiatry*, *80*(10), 765–774. doi: 10.1016/j.biopsych.2016.03.2100

Petrus, P., Bialesova, L., Checa, A., et al., (2017). Adipocyte Expression of SLC19A1 Links DNA Hypermethylation to Adipose Tissue Inflammation and Insulin Resistance. *The Journal of Clinical Endocrinology & Metabolism*. doi: 10.1210/jc.2017-01382

Pikaard, C. S., & Scheid, O. M. (2014). Epigenetic regulation in plants. *Cold Spring Harbor Perspectives in Biology*, *6*(12), a019315. doi: 10.1101/cshperspect.a019315

Pinney, S. (2014). Mammalian Non-CpG Methylation: Stem Cells and Beyond. *Biology*, *3*(4), 739–751. doi: 10.3390/biology3040739

Pirini, F., Rodriguez-Torres, S., Ayandibu, B. G., et al.,. (2018). INSIG2 rs7566605 single nucleotide variant and global DNA methylation index levels are associated with weight loss in a personalized weight reduction program. *Molecular Medicine Reports*, *17*(1), 1699–1709. doi: 10.3892/mmr.2017.803

Pirola, C. J., Gianotti, T. F., Burgueño, A. L., et al., (2013). Epigenetic modification of liver mitochondrial DNA is associated with histological severity of nonalcoholic fatty liver disease. *Gut*, *62*(9), 1356–63. doi: 10.1136/gutjnl-2012-302962

Pirola, C. J., Scian, R., Gianotti, T. F., et al., (2015). Epigenetic Modifications in the Biology of Nonalcoholic Fatty Liver Disease. *Medicine*, *94*(36), e1480. doi: 10.1097/MD.0000000000001480

Piyathilake, C. J., Macaluso, M., Chambers, M. M., et al., (2014). Folate and vitamin B12 may play a critical role in lowering the HPV 16 methylation-associated risk of developing higher grades of CIN. *Cancer Prevention Research (Philadelphia, Pa.)*, *7*(11), 1128–37. doi: 10.1158/1940-6207.CAPR-14-0143

Plusquin, M., Guida, F., Polidoro, S., et al., (2017). DNA methylation and exposure to ambient air pollution in two prospective cohorts. *Environment International*, *108*, 127–136. doi: 10.1016/j.envint.2017.08.006

Ponting, C. P., Oliver, P. L., & Reik, W. (2009). Evolution and Functions of Long Noncoding RNAs. *Cell*. doi: 10.1016/j.cell.2009.02.006

Portela, A., & Esteller, M. (2010). Epigenetic modifications and human disease. *Nature Biotechnology*. doi: 10.1038/nbt.1685

Powell, N. D., Sloan, E. K., Bailey, M. T., et al., (2013). Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via beta-adrenergic induction of myelopoiesis. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(41), 16574–16579. doi: 10.1073/pnas.1310655110

Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *American Journal of Clinical Nutrition*. American Society for Nutrition. doi: 10.1074/jbc.M505002200

Pusceddu, I., Herrmann, M., Kirsch, S. H., et al., (2016). Prospective study of telomere length and LINE-1 methylation in peripheral blood cells: the role of B vitamins supplementation. *European Journal of Nutrition*, *55*(5), 1863–1873. doi: 10.1007/s00394-015-1003-1

Quarmyne, M., Doan, P. L., Himburg, H. A., et al., (2015). Protein tyrosine phosphatase-σ regulates hematopoietic stem cell-repopulating capacity. *The Journal of Clinical Investigation*, *125*(1), 177–82. doi: 10.1172/JCI77866

Quinn, J. J., & Chang, H. Y. (2015). Unique features of long non-coding RNA biogenesis and function. *Nature Reviews Genetics*, *17*(1), 47–62. doi: 10.1038/nrg.2015.10

Racioppi, L., & Means, A. R. (2012). Calcium/calmodulin-dependent protein kinase kinase 2: Roles in signaling and pathophysiology. *Journal of Biological Chemistry*. doi: 10.1074/jbc.R112.356485

Ramos-Lopez, O., Arpón, A., Riezu-Boj, J. I., et al., (2018). DNA methylation patterns at sweet taste transducing genes are associated with BMI and carbohydrate intake in an adult population. *Appetite*, *120*, 230–239. doi: 10.1016/j.appet.2017.09.004

Reik, W. (2001). Epigenetic Reprogramming in Mammalian Development. *Science*, *293*(5532), 1089–1093. doi: 10.1126/science.1063443

Relton, C. L., Groom, A., St. Pourcain, B., et al., (2012). DNA Methylation Patterns in Cord Blood DNA and Body Size in Childhood. *PLoS ONE*, *7*(3), e31821. doi: 10.1371/journal.pone.0031821

Remely, M., Lovrecic, L., De La Garza, A. L., et al., (2015). Therapeutic perspectives of epigenetically active nutrients. *British Journal of Pharmacology*. doi: 10.1111/bph.12854

Rendo-Urteaga, T., García-Calzón, S., González-Muniesa, P., et al., (2015). Peripheral blood mononuclear cell gene expression profile in obese boys who followed a moderate energy-restricted diet: Differences between high and low responders at baseline and after the intervention. *British Journal of Nutrition*, *113*(2), 331–342. doi: 10.1017/S0007114514003584

Rhee, J. K., Lee, J. H., Yang, H. K., et al., (2017). DNA Methylation Profiles of Blood Cells Are Distinct between Early-Onset Obese and Control Individuals. *Genomics & Informatics*, *15*(1), 28–37. doi: 10.5808/GI.2017.15.1.28

Richard, M. A., Huan, T., Ligthart, S., et al., (2017). DNA Methylation Analysis Identifies Loci for Blood Pressure Regulation. *American Journal of Human Genetics*, 888–902. doi: 10.1016/j.ajhg.2017.09.028

Richards, J., Diaz, A. N., & Gumz, M. L. (2014). Clock genes in hypertension: novel insights from rodent models. *Blood Pressure Monitoring*, *19*(5), 249–54. doi: 10.1097/MBP.0000000000000060

Robertson, K. D. (2005). DNA methylation and human disease. *Nature Reviews Genetics*. doi: 10.1038/nrg1655

Roe, N. D., He, E. Y., Wu, Z., et al., (2013). Folic acid reverses nitric oxide synthase uncoupling and prevents cardiac dysfunction in insulin resistance: Role of Ca2+/calmodulin-activated protein kinase II. *Free Radical Biology and Medicine*, *65*, 234–243. doi: 10.1016/j.freeradbiomed.2013.06.042

Rondelet, G., & Wouters, J. (2017). Human DNA (cytosine-5)-methyltransferases: a functional and structural perspective for epigenetic cancer therapy. *Biochimie*. doi: 10.1016/j.biochi.2017.06.003

Rönn, T., Volkov, P., Davegardh, C., et al., (2013). A Six Months Exercise Intervention Influences the Genome-wide DNA Methylation Pattern in Human Adipose Tissue. *PLoS Genetics*, *9*(6), e1003572. doi: 10.1371/journal.pgen.1003572

Rönn, T., Volkov, P., Gillberg, L., et al., (2015). Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood. *Human Molecular Genetics*, *24*(13), 3792–3813. doi: 10.1093/hmg/ddv124

Rounge, T. B., Page, C. M., Lepistö, M., et al., (2016). Genome-wide DNA methylation in saliva and body size of adolescent girls. *Epigenomics*, *8*(11), 1495–1505. doi: 10.2217/epi-2016-0045

Rudkowska, I., Perusse, L., Bellis, C., et al., (2015). Interaction between Common Genetic Variants and Total Fat Intake on Low-Density Lipoprotein Peak Particle Diameter: A Genome-Wide Association Study. *Journal of Nutrigenetics and Nutrigenomics*, *8*(1), 44–53. doi: 10.1159/000431151

Rzehak, P., Covic, M., Saffery, R., et al., (2017). DNA-Methylation and Body Composition in Preschool Children: Epigenome-Wide-Analysis in the European Childhood Obesity Project (CHOP)-Study. *Scientific Reports*, *7*(1), 14349. doi: 10.1038/s41598-017-13099-4

Sabet, J. A., Park, L. K., Iyer, L. K., et al., (2016). Paternal B vitamin intake is a determinant of growth, hepatic lipid metabolism and intestinal tumor volume in female Apc1638N mouse offspring. *PLoS ONE*, *11*(3), e0151579. doi: 10.1371/journal.pone.0151579

Saeed, S., Quintin, J., Kerstens, H. H. D., et al., (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science*, *345*(6204), 1251086. doi: 10.1126/science.1251086

Sahar, S., & Sassone-Corsi, P. (2013). The epigenetic language of circadian clocks. *Handbook of Experimental Pharmacology, (217):29-4*(217), 29–44. doi: 10.1007/978-3-642-25950-0_2

Sakamoto, A., Nishimura, Y., Ono, H., et al., (2002). Betaine and homocysteine concentrations in foods. *Pediatrics International*, *44*(4), 409–413. doi: 10.1046/j.1442-200X.2002.01591.x

Salas-Salvadó, J., Rubio, M. A., Barbany, M., et al., (2007). Consenso SEEDO 2007 para la evaluación del sobrepeso y la obesidad y el establecimiento de criterios de intervención terapéutica. *Medicina Clínica*, *128*(5), 184–196. doi: 10.1016/S0025-7753(07)72531-9

Samaniego-Vaesken, M. L., Alonso-Aperte, E., & Varela-Moreiras, G. (2017). Voluntary folic acid fortification levels and nutrient composition of food products from the Spanish market: A 2011–2015 update. *Nutrients*, *9*(3). doi: 10.3390/nu9030234

San-Cristobal, R., Navas-Carretero, S., Milagro, F. I., et al., (2016). Gene methylation parallelisms between peripheral blood cells and oral mucosa samples in relation to overweight. *Journal of Physiology and Biochemistry*, *73*(3), 465–474. doi: 10.1007/s13105-017-0560-6

Sanchez-Guerra, M., Zheng, Y., Osorio-Yanez, C., et al., (2015). Effects of particulate matter exposure on blood 5-hydroxymethylation: Results from the Beijing truck driver air pollution study. *Epigenetics*, *10*(7), 633–642. doi: 10.1080/15592294.2015.1050174

Saxonov, S., Berg, P., & Brutlag, D. L. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proceedings of the National Academy of Sciences*, *103*(5), 1412–1417. doi: 10.1073/pnas.0510310103

Sazci, A., Ergul, E., Aygun, C., et al., (2008). Methylenetetrahydrofolate reductase gene polymorphisms in patients with nonalcoholic steatohepatitis (NASH). *Cell Biochemistry and Function*, *26*(3), 291–296. doi: 10.1002/cbf.1424

Seki, Y., Suzuki, M., Guo, X., et al., (2017). In Utero Exposure to a High-Fat Diet Programs Hepatic Hypermethylation and Gene Dysregulation and Development of Metabolic Syndrome in Male Mice. *Endocrinology*, *158*(9), 2860–2872. doi: 10.1210/en.2017-00334

Severin, P. M. D., Zou, X., Schulten, K., et al., (2013). Effects of cytosine hydroxymethylation on DNA strand separation. *Biophysical Journal*, *104*(1), 208–215. doi: 10.1016/j.bpj.2012.11.013

Sharma, A. (2017). Transgenerational epigenetics: Integrating soma to germline communication with gametic inheritance. *Mechanisms of Ageing and Development*. doi: 10.1016/j.mad.2016.12.015

Sharp, G. C., Lawlor, D. A., Richmond, R. C., et al., (2015). Maternal pre-pregnancy BMI and gestational weight gain, offspring DNA methylation and later offspring adiposity: Findings from the Avon Longitudinal Study of Parents and Children. *International Journal of Epidemiology*, *44*(4), 1288–1304. doi: 10.1093/ije/dyv042

Shaw, G. M., Carmichael, S. L., Yang, W., et al., (2004). Periconceptional dietary intake of choline and betaine and neural tube defects in offspring. *American Journal of Epidemiology*, *160*(2), 102–109. doi: 10.1093/aje/kwh187

Shen, L., Zhu, J., Robert Li, S. Y., et al., (2017). Detect differentially methylated regions using non-homogeneous hidden Markov model for methylation array data. *Bioinformatics*, *33*(23), 3701–3708. doi:

10.1093/bioinformatics/btx467

Shi, C., & Pamer, E. G. (2011). Monocyte Recruitment During Infection and Inflammation. *Nature Reviews Immunology*, *11*(11), 762–774. doi: 10.1038/nri3070

Shim, J. S., Oh, K., & Kim, H. C. (2014). Dietary assessment methods in epidemiologic studies. *Epidemiology and Health*, *36*, e2014009. doi: 10.4178/epih/e2014009

Shimizu, M. (2017). Multifunctions of dietary polyphenols in the regulation of intestinal inflammation. *Journal of Food and Drug Analysis*. doi: 10.1016/j.jfda.2016.12.003

Silva-Martínez, G. A., Rodríguez-Ríos, D., Alvarado-Caudillo, Y., et al., (2016). Arachidonic and oleic acid exert distinct effects on the DNA methylome. *Epigenetics*, *11*(5), 321–34. doi: 10.1080/15592294.2016.1161873

Silva Figueiredo, P., Carla Inada, A., Marcelino, G., et al., (2017). Fatty Acids Consumption: The Role Metabolic Aspects Involved in Obesity and Its Associated Disorders. *Nutrients*, *9*(10), 1158. doi: 10.3390/nu9101158

Singh, R. K., Kumar, P., & Mahalingam, K. (2017). Molecular genetics of human obesity: A comprehensive review. *Comptes Rendus - Biologies*. doi: 10.1016/j.crvi.2016.11.007

Smith, Z. D., & Meissner, A. (2013). DNA methylation: roles in mammalian development. *Nature Reviews Genetics*, *14*(3), 204–220. doi: 10.1038/nrg3354

Sobradillo, B., Aguirre, A., & Aresti, U. (2000). Curvas crecimiento Orbegozo. *Instituto de Investigación Sobre Crecimiento Y Desarrollo. Fundación Faustino Obegozo Eizaguirre*, 499–523. Retrieved from https://www.aepap.org/sites/default/files/curvas_orbegozo.pdf

Solas, M., Milagro, F. I., Ramírez, M. J., et al., (2017). Inflammation and gut-brain axis link obesity to cognitive dysfunction: plausible pharmacological interventions. *Current Opinion in Pharmacology*, *37*, 87–92. doi: 10.1016/j.coph.2017.10.005

Solini, A., Santini, E., & Ferrannini, E. (2006). Effect of short-term folic acid supplementation on insulin sensitivity and inflammatory markers in overweight subjects. *International Journal of Obesity*, *30*(8), 1197–202. doi: 10.1038/sj.ijo.0803265

Sookoian, S., Rosselli, M. S., Gemma, C., et al., (2010). Epigenetic regulation of insulin resistance in nonalcoholic fatty liver disease: Impact of liver methylation of the peroxisome proliferator-activated receptor γ coactivator 1α promoter. *Hepatology*, *52*(6), 1992–2000. doi: 10.1002/hep.23927

Soriano-Tárraga, C., Jiménez-Conde, J., Giralt-Steinhauer, E., et al., (2016). Epigenome-wide association study identifies TXNIP gene associated with type 2 diabetes mellitus and sustained hyperglycemia. *Human Molecular Genetics*, *25*(3), 609–619. doi: 10.1093/hmg/ddv493

Soubry, A., Murphy, S. K., Wang, F., et al., (2015). Newborns of obese parents have altered DNA methylation patterns at imprinted genes. *International Journal of Obesity*, *39*(4), 650–657. doi: 10.1038/ijo.2013.193

St-Onge, M. P. (2017). Sleep-obesity relation: underlying mechanisms and consequences for treatment. *Obesity Reviews*, *18*, 34–39. doi: 10.1111/obr.12499

Stead, L. M., Brosnan, J. T., Brosnan, M. E., et al., (2006). Is it time to reevaluate methyl balance in humans? *American Journal of Clinical Nutrition*. doi: 10.1093/ajcn/83.1.5

Stenvinkel, P., Karimi, M., Johansson, S., et al., (2007). Impact of inflammation on epigenetic DNA methylation - A novel risk factor for cardiovascular disease? *Journal of Internal Medicine*, *261*(5), 488–499. doi:

10.1111/j.1365-2796.2007.01777.x

Stepanow, S., Reichwald, K., Huse, K., et al., (2011). Allele-specific, age-dependent and BMI-associated DNA methylation of human MCHR1. *PLoS ONE*, *6*(5), e17711. doi: 10.1371/journal.pone.0017711

Stevenson, T. J. (2017). Epigenetic Regulation of Biological Rhythms: An Evolutionary Ancient Molecular Timer. *Trends in Genetics*. doi: 10.1016/j.tig.2017.11.003

Stewart, C. P., Christian, P., LeClerq, S. C., et al., (2009). Antenatal supplementation with folic acid + iron + zinc improves linear growth and reduces peripheral adiposity in school-age children in rural Nepal. *American Journal of Clinical Nutrition*, *90*(1), 132–140. doi: 10.3945/ajcn.2008.27368

Sun, S., Ji, Y., Kersten, S., et al,. (2012). Mechanisms of Inflammatory Responses in Obese Adipose Tissue. *Annual Review of Nutrition*, *32*(1), 261–286. doi: 10.1146/annurev-nutr-071811-150623

Tahira, K., Ueno, T., Fukuda, N., et al., (2011). Obesity alters the expression profile of clock genes in peripheral blood mononuclear cells. *Archives of Medical Science*, *7*(6), 933–940. doi: 10.5114/aoms.2011.26603

Tarquini, R., & Mazzoccoli, G. (2017). Clock Genes, Metabolism, and Cardiovascular Risk. *Heart Failure Clinics*. doi: 10.1016/j.hfc.2017.05.001

Teitell, M., & Richardson, B. (2003). Dna methylation in the immune system. *Clinical Immunology*, *109*(1), 2–5. doi: 10.1016/S1521-6616(03)00224-9

Thaler, L., & Steiger, H. (2017). Eating disorders and epigenetics. In *Advances in Experimental Medicine and Biology*, *978*, 93–103. doi: 10.1007/978-3-319-53889-1_5

Théry, C. (2011). Exosomes: secreted vesicles and intercellular communications. *F1000 Biology Reports*, *3*, 15. doi: 10.3410/B3-15

Thomas-Valdés, S., Tostes, M. D. G. V., AnunciaÇao P. C., et al., (2017). Association between vitamin deficiency and metabolic disorders related to obesity. *Critical Reviews in Food Science and Nutrition*, *57*(15), 3332–3343. doi: 10.1080/10408398.2015.1117413

Tian, H., Tian, D., Zhang, C., et al., (2017a). Efficacy of Folic Acid Therapy in Patients with Hyperhomocysteinemia. *Journal of the American College of Nutrition*, *36*(7), 528–532. doi: 10.1080/07315724.2017.1330162

Tian, T., Yang, K. Q., Cui, J. G., et al., (2017b). Folic Acid Supplementation for Stroke Prevention in Patients With Cardiovascular Disease. *The American Journal of the Medical Sciences*, *354*(4), 379–387. doi: 10.1016/j.amjms.2017.05.020

Tobi, E. W., Lumey, L. H., Talens, R. P., et al., (2009). DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human Molecular Genetics*, *18*(21), 4046–4053. doi: 10.1093/hmg/ddp353

Tong, Q., Dalgin, G., Xu, H., et al., (2000). Function of GATA transcription factors in preadipocyte-adipocyte transition. *Science*, *290*(5489), 134–138. doi: 10.1126/science.290.5489.134

Toohey, J. I. (2006). Vitamin B12 and methionine synthesis: A critical review. Is nature's most beautiful cofactor misunderstood? *BioFactors*. doi: 10.1002/biof.5520260105

Tremblay, B. L., Cormier, H., Rudkowska, I., et al., (2015). Association between polymorphisms in phospholipase A2 genes and the plasma triglyceride response to an n-3 PUFA supplementation: a clinical trial. *Lipids in Health and Disease*, *14*(1), 12. doi: 10.1186/s12944-015-0009-2

Tremblay, B. L., Guénard, F., Rudkowska, I., et al., (2017). Epigenetic changes in blood leukocytes following an

omega-3 fatty acid supplementation. *Clinical Epigenetics*, *9*, 43. doi: 10.1186/s13148-017-0345-3

Truswell, A. S. (2007). Vitamin B12. *Nutrition & Dietetics*, *64*(s4 The Role of), S120–S125. doi: 10.1111/j.1747-0080.2007.00198.x

Tryndyak, V. P., Han, T., Muskhelishvili, L., et al., (2011). Coupling global methylation and gene expression profiles reveal key pathophysiological events in liver injury induced by a methyl-deficient diet. *Molecular Nutrition and Food Research*, *55*(3), 411–418. doi: 10.1002/mnfr.201000300

Tsujiuchi, T., Tsutsumi, M., Sasaki, Y., et al., (1999). Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. *Japanese Journal of Cancer Research*, *90*(9), 909–913. doi: 10.1111/j.1349-7006.1999.tb00834.x

Tungtrongchitr, R., Pongpaew, P., Tongboonchoo, C., et al., (2003). Serum homocysteine, B12 and folic acid concentration in Thai overweight and obese subjects. *International Journal for Vitamin and Nutrition Research. Internationale Zeitschrift Fur Vitamin- Und Ernahrungsforschung. Journal International de Vitaminologie et de Nutrition*, *73*(1), 8–14. doi: 10.1024/0300-9831.73.1.8

Turner, M. D., Nedjai, B., Hurst, T., et al., (2014). Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochimica et Biophysica Acta - Molecular Cell Research*. Elsevier. doi: 10.1016/j.bbamcr.2014.05.014

Ueland, P. M. (2011). Choline and betaine in health and disease. *Journal of Inherited Metabolic Disease*. doi: 10.1007/s10545-010-9088-4

Vaag, A. A., Grunnet, L. G., Arora, G. P., et al., (2012). The thrifty phenotype hypothesis revisited. *Diabetologia*, *55*(8), 2085–2088. doi: 10.1007/s00125-012-2589-y

Vallée Marcotte, B., Cormier, H., Guénard, F., et al., (2016). Novel Genetic Loci Associated with the Plasma Triglyceride Response to an Omega-3 Fatty Acid Supplementation. *Journal of Nutrigenetics and Nutrigenomics*, *9*(1), 1–11. doi: 10.1159/000446024

van Dijk, S. J., Peters, T. J., Buckley, M., et al., (2017). DNA methylation in blood from neonatal screening cards and the association with BMI and insulin sensitivity in early childhood. *International Journal of Obesity*. doi: 10.1038/ijo.2017.228

van Dijk, S. J., Tellam, R. L., Morrison, J. L., et al., (2015). Recent developments on the role of epigenetics in obesity and metabolic disease. *Clinical Epigenetics*, *7*(1), 66. doi: 10.1186/s13148-015-0101-5

van Dijk, S. J., Zhou, J., Peters, T. J., et al., (2016). Effect of prenatal DHA supplementation on the infant epigenome: results from a randomized controlled trial. *Clinical Epigenetics*, *8*, 114. doi: 10.1186/s13148-016-0281-7

Vanamala, J. (2017). Food systems approach to cancer prevention. *Critical Reviews in Food Science and Nutrition*. Taylor & Francis. doi: 10.1080/10408398.2015.1028023

Vinkers, C. H., Kalafateli, A. L., Rutten, B. P., et al., (2015). Traumatic stress and human DNA methylation: a critical review. *Epigenomics*, *7*(4), 593–608. doi: 10.2217/epi.15.11

Volberg, V., Yousefi, P., Huen, K., et al., (2017). CpG Methylation across the adipogenic PPARγ gene and its relationship with birthweight and child BMI at 9 years. *BMC Medical Genetics*, *18*(1), 7. doi: 10.1186/s12881-016-0365-4

Volkov, P., Bacos, K., Ofori, J. K., et al., (2017). Whole-Genome bisulfite sequencing of human pancreatic islets

reveals novel differentially methylated regions in type 2 diabetes pathogenesis. *Diabetes*, *66*(4), 1074–1085. doi: 10.2337/db16-0996

Waddington, C. H. (1968). Towards a Theoretical Biology. *Nature*, *218*, 525-527. doi: 10.1038/218525a0

Wadhwa, P. D., Buss, C., Entringer, S., et al., (2009). Developmental origins of health and disease: Brief history of the approach and current focus on epigenetic mechanisms. *Seminars in Reproductive Medicine*. doi: 10.1055/s-0029-1237424

Wahl, S., Drong, A., Lehne, B., et al., (2016). Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature*, *541*(7635), 81–86. doi: 10.1038/nature20784

Wald, P., Liu, X. S., Pettit, C., et al., (2017). Prognostic value of microRNA expression levels in pancreatic adenocarcinoma: a review of the literature. *Oncotarget*, *8*(42), 73345–73361. doi: 10.18632/oncotarget.20277

Wang, D. D., & Hu, F. B. (2017). Dietary Fat and Risk of Cardiovascular Disease: Recent Controversies and Advances. *Annual Review of Nutrition*, *37*(1), 423–446. doi: 10.1146/annurev-nutr-071816-064614

Wang, F., Demura, M., Cheng, Y., et al., (2014). Dynamic CCAAT/enhancer binding protein-associated changes of DNA methylation in the angiotensinogen gene. *Hypertension*, *63*(2), 281–8. doi: 10.1161/HYPERTENSIONAHA.113.02303

Wang, G., Dai, J., Mao, J., et al., (2005). Folic acid reverses hyper-responsiveness of LPS-induced chemokine secretion from monocytes in patients with hyperhomocysteinemia. *Atherosclerosis*, *179*(2), 395–402. doi: 10.1016/j.atherosclerosis.2004.10.033

Wang, X., Cao, Q., Yu, L., et al., (2016). Epigenetic regulation of macrophage polarization and inflammation by DNA methylation in obesity. *JCI Insight*, *1*(19). doi: 10.1172/jci.insight.87748

Wang, X., Zhu, H., Snieder, H., et al., (2010). Obesity related methylation changes in DNA of peripheral blood leukocytes. *BMC Medicine*, *8*, 87. doi: 10.1186/1741-7015-8-87

Wankhade, U. D., Zhong, Y., Kang, P., et al., (2017). Enhanced offspring predisposition to steatohepatitis with maternal high-fat diet is associated with epigenetic and microbiome alterations. *PLOS ONE*, *12*(4), e0175675. doi: 10.1371/journal.pone.0175675

Wanunu, M., Cohen-Karni, D., Johnson, R. R., et al., (2011). Discrimination of methylcytosine from hydroxymethylcytosine in DNA molecules. *Journal of the American Chemical Society*, *133*(3), 486–492. doi: 10.1021/ja107836t

Warnick, G. R., Benderson, J., & Albers, J. J. (1982). Dextran sulfate-Mg2+ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clinical Chemistry*, *28*(6), 1379–1388.

Waterland, R. A., Kellermayer, R., Laritsky, E., et al., (2010). Season of conception in rural gambia affects DNA methylation at putative human metastable epialleles. *PLoS Genetics*, *6*(12), 1–10. doi: 10.1371/journal.pgen.1001252

Watts, L. M., Manchem, V. P., Leedom, T. A., et al., (2005). Reduction of hepatic and adipose tissue glucocorticoid receptor expression with antisense oligonucleotides improves hyperglycemia and hyperlipidemia in diabetic rodents without causing systemic glucocorticoid antagonism. *Diabetes*, *54*(6), 1846–1853. doi: 10.2337/diabetes.54.6.1846

Weaver, J. (2007). Classical endocrine diseases causing obesity. *Frontiers of Hormone Research*. doi: 10.1159/000115367

Weisberg, I. S., Park, E., Ballman, K. V., et al., (2003). Investigations of a common genetic variant in betaine-homocysteine methyltransferase (BHMT) in coronary artery disease. *Atherosclerosis*, *167*(2), 205–214. doi: 10.1016/S0021-9150(03)00010-8

What Is Metabolic Syndrome? - NHLBI, NIH. (2016). Retrieved November 12, 2017, from https://www.nhlbi.nih.gov/health/health-topics/topics/ms

WHO | Cardiovascular diseases (CVDs). (2017). *WHO*. https://doi.org/Fact sheet N°317

Wit, J. E. D. (2011). Part 1 : The role of vitamin B6 in one-carbon metabolism, oxidative stress, immune system regulation and carcinogenesis, 1–29. Retrieved from https://dspace.library.uu.nl/handle/1874/210236

Wolffe, A. P., & Matzke, M. A. (1999). Epigenetics: regulation through repression. *Science*, *286*(5439), 481–6. doi: 10.1126/SCIENCE.286.5439.481

Wu, S., Gennings, C., Wright, R. J., et al., (2018). Prenatal Stress, Methylation in Inflammation-Related Genes, and Adiposity Measures in Early Childhood: The Programming Research in Obesity, Growth Environment and Social Stress Cohort Study. *Psychosomatic Medicine*, *80*(1), 34–41. doi: 10.1097/PSY.0000000000000517

Xing, J., Kang, L., & Jiang, Y. (2011). Effect of dietary betaine supplementation on lipogenesis gene expression and CpG methylation of lipoprotein lipase gene in broilers. *Molecular Biology Reports*, *38*(3), 1975–1981. doi: 10.1007/s11033-010-0319-4

Yuan, Z., Syed, M. A., Panchal, D., et al., (2012). Curcumin mediated epigenetic modulation inhibits TREM-1 expression in response to lipopolysaccharide. *International Journal of Biochemistry and Cell Biology*, *44*(11), 2032–2043. doi: 10.1016/j.biocel.2012.08.001

Yusufov, M., Weyandt, L. L., & Piryatinsky, I. (2017). Alzheimer's disease and diet: a systematic review. *International Journal of Neuroscience*, *127*(2), 161–175. doi: 10.3109/00207454.2016.1155572

Zaidi, S. K., Young, D. W., Montecino, M., et al., (2010). Architectural Epigenetics: Mitotic Retention of Mammalian Transcriptional Regulatory Information. *Molecular and Cellular Biology*, *30*(20), 4758–4766. doi: 10.1128/MCB.00646-10

Zeisel, S. H. (2017). Choline, other methyl-donors and epigenetics. *Nutrients*, *9*(5). doi: 10.3390/nu9050445

Zeisel, S. H., Da Costa, K. A., Franklin, P. D., et al., (1991). Choline, an essential nutrient for humans. *FASEB Journal 5*(7), 2093–8.

Zeybel, M., Hardy, T., Robinson, S. M., et al., (2015). Differential DNA methylation of genes involved in fibrosis progression in non-alcoholic fatty liver disease and alcoholic liver disease. *Clinical Epigenetics*, *7*(1), 25. doi: 10.1186/s13148-015-0056-6

Zhang, G., & Pradhan, S. (2014). Mammalian epigenetic mechanisms. *IUBMB Life*. doi: 10.1002/iub.1264

Zhang, N. (2015). Epigenetic modulation of DNA methylation by nutrition and its mechanisms in animals. *Animal Nutrition*. doi: 10.1016/j.aninu.2015.09.002

Zhang, Y., Liu, H., Lv, J., et al., (2011). QDMR: A quantitative method for identification of differentially methylated regions by entropy. *Nucleic Acids Research*, *39*(9), e58. doi: 10.1093/nar/gkr053

Zhao, J., Goldberg, J., & Vaccarino, V. (2013). Promoter methylation of serotonin transporter gene is associated with obesity measures: a monozygotic twin study. *International Journal of Obesity (2005)*, *37*(1), 140–5. doi: 10.1038/ijo.2012.8

Zhong, J., Karlsson, O., Wang, G., et al., (2017). B vitamins attenuate the epigenetic effects of ambient fine particles in a pilot human intervention trial. *Proceedings of the National Academy of Sciences*, *114*(13), 3503–3508. doi: 10.1073/pnas.1618545114

Zulet, M. A., Bondia-Pons, I., Abete, I., et al., (2011). The reduction of the metabolyc syndrome in Navarra-Spain (RESMENA-S) study; A multidisciplinary strategy based on chrononutrition and nutritional education, together with dietetic and psychological control. *Nutricion Hospitalaria*, *26*(1), 16–26. doi: 10.3305/nh.2011.26.1.5050

APPENDICES

Appendix 1: Noncoding RNAs, cytokines, and inflammation-related diseases

The FASEB Journal, 2015
DOI: 10.1096/fj.14-260323

Noncoding RNAs, cytokines, and inflammation-related diseases

José Luiz Marques-Rocha,* Mirian Samblas,[†,‡] Fermin I. Milagro,[†,‡,§] Josefina Bressan,* J. Alfredo Martínez,[†,‡,§,¶] and Amelia Marti[†,‡,§,¶,1]

*Department of Nutrition and Health, Universidade Federal de Viçosa, Viçosa, Brazil; [†]Department of Nutrition, Food Science, and Physiology, [‡]Center for Nutrition Research, University of Navarra, Pamplona, Spain; [§]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición, Physiopathology of Obesity, Carlos III Institute, Madrid, Spain; and [¶]Instituto de Investigación Sanitaria de Navarra, Pamplona, Spain

ABSTRACT **Chronic inflammation is involved in the onset and development of many diseases, including obesity, atherosclerosis, type 2 diabetes, osteoarthritis, autoimmune and degenerative diseases, asthma, periodontitis, and cirrhosis. The inflammation process is mediated by chemokines, cytokines, and different inflammatory cells. Although the molecules and mechanisms that regulate this primary defense mechanism are not fully understood, recent findings offer a putative role of noncoding RNAs, especially microRNAs (miRNAs), in the progression and management of the inflammatory response. These noncoding RNAs are crucial for the stability and maintenance of gene expression patterns that characterize some cell types, tissues, and biologic responses. Several miRNAs, such as miR-126, miR-132, miR-146, miR-155, and miR-221, have emerged as important transcriptional regulators of some inflammation-related mediators. Additionally, little is known about the involvement of long noncoding RNAs, long intergenic noncoding RNAs, and circular RNAs in inflammation-mediated processes and the homeostatic imbalance associated with metabolic disorders. These noncoding RNAs are emerging as biomarkers with diagnosis value, in prognosis protocols, or in the personalized treatment of inflammation-related alterations. In this context, this review summarizes findings in the field, highlighting those noncoding RNAs that regulate inflammation, with emphasis on recognized mediators such as TNF-α, IL-1, IL-6, IL-18, intercellular adhesion molecule 1, VCAM-1, and plasminogen activator inhibitor 1. The down-regulation or antagonism of the noncoding RNAs and the administration of exogenous miRNAs could be, in the near future, a promising therapeutic strategy in the treatment of inflammation-related diseases.—Marques-Rocha, J. L., Samblas, M., Milagro, F. I., Bressan, J., Martínez, J. A., Marti, A. Noncoding RNAs, cytokines, and inflammation-related diseases. *FASEB J.* 29, 3595–3611 (2015). www.fasebj.org**

Key Words: circular RNA · lincRNA · microRNA · lncRNA · obesity

Inflammation is a complex protective process that requires a cross-talk between different types of immune cells to remove or neutralize harmful stimuli (1). In the classic view, the inflammatory process is induced by an invasion of foreign pathogens of biologic origin or by tissue damage. Neutrophils, dendritic cells, and macrophages express almost all types of TLRs participating in the transmission of a signal from the plasma membrane through a multistep cascade to responsive transcription factors. Members of the TLR family have emerged as the primary evolutionarily conserved sensors of pathogen-associated molecular patterns (1). Binding of the TLRs to their respective ligands initiates a wide spectrum of responses, from phagocytosis to production of a variety of cytokines, which in turn shape and enhance the inflammatory and adaptive immune responses. Typical transcription factors that activate inflammatory mediators are NF-κB (2), activator protein 1 (3), signal transducer and activator of transcription (STAT) (4), CCAAT enhancer binding protein (C/EBP) (5), and Ets-like gene 1 (6). The interactions between transcription factors that compete for binding sites in the promoter regions of specific target genes are highly complex. Usually the multistep signaling leads to a prompt transcription of genes resulting in accumulation of specific mRNAs coding for TNF-α, IL-1, IL-6, IL-8, monocyte chemotactic protein 1 (MCP-1), and other cytokines involved in inflammation (7).

Some cytokines may elicit a broad inflammatory response, while others act on specific cell types. The activation, proliferation, and recruitment phenomena of specific differentiated immune cells are involved in resolving the nonhomeostatic state [for a review, see Shi (8)]. Thus, macrophages stimulate the inflammatory responses of

Abbreviations: ADAM17, ADAM (A disintegrin and metalloproteinase) metallopeptidase domain 17; AKT/GSK, protein kinase B/glycogen synthase kinase; BMI, body mass index; C/EBP, CCAAT enhancer binding protein; circRNA, circular RNA; COX-2, cyclooxygenase 2; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule 1; IL-1R,

(*continued on next page*)

[1] Correspondence: Department of Nutrition, Food Science, and Physiology, University of Navarra, c/ Irunlarrea 1, 31008, Pamplona, Navarra, Spain. E-mail: amarti@unav.es

doi: 10.1096/fj.14-260323

Appendix 2: Congress communications

Cambios en la metilación de los genes clock, bmal1 y nr1d1 en células blancas de mujeres obesas sometidas a un tratamiento de pérdida de peso

Mirian Samblas 1,2, Purificación Gómez-Abellán 3, Marta Garaulet 3, J. Alfredo Martínez 1,2,4, Fermín I. Milagro1,2,4

1 Departamento de Ciencias de la Alimentación y Fisiología. Universidad de Navarra. Pamplona, España.
2 Centre for Nutrition Research, University of Navarra, Pamplona, Spain
3 Departamento de Fisiología, Facultad de Biología. Universidad de Murcia. Murcia, España.
4 CIBER Fisiopatología Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III. Madrid, España

Objetivos: Evaluar, en células blancas, los porcentajes de metilación de tres regiones génicas de los genes CLOCK, BMAL1 y NR1D1 (relacionados con la regulación del ritmo circadiano), antes y después de un tratamiento de pérdida de peso basado en la Dieta Mediterránea, y analizar las diferencias de metilación entre buenos y malos respondedores al tratamiento.

Método: La población estudiada fue de 61 mujeres que asistieron voluntariamente a cinco clínicas de nutrición de Murcia (España) con el fin de perder peso mediante un tratamiento dietético y de comportamiento basado en el principio de la dieta mediterránea y técnicas de comportamiento y cognitivos (Método Garaulet). El ADN se aisló a partir de células blancas totales obtenidas antes y después del tratamiento, se trató con bisulfito sódico y, mediante la técnica de MassArray (Sequenom), se analizaron los porcentajes de metilación en los tres genes. Además, la población fue estratificada en dos grupos: buenos respondedores (n=25; aquellos que bajaron de peso de manera continuada a lo largo de 30 semanas) y malos respondedores (n=36; el resto).

Resultados: El tratamiento redujo los valores de IMC (kg/m2), insulina, colesterol total, HDL y LDL. Además, la pérdida de peso indujo modificaciones en la metilación de diversos CpGs de los genes CLOCK, BMAL1 y NR1D1. No se encontraron diferencias significativas en los valores de metilación inicial de buenos y malos respondedores. Sin embargo, en buenos respondedores se observó una correlación negativa entre la metilación de un CpG del gen CLOCK y la grasa corporal inicial, y entre un CpG del gen BMAL1 y la pérdida total de peso y el IMC.

Conclusiones: La pérdida de peso indujo modificaciones en la metilación de diversos CpGs de los genes CLOCK, BMAL1 y NR1D1. No se encontraron diferencias en los niveles de metilación inicial entre los buenos y los malos respondedores.

Integrative studies for the identification of transcriptomic and epigenetic biomarkers within a weight-loss program

Mirian Samblas 1,2, Maria Luisa Mansego, Maria Angeles Zulet 1,2,3, Fermín I. Milagro 1,2,3, J. Alfredo Martínez 1,2,3

1 Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain;
2 Centre for Nutrition Research, University of Navarra, Pamplona, Spain;
3 CIBERobn, Fisiopatología de la Obesidad y la Nutrición, Carlos III Institute, Madrid, Spain

Objective: Identification of candidate biomarkers that distinguish individual response for a weight loss dietary treatment by using the integrative analysis of mRNA expression and DNA methylation arrays.

Methods: The study consisted on a randomized sample of 33 obese people (mean BMI = 35.8 ± 4.7 kg/m2) who participated in the Metabolic Syndrome Reduction in Navarra (RESMENA) retrospective trial. They were classified as low (n=23) or high responders (n=10) depending on their weight loss (>8% of basal weight). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples obtained at the beginning and at endpoint. Extracted DNA was sodium-bisulfite converted and fragmented by controlled enzyme digestion previous hybridization to probes of Infinium Human Methylation450 BeadChip kit (Illumina Inc.). RNA was isolated from white blood cells by TRIzol Reagent for microarray analysis, which was performed with the Illumina Human HT-12 v4 gene expression BeadChip.

Results: For the methylation analysis, a significance threshold of $p<0.05$ and 5% of methylation variation were used, whereas for the expression study a FDR<0.05 and a fold change>1.5 were accepted. 2,102 CpG sites in 1,785 genes showed differential methylation between low and high responders. In the expression analysis, 156 transcripts were differentially expressed between both groups, 20 of which were downregulated and 136 upregulated in low responders. The integrative analysis of both methylation and expression data identified four genes (CD44, FBXW5, MTSS1 and ITPR1) that appeared differentially methylated and expressed in low responders in comparison with high responders.

Conclusion: In summary, DNA methylation has been suggested as a powerful tool for diagnosis and prognosis. The combination of a high-throughput DNA methylation and expression microarray dataset can be a useful strategy to identify novel genes that might be considered as predictors of the dietary response. Future studies are needed for the validation of these genes as outcome indicators within a weight loss program.

Folic acid and others methyl donors attenuate LPS-induced inflammatory response in human macrophages

Mirian Samblas 1,2, Fermín I. Milagro 1,2,3, Maria Angeles Zulet 1,2,3, J. Alfredo Martínez 1,2,3

1 Department of Nutrition, Food Science and Physiology. University of Navarra. Spain.
2 Centre for Nutrition Research. University of Navarra. Spain.
3 CIBERobn, Biomedical Research Centre in Physiopathology of Obesity and Nutrition. Carlos III Institute. Spain.

Background and objectives: Methyl donors participate in the epigenetics mechanisms by modulating the methylation levels of DNA and proteins. Moreover, methylating compounds trigger essential roles in metabolism and cellular homeostasis. Dietary supplementation with folic acid and other methyl donors has been reported to elicit beneficial effects on obesity-related manifestations and cardiovascular diseases. The aim of this study was to investigate the effects of methyl donors on the inflammatory process in the context of obesity.

Methods: The samples were obtained from 97 obese subjects (mean BMI=35.8±4.7 kg/m^2) that participated in the Metabolic Syndrome Reduction in Navarra (RESMENA) controlled trial. Methyl donor levels and inflammatory markers were quantified in plasma. Expression of inflammatory genes was measured in white blood cells. THP-1 cells (human monocytes) were differentiated into macrophages with 25 ng/ml of phorbol 12-myristate 13-acetate (TPA) and stimulated with 100 µg/ml of lipopolysaccharide (LPS), followed by incubation with 11 µM folic acid or a mix of methyl donors (folic acid 11 µM, choline 100 µM, vitamin B_{12} 18 nM) for 24h.

Results: Inverse correlations were found between plasma folic acid and vitamin B_{12} concentrations, with *TNFα* and *SERPINE1* expression in white blood cells, as well as circulating PAI-1 levels. Incubation of THP-1 activated macrophages with folic acid or a mix of methyl donors inhibited significantly *IL1B*, *TNFα*, *SERPINE1* and *TLR4* gene expression. Moreover, methyl donors decreased IL1B protein levels.

Conclusions: This study reveals a negative association between methyl donor levels in blood and important inflammatory markers. Moreover, the addition of folic acid or a mix of methyl donors to human macrophages activated with LPS reduced inflammatory gene expression, suggesting a role of methyl donors in the inflammatory process.

Frontiers in epigenetic chemical biology
The Royal Society, London (2017)

Identification of novel transcriptomic and epigenetic biomarkers using genome-wide analyses within a weight-loss program

Mirian Samblas 1,2, Maria Luisa Mansego, Maria Angeles Zulet 1,2,3, J. Alfredo Martínez 1,2,3, Fermín I. Milagro 1,2,3

1 Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.
2 Centre for Nutrition Research, University of Navarra, Pamplona, Spain.
3 CIBERobn, Biomedical Research Centre in Physiopathology of Obesity and Nutrition, Carlos III Institute, Madrid, Spain.

The aim of the study was to identify candidate biomarkers that distinguish individual response to a weight loss dietary treatment by using the integrative analysis of gene expression and DNA methylation arrays. A randomized subsample of 24 obese people (BMI=35.8 kg/m2) participating in the RESMENA controlled trial, were classified as low (LR; n=12) or high responders (HR; n=12) depending on their weight loss (>8% of basal weight). PBMCs were isolated from blood samples. Genome-wide DNA methylation analyses were performed by Infinium Human Methylation450 BeadChip kit. Expression microarray analysis was performed with Human HT-12 v4 expression BeadChip. Gene expression results were subsequently validated by qRT-PCR. The integrative analysis of both methylation and expression data identified four genes (CD44, FBXW5, MTSS1 and ITPR1) that were differentially methylated and expressed in HR in comparison with LR. Expression of these genes was lower in HR than in LR. In summary, the combination of high-throughput DNA methylation and expression microarray datasets can be a useful strategy to identify novel genes that might be considered as predictors of the dietary response and used in personalized nutrition. Nevertheless, additional studies are needed to replicate DNA methylation of these genes, whereas validation in larger populations is advisable.

21th International Congress of Nutrition (IUNS) (2017)

Low folic acid intake is associated with SIK1 hypomethylation and insulin resistance in obese subjects

Omar Ramos-Lopez 1, Mirian Samblas 1,2, Fermín I. Milagro 1,2,3, María Luisa Mansego 1, María Angeles Zulet 1,2,3, Jose Ignacio Riezu Boj 1,2, J. Alfredo Martínez 1,2,3

1 Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain.
2 Centre for Nutrition Research, University of Navarra, Pamplona, Spain.
3 CIBERobn, Biomedical Research Centre in Physiopathology of Obesity and Nutrition, Carlos III Institute, Madrid, Spain.

Background and objectives: Folic acid is involved in the regulation of many biological processes including DNA methylation via the one-carbon metabolism pathway. Furthermore, folic acid deficiency has been putatively implicated in the onset of diverse metabolic diseases, including insulin resistance, by altering DNA methylation patterns on key regulatory genes. The aim of this study was to investigate the association between folic acid intake and metabolic features, with emphasis on gene-specific DNA methylation patterns.

Methods: A cross-sectional ancillary study was conducted in obese subjects (n=47) from the RESMENA study (Spain). Fat mass was measured by dual-energy X-ray absorptiometry (DXA). Dietary intake and the metabolic profile were assessed by standardized methods. DNA methylation in peripheral white blood cells was analyzed by microarray (Infinium Human Methylation 450K BeadChips).

Results: Subjects with a folic acid intake lower than 300 μg/day showed more fat mass (especially trunk fat), as well as higher levels of glucose, insulin, HOMA-IR index, cortisol and PAI-1 than those consuming at least or more than 300 μg/day. Noteworthy, low folic acid was related to salt inducible kinase 1 (SIK1) gene hypomethylation. Moreover, methylation levels of SIK1, a direct CREB target gene involved in glucose metabolism negatively correlated with HOMA-IR index.

Conclusions: These results suggest that SIK1 hypomethylation could be an epigenetic mechanism underlying low folic acid-induced insulin resistance in obese subjects.

Keywords: Folic acid, epigenetics, DNA methylation, obesity, insulin resistance

Role of microRNAs mir-155 and let-7B on inflammation in THP-1 cells: effects of pro- and anti-inflammatory fatty acids

Jose Luiz Marques Rocha 1, Marcos García-Lacarte 2,3, Mirian Samblas 2,3, Josefina Bressan 1, J. Alfredo Martínez 2,3,4, Fermín I. Milagro 2,3,4

1 Departmen of Integrated Education of Health. Federal University of Espirito Santo, Brazil.
2 Department of Nutrition, Food Science and Physiology. University of Navarra. Spain.
3 Centre for Nutrition Research. University of Navarra. Spain.
4 CIBERobn, Biomedical Research Centre in Physiopathology of Obesity and Nutrition. Carlos III Institute. Spain.

Background and objectives: Our group has previously shown that a Mediterranean-based nutritional intervention is able to induce changes in the expression of Let-7b and miR-155-3p in white blood cells of individuals with metabolic syndrome. In addition, a low consumption of lipids and saturated fat has been associated with higher expression of Let-7b. However, to our knowledge there are no evidences about the role of anti- and pro- inflammatory fatty acids (FAs) on the expression of these microRNAs. In this sense, the main objective of the current study was to investigate the regulatory roles of miR-155-3p and Let-7b on the expression of inflammation-related genes in monocytes, macrophages and LPS-activated macrophages (AcM). Moreover, we explored the regulatory role of pro- and anti- inflammatory fatty acids on the expression of these miRNAs in the three cell types.

Methods: Human acute monocytic leukemia cells (THP-1) were differentiated into macrophages and activated with LPS for 24 hours. Monocytes, macrophages and AcM were transfected with miR-Let-7b-5p and miR-155-3p mimics or a negative control. The expression of the miRNAs and selected genes involved in inflammatory pathways (TNF, IL6, SERPINE1 and TLR4) was measured by qRT-PCR. The three cell types were also incubated with palmitic, oleic, docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids.

Results: miR-155-3p mimic increased the expression of IL6 in the three cell types. In the same way, SERPINE1 was upregulated in monocytes and macrophages. However, TLR4 was downregulated in miR-155-3p-transfected monocytes and macrophages. Let-7b mimic downregulated TNF/IL6 in monocytes and SERPINE1 in AcM. However, TNF, IL6 and SERPINE1 were upregulated in macrophages. Oleic acid was able to increase the expression of miR-155 in monocytes when compared with DHA but not when compared with non-treated cells. On the other side, oleic acid increased the expression of Let-7b in macrophages and AcM.

Conclusions: Overall, these findings suggest a pro-inflammatory role for miR-155-3p and an anti-inflammatory role for Let-7b in THP-1 cells. However, these effects depended on the cell type. Moreover, some of the beneficial properties of oleic acid in non-activated and LPS-activated macrophages might be mediated by increasing Let-7b expression.

Keywords: TNF, SERPINE1, monocyte, macrophage, oleic acid