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Facultad de Farmacia y Nutrición

Epigenetic DNA methylation signatures in different physiological, metabolic and nutritional states

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Dña. Ana Arpón Miranda

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, 6 de Septiembre de 2019

VºBº Director	VºBº Co-Director
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que las relata para que no se olviden.

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List of abbreviations

ADHD: Attention Deficit Hyperactivity Disorder

AIR: Acute Insulin Release

ASD: Autism Spectrum Disorders

BMI: Body Mass Index

BSID: Bayley Scale of Infant Development

CRP: C-Reactive Protein

CSi: Calculated insulin Sensitivity index

DI: IVGTT-based Disposition Index

DNMT: DNA methyltransferase

DOHaD: Developmental Origins of Health and Disease

DXA: Dual-energy X-ray Absorptiometry

EDTA: Ethylenediaminetetraacetic Acid

ELISA: Enzyme-Linked Immunosorbent Assay

EWAS: Epigenome-Wide Association Studies

HELP: *HpaII* tiny fragment Enrichment by Ligation-mediated PCR

HOMA-IR: Homeostasis Model Assessment-Insulin Resistance

HOMA-S: Homeostasis Model Assessment-Sensitivity

HRM: High Resolution Melting

IL-6: Interleukin 6

IPA: Ingenuity Pathway Analysis

ISRCTN: International Standard Randomised Controlled Trial Number

IVGTT: Intravenous Glucose Tolerance Test

LC-MS/MS: Liquid Chromatography coupled with tandem Mass Spectrometry

LINE-1: Long Interspersed Nuclear Element 1

MCP-1: Monocyte Chemotactic Protein-1

MedDiet + EVOO: Mediterranean diet supplemented with Extra-Virgin Olive Oil

MedDiet + nuts: Mediterranean diet supplemented with nuts

MeDIP: Methylated DNA Immunoprecipitation

miRNA: MicroRNA

MSP: Methylation-Specific PCR

MUFA: Monounsaturated Fatty Acid

NAFLD: Non-Alcoholic Fatty Liver Disease

NGS: Next-Generation Sequencing

ncRNA: Non-coding RNA

PCR: Polymerase Chain Reaction

PUFA: Polyunsaturated Fatty Acid

RISC: RNA-Induced Silencing Complex

ROC: Receiver Operating Characteristic

RRBS: Reduced Representation Bisulfite Sequencing

SAM: S-Adenosylmethionine

SFA: Saturated Fatty Acid

siRNA: Small interfering RNA

SNP: Single Nucleotide Polymorphism

SQN: Subset Quantile Normalisation

SWAN: Subset-quantile Within Array Normalisation

TET: Ten Eleven Translocation

TNF-α: Tumour Necrosis Factor α

TyG: Triglyceride-Glucose index

T2D: Type 2 Diabetes

WC: Waist Circumference

Abstract

Epigenetic signatures are heritable changes that can alter gene expression without modifying the DNA sequence. One major epigenetic mechanism is the methylation of CpG sites, which involves the incorporation of a methyl group into a cytosine that is adjacent to a guanine. DNA methylation patterns can be influenced by environmental factors and can change stochastically as a result of ageing, and thereby, modulate gene expression. Modifications in DNA methylation marks can alter the susceptibility to certain diseases and health impairments, including neurological disorders, obesity, type 2 diabetes and cardiovascular disease. In this context, DNA methylation alterations have emerged as promising biomarkers for disease screening, detection, and prediction, in addition to being potential targets for the development of new therapeutic approaches. Thus, the general objective of the current research is to identify DNA methylation patterns associated with different physiological, metabolic and nutritional states that might contribute to the development of biomarker panels and potential therapeutic targets. In order to achieve this, epigenome-wide association studies were performed in different populations to determine candidate CpG sites related to factors and features that could influence and/or be associated with DNA methylation. These included prematurity, a Mediterranean diet, insulin sensitivity and resistance, abdominal obesity and epigenetic age. Altogether, the results of the current thesis demonstrate that alterations in DNA methylation marks depend on the screened physiological, metabolic and nutritional state of the individual. Due to the plasticity of the epigenetic system, environmental factors can influence DNA methylation signatures, both during the first stages of life and during the adulthood. As such, prematurity and nutrition are two determinant factors of DNA methylation levels. The findings of this investigation revealed that preterm newborns exhibit lower levels of methylation of a CpG located at the *SLC6A3* gene, which is related to neurodevelopment. Regarding nutrition, following a Mediterranean diet was associated with alterations in the methylation of genes related to inflammatory and metabolic pathways, where fat quality may also play a role. Moreover, some DNA methylation signatures were associated with insulin sensitivity, insulin resistance and abdominal adiposity, supporting an influence of epigenetics on metabolic pathways and

in the onset and progression of metabolic diseases. For instance, *LPL* and *CTNND2* were highly associated with insulin sensitivity, able to accurately discern between subjects with low and high insulin sensitivity values. Concerning insulin resistance, four CpGs located at genes involved in glucose- and insulin-related pathways, including the *SH3RF3* and *MAN2C1* genes, were capable of distinguishing low and high insulin resistance levels. On the other hand, differential DNA methylation values were also found for subjects with and without central obesity, which could be discriminated by four CpGs in women (*c13orf36, ZC3H12D, MYO9B, KCNG3*) and one in men (*TCP11L1*). Lastly, ageing was also related to changes in DNA methylation, confirming that an unhealthy metabolic state accelerates the epigenetic age, possibly leading to early health deterioration. Overall, this research provides new insights into the underlying epigenetic mechanisms associated with prematurity, metabolic diseases and ageing, suggesting different CpG sites as putative biomarkers for diagnosis and prognosis or as potential therapeutic targets for the prevention and treatment of chronic diseases.

Table of contents

INTRODUCTION

1. HEALTH AND DISEASE

The World Health Organization defined health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" [1], but this definition has been accused of being holistic and utopic [2]. In this context, other suggested definition is "a dynamic state of well-being characterized by a physical, mental and social potential, which satisfies the demands of a life commensurate with age, culture, and personal responsibility. If the potential is insufficient to satisfy these demands the state is disease" [3].

Diseases can be classified in communicable and noncommunicable. In the last years, deaths due to communicable diseases have been reduced, whereas the noncommunicable ones have globally increased [4]. According to the World Health Organization, these physiopathological conditions caused 71% of deaths globally in 2016, reaching the 88% in high-income countries [4]. The risk of suffering from these diseases, such as obesity, cardiovascular events, diabetes, inflammatory diseases, neurological disorders and cancer [5], is influenced by exogenous and endogenous factors [6] (Figure 1).

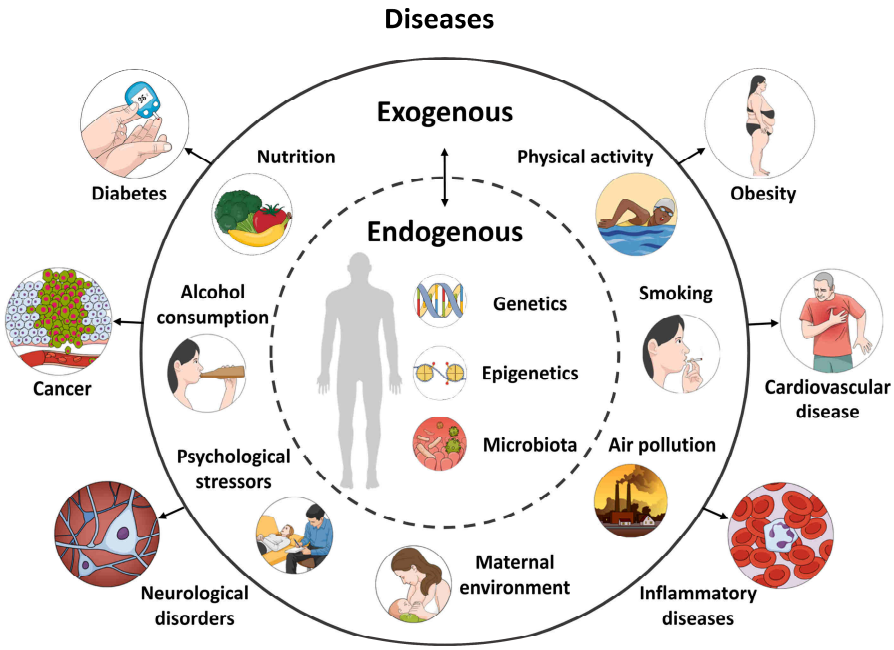

Figure 1: Exogenous and endogenous factors influencing the development of diseases.

1.1. Exogenous agents

Environmental factors, such as dietary patterns, sedentary habits, unhealthy lifestyles, and socio-demographic or cultural features greatly impact on diverse aspects of health and disease risk [5]. This influence starts at first developmental stages of life as hypothesised by the Developmental Origins of Health and Disease (DOHaD) theory [7, 8]. This hypothesis states that there is a programming of the foetus development in response to signals from the intrauterine environment in order to adapt to future challenges after birth [7]. That foetal programming might entail permanent changes in the phenotype of a developing organism, which will help to success in an adverse postnatal environment [9]. For example, in the presence of maternal undernutrition, foetus metabolism would be structurally and functionally impaired in a nutrient-deprived environment [10]. However, these adaptations might be detrimental under different postnatal environments, leading to an increased risk of developing chronic diseases such as cancer, cardiovascular disease, diabetes, obesity, asthma, and behavioural disorders in later life [11]. The underlying mechanisms that link environmental agents with disease risk include epigenetics, transcriptional mechanisms, cellular stresses, metabolic adaptations, alterations to the microbiome, and social determinants [12].

Environmental factors may also play a role in the onset of chronic diseases during the adulthood [6]. For instance, caloric diets rich in saturated fats, sugar, and refined grains, combined with a sedentary lifestyle, are major determinants of complex chronic diseases, such as obesity, diabetes, cardiovascular complications and cancer [6, 13, 14]. Following healthy dietary habits, such as a Mediterranean diet, combined with moderate physical activity, have demonstrated beneficial effects regarding cardiometabolic diseases, such as cardiovascular disease, obesity, Type 2 Diabetes (T2D) and metabolic syndrome [15-19]. The progress of disease is also influenced by smoking, alcohol abuse, exposure to environmental toxicants, as well as economic, psychosocial, reproductive, and pharmacologic factors [13, 20-22].

1.2. Endogenous factors

In addition to environmental factors, which are involved in disease onset and development, genetics, epigenetics and microbiota also play a crucial role in body homeostasis [23]. The interaction among genetics, epigenetics and microbiota, and their interplay with environmental factors, may adapt the physiological response to diverse stimuli [24].

1.2.1. Genetics

Genetic components may contribute to risk of diseases through interaction with environmental factors [5]. Genome-wide association studies and epidemiological studies have identified several Single Nucleotide Polymorphisms (SNPs) associated with complex diseases and traits such as obesity, diabetes, immunodeficiency, cancer, cardiovascular disease, among others [9, 25-27].

For instance, genetic variations in the genes *FTO, BDNF, MC4R, LEPR, TCF7L2, IRS1,* and *RPTOR*, among others, have been associated with obesity [25, 28, 29]. Cardiovascular disease and T2D have been associated with genetic variations in *loci* at *ADIPOQ, ADIPOR1, APOE, CDKN2A/2B, GLUL, HMGA1, HP, PHACTR1, CELSR2-PSRC1-SORT1, HNF1A, PCSK9, SOD2,* and *TCF7L2* genes [27]. Genetic variants in the *IL1RN* gene have been related to sepsis and acute respiratory distress syndrome [26]. Moreover, common single nucleotide variants in the *TLR1* gene have been involved in differences in the immune response [26]. Non-Alcoholic Fatty Liver Disease (NAFLD) has also been linked to genes, including *ADIPOQ, LEPR, APOC3, SREBP, PPAR, TM6SF2, MTTP, TNFA, SIRT1,* and *PNLPA3* [30, 31]. Other genes such as *BRAC1, BRAC2, ATM, CHEK2,* and *PALB2* have been related to increased breast cancer [32], and *loci* at *TERC, TERT, OBFC1,* and *RTEL1* have been associated with risk of multiple cancers [32].

Although the genetic make-up can explain part of the disease risk, there is a substantial proportion of the variation that remains unexplained [9, 33]. This missing heritability might be partly justified by epigenetic mechanisms and by interaction with environmental factors [34].

1.2.2. Epigenetics

Epigenetic mechanisms are heritable changes that can modify gene expression without altering the DNA sequence [35]. In contrast to the stability of the genome throughout the life course, the epigenetic system is plastic and reversible [36, 37] and it can be transmitted from generation to generation [11]. Environmental exposures can influence the epigenetic processes, altering gene expression, and subsequently, affecting disease risk [38]. Thus, epigenetics can be considered as the interface between genotype, environmental factors and phenotype [39, 40].

Epigenetic modifications are well-defined in rare developmental disorders and imprinting diseases, such as Prader-Willi and Angelman syndromes [22]. However, the role in complex diseases, where the environment is a fundamental factor, is more difficult to be determined [21]. Epigenetic modifications have been related to many diseases, such as cancer, obesity, diabetes, cardiovascular disease, neurodegenerative disorders, autoimmune diseases, among others [41-44].

1.2.3. Microbiota

The endogenous microbiome, particularly, the gut microbiota, plays a fundamental role in health and disease [45]. Indeed, alterations in the intestinal microbial ecosystem have been associated with neurodevelopmental illnesses, allergic diseases, inflammatory bowel disease, irritable bowel syndrome, and metabolic diseases, such as obesity and diabetes [46].

The gut microbiota is a complex ecosystem consisting of bacteria, fungi, yeasts, archaea, protists and viruses [47, 48]. The dominant bacterial phyla, that mainly colonise the large intestine [49], are *Firmicutes*, *Bacteroidetes*, which together account for approximately 90% of the total gut microbiota, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* [50]. The gut microbiota interacts with the host and regulates physiological processes, such as digestion, nutrient uptake and metabolism, synthesis of vitamins, modulation of mucosal immunity, and epithelial growth, among others [36].

Alterations in the gut microbiota alterations can affect genetic and epigenetic mechanisms [51]. In this context, there is growing evidence revealing that the expression of polymorphic genes associated with diseases, such as obesity and

colorectal cancer, was conditional upon exposure to the microbiota in primary colonic epithelial cells [52]. Moreover, the human variant rs878394, linked to *LYPLAL1* in humans and related to body fat distribution and insulin sensitivity, has been associated with the abundance of *Prevotella*, which belongs to the *Bacteroidetes* phylum [53]. The non-bacterial component of the gut microbiota has also been linked to the pathogenesis of some diseases, such as fungi, which might play a role in inflammatory bowel disease [54]. On the other hand, epigenetic modifications can also be influenced by gut microbiota imbalance [45]. For example, molecules and metabolites generated by the intestinal microbiome, such as butyrate or S-Adenosylmethionine (SAM), can have regulatory effects on epigenetic enzymes that control DNA methylation and histone modifications [45]. Interestingly, recent evidences have revealed that epigenetics can also regulate the composition of the gut microbiome, suggesting a bidirectional interaction between the host and the microbiome [51].

2. EPIGENETICS

2.1. Definition

The term epigenetics, which etymologically means "on top of/in addition to genetics" [55], was first introduced by Waddington in the 1940s to describe "the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being" [56, 57]. This initial definition denoted the molecular pathways regulating the expression of a genotype into a particular phenotype [58]. The meaning of epigenetics has evolved through time to the current accepted definition: ''the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence" [59].

DNA is packaged in the intracellular structure called chromatin [60]. Chromatin can be differentiated into heterochromatin, which is highly condensed and transcriptionally inert, and euchromatin, which is less compacted and more accessible to transcription factors and machinery [61]. The stability of these chromatin states are regulated by epigenetic signatures [62], avoiding the

recombination of repetitive sequences and transcription of transposable elements [63], preventing DNA damage and defining chromosomal organization in structural elements, such as the centromere or telomeres [61].

As aforementioned, epigenetics can be influenced by environmental factors [13]. Adverse environments might lead to alterations in epigenetic signatures, which can be transferred to the offspring in a mechanism denominated transgenerational inheritance [64]. Transgenerational epigenetic inheritance can be defined as the transmission of phenotypes over generations in the absence of exposure to the original agent, increasing the susceptibility to suffer from diseases [65, 66]. Although the epigenetic inheritance and the early-life environment are crucial in the establishment of epigenetic marks, environmental factors can alter the epigenetic status during the whole life of an organism [6].

2.2. Types of epigenetic modifications

Epigenetic processes, together with other transcriptional regulatory events, are implicated in the regulation of gene activity and expression during development and differentiation, or in response to environmental stimuli [67]. The main three recognized epigenetic mechanisms that regulate gene expression are covalent histone modifications, non-coding RNAs and DNA methylation [6] (Figure 2).

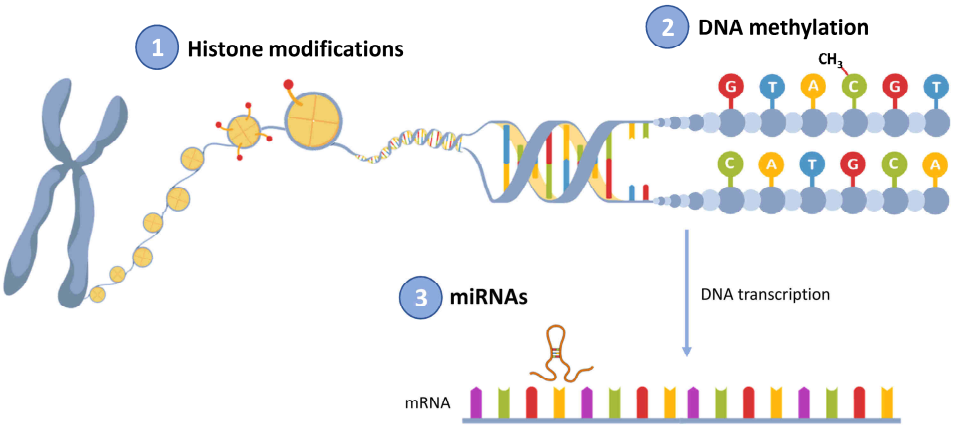

Figure 2: Types of epigenetic modifications. Adapted from Barrès & Zierath [68].

2.2.1. Histone modifications

Histones are proteins composed by a globular C-terminal domain and a flexible positively charged N-terminal tail [69]. An octamer of histones (two hetero-dimers of H2A-H2B and a tetramer of two homo-dimers of H3-H4) [40] and 147 bp of DNA wrapped around it comprise the nucleosome, which is the basic unit of chromatin [70].

Histones are subject to post-translational modification in their N-terminal tail, including acetylation, biotinylation, methylation, phosphorylation, ubiquitination, SUMOylation, ribosylation, isomerization, citrulination, butyrylation, propionylation, and glycosylation [43], although the most described in humans are acetylation, biotinylation, methylation, phosphorylation and ubiquitination [71] (Figure 3). These modifications regulate the gene expression by altering the chromatin assembly and compaction, changing the accessibility and providing binding sites for transcription factors and other effector proteins [72]. Histone modifications are reversible and regulated by the action of several enzymes, such as histone acetyltransferases, histone deacetylases, histone methyltransferases, histone demethylases, phosphatases, kinases, among others [73].

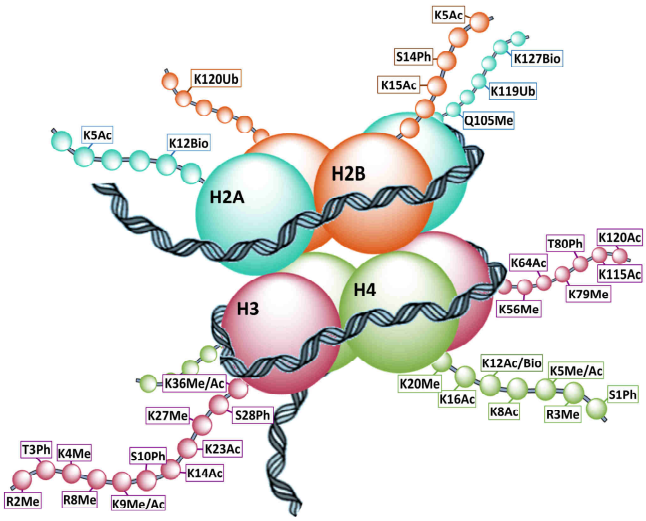

Figure 3: Example of histone modifications. Nomenclature: Aminoacid-position-modification (modifications: Ac: acetylation, Bio: biotinylation, Me: methylation, Ph: phosphorylation, Ub: ubiquitination). Modified from Tsankova *et al.* [74].

2.2.2. Non-coding RNAs

Non-coding RNAs (ncRNAs) are a cluster of RNAs that do not translate into functional proteins [75]. They can be classified into long ncRNAs, which contain >200 nucleotides, and short ncRNAs, made up of 20-300 nucleotides [76]. Long ncRNAs have been described as molecular guides, scaffolds, decoys, and allosteric modulators in the regulation of transcription and chromatin [77]. On the other hand, short ncRNAs, which are generally related to gene silencing by transcriptional repression or DNA degradation [76], include microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs and small nuclear RNAs [6, 75, 78].

The most extensively studied ncRNAs are the miRNAs [43]. They are processed RNAs strands of about 18-22 nucleotides, which have underwent a series of maturation steps from primary miRNAs [26]. Some miRNAs are located within introns of protein-coding genes and are transcribed along with the primary transcript, whereas others are transcribed from their own gene [79]. After transcription, the primary miRNA is processed by the RNase Drosha in the nucleus, generating the precursor miRNA, which is exported to the cytoplasm. Afterwards, the precursor miRNA is clove by Dicer, producing the mature miRNA [80] (Figure 4). The mature miRNA can regulate translation of mRNAs by forming part of the RNA-Induced Silencing Complex (RISC) and pairing with 3'- untranslated regions of the target messenger, resulting in translational inhibition if there is partial complementarity or degradation if the complementarity is complete [43, 79]. Hence, miRNAs affect their target genes at the posttranscriptional level, thus regulating many biological processes [27]. miRNAs have been proposed as biomarkers for diagnosis, prognosis, and personalisation of treatments for diseases, such as obesity [81]. Furthermore, accumulating evidence suggests that miRNA also target epigenetic effectors, such as histone deacetylases or DNA methyltransferases (DNMTs) [72], as well as be susceptible to epigenetic modulation [40].

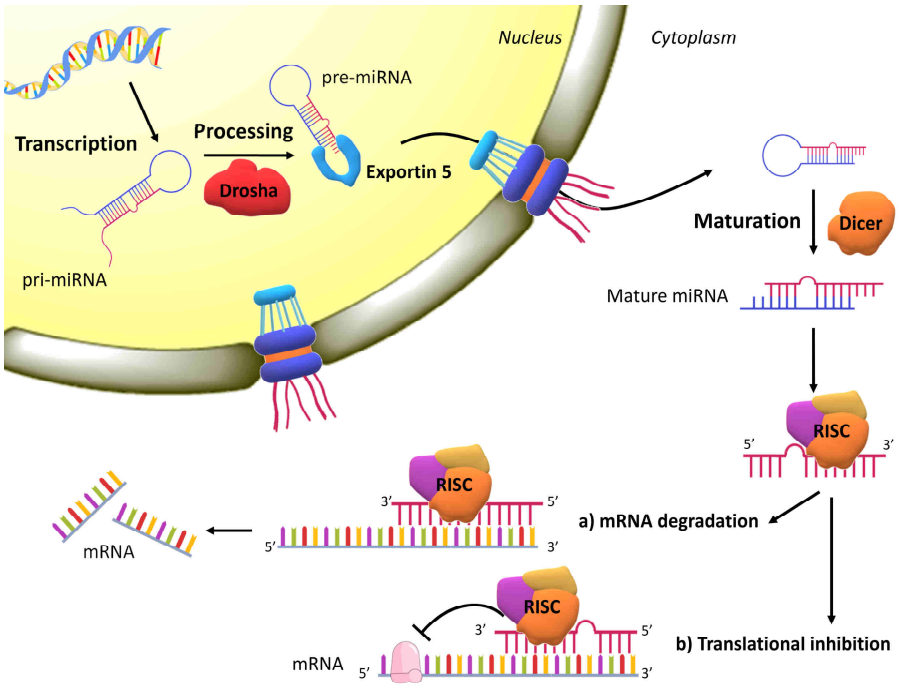

Figure 4: Biogenesis of miRNA. Modified from Castanotto & Rossi [82]. Abbreviations: pri-miRNA: primary miRNAs, pre-miRNA: precursor miRNA, RISC: RNA-Induced Silencing Complex.

2.2.3. DNA methylation

DNA methylation consists on the addition of a methyl group to the DNA molecule, either in adenine or cytosine nucleotide bases [83]. In mammals, the most common DNA methylation occurs in CpG sites, where a methyl group is incorporated at the 5th carbon of a cytosine adjacent to a guanine, resulting in 5-methylcytosine [77] (Figure 5).

DNA methylation can occur in different regions of the genome, such as repetitive sequences, gene body, promoter-related CpG islands and CpG island shores. CpG islands are regions of about 1000 bp with an elevated concentration in CG bases, mainly located at gene promoters (70% of total CpG islands) and generally, with low DNA methylation [84]. CpG island shores are located up to 2 kb upstream of the CpG island [85]. The majority of methylated CpGs are located within gene bodies [86].

DNA methylation can influence gene transcription in three different manners: (a) affecting the binding of methylation-specific recognition factors to promoters or gene bodies; (b) by modifying the affinity of a transcription factor to a gene promoter; (c) altering the chromatin structure and spatial accessibility of transcription factors and/or other DNA binding proteins [87]. In general, methylated DNA sequences are related to gene silencing, while unmethylated CpGs are associated with transcriptional activity [88]. However, there is evidence that DNA methylation can also positively correlate with gene expression [86].

Failure to maintain appropriate methylation patterns leads to abnormalities in DNA methylation levels and thus, changes in gene transcription that might influence the onset of diseases including cancer; neurological, neurodevelopmental and neurodegenerative diseases; autoimmune diseases; obesity and diabetes; among others [79, 87, 89].

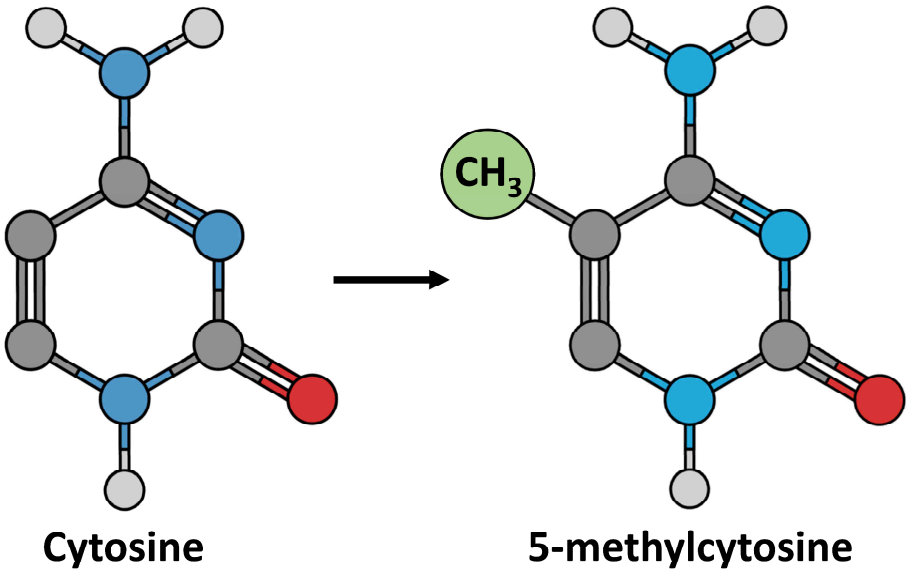

Figure 5: DNA methylation of cytosine forming 5-methylcytosine. Modified from Relton & Davey Smith [21].

3. DNA METHYLATION

3.1. Types of DNA methylation

Methyl groups can be added to both adenine and cytosine nucleotide bases [83]. On the one hand, adenine methylation consists on the addition of a methyl group to the 6th NH_2 of the adenine, forming N6-methyladenine [90]. This modification is dominant in bacteria and it was initially described as part of their defence mechanisms (restriction-modification systems) for discriminating between host and invader DNA [91]. Apart from that function, N6-methyladenine modification is involved in the regulation of chromosome replication, DNA segregation, mismatch repair, repression of transposable elements, and gene regulatory processes [90, 92]. Furthermore, not only has N6-methyladenine been observed in bacteria, but also in eukaryotic genomes, including ciliates, chlorophyte algae, dinoflagellates, and even, in some evolved organisms such as mosquito, plants or mammals [91] Recently, this type of modification was also reported in the human genome [93].

On the other hand, cytosine methylation can occur in the 4th NH_2 or in the 5th carbon of the cytosine, forming N4-methylcytosine or 5-methylcytosine, respectively [94]. Whereas N4-methylcytosine is confined to prokaryotes, 5- methylcytosine can also be observed in eukaryotes [94]. Two main different 5- methylcytosine methylations have been described: CpG and non-CpG methylation. CpG methylation consists on the incorporation of a methyl group to a cytosine that is adjacent to a guanine [95]. This modification is the most common DNA methylation in mammals [77]. On the contrary, non-CpG methylation is defined as the methylation of cytosines within CpC, CpT and CpA sequences [89]. In humans, non-CpG methylation has been found at CHG and CHH sites (where H can be an A, C or T) in stem cells [85] and brain [96]. In stem cells, this type of methylation seems to be related to the origin and maintenance of pluripotent state [97].

Different forms of 5-methylcytosine have also been described as part of the demethylation process [98]. The Ten Eleven Translocation (TET) enzymes progressively oxidize the 5-methylcytosine, leading to 5- hydroxymethylcytosine, followed by 5-formylcytosine, and then

5- carboxycytosine [99]. These less common forms might also have a regulatory function [96]. Indeed, 5-hydroxymethylcytosine methylation has been associated with an increase in the transcription of genes, due to the release of methyl binding proteins from the DNA when hydroxylated [79]. Moreover, 5- hydroxymethylcytosine methylation dynamically changed during postnatal neurodevelopment and ageing, being important in human neurological disorders [100] and other diseases, such as cancer [101].

3.2. DNA methylation process

DNA methylation at CpG sites is catalysed by DNMTs, which transfer a methyl group from SAM onto cytosine [37]. In mammals, five members of DNMTs have been reported: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L, although only DNMT1, DNMT3a and DNMT3b present methyltransferase activity [85].

DNMT1 is responsible for the maintenance and reproduction of the existing methylation patterns during DNA replication, from the hemimethylated CpG sites (when one CpG is methylated in one strand, but not on the other) to the new DNA strands generated [70]. This enzyme, which is the most abundant DNMT in the cell, exhibits a 30-40 fold preference for the hemimethylated substrates, but also presents *de novo* DNMT activity [85]. DNMT1 is constrictively expressed in dividing cells and mainly transcribed during the S-phase of the cell cycle [102].

DNMT3a and DNMT3b are *de novo* methyltransferases involved in the establishment of DNA methylation patterns during germline development and embryogenesis [73, 103]. The expression of these methyltransferases is increased in embryonic stem cells, whereas it is downregulated in differentiated cells [85]. However, they are also important for the stable inheritance and active remodelling of DNA methylation patterns in the differentiated ones [104]. DNMT3a seems to be more important in the late development or after birth, while DNMT3b may be implicated in early developmental stages and in particular regions of the genome [37].

DNMT3L stimulates the action of DNMT3a and DNMT3b, interacting and co-localizing with them in the nucleus [85]. In the case of DNMT2, it functions as a tRNA transferase rather than a DNA methyltransferase [104].

All the DNMTs use SAM as substrate [105], thus depending on methyl group donors and cofactors from ingested food, such as folate, choline, betaine or methionine [106]. These substances provide the methyl group necessary to methylate homocysteine and form methionine, which will be later transformed into SAM. Alternatively, when there is an excess of methionine, homocysteine can be converted into cysteine [107] (Figure 6).

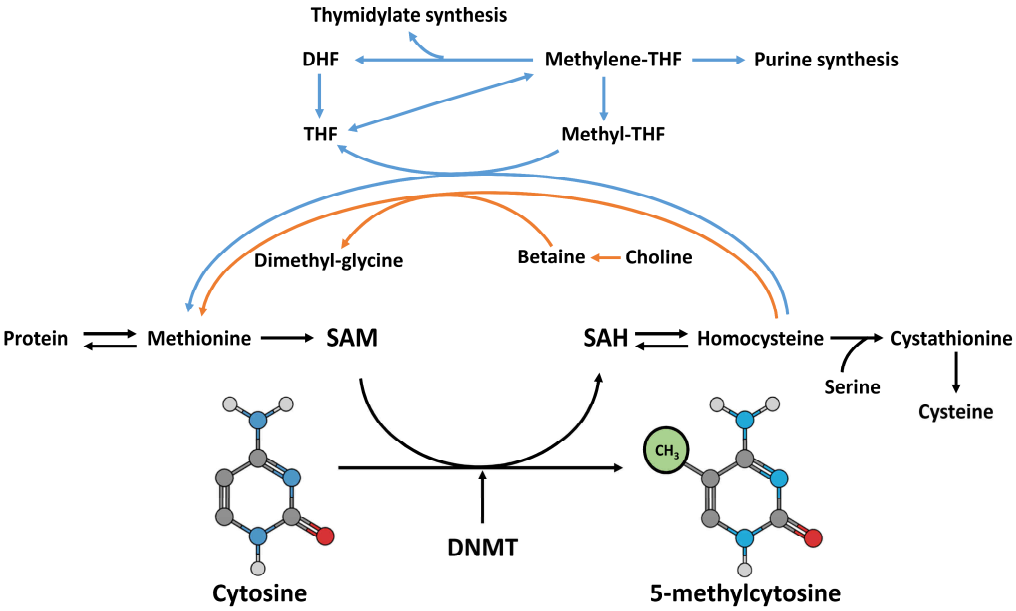

Figure 6: DNA methylation process and methyl substrates. Modified from Chmurzynska [106]. Abbreviations: DHF: Dihydrofolate; DNMT: DNA methyltransferase; SAH: S-Adenosylhomocysteine; SAM: S- Adenosylmethionine, THF: Tetrahydrofolate.

3.3. Techniques for DNA methylation assessment

Different technologies have been developed for DNA methylation analysis (Table 1). Nevertheless, any method is able to combine cost efficiency throughput, quantitative accuracy and sensitivity, capability for whole genome analysis, and precise exploration of individual CpG sites [108]. Hence, the election of the method might depend on the type of investigation [108].

DNA methylation assessment techniques can be classified, according to the resolution of the detection, in global, locus-specific or genome-wide methylation [109]. Furthermore, these different approaches are found after three pre-treatment methods: bisulfite conversion, enzyme restriction digestion and affinity enrichment [110]. Bisulfite conversion is based on the transformation of unmethylated cytosines into uracil due to deamination, whereas methylated cytosines remain intact, followed by a Polymerase Chain Reaction (PCR) amplification, where all the uracils are converted into thymines [111]. Enzyme restriction digestion consists on the distinction of methylated from unmethylated CpG sites by sensitive-methylation restriction endonucleases [110]. Finally, affinity enrichment is based on immunoprecipitation of genomic DNA with specific antibodies for methylated cytosine [109].

Combining pre-treatment methods and different techniques generates multitude of possibilities to measure and analyse DNA methylation.

3.3.1. Global quantification of DNA methylation

Global DNA methylation is defined as the total methylation status across the genome [112] and several methods employing the three different pre-treatments have been developed to measure it.

For instance, Long Interspersed Nuclear Element 1 (LINE-1) or Alu repetitive elements reflect global DNA methylation changes and can be measured using bisulfite conversion and subsequent amplification, followed by PCR-based methods such as pyrosequencing or High Resolution Melting (HRM) techniques [109]. Pyrosequencing is based on the measurement of the light generated after the phosphate release due to nucleotide incorporation [113], whereas HRM relies on the generation of DNA melt curve profiles [114]. Both techniques allow the detection of small methylation changes [115, 116].

Another methodology is Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS). This method requires digested DNA into single nucleosides which are separated using liquid chromatography. Then, methylation levels are measured with mass spectrometry allowing a detection rank from 0.05% to10%, and differences between samples as small as 0.25% of the total cytosine residues [117].

One affinity enrichment method to measure global DNA methylation is Enzyme-Linked Immunosorbent Assay (ELISA). This technique consists on capturing the DNA sample in a plate, subsequent incubations of primary and secondary antibodies, and detection by colorimetric or fluorometric reactions. A limitation of this assay is the high variability in the results [115].

Table 1: Techniques for DNA methylation assessment.

Pre-treatment	Resolution			
	Global quantitative	Locus-specific	Genome-wide	
			Array	NGS
Bisulfite conversion	Alu/LINE-1 PCR-pyrosequencing Alu/LINE-1 HRM	MSP qMSP SMART-MSP MethyLight MS-HRM MS-SnuPE Pyrosequencing MassARRAY	Infinium Golden Gate	RRBS WGSBS oxBS-Seq
Enzyme restriction	HPLC LC-MS/MS LUMA	HELP MS-MLPA MS-FLAG	DMH MCAM HELP CHARM MMASS	Methyl-Seq MCA-Seq MSCC HELP-Seq
Affinity enrichment	ELISA	MeDIP-PCR MIRA	MeDIP-chip MIRA-chip	MeDIP-Seq MIRA-Seq MethylCap-Seq

Modified from Mansego *et al.* [109]. Abbreviations: CHARM: Comprehensive High-throughput Arrays for Relative Methylation; DMH: Differential Methylation Hybridisation; ELISA: Enzyme-Linked Immunosorbent Assay; HELP: *HpaII* tiny fragment Enrichment by Ligation-mediated Polymerase Chain Reaction (PCR); HPLC: High Performance Liquid Chromatography; HRM: High Resolution Melting; LC-MS/MS: Liquid Chromatography coupled with tandem Mass Spectrometry; LUMA: Luminometric Methylation Assay; MCA-Seq: Melting Curve Analysis - Sequencing; MCAM: Methylated CpG island Amplification-Microarray; MeDIP: Methylated DNA Immunoprecipitation; MethylCap-Seq: Methyl-DNA binding domain - Capture - Sequencing; MIRA: Methylated CpG Island Recovery Assay; MMAS: Microarray-based Methylation Assessment of Single samples; MS-HRM: Methylation-Sensitive HRM; MS-MLPA: Methylation-Specific Multiplex Ligation-dependent Probe Amplification; MS-FLAG: Methylation-Specific Fluorescent Amplicon Generation; MS-SnuPE: Methylation-Sensitive Single nucleotide Primer Extension; MSCC: Methylation-Sensitive Cut Counting; MSP: Methylation-Specific PCR; oxBS-Seq: oxidative Bisulfite Sequencing; qMSP: quantitative MSP; RRBS: Reduced Representation Bisulfite Sequencing; SMART-MSP: Sensitive Melting Analysis after Real Time MSP; WGSBS: Whole Genome Shotgun Bisulfite Sequencing.

3.3.2. Locus-specific quantification of DNA methylation

Locus-specific identification of DNA methylation levels is a very useful approach in the case of investigations focused on particular CpGs [42].

One technique developed for that purpose is Methylation-Specific PCR (MSP), which is based on specific primers complementary to the methylated or the unmethylated bisulfite-treated products [41]. Although, MSP is sensitive to 0.1% methylated region of a given CpG island locus [109], the number of CpGs that can be analysed is low [115]. Pyrosequencing is another method able to determine the methylation level of specific CpG sites of a bisulfite-treated DNA sample and as aforementioned, the basis is the measurement of the light generated due to the release of phosphatase when nucleotides are incorporated to the sequencing primer [113]. Another example is MassARRAY, which consists on a non-fluorescent detection platform that measure PCR-derived amplicons with high sensitivity using mass spectrometry. The PCR products are obtained after the amplification with specific tagged primers of previously bisulfite-treated DNA regions. These products are transcribed into RNA *in vitro,* followed by base-specific cleavage [118].

HpaII tiny fragment Enrichment by Ligation-mediated PCR (HELP) is a technique based on the enzymatic digestion of two genomic DNA aliquots mediated by the methylation-sensitive *HpaII* and the methylation-insensitive isoschizomer *MspI* [119].

The Methylated DNA Immunoprecipitation (MeDIP)-PCR, an affinity enrichment method, is based on the immunoprecipitation of single-stranded DNA with anti-5-methylcytosine antibodies for enrichment for methylated sequences and then, analysed by real-time PCR [119].

3.3.3. Genome-wide DNA methylation

Genome-wide DNA methylation approaches allow the analysis of whole genome methylation and the development of Epigenome-Wide Association Studies (EWAS). Many of the methods developed for the analysis of locus-specific methylation have been combined with other techniques such as microarrays or sequencing in order to perform large-scale studies [110].

Methylation microarrays are based on the hybridisation of the DNA sample to a chip allowing the methylation analysis of multiple genes at the same time [120]. For example, Illumina has developed a method based on bisulfite conversion of DNA sequencing and hybridisation onto a microarray [109]. The last available platforms are Infinium HumanMethylation 450k BeadChip and MethylationEPIC BeadChip, which allow determining the methylation profile of approximately 450000 and 850000 CpGs, respectively [42].

Next-Generation Sequencing (NGS) approaches allow the detection of genome-wide differentially methylated CpGs using sequencing strategies [109]. For instance, some of the aforementioned techniques such as MeDIP or HELP can be combined with NGS in order to analyse methylation in the whole genome [110]. Other method is denominated Reduced Representation Bisulfite Sequencing (RRBS). RRBS consists on the isolation of short fragments generated after the cleavage with a methylation-insensitive restriction endonuclease with a CG-rich recognition sequence, such as *MspI* [115]. Afterwards, a standard library with these fragments is constructed using methylated adapters followed by bisulfite conversion [108]. RRBS covers approximately 80% of CpG islands, which corresponds to the 12% of the total CpGs in the human genome [109].

3.4. Methylation in different stages of life

Methylation levels vary from the germ cells to the late stages of life [95]. There are two crucial points for methylation reprogramming (demethylation/methylation processes): during primordial germ cell development and immediately after fertilization [121] (Figure 7).

Primordial germ cells suffer a process of methylation erasure and subsequently, *de novo* methylation occurs in both male and female germ cells, leading to mature gametes with methylation levels above somatic cells [70]. Upon fertilization, there is a demethylation process of both paternal and maternal genomes, being the maternal slower than the paternal [122]. This process of demethylation occurs before the implantation [122]. Nevertheless, current evidences suggest that there is an incomplete clearance of the epigenetic signatures, resulting in transgenerational epigenetic inheritance [123].

After implantation, at the blastocyst stage, there is a re-methylation of most of the genome [70]. Epigenetic patterns for different cell types are set at this point [6], as well as the process of X-chromosome inactivation [79], being the most critical period for the establishment of the epigenome [6]. After the establishment of the epigenome, the high methylation rate of the *de novo* methylation tends to decrease with differentiation [124].

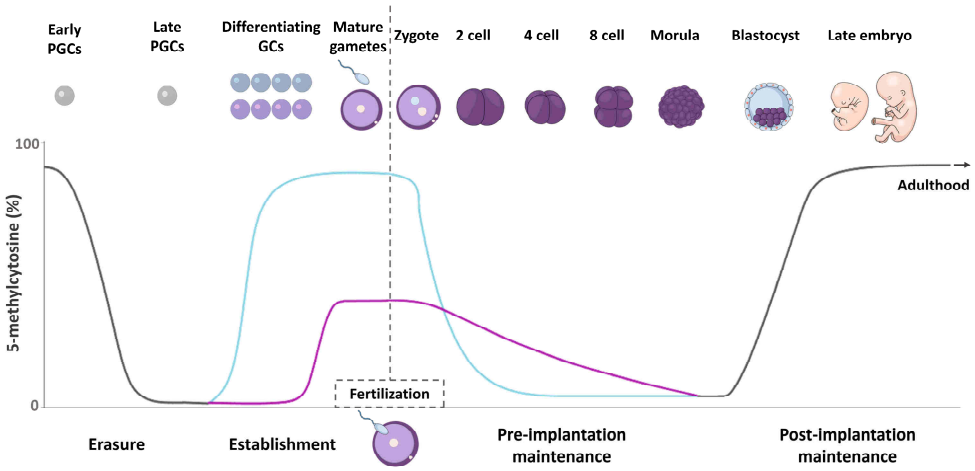

Figure 7: Whole genome methylation levels in the different developmental stages (blue line: paternal methylation levels, purple line: maternal methylation levels). Adapted from Monk *et al.* [122]. Abbreviations: GCs: Germ Cells, PGCs: Primordial Germ Cells.

Epigenetic signatures, which influence lineage and tissue-specific gene expression, are relatively stable and preserved throughout the life course of the organism [121]. However, alterations in DNA methylation in the germline and during pre-implantation could increase disease susceptibility later in life [125].

From the first stages of life after birth to the death, extrinsic an intrinsic factors could affect DNA methylation, altering the gene expression and also, affecting disease risk [38]. Furthermore, there is an epigenetic drift during ageing, involving a global DNA hypomethylation and a CpG hyper or hypomethylation in several gene promoters [55].

3.5. Factors influencing DNA methylation

Personal habits, life experiences and environmental exposures to nutrition, chemical and physical factors may affect DNA methylation, modifying gene expression and thereby, having an impact on health [11, 126].

3.5.1. Prematurity and prenatal environment

The establishment of an appropriate DNA methylation during foetal growth and development is crucial to reduce disease risk in the future [127]. Many factors can alter DNA methylation in these stages, such as the mother's health [128] or preterm birth [22].

Preterm newborns show an increased risk for morbidity and mortality, as well as higher probabilities of suffering from disabilities and chronic diseases such as neurological disorders, hypertension, T2D, and cardiovascular disease, among others [129]. Epigenetics seems to play a role in the relationship between prematurity and the development of these diseases [7]. Indeed, several studies have reported an association between epigenetic differences and gestational age and growth patterns [129]. For instance, low birth weight has been related to a methylation increase of *PPARGC1A* in muscle, leading to a higher prevalence of T2D [130]. Numerous CpGs, many of them related to developmental processes, have been identified associated with preterm birth in cord blood [131]. Moreover, a longitudinal study found differences in methylation at 18 years of age between individuals who were born preterm and born at term [132].

Different health issues during pregnancy such as maternal nutrition, alcohol and drug consumption, exposure to endocrine disruptors, diabetes, obesity, and psychological health, may have a great impact in the child's epigenetic signatures [133]. Epigenetic alterations produced before birth may increase the susceptibility to several diseases [134].

- **Maternal nutrition**

Prenatal nutrition deficiency may lead to alterations in the structure and function of the brain, development, growth, metabolic and biochemical processes [135, 136]. These alterations might be partially explained by epigenetic modifications that can be observed in the offspring, even in the adulthood [35].

A well-studied example is the maternal malnutrition during the Dutch Hunger Winter [76]. Children born from those malnourished mothers showed hypomethylation in the *IGF2* gene [137] and hypermethylation in *IL10, LEP, ABCA1, GNASAS* and *MEG3* genes [138], as well as an increased risk for cardiovascular and metabolic disorders, pulmonary diseases and breast cancer [139].

Overnutrition can also affect the epigenetic signatures of the offspring [24]. For instance, in mouse models, a maternal high-fat diet led to changes in the offspring methylation [10, 133]. One study reported changes in epigenetic regulators such as *Mecp2* in amygdala [140], whereas other scientists showed more than 1000 differentially methylated regions associated with the maternal high-fat diet [141].

Maternal changes in nutrient intake involve changes in DNA methylation [142]. Protein-restriction diets during pregnancy in rats have been related to methylation alterations in genes implicated in fat metabolism (*Ppargc1a*) and in response to stress (*Nr3c1*) in the offspring [143]. On the other hand, low-carbohydrate intake has been associated with an increased methylation in *RXRA* gene, leading to higher Body Mass Index (BMI) and fat mass in children [144]. Changes in the consumption of different compounds, such as minerals or heavy metals, may also alter DNA methylation [134]. For example, magnesium deficiency in pregnant rats can alter methylation of the *Hsd11b2* gene [145]. Moreover, a high intake of zinc during gestation has been associated with a reduced DNA methylation in the *A20* promoter of offspring chicks [146].

Maternal diet supplemented or restricted with methyl donor compounds, such as folate, choline, betaine and methionine, may influence not only the methylation status, but also the offspring phenotype, due to the availability of methyl groups [8]. A visual example is the range of colours of Agouti viable yellow mice coats depending on the amount of methyl donor intake [147]. In humans, supplementation with folic acid during pregnancy modifies the methylation of different CpGs at the *IGF2* gene in the offspring [148]. Furthermore, folate deficiency can increase the risk of overweight or obesity in children, while an adequate folate intake during pregnancy can attenuate this risk in children from obese mothers [149].

❖ **Obesity and diabetes**

Maternal obesity has been related to many complications during pregnancy and long-term consequences in the offspring [150]. Underlying this relationship, DNA methylation and other epigenetic processes may have a role [151]. Indeed, associations between maternal obesity and DNA methylation alterations have been reported in several CpGs [152]. For example, maternal BMI correlated with methylation changes at 104 CpG sites [153]. Another investigation showed differences in methylation levels in more than 5000 genes, mainly related to cardiometabolic pathways, between siblings born from obese mothers before and after a bariatric gastrointestinal bypass surgery [154]. Other studies showed that pre-pregnancy overweight or obesity increased the risk of Attention Deficit Hyperactivity Disorder (ADHD), cerebral palsy, and Autism Spectrum Disorders (ASD) [150].

Offspring of diabetic mothers have higher risk of developing hypertension, obesity and T2D [41]. Indeed, early exposure to hyperglycaemia, elevated insulin and lipid levels may dysregulate functions related to the development of T2D and obesity [121]. Maternal T2D during pregnancy has been linked to methylation changes in the offspring at CpG sites located at *LHX3*, *PCDHGA4* and *STC1,* and near *PRDM16* and *AK3* genes [155]. Hypermethylation at *PRDM16* predicted future risk of diabetes, whereas hypermethylation near *AK3* and hypomethylation at *PCDHGA4* and *STC1* were related to impairments in insulin secretion in the offspring [155].

❖ **Alcohol consumption and smoking**

Maternal alcohol consumption during gestation can cause developmental alterations in neural pathways, immune system, growth, etc. For instance, methylation patterns of genes such as *SLC6A3* and *DRD4,* which are members of the dopaminergic system, have been associated with maternal alcohol consumption [156].

Exposure to tobacco toxins during pregnancy leads to poor umbilical blood flow, oxidative stress and changes in gene expression, which can result in developmental problems such as foetal growth restriction [156]. Maternal smoking has been related to methylation of several CpGs, located in genes such as *AHRR, MYO1G, IGF2, CYP1A1,* and *CNTNAP2* [157-160]. Methylation

changes due to smoking have been observed in buccal cells, placenta, umbilical cord blood and blood cells from mother and child [157]. Furthermore, many of the differentially methylated genes associated with maternal smoking were related to pathways of cancer, cell cycle, angiogenesis, and immune system, among others [161].

- **Endocrine disruptors**

Environmental exposure to toxicants, such as phthalates, during pregnancy, has been related to many disorders such as low birth weight, obesity, prematurity, autism, and allergies [162], where DNA methylation alterations may have a role [163]. Indeed, decreases in methylation of LINE-1, *IGF2* and *PPARA* have been associated with a rise in phthalate concentration [164].

- **Psychological issues**

Early life stress and psychological threats are risk factors for neuropsychiatric disorders during the life course [165]. For instance, prenatal maternal depression has been associated with DNA methylation changes in *NR3C1* and *SLC6A4* in cord blood, infant saliva and adult venous blood [22]. Furthermore, maternal stress during pregnancy has been linked to methylation of a specific CpG site located at the *NR3C1* promoter [166]. Interestingly, *NR3C1* has been associated with adverse infant neurobehaviour, stress response, blood pressure and physical development [167].

3.5.2. Nutritional factors

Besides the influence of diet and nutrients in the prenatal environment, nutritional factors can also affect the DNA methylation status after birth and during the whole life of an organism [6]. Nutrients may affect the action of epigenetic enzymes and the bioavailability of substrates for methylating reactions [126].

Several studies have demonstrated that DNA methylation is responsive to caloric restriction [41]. For instance, a weight-loss intervention induced methylation changes in *KCNQ1* and *WT1* in obese patients with a previous stroke event [168]. A hypocaloric treatment in overweight or obese subjects showed differences in *ATP10A* and *WT1* methylation in peripheral blood

mononuclear cells after the intervention [169]. Methylation differences between high and low responders to a weight loss intervention were found in regions located in or near *AQP9*, *DUSP22*, *HIPK3*, *TNNT1*, and *TNNI3* genes in blood [170], whereas 644 genes showed differences in subcutaneous adipose tissue [171]. Furthermore, *LEP* and *TNF* promoter methylation in blood mononuclear cells was able to predict the hypocaloric diet-induced weight loss [172, 173].

High-fat diets increase adiposity and alter both epigenetic signatures and expression of key obesogenic genes [35]. In this regard, *Lep* promoter was hypermethylated in murine white adipose tissue after 11 [174] or 14 weeks with this type of diet [175], whereas *Gck* and *Lpk* were hypermethylated in liver after 8 weeks of diet [176]. On the other hand, *Mcr4* was hypomethylated in murine brain tissue after 32 weeks of high-fat diet [177]. Another study showed that a high fat diet induced changes in methylation levels of *FASN* and *NDUFB6* genes in rats [178]. In humans, a five-day high fat diet induced DNA methylation changes in skeletal muscle from young healthy men in more than 6500 genes, which were mainly related to inflammation, cancer and reproduction [179].

Mediterranean diet involves the consumption of a high amount of vegetables, fruits, cereals, legumes and nuts; a relatively high quantity of unsaturated fatty acids, mostly provided by extra-virgin olive oil; a moderate-high amount of fish; moderate quantity of poultry and dairy products; and low consumption of red meat and meat products [180]. This diet has been related to lower risk of cardiovascular disease, as well as to changes in DNA methylation [181]. For example, there is an association between adherence to Mediterranean diet and LINE-1 methylation in blood samples [182].

Moreover, the type of consumed fat is also determinant for DNA methylation [183]. In this context, several studies have been performed comparing diets rich in Saturated or Polyunsaturated Fatty Acids (SFA and PUFA, respectively). For example, differences in DNA methylation have been observed in 4875 CpGs in adipose tissue from healthy young subjects enrolled in a SFA- or PUFA- enriched diet [184]. Methylation levels of several CpGs were also different in blood from preadolescents depending on SFA, PUFA and Monounsaturated Fatty Acids (MUFA) intake [183]. Moreover, *CLOCK* methylation

has been negatively associated with MUFA intake and positively correlated with PUFA intake [185], whereas *TNF* methylation was lower after n-6 PUFA intake [186].

An adequate intake of methyl donors is necessary to maintain suitable DNA methylation levels [128]. Low levels of folate consumption have been related to a decrease in DNA methylation in elderly women [187], as well as an increased risk of colorectal and pancreatic cancers [188, 189]. Furthermore, a positive association was found between folate levels and DNA methylation in 236 CpGs, which were hypomethylated in liver biopsies from T2D subjects comparing to control subjects [190].

3.5.3. Ageing

Epigenetic alterations accumulate during lifespan, depending on genetic, stochastic and environmental factors [6]. Indeed, studies in monozygotic twins revealed differences in methylation in older twins in comparison to the first years of life, when methylation was indistinguishable [191].

Along with age, there is a general decrease of genomic DNA methylation and a hypermethylation of certain gene promoters throughout the genome [86, 192]. Intriguingly, this pattern is the same observed in cancer development, where there is a global hypomethylation in tumours but hypermethylation of certain tumour-suppressor genes [193]. Some genes reported to be hypermethylated with ageing are *ESR, IGF2, TUSC3, MYOD1, DAPK, CDH1, CDKN2A, KLF14, FHL2, GLRA2, ELOVL2, and TIMP3* [142, 194, 195]. Furthermore, methylation of CpG sites located at *CREB5, RELA* and *ULK1* genes have been associated with age in peripheral white blood cells [196].

Ageing also affects the expression of DNMTs [197]. Indeed, expression of DNMT1 and DNMT3a decreases, while DNMT3b increases with ageing [197]. The reduced expression of DNMT1 in the ageing process is consistent with the global hypomethylation of the genome, since DNMT1 is in charge of methylation maintenance [142].

These alterations in epigenetic patterns may contribute to age-related diseases, such as metabolic diseases (obesity, T2D, cardiovascular disease, etc.), cancer, and neurological diseases (Alzheimer, Parkinson, etc.) [103].

Recently, several investigators have developed epigenetic clocks, which are able to predict the biological age based on DNA methylation levels in many tissues [198] (Table 2). Hannum *et al.* developed an estimator based on 71 CpGs in leukocytes [199], whereas the group led by Horvath, designed DNAmAge, based on 353 CpGs in different tissues [200], PhenoAge for predicting lifespan based on regressing a phenotypic measure of mortality risk on CpGs [201]; and GrimAge, which used DNA methylation-based biomarkers for seven plasma proteins and smoking [202]. GrimAge has been demonstrated as the better predictor of lifespan among all the currently available predictors [202]. These models are useful to analyse the role of methylation in age-related diseases, helping to explain the relationship between the metabolic status and the onset of these diseases [103]. Accelerated epigenetic age, defined as the residual from regressing the epigenetic age on chronological age [203], can be understood as the ageing of epigenetic DNA regulation. Epigenetic age acceleration has been related to obesity [203], cardiovascular disease [204], T2D [205], Alzheimer disease [206], allergy and asthma [207], and cancer [208], among others.

Table 2: Characteristics of epigenetic clocks.

Approach	Number of CpGs	Characteristics of CpGs	Tissue	Prediction	Reference
Hannum	71	Biomarkers of chronological age	Blood	Biological ageing	Hannum 2013 [199]
DNAmAge	353	Biomarkers of chronological age	Multi-tissue	Biological ageing	Horvath 2013 [200]
PhenoAge	513	Biomarkers of chronological and phenotypical age	Multi-tissue	Morbidity and mortality outcomes	Levine 2018 [201]
GrimAge	1030	Biomarkers of smoking and 7 plasma proteins	Blood	Lifespan and health span	Lu 2019 [202]

3.5.4. Lifestyle

Physical activity, smoking, alcohol consumption, and shift-working are modifiable lifestyle habits that can influence methylation patterns and thereby, alter the susceptibility to suffer from certain diseases [209].

- **Physical activity**

Interventions with physical activity have demonstrated changes in DNA methylation levels. For example, acute exercise altered DNA methylation at *PPARGC1A, PDK4* and *PPARD* genes in skeletal muscle, as well as their expression [210]. Higher DNA methylation levels were found in *IRS1* in cultured human myotubes after *in vivo* exercise [211]. Moreover, numerous CpGs in adipose tissue were altered after a six-month exercise intervention, such as the located at *KCNQ1, TCF7L2, IGF2BP2,* and *JAZF1* genes [212]. Another six-month exercise intervention in individuals with a family history of T2D showed a differential methylation in CpGs located at genes, such as *MEF2A, RUNX1, NDUFC2, THADA, ADIPOR1, BDKRB2,* and *TRIB2* [213].

- **Alcohol consumption and smoking**

Alcohol abuse provokes liver injuries, such as steatosis, alcoholic steatohepatitis, fibrosis and cirrhosis, being one of the leading mortality causes [214]. Evidences suggest a relationship between alcohol consumption and changes in methylation levels [214]. For instance, alcohol interferes with folate bioavailability and metabolism and therefore, with accessibility to methyl groups [215]. Alcohol consumption has been related to hypermethylation of genes such as *AVP*, *HERP, OPRM1, SLC6A3,* and *SNAC,* and hypomethylation of *ANP* [216]. Furthermore, an EWAS showed widespread methylation modifications in lymphoblasts from females due to alcohol consumption [217]. One of those altered CpGs, *GDAP1*, was replicated by other group in alcohol-dependent patients [218].

Tobacco smoke contains toxins with carcinogenic, proinflammatory and proatherogenic properties [219]. Different studies have evidenced epigenetic changes associated with smoking [219]. For example, peripheral blood lymphocytes from smoking lung cancer patients showed *TP53* hypomethylation [220]. Moreover, more than 1500 CpGs in buccal cells were associated with smoking and this pattern was able to discriminate between normal and cancer tissue [221].

❖ **Shift-working**

Shift-work can negatively impact on the workers' health due to alterations in their circadian rhythms [222]. For example, DNA methylation changes in Alu repetitive elements and inflammatory genes, such as *IFNG* and *TNF,* have been found in blood cells from night-shift workers [223]. Additionally, methylation changes on CpG islands from clock genes, such as *CLOCK* and *CRY2*, are linked to breast cancer occurrence in long-term shift-workers [224].

3.5.5. Others

Many other factors may influence the methylation signatures such as air pollutants, endocrine disruptors, neurotoxic metals, psychological and behavioural stressors, infectious agents, oxidative stress, etc.

❖ **Psychological stressors**

Several studies have demonstrated that DNA methylation is sensitive to a range of psychological stressors [22]. For instance, an increase in methylation of *NR3C1* exon 1_F region was associated with military deployment and succeeding development of mental health problems and posttraumatic stress disorder [225]. This region was also altered in the brain of suicide victims with a history of childhood abuse [226].

❖ **Infectious agents**

Infectious agents are able to induce epigenetic changes [21]. In this regard, *Helicobacter pylori* has been associated with an increase in *THBD, LOX* and *HAND1* methylation [227] and the Epstein-Barr virus, with *CDH1* promoter hypermethylation [228].

❖ **Oxidative stress**

Oxidative DNA damage can alter DNA structure and provoke chromosomal rearrangements, interfering in the DNA methylation process [134]. In this context, guanines can be replaced by the oxidative damage by-product 8-hydroxy 2'-deoxy-guanosine, affecting the binding of methyl-CpG binding proteins and thereby, resulting in heritable epigenetic changes [229]. Furthermore, reactive oxygen species can interact with different factors such as Snail, which recruits DNMT1, stimulating promoter methylation of *CDH1* [230].

❖ **Environmental pollution**

Air pollutants, such as black carbon or airborne benzene, are particulate matter that can alter the epigenome and increase the prevalence of respiratory problems and lung cancers [76]. For instance, exposure to low-dose airborne benzene has been associated with reduced levels of LINE-1 and Alu methylation, as well as hypermethylation of *CDKN2B* and hypomethylation of *MAGE-1* [231]. Exposure to polycyclic aromatic hydrocarbons has also been related to *TP53* hypomethylation and to higher methylation levels in *IL6* and both LINE-1 and Alu elements [232]. Furthermore, exposure to elevated concentration of ozone has been associated with hypomethylation of *F3, ICAM1* and *TLR2,* and hypermethylation of *IFNG* and *IL6* [233].

❖ **Neurotoxic metals**

Metals including arsenic, lead and nickel are able to modify DNA methylation [76]. For example, arsenic exposure has been related to a hypermethylation of *TP53*, also observed in arsenic-induced skin cancer patients [234]. However, high doses of arsenic led to *TP53* hypomethylation in a small subgroup of patients and hypermethylation of *CDKN2A* [234]. In the same line, another study showed global hypermethylation after exposure to 250–500 μg/L of arsenic in drinking water, whereas higher levels (>500 μg/L) resulted in global hypomethylation [235].

3.6. Diseases and DNA methylation

Epigenetic aberrations play an important role in the onset and progression of many diseases, partly mediated by the effect of environmental factors [21]. Hence, understanding the underlying epigenetic mechanisms, the interactions and the modifications involved in health and disease has become a priority in the biomedical field [85] (Figure 8).

3.6.1. Neurological diseases

Epigenetic changes are able to modify the structure and function of the brain leading to psychopathology by three different ways: changing brain growth and development, altering signalling pathways and increasing the effects of neurotoxins [135]. In this regard, DNA methylation modulation is involved in brain

development, plastic changes and brain diseases [236], playing a critical role in learning and memory processes [237]. Evidences have revealed the association between DNA methylation alterations and neurodevelopmental impairments, mental disorders and neurodegenerative diseases [85].

For example, prevalence of neural tube defects during neurodevelopment increases with reductions in DNA methylation and it can be prevented by supplementation with folic acid during pregnancy [238].

Mental disorders, such as ASD or schizophrenia, have also been related to DNA methylation alterations [98]. For instance, differentially methylated regions have been found when comparing ASD patients with controls. Moreover, schizophrenia patients showed lower methylation levels of *COMT* than healthy controls [239].

Additionally, DNA methylation alterations have been described in neurodegenerative diseases, such as Alzheimer and Parkinson [85, 237]. Several studies have analysed global and specific CpG methylation in Alzheimer disease [236]. For example, global DNA hypermethylation has been reported in Alzheimer patients [240, 241], although one investigation showed no differences with controls [242]. Furthermore, an EWAS has reported differential methylation in CpGs between Alzheimer patients and controls: hypomethylation of genes involved in membrane transport and protein metabolism and hypermethylation of genes implicated in transcriptional processes [243]. Some specific genes widely described in relation to Alzheimer disease were *BDNF* (blood and brain) and *SORBS3* (brain), showing higher methylation than in control patients [236].

On the other hand, Parkinson patients showed a hypomethylation of *TNFA* promoter, which is, in turn, overexpressed, increasing the vulnerability and damage of neuronal cells [244]. Other studies reported hypomethylation of CpGs located at genes, including *CYP2E1, JAKMIP3* and *MYOM2*, and hypermethylation of CpGs at genes, such as *VAV2, MOG* and *MAPT* in Parkinson patients in comparison to controls [245, 246].

3.6.2. Metabolic diseases

Metabolic diseases are complex diseases caused by dysregulation in metabolic processes, where mitochondrial dysfunction, oxidative stress, inflammation and signalling pathways may play an important role [103]. Indeed, metabolites affect many cellular processes modulating gene expression directly or through epigenetic modifications [247]. Therefore, metabolic alterations might be influenced by the interrelationship between genetic, epigenetic and environmental factors [66].

Glucose homeostasis and insulin resistance are key players in the development of metabolic-related diseases. Insulin is secreted by pancreatic β-cells and it is responsible for the glucose uptake in different tissues, participating in the inhibition of liver glucose production and lipolysis suppression [248]. In an insulin-resistant condition, resulting from the expansion of adipose tissue, glucose accumulates in blood, leading to hyperglycaemia and hyperinsulinemia [248]. Some epigenetically regulated genes contributing to these states are *ABCG1, IRS1, IRS2, PPARGC1A, CPT1A, CCND2, FHL2, CDKN1A, PDE7B, SREBF1, IL18, CD44, CXCL1, IGF1R, LEF1, GIPR, GRB10, SIRT2, HDAC4, DNMT3A, LEPR,* and *LEP* [27, 249].

Furthermore, accompanying insulin resistance, there is a low-grade inflammation status derived from the increased secretion of different chemokines, mainly in the adipose tissue, such as Tumour Necrosis Factor α (TNF-α), Monocyte Chemotactic Protein-1 (MCP-1), C-Reactive Protein (CRP), and Interleukin 6 (IL-6) [250]. In this context, DNA methylation modifications associated with inflammatory processes have been reported in genes including *SOCS3, ADIPOQ, ABCG1* and *PPARGC1A* [249].

- **Obesity**

Regarding obesity, several EWAS have studied the association between DNA methylation and obesity traits, mainly BMI and Waist Circumference (WC) [251]. For instance, a study carried out in three different datasets found three CpGs in *HIF3A* gene that were associated with BMI in whole blood [38]. Another EWAS showed the correlation in whole blood between BMI or WC and the methylation of several CpGs, located at genes such as *HIF3A, CPT1A, SREBF1*

and *ABCG1* [252]. In another large-scale study, an association in peripheral blood cells between BMI and 287 CpGs was reported, distributed in 207 genetic loci, from which only 187 were replicated [253]. Among these genetic loci, 210 unique genes were identified, including *ABCG1, CPT1A, SOCS3, SREBF1* and *PHGDH* [253]. In CD4+ T-cells, methylation at genes, such as *CPT1A, PHGDH* and *CD38*, was associated with BMI and WC in whole blood samples [254]. Other scientists demonstrated the correlation between 94 CpGs and BMI, and between 49 CpGs and WC, including the located at *CUX1, DDAH2, ABCG1, CPT1A, SREBF1* and *SYNGAP1* genes [255]. Furthermore, another research found that 83 CpGs were related to BMI in peripheral blood cells and replicated in an independent cohort, such as the located at *ABCG1, CPT1A, SREBF1* and *DHCR24* genes [256]. In adipose tissue, another EWAS showed the association between BMI and the methylation and the expression of 2825 genes, including *FTO, ITIH5, CCL18, MTCH2, IRS1,* and *SPP1* [257].

- **Diabetes**

The relation between DNA methylation and T2D has also pointed out the involvement of several CpGs [251]. For example, differentially methylated CpGs located at the genes *ABCG1, PHOSPHO1, SOCS3, SREBF1* and *TXNIP* were found in peripheral blood cells comparing T2D and non-T2D Indian Asians and European subjects [258]. Another EWAS showed the association between *ABCG1* methylation and HOMA-IR and fasting insulin [259], whereas the same CpG was related to HOMA-IR, fasting insulin and glucose, and two-hour fasting insulin and glucose in another study [260]. Furthermore, *TXNIP* methylation was associated with both T2D and BMI in whole blood in an Arab population [261]. In human pancreatic islets, one study identified 1649 CpGs differentially methylated between T2D subjects and controls, including CpGs located at the genes *TCF7L2, FTO* and *KCNQ1* [262]; while another one reported more than 25000 differentially methylated regions located at genes such as *PDX1, TCF7L2,* and *ADCY5* [263]. Furthermore, another large-scale study showed differential DNA methylation in 276 CpGs between T2D and non-T2D pancreatic islets [264]. In adipose tissue, more than 15500 CpGs were differentially methylated comparing subjects with and without T2D, including the located at *PPARG, KCNQ1, TCF7L2* and *IRS1* genes[265]. In skeletal muscle, T2D subjects showed

differential methylation in CpGs located at genes such as *CDKN2A* and *HNF4A* [266].

- **Cardiovascular disease**

There are less EWAS concerning cardiovascular disease. For example, subjects with and without coronary artery disease exhibited differences in 369 CpGs in whole blood cells, highlighting the CpGs located at *COL4A2* and *MMP9* genes [267]. Another study reported hypermethylation of 72 differentially methylated regions in blood from coronary artery disease patients [268]. Moreover, the comparison between stroke patients and controls revealed methylation differences in 80 CpG sites and 59 CpG sites presenting interaction between stroke and obesity [269].

- **Metabolic syndrome**

Metabolic syndrome has also been associated with DNA methylation in some EWAS. One study in 614 African-Americans found two differentially methylated CpG sites at the *IGF2BP1* and *ABCG1* genes, although only *ABCG1* was replicated in another dataset [270]. Moreover, *SOCS3* methylation was inversely correlated with metabolic syndrome in 192 individuals and validated in 1092 subjects [271].

- **Non-alcoholic fatty liver disease**

The progression of NAFLD has been associated with methylation differences in CpGs [272]. For instance, 69247 CpGs were differentially methylated between patients with mild and advanced NAFLD [273]. The majority of those CpGs (76%) were hypomethylated with disease progression, such as the located at *FGFR2* or *CASP1* genes, while 24% were hypermethylated, including the located at *MAT1A* [273].

3.6.3. Others

Other diseases, such as cancer or autoimmune diseases, have been associated with aberrations in DNA methylation.

- **Cancer**

Cancer is accompanied by a global genomic hypomethylation and a hypermethylation of regions rich in CpGs [42]. Hypomethylation primarily occurs at repetitive regions of the genome, increasing chromosomal instability, translocations, and gene disruptions [85]. Furthermore, hypomethylation of specific CpG in promoters can increase the expression of proto-oncogenes [69]. On the other hand, hypermethylation of CpG regions is usually associated with gene silencing in cancer cells, decreasing expression of tumour suppressor genes and altering transcription of genes involved in cancer development affecting functions such as DNA repair, cell cycle control, apoptosis, angiogenesis, cell-to-cell interactions, etc. [274]. Therefore, hypermethylation and hypomethylation help tumour cells to acquire growth advantages, increasing genetic instability and aggressiveness [73].

These methylation modifications are potential biomarkers for prognosis, diagnosis and classification of cancer [42]. In this regard, several studies have demonstrated the potential of DNA methylation for profiling, distinguishing between subtypes and probabilities of relapse [275-279].

Several examples of hypermethylated genes in cancer are *BRCA1*, *HMLH1, MGMT, WRN, CDH1, CDH13, CDKN2A, RARB2, GATA4, GATA5, TP73, SYK, GSTP1, DAPK1, ER, PR, PRLR, TMS1, RUNX3,* and *RIZ1*, which are involved in DNA repair, tumour suppression, cell adherence, cell cycle inhibition, and signalling processes, among others [69, 85, 274]. Contrarily, genes that are hypomethylated in different types of cancer are *S100P, SNCG, DPP6,* and *IGF2* [69, 85].

Some compounds, such as resveratrol, curcumin, genistein, other polyphenols, and folic acid, have shown chemoprotective properties due to their action on intracellular signalling pathways via epigenetic mechanisms [237, 280].

- **Autoimmune diseases**

Autoimmune diseases, such as rheumatoid arthritis, lupus erythematosus and multiple sclerosis, are caused by an abnormal immune response of the organism to their own cells and tissues [98]. Several studies have revealed a relationship between DNA methylation and these diseases [98, 281].

Rheumatoid arthritis patients showed a region with differential methylation in the major histocompatibility complex in comparison with controls [282], as well as differences in *IL6* promoter methylation in blood cells [283]. Furthermore, rheumatoid arthritis has been related to more than 1000 methylation sites, including the ones located at *MX1*, *IFI44L*, *DTX3L* and *PARP9* genes [284].

Regarding lupus erythematosus, a hypomethylation of LINE-1 repetitive elements was found in neutrophils from patients [285], as well as associations between several CpGs methylation and autoantibody production [286]. Furthermore, low levels of *IL6* promoter in peripheral blood cells might be involved in the aetiology of lupus erythematosus [287].

Multiple sclerosis has been associated with hypermethylation of *HLA-DRB1*, *IL4* and *FOXP3* and demethylation of *IFNG* and *IL17A* in different types of T- cells [288]. In addition, hypermethylation and hypomethylation of several genes have been reported in demyelinated hippocampus of multiple sclerosis patients [289].

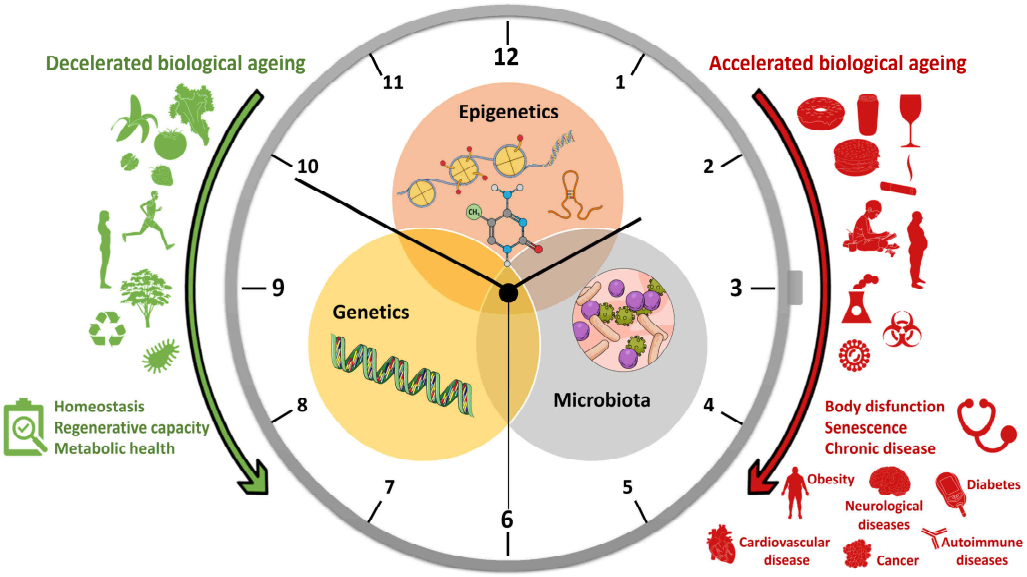

Figure 8: Endogenous factors and biological age are influenced by exogenous agents affecting the development of diseases. Modified from Twomey *et al.* [290].

HYPOTHESIS AND OBJECTIVES

1. HYPOTHESIS

This investigation is based on the hypothesis that epigenetic marks are plastic and reversible, and that they can be altered by different environmental factors. These modifications are crucial during the prenatal period and the early stages of life. However, epigenetic variations also occur during the rest of the lifespan. Impairments and aberrations in epigenetic signatures cause increased susceptibility to various diseases, such as neurodevelopmental disorders, cancer, obesity, T2D and cardiovascular disease. In fact, DNA methylation modifications can alter gene expression, influencing the onset and progression of several morbid complications.

The identification of novel epigenetic alterations may contribute to understand underlying epigenetic processes, contributing to the design of biomarker panels for early disease prediction, as well as potential targets for developing new approaches for the prevention and treatment of diseases.

Hence, it is hypothesised that different physiological, metabolic and nutritional states such as prematurity, dietary interventions, obesity, insulin sensitivity/resistance and ageing may be associated with DNA methylation modifications at specific nucleotide sites.

2. OBJECTIVES

2.1. General objective

The general aim of this research was to identify DNA methylation patterns in peripheral white blood cells associated with different physiological, metabolic and nutritional conditions, to further understand the epigenetic regulation and contribute to the development of biomarker panels and identification of potential therapeutic targets for disease management.

2.2. Specific objectives

1. To analyse the DNA methylation differences between preterm and full-term newborns, which could help to explain the adverse effects associated with prematurity (*Chapter 1*).
2. To evaluate whether the adherence to Mediterranean diet is related to DNA methylation changes (*Chapter 2*).
3. To investigate whether an intervention with two Mediterranean diets, one rich in extra-virgin olive oil and the other one in nuts, was influencing the DNA methylation status (*Chapter 3*).
4. To assess the DNA methylation association with measures of insulin sensitivity based on intravenous glucose tolerance tests in non-diabetic individuals (*Chapter 4*).
5. To explore DNA methylation levels in order to identify epigenetic signatures associated with insulin resistance and determining biomarkers related to hazardous HOMA-IR levels (*Chapter 5*).
6. To study DNA methylation changes in order to identify epigenetic methylation marks associated with waist circumference phenotypes (*Chapter 6*).
7. To investigate the interaction between ageing and epigenetic processes determining the role of visceral adipose tissue, insulin resistance and dyslipidaemia on epigenetic age acceleration (*Chapter 7*).

SUBJECTS AND METHODS

The study design of the current research was focused on DNA methylation and its putative relationship with prematurity (*Chapter 1*), Mediterranean diet (*Chapter 2* & *3*), insulin sensitivity (*Chapter 4*), insulin resistance (*Chapter 5*), central obesity (*Chapter 6*) and epigenetic age (*Chapter 7*) (Figure 9).

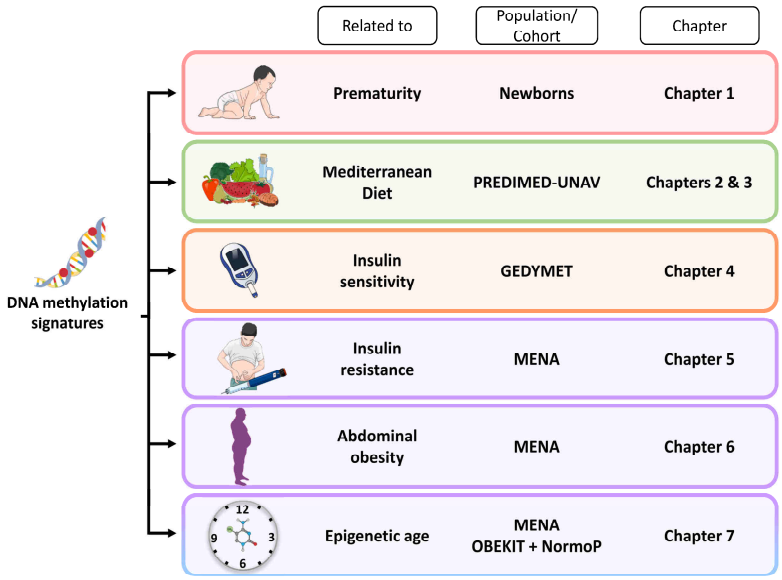

Figure 9: Experimental design including the investigated factors and features, population and assignment of chapters.

In order to investigate the interactions between methylation and different phenotypical, metabolic and nutritional states, data from several populations were obtained (Figure 10). Studies involved in this research were Newborns, PREDIMED-UNAV (Prevention with Mediterranean diet - Universidad de Navarra), GEDYMET (Genes, Diabetes and Metabolism), OBEKIT (Development of a nutrigenetic test for personalized prescription of body weight loss diets), NormoP (Normopesos) and the rest of studies included in the MENA (Methyl Epigenome Network Association) project: Food4Me-UNAV (Strategies for personalised nutrition - UNAV), DiOGenes-UNAV (Diet, Obesity and Genes-UNAV), ICTUS, NUGENOB-UNAV (Nutrient-gene interaction in human obesity: implications for dietary guidelines - UNAV), OBEPALIP (Effects of lipoic acid and eicosapentaenoic acid in human obesity), and RESMENA (Metabolic syndrome reduction in Navarra). The studies were submitted at Research Ethics Committees at all recruiting centres in compliance with the Helsinki Declaration

(50/2005, 14-281, 132/2015, KF01-267787/IHE 4-1-2.0091, 041/2012, 2/10, 5/04/2001, 007/2009, 065/2009). Written informed consent was provided by participants or by a direct familiar in the case of newborns or severe disabilities (ICTUS population) after receiving information about the protocols

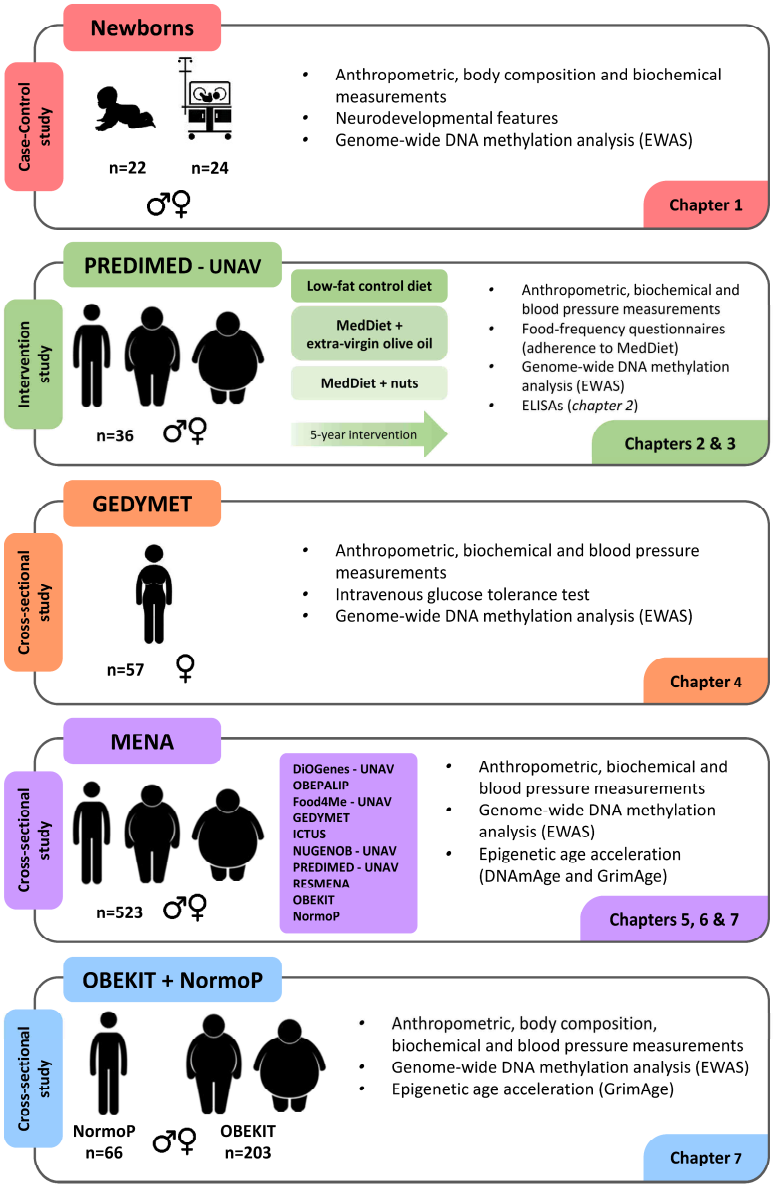

Figure 10: Experimental design for chapters 1 to 7. Abbreviations: ELISA: Enzyme-Linked Immunosorbent Assay, EWAS: Epigenome-Wide Association Studies, MedDiet: Mediterranean Diet.

1. STUDY POPULATIONS

1.1. Preterm and full-term newborns

The study of preterm and full-term newborns was conducted in collaboration with the La Paz University Hospital (Madrid) to analyse the differences in methylation levels between two groups of newborns that could explain some adverse effects related to prematurity.

1.1.1. Study population

The study population included 22 full-term newborns (>37 weeks) and 24 preterm newborns (<34 weeks and <1500 g of body weight) from the neonatal unit of the La Paz University Hospital (Madrid) fulfilling different criteria (Table 3). All the newborns were controlled postnatally in terms of nutrition and environment conditions during the first three years.

Table 3: Inclusion and exclusion criteria of the newborns study.

Inclusion criteria
Full-term newborns >37 weeks
Preterm newborns <34 weeks and <1500 g of body weight
Exclusion criteria
Presence of congenital diseases
Chromosomal abnormalities
Short bowel syndrome or other digestive disorders

1.1.2. Data collection

Anthropometric and body composition (weight, length, head circumference and fat mass), and biochemical (plasma glucose, total cholesterol, insulin, triglycerides, leptin, adiponectin, acylated ghrelin, IL-6 and cortisol) measurements were recorded at 12 months following standardised protocols. Developmental outcomes were assessed at 24 months of gestational age using the Bayley Scale of Infant Development version II (BSID-II) and at 36 months using the Bayley Scale of Infant Development version III (BSID-III) as described elsewhere [291, 292]. Perinatal complications of preterm newborns such as periventricular leukomalacia, retinopathy, sepsis, chronic lung disease and

intraventricular haemorrhage were recorded. Maternal and paternal data such as age, height, weight, BMI, educational level, smoking and presence of diabetes were collected at the La Paz University Hospital in Madrid following standardised protocols.

1.2. PREDIMED-UNAV

The PREDIMED trial was a randomized, primary prevention feeding trial with blinded assessment of end points conducted in several centres of Spain with the objective of evaluating the effects of the Mediterranean Diet on primary cardiovascular prevention (www.predimed.es). The specific aims in *Chapters 2 & 3* were to evaluate the relationship between Mediterranean Diet and DNA methylation, in terms of adherence to Mediterranean Diet or its specific components such as extra-virgin olive oil and nuts, respectively. *Chapters 5 & 6* included PREDIMED-UNAV subjects in the MENA study. This trial was registered with the International Standard Randomised Controlled Trial Number (ISRCTN of London, England: 35739639). The protocol and recruitment methods have been described in detail elsewhere [293, 294].

1.2.1. Study population

Eligible participants were selected from the recruitment centre at the University of Navarra following different criteria (Table 4).

A total of 36 participants from PREDIMED-UNAV, who were randomized into one of three nutritional interventions were selected (12 individuals for each group) for *Chapters 2 & 3*. For the MENA study (*Chapters 5 & 6*), 116 subjects were chosen.

1.2.2. Dietary intervention

The PREDIMED study involves three interventions: a Mediterranean Diet supplemented with Extra-Virgin Olive Oil (MedDiet + EVOO), a Mediterranean Diet supplemented with mixed nuts (MedDiet + nuts), and advice in reducing all sources of fat (Low-fat control group). Participants in the MedDiet + EVOO group received extra-virgin olive oil (approximately one litre per week) and individuals in the MedDiet + nuts group obtained 30 g of mixed nuts per day (15 g of walnuts, 7.5 g of hazelnuts, and 7.5 g of almonds) as described

elsewhere [293, 294]. Neither calorie restriction nor physical activity were promoted.

Table 4: Inclusion and exclusion criteria of the PREDIMED-UNAV study.

Inclusion criteria
Age between 60 and 70
Non-smokers or former smokers
Either **a)** or **b)** should be met:
a) Type 2 diabetes
b) Three or more of the following risk factors:
- Hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or under antihypertensive medication)
- LDL-cholesterol ≥160 mg/dL
- HDL-cholesterol ≤40 mg/dL independently of lipid-lowering therapy
- Body mass index ≥25 kg/m^2
- Family history of premature coronary heart disease
- If the HDL-cholesterol level was ≥60 mg/dL, one risk factor was subtracted
Exclusion criteria
Previous history of cardiovascular disease
Severe medical condition that may impair the ability of the person to participate in a nutrition intervention study
Other medical condition thought to limit survival to less than 1 year
Immunodeficiency or HIV-positive status
Illegal drug use, chronic alcoholism, or total daily alcohol intake >80g/d
Body mass index >40 kg/m^2
Difficulties or major inconvenience to change dietary habits
Impossibility to follow a Mediterranean-type diet
A low predicted likelihood to change dietary habits
History of food allergy to any of the components of olive oil or nuts
Participation in any drug trial or use of any investigational drug within the last year
Institutionalized patients for chronic care, those who lacked autonomy, were unable to walk, lacked a stable address, or were unable to attend visits in the Primary Care Health clinics every 3 months
Illiteracy
Patients with an acute infection or inflammation were allowed to participate in the study 3 months after the resolution of their condition

1.2.3. Data collection

Data were collected at baseline and at five years of intervention. All of the groups received dietary training and questionnaires about medical conditions, food consumption (such as a validated 14-item dietary screener (p14) to assess adherence to Mediterranean Diet at baseline and at the last visit [295] and a 137-item validated food-frequency questionnaire [296]) were completed as described elsewhere [294]. Data of anthropometric measures, body composition and blood pressure were collected in the same visit following standardised procedures [297]. Plasma, serum, and buffy-coat were stored at -80 ºC and biochemical features were evaluated, as described elsewhere [297].

In the MENA study, data included baseline anthropometrics (weight, height, WC), blood pressure and blood samples for methylation and biochemical (glucose, insulin, Homeostasis Model Assessment-Insulin Resistance (HOMA-IR), HDL-cholesterol, triglycerides, Triglyceride-Glucose (TyG) index) analyses.

1.3. GEDYMET

The GEDYMET study was conducted in collaboration with the Pontificia Universidad Católica de Chile in order to analyse the associations between methylation levels and biochemical measurements related to glucose and insulin in non-diabetic individuals (*Chapter 4*). Furthermore, *Chapters 5 & 6* included all the participants from GEDYMET as a part of the MENA study.

1.3.1. Study population

The study population included 57 women (age range 18–46 years) who fulfilled the criteria exposed in Table 5.

Table 5: Exclusion criteria of the GEDYMET study.

Exclusion criteria
Diabetes or family history of diabetes
Dyslipidaemia
Anaemia
Pregnancy

1.3.2. Data collection

The volunteers visited the UC Centre of Clinical Research for carrying out biochemical and anthropometric measurements such as weight, height, BMI, WC, glucose and fasting plasmatic insulin, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, TyG index, blood pressure. In another visit, an abbreviated version of minimal-model Intravenous Glucose Tolerance Test (IVGTT) was performed following the procedure explained elsewhere [298]. HOMA-Sensitivity (HOMA-S) index, Acute Insulin Release (AIR) index, Calculated insulin Sensitivity index (CSi) and IVGTT-based Disposition Index (DI) index were calculated.

1.4. OBEKIT

The OBEKIT study is a 10-month randomized, longitudinal and controlled intervention trial with the main objective of evaluating the response to a two hypocaloric diets with different macronutrient composition based on the genetic background. The OBEKIT protocol has been described in detail elsewhere [299] and the trial was registered at ClinicalTrials.gov (reg. NCT02737267).

1.4.1. Study population

Eligible participants were selected following the inclusion and exclusion criteria detailed (Table 6).

A total of 96 participants from OBEKIT were selected for the MENA study (*Chapters 5 & 6*). *Chapter 7* included 203 participants from OBEKIT as an independent cohort of the MENA study.

1.4.2. Data collection

Data from the study participants were collected at baseline including anthropometrical measurements (weight, height, WC, fat mass measured by Dual-energy X-ray Absorptiometry (DXA), lean mass DXA, trunk fat mass DXA, android fat mass DXA, gynoid fat mass DXA, visceral adipose tissue mass DXA), blood samples for methylation analysis, blood pressure and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG

index, and CRP, following standardised protocols explained in detail elsewhere [299].

Table 6: Inclusion and exclusion criteria of the OBEKIT study.

Inclusion criteria
Adults between 18 and 70 years old
Body mass index ≥25 kg/m^2 and <40 kg/m^2
Physical examination and vital signs normal, or is considered abnormal, but clinically insignificant by researcher
In the case of individuals with chronic stable dose drug treatment and during the last 3 previous months at baseline, the investigator will assess their possible inclusion
Exclusion criteria
Pregnancy
Breastfeeding period. If artificial feeding until 6 months after birth
Type 1 diabetes
Severe kidney and digestive system diseases
Electrolyte disorders (disorders of sodium, potassium, calcium, chlorine, phosphorus, magnesium)
Acute cardiovascular diseases
Cancer
Eating disorders
Recent prescription drug treatment (without stable doses scheduled)
Weight loss medications or others drugs that affect body weight (anti-psychotic or anti-depressant drugs, corticosteroids)
Some type of cognitive / psychic impairment
Subjects in which poor collaboration or, in the investigator's opinion, have difficulty following the procedures of the study is foreseen
Lack of commitment (at the discretion of the investigator) with the intervention, suspected non-compliance, or real difficulties to follow the development of the study

1.5. NormoP

The NormoP population was designed with the aim of collecting control data for the OBEKIT study.

1.5.1. Study population

In the MENA study, the NormoP group included 12 individuals who fulfilled the same criteria as OBEKIT subjects, except for the body mass index, which was ≥18.5 kg/m^2 and <25 kg/m^2. *Chapter 7* included 66 participants from NormoP as an independent cohort of the MENA study.

1.5.2. Data collection

Data from the study participants of NormoP were collected at baseline including (weight, height, WC, fat mass DXA, lean mass DXA, trunk fat mass DXA, android fat mass DXA, gynoid fat mass DXA, visceral adipose tissue mass DXA), blood samples for methylation analysis, blood pressure and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index, CRP), following standardised protocols.

1.6. MENA

The MENA study compiled methylation data from the following cohorts and studies: PREDIMED-UNAV, GEDYMET, OBEKIT, NormoP, Food4Me-UNAV, DiOGenes-UNAV, ICTUS, NUGENOB-UNAV, OBEPALIP, and RESMENA. The aim of this study was to determine DNA methylation sites that were related to different anthropometric and metabolic characteristics of the participants (*Chapters 5 & 6*). *Chapter 7* included all these studies and cohorts (without OBEKIT and NormoP, which were studied as an independent population) to assess the relationship between DNA methylation and epigenetic age acceleration.

1.6.1. PREDIMED-UNAV

The MENA study included 116 subjects from the PREDIMED-UNAV study. Baseline anthropometric (weight, height, WC) and blood pressure measurements, blood samples for methylation analysis and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) were collected. A further description has been detailed in *Subjects and Methods, section 1.2.*

1.6.2. GEDYMET

The MENA study included a total of 57 women from the GEDYMET study. Data employed in the MENA study included measurements of weight, height, WC, blood pressure and blood samples for methylation analysis and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index). Further details have been explained in *Subjects and Methods, section 1.3.*

1.6.3. OBEKIT

The OBEKIT study contributed with 96 individuals to the MENA study. Anthropometric (weight, height, WC) and blood pressure measurements, blood samples for methylation analysis and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) were employed. Further details have been explained in *Subjects and Methods, section 1.4.*

1.6.4. NormoP

The MENA study involved a total of 12 subjects from the NormoP study. Data employed in these works included measurements of weight, height, WC, and blood pressure, and blood samples for methylation analysis and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index). Further details have been explained in *Subjects and Methods, section 1.5.*

1.6.5. Food4Me-UNAV

The Food4Me study (http://www.food4me.org) was a European on-line randomized controlled intervention study with the aim of investigating the utility of a personalised nutrition approach for improving nutritional and diet-related outcomes. The Food4Me study was registered at ClinicalTrials.gov (reg. NCT01530139) and the protocol has been described in detail elsewhere [300].

1.6.5.1. Study population

A total of 39 participants were included in the MENA study from the Food4Me-UNAV study, who were selected following different criteria (Table 7).

Table 7: Inclusion and exclusion criteria of the Food4Me study.

Inclusion criteria
Age ≥18 years old
Exclusion criteria
Pregnant or lactating
No or limited access to the Internet
Following a prescribed diet for any reason, including weight loss, in the last 3 months
Diabetes, coeliac disease, Crohn's disease, or any metabolic disease or condition altering nutritional requirements such as thyroid disorders (if condition was not controlled), allergies or food intolerances

1.6.5.2. Data collection

Data from the study participants of Food4Me-UNAV were collected at baseline including anthropometrics (weight, height, WC), blood pressure and blood samples for methylation and biochemical (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) measurements following standardised protocols explained in detail elsewhere [300].

1.6.6. DiOGenes-UNAV

The DiOGenes study is a multicentre, randomized, dietary-intervention study primarily designed to assess the efficacy of moderate-fat diets that vary in protein content and glycaemic index in the prevention of weight regain and obesity-related risk factors after weight loss. For the MENA study, participants were selected from the recruitment centre at the University of Navarra (DiOGenes-UNAV). This trial was registered at ClinicalTrials.gov with the number: NCT00390637. The protocol and recruitment methods have been described in detail elsewhere [301, 302].

1.6.6.1. Study population

Eligible participants in the DiOGenes study were selected following different criteria (Table 8). A total of 52 participants were included in the MENA study.

1.6.6.1. Data collection

Data from the study participants of DiOGenes-UNAV were collected at baseline including anthropometrics (weight, height, WC), blood pressure, and blood

samples for methylation and biochemical (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) measurements, following standardised protocols explained elsewhere [301, 302].

Table 8: Inclusion and exclusion criteria of the DiOGenes study.

Inclusion criteria
Families with at least 1 overweight (body mass index >27 kg/m^2 and <45 kg/m^2) parent aged (18-65 years old), and at least 1 healthy child aged between 5 and 18 years, who was willing to participate in at least a minimum of the investigations
Families in which no biological relationship existed between parents and children
Exclusion criteria
Adult offspring above the age of 18 years still living at home
Adult members of the family who did not have a parental position
Subjects using prescription medication
Subjects suffering from diseases or conditions related to body weight regulation (malabsorption, untreated hypo/hyperthyroidism, eating disorders, systemic use of steroids, etc.) and obesity-related cardiovascular risk factors (heart disease, systolic and diastolic blood pressures ≥160/100 mmHg, blood glucose >6.1 mM/L, blood cholesterol >7 mM/L, blood triglycerides >3 mM/L)
Subjects with food allergies
Urinary protein, glucose, pH, ketone and haemoglobin levels outside accepted reference ranges
Marked alcohol habits >21 alcoholic units/week (male), or >14 alcoholic units/week (female)
Planned major changes in physical activity during the study to an extent that might interfere with the study outcome, as judged by the investigator
Blood donation within the past 2 months prior to the study
Adults with a weight change of >3 kg within 2 months prior to first clinical investigation day
Psychiatric disease (based on medical history only)
Pregnant or lactating women, pregnancy planned within the next 18 months
Surgically treated obesity
Participation in other clinical studies within the last 3 months
Drug abuse (based on clinical judgment)
Adults unable to give an informed consent
Adults unable to engage in an 8-week low-calorie diet
Individuals following a special diet (vegetarian, Atkins or other)

1.6.7. ICTUS

The ICTUS study is a case-control nutritional intervention focused on investigating the effect of the dietary treatment on anthropometric measurements, inflammation markers, lipid profile, insulin status and the methylation patterns of two stroke-related genes in obese individuals who have suffered an ischemic stroke insult.

1.6.7.1. Study population

The MENA study included a total of seven individuals from the ICTUS study, selected according the criteria explained in Table 9.

Table 9: Inclusion and exclusion criteria of the ICTUS study.

Inclusion criteria
Age 50-80 years old
Body mass index ≥30 kg/m^2
Case group: Patients with acute ischemic stroke hospitalized within 24 hours from the onset of symptoms
Control group: Patients not having suffered vascular or neurological disorders
Exclusion criteria
Major cardiac, renal, hepatic, endocrine disorders, skeletal disorders, cancer, inflammatory diseases and recent infections

1.6.7.2. Data collection

Data from the study participants of ICTUS were collected at baseline including anthropometrics (weight, height, WC), blood pressure and blood samples for methylation and biochemical (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) measurements following standardised protocols explained elsewhere [168].

1.6.8. NUGENOB-UNAV

The NUGENOB study is a randomized, parallel, two-arm, open-label 10-week dietary intervention of two hypoenergetic diets (high- versus low-fat diet) to examine if there is an interaction between the nutrient composition of the diet, specifically the fat content, and obesity related genes in response to the dietary treatment. The protocol and recruitment methods have been described in

detail elsewhere [303, 304]. The trial was registered at ISRCTN registry (ISRCTN25867281).

1.6.8.1. Study population

The MENA study included 22 participants from NUGENOB-UNAV. Participants from the NUGENOB study fulfilled the inclusion and exclusion criteria detailed below (Table 10).

1.6.8.2. Data collection

The MENA study included data from NUGENOB-UNAV collected at baseline of anthropometrical measurements (weight, height, WC), blood pressure, blood samples for methylation analysis and biochemical measurements (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index), following standardised protocols explained in detail elsewhere [303, 304].

Table 10: Inclusion and exclusion criteria of the NUGENOB study**.**

Inclusion criteria
Adults between 20 and 50 years old
Body mass index ≥30 kg/m^2
White European (by self-report)
Pre-menopausal (women)
Exclusion criteria
Weight change >3 kg within the 3 months before the beginning of the study
Drug-treated hypertension, diabetes, hyperlipidaemia or thyroid disease
Use of anorexigenic agents
Anti-epileptic or anti-parkinsonian drugs
Surgically treated obesity
Pregnancy
Use of barbiturates, benzodiazepines, beta-blockers, butyrophenones, carbonic anhydrase inhibitors, diuretics, dopamine reuptake inhibitors, digoxin, fibrates, fish oil supplement, glucocorticoids, immunosuppressives, insulin, laxatives, monoamine oxidase inhibitors, niacin (>150 mg per day), nicotine, oral hypoglycaemic, orlistat, phenothiazines, selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, statins, thyroid hormone, triamterene, tricyclic antidepressants, warfarin or zonisamide
Alcohol or drug abuse
Participation in other simultaneous ongoing trials

1.6.9. OBEPALIP

The OBEPALIP study is a parallel, short-term randomized double blind placebo-controlled trial designed with the objective of evaluating the potential body weight-lowering effects of dietary supplementation with eicosapentaenoic acid and α-lipoic acid separately or in combination, in healthy overweight/obese women during a hypocaloric diet. The trial was registered at ClinicalTrials.gov (reg. NCT01138774). The protocol and recruitment methods have been described in detail elsewhere [305].

1.6.9.1. Study population

Eligible participants in the OBEPALIP study were selected following different criteria (Table 11). A total of 52 participants were included for the MENA study.

Table 11: Inclusion and exclusion criteria of the OBEPALIP study.

Inclusion criteria
Women
Age between 20 and 45 years, and with regular menstrual cycles
Body mass index between 27.5 and 39.9 kg/m^2
Weight unchanged (±3 kg) for the last 3 months
All subjects should have an overall physical and psychological condition that the investigator believes is in accordance with the overall aim of the study
Exclusion criteria
Use of prescription medication
Any chronic metabolic- or obesity-related disease, hepatic or renal systemic disease: hypertension, dyslipidaemia, type 1 or 2 diabetes, thyroid function disorders, cirrhosis, fatty liver, etc.
Food allergies or food intolerance expected to come up during the study
Special diets (Atkins, vegetarian, etc.) prior 3 months the start of the study
Eating disorders
Surgically treated obesity
Pregnant or lactating women or planning to be pregnant in the next 2 months
Alcohol or drug abuse (based on clinical parameters)

1.6.9.2. Data collection

Collected baseline data from the OBEPALIP subjects included anthropometrical (weight, height, WC) and blood pressure measurements, as well as blood

samples for methylation and biochemical (glucose, insulin, HOMA-IR, HDL-cholesterol, triglycerides, TyG index) assessment, following standardised protocols explained elsewhere [305].

1.6.10. RESMENA

The RESMENA study was designed as a randomized, longitudinal and controlled intervention trial to compare the effects of two hypocaloric dietary strategies on metabolic syndrome comorbidities over a six-month period as main objective. The trial was registered at ClinicalTrials.gov (reg. NCT01087086) and the protocol has been described in detail elsewhere [306].

1.6.10.1. Study population

The MENA study included 44 subjects from the RESMENA study. The inclusion and exclusion criteria are described (Table 12).

Table 12: Inclusion and exclusion criteria of the RESMENA study.

Inclusion criteria
Adults between 35 and 65 years old
Metabolic syndrome according to International Diabetes Federation criteria
Exclusion criteria
Weight change >3 kg within the 3 months before the beginning of the study
Psychiatric or psychological disorders
Difficulty for changing dietary habits
Eating disorders
Chronic diseases related to the metabolism of energy and nutrients
Specific pharmacological treatments
Subjects with special diets
Food allergies or intolerances
Daily smokers
Having a serious disease
Consumption of vitamin or nutritional supplements

1.6.10.2. Data collection

Baseline data from the study participants of RESMENA included anthropometrics (weight, height, WC), blood pressure and blood samples for methylation and biochemical (glucose, insulin, HOMA-IR, HDL-cholesterol,

triglycerides, TyG index) measurements, and they were determined following standardised protocols explained elsewhere [306].

2. DNA METHYLATION ANALYSIS

2.1. DNA extraction

The DNA from peripheral white blood cells was extracted from venous blood samples drawn on Ethylenediaminetetraacetic Acid (EDTA) tubes. Samples were centrifuged at 2000xg, 4 °C, 15 or 20 min and buffy coats were collected (Figure 11). The extraction was performed using MasterPure™ DNA Purification Kit for Blood (Epicentre, Madison, WI, USA) according to manufacturer's instructions for all the studies except for the newborns samples, which were treated following a protocol specified in the pertinent chapter [307].

2.2. Genome-wide methylation analysis

Peripheral white blood cell DNA samples were shipped on dry ice to Unidad de Genotipado y Diagnóstico Genético from Fundación Investigación Hospital Clínico de Valencia (INCLIVA), where microarray preparation, hybridisation and scanning were performed. DNA quantification was carried out using PicoGreen® dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). Bisulphite conversion of 500 mg of genomic DNA was achieved using EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) and then, samples were amplified and hybridised with the Infinium Human Methylation 450k BeadChip or with Infinium MethylationEPIC BeadChip (for OBEKIT and NormoP) (Illumina, San Diego, CA, USA). They were scanned using the Illumina hiScanSQ platform and the intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, San Diego, CA, USA) as illustrated (Figure 11).

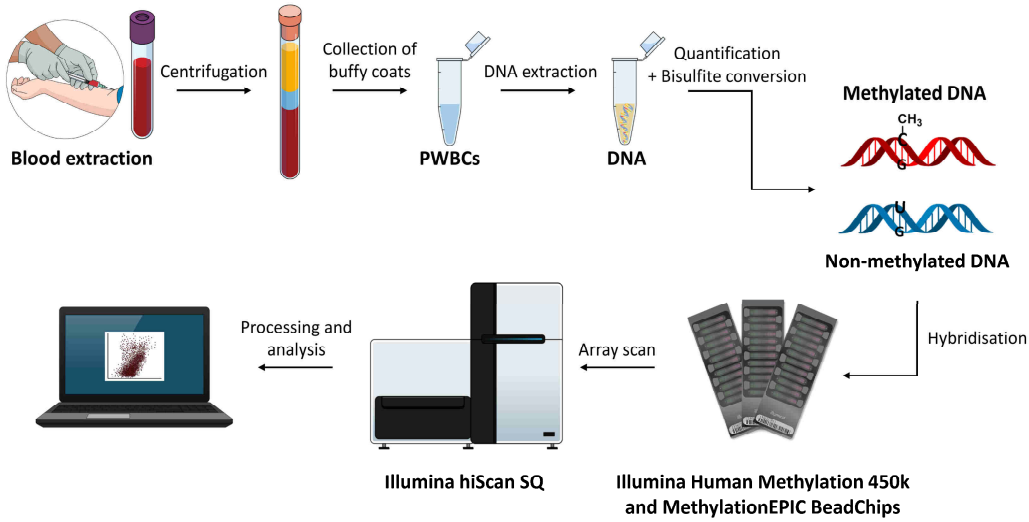

Figure 11: Complete process of DNA methylation analysis from blood extraction to data processing. Abbreviations: PWBCs: peripheral white blood cells.

2.3. Methylation data management

Methylation raw data was submitted to a pre-processing and analysis pipeline where quality of samples, quality of probes, and background correction were applied. In this process, a filtering of probes located on the X and Y chromosomes was also executed. Filtering of probes with SNPs was performed in *Chapters 4, 5 & 6.* Different normalisation approaches were performed depending on the objective of each study: Subset Quantile Normalisation (SQN) method by Touleimat & Tost [308] (*Chapters 1, 2, 3 & 4*), or the Subset-quantile Within Array Normalisation (SWAN) method developed by Maksimovic *et al.* [309] (*Chapters 5 & 6*). Methylation raw data in *Chapter 7* was only submitted to background correction with dye-bias normalisation without any filtering of probes [310]. When specified, the magnitude of batch effects was assessed after data normalisation and corrected using the ComBat normalisation method, which is an empirical Bayes based method to correct for technical variation related to the slide [311, 312] (*Chapters 5 & 6*).

Differences in methylation resulting from differences in cellular heterogeneity were corrected using the Houseman procedure [313] in *Chapters 1, 4, 5 & 6.* In the case of *Chapters 2 & 3*, the differences in the composition of types of

leukocytes were assessed in order to avoid a possible influence in methylation differences of some CpGs.

After this methylation data processing, Linear Models for Microarray Data (LIMMA) as published [314] for the R statistical software [315] were used to compute different analysis in some of the chapters (*Chapters 1, 3, 5 & 6*). Correction for multiple comparison tests (Bonferroni and Benjamini-Hochberg approaches) and different statistical filters were applied to avoid type I errors.

3. OTHER ANALYSES

3.1. Ingenuity Pathway Analysis

Data (*Chapters 1* - 6) were analysed by Ingenuity Pathway Analysis (IPA) software (Qiagen Redwood City, CA, USA, www.ingenuity.com). Associated pathways and gene regulatory networks were identified by predefined pathways and functional categories of the Ingenuity Knowledge Base [264]. Canonical pathway analyses were performed with IPA's Core Analysis module and selected if $p<0.05$ after Fisher's test as statistically significant.

3.2. ELISAs

Selected soluble proteins were measured using standard ELISA in EDTA plasma samples from PREDIMED study (*Chapter* 2) following the manufacturer's protocol. All ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MA, USA) and concentrations were measured by Multiskan Spectrum from Thermo Scientific (Waltham, MA, USA).

3.3. Epigenetic age acceleration

Epigenetic age acceleration, defined as the residual from regressing the epigenetic age on chronological age, was assessed using the website DNA methylation Age Calculator (https://dnamage.genetics.ucla.edu/home) developed by Steve Horvath and were applied accordingly to the software provider in *Chapter 7*. Two different methods were employed for calculating age acceleration. The first method, denominated DNAmAge and developed by Horvath [200], calculates the epigenetic age from human samples profiled with

the Illumina Infinium 450k platform based on the methylation levels of 353 CpGs. The second method, denominated GrimAge and developed by Lu *et al.* [202] predicted epigenetic age based on a linear combination of chronological age, sex, and DNAm-based surrogate biomarkers for seven plasma proteins and smoking pack-years.

4. STATISTICAL PROCEDURES

Statistical analyses are specifically explained in each chapter. Briefly, normality was assessed by Shapiro-Wilk test and homoscedasticity by Levene test. Comparisons between two independent groups were performed using Student's t-test for normal distribution, Mann-Whitney U test for not normal but similar distribution, and Median test for not normal but different distribution. Comparisons between two dependent groups were determined using Student's t-test for normal distribution, Wilcoxon signed-rank test for not normal but similar distribution, and Sign test for not normal but different distribution. In the case of more than two groups, comparisons were performed using ANOVA (Multiple comparison test: Tukey's test) for normal distribution or Kruskal-Wallis (Multiple comparison test: Mann-Whitney U test) for not normally distributed data. If the variables were qualitative, the comparisons were determined using Chi-square test (Contingency RxC, for independent samples), Fisher's exact test (Contingency 2x2, for independent samples) or Cochran test (three or more related samples). Correlations were assessed using Pearson for normal distribution or Spearman for not normal distribution. Simple or multivariable linear regressions were employed for two continuous variables and logistic regressions for a categorical and a continuous variable. Receiver Operating Characteristic (ROC) curves were also performed. Internal validation of ROC curves was performed using a correction for optimistic prediction according to Tibshirani's enhanced bootstrap method described by Harrel [316]. Furthermore, mediation analysis was assessed using structural equation modelling as described by Zhao *et al.* [317].

The software used for statistical analysis were Stata versions 12.0, 12.1 or 14.0 (StataCorp, College Station, TX, USA); GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA); and R Studio [315].

RESULTS

CHAPTER 1

Methylation changes and pathways affected in preterm birth: a role for *SLC6A3* in neurodevelopment

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Supplementary material

Methylation changes and pathways affected in preterm birth: a role for *SLC6A3* in neurodevelopment

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Table S1: Preterm parents' data and perinatal complications of newborns.

	Father					Mother									Child features					
	Age (y)	Height (cm)	Weight (kg)	BMI (kg/m²)	Educational level[a]	Age (y)	Height (cm)	Preconceptional weight (kg)	BMI (kg/m²)	Educational level[a]	Former smoker	Smoker (*n* of cigarettes)	Familiar history of diabetes type II	Maternal diabetes	Position in n of siblings	Periventricular leukomalacia	Retinopathy	Sepsis	Chronic lung disease	Intraventricular haemorrhage	Twins
1	36	179	75	23.4	Degree (Long)	31	158	45	18.0	Degree (Long)	Yes	No	No	No	1	Yes	Grade II	Yes	Yes	No	No
2	37	173	72	24.1	Bacc/AD	32	150	50	22.2	SE	No	No	No	Yes	1	Yes	Grade I	No	Yes	No	No
3	36	180	NA	NA	Degree (Short)	40	160	45	17.6	Degree (Long)	No	No	No	No	1	No	Grade I	Yes	Yes	No	No
4	33	175	70	22.9	Degree (Short)	29	152	49	21.2	Degree (Short)	No	No	No	No	2	No	No	No	No	No	Yes
5	35	177	82	26.2	Degree (Long)	35	162	52	19.8	Degree (Long)	Yes	No	No	No	NA	No	No	No	Yes	No	No
6	33	173	86	28.7	Bacc/AD	29	154	60	25.3	Degree (Long)	No	No	No	No	1	No	No	No	No	No	No
7	35	175	100	32.7	Bacc/AD	34	160	59	23.0	Degree (Long)	No	No	Yes	No	1	No	No	Yes	No	No	No
8	35	NA	82	NA	Degree (Long)	34	159	76	30.1	Degree (Long)	No	No	Yes	No	2	No	Grade II	Yes	No	No	No
9	30	180	75	23.1	SE	27	160	53	20.7	Bacc/AD	No	Yes (4)	Yes	No	NA	Yes	No	No	No	No	Yes
10	30	183	82	24.5	SE	26	161	79	30.5	Degree (Short)	Yes	No	No	No	1	No	No	No	No	No	No
11	36	172	75	25.4	Bacc/AD	39	163	55	20.7	Bacc/AD	No	No	No	No	2	No	No	No	No	No	No
12	NA	158	53	21.2	Bacc/AD	32	186	112	32.4	Bacc/AD	No	No	Yes	No	1	No	No	No	Yes	No	No
13	NA	190	88	24.4	SE	36	170	62	21.5	Degree (Long)	No	No	No	No	2	No	No	No	Yes	No	No
14	NA	179	73	22.8	Degree (Long)	NA	166	56	20.3	Degree (Short)	No	Yes (13)	No	No	3	Yes	No	No	No	Grade I	No
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	No	No	No	No	No	Yes
16	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	2	Yes	Grade II	Yes	Yes	No	Yes
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	2	No	No	No	Yes	No	Yes
18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	Yes	Grade II	No	Yes	Grade IV	Yes
19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	NA	No	No	No	No	No	No
20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	No	Grade III	Yes	Yes	No	No
21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	No	No	No	No	No	No
22	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	No	No	No	No	Grade II	No
23	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	NA	No	No	No	Yes	No	No
24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	No	No	No	No	1	Yes	No	No	No	Grade III	No

[a] Bacc/AD: Baccalaureate/Associate Degree, SE: Secondary education

NA: non-available

Table S2: Full-term parents' data.

	Father					Mother										Child features
	Age (y)	Height (cm)	Weight (kg)	BMI (kg/m²)	Educational level[a]	Age (y)	Height (cm)	Preconceptional weight (kg)	BMI (kg/m²)	Educational level[a]	Former smoker	Smoker (*n* of cigarettes)	Familiar history of diabetes type II	Maternal diabetes	Position in n of siblings	Twins
1	NA	182	NA	NA	Bacc/AD	23	165	NA	NA	Bacc/AD	Yes	No	Yes	No	1	No
2	NA	171	NA	NA	SE	32	154	NA	NA	Bacc/AD	Yes	No	No	No	2	No
3	NA	175	NA	NA	Bacc/AD	37	164	NA	NA	Bacc/AD	Yes	No	No	No	1	No
4	NA	162	NA	NA	Degree (Long)	27	154	NA	NA	Degree (Short)	No	No	No	No	1	No
5	NA	173	NA	NA	Degree (Long)	30	170	NA	NA	Degree (Short)	No	No	No	No	2	No
6	39	171	77	26.3	Bacc/AD	38	153	49	20.9	Degree (Short)	No	No	Yes	No	2	No
7	NA	173	NA	NA	Degree (Short)	32	160	NA	NA	Degree (Long)	No	No	Yes	No	2	No
8	NA	180	NA	NA	Degree (Short)	34	162	NA	NA	Degree (Short)	Yes	No	No	No	1	No
9	36	170	80	27.7	Bacc/AD	37	157	78	31.6	Bacc/AD	No	No	Yes	Yes	5	No
10	NA	168	NA	NA	Degree (Long)	35	162	NA	NA	Degree (Long)	No	No	No	No	2	No
11	NA	180	NA	NA	SE	34	166	NA	NA	Bacc/AD	Yes	No	No	No	1	No
12	31	181	72	22.0	SE	28	157	73	29.6	SE	No	No	No	No	2	No
13	31	182	81	24.5	Degree (Short)	29	165	63	23.1	Degree (Short)	No	No	Yes	No	1	No
14	24	181	90	27.5	SE	22	158	60	24.0	SE	Yes	No	Yes	No	1	No
15	32	171	71	24.3	SE	30	161	60	23.1	SE	No	Yes (15)	Yes	No	1	No
16	32	173	95	31.7	Bacc/AD	30	160	65	25.4	Bacc/AD	No	No	Yes	No	2	No
17	37	176	95	30.7	Bacc/AD	35	167	58	20.8	Degree (Long)	Yes	No	No	No	1	No
18	NA	175	88	28.7	Degree (Long)	31	163	69	26.0	Degree (Long)	No	No	No	No	NA	No
19	NA	178	NA	NA	SE	32	159	NA	NA	SE	Yes	No	No	No	1	No
20	NA	186	NA	NA	Degree (Long)	36	174	NA	NA	Degree (Long)	No	No	No	No	1	Yes
21	NA	186	NA	NA	Degree (Long)	36	174	NA	NA	Degree (Long)	No	No	No	No	2	Yes
22	37	175	72	23.5	Bacc/AD	30	155	64	26.6	Bacc/AD	No	Yes (12)	No	No	2	No

[a] Bacc/AD: Baccalaureate/Associate Degree, SE: Secondary education
NA: non-available

Table S3: Correlations between differentially methylated genes of *SLC6A3* network and BSID.

CpG	Gene	Variable	r	p
cg00217795	DIO2	BSID-II motor	-0.35	0.0188
cg00700487	NOS3	BSID-II motor	-0.37	0.0188
		BSID-III language	-0.32	0.0471
		BSID-III motor	-0.33	0.0402
cg06908474	CAT	BSID-II motor	-0.46	0.0015
		BSID-III fine motor sub-scale	-0.33	0.0374
cg07904028	PPP2R2C	BSID-III language	-0.35	0.0271
		BSID-III motor	-0.41	0.0100
cg10593400	DDAH1	BSID-II motor	-0.46	0.0016
		BSID-III motor	-0.41	0.0104
		BSID-III fine motor sub-scale	-0.33	0.0402
cg13411554	CACNA1D	BSID-III motor	-0.41	0.0104
		BSID-III fine motor sub-scale	-0.34	0.0355
cg13428066	KCNQ1;KCNQ1OT1	BSID-II motor	-0.35	0.0170
cg22549408	PMAIP1	BSID-II motor	-0.32	0.0325
		BSID-II mental	-0.40	0.0063
		BSID-III cognitive	-0.35	0.0284
		BSID-III language	-0.32	0.0466
		BSID-III expressive language sub-scale	-0.49	0.0016
		BSID-III motor	-0.35	0.0301
		BSID-III fine motor sub-scale	-0.40	0.0115
		BSID-III gross motor sub-scale	-0.33	0.0402
cg22932677	MAST1	BSID-III cognitive	-0.36	0.0241
		BSID-III motor	-0.49	0.0016
		BSID-III fine motor sub-scale	-0.40	0.0127
		BSID-III gross motor sub-scale	-0.42	0.0077
cg23268197	INSL3	BSID-II motor	-0.38	0.0092
cg27016307	HRC	BSID-II motor	-0.48	0.0008
cg27027427	IFIT3	BSID-II motor	-0.52	0.0003
		BSID-II mental	-0.30	0.0430
cg27046335	PRLR	BSID-II motor	-0.33	0.0287
		BSID-III language	-0.32	0.0469
		BSID-III fine motor sub-scale	-0.32	0.0465

Spearman or Pearson correlations were performed when appropriate. Significance considered p<0.05.
BSID: Bayley Scale of Infant Development (version II, BSID-II and version III, BSID-III)

CHAPTER 2

Adherence to Mediterranean diet is associated with methylation changes in inflammation-related genes in peripheral blood cells

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Supplementary material

Adherence to Mediterranean diet is associated with methylation changes in inflammation-related genes in peripheral blood cells

Available at:

https://link.springer.com/article/10.1007%2Fs13105-017-0552-6#SupplementaryMaterial

Table S1: p14 validated questionnaire for assessment of adherence to Mediterranean diet.

	Foods and frequency of consumption	Criteria for 1 point*
1	Do you use olive oil as main culinary fat?	Yes
2	How much olive oil do you consume in a given day (including oil used for frying, salads, out of house meals, etc.)?	4 or more tablespoons
3	How many vegetable servings do you consume per day? *(1 serving = 200g – consider side dishes as ½ serving)*	2 or more *(at least 1 portion raw or as salad)*
4	How many fruits units (including natural fruit juices) do you consume per day?	3 or more
5	How many servings of red meat, hamburger, or meat products (ham, sausage, etc.) do you consume per day? *(1 serving = 100-150g)*	Less than 1
6	How many servings of butter, margarine, or cream do you consume per day? *(1 serving = 12g)*	Less than 1
7	How many sweet/carbonated beverages do you drink per day?	Less than 1
8	How much wine do you drink per week?	7 or more glasses
9	How many servings of legumes do you consume per week? *(1 serving = 150g)*	3 or more
10	How many servings of fish or shellfish do you consume per week? *(1 serving – 100-150g fish or 4-5 units or 200g shellfish)*	3 or more
11	How many times per week do you consume commercial sweets or pastries (not homemade), such as cakes, cookies, biscuits, or custard?	Less than 3
12	How many servings of nuts (including peanuts) do you consume per week? *(1 serving = 30g)*	3 or more
13	Do you preferentially consume chicken, turkey, or rabbit meat instead of veal, pork, hamburger or sausage?	Yes
14	How many times per week do you consume vegetables, pasta, rice, or other dishes seasoned with *sofrito* (sauce made with tomato and onion, leek or garlic, simmered with olive oil?	2 or more

*0 points if these criteria are not met.

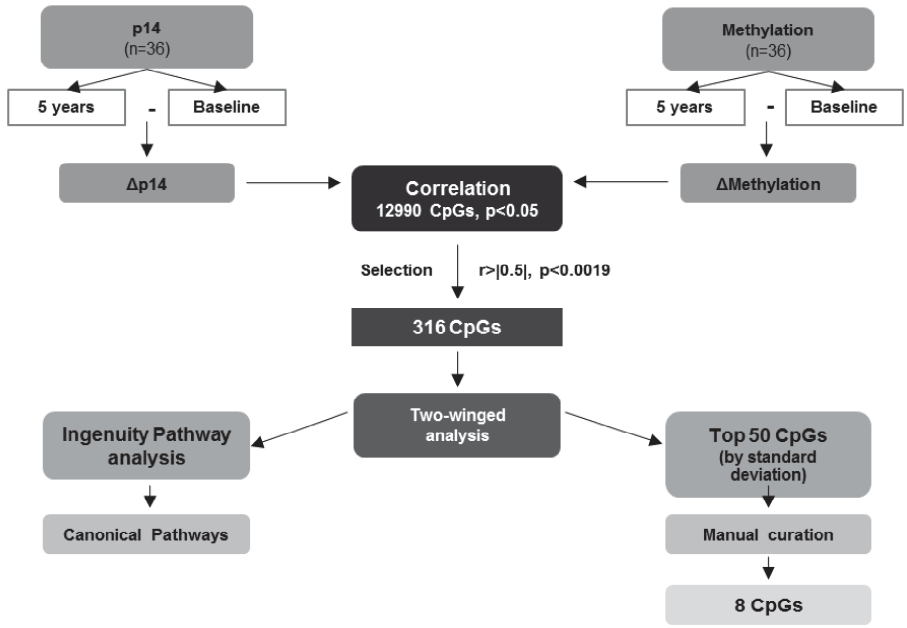

Figure S1: Steps followed in the selection of differentially methylated CpGs. p14 and methylation changes were calculated subtracting methylation data at baseline from data at 5 years to estimate Δp14 and ΔMethylation. Then, correlations between these two data were performed, obtaining 12990 CpGs with a $p<0.05$ (considered the limit of significance). From those CpGs, 316 ones were selected with an $r>|0.5|$ from the correlation, which corresponds to a $p<0.0019$. This 316 CpGs were submitted to a two-winged analysis. Firstly, all the CpGs were analysed by Ingenuity Pathway analysis, obtaining canonical pathways. Secondly, mean and standard deviation was calculated for all the 316 CpGs, and the 50 CpGs with higher standard deviation were selected. They were classified by manual curation in functional groups and finally, 8 CpGs were chosen for further analysis.

Table S2: Protein concentrations and methylation data of the study population between five year follow-up and baseline.

	Baseline (n=36)	5 years (n=36)	Δ (n=36)
TNF-α, pg/mL	0.614 (0.231)	0.680 (0.288)	0.065 (0.272)
CRP, ng/mL	2373 (1913)	2478 (2328)	106 (2236)
VCAM-1, ng/mL	2588 (842)	2449 (597)	-140 (708)
sICAM-1, ng/mL	245.3 (122.7)	223.7 (98.9)	-21.5 (128.0)
LEP, ng/mL	21.5 (6.8)	20.0 (6.6)	-1.4 (7.9)
Methylation cg24204847 (*EEF2*), %	62.1 (13.7)	68.2 (16.9)	6.1 (18.9)
Methylation cg01314574 (*COL18A1*) , %	43.8 (10.6)	42.1 (12.1)	-1.7 (16.2)
Methylation cg05213896 (*IL4I1*), %	28.6 (10.1)	26.8 (9.8)	-1.8 (12.2)
Methylation cg15466952 (*LEPR*), %	53.2 (9.3)	57.7 (8.6)	4.5 (12.1)
Methylation cg06271237 (*PLAGL1*), %	35.4 (7.6)	35.0 (9.0)	-0.4 (9.1)
Methylation cg25191332 (*IFRD1*), %	24.1 (6.0)	23.1 (5.3)	-1.0 (7.5)
Methylation cg24657977 (*MAPKAPK2*), %	21.1 (4.0)	22.7 (5.7)	1.5 (6.5)
Methylation cg15809858 (*PPARGC1B*), %	17.4 (7.3)	15.0 (5.0)	-2.4 (6.4)

Δ indicates changes between 5 years and baseline

COL18A1: Collagen Type XVIII Alpha 1; CRP: C-reactive protein; *EEF2*: Eukaryotic Elongation Factor 2; *IFRD1*: Interferon-related developmental regulator 1; *IL4I1*: Interleukin 4-induced gene-1; LEP: Leptin; *LEPR*: Leptin receptor, *MAPKAPK2*: Mitogen-activated protein kinase (MAPK)–activated protein kinase 2; *PLAGL1*: Pleiomorphic adenoma gene-like 1; *PPARGC1B*: Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Beta; sICAM-1: soluble intercellular cell adhesion molecule 1; TNF-α: Tumor necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.

Table S3: Selected CpGs (n=316) after correlation between methylation changes and p14 changes with criteria r>|0.5|, corresponding to p<0.0019. The table shows the CpGs selected with their corresponding genes, r and p-values from the correlations. These genes are studied by Ingenuity Pathway Analysis and a subsequent filter is applied to perform another selection.

ID	r	p	Gene
cg20393622	0.6856	3.95E-06	ACAA2;SCARNA17
cg09984518	0.6839	4.26E-06	AKAP5L
cg19413728	-0.6344	3.26E-05	CADPS
cg05492964	-0.6311	3.68E-05	PRR13
cg24602388	0.6230	4.95E-05	
cg16736964	0.6128	7.09E-05	
cg12100751	-0.6122	7.25E-05	C1orf59
cg27153655	0.6099	7.85E-05	SERPINA10
cg00543460	-0.6089	8.11E-05	TPM1
cg03641793	0.6077	8.46E-05	MAPK7
cg18627459	0.6069	8.69E-05	RFC5
cg01314574	0.6050	9.28E-05	COL18A1
cg12973315	0.6026	1.01E-04	GPR78
cg09112514	0.6016	1.04E-04	PDGFRA
cg15241390	0.6000	1.10E-04	ORMDL1;PMS1
cg19977494	-0.5993	1.12E-04	CCDC136
cg15234312	-0.5972	1.20E-04	MYADM
cg22079057	0.5956	1.27E-04	VGLL4
cg08248955	-0.5955	1.28E-04	ARPC1B
cg06479142	0.5930	1.38E-04	
cg13151517	0.5927	1.39E-04	PLSCR3
cg26887465	0.5866	1.70E-04	ITFG3
cg22980722	-0.5859	1.74E-04	PFKL
cg14134015	-0.5843	1.83E-04	ZNRD1
cg06440270	0.5835	1.87E-04	
cg20373416	0.5833	1.89E-04	USP5
cg09958967	0.5832	1.89E-04	SLC30A3
cg02913884	0.5831	1.90E-04	PRPF38B
cg21610508	-0.5809	2.03E-04	
cg07468062	0.5807	2.05E-04	KIAA1257
cg03794522	0.5801	2.09E-04	
cg03024135	-0.5799	2.10E-04	SPTB
cg24079455	-0.5798	2.11E-04	C10orf137
cg14355619	0.5770	2.30E-04	
cg25498327	-0.5769	2.31E-04	SLC30A5
cg01844866	-0.5758	2.38E-04	JPH3
cg00336475	-0.5751	2.43E-04	TMEM202
cg16616325	-0.5747	2.46E-04	TSNAX-DISC1
cg14971781	-0.5746	2.47E-04	ACBD3
cg22777832	-0.5735	2.55E-04	ARFRP1;ZGPAT
cg07522644	-0.5730	2.59E-04	SRF
cg05253165	0.5730	2.60E-04	NECAB2
cg13465554	-0.5726	2.63E-04	GRM5
cg22014986	0.5718	2.69E-04	FRMD8
cg00348647	-0.5711	2.75E-04	
cg02773041	0.5704	2.81E-04	PPIE
cg10583632	0.5702	2.83E-04	ZNF624
cg11229230	-0.5694	2.90E-04	FBLN7
cg17287814	-0.5670	3.11E-04	PPP2R2A
cg25191332	0.5667	3.14E-04	IFRD1
cg23779621	-0.5663	3.18E-04	TMEM64
cg02577881	0.5662	3.19E-04	FBXO34
cg21082272	-0.5662	3.19E-04	LOC100130872;LOC100130872-SPON2
cg04627110	-0.5651	3.29E-04	MOG
cg23033759	-0.5649	3.31E-04	C14orf109;MOAP1
cg18331249	-0.5642	3.39E-04	LAPTM4B
cg14494451	-0.5632	3.48E-04	ARL6IP6
cg01514075	-0.5619	3.62E-04	VAMP3
cg19436502	0.5613	3.68E-04	GK5
cg06927863	0.5612	3.70E-04	LSM1;BAG4
cg05771701	0.5610	3.72E-04	CCDC62
cg24059115	0.5609	3.73E-04	AKR1B1
cg17098965	-0.5607	3.75E-04	ZNF217
cg01240229	-0.5588	3.97E-04	SNRPN
cg23676961	0.5587	3.98E-04	OMA1
cg24204847	-0.5585	4.00E-04	EEF2
cg20795117	-0.5578	4.09E-04	SCAMP3
cg10602537	-0.5569	4.20E-04	ERGIC1
cg06082432	0.5566	4.23E-04	SOCS1
cg21028182	-0.5562	4.28E-04	NOP56;SNORD86;SNORA51
cg15988239	0.5557	4.34E-04	PDHX;APIP
cg01696193	0.5556	4.36E-04	BEND6;DST
cg05386508	-0.5543	4.51E-04	FAM117A
cg02426414	-0.5540	4.55E-04	SHMT1
cg14578366	-0.5539	4.57E-04	COMMD6
cg26135388	-0.5537	4.60E-04	
cg03267167	-0.5516	4.88E-04	
cg21552063	-0.5514	4.90E-04	
cg20966270	-0.5511	4.95E-04	
cg11240634	-0.5504	5.04E-04	FGF19
cg06291211	-0.5499	5.13E-04	CBLB
cg12233418	-0.5492	5.22E-04	CTDP1
cg19661819	-0.5492	5.22E-04	NUB1
cg03562592	0.5492	5.23E-04	SLC20A2
cg21135533	-0.5490	5.26E-04	PRDM14
cg24657977	0.5484	5.35E-04	MAPKAPK2
cg10750613	-0.5478	5.44E-04	
cg06348851	-0.5477	5.46E-04	NRD1

ID	r	p	Gene
cg02326500	-0.5219	1.10E-03	
cg12581598	-0.5218	1.10E-03	PPP1R12B
cg16679001	-0.5216	1.11E-03	
cg11589723	-0.5214	1.11E-03	GLTSCR1
cg21767402	0.5213	1.11E-03	ZFP64
cg14038214	0.5211	1.12E-03	
cg16462237	-0.5204	1.14E-03	ULK2
cg24782003	-0.5203	1.14E-03	
cg17401179	0.5199	1.15E-03	AHRR
cg01778842	0.5198	1.16E-03	LOC401127
cg05523882	-0.5198	1.16E-03	AARS
cg15794034	-0.5196	1.16E-03	AMICA1
cg07826642	0.5196	1.16E-03	SLC29A1
cg03172801	-0.5188	1.19E-03	MYL12A
cg17916407	0.5187	1.19E-03	KLHDC2
cg21030939	0.5186	1.19E-03	IFNGR1
cg07582993	-0.5185	1.20E-03	UBOX5;FASTKD5
cg25060829	-0.5185	1.20E-03	ZSCAN12
cg20539142	-0.5185	1.20E-03	CHRNA6
cg03135127	0.5181	1.21E-03	TRMT1
cg09034836	0.5176	1.22E-03	PDE12L1P
cg09329275	-0.5173	1.23E-03	
cg07052663	-0.5169	1.25E-03	
cg11051647	-0.5168	1.25E-03	DCX
cg15809858	-0.5168	1.25E-03	PPARGC1B
cg10785578	-0.5167	1.25E-03	DIP2C;C10orf108
cg23462152	-0.5167	1.25E-03	
cg03513762	0.5164	1.26E-03	
cg06786167	-0.5162	1.27E-03	DLG3
cg23573822	-0.5161	1.27E-03	WDR37
cg02678084	0.5161	1.27E-03	C16orf5
cg12115190	-0.5160	1.28E-03	ATP2B2;MIR885
cg10566699	0.5158	1.28E-03	MS4A10
cg19671717	-0.5156	1.29E-03	EBAG9
cg00127036	-0.5155	1.29E-03	ZNF346
cg07632106	0.5154	1.30E-03	MYO10
ch.14.44741640R	0.5154	1.30E-03	
cg19465033	0.5153	1.30E-03	
cg16231241	-0.5153	1.30E-03	
cg19408740	0.5153	1.30E-03	IGSF8
cg09846818	0.5152	1.30E-03	INPP5A
cg11453585	0.5151	1.31E-03	ACTR1A;SUFU
cg24410949	0.5150	1.31E-03	NLE1
cg20866393	-0.5147	1.32E-03	
cg13686143	-0.5146	1.32E-03	ORC2L
cg09452911	0.5145	1.33E-03	ANTXR1
cg00622149	0.5138	1.35E-03	BRMS1L
cg20999737	-0.5136	1.36E-03	KCNC4
cg11790551	0.5136	1.36E-03	INPP5A
cg03803325	0.5134	1.36E-03	PDCD6
cg02365767	0.5132	1.37E-03	ZNF180
cg17742781	-0.5129	1.38E-03	
cg14205952	0.5128	1.38E-03	ABL1
cg11062418	0.5128	1.39E-03	FAM59B
cg18724200	-0.5127	1.39E-03	
cg19268388	-0.5126	1.39E-03	EHMT2
cg14758065	0.5126	1.39E-03	
cg09868496	-0.5125	1.40E-03	RERE
cg15466952	0.5124	1.40E-03	LEPR;LEPROT
cg19640779	-0.5122	1.41E-03	
cg21511365	0.5120	1.41E-03	
cg05281603	-0.5115	1.43E-03	BTG4;C11orf88;MIR34C
cg25982743	-0.5113	1.44E-03	TIMP4;SYN2
cg11538920	-0.5111	1.45E-03	MAN1C1
cg04372079	-0.5110	1.45E-03	SNX25
cg17981675	0.5107	1.46E-03	SMNDC1
cg15704988	-0.5106	1.46E-03	STAT3
cg15065123	-0.5106	1.46E-03	
cg25830640	-0.5106	1.46E-03	OSBPL10;ZNF860
cg11357221	-0.5105	1.47E-03	
cg02385809	-0.5104	1.47E-03	EEF1E1
cg24274579	-0.5100	1.49E-03	TBX4
cg17287767	0.5098	1.49E-03	STAT4
cg13366849	-0.5095	1.50E-03	DNM2
cg10288772	0.5092	1.52E-03	NR3C2
cg18567268	-0.5091	1.52E-03	LIPE
cg09282201	0.5090	1.52E-03	PRDM16
cg21139564	-0.5090	1.52E-03	MIR220A
cg08946995	0.5089	1.53E-03	
cg13700458	-0.5089	1.53E-03	LOC90784
cg19886365	0.5086	1.54E-03	XKR4
cg08756033	-0.5084	1.55E-03	C13orf33
cg18047684	0.5083	1.55E-03	CHCHD3
cg13133084	-0.5081	1.56E-03	SH3D19
cg05474761	0.5081	1.56E-03	
cg10784414	0.5078	1.57E-03	UQCRC1
cg13701615	0.5078	1.57E-03	PDCD6
cg12879381	0.5077	1.57E-03	TUBB6

ID	r	p	Gene
cg09947274	-0.5463	5.67E-04	C2orf28
cg15136424	-0.5463	5.67E-04	ZAK
cg15459695	-0.5460	5.72E-04	AGAP1
cg26509211	0.5453	5.83E-04	BICC1
cg24879882	-0.5451	5.87E-04	GBX1
cg15960928	-0.5439	6.06E-04	TROAP
cg10216832	0.5432	6.18E-04	
cg26555052	-0.5429	6.24E-04	FLT4
cg06427838	0.5426	6.29E-04	RPSAP58
cg13671181	0.5422	6.35E-04	NCRNA00029
cg13257636	-0.5419	6.40E-04	CLIP4
cg07905888	-0.5418	6.43E-04	NCDN;KIAA0319L
cg18697991	-0.5415	6.49E-04	MIR548A2
cg20003494	0.5406	6.65E-04	SNCA
cg21152628	0.5405	6.66E-04	TMEM132A;SLC15A3
cg24717029	-0.5399	6.77E-04	PHLPP1
cg23877661	-0.5398	6.79E-04	GABRQ
cg20839145	-0.5398	6.80E-04	MYLK
cg04012719	0.5395	6.85E-04	
cg17258335	0.5395	6.86E-04	
cg06289844	0.5392	6.90E-04	HEY2
cg14332129	-0.5390	6.94E-04	TLE2
cg23521444	-0.5390	6.94E-04	PLAGL1
cg22162872	-0.5387	7.00E-04	MFSD6
cg19457802	-0.5383	7.08E-04	
cg15417498	0.5379	7.15E-04	NFIL3
cg19852607	0.5375	7.24E-04	CYP2C18
cg02611770	-0.5375	7.24E-04	
cg05152300	-0.5372	7.29E-04	XYLT1
cg14257319	0.5372	7.29E-04	SLC27A4
cg13670531	-0.5369	7.36E-04	ARAP1L2
cg10084289	0.5367	7.39E-04	KIAA1919
cg17686973	0.5357	7.59E-04	MIR515-2;MIR515-1
cg13617521	-0.5357	7.59E-04	DKC1;SNORA36A
cg01012666	0.5350	7.75E-04	PANK4
cg00493358	0.5341	7.94E-04	CBFA2T3
cg17743719	0.5334	8.07E-04	STX11
cg07999668	-0.5334	8.09E-04	AICDA
cg05788089	-0.5333	8.11E-04	ADPRHL1
cg04468568	0.5333	8.11E-04	C11orf45;KCNJ5
cg10318066	-0.5332	8.13E-04	
cg27494615	0.5328	8.22E-04	IAH1
cg05709655	-0.5326	8.27E-04	MRPL10;LRRC46
cg07079724	-0.5324	8.30E-04	CPLX1
cg20974492	0.5322	8.35E-04	
cg13598434	-0.5321	8.37E-04	HEXIM1
cg06124285	-0.5320	8.40E-04	GJA5
cg18729664	-0.5319	8.41E-04	FILIP1
cg01185530	-0.5319	8.41E-04	DNAJC3
cg24360993	-0.5319	8.42E-04	HDC
cg03030717	0.5314	8.54E-04	
cg24567600	0.5312	8.57E-04	
cg08954417	0.5312	8.59E-04	SNTG1
cg25750469	-0.5312	8.59E-04	TTLL1
cg07541200	-0.5311	8.60E-04	
cg22889316	-0.5307	8.70E-04	LYRM4
cg06271287	0.5303	8.78E-04	PLAGL1
cg18627852	0.5300	8.86E-04	SNRPF
cg06775939	0.5294	8.99E-04	
cg08980396	-0.5292	9.06E-04	C11orf54
cg13626505	-0.5287	9.18E-04	ETFA
cg14016806	0.5285	9.21E-04	GLT1D1
cg24474822	0.5283	9.25E-04	C7orf50
cg16563178	-0.5284	9.25E-04	HYAL2
cg12878400	-0.5281	9.32E-04	KRTAP9-2
cg20787969	0.5281	9.33E-04	CRBN
cg09380198	-0.5275	9.46E-04	MTRF1
cg21291347	0.5275	9.47E-04	TDP1
cg13924580	-0.5271	9.55E-04	NIPAL2
cg05213896	-0.5269	9.61E-04	IL4I1
cg02048812	-0.5261	9.82E-04	
cg22853713	0.5261	9.82E-04	CASZ1
cg10012394	0.5256	9.95E-04	RNF180
cg21647375	0.5255	9.97E-04	RANBP3
cg06171619	0.5255	9.97E-04	C16orf35
cg26249168	-0.5250	1.01E-03	
cg11805252	0.5247	1.02E-03	
cg22525069	0.5245	1.02E-03	
cg13557922	0.5245	1.02E-03	
cg10188592	0.5242	1.03E-03	HSPE1;HSPD1
cg01109867	0.5240	1.04E-03	UBE2G2;UBE2G2
cg24023847	-0.5239	1.04E-03	MCM9
cg05646865	0.5233	1.06E-03	SLFN11
cg27657685	-0.5231	1.06E-03	
cg24209994	0.5224	1.08E-03	CLPB
cg24751129	0.5224	1.08E-03	GNMT
cg15606388	-0.5221	1.09E-03	C17orf53
cg02066650	-0.5219	1.09E-03	BUB1

ID	r	p	Gene
cg02324529	-0.5074	1.58E-03	KIAA1211
cg03855588	-0.5074	1.58E-03	CPT1A
cg26465251	0.5067	1.61E-03	MYH9
cg19275235	-0.5066	1.62E-03	SFRS14
cg17960442	-0.5064	1.63E-03	CAMK4
cg20000847	-0.5063	1.63E-03	C3orf21
cg17701373	-0.5063	1.63E-03	SGCE;PEG10
cg09712135	0.5063	1.63E-03	UNC5CL
cg17177931	0.5060	1.64E-03	ZBTB12
cg24134880	-0.5059	1.64E-03	GATAD1
cg22573731	-0.5057	1.65E-03	
cg00816936	-0.5057	1.66E-03	GPR177
cg20874666	-0.5056	1.66E-03	
cg23517298	-0.5056	1.66E-03	DGKG
cg08440550	-0.5056	1.66E-03	FNIP1
cg06520846	-0.5054	1.67E-03	PPCS;ZMYND12
cg03216491	0.5051	1.68E-03	MRAP
cg22020155	0.5050	1.68E-03	MTFR1
cg27023962	0.5049	1.69E-03	
cg03424826	-0.5048	1.69E-03	RNF152
cg04496233	-0.5046	1.70E-03	C11orf2
cg20122491	0.5045	1.70E-03	ZKSCAN5
cg01329511	0.5043	1.71E-03	SMYD5
cg05325061	-0.5040	1.72E-03	CA5A
cg12648677	-0.5037	1.74E-03	ATAD3A
cg13454935	-0.5032	1.76E-03	NIT2
cg24941405	-0.5031	1.76E-03	AURKA;CSTF1
cg14922895	0.5031	1.76E-03	TNRC6B
cg19577697	-0.5031	1.76E-03	ARMC9
cg24992690	-0.5026	1.79E-03	BAZ2B
cg18425638	-0.5024	1.79E-03	GNB1L
cg14481079	-0.5024	1.79E-03	
cg01709766	-0.5021	1.81E-03	TRIM6-TRIM34;TRIM34
cg22281363	-0.5019	1.82E-03	TCEA1
cg09069150	-0.5019	1.82E-03	SORCS1
cg04231888	-0.5016	1.83E-03	NAV2
cg07264818	0.5014	1.84E-03	CHD3;SCARNA21
cg22623080	0.5014	1.84E-03	AMOTL1
cg12599581	-0.5013	1.84E-03	TUBGCP4;ZSCAN29
cg22546565	-0.5011	1.85E-03	KIF3B
cg14454942	-0.5010	1.86E-03	MUTED
cg07706053	-0.5009	1.86E-03	PAX7
cg19772651	-0.5009	1.86E-03	RDH16
cg14586363	-0.5006	1.87E-03	CTBP1
cg24350392	-0.5003	1.89E-03	LASP1
cg05135942	0.5003	1.89E-03	CCDC41;LOC144486
cg14263021	0.5003	1.89E-03	
cg22668906	-0.5003	1.89E-03	
cg10162958	-0.5002	1.89E-03	MCF2L
cg23197280	-0.5002	1.89E-03	TSTA3
cg01730945	-0.5002	1.89E-03	PLEKHA6

Table S4: Top fifty methylated genes (expressed in blood) that correlate with the adherence to MedDiet. These genes present the highest standard deviation of methylation changes. The eight genes selected by bibliographic research afterwards are presented in bold.

ID	Gene	Standard deviation	r	p
cg24204847	**EEF2**	18.88	-0.5585	4.00E-04
cg01314574	**COL18A1**	16.15	0.6050	9.28E-05
cg11589723	GLTSCR1	15.57	-0.5214	1.11E-03
cg24134880	GATAD1	14.05	-0.5059	1.64E-03
cg07706053	PAX7	12.98	-0.5009	1.86E-03
cg05213896	**IL4I1**	12.15	-0.5269	9.61E-04
cg15466952	**LEPR; LEPROT**	12.13	0.5124	1.40E-03
cg13617521	DKC1	12.11	-0.5357	7.59E-04
cg01329511	SMYD5	11.65	0.5043	1.71E-03
cg09947274	CAD	11.56	-0.5463	5.67E-04
cg13366849	DNM2	10.88	-0.5095	1.50E-03
cg04372079	SNX25	10.87	-0.5110	1.45E-03
cg27153655	SERPINA10	10.20	0.6099	7.85E-05
cg24751129	GNMT	10.18	0.5224	1.08E-03
cg23877661	GABRQ	10.02	-0.5398	6.79E-04
cg19661819	NUB1	9.73	-0.5492	5.22E-04
cg05523882	AARS	9.59	-0.5198	1.16E-03
cg00543460	TPM1	9.32	-0.6089	8.11E-05
cg12973315	GPR78	9.29	0.6026	1.01E-04
cg05253165	NECAB2	9.20	0.5730	2.60E-04
cg06271237	**PLAGL1**	9.13	0.5303	8.78E-04
cg10602537	ERGIC1	8.96	-0.5569	4.20E-04
cg10785578	DIP2C	8.81	-0.5167	1.25E-03
cg15136424	ZAK	8.73	-0.5463	5.67E-04
cg07582993	FASTKD5	8.34	-0.5185	1.20E-03
cg21152628	TMEM132A;SLC15A3	8.00	0.5405	6.66E-04
cg19268388	EHMT2	7.75	-0.5126	1.39E-03
cg04231888	NAV2	7.71	-0.5016	1.83E-03
cg07079724	CPLX1	7.64	-0.5324	8.30E-04
cg25191332	**IFRD1**	7.53	0.5667	3.14E-04
cg17401179	AHRR	7.52	0.5199	1.15E-03
cg01514075	VAMP3	7.49	-0.5619	3.62E-04
cg09282201	PRDM16	7.29	0.5090	1.52E-03
cg22889316	LYRM4	7.06	-0.5307	8.70E-04
cg19852607	CYP2C18	6.87	0.5375	7.24E-04
cg26021810	GPR25	6.84	0.5075	1.58E-03
cg09868496	RERE	6.71	-0.5125	1.40E-03
cg12115190	ATP2B2	6.60	-0.5160	1.28E-03
cg24657977	**MAPKAPK2**	6.54	0.5484	5.35E-04
cg15809858	**PPARGC1B**	6.44	-0.5168	1.25E-03
cg05135942	CCDC41	6.40	0.5003	1.89E-03
cg18225258	GNB1L	5.80	-0.5024	1.79E-03
cg06171619	C16orf35	5.77	0.5255	9.97E-04
cg20003494	SNCA	5.67	0.5406	6.65E-04
cg12581598	PPP1R12B	5.48	-0.5218	1.10E-03
cg26509211	BICC1	5.48	0.5453	5.83E-04
cg00816936	GPR177	5.34	-0.5057	1.66E-03
cg17098965	ZNF217	5.26	-0.5607	3.75E-04
cg09069150	SORCS1	5.21	-0.5019	1.82E-03
cg06786167	DLG3	5.01	-0.5162	1.27E-03

CHAPTER 3

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

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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

Ana Arpón [1,2], Fermín I. Milagro [1,2,3], Cristina Razquin [3,4,5], Dolores Corella [3,6], Ramón Estruch [3,7], Montserrat Fitó [3,8], Amelia Marti [1,3,5], Miguel A. Martínez-González [3,4,5], Emilio Ros [3,9], Jordi Salas-Salvadó [3,10], José-Ignacio Riezu-Boj [1,2,5,†] and J. Alfredo Martínez [1,2,3,5,11,*,†]

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Abstract: DNA methylation could be reversible and mouldable by environmental factors, such as dietary exposures. The objective was to analyse whether an intervention with two Mediterranean diets, one rich in extra-virgin olive oil (MedDiet + EVOO) and the other one in nuts (MedDiet + nuts), was influencing the methylation status of peripheral white blood cells (PWBCs) genes. A subset of 36 representative individuals were selected within the PREvención con DIeta MEDiterránea (PREDIMED-Navarra) trial, with three intervention groups in high cardiovascular risk volunteers: MedDiet + EVOO, MedDiet + nuts, and a low-fat control group. Methylation was assessed at baseline and at five-year follow-up. Ingenuity pathway analysis showed routes with differentially methylated CpG sites (CpGs) related to intermediate metabolism, diabetes, inflammation, and signal transduction. Two CpGs were specifically selected: cg01081346–*CPT1B/CHKB-CPT1B* and cg17071192–*GNAS/GNASAS*, being associated with intermediate metabolism. Furthermore, cg01081346 was associated with PUFAs intake, showing a role for specific fatty acids on epigenetic modulation. Specific components of MedDiet, particularly nuts and EVOO, were able to induce methylation changes in several PWBCs genes. These changes may have potential benefits in health; especially those changes in genes related to intermediate metabolism, diabetes, inflammation

and signal transduction, which may contribute to explain the role of MedDiet and fat quality on health outcomes.

Keywords: Mediterranean diet; DNA methylation; nuts; olive oil; blood cells

1. Introduction

Epigenetics has been defined as the study of heritable changes that cannot be explained through variations in DNA nucleotide sequence [1], but can result in alteration of gene expression, providing a connection among genetics, diseases, and the environment [2]. Actually, epigenetic alterations have been associated to several diseases and complications, such as obesity, type 2 diabetes, adverse cardiovascular events, or immune diseases [3]. Accumulating evidence suggests that epigenetic marks are reversible [2,4] and they can be modulated by environmental factors [5,6]. Interestingly, nutrients and specific dietary components of the diet are able to modify gene expression through changes in DNA methylation [3]. Thus, the study of nutritional biomarkers and the progress in the epigenetics field are contributing not only to define new nutrient roles in health and disease, but also to the implementation of precision nutrition strategies [7].

Mediterranean diet (MedDiet) has been associated with a decrease in the risk of cardiovascular events, with a favourable effect on blood pressure, insulin sensitivity, lipid profile, inflammation, oxidative stress, and metabolic syndrome features [8–10]. Interestingly, MedDiet has been involved in changes in DNA methylation. For instance, a preliminary study within the PREvención con DIeta MEDiterránea (PREDIMED) trial reported associations between MedDiet and methylation at 1-year in *FTO* (Alpha-ketoglutarate dependent dioxygenase) and *TCF7L2* (Transcription factor 7 like 2) genes [11]. Another ancillary study within PREDIMED-Navarra study described an association between adherence to MedDiet and methylation of several inflammation-related genes in peripheral white blood cells [12].

Although extra-virgin olive oil (EVOO) and nuts have been previously related to DNA methylation changes [13,14], no new relationships between the different MedDiets (enriched in either EVOO or nuts) from the PREDIMED trial and methylation have been described before. Therefore, the aim of the current investigation was to explore methylation changes, which were caused by two diets with Mediterranean profiles, in genes of peripheral white blood cells (PWBCs) from participants in the PREDIMED-Navarra trial with emphasis on the impact of fat-quality.

2. Materials and Methods

2.1. Study Design and Participants

The current study was conducted within the framework of the PREDIMED trial. Briefly, PREDIMED was a multicentre, randomized, primary prevention feeding trial with blinded assessment of end points carried out in Spain with the aim of evaluating the effects of the MedDiet on primary cardiovascular prevention (www.predimed.es). The study design has been described elsewhere [15,16], and it was approved by the Research Ethics Committees at all of the recruiting centres in compliance with the Helsinki Declaration. The Institutional Review Board of the Navarra recruitment centre approved the study protocol (protocol 50/2005). All participants provided written informed consent. This trial was registered with the International Standard Randomised Controlled Trial Number system 35739639.

Eligible participants in PREDIMED trial were men aged 55–80 and women aged 60–80 years without any previous history of cardiovascular disease. Inclusion and exclusion criteria have been described elsewhere [15,16]. Participants were randomized to one of three nutrition interventions: a MedDiet supplemented with EVOO (MedDiet + EVOO), a MedDiet supplemented with mixed nuts

(MedDiet + nuts), or advice in reducing all sources of fat, which was the control group. All of the groups received dietary training and questionnaires about medical conditions, food consumption (such as 137-item validated food-frequency questionnaire) [17], and physical activity (Minnesota Leisure-Time Physical Activity Questionnaire) [16] were completed as described elsewhere [15]. Data of anthropometric measures, body composition and blood pressure were collected in the same consultations following standardized procedures [10]. Plasma, serum, and buffy-coat were stored (−80 °C) and biochemical features were evaluated, as described elsewhere [10].

For this study, participants were selected from the recruitment centre at the University of Navarra following different criteria: non-smokers or former-smokers, same proportion of women and men, and aged between 60 and 70 years old, enrolling a total of 36 participants (12 from each diet) with data at baseline and at five years of intervention.

2.2. DNA Extraction and DNA Methylation Analysis

The procedures for DNA extraction from buffy coat (PWBCs) of venous blood samples have been described elsewhere [12]. The extracted DNA was sent on dry ice to Unidad de Genotipado y Diagnóstico Genético from Fundación Investigación Clínico de Valencia, where bisulphite treatment was performed and Infinium HumanMethylation450K bead chip (Illumina, San Diego, CA, USA) was used for DNA methylation analysis as described elsewhere [12].

2.3. Treatment of Methylation Raw Data

Normalization of microarray data was performed in R by a categorical subset quantile normalization method using the pipeline developed by Touleimat & Tost [18]. After data normalization, Linear Models for Microarray Data (LIMMA) [19] was used to identify the probes with significant differential methylation changes (five years–baseline) between two diets, being the comparisons: MedDiet + EVOO vs. low-fat control diet and MedDiet + nuts vs. low-fat control diet. The linear model was adjusted for age, sex, and microarray chip. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [20] and are accessible through GEO Series accession number GSE107205 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE107205).

2.4. Ingenuity Pathway Analysis

From all the significant CpGs that were obtained after LIMMA analysis, a selection was carried out in order to have an appropriate number of CpGs for obtaining relevant results in the analysis of canonical pathways by Ingenuity Pathway Analysis (IPA) platform (Qiagen Redwood City, CA, USA, www.ingenuity.com). CpGs with a $p < 0.005$ and effect size ≤ -9 or ≥ 9 (the effect size represents the methylation change for the CpG site in %) were selected. Predefined pathways and functional categories of the Ingenuity Knowledge Base were used in order to detect associated pathways and relevant gene regulatory networks [21]. Canonical pathways with a $p < 0.05$ after Fisher's test were defined as a statistically significant overrepresentation of input genes in a given process.

2.5. Differential CpG Site Selection

In order to select CpGs presenting a higher effect on methylation with biological implications that could be noticeable and minimize the type I error rate, a series of statistically restricted selections with stringent criteria were performed. CpG sites were selected by B-values (statistic of differential methylation generated in LIMMA package) >0 and effect size <-9 or >9 for both of the comparisons. Moreover, methylation values must not be significantly different among the three dietary groups at baseline, but show differences throughout the intervention. The tests that were used in these cases were ANOVA with Tukey's multiple comparison test or Kruskal-Wallis with Mann-Whitney's U test, when appropriate, and Student-*t* test or sign test, when appropriate. Then, regression models between selected CpGs methylation and foods or their components, adjusted for diabetes and BMI, were performed.

2.6. Blood Cell Type Composition

Differences in the composition of types of leukocytes between five years and baseline were correlated (Pearson) with nuts intake at five years, EVOO consumption at five years and with methylation of the CpGs selected. Differences among dietary groups in the composition of blood cells were also assessed.

2.7. Statistical Analysis of Participants

Anthropometric and biochemical features of participants and food consumption were characterised and tested comparing the three groups at baseline, at five years and their differences (five years-baseline) using ANOVA or Kruskal-Wallis test (for quantitative variables) and Chi-square test, Fisher's exact test, or Cochran test (for qualitative variables), as appropriate. The values were also compared for each diet between five years and baseline using Student *t*-test, Wilcoxon signed-rank test, Sign test, or Cochran test, when advised. The Shapiro-Wilk analysis was used to test normality.

Statistical calculations and graphs were performed using STATA version 12.0 (Stata Corp, College Station, TX, USA) and GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA), respectively. The statistically significant level was set at $p < 0.05$.

3. Results

3.1. Participants' Differences in Anthropometric and Biochemical Features, Food Consumption and Blood Cell Type Composition

The three dietary groups showed no differences in anthropometric and biochemical features, except for HDL cholesterol levels measured at five years. Concerning clinical manifestations, the three groups exhibited a significant decrease in diabetes, hypercholesterolemia, and arterial hypertension cases after five years of intervention, except for diabetes in low-fat control diet and for arterial hypertension in MedDiet + nuts group, which did not present significant differences (Table 1).

Energy and nutrient intakes were also recorded (Table 2). At the baseline, no significant differences between groups were noted. The highest significant differences were observed for nuts and EVOO consumption at five years among the three groups. Moreover, EVOO consumption was statistically different between five years and baseline in MedDiet + EVOO group. Some differences were also observed in fruit intake between five years and baseline in low-fat control diet and MedDiet + nuts group, as well as in red meat intake in MedDiet + EVOO group. There were also differences in legumes intake between five years and baseline in MedDiet + nuts group and among the three groups.

Since methylation measured in PWBCs can vary by blood cell type, and thus the methylation changes that are associated with the variables investigated in this study may reflect an alteration in blood cell composition, correlation studies were performed between the relative proportions of granulocytes, lymphocytes, or mid cells and nuts intake, EVOO intake or methylation of the selected CpGs, but no associations for any comparison were found (Supplementary Materials Table S1). There were also no differences in blood cell types among the three dietary groups (Supplementary Materials Table S2).

Table 1. Anthropometric, clinical and biochemical characteristics of the study population, with the statistical differences among the three dietary groups at baseline and after five-year follow-up.

	Low-Fat Control Diet (n = 12)		p^2	MedDiet + EVOO (n = 12)		p^2	MedDiet + nuts (n = 12)					
	Baseline	5 Years		Baseline	5 Years		Baseline	5 Years	p^2	$p\,0^3$	$p\,5^3$	$p\,\Delta^3$
Female, *n* (%)	6 (50)			6 (50)			6 (50)			ns		
Age(years) [1]	64.3 (3.9)	NA		63.5 (1.7)	NA		63.2 (2.1)	NA		ns		
Weight(kg) [1]	73.0 (10.9)	73.1 (9.6)	ns	73.3 (9.4)	74.6 (10.7)	ns	71.6 (7.5)	73.2 (7.6)	ns	ns	ns	ns
Waist circumference(cm) [1]	92.4 (9.6)	92.4 (9.5)	ns	90.1 (7.1)	93.8 (6.4)	ns	93.5 (6.3)	94.3 (6.7)	ns	ns	ns	ns
BMI(kg/m^2) [1]	27.3 (3.2)	27.4 (2.9)	ns	27.9 (1.5)	28.3 (1.8)	ns	28.1 (1.5)	28.8 (1.8)	ns	ns	ns	ns
Glycemia(mg/dL) [1]	106.0 (27.6)	102.2 (18.0)	ns	129.5 (67.1)	120.8 (52.7)	ns	131.5 (57.4)	127.0 (34.9)	ns	ns	ns	ns
HDL cholesterol(mg/dL) [1]	52.7 (14.5)	51.5 (13.3)	ns	55.2 (8.3)	55.1 (10.4)	ns	60.9 (9.1)	64.6 (12.0)	ns	ns	0.022	ns
LDL cholesterol(mg/dL) [1]	132.0 (15.5)	112.3 (27.1)	ns	122.0 (30.5)	113.0 (26.3)	ns	119.1 (23.2)	123.1 (35.2)	ns	ns	ns	ns
Total cholesterol(mg/dL) [1]	209.8 (21.0)	188.6 (27.7)	ns	203.4 (28.7)	191.6 (29.1)	ns	199.2 (24.3)	210.4 (41.2)	ns	ns	ns	ns
Triglycerides(mg/dL) [1]	126.1 (49.6)	124.1 (40.7)	ns	131.2 (78.2)	117.6 (47.9)	ns	95.7 (24.6)	113.4 (40.0)	ns	ns	ns	ns
Systolic arterialpressure (mmHg) [1]	148.9 (17.2)	150.1 (22.4)	ns	158.5 (19.1)	157.6 (20.5)	ns	142.9 (16.0)	147.7 (21.6)	ns	ns	ns	ns
Diastolic arterialpressure (mmHg) [1]	85.7 (8.6)	85.8 (11.4)	ns	88.1 (8.4)	83.9 (10.0)	ns	87.9 (7.2)	87.2 (14.0)	ns	ns	ns	ns
Diabetes, *n* (%)	1 (8)	0 (0)	ns	4 (33)	0 (0)	0.046	6 (50)	0 (0)	0.014	ns	ns	0.022
Hypercholesterolemia, *n* (%)	8 (67)	0 (0)	0.005	9 (75)	1 (8)	0.005	10 (83)	1 (8)	0.003	ns	ns	ns
Arterial hypertension, *n* (%)	12 (100)	0 (0)	<0.001	11 (92)	1 (8)	0.004	10 (83)	4 (33)	ns	ns	ns	0.010
Hypertriglyceridemia, *n* (%)	1 (8)	2 (17)	ns	2 (17)	0 (0)	ns	0 (0)	0 (0)	ns	ns	ns	ns

[1] Values are represented as Mean (SD).[2] Values obtained by Student *t*-test, Wilcoxon signed-rank test, Sign test or Cochran test, as appropriate, comparing data from 5 years and baseline for each diet.[3] Values obtained by chi-square test, Fisher's exact test or Cochran test (for qualitative variables), and ANOVA test or Kruskal-Wallis test (for quantitative variables), as appropriate, from baseline data (p 0), from five years data (p 5) or from the difference between 5 years and baseline data ($p\,\Delta$) among the three groups. $p < 0.05$ is considered significant. EVOO: extra-virgin olive oil; HDL: high-density lipoprotein; LDL: low-density lipoprotein; MedDiet: Mediterranean diet; NA: non-applicable; ns: non-significant.

Table 2. Energy and nutrient intake of participants and statistical differences among the three dietary groups at baseline and after five-year follow-up.

Food/component (g/day)	Low-Fat Control Diet (n = 12)		p^2	MedDiet + EVOO (n = 12)		p^2	MedDiet + nuts (n = 12)		p^2	$p\ 0^3$	$p\ 5^3$	$p\ \Delta^3$
	Baseline	5 Years		Baseline	5 Years		Baseline	5 Years				
Carbohydrates	230.4 (46.7)	267.9 (60.1)	ns	239.4 (82.1)	248.7 (76.2)	ns	224.9 (52.3)	240.8 (44.9)	ns	ns	ns	ns
Proteins	89.9 (23.9)	96.8 (16.1)	ns	88.9 (16.0)	88.7 (14.8)	ns	91.5 (16.0)	95.0 (18.9)	ns	ns	ns	ns
Fat	100.5 (24.2)	97.2 (19.5)	ns	108.2 (30.3)	117.3 (25.6)	ns	108.9 (19.4)	124.1 (25.1)	ns	ns	0.017	ns
MUFA	51.8 (11.8)	51.3 (11.0)	ns	56.6 (16.4)	63.9 (10.6)	ns	55.3 (12.4)	64.6 (13.0)	ns	ns	0.014	ns
SFA	23.3 (7.2)	22.5 (7.3)	ns	26.7 (8.9)	25.0 (6.8)	ns	24.8 (5.3)	28.8 (8.1)	ns	ns	ns	ns
PU FA	15.8 (5.5)	15.5 (5.0)	ns	16.8 (6.3)	16.9 (4.2)	ns	18.6 (4.7)	19.7 (4.1)	ns	ns	ns	ns
Vegetables	302.1 (90.2)	352.2 (104.2)	ns	302.3 (117.8)	370.2 (58.2)	ns	298.9 (71.1)	372.7 (129.1)	ns	ns	ns	ns
Fish	73.5 (32.6)	93.6 (37.5)	ns	90.2 (33.8)	82.6 (25.7)	ns	91.2 (33.5)	100.4 (39.9)	ns	ns	ns	ns
Fruits	291.6 (145.1)	431.4 (237.1)	0.009	319.8 (181.9)	459.3 (109.9)	ns	367.2 (163.2)	500.8 (186.7)	0.034	ns	ns	ns
Legumes	17.9 (9.9)	14.7 (6.4)	ns	16.0 (4.7)	19.6 (7.5)	ns	15.5 (3.5)	20.4 (5.8)	0.019	ns	ns	0.034
Nuts	12.2 (18.2)	5.5 (8.8)	ns	9.9 (16.4)	11.3 (8.3)	ns	20.5 (15.1)	28.2 (14.2)	ns	ns	<0.001	ns
EVOO	29.6 (26.3)	32.9 (26.2)	ns	39.6 (24.7)	66.4 (8.1)	0.003	25.0 (30.0)	33.8 (32.4)	ns	ns	0.002	0.049
Rest of olive oils	16.7 (24.6)	16.7 (24.6)	ns	10.0 (15.8)	0 (0)	0.049	26.3 (27.6)	25.8 (32.6)	ns	ns	ns	ns
Red meat	63.2 (42.5)	59.8 (38.1)	ns	71.7 (42.3)	39.5 (28.0)	0.045	57.5 (29.8)	54.8 (32.6)	ns	ns	ns	ns
White meat	54.9 (28.6)	63.0 (25.4)	ns	39.5 (22.4)	36.7 (26.8)	ns	43.1 (23.2)	47.7 (26.4)	ns	ns	ns	ns
Pastries	35.3 (18.2)	36.9 (27.3)	ns	43.4 (24.5)	29.8 (21.4)	ns	23.9 (21.1)	37.6 (27.6)	ns	ns	ns	ns
Total energy (kcal/day)	2310 (420)	2412 (475)	ns	2378 (651)	2470 (513)	ns	2349 (432)	2535 (452)	ns	ns	ns	ns

Values are represented as Mean (SD).[1] Values obtained by Student t-test, Wilcoxon signed-rank test or Sign test, as appropriate, comparing data from 5 years and baseline for each diet.[2] Values obtained by ANOVA test or Kruskal-Wallis test, as appropriate, from baseline data (p 0), from 5 years data (p 5) or from the difference between 5 years and baseline data ($p\ \Delta$) among the three groups. $p < 0.05$ is considered significant. EVOO: extra-virgin olive oil; MedDiet: Mediterranean diet; ns: non-significant; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.

3.2. Relevant Differentially Methylated CpGs Were Related to Metabolism, Inflammation, Intracellular Signals and Diabetes

For IPA analysis, 223 CpGs from MedDiet + EVOO vs. low-fat control diet comparison and 359 CpGs from MedDiet + nuts vs. low-fat control diet were analysed. The obtained canonical pathways were collected for both comparisons (Supplementary Materials Table S3 and Table S4, respectively). Some CpGs from both of the comparisons were related to metabolism, such as Fatty Acid Activation, γ-linolenate Biosynthesis II, Fatty Acid β-oxidation I, Fatty Acid α-oxidation, and Adipogenesis pathway. CpGs from MedDiet + EVOO vs. low-fat control diet comparison were related to pathways that were involved in inflammation, such as Leukocyte extravasation signaling, LPS/IL-1 Mediated Inhibition of retinoid acid receptor function, and Crosstalk between Dendritic Cells and Natural Killer Cells. On the other hand, CpGs from MedDiet + nuts vs. low-fat control diet comparison were related to a variety of intracellular signals (apoptosis and DNA damage, Protein kinase A, Notch, G-Protein coupled receptor, Stress-activated protein kinase/c-Jun NH(2)-terminal kinase, etc.), and with diabetes (Type II Diabetes Mellitus Signalling).

3.3. Two Differentially Methylated CpGs (cg01081346 and cg17071192) Were Selected

After those preliminary analyses, CpGs were selected by B-values >0 and effect size ≤ -9 or ≥ 9 for both comparisons, as well as by showing non-significant differences in methylation values among the three dietary groups at baseline and showing significant differences throughout the intervention. Two CpGs were obtained: cg01081346 and cg17071192. The CpG cg01081346 presented an increase in methylation in MedDiet + nuts after five years of intervention (Figure 1a), and showed significant higher methylation changes in MedDiet + nuts subjects when comparing with the other two dietary groups (Figure 1b). In addition, nuts intake (g/day) at five years of follow-up was associated with an increase in cg01081346 methylation (Figure 1c). The other CpG (cg17071192) presented a methylation decrease in MedDiet + EVOO throughout the intervention (Figure 2a) and showed significant differences in methylation changes between the MedDiet + EVOO group and the other two groups (Figure 2b). The corresponding genes for these CpGs, according to the Illumina CG database, were *CPT1B/CHKB-CPT1B* (Carnitine palmitoyltransferase 1B/Choline kinase-like, Carnitine palmitoyltransferase 1B) for cg01081346, located at the TSS220, TSS1500 (*CPT1B*) and body of the gene (*CHKB-CPT1B*), and *GNAS/GNASAS* (Guanine Nucleotide Binding Protein (G Protein), Alpha Stimulating Activity Polypeptide 1/*GNAS* Antisense RNA 1) for cg17071192, located at the body of the gene (*GNASAS*) and TSS1500 (*GNAS*).

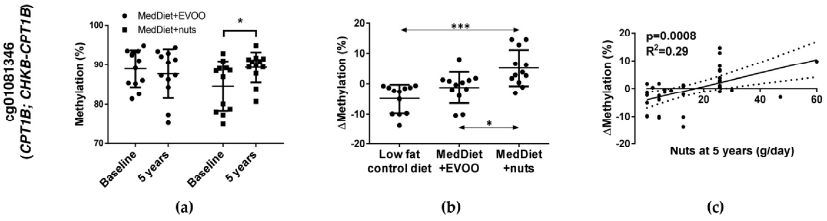

Figure 1. Representation of significant methylation changes of cg01081346. (**a**) Methylation mean and SD of each dietary group at baseline and at 5 years. Statistical analysis was performed by a Student-*t* test between 5 years and baseline for each diet. (**b**) Methylation changes (Mean and SD) for each participant and diet. Statistical analysis of differences in methylation changes among diets was performed by ANOVA (+Tukey's multiple comparison test). Significance is considered * $p < 0.05$, *** $p < 0.001$. (**c**) Regression graph representing the relation between methylation changes and nuts intake at 5 years. Dot lines on both sides of the solid line (linear regression line) represent 95% confidence band. MedDiet: Mediterranean diet.

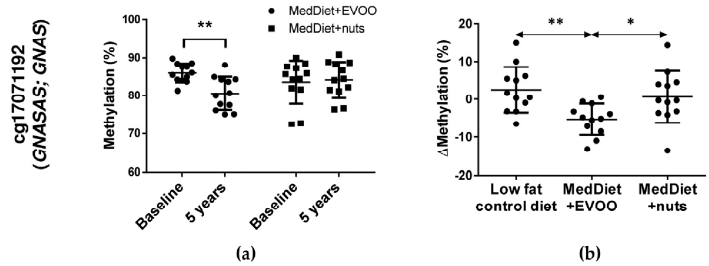

Figure 2. Representation of significant methylation changes of cg17071192. (**a**) Methylation mean and SD of each dietary group at baseline and at 5 years. Statistical analysis was performed by a Student-*t* test between 5 years and baseline for each diet. (**b**) Methylation changes (Mean and SD) for each participant and diet. Statistical analysis of differences in methylation changes among diets was performed by ANOVA (+Tukey's multiple comparison test) or Kruskal-Wallis (+Mann-Whitney's U), when appropriate. Significance is considered * $p < 0.05$, ** $p < 0.01$. EVOO: extra-virgin olive oil; MedDiet: Mediterranean diet.

3.4. CpG cg01081346 Was Associated with PUFAs

In order to check whether fatty acids were related to methylation changes, other regression analyses were performed between methylation changes of CpGs and total lipids, saturated fatty acids, monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) that were consumed at five years. Results showed that methylation of cg01081346 was associated with PUFAs (Figure 3), whereas cg17071192 presented no association.

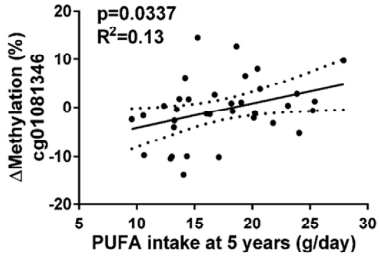

Figure 3. Regression graph between cg01081346 methylation and polyunsaturated fatty acids (PUFA) consumption at five years. Dot lines on both sides of the solid line (linear regression line) represent 95% confidence band.

4. Discussion

The current study demonstrates that a MedDiet pattern plus specific food supplements rich in different fat quality, in this case nuts and EVOO, could induce changes in methylation levels in specific CpGs of PWBCs. Therefore, further insights are added to previous reports where environmental factors, including the diet, were able to modify the epigenome [22].

MedDiets supplemented with nuts or EVOO have evidenced beneficial effects in individuals with cardiovascular risk [8]. Among these effects, decreased levels of LDL cholesterol and increased levels of HDL cholesterol were observed [23]. In the subset of participants selected for this study, there were differences among the three groups in HDL cholesterol. In fact, the low-fat dietary control group decreased HDL levels, whereas MedDiet + EVOO maintained the levels and MedDiet + nuts increased

them. In most cases, there was a reduction in the incidence of diabetes, hypercholesterolemia and arterial pressure, indicating a general improvement in health status after each dietary intervention.

Regarding MedDiet supplements intake, although MedDiet + EVOO group presented differences in consumption of EVOO at five years and in changes between five years and baseline, MedDiet + nuts only showed differences in nuts intake at five years. This result could be influenced by the initial values of nuts consumption of the MedDiet + nuts group, which were higher than in the other two groups, although not statistically significant. Some dietary groups presented differences in other foods intake, probably due to dietary advice.

The IPA strategy revealed that the selected differentially methylated genes were mainly related to intermediate metabolism, inflammation, intracellular signals, and diabetes phenomena. These areas are interconnected and MedDiets supplemented with EVOO or nuts have been described as having a favourable impact in different features related to them, such as inflammatory parameters, hypertension, hyperlipidaemia, among others [8,9]. Thus, MedDiet, and especially the food supplements employed, EVOO and nuts, could be inducing changes in the DNA methylation, which in turn, may cause changes in the expression of some genes that are associated with these processes and related diseases. For instance, Rodríguez-Miguel et al. showed that an olive oil-enriched diet increased levels of global methylation in mammary glands and tumours [13]. In addition, adherence to MedDiet has been associated with changes in methylation in inflammatory-related genes in a previous study from this group [12]. Further studies of the relationship between significant genes and the pathways from IPA would be interesting to elucidate their putative involvement in MedDiet health beneficial effects.

After an exhaustive analytical selection in order to choose the most relevant CpGs that had diet-induced methylation changes, two CpGs were specifically analysed: cg01081346–*CPT1B/CHKB-CPT1B* and cg17071192-*GNAS/GNASAS*. Interestingly, three surrounding CpGs of 27 for *CPT1B/CHKB-CPT1B* and 13 CpGs of 129 for *GNAS/GNASAS* also showed significant differences ($p < 0.05$). These CpGs may be related to the expression of genes performing actions that were in agreement with the differentially methylated areas obtained in the IPA. For instance, *CPT1B* has been related to insulin sensitivity and cardiac risk [24,25]. This gene is implicated in the conversion of acyl-CoA and carnitine into acyl-carnitine in the outer membrane of mitochondria, determining the balance between glucose and fatty acid metabolism [26]. The levels of these molecules are very important in lymphocytes and mononuclear phagocytes metabolism [27]. On the other hand, *GNASAS* encodes for an antisense RNA transcript that regulates *GNAS*, which has been involved in glucose and energy regulation [28,29]. Although methylation levels were measured in PWBCs, which are not very related to some of the aforementioned functions, these cells could be proxies for less accessible tissues [30]. Indeed, differentially methylated CpGs in blood cells have been identified as mirroring the adipose tissue methylation pattern [31].

Nuts and EVOO are both rich in MUFAs and PUFAs [32,33]. In fact, in this study, methylation of CpG cg01081346 was associated with PUFA uptake. It is likely that this CpGs could be influenced by fat quality due to PUFAs consumed in nuts. Indeed, it has been described that the quality of dietary fat influences the methylation of genes [34]. Nevertheless, the CpG cg17071192 might change the methylation pattern due to other MedDiet nutrients or other compounds that are present in EVOO. For instance, polyphenols from EVOO have been described down-regulating proatherogenic genes [35], while some derivatives (hydroxityrosol, tyrosol, and their secoiridoid derivatives) have shown strong antioxidant and anti-inflammatory activity in vitro [36,37]. In addition, the stimulatory effect of phenolic extracts from EVOO and hydroxityrosol on cannabinoid receptor 1 (CB1) expression inversely correlated with DNA methylation at cannabinoid receptor 1 promoter and it was associated with reduced proliferation of human colon cancer cells [38].

The present investigation was not devoid of limitations. Firstly, the sample size is relatively low, but the results are plausible. Secondly, mRNA levels could be evaluated in order to determine EVOO and nuts roles on the modification of the methylation levels and in turn, the effect on the expression of genes with a function in the maintenance of a good health status.

5. Conclusions

Summing up, this study shows that an intervention with MedDiet + EVOO and MedDiet + nuts in the PREDIMED-Navarra trial are influencing the methylation of genes in PWBCs. This effect might be, in part, due to the fat profile of either EVOO or nuts. Specifically, methylation changes occurs in genes related to intermediate metabolism, diabetes, inflammation, and signal transduction process, such as *CPT1B* and *GNAS*, which are specifically involved in acyl-carnitine process in mitochondria, and glucose and energy regulation, respectively. Hence, the potential beneficial effects on health of Mediterranean dietary pattern supplemented with EVOO or nuts could be mediated, at least in part, through epigenetic mechanisms, where the quality of fat intake, such as PUFA consumption, is playing a mediating role.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/1/15/s1, Table S1: Pearson correlation values of differences in types of leukocytes and consumption at 5 years of extra-virgin olive oil (EVOO) and nuts, and methylation changes of cg01081343 and cg17071192, Table S2: Differences among dietary groups in the composition of blood cells, Table S3: Canonical pathways (Ingenuity Pathway Analysis) associated with differentially methylated CpGs selected from MedDiet + EVOO vs. low-fat control diet LIMMA analysis, Table S4: Canonical pathways (Ingenuity Pathway Analysis) associated with differentially methylated CpGs selected from MedDiet + nuts vs. low-fat control diet LIMMA analysis.

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Supplementary material

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

Available at: https://www.mdpi.com/2072-6643/10/1/15#supplementary

Table S1. Pearson correlation values of differences in types of leukocytes and consumption at 5 years of extra-virgin olive oil (EVOO) and nuts, and methylation changes of cg01081343 and cg17071192.

Correlation	r	p-value
cg01081343 vs.		
Leukocytes	0.042	0.842
Lymphocytes	0.048	0.821
Monocytes	0.250	0.228
Neutrophils	-0.134	0.522
Eosinophils	0.060	0.777
Basophils	0.096	0.649
cg17071192 vs.		
Leukocytes	0.245	0.237
Lymphocytes	-0.289	0.161
Monocytes	0.196	0.348
Neutrophils	0.184	0.378
Eosinophils	0.148	0.481
Basophils	-0.016	0.939
Nuts vs.		
Leukocytes	0.386	0.062
Lymphocytes	0.023	0.916
Monocytes	0.302	0.152
Neutrophils	-0.078	0.719
Eosinophils	-0.115	0.593
Basophils	0.012	0.955
Extra-virgin olive oil vs.		
Leukocytes	-0.097	0.653
Lymphocytes	-0.103	0.634
Monocytes	-0.235	0.268
Neutrophils	-0.227	0.286
Eosinophils	-0.249	0.242
Basophils	0.003	0.989

Correlation values were obtained after Pearson correlations.
$p<0.05$ is considered significant.

Table S2. Differences among dietary groups in the composition of blood cells.

	p-value ANOVA	p-value Low-fat vs. MedDiet+EVOO	p-value Low-fat vs. MedDiet+nuts	p-value MedDiet+EVOO vs. MedDiet+nuts
Leukocytes	0.118			
Lymphocytes	0.065			
Monocytes	0.963			
Neutrophils	0.049	0.077	0.997	0.089
Eosinophils	0.290			
Basophils	0.354			

Statistical analysis was performed using ANOVA (+ Tukey's multiple comparison test). $p<0.05$ is considered significant.

Table S3. Canonical pathways (Ingenuity Pathway Analysis) associated with differentially methylated CpGs selected from MedDiet+EVOO vs. low-fat control diet LIMMA analysis.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Genes
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2,76E00	3,83E-02	TLR4,COL19A1,PROK1,IFNGR2,COL20A1,COL11A2,TNFRSF1B
Fatty Acid Activation	2,20E00	1,54E-01	SLC27A5,ACSBG2
Crosstalk between Dendritic Cells and Natural Killer Cells	2,01E00	4,49E-02	TLR4,FSCN2,TNFRSF1B,CAMK2B
Aryl Hydrocarbon Receptor Signaling	1,99E00	3,57E-02	AHRR,NFIC,POLA1,ALDH1A2,ALDH3B2
γ-linolenate Biosynthesis II (Animals)	1,97E00	1,18E-01	SLC27A5,ACSBG2
Mitochondrial L-carnitine Shuttle Pathway	1,97E00	1,18E-01	SLC27A5,ACSBG2
LPS/IL-1 Mediated Inhibition of RXR Function	1,73E00	2,70E-02	TLR4,SLC27A5,ALDH1A2,ACSBG2,TNFRSF1B,ALDH3B2
Fatty Acid β-oxidation I	1,44E00	6,25E-02	SLC27A5,ACSBG2
Uracil Degradation II (Reductive)	1,43E00	2,50E-01	DPYD
Thymine Degradation	1,43E00	2,50E-01	DPYD
Leukocyte Extravasation Signaling	1,32E00	2,38E-02	MMP28,RAP1GAP,CLDN16,DLC1,CLDN22

Ratio represents differentially methylated genes/genes in the pathway. A –log(p-value)>1.301 is considered significant, which corresponds to a p<0.05. CpG: CG site; EVOO: Extra-Virgin Olive Oil; LIMMA: Linear Models for Microarray Data; MedDiet: Mediterranean Diet.

Table S4. Canonical pathways (Ingenuity Pathway Analysis) associated with differentially methylated CpGs selected from MedDiet+nuts vs. low-fat control diet LIMMA analysis.

Ingenuity Canonical Pathways	-log(p-value)	Ratio	Genes
Axonal Guidance Signaling	2,78E00	3,56E-02	PIK3C2B,FYN,PLXNC1,GNAS,SEMA5A,EPHA4,PLCH2,PRKCZ,NFATC1, WNT10A,SRGAP1,PRKAG2,PLXNB2,ABLIM2,LNPEP,SHANK2
Sperm Motility	2,49E00	5,60E-02	NPPB,PDE2A,GNAS,CACNA1H,PRKAG2,PLCH2,PRKCZ
Sumoylation Pathway	2,44E00	6,25E-02	TP53,ETS1,DAXX,AR,CEBPA,MAP3K5
Role of p14/p19ARF in Tumor Suppression	2,38E00	9,30E-02	TP53,RB1,PIK3C2B,TTF1
Myc Mediated Apoptosis Signaling	2,35E00	7,14E-02	TP53,PIK3C2B,IGF1R,PRKCZ,BCL2
Adipogenesis pathway	2,33E00	5,22E-02	TP53,HDAC9,ZNF423,NR1D2,EBF1,HDAC4,CEBPA
SAPK/JNK Signaling	2,27E00	5,77E-02	TP53,PIK3C2B,DAXX,DUSP10,MAP3K5,NFATC1
Molecular Mechanisms of Cancer	2,27E00	3,48E-02	TP53,RB1,FYN,DAXX,SYNGAP1,PIK3C2B,GNAS,WNT10A,PRKAG2, RBPJ,MAP3K5,PRKCZ,BCL2
p53 Signaling	2,13E00	5,41E-02	TP53,HDAC9,RB1,PIK3C2B,COQ8A,BCL2
Telomerase Signaling	2,13E00	5,41E-02	TP53,ETS1,HDAC9,RB1,PIK3C2B,HDAC4
Growth Hormone Signaling	2,09E00	6,17E-02	PIK3C2B,CEBPA,IGF1R,RPS6KA2,PRKCZ
Neuropathic Pain Signaling In Dorsal Horn Neurons	2,08E00	5,26E-02	PIK3C2B,GRM8,GRIA1,PRKAG2,PLCH2,PRKCZ
Unfolded protein response	2,03E00	7,41E-02	PDIA2,CEBPA,MAP3K5,BCL2
Role of NFAT in Cardiac Hypertrophy	2,01E00	4,17E-02	HDAC9,PIK3C2B,HDAC4,GNAS,PRKAG2,IGF1R,PLCH2,PRKCZ
Synaptic Long Term Potentiation	1,98E00	5,00E-02	GRM8,GRIA1,PRKAG2,PPP1R14A,PLCH2,PRKCZ
Induction of Apoptosis by HIV1	1,87E00	6,67E-02	TP53,DAXX,MAP3K5,BCL2
Prostate Cancer Signaling	1,83E00	5,32E-02	TP53,RB1,PIK3C2B,AR,BCL2
Melanocyte Development and Pigmentation Signaling	1,81E00	5,26E-02	PIK3C2B,GNAS,PRKAG2,RPS6KA2,BCL2
Cell Cycle: G1/S Checkpoint Regulation	1,77E00	6,25E-02	TP53,HDAC9,RB1,HDAC4
Protein Kinase A Signaling	1,73E00	3,06E-02	PDE2A,GNAS,PPP1R1B,DUSP10,MYLK2,PTPRS,PRKAG2,PPP1R14A, PLCH2,NFATC1,PRKCZ,DUSP16
Notch Signaling	1,69E00	7,89E-02	NOTCH4,RBPJ,DLL3
Aryl Hydrocarbon Receptor Signaling	1,67E00	4,29E-02	TP53,AHRR,RB1,RARA,POLA1,ALDH3A1
Netrin Signaling	1,66E00	7,69E-02	PRKAG2,ABLIM2,NFATC1
Chronic Myeloid Leukemia Signaling	1,66E00	4,81E-02	TP53,HDAC9,RB1,PIK3C2B,HDAC4
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1,64E00	3,83E-02	COL19A1,COL23A1,COL9A3,IGF1R,COL18A1,COL11A2,BCL2
CREB Signaling in Neurons	1,62E00	3,80E-02	PIK3C2B,GNAS,GRM8,GRIA1,PRKAG2,PLCH2,PRKCZ
Ovarian Cancer Signaling	1,62E00	4,17E-02	TP53,RB1,PIK3C2B,WNT10A,PRKAG2,BCL2
Synaptic Long Term Depression	1,60E00	4,11E-02	GNAS,GRM8,GRIA1,IGF1R,PLCH2,PRKCZ
Glioma Signaling	1,56E00	4,55E-02	TP53,RB1,PIK3C2B,IGF1R,PRKCZ
Mitochondrial L-carnitine Shuttle Pathway	1,56E00	1,18E-01	SLC27A2,CPT1B
Superpathway of Inositol Phosphate Compounds	1,55E00	3,42E-02	PIK3C2B,FYN,PHOSPHO1,PPP1R1B,DUSP10,PPP1R14A,PLCH2,DUSP16
β-alanine Degradation I	1,52E00	5,00E-01	ABAT
Valine Degradation I	1,52E00	1,11E-01	ABAT,HIBADH
Cyclins and Cell Cycle Regulation	1,49E00	5,13E-02	TP53,HDAC9,RB1,HDAC4
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	1,48E00	6,52E-02	TP53,RB1,RARA
DNA damage-induced 14-3-3σ Signaling	1,47E00	1,05E-01	TP53,HUS1
3-phosphoinositide Biosynthesis	1,46E00	3,52E-02	PIK3C2B,FYN,PHOSPHO1,PPP1R1B,DUSP10,PPP1R14A,DUSP16
NGF Signaling	1,46E00	4,27E-02	TP53,PIK3C2B,RPS6KA2,MAP3K5,PRKCZ
p38 MAPK Signaling	1,46E00	4,27E-02	TP53,DAXX,DUSP10,RPS6KA2,MAP3K5
Glioblastoma Multiforme Signaling	1,44E00	3,77E-02	TP53,RB1,PIK3C2B,WNT10A,IGF1R,PLCH2
D-myo-inositol-5-phosphate Metabolism	1,42E00	3,73E-02	PHOSPHO1,PPP1R1B,DUSP10,PPP1R14A,PLCH2,DUSP16
Dopamine-DARPP32 Feedback in cAMP Signaling	1,41E00	3,70E-02	GNAS,PPP1R1B,PRKAG2,PPP1R14A,PLCH2,PRKCZ
Role of Tissue Factor in Cancer	1,40E00	4,10E-02	TP53,PDIA2,PIK3C2B,FYN,RPS6KA2
Small Cell Lung Cancer Signaling	1,39E00	4,76E-02	TP53,RB1,PIK3C2B,BCL2
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	1,38E00	4,71E-02	GNAS,PRKAG2,PLCH2,PRKCZ
Fatty Acid α-oxidation	1,35E00	9,09E-02	ALOX12B,ALDH3A1
Thyroid Hormone Biosynthesis	1,34E00	3,33E-01	TPO
4-aminobutyrate Degradation I	1,34E00	3,33E-01	ABAT
Docosahexaenoic Acid (DHA) Signaling	1,34E00	5,77E-02	PIK3C2B,BIK,BCL2
Type II Diabetes Mellitus Signaling	1,33E00	3,94E-02	PIK3C2B,SLC27A2,PRKAG2,MAP3K5,PRKCZ
HER-2 Signaling in Breast Cancer	1,33E00	4,55E-02	TP53,PIK3C2B,MAP3K5,PRKCZ
Gap Junction Signaling	1,32E00	3,53E-02	PIK3C2B,DBN1,GNAS,PRKAG2,PLCH2,PRKCZ

Ratio represents differentially methylated genes/genes in the pathway. A –log(p-value)>1.301 is considered significant, which corresponds to a p<0.05.
CpG: CG site; LIMMA: Linear Models for Microarray Data; MedDiet: Mediterranean Diet

CHAPTER 4

Insulin sensitivity is associated with lipoprotein lipase (*LPL*) and catenin delta 2 (*CTNND2*) DNA methylation in peripheral white blood cells in non-diabetic young women

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Insulin Sensitivity Is Associated with Lipoprotein Lipase (*LPL*) and Catenin Delta 2 (*CTNND2*) DNA Methylation in Peripheral White Blood Cells in Non-Diabetic Young Women

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Abstract: Hyperglycaemia and type 2 diabetes (T2D) are associated with impaired insulin secretion and/or insulin action. Since few studies have addressed the relation between DNA methylation patterns with elaborated surrogates of insulin secretion/sensitivity based on the intravenous glucose tolerance test (IVGTT), the aim of this study was to evaluate the association between DNA methylation and an insulin sensitivity index based on IVGTT (calculated insulin sensitivity index (CSi)) in peripheral white blood cells from 57 non-diabetic female volunteers. The CSi and acute insulin response (AIR) indexes, as well as the disposition index (DI = CSi × AIR), were estimated from abbreviated IVGTT in 49 apparently healthy Chilean women. Methylation levels were assessed using the Illumina Infinium Human Methylation 450k BeadChip. After a statistical probe filtering, the two top CpGs whose methylation was associated with CSi were cg04615668 and cg07263235, located in the catenin delta 2 (*CTNND2*) and lipoprotein lipase (*LPL*) genes, respectively. Both CpGs conjointly predicted insulin sensitivity status with an area under the curve of 0.90. Additionally, cg04615668 correlated with homeostasis model assessment insulin-sensitivity (HOMA-S) and AIR, whereas cg07263235 was associated with plasma creatinine and DI. These results add further insights into the epigenetic regulation of insulin sensitivity and associated complications, pointing the *CTNND2* and *LPL* genes as potential underlying epigenetic biomarkers for future risk of insulin-related diseases.

Keywords: insulin resistance; diabetes; epigenetics; insulin sensitivity index; EWAS

1. Introduction

Diabetes is defined as "a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both" [1]. Although the relative contribution of

insulin secretion versus insulin action impairments in type 2 diabetes (T2D) depends on many factors, it has been extensively reported that obesity-related insulin resistance plays an important role in the onset and development of T2D [2]. Insulin resistance (or its inverse, insulin sensitivity) shows high inter-individual variability. Therefore, it is important to assess the performance of biomarkers of insulin sensitivity in the absence of hyperglycaemia, before inflammation and other obesity-related impairments of metabolism appear, in order to adequately evaluate the initial stages and directionality of the relation between the proposed biomarkers with insulin sensitivity. Epidemiologic studies have focused on simple measurements of insulin sensitivity based on glucose and insulin fasting plasma samples, such as the homeostasis model assessment insulin-sensitivity (HOMA-S) index, which is the inverse of the commonly used HOMA-insulin resistance (HOMA-IR) index [3]. Given that the main contributor of circulating glucose in fasting conditions is the liver, it is generally accepted that the HOMA-S index predominantly represents a measure of hepatic insulin sensitivity [4]. In contrast, other insulin sensitivity measurements, such as the M-value of the hyperinsulinemic-euglycaemic clamp, are obtained under conditions where a constant high level of circulating insulin is maintained and then, endogenous hepatic glucose production is inhibited [5]. Thus, the M-value can be considered mainly a measure of systemic and/or muscle/adipose insulin sensitivity. Alternatively, other general measures of insulin sensitivity have been derived from the oral glucose tolerance test (OGTT), that allows the calculation of the Matsuda-ISICOMP index and other insulin-related indexes, or from the intravenous glucose tolerance test (IVGTT) [6]. The IVGTT is a procedure that has the interesting operational advantage of allowing the simultaneous measurement of insulin secretion and insulin sensitivity in the same test [7,8]. The specific use of an abbreviated version of the IVGTT (1 hour test, instead of the extended 3 hour IVGTT test) provides gold-standard measurements of acute insulin release (AIR), using the area under the curve (AUC) of plasma insulin during the first 10 min of the IVGTT, and adequate estimations of insulin sensitivity through the calculated insulin sensitivity (CSi), using the plasma insulin and glucose measurements during the second part, 10 to 50 min of the abbreviated IVGTT [9]. Interestingly, a hyperbolic relation has been described for insulin secretion and sensitivity indexes derived from IVGTT in such a way that it is possible to calculate the disposition index (DI) as the product between insulin secretion and insulin sensitivity (DI = AIR × CSi) [7,10]. DI is considered a measure of insulin secretion adjusted by systemic insulin sensitivity representing a marker of glucose homeostasis dysregulation [11]. Additionally, it has been reported that both DI and the oral disposition index (ODI) based on OGTT are relevant predictors of future T2D development [12].

In the prediabetes status, the increased plasma glucose levels and the hyperinsulinemia are triggered by a failure in normal glucose homeostasis that have been related, among many other factors, with transcriptional variations in key metabolic organs that may be explained by epigenetic regulation [13]. Indeed, epigenome-wide association studies (EWASs) have revealed an influence of DNA methylation in genes related to T2D and glucose homeostasis [14–20]. These changes directly influence both insulin-producing pancreatic β-cells, as well as other organs involved in glucose homeostasis. Changes in methylation patterns related to T2D development are also accompanied by variations of methylation patterns in blood cells [21,22]. There are no studies in the literature analysing the relations between leukocyte DNA methylation across the genome and insulin sensitivity measured by IVGTT or studies specifically focused on the DI.

Since diabetes is not usually diagnosed until several years after the appearance of insulin and glucose deregulation, it is crucial to detect the early stages of the disease through the use of adequate biomarkers of reduced insulin sensitivity [23]. In this context, studies conducted in non-diabetic subjects are useful in evaluating novel biomarkers to identify the susceptibility to develop T2D through the evaluation of intermediate phenotypes such as the insulin sensitivity. Therefore, the aim of this study was to assess the association between DNA methylation patterns in peripheral white blood cells (PWBCs) with measures of insulin sensitivity based on the intravenous glucose tolerance tests in non-diabetic women.

2. Results

2.1. Anthropometric and Biochemical Characteristics of the Participants

Summary statistics for anthropometric and biochemical variables, as well as insulin sensitivity measurements, are reported in Table 1.

Table 1. Anthropometric and biochemical measurements, and insulin sensitivity indexes of $n = 57$ participants of this study.

Variable	N	Median (IQR)
Age (y)	57	25 (22–30)
Weight (kg)	57	59.5 (56.0–64.2)
Height (m)	57	1.59 (1.56–1.63)
Body mass index (kg/m^2)	57	23.4 (21.8–25.4)
Plasma total cholesterol (mg/dL)	56	167.5 (148.0–196.5)
Plasma HDL cholesterol (mg/dL)	56	63.0 (51.5–71.5)
Plasma LDL cholesterol (mg/dL)	56	84.0 (67.5–103.5)
Plasma triglycerides (mg/dL)	56	92.5 (68.0–131.0)
Systolic blood pressure (mmHg)	57	113 (104–119)
Diastolic blood pressure (mmHg)	57	70 (65–75)
Fasting glucose (mg/dL)	49	78 (74–82)
Fasting insulin (IU/µmL)	49	7.0 (5.8–9.1)
HOMA-S	48	71.5 (55.5–88.5)
Calculated insulin sensitivity (CSi)	49	5.7 (4.0–8.2)
Acute insulin release (AIR)	49	538.7 (398.5–718.6)
IVGTT-based disposition index (DI)	49	2792.6 (2023.4–4136.4)

HDL: High density lipoprotein; HOMA-S: Homeostasis model assessment-insulin sensitivity; IQR: Interquartile range; IVGTT: Intravenous glucose tolerance test; LDL: Low density lipoprotein.

2.2. CpG Sites Selection and Ingenuity Pathway Analysis

In order to identify the CpG sites with the highest methylation variability that may have a biological implication, an initial selection by the slope between methylation and CSi was performed to discard multiple CpG sites showing lack of intrinsic variation. Then, 1416 CpGs with a slope >|0.005| were further analysed (Figure 1, Table S1) because of their correlation with CSi. The raw p-values from non-parametric correlational analysis were subsequently adjusted by the Benjamini–Hochberg method, resulting in 253 CpG sites significantly associated with CSi (false discovery rate (FDR) < 0.05) (Table S2). These 253 CpGs were analysed for canonical pathways from Ingenuity Pathway Analysis (IPA) (Table S3). Some of the obtained canonical pathways were related to insulin and glucose (Figure 2), such as opioid signalling pathway, G-protein coupled receptor, glycine betaine degradation, nitric oxide signalling in the cardiovascular system, gustation pathway or type 2 diabetes mellitus.

The 10 top most significant CpGs of the 253 CpGs selected by FDR < 0.05 were cg04615668-*CTNND2* (corresponding gene according to the Illumina CG Database), cg07263235-*LPL*, cg09620718-*ACSM1*, cg23760585-*FLJ22536*, cg23874746-*PDE1A*, cg27385193-NA, cg10687107-NA, cg17270100-NA, cg07737566-*GRB10*, and cg05992904-*FAM19A5* (Figure 3, Figure S1). Further analyses where performed with the two most significant CpGs, cg04615668 and cg07263235, which are located in the genes catenin delta 2 (*CTNND2*) and lipoprotein lipase (*LPL*), respectively. Correlations between DNA methylation and CSi for both CpGs are plotted (Figure 4A).

Int. J. Mol. Sci. **2019**, *20*, 2928

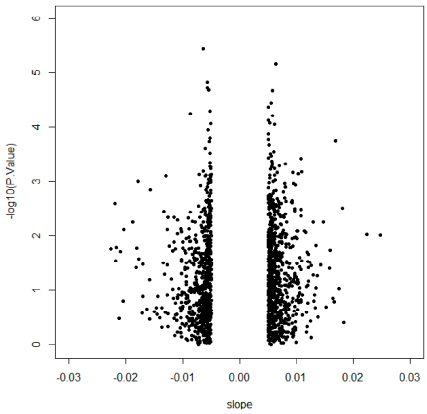

Figure 1. Volcano plot representing 1416 CpGs selected by slope >|0.005| according to the logarithm of the *p*-value obtained from Spearman correlation with calculated insulin sensitivity index (CSi).

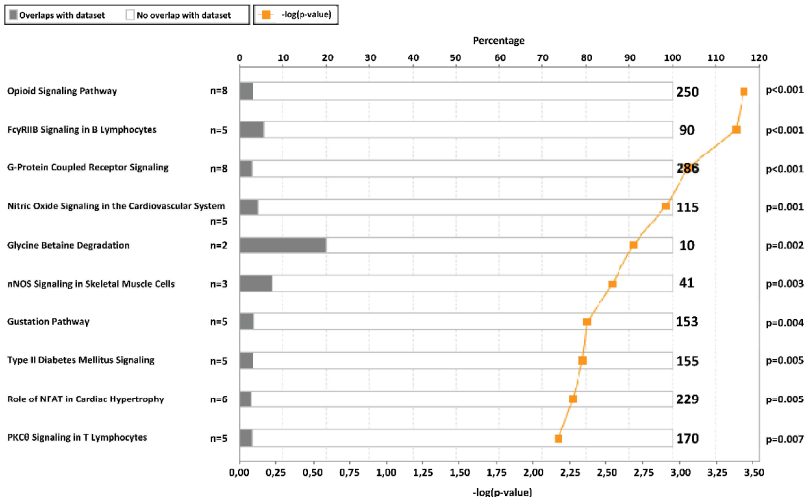

Figure 2. Top 10 canonical pathways from ingenuity pathway analysis of 253 CpGs selected by Spearman false discovery rate (FDR) < 0.05. The graph presents the canonical pathways ordered by −log(*p*-value) and the percentage of genes from our list that are in one specific pathway (total number of genes in the pathway at the right part of the graph). The *p*-value adjusted by Fisher's test is also indicated at the right side.

Int. J. Mol. Sci. 2019, 20, 2928

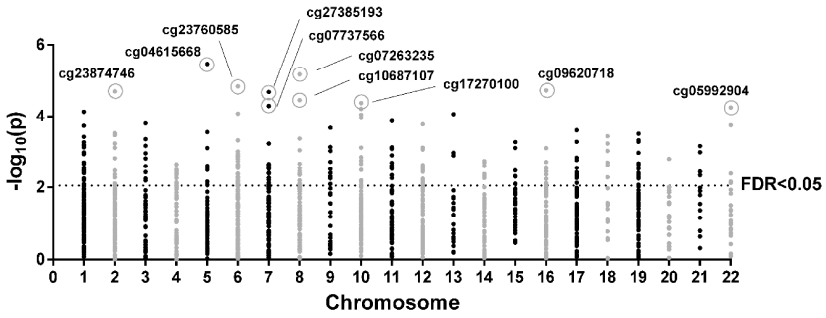

Figure 3. Manhattan plot of 1416 CpGs selected by slope >|0.005| in each chromosome. Points above the horizontal line are false discovery rate (FDR) < 0.05. The top 10 CpGs are indicated.

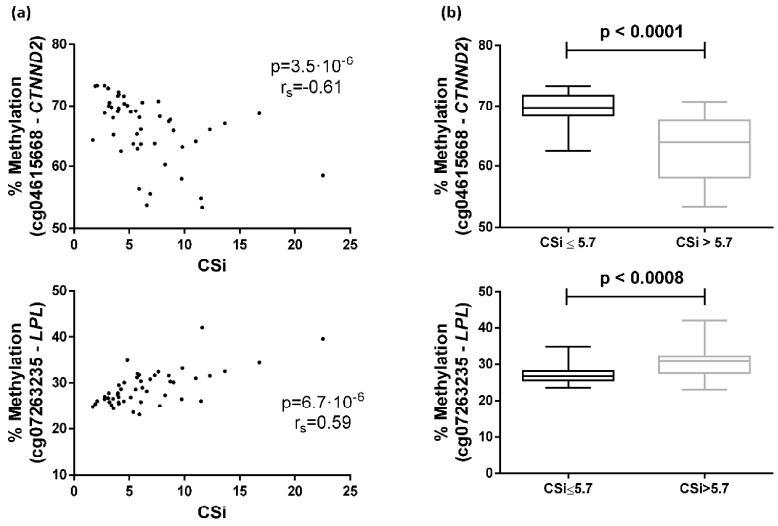

Figure 4. Associations between the calculated insulin sensitivity index (CSi) and DNA methylation in peripheral white blood cells. (**a**) Spearman correlation between CSi and cg04615668-*CTNND2* or cg07263235-*LPL* methylation; (**b**) Differential methylation of cg04615668-*CTNND2* or cg07263235-*LPL* between individuals separated by the median of CSi.

2.3. Differences between Groups Separated by the Median of CSi Values

Participants of the GEDYMET (genetics, dysglycemia and metabolism) study were also separated by the median CSi values (cut-off value = 5.7) to categorise the subjects into insulin-sensitive (higher CSi values, n = 24) and insulin-resistant (lower CSi values, n = 25) groups. The group with higher CSi showed a methylation mean and SD of 63.2(5.4) for cg04615668 and 30.7(4.2) for cg07263235, whereas the group with lower CSi presented a methylation mean and SD of 69.4(3.0) and 27.1(2.4) for cg04615668 and cg07263235, respectively. Significant differences in methylation percentage of cg04615668 and cg07263235 were found when comparing both groups (Figure 4B).

Additionally, logistic regressions and receiver operating characteristic (ROC) curves, both adjusted by age, were carried out to determine whether both CpG site methylation levels were able to predict

Int. J. Mol. Sci. 2019, 20, 2928

the CSi group. Logistic regressions showed an odds ratio (OR) = 0.67 for cg04615668 (pseudo R^2 = 0.34, $p < 0.0001$) and OR = 1.43 for cg07263235 (pseudo R^2 = 0.21, $p = 0.0009$). The AUCs were estimated as 0.86 (95% confidence interval 0.75–0.96) for cg04615668 and 0.81 (95% confidence interval 0.68–0.94) for cg07263235. Interestingly, multiple logistic regression including both CpGs adjusted by age significantly improved the model (OR cg04615668 = 0.68, OR cg07263235 = 1.36, pseudo R^2 = 0.44, $p < 0.0001$), reaching an AUC of 0.90 for predicting CSi (Figure 5).

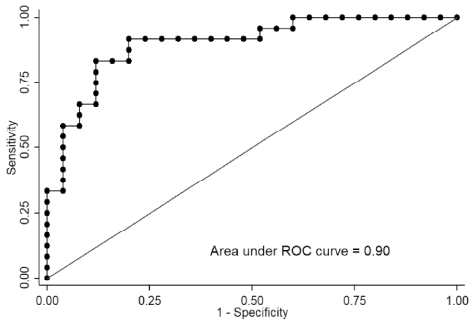

Figure 5. Receiver operating characteristic (ROC) curve of the logistic regression of cg04615668-*CTNND2* and cg07263235-*LPL* adjusted by sex allows the discrimination of subjects with the calculated insulin sensitivity index (CSi) ≤5.7 (insulin-resistant) versus >5.7 (insulin-sensitive).

2.4. Correlation with Other Variables

Furthermore, methylation values at the cg04615668 site significantly correlated with AIR ($p = 0.0098$) and HOMA-S ($p = 0.0483$) (Figure 6A), while methylation values at the cg07263235 site were significantly associated with plasma creatinine ($p = 0.0314$) and DI ($p = 0.0120$) (Figure 6B).

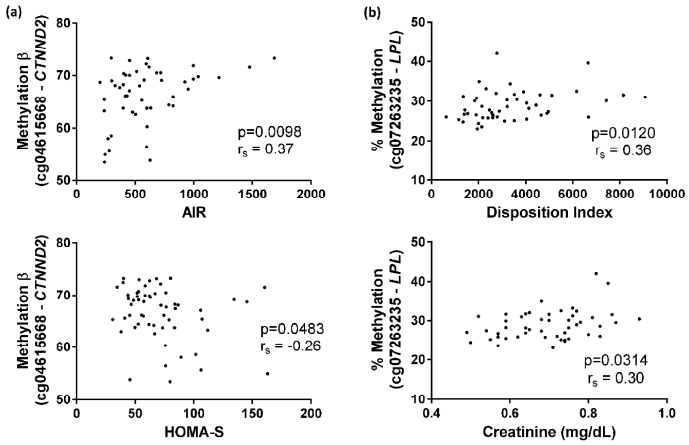

Figure 6. Spearman correlations of cg04615668 and cg07263235 methylation. (**a**) Correlation between cg04615668-*CTNND2* methylation and acute insulin response (AIR) index or homeostasis model assessment insulin-sensitivity (HOMA-S) index; (**b**) Correlation between cg07263235-*LPL* methylation and disposition index (DI) or plasma creatinine.

3. Discussion

The CpG sites cg04615668 and cg07263235, located in the *CTNND2* and *LPL* genes, respectively, achieved the most significant signals of association between DNA methylation levels in PWBCs and IVGTT-based insulin sensitivity measurements (CSi). Methylation of these specific CpGs was clearly different in the two groups separated by the median CSi of the whole group. Furthermore, both CpGs together were able to predict CSi using ROC curve analysis. The CpG cg04615668 was also associated with the AIR and HOMA-S indexes, whereas cg07263235 was correlated with the DI (defined as the product between AIR × CSi) and plasma creatinine levels. To our knowledge, this study is the first to relate CSi with DNA methylation, adding further insights into the epigenetic regulation of systemic insulin sensitivity and related traits.

There is a need to develop biomarkers to detect early steps in the pathophysiologic progression of T2D, as well as to elucidate underlying mechanisms of the disease [24]. Genetics, epigenetics, as well as non-genetic factors (diet, lifestyle) are involved in the pathogenesis of dysglycaemia and T2D [25]. On the other hand, deregulations in insulin sensitivity and secretion might be associated with epigenetic modifications [13]. Previous EWAS showed an association between DNA methylation patterns in PWBCs and T2D and glucose homeostasis traits [14–20]. Additionally, different studies have proposed potential DNA methylation biomarkers in relation to plasma insulin levels, insulin secretion and insulin resistance such as those located in *PPARGC1A*, *HTR2A*, *LY86*, *TFAM*, *GIPR*, *ADIPOQ*, and *IGFBP3* genes [21]. Our study has found a relation between the insulin sensitivity index CSi, based on IVGTT [9], and methylation of CpGs in several genes. According to IPA, some of these genes were related to insulin-related pathways and T2D signalling, such as type 2 diabetes mellitus signalling. In the case of the opiod signalling pathway, opioid μ-receptors may be activated by β-endorphin to improve insulin resistance [26] and opiates can inhibit insulin signalling through direct crosstalk between the downstream signalling pathways of the opioid receptor and the insulin receptor [27]. As for the G-protein coupled receptor signalling, insulin and glucagon secretion is affected by factors binding to G-protein coupled receptors on the surface of β- and α-cells [28]. Regarding the glycine betaine degradation pathway, glycine betaine improves glucose tolerance and has been associated with reduced incidence of diabetes [29]. Furthermore, the pathway nitric oxide signalling in the cardiovascular system involves nitric oxide, which represents a central regulator of energy metabolism and body composition [30], and it is also a component of the insulin-signalling cascade [31]. The gustation pathway may also be related since inhibition of sweet chemosensory receptors alters insulin responses during glucose ingestion [32]. Specifically, statistically significant CpGs (FDR < 0.05) from our study that were previously related to insulin were located in the genes *LPL* [33], *GRB10* [34], *WISP1* [35], *PRDM16* [36], *TMEM132C* [37], *ADAMTS9* [38], and *NOX4* [39].

The CpG cg04615668 is located in the gene *CTNND2* (according to Illumina CG database), which encodes an adhesive junction associated protein called catenin delta 2, δ-catenin, NPRAP or neurojungin. This protein functions in Wnt signalling to regulate gene expression [40] and has been reported to be involved in the pathogenesis of cancer, cortical cataract-linked Alzheimer's disease, autism, schizophrenia, mental retardation, myopia, and infectious diseases [40]. For example, *CTNND2* plays a critical role in neuronal development since it has been observed that it is likely rate-limiting for dendritic morphogenesis and maintenance, and its haploinsufficiency is common in autism [41]. However, little is known about the implication of *CTNND2* in metabolic diseases. In this context, a polymorphism located at this gene (rs6873671) has been significantly associated with human type 2 diabetes in two independent genome-wide studies [42,43], suggesting that *CTNND2* is involved in the regulation of glucose metabolism. Another polymorphism (rs10513097) has appeared in a genome-wide association study (GWAS) related to body mass index [44]. For this reason, it is necessary to highlight the importance of the present study, because it is the first time that methylation of this gene (in this case, DNA methylation in one CpG) has been linked with impairments in insulin sensitivity and glucose metabolism.

According to the current investigation, the association between cg04615668 methylation and CSi is negative, suggesting that hypomethylation of this site in PWBCs is related to higher insulin sensitivity. Moreover, methylation level in this CpG site is also correlated with two other insulin-related parameters such as the AIR index and HOMA-S, reflecting its involvement in insulin and glucose pathways.

On the other hand, the enzyme encoded by the *LPL* gene hydrolyses triglycerides in circulating chylomicrons, low density lipoproteins and very low density lipoproteins to render free unesterified fatty acids to the circulation [45]. *LPL* is synthesized in parenchymal cells such as skeletal muscle cells, adipocytes, macrophages and mammary gland cells, among other tissues and cell types [46]. After maturation in the rough endoplasmic reticulum (mainly driven by the lipase maturation factor-1 or LMF1), *LPL* is secreted and binds to heparan sulphate proteoglycans which are crucial in the translocation of the enzyme from its site of synthesis to the endothelium, also acting as cofactors in enzymatic reactions [47]. *LPL* activity is additionally regulated by apolipoproteins, angiopoietins, miRNAs and hormones. Insulin is considered a major regulator of adipose tissue *LPL*, through its effect on *LPL* transcription during adipocyte differentiation and through increasing *LPL* mRNA levels [48]. Initially, a tissue-specific regulation of *LPL* action by insulin was reported in such a way that *LPL* activity in adipose tissue was stimulated by acute infusions of insulin (leading to free fatty acids for storage) while *LPL* activity in skeletal muscle was decreased by this hormone [49]. However, nutritional studies involving 2 weeks of a high-carbohydrate diet or high-fat diet in human volunteers seemed to increase the *LPL* response to carbohydrate feeding in both adipose tissue and skeletal muscle [50]. Moreover, it is also important to remark that mice with muscle-specific *LPL* overexpression generated a muscle-selective insulin resistance [51]. In contrast, the disruption of *LPL* in skeletal muscle results in reductions in lipid storage and increased myocyte insulin signalling, together with marked insulin resistance in other tissues, leading finally to obesity and systemic insulin resistance. In support of a mediation role of *LPL* in systemic insulin sensitivity, Goodarzi et al. (2004) [52] and Goodarzi et al. (2007) [33] found that common *LPL* gene variation was involved in insulin resistance measured through hyperinsulinemic-euglycaemic clamps and intravenous glucose tolerance tests in Mexican Americans.

According to current research, the association between cg07263235 methylation and CSi is positive. Therefore, hypermethylation of this site in PWBCs might display higher insulin sensitivity. Methylation level in this CpG site is also positively correlated with the DI (CSi × AIR), showing that the hypomethylation in this site may indicate an impaired relative insulin secretion. Houde et al. described that *LPL* methylation in one specific CpG was lower in placentae of women with gestational diabetes mellitus [53]. However, there are other studies showing that an increase in *LPL* methylation was detrimental. Indeed, Castellano-Castillo et al. have described higher levels of *LPL* methylation in adipose tissue from patients with metabolic syndrome [54] and Drogan et al. showed an association between *LPL* methylation in adipose tissue and regional body fat distribution [55]. The disparity in the results from studies in the scientific literature is difficult to interpret given the multiple differential patterns of methylation in CpG sites of different cells and tissues.

Since the cg07263235 is located at the *LPL* promoter, a complementary analysis of putative transcription factors that bind on this CpG was performed using the software TRANSFAC (v2019.1) (GeneXplain, Wolfenbüttel, Germany). This software showed that cyclic AMP-responsive element-binding protein 1 (CREB1) may act in the regulation of *LPL* expression. Other investigators have demonstrated that glucose-dependent insulinotropic polypeptide (GIP), in the presence of insulin, upregulates adipocyte *LPL* gene transcription through CREB/cAMP-responsive CREB coactivator 2 (TORC2) activation [56]. Thus, we again speculate that the regulation of *LPL* by CREB might be mediated by cg07263235 methylation. Furthermore, it is worth noting that cg07263235 methylation also correlated with circulating creatinine in our study in a positive manner. Serum creatinine is a surrogate marker for muscle mass in healthy subjects [57]. Since skeletal muscle mass is inversely associated with T2D [58], low creatinine would represent a proxy of low muscle mass and possibly be linked to a higher risk of developing T2D [59].

Remarkably, both CpGs together allowed the distinction of individuals with low and high CSi with an AUC of 0.90. Since these CpGs are associated with different insulin-related parameters, it seems that although they are related to different glucose-related metabolic mechanisms, they complement each other to differentiate CSi groups. Therefore, we speculate on the hypothesis that the methylation at specific sites of the insulin-sensitive genes *CTNND2* and *LPL* may act as biomarkers of whole body insulin resistance, given a possible effect of DNA methylation on gene expression, with subsequent consequences in insulin resistance-related diseases. It must be noted that our population is very specific (healthy non-diabetic young women) and that the CSi is not usually measured in other investigations. Hence, these CpGs are more likely to be biomarkers of early diagnosis of possible insulin-related problems in a healthy population and not in diabetic or metabolic-impaired subjects. Indeed, after an exhaustive search of methylation databases and in a subpopulation of the Methyl Epigenome Network Association (MENA) study (n = 417, females = 59%, T2D = 59, non-T2D = 358), we have not been able to validate our CpGs in insulin-resistant individuals or with T2D (data not shown).

Our study presents several methodological limitations. The sample size is relatively small, which is partially a consequence of the complex IVGTT procedure. As it happens in association studies involving massive measurements, type I and type II errors cannot be discarded, although data preprocessing and strict CpG selections have been carried out to avoid them. Although methylation is tissue-specific, and methylation patterns and the study of insulin-sensitive organs, such as muscle or adipose tissue, are more appropriate to find epigenetic biomarkers for insulin sensitivity, the measure of DNA methylation biomarkers in white blood cells has the advantage of accessibility to the biological sample. Other studies have demonstrated that blood cells can act as proxies for these tissues [21,60,61]. Gene expression analysis would have been helpful to reveal the relationship between methylation and gene function in this particular study, but unfortunately, there was no biological sample available for this purpose. However, the association between the level of methylation in the CpG site cg04615668 and *CTNND2* gene expression is generally described as direct, whereas for cg07263235 methylation and *LPL* gene expression the relation was generally inverse, when assessed in the MEXPRESS online utility (https://mexpress.be/) based on multiple tissue gene expressions in several cancer types. Finally, causality cannot be established due to the transversal nature of the study. DNA methylation can either be a consequence, a cause, or a proxy of insulin action impairment.

In conclusion, this study reports for the first time an association between DNA methylation patterns with the insulin sensitivity index CSi measured through an intravenous glucose challenge. The most significant signals of association correspond to two CpGs located in the *CTNND2* (cg04615668) and *LPL* (cg07263235) genes. These findings may contribute to identifying potential biomarkers and new regulatory mechanisms in insulin-related diseases.

4. Materials and Methods

A cross-sectional study was carried out on 57 non-diabetic nulliparous, non-pregnant women volunteers without parental family history of diabetes. They were recruited for a metabolic study to assess future gestational diabetes (Table 1) (The GEDYMET Chilean study) [11]. Exclusion criteria were previous or in situ diagnosis of diabetes, family history of diabetes, dyslipidaemia, anaemia or pregnancy. The volunteers visited the Centre of Clinical Research (School of Medicine, Pontificia Universidad Católica de Chile) to carry out an abbreviated version of minimal-model IVGTT after the administration of 0.3 grams of glucose per kg of body weight, as a 50% water solution infused for 60 s [9]. As part of the abbreviated IVGTT protocol, plasma glucose and insulin levels were measured at −15, −5, 2, 3, 4, 5, 6, 8 and 10 min to calculate the AIR index as the area under the curve of plasma insulin [62]. After AIR, additional plasma glucose and insulin levels were measured at 10, 15, 20, 30, 40 and 50 min to complete IVGTT and to estimate the CSi using the website http://webmet.pd.cnr.it/csi/ [9]. CSi is considered a surrogate of insulin sensitivity showing strong association with the hyperinsulinemic-euglycaemic clamp. The IVGTT-based DI, represents a measure of insulin secretion adjusted by systemic insulin sensitivity and was calculated as the product of AIR × CSi [63]. Plasma glucose and insulin levels

measured at −15 and −5 min before IVGTT were used to calculate the HOMA-S index, which is the inverse of the HOMA-IR index (HOMA-S = 1/HOMA-IR = 1/(fasting insulin (μUI/mL) × fasting glucose (mg/dL)/405)). This research was approved by the Ethics Committee of the School of Medicine, Pontificia Universidad Católica de Chile (Santiago, Chile) in compliance with the Helsinki Declaration of ethical principles for medical research involving human subjects (code 14-281, date 4th June 2015). All participants provided written informed consent.

4.1. Anthropometry, Blood Pressure and Biochemical Determinations

Anthropometric measurements were carried out by trained personnel in light clothing and without shoes, using a calibrated set of stadiometers, scales and tapes. Weight (kg) and height (m) were used to calculate BMI (kg/m^2). Systolic and diastolic blood pressure (mmHg) were measured with digital sphygmomanometer as an average of three measurements. Venous blood samples were drawn by venipuncture in EDTA tubes. Plasma was separated from whole blood by centrifugation at 3500 rpm at 5 °C for 15 min, and frozen immediately at −80 °C until assay. Plasma levels of insulin (μU/mL) and glucose (mg/dL) were measured in the central laboratory of the Pontificia Universidad Católica de Chile by standard electro-chemiluminescence and colorimetric methods (http://redsalud.uc.cl/ucchristus/laboratorio-clinico/).

4.2. DNA Methylation Analysis

Genomic DNA was extracted from PWBCs using the MasterPureTM DNA purification kit (Epicenter, Madison, WI, USA) and quantified with the Pico Green dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). In order to convert cytosine into uracil, high-quality DNA samples (500 ng) were treated with sodium bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol. Illumina Infinium Human Methylation 450k BeadChip (Illumina, San Diego, CA, USA) was employed to measure DNA methylation levels of CpG sites across the human genome. This analysis was conducted in the Unidad de Genotipado y Diagnóstico Genético from the Fundación de Investigación Clínico de Valencia, as detailed elsewhere [64].

4.3. Treatment of Methylation Raw Data

Signal measurement intensities were scanned in the 450k array using the Illumina iScanSQ platform. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina). Methylation raw data are available in NCBI's gene expression omnibus [65] as part of the MENA study through GEO series accession number GSE115278 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115278).

β-Values were computed using the formula β-Value = M/(U + M) where M and U are the raw "methylated" and "unmethylated" signals, respectively. β-Values were corrected for type I and type II bias using the peak-based correction. Data were normalized in R using a categorical subset quantile normalization method (SQN) and probes associated with X and Y chromosomes were filtered out using the pipeline developed by Touleimat and Tost [66]. Probes with single nucleotide polymorphisms (SNPs) were also filtered out. Differences in methylation resulting from differences in cellular heterogeneity were corrected using estimateCellCounts function from minfi package for R statistical software [67], based on the Houseman method [68].

4.4. Statistical Analysis

After pre-processing, in order to select CpGs with a higher effect that may present biological noticeable implications, 1416 CpGs were selected with a slope >|0.005| calculated from the relationship between methylation and CSi. The methylation of the 1416 CpGs was correlated with CSi using Spearman's correlation coefficients. *p*-values were adjusted for multiple testing through the Benjamini–Hochberg method. Afterwards, the top 10 significant CpGs were selected, and the

first two (cg04615668 and cg07263235) were further analysed. The Mann–Whitney U test was employed for evaluating the differences between two groups of individuals generated using the median of CSi. Logistic regressions and ROC curve AUCs, both adjusted by age, were calculated to determine if the CpGs were able to predict the median group of each individual. Correlations and the volcano plot were performed using the R statistical software [67]. Other statistical calculations, as well as the ROC curve, were performed with STATA version 12.0 (Stata Corp, College Station, TX, USA). The Manhattan plot, correlation graphs and box plots were generated using GraphPad Prism 6 (Graph-Pad Software, San Diego, CA, USA).

4.5. Ingenuity Pathway Analysis

After the selection of 1416 CpGs (see above), an adequate number of CpGs were selected by having Spearman correlations' FDR < 0.05 (253 CpGs) and then, analysed using IPA software (Qiagen, Redwood City, CA, USA, www.ingenuity.com). Associated pathways and gene regulatory networks were identified by predefined pathways and functional categories of the ingenuity knowledge base [69]. Canonical pathway analyses were performed with IPA's core analysis module and selected if $p < 0.05$ after Fisher's test for multiple comparison was statistically significant.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/12/2928/s1.

Author Contributions: Conceptualization, A.A, J.L.S., F.I.M., J.-I.R.-B. and J.A.M.; formal analysis, A.A.; funding acquisition, J.L.S., F.I.M. and J.A.M.; Investigation, L.R.C. and C.B.; Methodology, A.A., J.L.S. and J.-I.R.-B.; project administration, J.L.S. and J.A.M.; resources, J.L.S., L.R.C. and C.B.; supervision, J.-I.R.-B. and J.A.M.; writing—original draft, A.A. and J.L.S.; writing—review & editing, F.I.M., J.-I.R.-B. and J.A.M.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AIR	Acute insulin response
AUC	Area under the curve
CREB1	Cyclic AMP-responsive element-binding protein 1
CSi	Calculated insulin sensitivity index
DI	Disposition index
EWAS	Epigenome-wide association study
FDR	False discovery rate
GEDYMET	Genetics, dysglycemia and metabolism
GIP	Glucose-dependent insulinotropic polypeptide
GWAS	Genome-wide association study
HOMA-IR	Homeostasis model assessment - insulin resistance index
HOMA-S	HOMA-insulin sensitivity index
IPA	Ingenuity pathway analysis
IVGTT	Intravenous glucose tolerance test
LPL	Lipoprotein lipase
MENA	Methyl Epigenome Network Association
PWBCs	Peripheral white blood cells
ROC	Receiver operating characteristic
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
TORC2	CREB/cAMP-responsive CREB coactivator 2

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Supplementary material

Insulin sensitivity is associated with lipoprotein lipase (*LPL*) and catenin delta 2 (*CTNND2*) DNA methylation in peripheral white blood cells in non-diabetic young women

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Table S1: Selection of 1416 CpGs by slope > |0.005|.

CG_ID	UCSC_RefGene_Name	UCSC_RefGene_Accession	UCSC_RefGene_Group
cg00009523	SNTG2	NM_018968	Body
cg00019495	HOPX;HOPX;HOPX;HOPX;HOPX;HOPX	NM_139212;NM_139212;NM_032495;NM_001145460;NM_032495;NM_001145460	5UTR;1stExon;1stExon;5UTR;5UTR;1stExon
cg00028022	NA	NA	NA
cg00041311	SIAH1	NM_003031	5UTR
cg00044796	NA	NA	NA
cg00063006	PPP1R2P1	NR_027771	Body
cg00079898	OR52N5	NM_001001922	TSS1500
cg00098175	SLC12A7	NM_006598	Body
cg00101154	MRPL28	NM_006428	Body
cg00123214	RWDD3;RWDD3	NM_001128142;NM_015485	Body;Body
cg00124993	MIR886	NR_030583	TSS200
cg00129273	NA	NA	NA
cg00143986	GPR133	NM_198827	Body
cg00146004	NA	NA	NA
cg00234027	NA	NA	NA
cg00245057	NA	NA	NA
cg00255732	ANO7	NM_001001891	Body
cg00256329	NXN	NM_022463	Body
cg00260937	KCP	NM_001135914	Body
cg00290086	DMRT2;DMRT2;DMRT2	NM_001130865;NM_006557;NM_181872	5UTR;5UTR;5UTR
cg00313914	NAV1	NM_020443	Body
cg00321709	CYP2E1	NM_000773	Body
cg00322927	OPCML;OPCML	NM_002545;NM_001012393	Body;Body
cg00329052	NA	NA	NA
cg00340349	CDK9	NM_001261	Body
cg00347798	NA	NA	NA
cg00366190	WDR88	NM_173479	TSS200
cg00373114	POTEA;POTEA	NM_001002920;NM_001005365	TSS1500;TSS1500
cg00377727	SEC23A	NM_006364	TSS1500

(The rest of the table is available at: https://www.mdpi.com/1422-0067/20/12/2928#supplementary)

Table S2: CpG information and correlation adjusted p-values (FDR) of the 253 CpGs that were significantly associated with CSi (FDR<0.05).

CG_ID	UCSC_RefGene_ Name	UCSC_RefGene_ Accession	UCSC_RefGene_ Group	slope	FDR spearman indice_ivgtt_CSI_ mgdL_ulUmL
cg04615668	CTNND2	NM_001332	Body	-0.00639884	0.00474066
cg07263235	LPL	NM_000237	TSS200	0.00638642	0.00474066
cg09620718	ACSM1	NM_052956	TSS1500	-0.00564231	0.00489905
cg23760585	FLJ22536	NR_015410	Body	-0.00567925	0.00489905
cg23874746	PDE1A;PDE1A	NM_005019;NM_001003683	1stExon;Body	-0.00543908	0.00489905
cg27385193	NA	NA	NA	0.00579727	0.00489905
cg10687107	NA	NA	NA	0.00549235	0.00709231
cg17270100	NA	NA	NA	0.00501286	0.00739364
cg05992904	FAM19A5	NM_001082967	Body	-0.00864043	0.00787237
cg07737566	GRB10	NM_001001555	5UTR	-0.00519965	0.00787237
cg14251734	HPSE2;HPSE2;HPSE2;HPSE2	NM_001166244; NM_001166245; NM_021828;NM_001166246	Body;Body;Body;Body	0.00576695	0.00787237
cg03459415	NA	NA	NA	0.00504936	0.00836765
cg05202389	TPP2	NM_003291	Body	-0.00509172	0.00836765
cg13906701	UNC93A;UNC93A	NM_018974;NM_001143947	Body;Body	0.00533558	0.00836765
cg20831708	SEC31B	NM_015490	5UTR	0.00606676	0.00836765
cg19584136	MXI1;MXI1	NM_130439;NM_001008541	Body;5UTR	-0.00561186	0.00969053
cg24011351	SLC22A8	NM_004254	5UTR	0.0050268	0.01098497
cg07034145	NA	NA	NA	-0.00524399	0.01226174
cg03212480	TMCC2	NM_014858	Body	-0.00532639	0.01228382
cg06613286	TMEM132C	NM_001136103	Body	0.00506578	0.01228382
cg13306870	CBX7	NM_175709	3UTR	0.01689719	0.01228382
cg13728834	TMEM8B;TMEM8B;TMEM8B	NM_001042590; NM_016446;NM_001042589	Body;Body;Body	0.0051094	0.01332671
cg18341478	CCDC40	NM_017950	Body	-0.00604918	0.0149661
cg02359712	NA	NA	NA	0.00604797	0.01641086
cg03162314	CTU1	NM_145232	3UTR	0.00545161	0.01646512

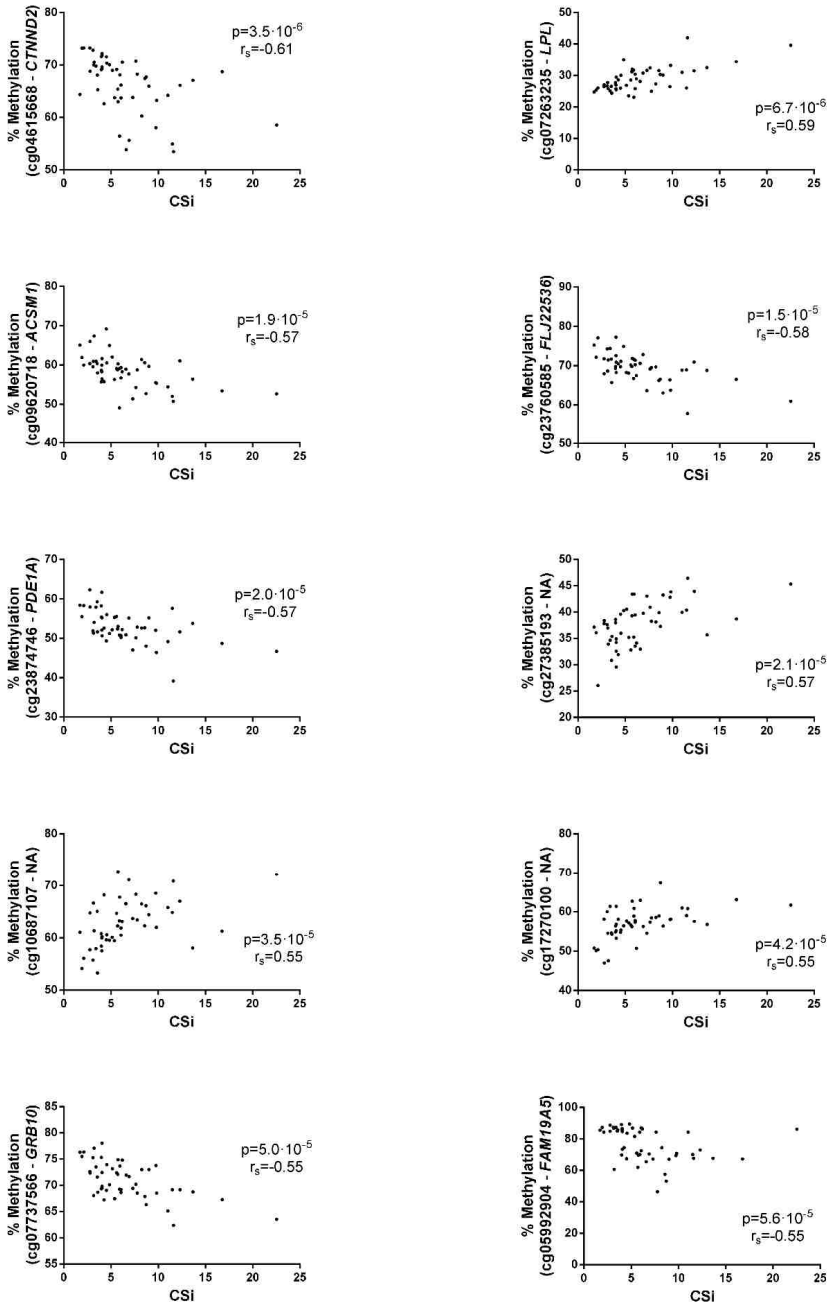
(The rest of the table is available at: https://www.mdpi.com/1422-0067/20/12/2928#supplementary)

Table S3: Significant Ingenuity Canonical Pathways obtained from the 253 CpGs selected by Spearman FDR <0.05.

Ingenuity Canonical Pathways	-log(p-value)
Opioid Signaling Pathway	3.44E+00
FcγRIIB Signaling in B Lymphocytes	3.39E+00
G-Protein Coupled Receptor Signaling	3.06E+00
Nitric Oxide Signaling in the Cardiovascular System	2.91E+00
Glycine Betaine Degradation	2.68E+00
nNOS Signaling in Skeletal Muscle Cells	2.54E+00
Gustation Pathway	2.37E+00
Type II Diabetes Mellitus Signaling	2.34E+00
Role of NFAT in Cardiac Hypertrophy	2.28E+00
PKCθ Signaling in T Lymphocytes	2.18E+00
Thio-molybdenum Cofactor Biosynthesis	2.16E+00
Apelin Cardiomyocyte Signaling Pathway	2.07E+00
Netrin Signaling	1.98E+00
Melanoma Signaling	1.97E+00
Endocannabinoid Neuronal Synapse Pathway	1.91E+00
Cellular Effects of Sildenafil (Viagra)	1.88E+00
Cardiac β-adrenergic Signaling	1.79E+00
Apelin Adipocyte Signaling Pathway	1.74E+00
Role of JAK1 and JAK3 in γc Cytokine Signaling	1.73E+00
CREB Signaling in Neurons	1.71E+00
Methionine Salvage II (Mammalian)	1.69E+00
AMPK Signaling	1.68E+00
cAMP-mediated signaling	1.68E+00
IL-15 Signaling	1.65E+00
Angiopoietin Signaling	1.62E+00
Antiproliferative Role of Somatostatin Receptor 2	1.61E+00
Relaxin Signaling	1.57E+00
CCR5 Signaling in Macrophages	1.56E+00

(The rest of the table is available at: https://www.mdpi.com/1422-0067/20/12/2928#supplementary)

Supplementary Figure S1: Correlation of the methylation of the top 10 CpGs with the Calculated Sensitivity index (CSi).



CHAPTER 5

Epigenome-wide association study in peripheral white blood cells involving insulin resistance

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Epigenome-wide association study in peripheral white blood cells involving insulin resistance

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Insulin resistance (IR) is a hallmark of type 2 diabetes, metabolic syndrome and cardiometabolic risk. An epigenetic phenomena such as DNA methylation might be involved in the onset and development of systemic IR. The aim of this study was to explore the genetic DNA methylation levels in peripheral white blood cells with the objective of identifying epigenetic signatures associated with IR measured by the Homeostatic Model Assessment of IR (HOMA-IR) following an epigenome-wide association study approach. DNA methylation levels were assessed using Infinium Methylation Assay (Illumina), and were associated with HOMA-IR values of participants from the Methyl Epigenome Network Association (MENA) project, finding statistical associations for at least 798 CpGs. A stringent statistical analysis revealed that 478 of them showed a differential methylation pattern between individuals with HOMA-IR ≤ 3 and > 3. ROC curves of top four CpGs out of 478 allowed differentiating individuals between both groups (AUC≈0.88). This study demonstrated the association between DNA methylation in some specific CpGs and HOMA-IR values that will help to the understanding and in the development of new strategies for personalized approaches to predict and prevent IR-associated diseases.

Type 2 diabetes (T2D) is a worldwide major health concern and the most predominant type of diabetes[1]. According to the World Health Organisation, the global prevalence of diabetes among adults over 18 years old has risen from 4.7% in 1980 to 8.5% in 2014. Furthermore, in 2015 about 1.6 million deaths were directly attributed to diabetes[1].

T2D is a multifactorial disease defined by the interaction of genetics and environmental factors[2]. The heritability for T2D is estimated to be between 15 and 85%. However, the genetic loci identified to date only explain 5–10% of this heritability[3]. In this context, available evidences suggest that epigenetics may be contributing to variations in gene expression and the risk for this metabolic disease[4]. In fact, recent investigations have associated the onset and progression of diabetes with specific changes in the epigenome[3,5].

Insulin resistance (IR) is a pathological condition in which cells fail to respond properly to insulin[6]. IR is one of the most important precursors of T2D and other adversely associated cardiometabolic conditions, such as obesity, hypertension, cardiovascular disease (CVD)[7], and metabolic syndrome[8]. IR is specifically associated with a low-grade inflammation, as well as with chronic enhancement of oxidative stress, triggering endothelial dysfunction and promoting atherogenesis[4]. Furthermore, both genetic and epigenetic factors are involved in the development of systemic IR[9]. The validated method Homeostatic Model Assessment of IR (HOMA-IR) is usually employed for measuring IR and β-cell function[10].

Epigenetic marks are heritable changes that cannot be explained through variations in DNA nucleotide sequence[11]. These modifications are potentially reversible and can be altered by environmental factors[2], resulting in alterations of gene expression and providing an interactive connection among genetics, specific diseases and the environment[12].

Among the different epigenetic modifications, DNA methylation has been widely searched[13]. Some epigenome-wide association studies (EWASs) have revealed significant associations between DNA methylation

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	TOTAL		ADULTS (n = 474)*																			
			DiOGenes-UNAV		OBEPALIP		Food4Me-UNAV		GEDYMET		ICTUS		NUGENOB-UNAV		PREDIMED-UNAV		RESMENA		NormoP		OBEKIT	
Variables	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values	n	Values
Sex (females)	474	303 (64)	52	27 (52)	29	29 (100)	39	21 (54)	57	57 (100)	7	5 (71)	22	14 (64)	116	59 (51)	44	22 (50)	12	6 (50)	96	63 (66)
Age (years)	474	47.0 (14.3)	52	42.7 (5.8)	29	37.4 (7.3)	39	41.7 (10.0)	57	27.0 (6.2)	7	57.1 (7.4)	22	34.7 (9.7)	116	65.0 (3.7)	44	48.6 (10.1)	12	39.4 (5.6)	96	46.8 (9.6)
Weight (kg)	474	81.7 (19.1)	52	95.3 (17.7)	29	83.1 (9.5)	39	74.4 (14.6)	57	60.7 (8.8)	7	121.9 (15.2)	22	87.3 (20.8)	116	71.7 (9.2)	44	103.0 (18.1)	12	65.8 (9.3)	96	89.2 (13.6)
BMI (kg/m^2)	474	30.0 (5.7)	52	33.9 (5.8)	29	31.6 (3.1)	39	26.0 (5.3)	57	24.1 (3.5)	7	44.3 (4.0)	22	31.1 (8.2)	116	27.7 (2.3)	44	36.5 (3.7)	12	22.8 (1.5)	96	31.9 (3.7)
Glucose (mg/dL)	443	102.3 (29.8)	37	99.0 (12.1)	29	89.9 (5.9)	39	91.8 (10.3)	57	78.1 (5.7)	7	120.6 (29.5)	12	102.3 (23.4)	110	121.5 (42.5)	44	122.2 (33.6)	12	85.1 (7.3)	96	95.8 (11.9)
Insulin (μUI/mL)	332	9.7 (7.0)	37	13.0 (7.1)	29	6.3 (3.3)	39	6.0 (4.6)	57	8.3 (2.7)	7	23.0 (12.2)	11	11.3 (6.4)	0	NA	44	15.8 (9.7)	12	3.6 (2.2)	96	8.5 (5.3)
HOMA-IR	332	2.4 (2.3)	37	3.2 (2.0)	29	1.4 (0.7)	39	1.4 (1.0)	57	1.6 (0.6)	7	7.1 (4.5)	11	3.0 (2.3)	0	NA	44	4.9 (3.4)	12	0.8 (0.5)	96	2.1 (1.5)
HOMA IR > 3	78	5.7 (2.7)	19	4.7 (1.7)	1	3.3 (NA)	1	6.2 (NA)	1	3.2 (NA)	7	7.1 (4.5)	3	6.1 (2.1)	0	NA	28	6.7 (3.1)	0	NA	18	4.6 (1.5)
HOMA-IR ≤ 3	254	1.5 (0.7)	18	1.7 (0.7)	28	1.3 (0.6)	38	1.2 (0.7)	56	1.6 (0.5)	0	NA	8	1.9 (0.9)	0	NA	16	1.7 (0.4)	12	0.8 (0.5)	78	1.5 (0.7)

Table 1. Anthropometric, clinical and biochemical characteristics of the study population and by project/consortium. Values are Mean (SD), except for Sex, which is represented as number of cases (%). *474 individuals obtained after processing the methylation raw data of 523 initial samples. BMI: Body mass index; HOMA-IR: Homeostatic model assessment of Insulin resistance; NA: not applicable.

Variable (x100)[a]	β	SE	p	[95% CI]
cg16462528	−0.046	0.011	<0.001	−0.067, −0.025
cg13133503	−0.080	0.036	0.028	−0.151, −0.009
cg07638362	−0.135	0.050	0.007	−0.234, −0.037

Table 2. Significant adjusted linear regression models of the top CpGs selected by a slope ≥ |0.1| and False Discovery Rate (FDR) < 0.05 and Spearman's rho. Adjusted by study, sex, age and body mass index. CI: confidence interval; SE: standard error. [a]β coefficients for those variables reflect increases in 0.01 units.

and glucose homeostasis[5,14–20], but only four of them studied some relationships with IR in different populations and approaches[5,14,15,18]. Therefore, the aim of the current work was to explore DNA methylation levels in peripheral white blood cells (PWBCs) by using an EWAS strategy with the objective of identifying epigenetic signatures associated with HOMA-IR and specifically identifying potential biomarkers that allow the discrimination of potentially hazardous HOMA-IR levels.

The assessment of epigenetic phenomena may help to understand the basis of metabolic pathway regulation, as well as the relationships between genomics and the environment influence, to promote new strategies to better understand human health and to develop novel biomarker panels related to T2D, obesity and accompanying comorbidities[20,21].

Results

Participant characteristics. Anthropometric and biochemical characteristics of the participants are reported (Table 1).

DNA methylation was significantly associated with HOMA-IR. Methylation values of CpGs were analysed for Linear Models for Microarray Data (LIMMA) regression with HOMA-IR in 332 subjects. Significant CpGs were selected by a False Discovery Rate (FDR) cut-off of 0.05 and a slope > |0.1|, obtaining 798 CpGs (Supplementary Material Table S1). The top 10 CpGs were further analysed for robustness. Spearman correlations were performed, and six CpGs were selected by having higher rho coefficient. Then, multiple linear regressions were performed adjusting by sex, age, study and body mass index (BMI), remaining three of the six CpGs significant (Table 2). These CpGs were cg13133503 (corresponding gene according to Illumina CG database *CLCA4*), cg07638362 (NA), and cg16462528 (*LECT1*), which are highlighted in a Manhattan plot (Fig. 1). Linear regression graphs between methylation values and HOMA-IR for these three CpGs are also represented (Fig. 2).

Individuals with HOMA-IR > 3 showed a differential methylation pattern. Participants were classified according to the HOMA-IR cut-off of 3 in order to analyse whether methylation was differential between both groups. There were 78 individuals with HOMA-IR > 3 and 254 with HOMA-IR ≤ 3 (Table 1). Methylation

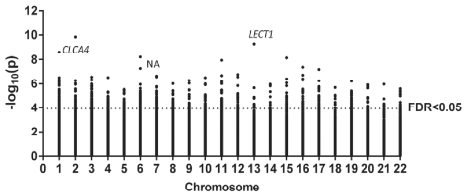

Figure 1. Manhattan plot of HOMA-IR-associated CpGs selected by a slope ≥|0.1|. Points above the dot line showed a False Discovery Rate (FDR) < 0.05. The three CpGs selected by slope ≥|0.1|, FDR < 0.05, Spearman's rho, and by multiple linear regressions adjusting by sex, age, study and body mass index are marked.

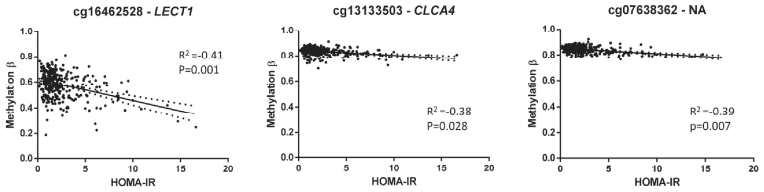

Figure 2. Linear regression graphs between HOMA-IR and methylation β values of the significant three CpGs selected by slope ≥|0.1|, False Discovery Rate (FDR) <0.05, and Spearman's rho. Adjusted by study, sex, age and body mass index. Dot lines on both sides of the solid line (linear regression for correlation) represent 95% confidence band.

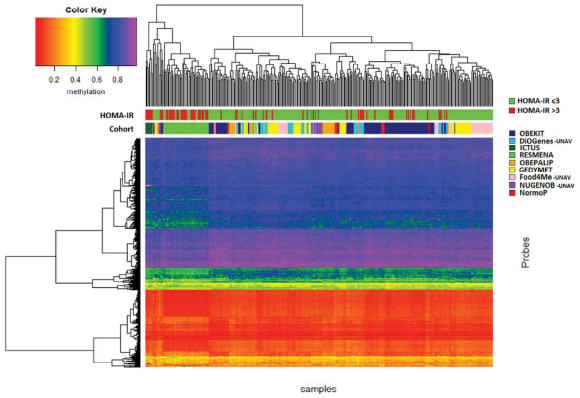

Figure 3. Heat map of 478 CpGs selected by Student's *t*-test between HOMA-IR ≤3 and >3 ($p < 6.26 \cdot 10^{-5}$ after Bonferroni correction).

values of the 798 CpGs were compared between both HOMA-IR groups. After applying the Bonferroni correction for multiple comparisons, a total of 478 CpGs showed statistically significant differences (Supplementary Material Table S2).

The resulting 478 CpGs were clustered in a heat map according to methylation patterns (Fig. 3). Two main clusters of 61 and 271 individuals were generated. The first cluster contained 62.3% of individuals with HOMA-IR > 3. However, the second cluster only included 14.8% of HOMA-IR > 3. The difference in HOMA-IR proportions of the clusters was statistically significant ($p < 0.001$).

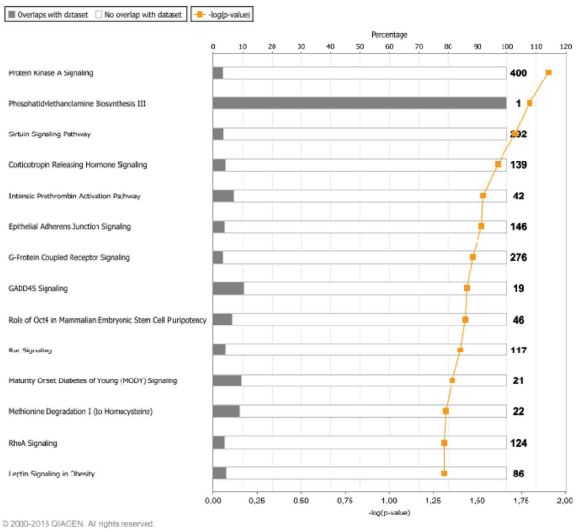

Figure 4. Canonical pathways from Ingenuity Pathway Analysis of 478 CpGs selected by Student's *t*-test between HOMA-IR ≤ 3 and >3 ($p < 6.26 \cdot 10^{-5}$ after Bonferroni correction).

Differentially methylated CpGs between HOMA-IR groups were related to glucose and insulin pathways. Canonical pathways were obtained from Ingenuity Pathway Analysis (IPA) for these 478 CpGs (Fig. 4). Some of the statistically significant pathways were related to insulin and glucose, such as *Protein Kinase A Signalling*, *Sirtuin Signalling Pathway*, *G-Protein Coupled Receptor Signalling*, *Rac Signalling*, *Mature Onset Diabetes of Young (MODY) Signalling*, *RhoA Signalling*, and *Leptin Signalling in Obesity*. The top four CpGs differentially methylated between HOMA-IR ≤ 3 and >3 were cg23475244 (NA), cg06115835 (*SH3RF3*), cg16278828 (*MAN2C1*) and cg16639311 (NA) as illustrated (Fig. 5).

The top four CpGs allow to differentiate between HOMA-IR ≤ 3 and >3. In order to further analyse whether methylation could differentiate between both HOMA-IR groups, the Receiver Operating Characteristic (ROC) curves adjusted by study, sex, age and BMI for the top four CpGs (cg23475244, cg06115835, cg16278828, and cg16639311) were calculated. The areas under the curve (AUC) of these CpGs were around 0.90 (AUC cg23475244 = 0.8965, AUC cg06115835 = 0.9026, AUC cg16278828 = 0.8989, and AUC cg16639311 = 0.8952), and after an internal validation (Fig. 6), the values were around 0.88 (AUC cg23475244 = 0.8865, AUC cg06115835 = 0.8919, AUC cg16278828 = 0.8893, and AUC cg16639311 = 0.8826).

Discussion

This study involving the Methyl Epigenome Network Association (MENA) project demonstrated the association between DNA methylation in specific CpGs and HOMA-IR values. Our results also provided evidence of a differential methylation pattern between individuals with a HOMA-IR ≤ 3 and >3. Additionally, these data have led to the identification of four CpGs that allow us to differentiate individuals between HOMA-IR ≤ 3 and >3 with an approximate AUC of 0.88. This assay adds further insights and knowledge about the relationship between T2D-related traits and epigenetic DNA modifications.

As aforementioned, IR is a hallmark of several diseases and unhealthy cardiometabolic conditions such as T2D, CVD, hypertension, obesity[7] and metabolic syndrome[8]. Epigenetic mechanisms have been involved in the onset and development of IR[9]. Indeed, several studies have related methylation of specific genes with HOMA-IR[3,7,8,22–31]. Nevertheless, few EWAS have been performed to date[5,14,15,18]. In line with these studies, this EWAS of the MENA project showed an association of 798 CpGs with HOMA-IR (slope $\geq |0.1|$ and FDR < 0.05). In our study, from the top 10 CpGs, selected ones with better association and significant after linear regressions adjusting by study, age, sex, and BMI were cg07638362 (according to Illumina CG database this CpG was not associated to any gene), cg13133503 (*CLCA4*) and cg16462528 (*LECT1*). These CpGs, to our knowledge, have not been previously described in other EWAS. However, some of the mentioned genes have been found in the list of one study. Specifically, differentially methylated regions of *LECT1* and *CLCA4* have been significantly different between diabetics and non-diabetics[32]. Both *CLCA4* and *LECT1* have been related to methylation regulation[33–35]. *CLCA4* has been involved in the activation of cAMP-dependent protein kinase A [www.genecards.org]. This

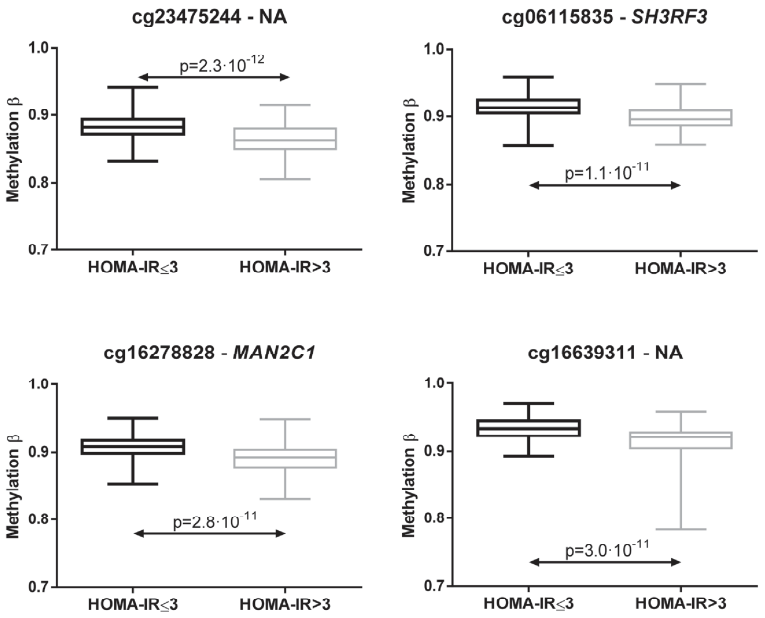

Figure 5. Box plots of top four CpGs selected by Student's *t*-test between HOMA-IR ≤3 and >3 ($p < 6.26 \cdot 10^{-5}$ after Bonferroni correction). Whiskers represent minimum and maximum values.

pathway is intimately connected to glucose homeostasis[36]. On the other hand, *LECT1* plays a role as antiangiogenic factor in cardiac valves, preventing valvular heart diseases[37]. Methylation of this gene may be associating IR with CVD. Thus, the association of several CpGs between DNA methylation and IR detected in our study adds further support for a potential role of abnormal DNA methylation in IR[7].

Since IR is a key feature of T2D, obesity and metabolic syndrome[7,8], it is interesting to analyse other EWAS and methylation studies related to these adverse metabolic conditions. These investigations have been performed in several tissues such as pancreatic islets, liver, adipose tissue, skeletal muscle and blood cells[38]. There are five genes in our list that were previously related to insulin resistance (*CXCR1*, *HDAC4*, *IGFR1*, *LEPR*, and *ABCG1*)[4,5,18]. On the other hand, T2D and glycaemic traits have been associated with the following genes found in our selection *NR4A3*[32], *KCNQ1*[39], *IRS1*[39,40], *SREBF1*[14,16,17,20], *SOCS3*[14,16,17,20], *ZNF518B*[8], *SAMD12*[15,19], *LY6G6E*[16], *PHGDH*[20], and *ABCG1*[5,14–16,18,41]. Additionally, *IRS1*[40], *SREBF1*[18,20,42], *ABCG1*[17,20,43–45], *SOCS3*[17,44,46], *LY6G6E*[43] and *PHGDH*[45,47] have also been found in EWAS analysing BMI or obesity traits. Other genes from our list that are related to obesity or BMI were *AOC3*[48], *c7orf50*[43], *NOD2*[20,42], and *SLC1A5*[42]. Regarding genes associated with age, *ZNF423*[49] and *THRB*[50] were found in our list. In the case of smoking-associated genes, *ECE1*, *ATP8B2*, *c7orf50*, *IGF1R*, *RPL23A*, *SFRS1*[51], *RPTOR*, *RARA*[52], *c6orf48*[53], and *IER3*[54] appeared in the selection. Interestingly, the specific CpGs described for *ABCG1* (cg06500161)[5,14–18,20,42–45], *SREBF1* (cg11024682)[14,16–18,20,42,43], *SOCS3* (cg18181703)[14,16,17,20,46], and *PHGDH* (cg14476101)[20,42,45,47] were also found in our list. These four mentioned CpGs probably represent the widest described ones in relationship with T2D, obesity and other metabolic impairments in several studies with different tissues such as skeletal muscle, liver, pancreas and blood cells. Our investigation adds some new CpGs and genes to the previously described list, contributing to the knowledge and the management of IR-associated diseases.

As a novelty, our results have shown that individuals with HOMA-IR ≤ 3 or >3 exhibited a differential methylation pattern for at least 478 CpGs. Furthermore, the clustering showed that 62.3% of individuals in the first cluster had a HOMA-IR > 3. Thus, more than half of the people with similar methylation patterns presented a HOMA IR > 3. However, the distribution of some cohorts was not heterogeneous. This situation is due to the specific recruitment requirements for each cohort. Indeed, cohorts such as RESMENA, where all the patients had metabolic syndrome, is completely found in the first cluster.

Furthermore, these 478 CpGs corresponded to some genes involved in glucose and insulin-related pathways according to IPA. For example, *Protein Kinase A Signalling*, where protein kinase A activation triggers insulin secretion in β-cells[55]; *Sirtuin Signalling Pathway*, where sirtuins influence many steps of glucose metabolism in liver, pancreas, muscle and adipose tissue[56]; and *G-Protein Coupled Receptor Signalling*, where insulin and

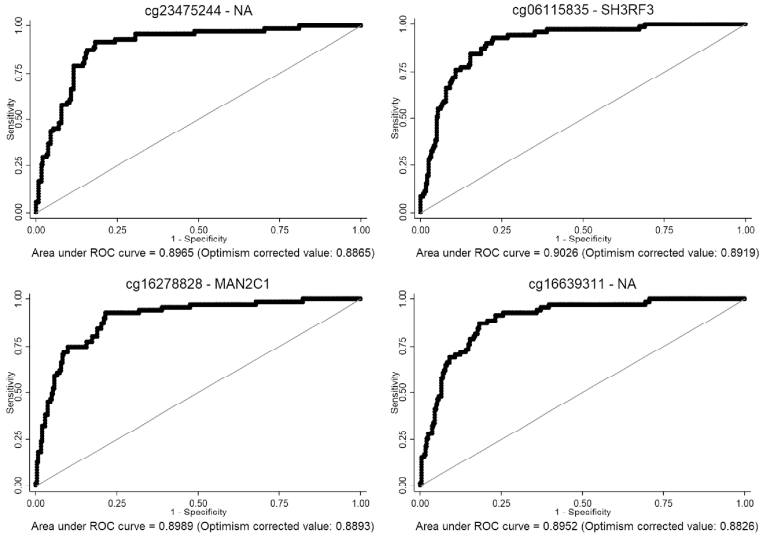

Figure 6. ROC curves of the top four CpGs (cg23475244, cg06115835, cg16278828, and cg16639311). Optimism corrected value was calculated using the Tibshirani's enhanced bootstrap method described by Harrell[64].

glucagon secretion is affected by factors binding to G-protein coupled receptors on the surface of β- and α-cells[57]. Other pathways were *Rac Signalling*, which is involved in the regulation of insulin-stimulated glucose uptake[58]; *RhoA Signalling*, pathway that has been implicated in the pathogenesis of diabetes[59]; and *Leptin Signalling in Obesity*, since leptin is a regulator of glycaemic control[60]. Furthermore, *Maturity Onset Diabetes of Young (MODY) Signalling* represents the pathway of another type of diabetes that accounts for less than 2% of all diabetic cases. MODY is a monogenic form of diabetes characterized by an early onset, autosomal dominant mode of inheritance and a primary defect in pancreatic β-cell function[61].

Only two of the top four CpGs with statistically significant differences between HOMA-IR ≤3 and >3 individuals presented associated genes according to Illumina CG database. Those genes were *SH3RF3* and *MAN2C1*. The function of *SH3RF3* is not well known, whereas *MAN2C1* is related to glycosaminoglycan (GAG) metabolism. The GAGs are heteropolysaccharides formed by a chain of repeating disaccharide units[62]. Changes in GAGs structure and function have been reported in the kidney, liver, arteries and retinal vessels of diabetics[63].

Since methylation patterns of the 478 CpGs were able to cluster HOMA-IR individuals, we analysed the ability of the top four CpGs to differentiate between HOMA-IR ≤3 and >3 individuals. These top four CpGs distinguished HOMA-IR groups with a valuable AUC around 0.88 after an internal validation based on the optimistic correction model described by Harrell[64], suggesting these CpGs as potential valuable biomarkers of IR.

This study was not devoid of limitations. Firstly, methylation is tissue-specific and the ideal tissue for this study would have been the pancreatic β-cells or cells from recognized insulin sensitive tissues such as skeletal muscle or white adipose tissue[65]. However, peripheral blood is the best non-invasive alternative tissue that reflects multiple metabolic and inflammatory pathways[66], and relevant studies have demonstrated that epigenetic reprogramming may serve as a surrogate marker for metabolic disorders[41]. Interestingly, gene methylation parallelisms between peripheral blood cells and pancreatic islets have been recently reported, suggesting that blood may be used as a marker for islet DNA methylation[67]. Secondly, type I and type II error cannot be discarded, although multiple comparison tests and statistical adjustments for potential confounding factors such as sex, age, cohorts, DNA methylation chips, and cell composition heterogeneity have been performed. Thirdly, a validation sample would have been useful to corroborate the results in the selected genes. Unfortunately, this sample was not available. However, in order to resolve this issue and correct the overestimation of AUC, an internal validation using a bootstrap method[64] was performed, obtaining similar results. Further studies are needed to verify the relationship between the selected CpGs and HOMA-IR. Finally, due to the cross-sectional feature of the study, methylation cannot be defined as a cause or consequence of cardiometabolic conditions. Remarkably, although there is an epigenetic programming during the first stages of human development[68], Wahl *et al.* have described methylation alterations as a cause of higher BMI and adiposity[20].

Epigenetic gene regulation, and specifically, DNA methylation, is playing a role in the pathogenesis of many complex disorders, including T2D, obesity or metabolic syndrome[37]. There is great interest to perform

methylation profiling in peripheral blood to find potential methylation disease-related associations and use specific DNA methylated regions as biomarkers[69]. In summary, this study found associations between DNA methylation and IR, a hallmark of T2D, with a differential methylation pattern between individuals with HOMA-IR ≤ 3 and >3 in genes that are mainly involved in glucose and insulin-related pathways, and suggested four CpGs as biomarkers of IR. These results will hopefully contribute to the understanding of some epigenetic mechanisms that may regulate glycaemic traits, such as HOMA-IR, and the risk of T2D, as well as provide the basis for creating personalized strategies to predict, prevent and treat IR-associated diseases.

Subjects and Methods

Participants. The MENA project was conducted in 523 adult participants from available cohorts at the University of Navarra (UNAV): DiOGenes-UNAV with n = 58[70], OBEPALIP with n = 29[71], Food4Me-UNAV with n = 42[72], GEDYMET with n = 57[73], ICTUS with n = 7[74], NUGENOB-UNAV with n = 42[75], PREDIMED-UNAV with n = 129[76,77], RESMENA with n = 47[78], OBEKIT with n = 100[79] and NormoP with n = 12. However, only 474 final samples were available after the data processing explained in detail below.

Study designs, characteristics, inclusion and exclusion criteria were described for each study cohort, except for NormoP, whose design has not yet been described. All of them were approved by the Research Ethics Committee of the University of Navarra (CEI UN, Pamplona, Spain), except for GEDYMET, which was approved by the Ethics committee of the School of Medicine, Pontificia Universidad Católica de Chile (Santiago, Chile), in compliance with the Helsinki Declaration of ethical principles for medical research involving human subjects. All participants provided written informed consent.

The NormoP cohort participants recruitment started in 2016 in the University of Navarra (Pamplona, Spain). Eligible participants were self-declared healthy individuals, >18 years old, and had a BMI of between 18.5 and 24.9 kg/m^2. Exclusion criteria included pregnancy, type I diabetes, severe renal and digestive diseases, hydroelectrolitic disorders, acute CVD, cardiac arrhythmias, ictus, neoplasia, anaemia, eating disorders, pharmacological treatment, and dietary supplements that may affect the results.

Study variables. Anthropometric measurements and the metabolic profile were obtained from databases of the aforementioned cohorts, which followed validated protocols. Data of some characteristics were not available for all the 474 participants. IR was estimated using the validated HOMA-IR index method[10].

DNA extraction and DNA methylation analysis. Venous blood samples were drawn on EDTA tubes. Genomic DNA was extracted from PWBCs using the MasterPure™ DNA Purification kit (Epicenter, Madison, WI), whose quality was assessed with the Pico Green dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). High-quality DNA samples (500 ng) were treated with bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions, converting cytosine into uracil. DNA methylation levels were measured by microarray with the Infinium Human Methylation 450 K bead chip technology (Illumina, San Diego, CA, USA) in all the cohorts, except OBEKIT, which was performed with Infinium MethylationEPIC beadchip (Illumina). This analysis was conducted in the Unidad de Genotipado y Diagnóstico Genético from Fundación Investigación Clínico de Valencia, as detailed elsewhere[80].

Treatment of methylation raw data. Beta-values have been used as metrics to measure methylation levels. Beta-value in methylation experiments is the estimate of the methylation level using the ratio of the methylation probe intensity and the overall intensity, corresponding to the percentage of methylation on a specific site[81]. After obtaining intensity data using ChAMP package for R v.1.11.0[82] as described elsewhere[83], the filtering process was performed in probes with a detection p-value above 0.01 in one or more samples, probes with a bead-count <3 in at least 5% of samples, non-CpG probes, probes with SNPs[84], probes that align to multiple locations[84] and probes located on the X or Y chromosomes.

From the 523 initial participants, samples with a failed CpG fraction above 0.01 were eliminated (n = 20), leaving 503 individuals. After filtering probes, intra-cell type normalization was done using Subset-quantile Within Array Normalization (SWAN) method to avoid the bias introduced by the Infinium type 2 probe design[85]. In order to assess the similarity of normalized methylation samples in both batches and the pooled data, multidimensional scaling plots based on top of 1000 most variable probes were performed. A total of 29 samples failed to fulfil this requisite, which left 474 participants for the subsequent analyses.

After SWAN normalization, magnitude of batch effects were assessed and corrected using the ComBat normalization method, which is an empirical Bayes based method to correct for technical variation related to the slide[86,87]. Furthermore, differences in methylation resulting from differences in cellular heterogeneity were corrected using the Houseman procedure[88].

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[89] and are accessible through GEO Series accession number GSE115278 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115278).

Statistical analysis. After pre-processing, LIMMA package from the R statistical software[82] was used to compute a linear regression between DNA methylation values and HOMA-IR. A total of 332 subjects from the MENA project showed data for both variables (Table 1). This analysis was adjusted by the effect of confounding factors such as sex, age, study and bead chip. Raw p-values were corrected using the Benjamini-Hochberg procedure for multiple comparisons, and a FDR cut-off of 0.05 and a slope $\geq |0.1|$ were used as statistically significant thresholds. The top 10 CpGs were analysed for robustness with Spearman correlations and then, linear regressions between HOMA-IR and methylation adjusted for study, sex, age, and BMI were also performed for the six selected CpGs.

The cut-off for HOMA-IR differs for different races, ages, genders, diseases, complications, etc.[90] and no reference value has been established[91]. Since there is no consensus for the HOMA-IR cut point and in order to facilitate the analysis of this metabolically heterogeneous group, a cut-off of HOMA-IR = 3 was chosen, corresponding to a value between the 75th and 80th percentiles, which are established as cut points by International Diabetes Federation (IDF) and Adult Treatment Panel III (ATPIII) for metabolic syndrome[92]. No influences in terms of races were considered, since more than 92% of the individuals were Caucasian in the MENA project and additionally, the study has been considered as a covariate in the analyses. Moreover, some studies have previously used this cut-off for HOMA-IR[93,94]. Differentially methylated CpGs between individuals with HOMA-IR > 3 and HOMA-IR ≤ 3 were explored using two-tailed Student's t-test with Bonferroni correction. A p-value $< 6.26 \cdot 10^{-5}$ was considered significant. Adjusted (for study, sex, age, and BMI) ROC curves were performed to determine the AUC of the top selected CpGs distinguishing individuals between HOMA-IR ≤ 3 or > 3. Furthermore, an internal validation using a correction for optimistic prediction was performed according to Tibshirani's enhanced bootstrap method described by Harrell[64] in order to evaluate the overestimation of the model.

Statistical calculations were performed with STATA version 12.0 (Stata Corp, College Station, TX, USA), unless otherwise indicated. Manhattan plots, correlation graphs and box plots were produced using GraphPad Prism 6 (Graph-Pad Software, CA, USA). The heat map was created with the R software[82] using library gplots and the heatmap.2 function.

Ingenuity Pathway Analysis. Differentially methylated CpGs between individuals with HOMA-IR > 3 and HOMA-IR ≤ 3 were analysed by IPA software (Qiagen Redwood City, CA, USA, www.ingenuity.com) as defined in the package. Predefined pathways and functional categories of the Ingenuity Knowledge Base were used in order to detect associated pathways and relevant gene regulatory networks[95]. Pathway analyses were performed with IPA's Core Analysis module. Canonical pathways with a $p < 0.05$ after Fisher's test were defined as a statistically significant overrepresentation of input genes in a given process.

Data Availability

The data have been deposited in NCBI's Gene Expression Omnibus[89] and are accessible through GEO Series accession number GSE115278 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115278).

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Author Contributions

A.A. performed most of the data analysis and wrote the first version of the paper. O.R.L. and M.L.M. helped in the analysis. J.L.S. critical discussed all aspects of the manuscript and contributed with a cohort. J.I.R.B., F.I.M. and J.A.M. supervised data analysis and helped with interpretation and with manuscript elaboration, as well as provide the conceptual design and financial support. All authors read and approved the final manuscript.

Additional Information

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Supplementary material

Epigenome-wide association study in peripheral white blood cells involving insulin resistance

Available at: https://www.nature.com/articles/s41598-019-38980-2#Sec16

Table S1: Selection of 798 CpGs after linear regression by FDR<0.05 and slope ≥ |0.1|.

CpG	UCSC_RefGene_ Accession	UCSC_RefGene_ Group	UCSC_CpG_ Islands_Name	p	FDR
cg22051399	NA	NA	NA	1.44E-10	4.90E-05
cg16462528	LECT1;LECT1	NM_001011705;NM_007015	Body;Body	4.96E-10	8.46E-05
cg13133503	CLCA4;CLCA4	NR_024602;NM_012128	Body;Body	2.31E-09	0.00026276
cg13108601	FRS3;PRICKLE4	NM_006653;NM_013397	TSS1500;TSS1500	5.88E-09	0.00048823
cg02613818	IGF1R	NM_000875	Body	7.16E-09	0.00048823
cg19749898	HCCA2	NM_053005	Body	1.14E-08	0.00064765
cg02793828	NA	NA	NA	4.27E-08	0.00207921
cg07638362	NA	NA	NA	5.32E-08	0.00226558
cg14315232	NA	NA	NA	6.47E-08	0.00245104
cg04559178	NA	NA	NA	1.72E-07	0.00556053
cg10293354	NA	NA	NA	1.89E-07	0.00556053
cg05935374	RAB30	NM_014488	TSS200	2.08E-07	0.00556053
cg20703375	ARPC1A	NM_006409	Body	2.24E-07	0.00556053
cg19260567	CNTNAP2	NM_014141	Body	2.72E-07	0.00556053
cg03806828	RAB21	NM_014999	TSS200	2.72E-07	0.00556053
cg09242721	TRNT1	NM_182916	TSS200	2.74E-07	0.00556053
cg22964775	LPHN3	NM_015236	TSS1500	3.02E-07	0.00556053
cg07908574	SLC45A1	NM_001080397	Body	3.07E-07	0.00556053
cg04238871	FAM53B	NM_014661	5'UTR	3.10E-07	0.00556053
cg04737885	ADCY9	NM_001116	3'UTR	3.88E-07	0.00636839
cg15924985	NA	NA	NA	3.92E-07	0.00636839
cg04724873	KIAA1751	NM_001080484	Body	4.66E-07	0.00650242
cg11390741	ITGA9	NM_002207	Body	4.69E-07	0.00650242

(The rest of the table is available at: https://www.nature.com/articles/s41598-019-38980-2#Sec16)

Table S2: Selection of 478 CpGs after comparing individuals with HOMA≤3 and >3 (Student's t-test + Bonferroni correction).

CpG	Student's t-test	UCSC_RefGene_Accession	UCSC_RefGene_Group	UCSC_CpG_Islands_Name
cg23475244	2.27366E-12	NA	NA	NA
cg06115835	1.05058E-11	SH3RF3	NM_001099289	Body
cg16278828	2.75829E-11	MAN2C1	NM_006715	Body
cg16639311	2.96689E-11	NA	NA	NA
cg00882175	6.86666E-11	NA	NA	NA
cg20009378	1.31144E-10	GRK1	NM_002929	Body
cg03438024	1.50385E-10	NA	NA	NA
cg11565042	1.57477E-10	GPR45	NM_007227	1stExon
cg07638362	1.67285E-10	NA	NA	NA
cg08913530	2.63749E-10	C10orf129	NM_207321	Body
cg13875763	3.558E-10	NA	NA	NA
cg02951880	4.2286E-10	VANGL2	NM_020335	Body
cg19450145	4.28193E-10	TULP4;TULP4	NM_020245;NM_001007466	Body;Body
cg06199907	4.28514E-10	MARVELD3	NM_001017967	Body
cg00472528	5.89409E-10	NA	NA	NA
cg10857489	5.98209E-10	SLC12A7	NM_006598	Body
cg23162967	6.9311E-10	CBLB	NM_170662	TSS200
cg06609415	6.95911E-10	NA	NA	NA
cg15537788	1.02976E-09	SIPA1L3	NM_015073	3'UTR
cg16276063	1.26828E-09	SOX2OT	NR_004053	Body
cg04724873	1.50316E-09	KIAA1751	NM_001080484	Body
cg21963643	2.07292E-09	CACNA1E	NM_000721	TSS1500
cg24002010	2.4842E-09	TMEM86B;SAPS1	NM_173804;NM_014931	TSS1500;3'UTR
cg09991951	2.64527E-09	NA	NA	NA
cg09984469	2.8116E-09	CYP26B1	NM_019885	Body
cg12456777	2.81964E-09	SNORD42B;RPL23A	NR_000013;NM_000984	TSS1500;1stExon
cg18062721	3.19762E-09	VGLL4;VGLL4	NM_014667;NM_001128219	Body;Body
cg09242721	3.24334E-09	TRNT1	NM_182916	TSS200
cg20376082	3.93989E-09	NA	NA	NA

(The rest of the table is available at: https://www.nature.com/articles/s41598-019-38980-2#Sec16)

CHAPTER 6

Methylome-wide association study in peripheral white blood cells focusing on central obesity and inflammation

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Methylome-Wide Association Study in Peripheral White Blood Cells Focusing on Central Obesity and Inflammation

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Abstract: Epigenetic signatures such as DNA methylation may be associated with specific obesity traits in different tissues. The onset and development of some obesity-related complications are often linked to visceral fat accumulation. The aim of this study was to explore DNA methylation levels in peripheral white blood cells to identify epigenetic methylation marks associated with waist circumference (WC). DNA methylation levels were assessed using Infinium Human Methylation 450K and MethylationEPIC beadchip (Illumina) to search for putative associations with WC values of 473 participants from the Methyl Epigenome Network Association (MENA) project. Statistical analysis and Ingenuity Pathway Analysis (IPA) were employed for assessing the relationship between methylation and WC. A total of 669 CpGs were statistically associated with WC (FDR < 0.05, slope ≥ |0.1|). From these CpGs, 375 CpGs evidenced a differential methylation pattern between females with WC ≤ 88 and > 88 cm, and 95 CpGs between males with WC ≤ 102 and > 102 cm. These differentially methylated CpGs are located in genes related to inflammation and obesity according to IPA. Receiver operating characteristic (ROC) curves of the top four significant differentially methylated CpGs separated by sex discriminated individuals with presence or absence of abdominal fat. ROC curves of all the CpGs from females and one CpG from males were validated in an independent sample (n = 161). These methylation results add further insights about the relationships between obesity, adiposity-associated comorbidities, and DNA methylation where inflammation processes may be involved.

Keywords: waist circumference; epigenetics; DNA methylation

1. Introduction

Obesity has increased worldwide to reach epidemic proportions [1]. According to the World Health Organization, the prevalence of obesity nearly doubled between 1980 and 2014, reaching an alarming percentage of 11% in men and 15% in women worldwide [2]. This rising burden is accompanied by the increased prevalence of obesity-linked comorbidities, such as cardiovascular

diseases, diabetes, cancer, and musculoskeletal disorders, among others [2]. The development of these obesity-related complications are more often associated with visceral fat instead of total body fat [3]. In this context, waist circumference (WC) measurements can be a useful tool to assess abdominal obesity, anatomically discriminating between visceral and subcutaneous fat depositions in individuals with obesity [4].

Obesity is a multifactorial disease resulting from the interaction of environmental and lifestyle factors with the genetic make-up [5]. Genetic variations contribute to the individual susceptibility to developing diseases, but accumulating evidences suggest that epigenetic phenomena are also involved [6]. Epigenetic marks and modifications may play a causative role in the development of obesity by altering the expression of obesity-related genes, or alternatively, be a consequence of obesity, predisposing some subjects to associated comorbidities [7]. Indeed, several epigenome-wide association studies (EWASs) have revealed that obesity traits are associated with DNA methylation shifts in different tissues [6,8,9]. However, most of these studies use body mass index (BMI) instead of WC, but WC appears to be a more accurate and convenient surrogate for central obesity assessment [4], being therefore more associated with obesity-related diseases [3]. Indeed, there are only few EWAS analyzing the association of methylation status and WC [10–16].

The aim of the current research was to explore DNA methylation levels in peripheral white blood cells to identify epigenetic methylation signatures associated with WC.

2. Subjects and Methods

2.1. Participants

This study was performed in 523 adult participants from cohorts composing the Methyl Epigenome Network Association (MENA) project such as DiOGenes-UNAV with n = 58 [17], OBEPALIP with n = 29 [18], Food4Me-UNAV with n = 42 [19], GEDYMET with n = 57 [20], ICTUS with n = 7 [21], NUGENOB-UNAV with n = 42 [22], PREDIMED-UNAV with n = 129 [23], RESMENA with n = 47 [24], OBEKIT with n = 100 [25], and NormoP with n = 12 [26], whose providers are gratefully acknowledged. Study design, characteristics, and exclusion and inclusion criteria of each of these cohorts have been previously described. All of these studies were approved by the research ethics committees at all recruiting centers in compliance with the Helsinki Declaration of ethical principles for medical research involving human subjects (DiOGenes KF01-267787 IHE 4-1-2.0091 dd. 23-03-2006, OBEPALIP 007/2009, Food4Me-UNAV 041/2012, GEDYMET 14-281, ICTUS 2/10, NUGENOB-UNAV 5/04/2001, PREDIMED-UNAV 50/2005, RESMENA 065/2009, OBEKIT and NormoP 132/2005). All participants provided written informed consent.

A validation was performed in an independent sample of individuals (n = 161) from the OBEKIT and NormoP studies (not included in the MENA study) (Supplementary Table S1).

2.2. Study Variables

Anthropometric measurements at baseline were obtained from databases of the aforementioned cohorts following standardized validated protocols [27]. BMI was calculated dividing weight in kg by height in meters squared (kg/m^2). WC was measured at the mid-point between the lower rib and the iliac crest using a tape measure. Blood samples for methylation analysis were obtained at the same visit. A summary of the studies design and collected samples are reported (Supplementary Table S2).

2.3. DNA Extraction, DNA Methylation Analysis, and Treatment of Methylation Raw Data

Procedures explaining DNA extraction and DNA methylation analysis have been detailed elsewhere [26]. Briefly, Infinium Human Methylation 450K bead chip technology (Illumina, San Diego, CA, USA) was employed to measure DNA methylation levels in all the cohorts, except OBEKIT, which was performed with Infinium MethylationEPIC beadchip (Illumina).

2.4. Treatment of Methylation Raw Data

Beta-values were employed to assess methylation levels in order to estimate the methylation degree using the ratio of the methylation probe intensity and the overall intensity, corresponding to the percentage of methylation on a specific site [28]. Intensity data were obtained using the ChAMP package for R v.1.11.0 [29] as described elsewhere [30]. Then, the filtering process was performed in probes with a detection p-value > 0.01 in one or more samples, probes with a beadcount < 3 in at least 5% of samples, non-CG site (CpG) probes, probes with single nucleotide polymorphisms [31], probes that align to multiple locations [31], and probes located on the X or Y chromosomes.

Out of the 523 initial participants, 20 samples with a failed CpG fraction above 0.01 were eliminated, leaving 503 individuals. Afterwards, intra-cell type normalization using the subset-quantile within array normalization method was performed to avoid any bias introduced by the Infinium type 2 probe design [32]. Then, multidimensional scaling plots based on top 1000 most variable probes were carried out to evaluate the similarity of normalized methylation samples in both batches and the pooled data. A total of 29 samples failed to accomplish this requirement, leaving 474 participants for the subsequent analyses.

Since the methylation data was obtained at different times in the lab, following subset-quantile within array normalization, the ComBat normalization method was used to assess and correct the magnitude of batch effects [33,34]. Furthermore, the Houseman procedure [35] was employed to correct differences in methylation resulting from cellular heterogeneity.

The data have been deposited in NCBI's Gene Expression Omnibus [36] and are accessible through GEO Series accession number GSE115278 [37].

2.5. Statistical Analysis

After pre-processing, the linear models for microarray data (LIMMA) package for the R statistical software [29] was used to compute a linear regression between DNA methylation and WC (n = 473, since data of WC was not available for one participant) adjusted by the effect of confounding factors, such as sex, age, study, and batch effect. Raw p-values were corrected employing the Benjamini–Hochberg procedure as a correction for multiple comparisons. A quantile–quantile plot showed that there was a small inflation of the adjusted p-values, which were corrected [38] (Supplementary Figure S1). The statistically significant threshold for CpG selection was set by a false discovery rate cut-off of 0.05 and a slope ≥ |0.1|.

In order to ascertain if there were methylation differences between subjects with high and low WC, and due to gender differences in the established cut points for WC, the selected CpGs were categorized by sex. WC was used as an anthropometric marker of abdominal obesity and was dichotomized following the National Cholesterol Education Program Adult Treatment Panel III. The cut-off for WC in females was 88 cm, whereas, a WC of 102 cm was considered for males [39]. Differentially methylated CpGs were explored using Student's t-test with the Bonferroni correction (in order to avoid type I errors) between females with WC ≤ 88 and > 88 cm, and between males with WC ≤ 102 and > 102 cm. A p-value < $7.47 \cdot 10^{-5}$ was considered statistically significant. Receiver operating characteristic (ROC) curves adjusted by age were calculated for the top four significant differentially methylated CpGs for each sex. ROC curves were validated in an independent sample of individuals (n = 161).

Statistical calculations were performed with Stata version 12.1 (StataCorp 2011, College Station, TX, USA), unless otherwise indicated. Correlation graphs and box plots were generated using GraphPad Prism 6 (Graph-Pad Software, San Diego, CA, USA). The volcano plot, quantile–quantile plot, and heat map (using library gplots and the heatmap.2 function) were created with the R software [29].

2.6. Ingenuity Pathway Analysis

The genes corresponding to the differentially methylated CpGs between females with WC ≤ 88 and > 88 cm, or males with WC ≤ 102 and > 102 cm, were analyzed by Ingenuity Pathway Analysis

(IPA) software (Qiagen Redwood City, CA, USA, www.ingenuity.com). Associated pathways and gene regulatory networks were identified by predefined pathways and functional categories of the Ingenuity Knowledge Base [40]. Canonical pathway analyses were performed with IPA's core analysis module and selected if $p < 0.05$ after Fisher's test as statistically significant.

3. Results

Anthropometric characteristics including weight, BMI, and WC of the participants are reported (Table 1). The proportion of females is higher than men and the average age is 47 years, although there are studies with a mean of 27 years, such as GEDYMET or 65 years, such as PREDIMED-UNAV. Furthermore, mean BMI indicates that the average population is obese, but the range of means of the studies goes from 22.8 to 44.3 kg/m^2. WC values showed a higher number of individuals from both sexes with central adiposity, although it also depends on the study.

Linear regressions (LIMMA) between methylation values and WC were performed and 669 significant CpGs were selected after applying false discovery rate cut-off of 0.05 and a slope ≥ |0.1|, and correcting for inflation (Supplementary Table S3). The six top CpGs were cg11649376 (corresponding gene according to Illumina CG database *ACSS3*), cg23304023 (*TACC2*), cg20401786 (*TSNARE1*), cg02813542 (*TCP11L1*), cg01243823 (*NOD2*), and cg09499256 (*FPR2*), which are plotted in Figure 1. Linear regression graphs adjusted by sex and age between methylation values and WC for these six CpGs are depicted in Figure 2.

Participants were classified according to sex and WC, separating females with WC ≤ 88 and > 88 cm, and males with WC ≤ 102 and > 102 cm, in order to analyze whether methylation was differential between both groups for each sex. There were 121 females with WC ≤ 88 cm and 182 with WC > 88 cm, and 82 males with WC ≤ 102 cm and 88 with WC > 102 cm (Table 1). Methylation values of the 669 CpGs were compared between both WC groups for each sex. For females, 375 CpGs showed statistically significant differences and 95 CpGs for males ($p < 7.47\times10^{-5}$) (Supplementary Table S4A and S4B, respectively).

The respective 375 and 95 CpGs were clustered in a heat map according to methylation patterns (Figure 3). For females (Figure 3A), three main clusters of 35, 84, and 184 individuals were generated. The first and third clusters contained 51.1% of individuals with WC > 88 cm. However, the second cluster included 83.3% of WC > 88 cm. On the other hand, males (Figure 3B) were clustered in two groups of 71 and 99 individuals. The first cluster group showed a percentage of 77.5% of males with WC > 102 cm, while in the second group, the percentage was 33.3%. The difference in WC proportions of the clusters was statistically significant ($p < 0.001$) for both sexes.

Canonical pathways were obtained from IPA for the significant 375 and 95 CpGs for females and males, respectively (Supplementary Figure S2). Some of the statistically significant pathways were related to inflammation and obesity in both sexes. In females, some of these pathways were lipoate biosynthesis and incorporation II, role of *JAK2* in hormone-like cytokine signaling, inflammasome pathway, growth hormone signaling, and acetate conversion to acetyl-CoA. In the case of males, some of the pathways were inflammasome pathway, *TREM1* signaling, intrinsic prothrombin activation pathway, pentose phosphate pathway, Gai signaling, *STAT3* pathway, and *GP6* signaling pathway.

The top four CpGs showing differences between WC ≤ 88 or > 88 cm in females were cg09907509 (*c13orf36*), cg17478979 (*ZC3H12D*), cg24679890 (*MYO9B*), and cg06638795 (*KCNG3*) (Supplementary Table S4A). In males, the top four CpGs with differences between WC ≤ 102 or > 102 cm were cg01807303 (NA), cg03325085 (*JPH3*), cg02813542 (*TCP11L1*), and cg16379885 (*GRIK3*) (Supplementary Table S4B).

Table 1. Anthropometric, clinical, and biochemical characteristics of the study population categorized by project/consortium.

	TOTAL		ADULTS (n = 474)																	
			DiOGenes-UNAV		OBEPALIP		Food4Me-UNAV		GEDYMET		ICTUS		NUGENOB-UNAV		PREDIMED-UNAV		RESMENA		NormoP		OBEKIT	
Variables	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**	n	**Values**
Sex (females)	474	303 (64)	52	27 (52)	29	29 (100)	39	21 (54)	57	57 (100)	7	5 (71)	22	14 (64)	116	59 (51)	44	22 (50)	12	6 (50)	96	63 (66)
Age (years)	474	47.0 (14.3)	52	42.7 (5.8)	29	37.4 (7.3)	39	41.7 (10.0)	57	27.0 (6.2)	7	57.1 (7.4)	22	34.7 (9.7)	116	65.0 (3.7)	44	48.6 (10.1)	12	39.4 (5.6)	96	46.8 (9.6)
Weight (kg)	474	81.7 (19.1)	52	95.3 (17.7)	29	83.1 (9.5)	39	74.4 (14.6)	57	60.7 (8.8)	7	121.9 (15.2)	22	87.3 (20.8)	116	71.7 (9.2)	44	103.0 (18.1)	12	65.8 (9.3)	96	89.2 (13.6)
BMI (kg/m^2)	474	30.0 (5.7)	52	33.9 (3.8)	29	31.6 (3.1)	39	26.0 (5.3)	57	24.1 (3.5)	7	44.3 (4.0)	22	31.1 (8.2)	116	27.7 (2.3)	44	36.5 (3.7)	12	22.8 (1.5)	96	31.9 (3.7)
Waist circumference (cm)	473	95.8 (16.1)	52	107.5 (11.5)	29	95.4 (6.8)	39	87.9 (12.4)	57	72.7 (7.9)	7	125.3 (11.1)	22	93.7 (19.4)	115	91.8 (8.2)	44	112.5 (12.4)	12	78.2 (7.5)	96	104.1 (10.5)
Female ≤ 88 (cm)	121	76.3 (7.8)	0	NA	2	81.6 (3.2)	14	77.5 (7.4)	55	71.9 (6.7)	0	NA	5	72.0 (4.6)	35	82.3 (4.9)	0	NA	6	74.4 (8.0)	4	85.1 (3.0)
Female > 88 (cm)	182	100.9 (10.0)	27	102.9 (9.1)	27	96.4 (5.8)	7	97.9 (10.0)	2	95.0 (1.4)	5	120.6 (8.9)	9	102.9 (10.7)	24	92.8 (3.5)	22	105.7 (10.7)	0	NA	59	102.0 (9.7)
Male ≤ 102 (cm)	82	92.8 (7.4)	5	97.6 (3.0)	0	NA	16	90.1 (9.2)	0	NA	0	NA	5	81.0 (4.4)	47	95.6 (4.5)	1	94.0 (NA)	6	82.0 (4.8)	2	95.4 (4.0)
Male > 102 (cm)	88	114.8 (10.0)	20	116.0 (10.5)	0	NA	2	109.0 (8.5)	0	NA	2	137.2 (5.4)	3	123.2 (13.0)	9	105.6 (2.6)	21	120.5 (8.5)	0	NA	31	111.0 (7.2)

Values are mean (SD), except for sex, which is represented as number of cases (%). BMI: body mass index; NA: not applicable.

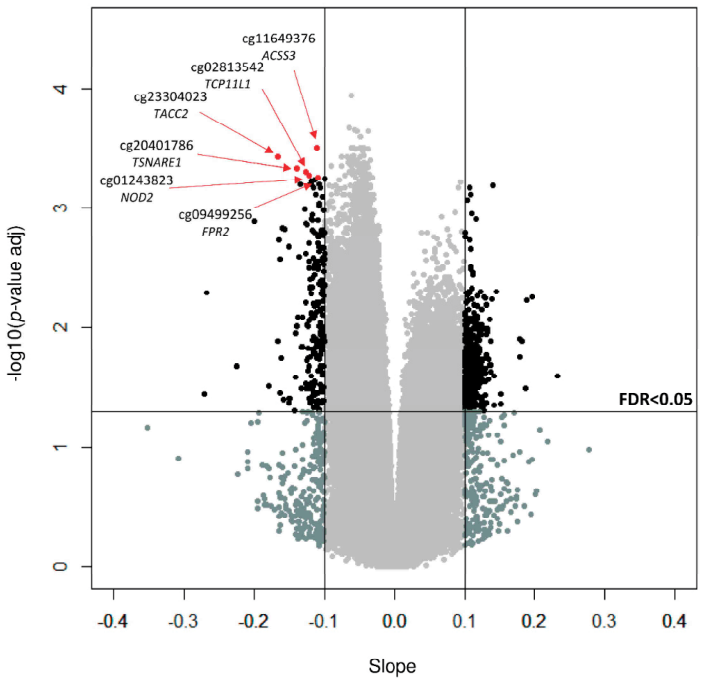

Figure 1. Volcano plot of waist circumference-associated CpGs (corresponding gene according to Illumina). Points above the horizontal line showed a false discovery rate (FDR) < 0.05 and outside the vertical lines represented a slope ≥ |0.1|.

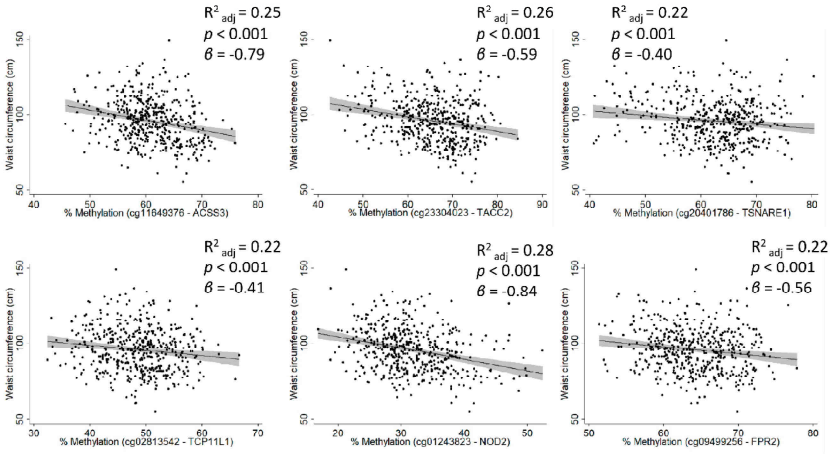

Figure 2. Linear regression graphs adjusted by sex and age representing the association between waist circumference and methylation β values of top six CpGs (corresponding gene according to Illumina) selected by a slope ≥ |0.1| and false discovery rate < 0.05. The grey stripe represent a 95% confidence band.

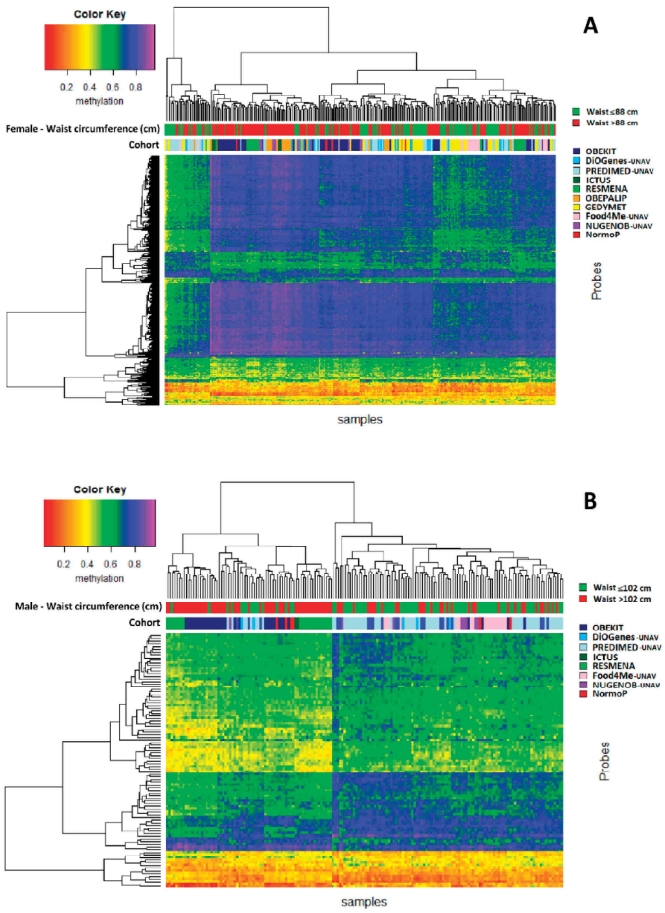

Figure 3. Heat maps representing the clusters between methylation levels (rows) and subjects from the different cohorts (columns). (**A**) Heat map of 375 CpGs selected by Student's *t*-test between females with waist circumference ≤ 88 and > 88 cm. (**B**) Heat map of 95 CpGs selected by Student's *t*-test between males with waist circumference ≤ 102 and > 102 cm. Significance: $p < 7.47 \times 10^{-5}$ after Bonferroni correction.

In order to analyze whether the four CpGs were valuable for assessing WC, areas under the curve (AUC) of the ROC curve were calculated for each CpG. For females, the AUCs were cg09907509 = 0.74, cg17478979 = 0.73, cg24679890 = 0.72, and cg06638795 = 0.72 (Figure 4A). For males, the AUCs were cg01807303 = 0.76, cg03325085 = 0.75, cg02813542 = 0.75, and cg16379885 = 0.75 (Figure 4B). Validation of these results was performed in an independent sample ($n = 161$). Results showed valuable AUCs (AUC > 0.70) for the four CpGs in females, confirming the results of the current investigation, whereas only cg02813542 was validated in males (Table 2).

Genes **2019**, *10*, 444

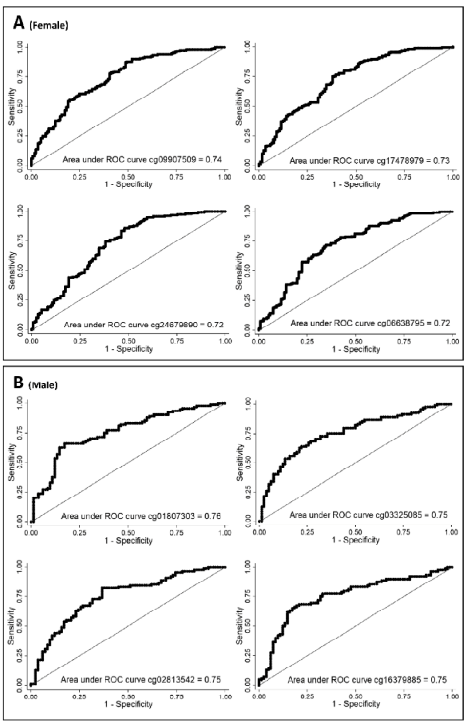

Figure 4. Receiver operating characteristic (ROC) curves. (**A**) Top four significant differentially methylated CpGs for females adjusted by age; (**B**) Top four significant differentially methylated CpGs for males adjusted by age.

Table 2. Validation of ROC curves in an independent sample (n = 161) for the top four significant differentially methylated CpGs for each sex adjusted by age.

CpG	AUC
Women	
cg09907509	0.73
cg17478979	0.77
cg24679890	0.72
cg06638795	0.72
Men	
cg01807303	0.63
cg03325085	0.60
cg02813542	0.71
cg16379885	0.62

4. Discussion

This study involving the MENA project reports the association between DNA methylation and WC in specific CpGs of several genes, mainly related to inflammation, obesity, and related comorbidities. Furthermore, current analyses showed the differential pattern in methylation depending on the degree of abdominal fat in both sexes, which can be predicted by several CpGs with AUCs > 0.70. This assay

adds further insights into the relationship between obesity, associated comorbidities, and epigenetic DNA methylation.

Obesity is usually classified according to BMI, which is an index widely employed for measuring the overall body size or generalized adiposity [12]. However, WC has been suggested as a better estimator of metabolic complications accompanying excessive adiposity [1], being associated with metabolic diseases, such as type 2 diabetes (T2D) and cardiovascular events [1]. Indeed, few EWAS have studied the putative association between DNA methylation and WC values [10–16], in contrast to the number of EWAS correlating methylation with BMI. In this sense, we found a relationship between DNA methylation levels of 669 CpGs and WC (slope ≥ |0.1| and false discovery rate < 0.05). The top six CpGs corresponded to the genes *ACSS3*, *TACC2*, *TSNARE1*, *TCP11L1*, *NOD2*, and *FPR2* (according to the Illumina CG database). Interestingly, methylation of all these genes was also associated with BMI (data not shown), indicating that both general and central adiposity might be related to epigenetic modifications in these genes [41]. Moreover, in a subanalysis (n = 108) concerning the current study, WC was associated with circulating levels of TNF-α and C-reactive protein (CRP), and some of the selected CpGs were also related to these inflammatory molecules (data not shown). These associations confirm that inflammatory processes are involved in the obesity status as previously described [41], which may be partly mediated by epigenetic mechanisms.

Remarkably, five of the six selected genes have been found related to different traits in previous EWAS. For instance, BMI has been associated with methylation in CpGs of the genes *FPR2*, *TACC2*, *TSNARE1*, *TCP11L1*, and *NOD2* [42]. Interestingly, the selected CpG cg01243823 (*NOD2*) from this study was related to BMI in different investigations [9,42–44]. Moreover, methylation in some CpGs located in the genes *TACC2*, *TSNARE1*, *TCP11L1*, and *NOD2*, including the CpGs cg02813542 (*TCP11L1*) and cg01243823 (*NOD2*) selected in this study, was statistically different when comparing diabetic and non-diabetic subjects [45]. Furthermore, the CpG cg02813542 was associated with BMI in adipose tissue from non-diabetic subjects, and the CpG cg01243823 was related to fasting glucose in the same samples [45]. Other CpGs in the genes *NOD2* [46] and *TCP11L1* [47] have been linked to atherosclerosis, and in the *TSNARE1* gene, with CRP [48]. The inflammation-related molecule CRP was also connected to the CpG cg01243823 selected in this research [48].

Although DNA methylation in the selected CpGs might not be exerting changes in the expression of the genes where they are located, their involvement in metabolic and inflammatory processes might indicate a putative connection. Specifically, the gene *ACSS3* encodes a protein that converts propionate to propionyl-CoA allowing it to enter mitochondrial respiration and the Krebs cycle [49]. Proteins of the same family (acyl-CoA synthases) have been related to inflammatory processes [50,51]. The gene *TACC2* has been found hypomethylated before weight loss in the top 20 differentially methylated CpGs in a study comparing before and after weight loss [52]. In the case of *TSNARE1*, differential methylation levels between basal and insulin-stimulated muscle has been described [53], as well as the association between methylation degree and C-reactive protein, which is a sensitive marker for low-grade inflammation [48]. The *NOD2* gene has been related to inflammation, since a higher expression of *NOD2* may contribute to an increased inflammatory response of immune cells in diet-induced obesity [54]. Genetic variants of *NOD2* may also influence the risk of chronic inflammation, insulin resistance, and T2D [55]. The gene *FPR2* binds to lipid mediators such as resolvin D1 for promoting resolution of inflammation [56], including the resolution of obesity-induced chronic low-grade inflammation [57]. These lipid mediators have been found decreased in obese mice [58]. In the case of the *TCP11L1* gene, two SNPs (rs3168277 and rs2273553) showed significant association with fasting blood glucose and HDL-C, respectively [59].

Abdominal obesity is a major risk factor concerning systemic inflammation, hyperlipidemia, insulin resistance, and cardiovascular disease [60]. Our study revealed that individuals with abdominal obesity traits (WC females > 88 cm, males > 102 cm) exhibited a differential methylation pattern for at least 375 and 95 CpGs, respectively. Remarkably, more than 75% of the males and more than 80% of the females that were in the same cluster with similar methylation patterns presented abdominal

obesity. Therefore, specific epigenetic modifications may be representative for people with central obesity and might potentially influence the onset and development of other adiposity-associated complications, such as T2D and cardiovascular events. Indeed, these differentially methylated CpGs were related to pathways of inflammation, obesity, and related comorbidities for both males and females, such as the pathway lipoate biosynthesis and incorporation II, since lipoic acid has anti-inflammatory and anti-oxidant actions, and it has been reported to be helpful against insulin resistance and hypertriglyceridemia [61]. Additionally, the role of *JAK2* in the hormone-like cytokine signaling pathway, where *JAK2* is involved in binding to receptors such as *GHR*, activating the signal transduction cascade, and growth hormone signaling. The expression of *GHR* has been found to be increased in adipose tissue abdominal depots, compared to gluteal depots, suggesting an effect of growth hormone to specifically reduce abdominal adipose tissue mass [62]. Moreover, growth hormone secretion is usually impaired in obesity [63]. Furthermore, in the inflammasome pathway, inflammasomes play the role of central regulators connecting the induction and the progression of autoinflammatory disease with cellular stress from obesity-induced inflammation, metabolic distress, and other stress signals [64]. The *TREM-1* gene in TREM1 signaling acts as an amplifier of inflammation expressed in macrophages. *TREM-1* has been found to be overexpressed in patients with obesity, predisposing pre-diabetics to obesity-induced insulin resistance [65]. The rate-limiting enzyme in the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD), is an important mediator of adipose tissue inflammation and insulin resistance in subjects with obesity [66]. In the case of the intrinsic prothrombin activation pathway, prothrombin activity is higher in individuals with central obesity [67], and weight loss interventions reduce the circulating levels of prothrombic markers [68].

The top four CpGs that were found to be differentially methylated between individuals with and without abdominal obesity were located at the genes *c13orf36*, *ZC3H12D*, *MYO9B*, and *KCNG3* in females; and *JPH3*, *TCP11L1*, and *GRIK3* in males (one CpG had no annotated gene). As aforementioned, the observed DNA methylation differences might not be indicative of changes in the corresponding gene expression, but their relationship with obesity and other metabolic processes might suggest a possible link. For instance, the gene *ZC3H12D* is involved in inflammatory diseases and in immune response [69]. CpG cg24679890 (*MYO9B*) has been previously related to BMI in various EWAS [16]. In the case of *JPH3*, it is necessary for glucose-stimulated insulin secretion [70]. Additionally, the gene *GRIK3*, involved in glutamate receptor signaling, was one of the genes differentially methylated related to inflammation and T2D in siblings born before and after maternal bariatric surgery [71]. Interestingly, cg02813542 (*TCP11L1*) also appeared in the top six CpGs after the association between DNA methylation and WC, as previously mentioned. Nevertheless, the *c13orf36* and *KCNG3* genes have not already been described in relation to obesity.

Since the methylation patterns of some CpGs were able to characterize individuals according to whether they do or do not have abdominal obesity, the ability of the four CpGs with higher AUC to differentiate between both WC groups for each sex was analyzed. All the CpGs distinguished WC groups with AUCs above 0.70. However, validation in an independent sample showed that cg09907509-*c13orf36*, cg17478979-*ZC3H12D*, cg24679890-*MYO9B*, and cg06638795-*KCNG3* for females, and cg02813542-*TCP11L1* for males were capable of discriminating individuals regarding their central adiposity. Although these CpGs cannot be confirmed as a cause or consequence of central adiposity, they might be considered for futures studies as potential biomarkers for early prediction or putative targets for the development of therapeutic approaches in the prevention and treatment of diseases, such as obesity or associated comorbidities.

The present study presents some limitations. The most important is the cross-sectional nature of the study, which does not allow the establishment of causality. Longitudinal studies are necessary to evaluate the direction of the association between DNA methylation and WC, although a validation in an independent population (n = 161) confirmed the general trends. In addition, RNA assays would have been interesting to relate methylation to gene expression. Another limitation is that methylation is tissue-specific [72]. The ideal tissue for this study would have been visceral adipose

tissue, but peripheral blood is the best non-invasive alternative tissue that reflects multiple metabolic and inflammatory pathways. Indeed, multiple studies have analyzed DNA methylation in blood in relation to obesity [9,10,73] and inflammation [74,75]. Furthermore, some studies have demonstrated that blood can reflect epigenetic changes in other tissues, such as adipose tissue [73,76]. Finally, though multiple comparison tests and statistical adjustments for potential confounding factors such as sex, age, cohorts, bead chips, and cell composition heterogeneity were performed, type I and type II errors could not be discarded. However, correction of inflation was performed to avoid type I errors. Despite these limitations, we understand that our study contributes to the growing body of evidence in support of epigenetic methylation mechanisms correlating with obesity features in a relative wide sample (n = 473), followed by a validation (n = 161).

In conclusion, this study found associations between DNA methylation in several CpGs and an excessive central adiposity in peripheral white blood cells. In addition, novel loci with differential DNA methylation variations in individuals with abdominal adiposity were identified. Further studies, especially longitudinal studies, are needed to assess causality and identify additional putative methylation biomarkers and early predictors of obese phenotypes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/10/6/444/s1: Figure S1: Q–Q plot of corrected adjusted p-values, Figure S2: Canonical pathways from Ingenuity Pathway Analysis. A) Canonical pathways from 375 CpGs selected by Student's t-test between females with waist circumference ≤ 88 and > 88 cm. B) Canonical pathways from 95 CpGs selected by Student's t-test between males with waist circumference ≤ 102 and > 102 cm. Significance: $p < 7.47 \cdot 10^{-5}$ after Bonferroni correction, Table S1: Anthropometric, clinical and biochemical characteristics of the validation population, Table S2: Summary of the studies and the collected anthropometric and DNA methylation measurements, Table S3: Selection of 669 CpGs applying false discovery rate cut-off of 0.05 and a slope ≥ |0.1|, Table S4: Selection of CpGs separating by waist circumference cut-points. (A) Selection of 375 CpGs for females after comparing waist circumference ≤ 88 and > 88 cm. (B) Selection of 95 CpGs for males after comparing waist circumference ≤ 102 and > 102 cm.

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Supplementary material

Methylome-wide association study in peripheral white blood cells focusing on central obesity and inflammation

Available at: https://www.mdpi.com/2073-4425/10/6/444#supplementary

Table S1: Anthropometric, clinical and biochemical characteristics of the validation population.

	TOTAL		ADULTS (n=161)			
			NormoP		OBEKIT	
Variables	**n**	**Values**	**n**	**Values**	**n**	**Values**
Sex (females)	161	122 (76)	54	42 (78)	107	80 (75)
Age (years)	161	43.8 (10.5)	54	40.1 (10.0)	107	45.6 (10.3)
Weight (kg)	161	77.8 (17.0)	54	60.5 (9.4)	107	86.5 (12.7)
BMI (kg/m²)	161	28.3 (5.4)	54	21.8 (1.9)	107	31.5 (3.3)
Waist circumference (cm)	161	91.9 (15.4)	54	74.7 (7.2)	107	100.6 (10.3)
Female ≤88 (cm)	54	75.3 (7.2)	42	72.6 (5.7)	12	84.5 (2.2)
Female >88 (cm)	68	100.7 (7.8)	0	NA	68	100.7 (7.8)
Male ≤102 (cm)	19	87.1 (9.0)	12	82.1 (6.9)	7	95.6 (4.4)
Male >102 (cm)	20	111.8 (7.7)	0	NA	20	111.8 (7.7)

Values are Mean (SD), except for Sex, which is represented as number of cases (%).
BMI: Body mass index; NA: not applicable

Table S2: Summary of the studies and the collected anthropometric and DNA methylation measurements.

		Study design	Anthropometric measurements		DNA methylation		
Study	n		Timing	WC measurement	Timing	Tissue	Illumina array
DiOGenes-UNAV	52	Assess the efficacy of moderate-fat diets that vary in protein content and glycaemic index in the prevention of weight regain and obesity-related risk factors after weight loss.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
OBEPALIP	29	Parallel, short-term randomized double blind placebo-controlled trial designed with the objective of evaluating the potential body weight-lowering effects of dietary supplementation with eicosapentanoic acid and α-lipoic acid separately or in combination, in healthy overweight/obese women during a hypocaloric diet.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
Food4Me-UNAV	39	Investigate the utility of a personalised nutrition approach for improving nutritional and diet-related outcomes.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
GEDYMET	57	Analyse the associations between methylation levels and biochemical measurements related to glucose and insulin in non-diabetic individuals.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
ICTUS	7	Case-control nutritional intervention with the objective of investigating the effect of the dietary treatment on anthropometric measurements, inflammation markers, lipid profile, insulin status and the methylation patterns of two stroke-related genes in obese individuals who have suffered an ischemic stroke insult.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
NUGENOB-UNAV	22	Randomized, parallel, 2-arm, open-label 20-week dietary intervention of 2 hypo-energetic diets (high- versus low-fat diet) to mainly examine if there is an interaction between the nutrient composition of the diet, specifically the fat content, and obesity related genes in response to the dietary treatment.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
PREDIMED-UNAV	116	Randomized, primary prevention feeding trial with blinded assessment of end points conducted in several centres of Spain with the objective of evaluating the effects of the MedDiet on primary cardiovascular prevention.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
RESMENA	44	Randomized, longitudinal and controlled intervention trial to compare the effects of 2 hypocaloric dietary strategies on metabolic syndrome comorbidities over a 6-month period as main objective.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Human Methylation 450k
NormoP	12	Collecting control data for the OBEKIT study.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Methylation EPIC
OBEKIT	96	Evaluate the response to a 2 hypocaloric diets with different macronutrient composition based on the genetic background.	Baseline	The mid-point between the lower rib and the iliac crest using a tape measure	Baseline	PWBC	Methylation EPIC

PWBC: peripheral white blood cells; WC: Waist circumference

Supplementary Figure S1: Q-Q plot of corrected adjusted p-values.

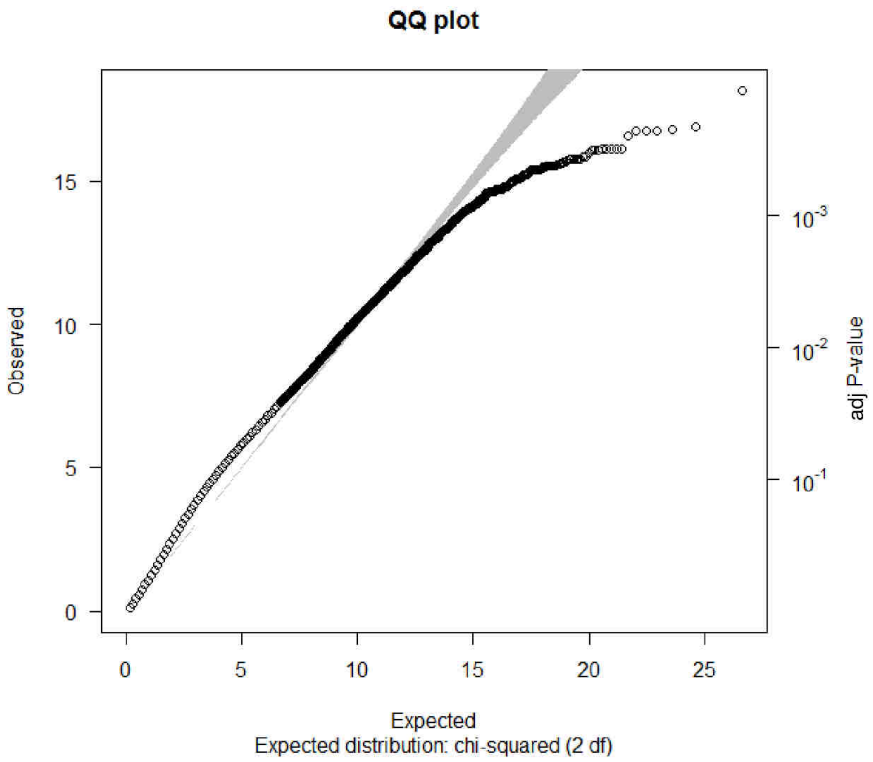


Table S3: Selection of 669 CpGs applying False Discovery Rate cut-off of 0.05 and a slope ≥ |0.1|.

CG_ID	adj pvalue correc	slope	Ave_ Methylation	CHR	MAP INFO	UCSC_ RefGene_ Name	UCSC_ RefGene_ Accession	UCSC_ RefGene_ Group
cg11649376	0.00031777	-0.11064177	60.7919961	12	81473234	ACSS3	NM_024560	Body
cg23304023	0.00037758	-0.16659719	65.5262402	10	123949949	TACC2;TACC2;TACC2	NM_206862;NM_006997;NM_206861;NM_206860	Body;Body;Body;Body
cg20401786	0.00046145	-0.13895546	62.4224121	8	143472994	TSNARE1	NM_145003	5'UTR
cg02813542	0.00049817	-0.12712325	48.4997434	11	33084877	TCP11L1;TCP11L1	NM_001145541;NM_018393	Body;Body
cg01243823	0.00053187	-0.1225277	31.5644568	16	50732212	NOD2	NM_022162	Body
cg09499256	0.00055046	-0.10881852	64.0887002	19	52263842	FPR2	NM_001005738	TSS1500
cg18391611	0.00056554	-0.10027695	73.991414	19	56042991	SBK2	NM_001101401	Body
cg06186457	0.00057587	-0.11342644	55.8815692	6	4504273	NA	NA	NA
cg13867274	0.00059083	-0.11820995	47.0346483	9	98281968	NA	NA	NA
cg22964775	0.00063166	-0.11867195	47.6663059	4	62362347	LPHN3	NM_015236	TSS1500
cg05223392	0.00063367	-0.13551048	71.6487031	7	2647927	IQCE;IQCE	NM_001100390;NM_152558	Body;Body
cg13741781	0.00063367	-0.1200258	55.1790653	14	106408885	NA	NA	NA
cg19824396	0.00063367	-0.10744884	74.298365	1	203018871	PPFIA4	NM_015053	TSS1500
cg16379885	0.00064097	0.13987836	22.7553199	1	37500369	GRIK3	NM_000831	TSS1500
cg17461336	0.00066952	-0.10600461	50.5063856	7	99441559	CYP3A43;CYP3A43;CYP3A43	NM_022820;NM_057096;NM_057095	Body;Body;Body
cg23768702	0.00067494	-0.11554175	55.2481212	18	44557673	TCEB3CL;TCEB3C;KATNAL2	NM_001100817;NM_145653;NM_031303	TSS1500;TSS1500;5'UTR
cg19265948	0.00067499	0.10745866	66.2677961	12	23229286	NA	NA	NA
cg08145495	0.0007611	-0.11258615	59.1694554	19	5834725	FUT6;FUT6	NM_001040701;NM_000150	5'UTR;5'UTR
cg00470882	0.0007677	0.10790901	79.1752165	3	122380166	NA	NA	NA
cg05956076	0.00079138	-0.1023847	75.2061406	6	32074934	TNXB	NM_019105	5'UTR
cg02541778	0.0008577	0.10432121	24.9990472	8	55382901	NA	NA	NA
cg07195224	0.00090404	-0.10824672	27.4994219	1	159047034	AIM2	NM_004833	TSS1500
cg05635169	0.00094914	-0.10807901	59.5204494	5	113589213	NA	NA	NA
cg10636246	0.00100719	-0.12870173	33.6059984	1	159046973	AIM2	NM_004833	TSS1500
cg02180424	0.00103593	-0.10119307	73.2727292	14	48095779	MDGA2	NM_001113498	Body
cg06745955	0.00110401	0.11021538	25.2029091	17	27038470	PROCA1	NM_152465	Body
cg15498134	0.00111724	-0.11655323	64.4915783	1	25246854	RUNX3;RUNX3	NM_001031680;NM_004350	Body;Body
cg17929770	0.00122527	0.11634104	24.2454443	19	46318514	RSPH6A;RSPH6A	NM_030785;NM_030785	1stExon;5'UTR
cg22351824	0.001231	-0.11964649	56.6347306	22	24084185	ZNF70	NM_021916	3'UTR

(The rest of the table is available at: https://www.mdpi.com/2073-4425/10/6/444#supplementary)

Table S4A: Selection of 375 CpGs for females after comparing waist circumference ≤88 and >88 cm.

CG_ID	Tstudent	UCSC_RefGene_Name	UCSC_RefGene_Accession	UCSC_RefGene_Group
cg09907509	1.1919E-13	C13orf36;C13orf36	NM_203451;NM_203451	5'UTR;1stExon
cg17478979	2.2136E-12	ZC3H12D	NM_207360	Body
cg24679890	4.5248E-12	MYO9B;MYO9B	NM_001130065;NM_004145	Body;Body
cg06638795	7.317E-12	KCNG3;KCNG3	NM_133329;NM_172344	Body;Body
cg14603345	8.4133E-12	BTBD3	NM_181443	TSS200
cg24799710	9.4942E-12	GHR	NM_000163	5'UTR
cg05968268	4.3646E-11	NA	NA	NA
cg19824396	4.9065E-11	PPFIA4	NM_015053	TSS1500
cg11649376	5.7439E-11	ACSS3	NM_024560	Body
cg14523238	1.0572E-10	GABBR2	NM_005458	Body
cg06745955	1.4915E-10	PROCA1	NM_152465	Body
cg19927816	1.6479E-10	SYMPK;RSPH6A	NM_004819;NM_030785	Body;TSS1500
cg17929770	1.662E-10	RSPH6A;RSPH6A	NM_030785;NM_030785	1stExon;5'UTR
cg02541778	1.829E-10	NA	NA	NA
cg04624110	2.0654E-10	MACROD2	NM_080676	TSS200
cg23304023	2.4871E-10	TACC2;TACC2;TACC2;TACC2	NM_206862;NM_006997;NM_206861;NM_206860	Body;Body;Body;Body
cg05336395	3.8826E-10	PCDH8;PCDH8	NM_002590;NM_032949	1stExon;1stExon
cg11653966	4.0695E-10	NPY	NM_000905	Body
cg19265948	5.4978E-10	NA	NA	NA
cg16379885	8.2655E-10	GRIK3	NM_000831	TSS1500
cg05223392	9.8839E-10	IQCE;IQCE	NM_001100390;NM_152558	Body;Body
cg24005196	1.0283E-09	PRL;PRL	NM_001163558;NM_000948	Body;Body
cg00470882	1.041E-09	NA	NA	NA
cg13300273	1.1518E-09	GPR25	NM_005298	1stExon
cg11311843	1.2818E-09	PHOSPHO1;PHOSPHO1	NM_178500;NM_001143804	Body;Body
cg03782202	1.8034E-09	HOXD11	NM_021192	TSS1500
cg00999410	1.8646E-09	OR6C1	NM_001005182	TSS200
cg22655988	1.9055E-09	CRMP1;CRMP1	NM_001014809;NM_001313	Body;Body
cg02831260	2.386E-09	NA	NA	NA

(The rest of the table is available at: https://www.mdpi.com/2073-4425/10/6/444#supplementary)

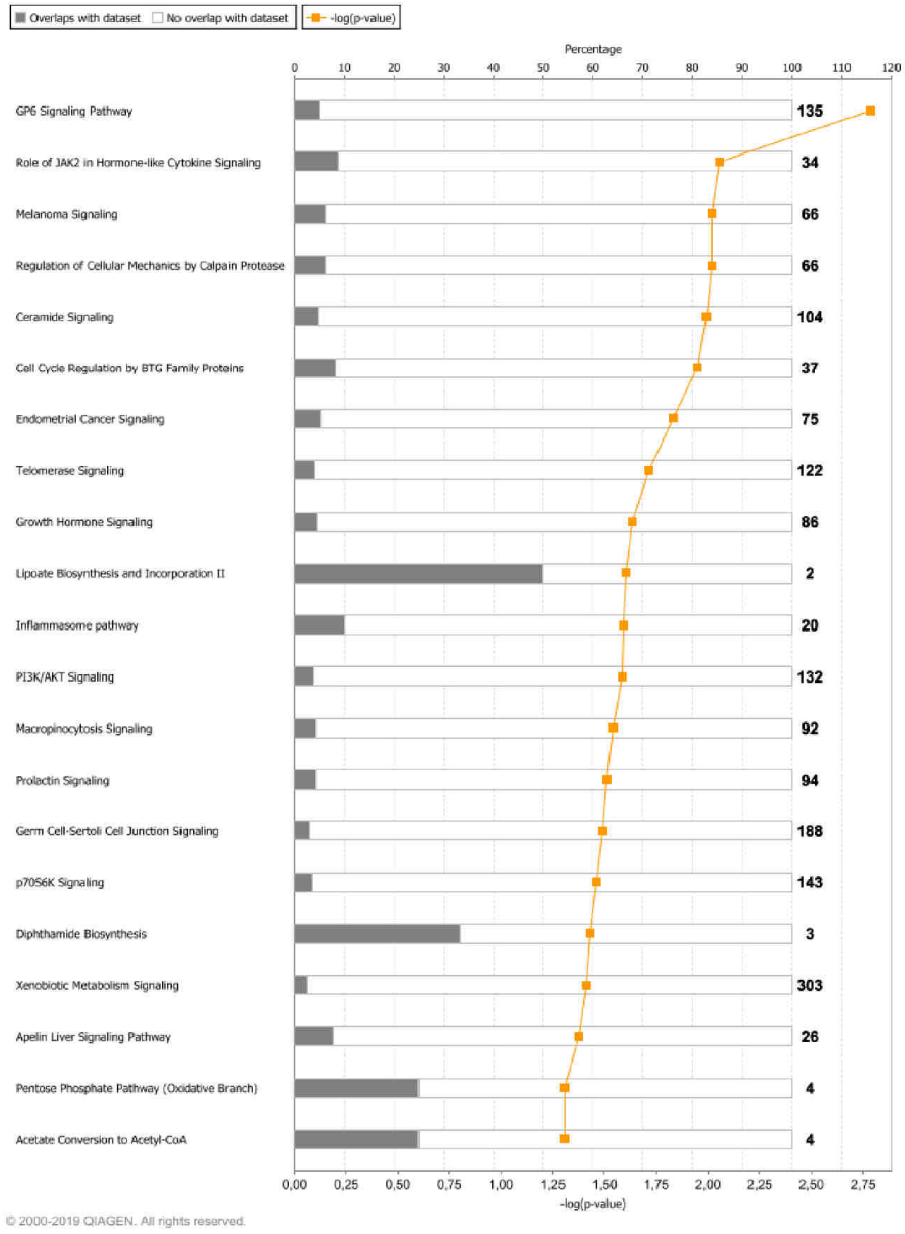
Table S4B: Selection of 95 CpGs for males after comparing waist circumference ≤102 and >102 cm.

CG_ID	T student	UCSC_RefGene_Name	UCSC_RefGene_Accession	UCSC_RefGene_Group
cg01807303	1.5859E-08	NA	NA	NA
cg03325085	4.4713E-08	JPH3	NM_020655	Body
cg02813542	1.1916E-07	TCP11L1;TCP11L1	NM_001145541;NM_018393	Body;Body
cg16379885	1.3091E-07	GRIK3	NM_000831	TSS1500
cg18989499	1.3323E-07	NA	NA	NA
cg09460490	1.7218E-07	NA	NA	NA
cg15624109	2.2665E-07	NA	NA	NA
cg01091079	2.3947E-07	NA	NA	NA
cg09858655	2.4879E-07	NA	NA	NA
cg21908038	2.6011E-07	DNASE2	NM_001375	3'UTR
cg09499256	4.5589E-07	FPR2	NM_001005738	TSS1500
cg01996643	6.4333E-07	KLC1;KLC1;KLC1	NM_001130107;NM_005552;NM_182923	5'UTR;5'UTR;5'UTR
cg15498134	6.7964E-07	RUNX3;RUNX3	NM_001031680;NM_004350	Body;Body
cg20980653	1.3616E-06	COL11A2;COL11A2;COL11A2	NM_080679;NM_080681;NM_080680	Body;Body;Body
cg05223392	1.3661E-06	IQCE;IQCE	NM_001100390;NM_152558	Body;Body
cg13800700	1.5537E-06	NA	NA	NA
cg06745955	1.7893E-06	PROCA1	NM_152465	Body
cg10739132	1.8184E-06	FXR1;FXR1;FXR1	NM_001013438;NM_001013439;NM_005087	Body;5'UTR;Body
cg09105334	1.9106E-06	NA	NA	NA
cg22351824	1.9623E-06	ZNF70	NM_021916	3'UTR
cg15102655	2.1775E-06	NA	NA	NA
cg08907282	2.7143E-06	LOC441089	NR_003665	TSS1500
cg13741781	2.7438E-06	NA	NA	NA
cg03660952	2.7455E-06	KCNQ1;KCNQ1	NM_000218;NM_181798	Body;Body
cg05784856	2.7498E-06	NA	NA	NA
cg24152845	2.7736E-06	SNORD115-1	NR_001291	TSS1500
cg05956076	2.822E-06	TNXB	NM_019105	5'UTR
cg02347487	2.8826E-06	NLRP14	NM_176822	5'UTR
cg23795893	3.5161E-06	PGR	NM_000926	TSS200

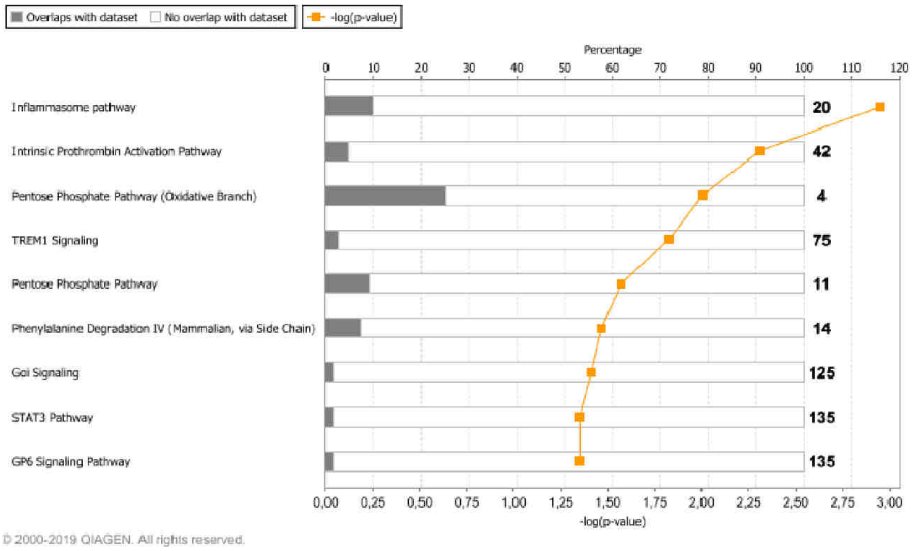
(The rest of the table is available at: https://www.mdpi.com/2073-4425/10/6/444#supplementary)

Supplementary Figure S2: Canonical pathways from Ingenuity Pathway Analysis. A) Canonical pathways from 375 CpGs selected by Student's t-test between females with waist circumference ≤88 and >88 cm. B) Canonical pathways from 95 CpGs selected by Student's t-test between males with waist circumference ≤102 and >102 cm. Significance: $p<7.47 \cdot 10^{-5}$ after Bonferroni correction.

A (Fem)



B (Male)



CHAPTER 7

Interaction among sex, aging and epigenetic processes concerning visceral fat, insulin resistance and dyslipidaemia

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Interaction Among Sex, Aging, and Epigenetic Processes Concerning Visceral Fat, Insulin Resistance, and Dyslipidaemia

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The distribution of adipose tissue is influenced by gender and by age, shifting from subcutaneous to visceral depots with longevity, increasing the development of several aging-related diseases and manifestations such as obesity, metabolic syndrome, and insulin resistance. Epigenetics might have an important role in aging processes. The aim of this research was to investigate the interactions between aging and epigenetic processes and the role of visceral adipose tissue, insulin resistance, and dyslipidaemia. Two different study samples of 366 and 269 adult participants were analyzed. Anthropometric, biochemical (including the triglycerides-glucose (TyG) index), and blood pressure measurements were assessed following standardized methods. Body composition measurements by Dual-energy X-ray absorptiometry (DXA) were also performed for the second sample. Methylation data were assessed by Infinium Human Methylation BeadChip (Illumina) in peripheral white blood cells. Epigenetic age acceleration was calculated using the methods DNAmAge (AgeAcc) and GrimAge (AgeAccGrim). Age acceleration (AgeAccGrim) showed better correlations than AgeAcc with most of the measured variables (waist circumference, glucose, HOMA IR, HDL-cholesterol, triglycerides, and TyG index) for the first sample. In the second sample, all the previous correlations were confirmed, except for HOMA-IR. In addition, many of the anthropometrical measurements assessed by DXA and C-reactive protein (CRP) were also statistically associated with AgeAccGrim. Associations separated by sex showed statistically significant correlations between AgeAccGrim and HDL-cholesterol or CRP in women, whereas, in men, the association was with visceral adipose tissue mass DXA, triglycerides and TyG index. Linear regression models (model 1 included visceral adipose tissue mass DXA and TyG index and model 2 included HDL-cholesterol and CRP) showed a significant association for men concerning visceral adipose tissue mass DXA and TyG index, while HDL-cholesterol and CRP were associated in women. Moreover, structural equation modeling showed that the TyG index was mediating the majority of the visceral

adipose tissue mass action on age acceleration. Collectively, these findings showed that there are different mechanisms affecting epigenetic age acceleration depending on sex. The identified relationships between epigenetic age acceleration and disease markers will contribute to the understanding of the development of age-related diseases.

Keywords: DNA methylation, visceral adipose tissue, C-reactive protein, HDL-cholesterol, TyG index

INTRODUCTION

Adipocytes are not only cells responsible for fuel storage triggered by a chronic imbalance between energy intake and energy expenditure, but they also participate in hormonal regulatory functions as well as in the synthesis of several endocrine/autocrine factors implicated in body homeostasis (1).

Excessive adipose tissue mass, particularly in the visceral compartment, has been related to hyperglycaemia, hypertension, hypercholesterolemia, and inflammatory processes (2). Indeed, obesity is associated with insulin resistance (IR), which, in turn, is also related to increased circulating levels of non-esterified fatty acids (3). This excess of lipids not only accumulates in different tissues, but it is also transported as very low-density lipoprotein triglycerides and secreted by the liver, leading to dyslipidaemia, which is a common feature of obesity and diabetes (4). These metabolic dysregulations are usually accompanied by changes in the endocrine balance (5) and in the secretory pattern of the adipose tissue (6, 7). Most of these metabolic processes are also related to aging, since nutrient-regulated pathways and energy balance may have a role in accelerating or delaying senescence (8).

On the other hand, the distribution of adipose tissue changes during the course of a lifetime, shifting from subcutaneous depots to intra-abdominal and ectopic fat deposition (9), and it is also influenced by sex (10). This fat/fuel redistribution has also been associated with the development of several aging-related diseases and manifestations, including obesity, metabolic syndrome and IR (11). Inadequate dietary habits and sedentarism, which are intimately related to increases in visceral fat, also contribute to the aging process, partly mediated by epigenetic mechanisms (12, 13).

Epigenetics involves a number of processes that modulate gene expression without altering the DNA sequence, such as DNA methylation, histone modifications and miRNAs (14). Several reports have related epigenetic signatures with longevity and aging. For example, Salas-Pérez et al., identified several differentially methylated sites that could be implicated in longevity and the development of metabolic disturbances (15). Furthermore, Horvath et al. have published several investigations relating DNA methylation with aging, and subsequently, developing different epigenetic clocks to measure "biological age" (16–18). Additionally, there is a general loss of histones in aging cells, and several modifications have been related to lifespan, such as histone methylation or histone acetylation (19). Moreover, miRNAs have also been involved in aging processes; for example, miR-34a has been described as an aging marker in several tissues and systems since it is upregulated in the aging heart, and its inhibition reduces cell death and fibrosis after an acute myocardial infarction (20).

The aim of this research was to investigate the interactions between aging and epigenetic processes, as well as the role of visceral adipose tissue, IR and dyslipidaemia on epigenetic age acceleration, together with putative mediating effects.

SUBJECTS AND METHODS

Participants

This research was performed in two different study samples. The first study involved 366 adult participants from studies and cohorts belonging to the Methyl Epigenome Network Association (MENA) project such as DiOGenes-UNAV with $n =$ 52 (21), OBEPALIP with $n = 29$ (22), Food4Me-UNAV with $n = 39$ (23), GEDYMET with $n = 57$ (24), ICTUS with n=7 (25), NUGENOB-UNAV with $n = 22$ (26), PREDIMED-UNAV with $n = 116$ (27), and RESMENA with $n = 44$ (28), whose providers are gratefully acknowledged.

The second study involved 269 adult participants from the OBEKIT study with $n = 203$ (29) and the NormoP study with $n = 66$ (30). Study design, characteristics, inclusion and exclusion criteria of each of these studies have been previously described. This research was carried out in accordance with the recommendations of the Research Ethics Committee of the University of Navarra (CEI-UN, Pamplona, Spain). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Research Ethics Committee of the University of Navarra (CEI-UN, Pamplona, Spain), except for GEDYMET, which was approved by the ethics committee of the School of Medicine, Pontificia Universidad Católica de Chile (Santiago, Chile).

Study Variables

Anthropometric (waist circumference), blood pressure and biochemical measurements [glucose, HOMA-IR, HDL-cholesterol, triglycerides, TyG index, C-reactive protein (CRP)] were retrieved from databases of the aforementioned studies following validated protocols. The HOMA-IR index was calculated as fasting insulin (μUI/mL) × fasting glucose (mg/dL)/405. The triglycerides-glucose (TyG) index was calculated as Ln(triglycerides [mg/dL] x glucose [mg/dL]/2) as an indicator of IR (31). Body composition in the second study was assessed by Dual energy X-ray absorptiometry (DXA) according to the manufacturer's instructions (Lunar iDXA, enCORE 14.5, GE Healthcare, Madison, WI, USA). Visceral adipose tissue mass was assessed with the CoreScan application for the software enCORE (GE Healthcare, Madison, WI, USA).

DNA Extraction and DNA Methylation Analysis

Venous blood samples were collected in EDTA tubes. Genomic DNA was extracted from peripheral white blood cells using the MasterPure™ DNA Purification kit (Epicenter, Madison, WI) and quantified with Pico Green dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). In order to convert cytosine into uracil, high-quality DNA samples (500 ng) were treated with bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's protocol. Infinium Human Methylation 450K BeadChip technology (Illumina, San Diego, CA, USA) was employed to measure DNA methylation levels in all the studies, except for OBEKIT and NormoP studies, which were performed with Infinium MethylationEPIC BeadChip (Illumina). The analyses were conducted in the Unidad de Genotipado y Diagnóstico Genético from Fundación Investigación Clínico de Valencia, as detailed elsewhere (32).

Treatment of Methylation Raw Data

Beta-values have been used to assess methylation levels in order to estimate the methylation degree using the ratio of the methylation probe intensity and the overall intensity, corresponding to the percentage of methylation on a specific site (33). Intensity data were obtained using the ChAMP package for R v.1.11.0 (34) as described elsewhere (35). Normalization of methylation samples from both studies was performed with the function preprocessNoob of Minfi package from Bioconductor. This function allows background correction with dye-bias normalization (36).

Estimation of Epigenetic Age Acceleration

Epigenetic age accelerations were defined as the residual (difference between the observed and the predicted value) from regressing the epigenetic age on chronological age (AgeAcc for DNAmAge method and AgeAccGrim for GrimAge method) as described by (37). After pre-processing, epigenetic age accelerations were calculated using two different methods available on the website DNA methylation Age Calculator (https://dnamage.genetics.ucla.edu/home) (16). The first method, denominated DNAmAge and developed by Horvath (16), was designed to calculate the epigenetic age (DNAm age) from human samples profiled with the Illumina Infinium 450K platform. This procedure was based on the DNA methylation levels of 353 CpGs. The second method, denominated GrimAge, was recently created by Horvath and collaborators (18). Briefly, this epigenetic clock was a method of epigenetic age prediction based on a linear combination of chronological age, sex, and DNAm-based surrogate biomarkers for seven plasma proteins and smoking pack-years. As indicated by Horvath on the DNA methylation Age Calculator website, samples with a correlation coefficient <0.8 with the gold standard (defined by averaging the beta values across the samples from the largest blood data set) were excluded (data provided by the website application).

Statistical Analysis

Variables with skewness >1 were log2-transformed [glucose, HOMA-IR, triglycerides and CRP (adding +1 to avoid the logarithm of zero)] as previously described (38). Age accelerations (AgeAcc and AgeAccGrim) from the first study were correlated (Pearson's r) with different variables including waist circumference, glucose (log2), HOMA-IR (log2), HDL-cholesterol, triglycerides (log2), systolic and diastolic blood pressure and the TyG index. Differences between women and men in the second study (OBEKIT + NormoP) were calculated using two-independent samples Student *t*-test. AgeAccGrim data from the second study were correlated (Pearson's r) with different anthropometric measurements determined by DXA (fat mass, lean mass, trunk fat mass, android fat mass, gynoid fat mass, visceral adipose tissue mass), the metabolic syndrome variables as measured in the MENA study, and the CRP (log2) as a marker of inflammation. Multiple linear regression models between AgeAccGrim and some variables from the second study (model 1: visceral adipose tissue mass and TyG index, model 2: HDL-cholesterol and CRP) were fitted, separating by sex. Mediation by TyG index in the relationship between AgeAccGrim and visceral adipose tissue mass DXA in men was assessed using structural equation modeling following the Zhao et al. approach (39).

p-values were considered statistically significant if $p<0.05$. Statistical calculations were performed with Stata version 12.1 (StataCorp 2011, College Station, TX, USA).

RESULTS

Correlations in the First Study Sample

Anthropometric, biochemical and blood pressure measurements of the first study are reported (**Table 1**).

Correlations between age accelerations measured by Horvath (AgeAcc) or by Lu et al. (AgeAccGrim) and the anthropometric, biochemical and blood pressure variables were performed for participants from the first study (**Table 2**). Results showed statistically significant associations between both

TABLE 1 | First study phenotypical and clinical characteristics.

	First study	
Variable	***n***	**Values***
Sex (females)	366	234 (63.9%)
Age (years)	366	47.3 (15.4)
Waist circumference (cm)	365	94.2 (16.5)
Glucose (mg/dL)	335	105 (33)
HOMA-IR	224	2.7 (2.5)
HDL-cholesterol (mg/dL)	339	54 (14)
Triglycerides (mg/dL)	339	125 (77)
TyG index	334	8.6 (0.7)
Systolic blood pressure (mmHg)	336	132 (22)
Diastolic blood pressure (mmHg)	336	78 (12)

*Values are represented as Mean (SD) except for sex which is represented as number of females(%).

age accelerations and all the anthropometric and biochemical measurements, except for glucose and triglycerides, which were only significant for AgeAccGrim. In addition, AgeAccGrim showed higher correlation coefficients and statistical significance with most of the variables, while no statistical associations were found with blood pressure.

Correlations in the Second Study Sample

Anthropometric, biochemical, and blood pressure measurements of the second study are reported (**Table 3**). Since several variables showed differences between women and men (**Table 3**), further analyses were performed separating by sex.

Correlations in the second study for both sexes and separated by sex were calculated with AgeAccGrim (**Table 4**) since it was the epigenetic age acceleration that showed better correlation coefficients and significance with many variables analyzed in the first study (**Table 2**). Correlations in the second study were statistically significant for the same variables as those obtained from the first study, except for HOMA-IR, which was not significant in the OBEKIT | NormoP study. Moreover, both CRP and many of the anthropometrical measurements assessed by DXA were also significantly associated with AgeAccGrim. When analyzing by sex, women showed statistically significant correlations between AgeAccGrim and HDL-cholesterol or CRP, whereas in men the identified associations were significant with visceral adipose tissue mass measured by DXA, triglycerides and TyG index. In order to analyse whether these significant correlations were different between both sexes, comparison of both correlation coefficients was performed. Significant differences were only found for triglycerides and TyG index (**Table S1**).

TABLE 2 | Correlations in the first study sample between epigenetic age accelerations calculated by two different methods and anthropometric, biochemical, and blood pressure variables.

		AgeAcc		AgeAccGrim	
Variable	***n***	***r***	***p***	***r***	***p***
Waist circumference (cm)	365	0.22	**<0.001**	0.37	**<0.001**
log2(Glucose) (mg/dL)	335	0.09	0.104	0.19	**<0.001**
log2(HOMA-IR)	224	0.15	**0.029**	0.41	**<0.001**
HDL-cholesterol (mg/dL)	339	−0.19	**<0.001**	−0.31	**<0.001**
log2(Triglycerides)(mg/dL)	339	0.11	0.051	0.23	**<0.001**
TyG index	334	0.11	**0.042**	0.26	**<0.001**
Systolic blood pressure (mmHg)	336	0.10	0.062	0.09	0.118
Diastolic blood pressure (mmHg)	336	0.08	0.125	−0.04	0.493

A significant p-value is considered p < 0.05 (in bold).

Linear Regression Models in the Second Study

Linear regression models were then fitted for the second study separated by sex (**Table 5**). Two different models were applied: model 1 included visceral adipose tissue mass measured by DXA and TyG index, whereas model 2 included HDL-cholesterol and

TABLE 3 | Second study phenotypical and clinical characteristics.

	Second study						
Variable	**Total**		**Women**		**Men**		**Women vs. Men**
	n	**Values[a]**	***n***	**Values[a]**	***n***	**Values[a]**	**p[b]**
Sex (females)	268	189 (70.5)					
Age (years)	268	44.8 (10.2)	189	44.7 (10.4)	79	44.8 (9.7)	0.946
Waist circumference (cm)	268	95.6 (15.2)	189	92.7 (14.7)	79	102.7 (14.0)	**<0.001**
Fat mass DXA (kg)	267	27.7 (17.1)	189	28.0 (17.6)	78	16.3 (9.9)	0.693
Lean mass DXA (kg)	267	36.2 (22.4)	189	31.9 (19.1)	78	27.0 (16.2)	**<0.001**
Trunk fat mass DXA (kg)	267	15.0 (15.2)	189	14.4 (9.3)	78	46.6 (26.2)	0.143
Android fat mass DXA (kg)	267	2.7 (1.6)	189	2.6 (1.5)	78	3.0 (1.8)	**0.041**
Gynoid fat mass DXA (kg)	267	4.6 (2.9)	189	4.9 (3.1)	78	3.9 (2.4)	**0.012**
Visceral adipose tissue mass DXA (kg)	264	1.1 (0.9)	186	0.9 (0.6)	78	1.8 (1.1)	**<0.001**
Glucose (mg/dL)	268	93 (11)	189	91 (10)	79	97 (11)	**<0.001[c]**
HOMA-IR	268	1.7 (1.2)	189	1.6 (1.2)	79	1.8 (1.4)	0.513[c]
HDL-cholesterol (mg/dL)	268	57 (13)	189	60 (13)	79	49 (10)	**<0.001**
Triglycerides (mg/dL)	268	93 (50)	189	85 (39)	79	113 (65)	**<0.001[c]**
TyG index	268	8.3 (0.5)	189	8.2 (0.5)	79	8.5 (0.5)	**<0.001**
C-reactive protein (μg/mL)	268	2.5 (3.6)	189	2.7 (3.9)	79	2.0 (2.7)	0.178[c]
Systolic blood pressure (mmHg)	260	124 (17)	184	120 (15)	76	133 (16)	**<0.001**
Diastolic blood pressure (mmHg)	260	77 (11)	184	75 (10)	76	81 (11)	**<0.001**

[a] *Values are represented as Mean (SD) except for sex which is represented as number of females(%).*
[b] *Values obtained by Student t-test comparing women and men. A significant p-value is considered p < 0.05 (in bold).*
[c] *The calculations have been performed with the log-transformed variable in base 2. For C-reactive protein, +1 was added to avoid forming the logarithm of zero.*

TABLE 4 | Correlations in the second study between epigenetic age acceleration estimated by AgeAccGrim and anthropometric, body composition, biochemical and blood pressure variables.

	AgeAccGrim								
	Total			Women			Men		
Variable	***n***	***r***	***p***	***n***	***R***	***p***	***n***	***r***	***p***
Waist circumference (cm)	268	0.17	**0.006**	189	0.09	0.208	79	0.15	0.200
Fat mass DXA (kg)	267	0.07	0.256	189	0.07	0.343	78	0.10	0.385
Lean mass DXA (kg)	267	0.20	**0.001**	189	0.06	0.407	78	0.10	0.398
Trunk fat mass DXA (kg)	267	0.13	**0.029**	189	0.09	0.221	78	0.12	0.312
Android fat mass DXA (kg)	267	0.13	**0.032**	189	0.08	0.262	78	0.13	0.250
Gynoid fat mass DXA (kg)	267	0.004	0.942	189	0.06	0.435	78	0.09	0.448
Visceral adipose tissue mass DXA (kg)	264	0.21	**<0.001**	186	0.13	0.073	78	0.23	**0.043**
log2(Glucose) (mg/dL)	268	0.17	**0.006**	189	0.11	0.146	79	0.13	0.243
log2(HOMA-IR)	268	0.09	0.165	189	0.08	0.283	79	0.05	0.672
HDL-cholesterol (mg/dL)	268	−0.26	**<0.001**	189	−0.21	**0.004**	79	−0.16	0.161
log2(Triglycerides)(mg/dL)	268	0.19	**0.002**	189	0.07	0.356	79	0.35	**0.001**
TyG index	268	0.22	**<0.001**	189	0.08	0.245	79	0.34	**0.002**
log2(C-reactive protein+1) (μg/mL)	268	0.14	**0.020**	189	0.17	**0.017**	79	0.21	0.068
Systolic blood pressure (mmHg)	260	0.07	0.249	184	−0.03	0.645	76	0.09	0.453
Diastolic blood pressure (mmHg)	260	0.06	0.350	184	0.01	0.885	76	−0.03	0.789

A significant p-value is considered p<0.05 (in bold).

TABLE 5 | Linear regression models of age acceleration AgeAccGrim separated by sex.

	AgeAccGrim					
	Women			Men		
	β	***p***	R^2_{adj}	β	***p***	R^2_{adj}
Model 1		0.199	0.01		**0.007**	0.10
Visceral adipose tissue mass DXA (kg)	0.803	0.158		0.037	0.944	
TyG index	0.114	0.882		2.534	**0.017**	
Model 2		**0.004**	0.06		0.050	0.03
HDL-cholesterol (mg/dL)	−0.054	**0.022**		−0.039	0.436	
log2(C-reactive protein+1) (μg/mL)	0.474	0.092		0.740	0.163	

A significant p-value is considered p < 0.05 (in bold).

CRP. Model 1 was statistically significant for men, but not for women; on the contrary, model 2 was statistically significant for women, but not for men.

Mediation Model in the Second Study

Since visceral adiposity has been suggested as the key point for the onset of IR, a possible mediation by TyG index in the relationship between visceral adipose tissue mass DXA and AgeAccGrim was assessed in men. Results showed that TyG index was mediating the majority of the effects of visceral adipose tissue mass accumulation on age acceleration (**Figure 1**).

DISCUSSION

Aging is an irreversible and progressive process that occurs from birth (40). Many theories have been proposed about aging (41), including wear-and-tear theories, the free radical hypothesis of aging, the cross-linking theory, and the neuroendocrine theory, among others (40). Interestingly, newer studies have suggested a role of epigenetic processes in lifespan regulation that should be further investigated (42). In this context, aging-related DNA methylation has been hypothesized as a biomarker that predicts both cellular age and chronological age (42).

Fat accumulation, and specifically, excessive visceral fat deposition, is common in elderly individuals. Excessive visceral fat deposition usually increases with age, as it is related to obesity, diabetes, cardiovascular diseases, cancer and fatty liver disease (11, 43). Visceral fat has an important role in the onset of IR (44). Subjects with accumulation of abdominal fat presented enhanced lipolysis and alteration of the flux of free fatty acids (44). The rise of circulating free fatty acids in turn increases hepatic gluconeogenesis and glycogenolysis, as well as increasing insulin secretion by the pancreas, resulting in hyperglycaemia (45). In addition, in the liver, there is a higher

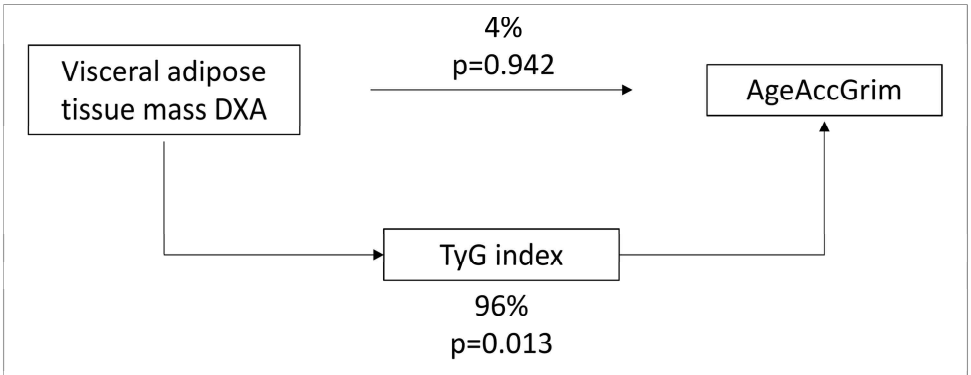

FIGURE 1 | TyG index-mediated relationship between visceral adipose tissue mass measured by DXA and age acceleration GrimAge in men.

production of triglycerides and less insulin breakdown, leading to hyperinsulinemia (45). Hyperglycaemia and hyperinsulinemia promote IR (46). Interestingly, the TyG index has been determined as a useful marker of IR (44). Indeed, it has been demonstrated as a better marker for the diagnosis of metabolic syndrome than HOMA-IR, since it involves measures of both triglycerides and glucose (47).

Aging is an important contributor to metabolism and health impairments, increasing the risk for several diseases (48). Numerous mechanisms related to longevity and age-related metabolic dysfunctions occur in adipose tissue (48). Therefore, the investigation of the relationships between adipose tissue, IR and aging and the role of epigenetics in this process is a challenging task that could provide new understanding about the putative interactions among them.

The assessment of aging has received attention because chronological age is sometimes different from biological and metabolic age (49). Therefore, different methods based on epigenetic measurements have been designed for that purpose: an estimator developed by Hannum et al. (50) based on 71 CpGs in leukocytes; DNAmAge clock created by Horvath (16) based on 353 CpGs in different tissues; PhenoAge designed by Levine et al. (17) for predicting lifespan based on regressing a phenotypic measure of mortality risk on CpGs; and the newest one, GrimAge, developed by Lu et al. (18) using DNAm-based biomarkers for seven plasma proteins and smoking. GrimAge has been reported as a better predictor of lifespan than currently available DNAm-based predictors (18). Studies with these epigenetic clocks have demonstrated epigenetic age acceleration in obesity (37, 51), diabetes (51, 52), non-alcoholic fatty liver disease (53) and different metabolic and inflammatory biomarkers (54). In this sense, epigenetic age acceleration could be defined as the aging of epigenetic DNA regulation, which may lead to longevity functions impairments earlier than expected.

A number of factors, such as sex, could mediate the interactions between epigenetic aging and adiposity biomarkers, since it is known that visceral fat accumulation is more common in men than in women (10). Furthermore, sex differences elicited by sexual hormones affect this adiposity dimorphism. In this context, estrogens may direct the expansion of fat through the increase of adiposity progenitor cells (hyperplasia) as well as regulate the vascular supply into adipose tissues (55) with impact on epigenetic mechanisms (56).

Indeed, our investigation demonstrated that sex is important in the analysis of the relationship between epigenetic age acceleration and adiposity biomarkers. Men showed a tighter association of epigenetic age acceleration with visceral adipose tissue mass and the TyG index, whereas HDL-cholesterol and CRP were associated with epigenetic age acceleration in women. However, we cannot conclude that the correlations observed were statistically different between sex groups, except for TyG index. These results are also in agreement with the fact that aging is usually accompanied by a deterioration of metabolic markers (57, 58). Furthermore, we found that the relationship between visceral adipose tissue and epigenetic age acceleration in men is mainly mediated by the TyG index. Thus, the accumulation of abdominal fat, through TyG index mediation, suggest an older biological age in men than the expected. This result is in accordance with the fact that visceral adipose tissue contributes to the development of IR (44) and that both are influencing aging processes (37, 59).

Other possible factors that might influence epigenetic aging are HDL-cholesterol or CRP, as described in our research. HDL-cholesterol relationship with epigenetic age acceleration was negative, thereby suggesting a deceleration of biological age with higher levels of this lipoprotein. Thus, HDL-cholesterol might modulate epigenetic aging processes due to its antiatherogenic effects such as the removal of lipid deposits, which is accompanied by the reduction of cytotoxic effects (60). Furthermore, HDL reduces the oxidative stress in plasma and cellular compartments, and the signaling pathways which it participates in are interconnected with stress response

and survival pathways (60). On the other hand, higher CRP levels were related to accelerated biological age, suggesting a negative influence of inflammation on aging-related epigenetic marks. Indeed, CRP is a molecule involved in inflammatory and immune processes (61), which has been associated with aging-related diseases such as diabetes, cardiovascular diseases and hypertension (62). Additionally, previous research showed that after adjusting for age, sex, body mass index, lipid levels and smoking status, increased levels of CRP were associated with cognitive and poor physical performances and reduced survival (63).

This research did have some limitations. The ideal tissue assessed in this study would have been adipose tissue since the relationship was established between visceral adipose tissue mass and age acceleration. However, several studies have demonstrated that peripheral blood is a valid non-invasive alternative tissue that reflects metabolic and inflammatory pathways and serves as a surrogate for assessing methylation (64–67). Furthermore, DNAm age performs well in blood, (16) and GrimAge has been developed with methylation data from blood samples (18). Another possible limitation is that methylation samples in the MENA and in the OBEKIT+NormoP studies were assessed with different Illumina arrays (Infinium 450K BeadChip and EPIC, respectively). Nevertheless, McEwen *et al.* revealed that both methods were equivalent in the determination of epigenetic age (68). Moreover, it must be noted that the percentage of contribution of our variables to epigenetic age acceleration is at maximum of 10%. Thus, there must be other factors influencing the progression of aging.

In summary, our research has revealed that the mechanisms that affect epigenetic age acceleration are different in women and men. Whereas, in men the accumulation of visceral adipose tissue accelerates the epigenetic age through mechanisms mostly mediated by IR, mechanisms in women seemed to be more related to HDL-cholesterol and CRP. These findings support the hypothesis that obesity and metabolic syndrome features, intimately related to adiposity and dyslipidaemia, are associated with accelerated aging effects (43), although in a different manner for each sex. Further research about the relationship between epigenetic age acceleration and disease markers will allow for a better understanding of the molecular mechanisms involved in the development of age-related diseases. This knowledge is needed to create new useful clinical and public health tools based on DNA methylation biomarkers and epigenetic age acceleration. Furthermore, sex differences concerning the factors influencing epigenetic age acceleration should be taken into account in future development of precision strategies and management of healthy aging.

DATA AVAILABILITY

The data of the first study have been deposited in NCBI's Gene Expression Omnibus (69) and are accessible through GEO Series accession number GSE115278 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115278). The remaining data will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Research Ethics Committee of the University of Navarra (CEI-UN, Pamplona, Spain). All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Research Ethics Committee of the University of Navarra (CEI-UN, Pamplona, Spain), except for GEDYMET, which was approved by the Ethics committee of the School of Medicine, Pontificia Universidad Católica de Chile (Santiago, Chile).

AUTHOR CONTRIBUTIONS

AA performed data analysis and wrote the first version of the paper. JS helped with data interpretation. MG-G helped with the statistical analysis. J-IR-B, FM, and JM supervised data analysis and helped with interpretation and with manuscript elaboration, as well as provided the conceptual design and financial support. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00496/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Interaction among sex, aging and epigenetic processes concerning visceral fat, insulin resistance and dyslipidaemia

Available at:

https://www.frontiersin.org/articles/10.3389/fendo.2019.00496/full#supplementary-material

Table S1: Differences in the correlations (AgeAccGrim vs. Variable) between sex groups in the second study.

Variable	Women		Men		p-value*
	n	r	n	r	
Visceral adipose tissue mass DXA (kg)	186	0.13	78	0.23	0.451
HDL-cholesterol (mg/dL)	189	-0.21	79	-0.16	0.704
log2(Triglycerides) (mg/dL)	189	0.07	79	0.35	0.030
TyG index	189	0.08	79	0.34	0.044
log2(C-reactive protein+1)	189	0.17	79	0.21	0.760

*p-value is calculated using a Z test on the Fisher-transformed coefficients.

GENERAL DISCUSSION

Epigenetic signatures are defined as heritable changes that can alter gene expression without modifying the DNA sequence [59]. Epigenetic marks can be influenced by environmental factors, leading to the modulation of gene expression [35]. Thus, epigenetics is considered an intermediate between environmental agents and genetics [134]. Changes and aberrations in epigenetic marks, caused by environmental factors or stochastically with ageing, can influence the development and progression of impairments and diseases, such as neurological disorders, obesity, T2D and cardiovascular disease [44]. The main epigenetic mechanisms regulating gene expression in mammals are: (1) histone modifications, such as acetylation, biotinylation, methylation, phosphorylation and ubiquitination [43], (2) non-coding RNAs, highlighting miRNAs, which regulate mRNA translation into proteins [78], and (3) DNA methylation at CpG sites, which consists on the incorporation of a methyl group to a cytosine adjacent to a guanine [77].

In the last years, epigenetic studies have joined efforts to develop epigenetic biomarker panels for diagnosis and prognosis, as well as possible treatments for diseases [318, 319]. Advances in high-throughput technologies are allowing the determination of methylation levels of the whole genome and the performance of large-scale EWAS, establishing relationships between DNA methylation levels and different features and states [34]. For example, microarray technology, such as the developed by Illumina, provides information about methylation levels in more than 450000 CpGs [109], or even 850000 CpGs in the last version. This technique has been validated [320], although it exhibits several drawbacks comparing to NGS: lower resolution, more difficulties in distinguishing repetitive elements due to hybridisation, and lack of information about methylation in repetitive genomic regions [321]. However, the easiness of use and interpretation, as well as the cheaper costs in comparison to NGS [109, 321], makes the array-based technique a useful tool in DNA methylation assessment.

Microarray approaches are appropriate for DNA methylation measurements in different tissues [322]. Due to the tissue-specificity of this epigenetic signature, the best approach is to measure methylation levels in the physiopathological relevant tissue [136]. Nevertheless, blood cells have been demonstrated as mirrors of DNA methylation concerning other tissues [21].

DNA methylation can modulate gene transcription by altering the affinity of transcription factors to gene promoters, by affecting the binding of methylation-specific recognition factors to promoters and gene bodies, and by hindering the accessibility of transcription factors and/or binding proteins [87]. DNA methylation is usually associated with gene silencing, although a positive correlation with gene expression has also been described [86]. Changes in gene expression due to DNA methylation alterations may be related to physiological impairments and thereby, CpG sites might be considered as biomarkers for diagnosis or therapeutic targets in the treatment of noncommunicable diseases [67].

The general objective of the present dissertation was to identify DNA methylation patterns associated with different physiological, metabolic and nutritional conditions, deepening in the comprehension of the epigenetic regulation and helping in the development of biomarker panels and potential therapeutic targets. EWAS in different populations were performed to determine candidate CpGs in relation to factors and features that could influence and/or be associated with DNA methylation, such as prematurity (*Chapter 1*), Mediterranean diet (*Chapter 2 & 3*), insulin sensitivity (*Chapter 4*), insulin resistance (*Chapter 5*), abdominal obesity (*Chapter 6*) and epigenetic age (*Chapter 7*).

DNA methylation, prematurity and neurodevelopment

Prematurity involves to be born before achieving a developmentally mature status and being physically ready for autonomous life [323]. Despite decades of investigation and the recent advances in health care, preterm births continue to be a worldwide health issue [129, 324]. Preterm newborns are predisposed to suffer from early-onset chronic diseases, including intellectual impairment, cerebral palsy, cardiovascular disease, obesity, respiratory complications, vision and hearing loss, among others [129, 325, 326].

Despite the epidemiological evidence, the underlying molecular mechanisms and aetiology behind these phenotypes are still not clear [326]. Preterm birth is influenced by environmental, social and genetic factors, though epigenetics

has been proposed to be in the interplay among them [7, 327]. Indeed, DOHaD hypothesis states that environmental signals induce a programming in the foetus development to face future challenges after birth, and epigenetics may be a fundamental part of this process [328]. Epigenetic alterations in preterm birth have been mainly explored in placentae and cord blood [327, 329-335], some employed buccal samples [336, 337] or mother's blood [131, 332, 338-340], whereas only a few studies analysed child's blood [341].

As previously mentioned, epigenetic alterations may mediate neurodevelopmental impairments in preterm newborns [342], who are prone to disruptions in cortical connectivity, myelination disorders and cell death, leading to loss of synaptic connections [343]. In this context, reduced grey matter volume in orbitofrontal cortex, decreased volume of amygdale, insula, hippocampus, cerebellum and fusiform gyrus have been reported in later stages of life [343]. These neurological disturbances are reflected in evidences of behavioural and physiological disorders during childhood and adolescence, such as social withdrawal, anxiety, depression, attention deficits, and increased risk for autism [344].

Several studies have reported a relationship between epigenetic modifications and neurological impairments in preterm individuals [342]. For instance, higher methylation at *NR3C1* gene was found in preterm newborns in two different studies [341, 344]. Furthermore, hypomethylation was observed in preterm newborns in the *HSD11B2* gene [344]. Interestingly, preterm methylation levels in *NR3C1* and *HSD11B2* genes were associated with neurobehavioural problems at discharge [341, 344]. Another investigation demonstrated higher methylation levels in preterm children in *SLC6A4* gene, which is a serotonin transporter [345], intimately related to pain [342] and socio-emotional stress [346]. Moreover, lower methylation levels were reported for *SLC7A5* and *SLC1A2* [337], which are involved in thyroid hormone and glutamate transport, respectively, in different parts of the neurological system, regulating the synaptic process [342].

In line with these investigations, *Chapter 1* reported several differentially methylated CpG sites between preterm and full-term newborns that were mainly associated with nervous system functions and general body development. Several CpGs were associated with developmental scales

(measured by BSID), probably creating a network of changes in gene expression that could influence the development of newborns. Particularly, the most significant CpG was located at the *SLC6A3* gene, which encodes for the dopamine transporter DAT-1, belonging to the sodium- and chloride-dependent neurotransmitter transporter family [347]. In this investigation, *SLC6A3* methylation, together with gestational age, seemed to have an effect on neurodevelopment and triglycerides levels.

The relationship between *SLC6A3* methylation and triglycerides might be supported by the involvement of the dopaminergic system in food reward, which regulates food intake [348]. Triglycerides directly affect cognitive and reward processes, contributing to obesity [349], which is, in turn, a prematurity risk [129], thereby explaining the connection between prematurity, *SLC6A3* methylation and triglycerides.

On the other hand, there is an intimately relationship between dopamine and neurological and behavioural disorders, including Parkinson, Tourette syndrome, depression, behavioural impairments, and ADHD [350-353]. For example, ADHD patients, which have shown a lower score on the Bayley Mental Scale [354], exhibited reduced cortical dopamine or dysregulations in the dopaminergic system [355, 356]. Furthermore, in ADHD patients, methylation levels of *SLC6A3* gene have been previously correlated with age [357] and involved in the modulation of the response to methylphenidate treatment [355]. Finally, *SLC6A3* DNA methylation changes were also associated with a maternal intake of a high-fat diet [358] and alcohol consumption [156] during pregnancy.

Altogether, current findings demonstrates a relationship between prematurity and methylation alterations, which might lead to different outcomes, such as neurodevelopmental and behavioural impairments, in later stages of life.

DNA methylation, nutrition and metabolic features

First developmental stages are crucial for the establishment of an appropriate DNA methylation pattern, but environmental factors also affect epigenetic signatures during the adulthood [6].

Healthy habits such as balanced nutrition or physical activity are recommended to maintain body homeostasis and functioning, preventing future diseases [359]. In this context, Mediterranean diet has been postulated as a healthy and balanced diet [360], which prevents from cardiovascular disease and associated risks, such as inflammation, hypertension, and hyperlipidaemia, among others [15, 361]. The usual intake of a classical Mediterranean diet includes a high amount of vegetables, fruits, cereals, legumes and nuts; a relatively high quantity of unsaturated fatty acids, mostly provided by extra-virgin olive oil; a moderate-high amount of fish; moderate quantity of poultry and dairy products; and low consumption of red meat and meat products [180].

Foods have been widely demonstrated as factors influencing epigenetics, and specifically, DNA methylation [6]. Indeed, Mediterranean diet has been related to changes in DNA methylation [181], such as the association between adherence to this diet and LINE-1 methylation in blood samples [182]. Another study demonstrated the relationship between adherence to Mediterranean diet in pregnant women and infant DNA methylation at *MEG3-IG* [362], *MEG3, IGF2, and SGCE/PEG10* regions [363]. Other preliminary studies reported that an intervention with Mediterranean diet was associated with changes in methylation at one year in *FTO* and *TCF7L2* genes [364], and a higher adherence to this diet at baseline was associated with statistically significant differences in *FNDC5* methylation [365].

Trying to shed more light on this issue, *Chapter 2 & 3* aimed to analyse the relationship between a five-year intervention with a Mediterranean diet and DNA methylation alterations.

In *Chapter* 2, adherence to Mediterranean diet was associated with DNA methylation levels. In order to assess adherence to Mediterranean diet, several scores have been developed, such as t-MED, m-MED, and r-MED [360], although in this investigation, it was estimated by a validated 14-item dietary screener based on 14 questions about frequency of consumption of several recommended foods in Mediterranean diet [295]. This score was assessed before and after the intervention and was correlated with methylation changes, obtaining several differentially methylated CpGs. The most significant CpGs were related to inflammation and metabolic pathways, confirming the

association between DNA methylation and metabolic traits and a putative influence on the development of metabolic diseases. Similar to our study, other research evidenced a relationship between DNA methylation, Mediterranean diet and colon cancer, finding several CpG sites in inflammation-related genes with a protective effect [366].

Accompanying metabolic diseases, such as obesity and metabolic syndrome, there is a low-grade inflammation as a consequence of the augmented secretion of different chemokines, mainly in the adipose tissue, including TNF- α, MCP-1, CRP, IL-6 [250], where epigenetic changes may be involved. Hence, *Chapter 2* focused on genes related to inflammation and immunocompetence. Moreover, one of those genes, *EEF2,* apart from being associated with the adherence to Mediterranean diet, showed a relation with inflammatory molecules, such as TNF-α and CRP. Remarkably, many of these genes were also related to adipogenesis and metabolic pathways, underlining the broad potential role of DNA methylation alterations in different metabolic traits and diseases.

Furthermore, certain foods and nutrients are known to alter epigenetic marks [68, 367]. For instance, an olive oil-enriched diet has been associated with increased levels of global methylation in mammary glands and tumours [368]. As aforementioned, a Mediterranean dietary pattern, known to be rich in vegetables, fruit, legumes, whole-grain cereals, nuts, and olive oil [180], is associated with DNA methylation modifications. In this context, *Chapter 3* aimed to study how a Mediterranean pattern supplemented with either nuts or extra-virgin olive oil influenced DNA methylation signatures in comparison with a low-fat control diet.

In our study, differentially methylated CpGs were related to metabolism, inflammation, intracellular signals and diabetes. Furthermore, specific changes were observed for two CpGs located at the genes *CPT1B/CHKB-CPT1B* and *GNAS/GNASAS,* which were related to a Mediterranean diet supplemented with nuts or extra-virgin olive oil, respectively. Particularly, extra-virgin olive oil is rich in polyphenols, MUFAs, PUFAs, Vitamin E and carotenoids, whereas nuts are rich in phytosterols, PUFAs, fibre, polyphenols and proteins [360]. In relation to these observations, the type of consumed fat is also a determinant of DNA

methylation [183] and several studies have reported differences in DNA methylation when using SFA or PUFA. For instance, 4875 CpGs were differentially methylated after consuming SFA- or PUFA-enriched diets by healthy young subjects [184]. In the same line, SFA, PUFA or MUFA intake was associated with methylation levels in several CpGs in blood [183]. Furthermore, *CLOCK* methylation inversely correlated with MUFA intake and was positively associated with PUFA intake [185]. In line with these investigations, methylation of the selected CpG at *CPT1B/CHKB-CPT1B* gene was associated with PUFA uptake in our study. Thus, this CpGs might be influenced by fat quality due to high PUFAs proportion in nuts. On the other hand, alterations in methylation at *GNAS/GNASAS* might be due to other nutrients or compounds from extra-virgin olive oil or from the Mediterranean dietary pattern. Indeed, polyphenols from extra-virgin olive oil exhibit proatherogenic properties by down-regulation of several genes [369], and some derivatives have demonstrated strong antioxidant and anti-inflammatory activity in vitro [370, 371]. Evidences suggest a role of polyphenols on DNA methylation by inhibiting DNMTs or reversing aberrant CpG island methylation, such as the green tea epigallocatechin-3-gallate polyphenol, which has shown DNMT inhibitory effects [372, 373]. Furthermore, cooking with olive oil has beneficial effects over other foods, since it can increase the bioavailability of some nutrients, such as phenolic compounds [374], and even reduce the formation of toxic compounds in high-temperature frying comparing with other fats [375]. In human colon cancer cells, DNA methylation and expression of the cannabinoid receptor was influenced by phenolic extracts found in extra-virgin olive oil and hydroxytyrosol [367].

Altogether, current research demonstrated that a Mediterranean dietary pattern and supplementations with extra-virgin olive oil and nuts, could induce alterations in DNA methylation, which in turn, may cause changes in the expression of some genes that are associated with metabolic-related processes and diseases, including inflammation, hyperlipidaemia, obesity, diabetes and cardiovascular disease.

Nutrition and lifestyle are reflected in phenotypical features [11]. Moreover, phenotypical traits, both in healthy or metabolically-impaired subjects, are associated with DNA methylation signatures [126]. In relation to this finding, the current dissertation aimed to study the relationship between DNA methylation

and metabolic features, including insulin sensitivity (*Chapter 4*), insulin resistance (*Chapter 5*) and abdominal adiposity (*Chapter 6*), in different populations.

Pancreatic β-cells secrete insulin, which is involved in the maintenance of glucose levels by facilitating glucose uptake by different tissues, and in the regulation of carbohydrate, lipid and protein metabolism [248, 376]. When tissues do not respond properly to insulin, insulin sensitivity is impaired, and then, an insulin-resistant condition is developed [376], which is a key feature of several diseases and unhealthy cardiometabolic disorders such as T2D, cardiovascular disease, hypertension, obesity and metabolic syndrome [377, 378]. There is the necessity of developing biomarkers to detect early steps in the pathophysiological progression of those diseases, as well as to elucidate underlying mechanisms [209]. Current evidences suggest that epigenetics are involved in these processes [195]. For example, methylation of *FGF21* is increased in human adipocytes from diabetic subjects and in cultured Dnmt3a overexpressor 3T3-L1 adipocytes [379]. This gene is known to facilitate glucose uptake in adipocytes and it is negatively regulated by DNMT3a, possibly mediating insulin resistance [379].

Since epigenetic mechanisms might be influencing deregulations in insulin sensitivity and secretion and the development of insulin resistance [380, 381], *Chapter 4 & 5* aimed to assess the association between DNA methylation and insulin sensitivity in health young women, or insulin resistance in a heterogeneous population.

Although several locus-specific approaches have proposed potential DNA methylation biomarkers in relation to plasma insulin levels, insulin secretion and insulin resistance, such as those located at *PPARGC1A, HTR2A*, *LY86, TFAM, GIPR, ADIPOQ, IGFBP3* [382], *TCF7L2* [383], *LEP* [384] genes, few EWAS have studied the relationships between DNA methylation and insulin sensitivity and resistance to date [258-260, 385]. In line with these studies, the two EWAS performed in *Chapters 4 & 5* demonstrated that the methylation of numerous CpGs is related to insulin secretion and insulin resistance measurements.

On the one hand, in *Chapter 4,* insulin sensitivity was measured using the CSi index [386]. This index allows an accurate assessment of insulin sensitivity based

on insulin and glucose measurements taken during one hour IVGTT test [386], instead of an hyperglycaemic-euglycaemic clamp, which is the gold-standard method [387]. Furthermore, the analysis was performed in a very specific population, young healthy women, which allows the assessment of alterations in DNA methylation prior to possible future insulin impairments. The most significant associated CpGs were located at genes involved in insulin-related pathways and some of them were previously reported linked to insulin, such as *LPL* [388], *GRB10* [389], *WISP1* [390], *PRDM16* [391], *TMEM132C* [392], *ADAMTS9* [393], and *NOX4* [394]. Interestingly, *CTNND2* and *LPL* exhibited the most significant associations with CSi and together allowed the discrimination of subjects with high and low CSi, suggesting that methylation at specific sites of these two insulin-sensitive genes may act as biomarkers for early diagnosis of insulin resistance-related diseases. Whereas little is known about the involvement of *CTNND2* in metabolic processes, except for two polymorphisms associated with T2D and BMI [395-397], *LPL* is intimately connected to them. Indeed, LPL hydrolyses triglycerides in circulating chylomicrons, low density lipoproteins and very low-density lipoproteins to render free unesterified fatty acids to the circulation [398]. Moreover, insulin is known to regulate *LPL* activity in adipose tissue and skeletal muscle [399] and two studies [388, 400] found that common *LPL* gene variation was involved in insulin resistance measured through hyperinsulinemic-euglycaemic clamps and IVGTT in Mexican-Americans, supporting the mediation role of *LPL* in systemic insulin sensitivity.

On the other hand, in *Chapter* 5, insulin resistance was assessed using HOMA- IR and was associated with 798 CpGs, among which *CXCR1*, *HDAC4*, *IGFR1*, *LEPR*, and *ABCG1* had been previously reported [27, 259, 260]. When comparing methylation of individuals with low and high values of HOMA-IR, the methylation pattern for many of them was different. Moreover, differentially methylated CpGs were located at some genes implicated in glucose and insulin-related pathways and four of them were suggested as putative biomarkers of insulin resistance, including two CpGs located at *SH3RF3* and *MAN2C1* genes.

Insulin is also involved in lipolysis suppression [248] and therefore, an insulin-resistant condition results in an expansion of adipose tissue and accumulation of glucose in blood, leading to hyperglycaemia and hyperinsulinemia [248].

Additionally, the accumulation of adipose tissue increases the release of fatty acids to the circulation, leading to a dyslipidaemia status [401], as well as an increased secretion of different chemokines that induce a low-grade inflammation state [250]. All these metabolic impairments can lead to higher risk of suffering from T2D, obesity and metabolic syndrome [376], where methylation is involved [66].

Obesity is characterised by an excessive adiposity [402]. However, metabolic disorders, such as systemic inflammation, hyperlipidaemia, insulin resistance, and cardiovascular disease [403], have been associated with visceral adipose tissue rather than subcutaneous fat [404]. Actually, WC has been suggested as a better estimator of metabolic complications accompanying excessive adiposity than BMI [405]. EWAS establishing a relationship between DNA methylation and BMI have been extensively performed, in contrast to the lower number of studies analysing WC [252, 254, 255, 402, 406-408]. Contributing to this field, *Chapter 6* demonstrated the association between WC and DNA methylation levels of 669 CpGs, as well as a differential methylation pattern between individuals with and without abdominal adiposity. Furthermore, several CpGs, located at *c13orf36, ZC3H12D, MYO9B, KCNG3* genes for females and *TCP11L1* for males, allowed the discrimination between both WC groups, suggesting that specific epigenetic modifications may be representative for people with central obesity.

Many of the significant CpGs associated with WC and differentially methylated between subjects with and without abdominal adiposity in this investigation were also related to inflammation, confirming the connection between metabolic and inflammatory processes [250]. Moreover, several genes of *Chapters 5 & 6* have been found in EWAS of different metabolic traits. For example, genes whose CpG methylation was associated with HOMA-IR were also related to BMI or obesity traits, highlighting the CpGs described for *ABCG1* [252, 253, 256, 258-261, 385, 409-411], *SREBF1* [252, 253, 256, 258, 260, 261, 409], *SOCS3* [253, 258, 261, 271, 409] and *PHGDH* [253, 254, 256, 411], which have been widely reported in the literature. On the contrary, genes whose CpG methylation was related to WC were also differential between T2D and non-T2D subjects, underlining *NOD2* (cg01243823) [265]. Moreover, other studies in the same population of *Chapters 5 & 6* found associations between obesity features and genes related to olfactory pathways [412], dopaminergic synapse transmission [413]; circadian

rhythm [414], as well as to endoplasmic reticulum stress, which also correlated with insulin resistance [415].

Additionally, comparing differentially methylated/associated CpGs among studies of this thesis, several common CpGs were found. For instance, 26 CpGs were shared comparing the significant CpGs obtained for the groups of Mediterranean diet supplemented with nuts and Mediterranean diet supplemented with extra-virgin olive oil, remarking the CpG located at *AHRR* gene. This gene has been previously related to obesity and triglycerides, as well as included in a methylation risk score associated with the incidence of cardiovascular events [416]. Moreover, both groups exhibited one shared CpG, located at *c2orf28* gene, whose methylation was also found associated with adherence to Mediterranean diet. On the other hand, 37 CpGs were differentially methylated between high and low WC in both men and women, highlighting the ones located at *GHR, TSNARE1, GRIK3* and *TCP11L1* genes, which have been related to metabolic and inflammatory pathways [190, 257, 417-419]. Both groups also shared the CpG located at *NLRP14* gene with the study in Mediterranean diet supplemented with extra-virgin olive oil. Intriguingly, a decreased *NLRP14* methylation was associated with lower WC and with consumption of Mediterranean diet supplemented with extra-virgin olive oil. The gene *NLRP14* is involved in the immune system, suggesting a relationship between inflammation, central adiposity and olive oil actions [347]. Furthermore, the CpG located at *NOD2* gene was found in both comparisons of WC and differentially methylated between HOMA-IR groups. This CpG located at *NOD2* has been previously described in the scientific literature in relation to T2D, fasting glucose, BMI and inflammation [253, 256, 257, 265, 411, 420]. Therefore, methylation alterations are influenced by nutrient intake and are related to many metabolic processes, such as obesity, inflammation and insulin resistance, reinforcing the connection between nutrition, epigenetics and metabolic impairments [40].

DNA methylation and ageing

Several mechanisms are involved in longevity and age-related metabolic dysfunctions [421], playing an important role in the onset and progression of

metabolic and health impairments [422]. In this context, there are numerous evidences suggesting that epigenetic marks are key players in ageing and lifespan regulation [423]. In fact, ageing-related DNA methylation has been hypothesised as a biomarker that predicts both cellular age and chronological age [424]. In this regard, different methods based on epigenetic measurements have been designed for estimating biological age: Hannum's clock, DNAmAge, PhenoAge and GrimAge [199-202]. Among them, GrimAge, which uses DNA methylation-based biomarkers for seven plasma proteins and smoking, has been demonstrated as the better predictor of lifespan [202]. Age acceleration is one of the estimates provided by these epigenetic clocks, defined as the residual from regressing the epigenetic age on chronological age, which has been associated with metabolic diseases, such as obesity [203, 425], diabetes [205, 425], NAFLD [426], and with different metabolic and inflammatory biomarkers [427].

Thus, establishing connections among ageing, epigenetics, adipose tissue and insulin resistance, which was the aim in *Chapter 7,* can deepen the understanding about the putative interactions among them and their joint role in the development of metabolic disorders.

Nonetheless, sex is a factor that can influence epigenetic ageing and its relationship with adiposity biomarkers, due to the differences between both sexes in visceral fat accumulation, which is more common in men [428]. As a matter of fact, *Chapter 7* has demonstrated sex differences in the mechanisms affecting age acceleration. Studies in men showed that the relationship between visceral adipose tissue and epigenetic age acceleration was mainly mediated by the TyG index, which has been determined as a useful marker of insulin resistance [429]. This finding is in agreement with the contribution of visceral adipose tissue to the development of insulin resistance and their relationship with ageing [200, 430]. On the other hand, studies in women displayed an influence of HDL-cholesterol and CRP on epigenetic age acceleration. The relationship of HDL-cholesterol with epigenetic age acceleration was negative, indicating a deceleration of biological age with higher levels of this lipoprotein, probably due to its antiatherogenic effects [431]. However, CRP, which is involved in inflammatory processes [432] was affecting epigenetic age in a positive manner, suggesting a negative influence of inflammation on ageing-related epigenetic marks.

Altogether, *Chapter 7* findings support the hypothesis that obesity and metabolic syndrome features, intimately associated with adiposity and dyslipidaemia, are related to accelerated ageing effects [196, 423], although in a sex-dependent manner.

Overall, current findings demonstrated that metabolic traits such as insulin sensitivity, insulin resistance and central obesity are linked to DNA methylation levels in numerous CpGs located at metabolic-related genes, probably playing a role in the pathogenesis of many complex disorders, such as T2D, obesity or metabolic syndrome, or as their consequence. Furthermore, metabolic features are also influencing acceleration of epigenetic age, suggesting that metabolic unhealthy people exhibit altered DNA methylation levels and are more prone to suffer from diseases at early stages.

Limitations and strengths

The current investigation have some limitations and strengths. One of the main drawbacks is the sample size in some of the studies performed, increasing the probability for false negatives (type II error), rejecting some potential true associations [433]. This issue is unavoidable in some samples, due to the fact that they involve a highly specific population, such as *Chapter 1* or *Chapter 4*, whose recruitment is complicated. However, in other cases, such as *Chapters 5, 6 & 7,* a relatively big sample has been analysed. Another limitation is the use of food frequency questionnaires for determining food intake, which is a subjective dietary assessment method with low accuracy [434]. One of the main strengths is that all the studies involved EWAS, analysing more than 450000 CpGs. Illumina array technology for assessing methylation (Infinium Human Methylation 450k BeadChip and Infinium MethylationEPIC BeadChip) allows a DNA methylation profiling of the whole genome [42]. Furthermore, this technology is widely employed in published EWAS and thereby, it allows comparisons with other investigations. Nevertheless, although the prices are decreasing, these microarray approaches are still expensive, which increases the difficulty of processing a big number of samples.

Furthermore, although corrections for multiple comparisons tests have been applied, false positives (type I error) cannot be discarded due to the high number of tests performed in all the studies. Nevertheless, different robust biological criteria and statistical strategies were applied in order to select the most significant probes.

Regarding the methylation analysis, there are several issues that must be taken into account. Firstly, DNA methylation is tissue specific and different levels can be observed between the relevant pathophysiological tissue and other tissues [99]. Additionally, the dysregulation detected with ageing is also tissue-dependent [21]. Therefore, methylation analyses should be ideally measured in metabolically relevant tissues [136]. However, many of these tissues are difficult to access, but there are evidences suggesting that epigenetic signatures on easily accessible tissues may serve as potential biomarkers of exposure or disease risk [21]. All the studies in this thesis were performed in peripheral white blood cells, which is a non-invasive accessible tissue that reflects metabolic and inflammatory pathways [382]. Numerous studies have demonstrated that blood cells can act as proxies of other tissues, such as brain, saliva, adipose tissue, muscle, and pancreatic islets [38, 156, 382, 435-438].

Secondly, DNA methylation can vary depending on blood cell type composition [112]. One of the main strengths in this investigation is the implementation of cell-type correction based on Houseman's method to avoid that problem, which identifies the distribution of different types of white blood cells using differentially methylated regions [313]. This correction was applied in all the studies, except for *Chapter 2 & 3*. Nonetheless, selected CpGs in those chapters were checked and none of them showed variations with cell type.

Thirdly, sequence variation caused by SNPs can be reflected in DNA methylation levels if located at the same pair bases [99, 439]. This issue has been taken into account in the majority of the studies and CpGs with SNPs have been removed in the analyses. However, *Chapter 1, 2 & 3,* included CpGs with SNPs, and therefore, some of the selected CpGs might be influenced by those polymorphisms.

Lastly, there is a concern about the relevance of small epigenetic changes in biological processes [95], although there are evidences of small methylation

variations which reflect alterations in biological pathways [95]. Nevertheless, all the analyses performed in this thesis have taken into account this issue, and CpGs were selected filtering by high methylation variability.

Furthermore, there are other issues that must be considered. Validation and independent replication is necessary for robust findings [99]. Although this validation has not been possible in some of the studies due to a lack of an independent population, several validations have been performed with the objective of corroborating the results. In fact, *Chapter 2* included the association with inflammatory molecules measured by ELISA; *Chapter 5* incorporated an internal validation to correct the overestimation of AUC using a bootstrap method [316], and *Chapter 6* corroborated the results in an independent population.

Moreover, analysis of expression arrays may be an interesting and helpful approach to evaluate the relationship between methylation changes and gene expression. This is a drawback of all the studies performed in this thesis since RNA samples were not available.

Also, DNA methylation levels can be influenced by external confounding factors such as tobacco, alcohol, diet, medications and other chemicals [99], which must be taken into account in the analyses. This lack of information is another weakness in this investigation, since environmental factors in human interventions and studies are difficult to control [98]. Nevertheless, when available, potential confounding factors have been included in the statistical analyses.

Finally, establishing the causal role of methylation changes is difficult [98], mainly due to the transversal nature of most of the studies. Thus, methylation cannot be defined as cause or consequence of metabolic impairments and diseases [44]. In order to overcome that challenge, longitudinal studies can be designed in order to determine methylation alterations occurring before or in early stages of diseases, which are more likely to be related to their aetiology [98]. Furthermore, mediation analysis can help to understand the mechanisms involved and the causes of diseases [440]. Interestingly, this type of analysis was performed in *Chapter 7* to find factors involved in acceleration of epigenetic age.

Corollary

Summing up, the results of the experimental investigation have demonstrated alterations in DNA methylation marks depending on the physiological, metabolic and nutritional states of the individuals. Since epigenetics is a plastic system, the effects of environmental factors on DNA methylation signatures are not only important in the first stages of life, but also during the adulthood [6]. Indeed, prematurity and nutrition are two determinant factors in DNA methylation levels, being related to neurodevelopment and inflammatory and metabolic pathways, respectively [129, 134]. These alterations can lead to the development of several impairments and diseases, such as neurobehavioural disorders, obesity, diabetes, cardiovascular disease and metabolic syndrome, although they can also be a consequence of these metabolic conditions [41, 43, 44]. Furthermore, DNA methylation is related to abdominal adiposity and insulin sensitivity and resistance, corroborating the influence in metabolic pathways and their connection with metabolic diseases. This association is also noted in ageing, since an unhealthy metabolic state accelerates the epigenetic age, possibly leading to an early decline of body functions.

This thesis adds further insights in the underlying epigenetic mechanisms of prematurity, metabolic diseases and ageing. Selected CpGs might be considered for future studies as potential biomarkers for diagnosis or putative targets for the development of therapeutic approaches in the prevention and treatment of diseases.

CONCLUSIONS

1. Epigenomic assessment on preterm newborns demonstrated that they exhibit different epigenetic patterns of methylation in comparison to full-term newborns. The largest methylation differences were observed at a CpG located at the dopamine transporter DAT-1 (*SLC6A3*), which was associated with motor and mental neurodevelopment, suggesting the involvement of epigenetic signatures in the adverse effects of prematurity such as neurodevelopmental impairment.
2. Adherence to a Mediterranean diet was associated with methylation differences in CpGs, some of them located at inflammation-related genes. Furthermore, one CpG located at *EEF2* gene was also correlated with concentration of the inflammatory molecules TNF-α and CRP.
3. A Mediterranean dietary pattern with foods rich in different types of fat was associated with specific changes in the levels of DNA methylation in genes related to intermediate metabolism, inflammation, and diabetes, such as *CTP1B* and *GNAS*.
4. Insulin sensitivity measured by an intravenous glucose challenge (CSi index) was associated with the level of DNA methylation of CpGs at genes such as *LPL* and *CTNND2,* which were able to discriminate subjects with lower and higher insulin sensitivity.
5. Insulin resistance was associated with DNA methylation, with individuals with higher and lower levels of HOMA-IR displaying a differential methylation pattern. Four CpGs, including those located at *SH3RF3* and *MAN2C1* genes*,* were the best estimators for discerning between the two HOMA-IR groups.
6. Waist circumference was associated with CpG methylation signatures at genes mainly related to inflammation, obesity, and related comorbidities. Subjects with the presence or absence of central obesity showed differential methylation patterns according to their sex. Of the top eight CpGs capable of distinguishing between the two abdominal fat groups, five CpGs, located at the *c13orf36, ZC3H12D, MYO9B, KCNG3* and *TCP11L1* genes, were validated in another population.
7. Epigenetic age acceleration, measured using the GrimAge approach, was associated with adiposity, metabolic and inflammatory markers in a sex-dependent manner. Thus, in males, the accumulation of visceral adipose tissue caused an acceleration of epigenetic age through mechanisms that appeared to be mostly mediated by insulin resistance, while, in females, these mechanisms seemed to be more related to HDL-cholesterol and C- reactive protein.

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APPENDIX

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DNA methylation patterns at sweet taste transducing genes are associated with BMI and carbohydrate intake in an adult population

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ABSTRACT

Individual differences in taste perception may influence appetite, dietary intakes, and subsequently, disease risk. Correlations of DNA methylation patterns at taste transducing genes with BMI and dietary intakes were studied. A nutriepigenomic analysis within the Methyl Epigenome Network Association (MENA) project was conducted in 474 adults. DNA methylation in peripheral white blood cells was analyzed by a microarray approach. KEGG pathway analyses were performed concerning the characterization and discrimination of genes involved in the taste transduction pathway. Adjusted FDR values ($p < 0.0001$) were used to select those CpGs that showed best correlation with BMI. A total of 29 CpGs at taste transducing genes met the FDR criteria. However, only 12 CpGs remained statistically significant after linear regression analyses adjusted for age and sex. These included cg15743657 (*TAS1R2*), cg02743674 (*TRPM5*), cg01790523 (*SCN9A*), cg15947487 (*CALHM1*), cg11658986 (*ADCY6*), cg04149773 (*ADCY6*), cg02841941 (*P2RY1*), cg02315111 (*P2RX2*), cg08273233 (*ITR1E*), cg14523238 (*GABBR2*), cg12315353 (*GABBR1*) and cg05579652 (*CACNA1C*). Interestingly, most of them were implicated in the sweet taste signaling pathway, except *CACNA1C* (sour taste). In addition, *TAS1R2* methylation at cg15743657 was strongly correlated with total energy ($p < 0.0001$) and carbohydrate intakes ($p < 0.0001$). This study suggests that methylation in genes related to sweet taste could be an epigenetic mechanism associated with obesity.

1. Introduction

The increasing prevalence of obesity constitutes an important public health and economic issue in developed and emerging countries (Tremmel, Gerdtham, Nilsson, & Saha, 2017). Globally, it has been estimated that about 2 billion people present overweight and obesity according to body mass index (BMI) measurements (Ng et al., 2014). Obesity has a pronounced impact on morbidity and mortality since contributes to the global incidence of common chronic diseases and morbid manifestation including type 2 diabetes and cardiovascular events (Seidell, 2015). Moreover, systematic analyses have revealed that overweight and obesity cause more than 3 million deaths each year (Lim et al., 2012).

Obesity epidemic is the result of environmental influences (diet and sedentary lifestyles) promoting positive energy balances and weight gains, which may interact with a genetic predisposition (Marti, Martinez-González, & Martinez, 2008). In this sense, the increased consumption of sweet energy-dense foods is thought to be a major contributor to the rising obesity rates worldwide (Bray, 2014; Ruanpeng, Thongprayoon, Cheungpasitporn, & Harindhanavudhi, 2017). Accordingly, observational studies have shown positive relationships between sweetened beverage intake and adiposity in some cases (Woodward-Lopez, Kao, & Ritchie, 2011), but not in others (Brand-Miller, 2017). In addition, there is growing evidence about the involvement of genetic and epigenetic factors in the development of obesity and associated complications by influencing individual responses to environment (Martinez, Milagro, Claycombe, & Schalinske, 2014; Milagro, Mansego, De

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Appendix

Abbreviations

5-HT	serotonin
ADCY6	adenylate cyclase 6
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
CACNA1C	calcium voltage-gated channel subunit alpha1 C
CALHM1	calcium homeostasis modulator 1
cAMP	cyclic adenine monophosphate
ChAMP	450k Chip Analysis Methylation Pipeline
CpG	cytosine-phosphate-guanine site.
DiOGenes	Diet, Obesity and Genes Dietary trial
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
ENaCs	epithelial Na + channels
FDR	false discovery rate
FOOD4ME	Food For Me study trial
GABA	gamma-aminobutyric acid
GABBR1	gamma-aminobutyric acid type B receptor subunit 1
GABBR2	gamma-aminobutyric acid type B receptor subunit 2
GEDYMET	Genetics, dysglycemia and metabolism
HDL-c	high-density lipoprotein cholesterol
HOMA-IR index	homeostatic model assessment-insulin resistance
HTR1E	5-hydroxytryptamine receptor 1E
ICTUS	International Citicoline Trial on Acute Stroke
IP3	inositol 1,4,5-trisphosphate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDL c	low density lipoprotein cholesterol
LIMMA	linear models for microarray data
MENA	Methyl Epigenome Network Association project
NUGENOB	Nutrient-Gene Interactions in Human Obesity trial
OBFKIT	Nutrigenetic test for personalized prescription of body weight loss diets trial
OBEPALIP	Eicosapentaenoic Acid in Human Obesity trial
P2RX2	purinergic receptor P2X2
P2RY1	purinergic receptor P2Y1
PKD1L3	polycystin 1 like 3, transient receptor potential channel interacting
PKD2L1	polycystin 2 like 1, transient receptor potential cation channel
PREDIMED	Prevención con Dieta Mediterránea trial
RESMENA	Reduction of the Metabolyc Syndrome in Navarra-Spain trial
SCN9A	sodium voltage-gated channel alpha subunit 9
SPSS	statistical package for the social sciences
SWAN	Subset-quantile Within Array Normalization
T1R	taste receptor type 1
T1R2	taste 1 receptor member 2
T1R3	taste 1 receptor member 3
T2R	taste receptor type 2
TAS1R2	taste 1 receptor member 2
TRC	taste receptor cells
TRPM5	transient receptor potential cation channel subfamily member 5
TyG	triglyceride-glucose index
VGCC	voltage-gated calcium channels
VGNC	voltage-gated sodium channels
WC	waist circumference

Miguel, & Martínez, 2013).

In this context, taste perception may play an important role in determining food preferences and eating behaviors (Galindo, Schneider, Stähler, Töle, & Meyerhof, 2012). Thus, individual differences in the ability to detect diverse taste qualities in the oral cavity may influence appetite, dietary intakes, and subsequently disease risk (Loper, La Sala, Dotson, & Steinle, 2015). Indeed, some studies have reported differences in sensitivity, perception and implicit attitude towards certain basic tastes between obese and lean subjects (Hardikar, Höchenberger, Villringer, & Ohla, 2017; Sartor et al., 2011), although others have not found such variation (Martinez-Cordero, Malacara-Hernandez, & Martinez-Cordero, 2015; Tucker, Nuessle, Garneau, Smutzer, & Mattes, 2015). Taste perception in humans is mediated by several specialized taste receptors within taste bud cell membranes: the G-protein coupled receptor families T2R involved in bitter taste (Chandrashekar et al., 2000), and T1R implicated in sweet and umami tastes (Zhao et al., 2003); other include the channel-type receptors PKD1L3/PKD2L1 involved in sour taste (Ishimaru et al., 2006), and ENaCs regulating salty taste (Chandrashekar et al., 2010). The activation of taste receptors mobilizes cellular pathways of transmission from taste bud cells to the central nervous system involving multiple mediators and effectors (Iwata, Yoshida, & Ninomiya, 2014; Kikut-Ligaj, 2015).

To date, several common polymorphisms within taste transducing genes have been associated with dietary intakes as well as on diverse metabolic disorders and chronic diseases including obesity (Ramos-Lopez et al., 2015b, 2016a, 2016b, 2015a; Chamoun et al., 2016). However, the role of epigenetic signatures in taste perception and obesity susceptibility has not been apparently explored. Advances in the understanding of epigenetic mechanisms underlying weight control may lead to the implementation of precision intervention strategies focused on early detection, diagnosis and treatment of obesity (Goni, Cuervo, Milagro, & Martínez, 2016). The aim of this study was to investigate the correlation between DNA methylation patterns at taste transducing genes and BMI in an adult population.

2. Methods

2.1. Subjects

A nutriepigenomic analysis within the Methyl Epigenome Network Association (MENA) project was conducted in 474 adults from previous cohorts (Abete et al., 2015; Huerta, Navas-Carretero, Prieto-Hontoria, Martínez, & Moreno-Aliaga, 2015; Larsen et al., 2010; Martínez-González et al., 2014; Petersen et al., 2006; San-Cristobal et al., 2015; Santos et al., 2016; Zulet et al., 2011) which constitutes the MENA project. The study protocol was in accordance with the ethical principles of the Helsinki Declaration. Subject's data were codified to guarantee anonymity.

2.2. Study variables

Anthropometric measurement and the metabolic profile were obtained from databases of the aforementioned cohorts. BMI was calculated dividing weight in kg by height in meters squared (kg/m^2). Insulin resistance was estimated using the homeostatic model assessment-insulin resistance (HOMA-IR) index (Aller, Abete, Astrup, Martinez, & van Baak, 2011). The triglyceride-glucose (TyG) index was subsequently calculated as a predictor of diabetes (Navarro-González, Sánchez-Íñigo, Pastrana-Delgado, Fernández-Montero, & Martinez, 2016) and cardiovascular events

(Sánchez-Íñigo, Navarro-González, Fernández-Montero, Pastrana-Delgado, & Martínez, 2016).

2.3. Dietary assessment

Dietary data was available from 212 subjects of the PREDIMED an OBEKIT cohorts, which presented similar characteristics in relation to the total sample. Habitual dietary intake was assessed using a validated food frequency questionnaire that included 137 food items (Martin-Moreno et al., 1993). For this purpose, individuals were asked to provide information about the number of times they had consumed each food item during the previous year base on the following frequency categories: daily, weekly, monthly or never. Accordingly, the Spanish food composition tables (Moreiras, Carbajal, Cabrera, & Cuadrado, 2016) were used to convert food portions to energy and macronutrient intakes.

2.4. DNA methylation analyses

Genomic DNA was extracted from peripheral white blood cells using the Master Pure kit (Epicenter, Madison, WI), whose quality was assessed with the Pico Green dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). High-quality DNA samples (500 ng) were treated with bisulfite using the EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, CA) according to the manufacturer's instructions, thus converting cytosine into uracil. DNA methylation analyses were performed by microarray with the Infinium Human Methylation 450K bead chip technology (Illumina, San Diego, CA, USA), as detailed elsewhere (Mansego, Garcia-Lacarte, Milagro, Marti, & Martinez, 2017).

The 450k Chip Analysis Methylation Pipeline (ChAMP) package for R software (version 1.11.0) was used for obtaining probes intensity data (Morris et al., 2014). Subsequently, probes were filtered based on the following criteria: detection p-values above 0.01 in one or more samples, probes with a beadcount <3 in at least 5% of samples, non-CpG probes, probes with SNPs, probes that align to multiple locations (Nordlund et al., 2013) and those located on the X or Y chromosomes. After filtering of probes, intra-array normalization was performed using the Subset-quantile Within Array Normalization (SWAN) method (Maksimovic, Gordon, & Oshlack, 2012) to adjust the bias introduced by the Infinium type 2 probe design. To the correction of non-biological experimental variation or "batch effects", the ComBat normalization method was applied (Johnson, Li, & Rabinovic, 2007; Teschendorff, Zhuang, & Widschwendter, 2011). Furthermore, DNA methylation data were corrected by cell composition heterogeneity using the Houseman algorithm (Houseman et al., 2012). This method allows quantifying the different peripheral white blood cell subtypes (granulocytes, monocytes, B cells, T cells- $CD8^+$, T-cells-$CD4^+$ and natural killer cells) using as reference a group of CpG signatures, which were validated with purified subpopulations. Beta values have been used as the metric for DNA methylation data. Beta values are calculated as the ratio between the Illumina methylated probe intensities and the overall probe intensities (sum of methylated and unmethylated probe intensities). The beta-value is a number between 0 and 1, or 0 and 100%, corresponding to unmethylated and completely methylated (Du, Kibbe, & Lin, 2008; Weinhold, Wahl, Pechlivanis, Hoffmann, & Schmid, 2016).

2.5. Pathway analyses

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.jp/kegg/pathway.html) (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017) was used to identify the genes implicated in the complete taste transduction pathway (map04742), and subsequently to discriminate the specific taste signaling pathway, in which these genes were involved.

2.6. Statistical analyses

Quantitative variables were expressed as means ± standard deviations and qualitative variables as number and percentages. After pre-processing, the LIMMA package for the R statistical software (version 3.3.2) was used to compute a lineal regression adjusted by the effect of confounding factors, such as age, sex, study cohorts and DNA methylation chips. Raw p values were adjusted using the Benjamini Hochberg procedure, and a False Discovery Rate (FDR) cut-off of 0.05 and B-statistic above 0 in the outcome-related analyses was used as statistical significant threshold. Adjusted FDR values < 0.0001 were used to select those CpGs that showed best correlation with BMI. Furthermore, linear regression analyses were performed to evaluate correlations of methylation values at taste transducing genes with BMI and dietary intakes, with age and sex as covariates. Statistical differences between DNA methylation values by BMI categories were analyzed by one-way ANOVA test adjusted for age and sex. Subsequently, *post-hoc* tests were run to define intergroup differences according to the homogeneity of variances. Bonferroni's test assuming equal variances and Dunnett's T3 test assuming unequal variances were used. Statistical analyses were performed using the IBM SPSS software, version 20 (IBM Inc., Armonk, NY, USA). Figure plots were created using the GraphPad Prism® software, version 6.0C (La Jolla, CA, USA) in order to illustrate significant correlations.

3. Results

Demographic, anthropometric, and metabolic characteristics of the total sample and by BMI categories are reported (Table 1). Overall, the mean values of glucose, total cholesterol and the TyG index were above the reference limits. Normal weight subjects were younger, had a higher proportion of men and better metabolic profile compared with the total population. Subjects with obesity had higher levels of insulin and HOMA-IR index, as well as lower levels of HDL-c than overweight and normal weight individuals (Table 1).

A total of 7457 CpG sites met the FDR criteria and were correlated with BMI (adjusted FDR <0.0001). Of these, 29 CpGs were implicated in the taste transduction pathway; however, only 12 CpGs remained statistically significant after linear regression analyses adjusted for age and sex (Table 2). These included cg15743657 (*TAS1R2*), cg02743674 (*TRPM5*), cg01790523 (*SCN9A*), cg15947487 (*CALHM1*), cg11658986 (*ADCY6*), cg04149773 (*ADCY6*), cg02841941 (*P2RY1*), cg02315111 (*P2RX2*), cg08273233 (*HTR1E*), cg14523238 (*GABBR2*), cg12315353 (*GABBR1*) and cg05579652 (*CACNA1C*).

The correlations between methylation levels at the aforementioned 12 CpG sites and BMI values were plotted (Fig. 1). Highest regression coefficients were observed for cg15743657 (*TAS1R2*), cg02841941 (*P2RY1*), cg14523238 (*GABBR2*) and cg12315353 (*GABBR1*). In addition, 10th, 25th, 50th, 75th, and 90th percentiles of methylation levels at taste transducing genes in relation to BMI categories are presented (Fig. 2). Overall, subjects with obesity presented lower methylation levels than normal weight and overweight individuals (Fig. 2). In addition, methylation at cg02743674 (*TRPM5*), cg15947487 (*CALHM1*), and cg14523238 (*GABBR2*) differed between overweight and normal weight subjects (Fig. 2).

Pathway analyses revealed that taste transducing genes whose methylation patterns were correlated with BMI, were mainly implicated in the sweet taste transduction pathway in type II receptor cells, including the sweet taste receptor *TAS1R2*, and several downstream signaling mediators and effectors such as *TRPM5*,

Appendix

Table 1
Demographic, anthropometric, and metabolic characteristics of the study population.

Variable	Total	Normal weight	Overweight	Obesity
n (%)	474 (100)	84 (17.7)	155 (32.7)	235 (49.6)
Age (years)	47.2 ± 14.1	35.2 ± 14.1[c]	53.2 ± 14.6	47.1 ± 11.2[a]
Men/women	171/303	20/64	64/91[b]	87/148
Anthropometrics				
Weight (kg)	81.6 ± 19.1	60.7 ± 8.1[c]	73.2 ± 8.9	94.6 ± 16.9[a]
BMI (kg/m^2)	30.1 ± 5.6	22.3 ± 1.5[c]	27.3 ± 1.3	35.4 ± 4.0[a]
WC (cm)	95.7 ± 16.1	73.8 ± 7.7[c]	91.2 ± 8.2	106.6 ± 12.2[a]
Metabolic profile				
Glucose (mg/dL)	102.3 ± 29.8	88.2 ± 25.3[c]	103.3 ± 31.7[b]	106.3 ± 28.6
Insulin (mIU/L)	9.6 ± 7.0	6.2 ± 3.0	6.5 ± 3.3	12.0 ± 8.0[a]
HOMA-IR index	2.4 ± 2.2	1.2 ± 0.6[c]	1.5 ± 0.8	3.2 ± 2.6[a]
Total cholesterol (mg/dL)	204.7 ± 40.3	185.0 ± 42.4[c]	210.0 ± 38.8[b]	207.7 ± 38.9
HDL-c (mg/dL)	53.5 ± 13.5	62.0 ± 12.4[c]	56.1 ± 12.6	48.9 ± 12.5[a]
LDL-c (mg/dL)	127.5 ± 36.7	106.1 ± 41.4[c]	130.6 ± 36.1[b]	132.6 ± 33.0
Triglycerides (mg/dL)	119.8 ± 72.5	92.6 ± 57.2[c]	116.8 ± 61.9[b]	131.0 ± 80.9
TyG index	4.61 ± 0.32	4.43 ± 0.27[c]	4.62 ± 0.29[b]	4.67 ± 0.33

Categorical variables are presented as number of cases and percentage and continuous variables as means ± standard deviations. BMI: body mass index; WC: waist circumference; HOMA-IR: homeostatic model assessment-insulin resistance index; HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; TyG: triglyceride-glucose index. [a]Obesity vs. overweight and normal weight, $p < 0.001$. [b]Overweight vs. normal weight, $p < 0.05$. [c]Normal weight vs. total, $p < 0.001$. Statistical test: One-way ANOVA.

Table 2
Genomic and statistical data of the 12 CpG sites at taste transducing genes associated with BMI.

CpG_ID	Gene name	Gene symbol	CHR position	Genomic region	FDR	Adj. FDR	B	R	r^2
cg02841941	Purinergic receptor P2Y1	*P2RY1*	3:152831269	TSS200	2.8E-12	2.5E-09	16.2	−0.33	0.11
cg15743657	Taste 1 receptor member 2	*TAS1R2*	1:18856736	TSS1500	1.5E-10	5.1E-08	12.2	−0.32	0.10
cg14523238	Gamma-aminobutyric acid type B receptor subunit 2	*GABBR2*	9:98427001	Body	1.9E-10	5.9E-08	12.1	−0.34	0.12
cg12315353	Gamma-aminobutyric acid type B receptor subunit 1	*GABBR1*	6:29601755	Body	2.5E-09	4.1E-07	9.5	−0.31	0.09
cg11658986	Adenylate cyclase 6	*ADCY6*	12:48780265	Exon 1	1.6E-08	1.6E-06	7.7	−0.29	0.08
cg04149773	Adenylate cyclase 6	*ADCY6*	12:48782583	5′UTR	1.4E-07	7.7E-06	5.6	−0.24	0.06
cg01790523	Sodium voltage-gated channel alpha subunit 9	*SCN9A*	2:166369637	5′UTR	5.9E-07	2.2E-05	4.2	−0.24	0.06
cg15947487	Calcium homeostasis modulator 1	*CALHM1*	10:103455020	Exon 1	1.1E-06	3.3E-05	3.6	−0.26	0.07
cg08273233	5-hydroxytryptamine receptor 1E	*HTR1E*	6:87011704	5′UTR	1.2E-06	3.7E-05	3.5	−0.24	0.06
cg05579652	Calcium voltage-gated channel subunit alpha1 C	*CACNA1C*	12:2060676	Body	2.3E-06	5.8E-05	2.9	−0.27	0.08
cg02743674	Transient receptor potential cation channel subfamily member 5	*TRPM5*	11:2408681	Body	3.4E-06	7.5E-05	2.5	−0.25	0.06
cg02315111	Purinergic receptor P2X2	*P2RX2*	12:132613864	TSS1500	4.6E-06	9.5E-05	2.2	−0.29	0.09

Genome assembly: GRCh38.p10. BMI: body mass index. Data are sorted by adj. FDR values. FDR: False Discovery Rate.

SCN9A, *CALHM1*, *ADCY6*, *P2RY1*, *P2RX2*, *HTR1E*, *GABBR2* and *GABBR1*. On the other hand, *CACNA1C* gene was located in type III presynaptic cells, which are involved in the regulation of sour taste (Fig. 3).

The average values of nutrient intakes in a subsample of study population are presented (Supplementary Table 1). Furthermore, relationships between taste methylation patterns and dietary intakes were screened in 212 individuals (Fig. 4). Noteworthy, significant correlations ($p < 0.0001$) between *TAS1R2* methylation at cg15743657 and dietary intakes of total energy (Fig. 4A) and carbohydrates (Fig. 4B) were found. On the other hand, consumptions of protein and fats were no apparently related to taste methylation status (data not shown).

4. Discussion

Sweet taste perception is considered one of the most important sensorial factors modulating food acceptance and appetite (Loper et al., 2015), which may influence individual obesity predisposition (Low, Lacy, & Keast, 2014). Indeed, longitudinal analysis in twins suggests that sweet taste perception is associated with BMI at the phenotypic and genotypic level (Hwang et al., 2016). The present research shows, apparently for the first time, correlations between methylation patterns at taste transducing genes and BMI values, which were independent of potential confounding factors such as sex, age and study cohorts. Interestingly, subjects with obesity had the lowest methylation levels of these genes compared to those who were overweight or normal weight. These findings may account for some of the interindividual differences reported in taste sensitivity between obese and lean subjects (Hardikar et al., 2017; Sartor et al., 2011). Moreover, establishing an epigenetic basis for taste function may help to explain and understand, at least in part, some of the inconsistencies among observational studies relating taste perception to obesity features (Feeney, O'Brien, Scannell, Markey, & Gibney, 2017; Low, Lacy, McBride, & Keast, 2016; Martinez-Cordero et al., 2015; Tucker et al., 2015).

Noteworthy, pathway analyses performed in this study revealed that BMI-associated genes were mainly implicated in the regulation of sweet taste perception including *TAS1R2*, *TRPM5*, *SCN9A*, *CALHM1*, *ADCY6*, *P2RY1*, *P2RX2*, *HTR1E*, *GABBR2* and *GABBR1*. At the molecular level, sweet taste perception in type II taste receptor cells (TRC) begins by the activation of the heterodimeric T1R2-T1R3 receptor, which recognize all compounds perceived as sweet by humans, including natural sugars and artificial sweeteners (Li et al., 2002). Nevertheless, T1R2 (encoded by the *TAS1R2* gene) is identified as the specific regulator of sweet taste sensations since T1R3 also mediates responses to L-amino acids (umami taste) in dimerization with the T1R1 receptor (Nelson et al., 2002). Signal transduction of sweet taste occurs via two distinct signaling pathways, one involves cAMP and the second one implicates inositol 1,4,5-trisphosphate (IP3). In the first case, natural sugars binding to the

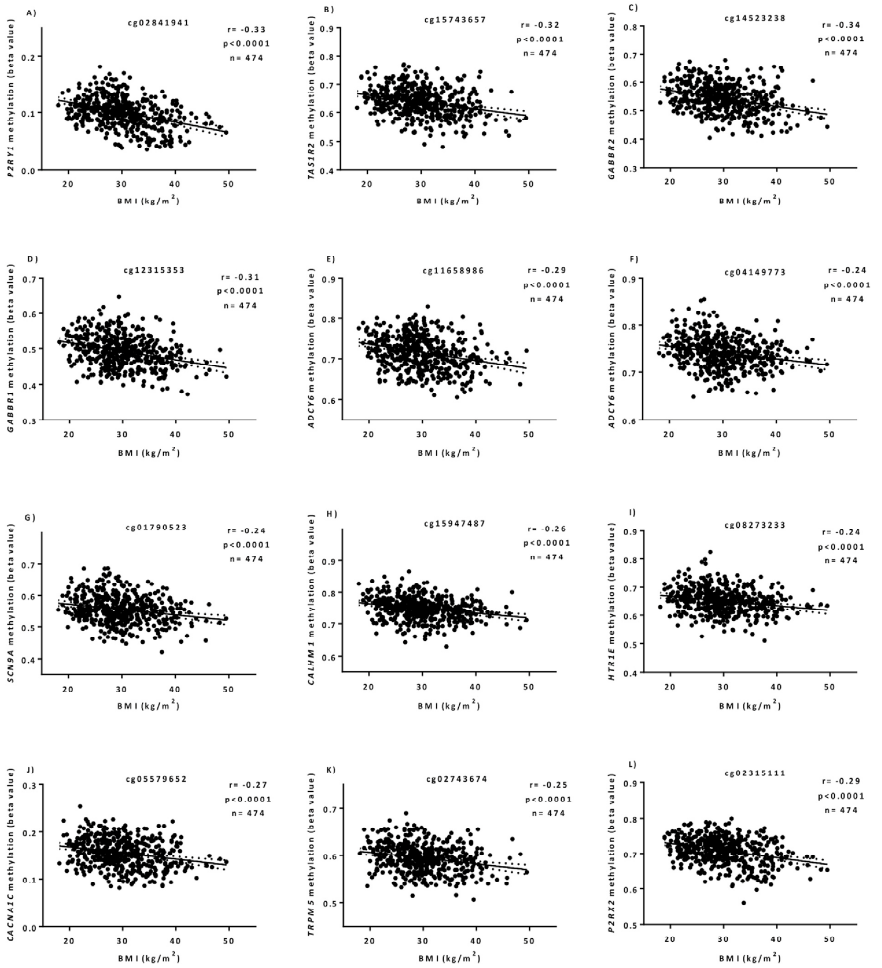

Fig. 1. Correlations between methylation levels at taste transducing genes and BMI values after adjustments for age and sex. (A) cg02841941, *P2RY1*, (B) cg15743657, *TAS1R2*, (C) cg14523238, *GABBR2*, (D) cg12315353, *GABBR1*, (E) cg11658986, *ADCY6* (F) cg04149773, *ADCY6*, (G) cg01790523, *SCN9A*, (H) cg15947487, *CALHM1*, (I) cg08273233, *HTR1E*, (J) cg05579652, *CACNA1C*, (K) cg02743674, *TRPM5*, (L) cg02315111, *P2RX2*. Statistical test: linear regression.

T1R2-T1R3 receptor activates adenylate cyclases (including ADCY6) to converts ATP to cAMP, which leads to inactivation of potassium channels in the basal membrane an consequently to TRC depolarization (Naim, Ronen, Striem, Levinson, & Zehavi, 1991; Striem, Pace, Zehavi, Naim, & Lancet, 1989). On the other hand, sweetener binding to the T1R2-T1R3 receptor induces IP3 elevation causing the release of calcium from intracellular stores to stimulates the cation channel TRPM5, resulting in sodium influx and the subsequent cell membrane depolarization (Zhang, Zhao, Margolskee, & Liman, 2007). Animal studies have suggested that *trpm5* ablation might result in reduced body weight gain and protected from excessive consumption of sweet palatable foods after long-term feeding with high caloric diets (Larsson, Håkansson, Jansen, Magnell, & Brodin, 2015). Thus, it has been highlighted that TRPM5 could be a target for therapeutic intervention in obesity by impacting taste sensory signals controlling energy consumption (Palmer, 2013). Moreover, polymorphisms within the *TRMP5* gene have been associated with obesity-related metabolic syndrome (Tabur et al., 2015) and prediabetic phenotypes in humans (Ketterer et al., 2011).

Within TRC, specialized voltage-gated sodium channels (VGNC) including SCN9A, detect cell membrane depolarization and initiate

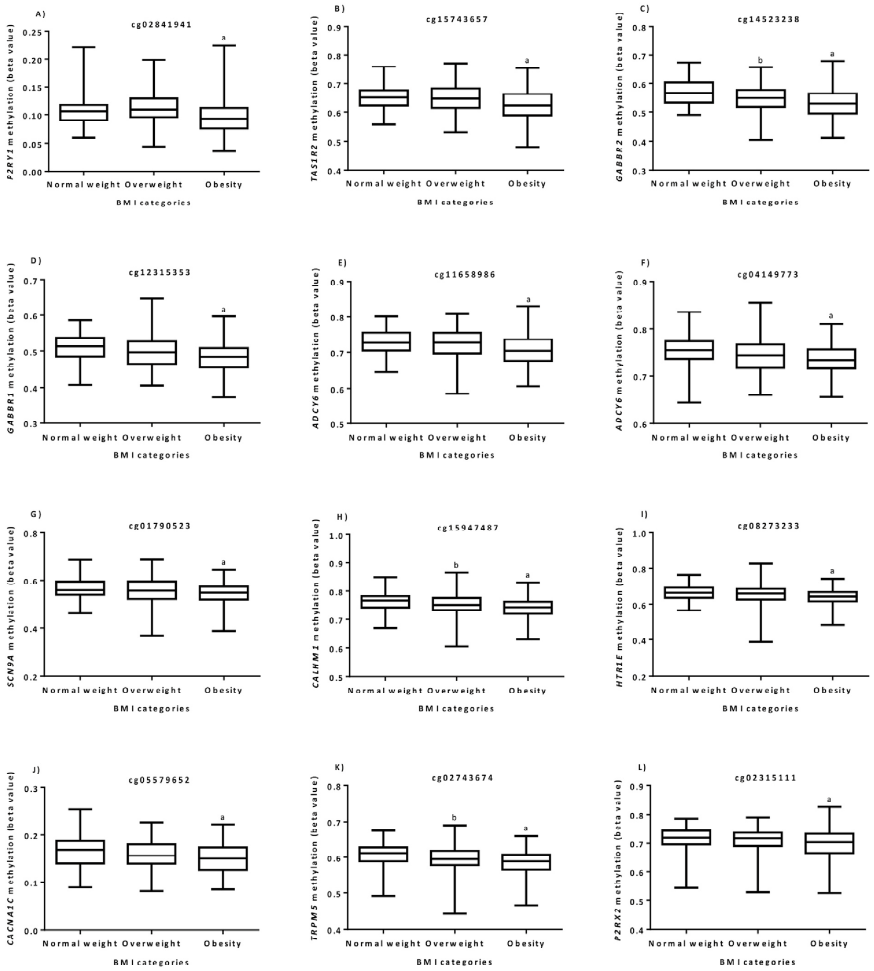

Fig. 2. Tenth, 25th, 50th, 75th, and 90th percentiles of methylation levels at taste transducing genes in relation to BMI categories after adjustments for age and sex. (A) cg02841941, *P2RY1*, (B) cg15743657, *TAS1R2*, (C) cg14523238, *GABBR2*, (D) cg12315353, *GABBR1*, (E) cg11658986, *ADCY6* (F) cg04149773, *ADCY6*, (G) cg01790523, *SCN9A*, (H) cg15947487, *CALHM1*, (I) cg08273233, *HTR1E*, (J) cg05579652, *CACNA1C*, (K) cg02743674, *TRPM5*, (L) cg02315111, *P2RX2*. [a]Obesity vs. overweight and normal weight, $p < 0.001$. [b]Overweight vs. normal weight. $p < 0.05$. Statistical test: one-way ANOVA.

the rising phase of action potentials encoding tasting information to afferent gustatory nerve fibers innervating TRC, where ATP acts as a neurotransmitter (Gao et al., 2009). The non-vesicular secretion of ATP from TRC requires CALHM1, an essential voltage-gated ion channel (Taruno et al., 2013). In this context, it has been suggested that Calhm1 knockout mice led to loss of sweet perception and to impaired gustatory nerve responses for this taste quality (Taruno et al., 2013). Also, *Calhm1* knockout mice had severely impaired perception of sweet, bitter, and umami tastes, whereas their sour and salty tasting abilities remained unaltered (Hellekant, Schmolling, Marambaud, & Rose-Hellekant, 2015). Furthermore, the secreted ATP transmits the taste signals to the afferent taste nerves by activating purinergic receptors such as P2RY1 and P2RX2 (Finger et al., 2005). Interestingly, it has been reported that obesity may exert its effects by promoting a decrease in P2RX2 expression in mice (Mizuno et al., 2014). In addition to ATP, other neurotransmitter candidates have been identified in taste buds cells including serotonin (5-HT) and GABA and their corresponding receptors (i.e. HT1RE, GABBR1, GABBR2), especially in type III presynaptic cells (Huang, Pereira, & Roper, 2011). Experimental studies

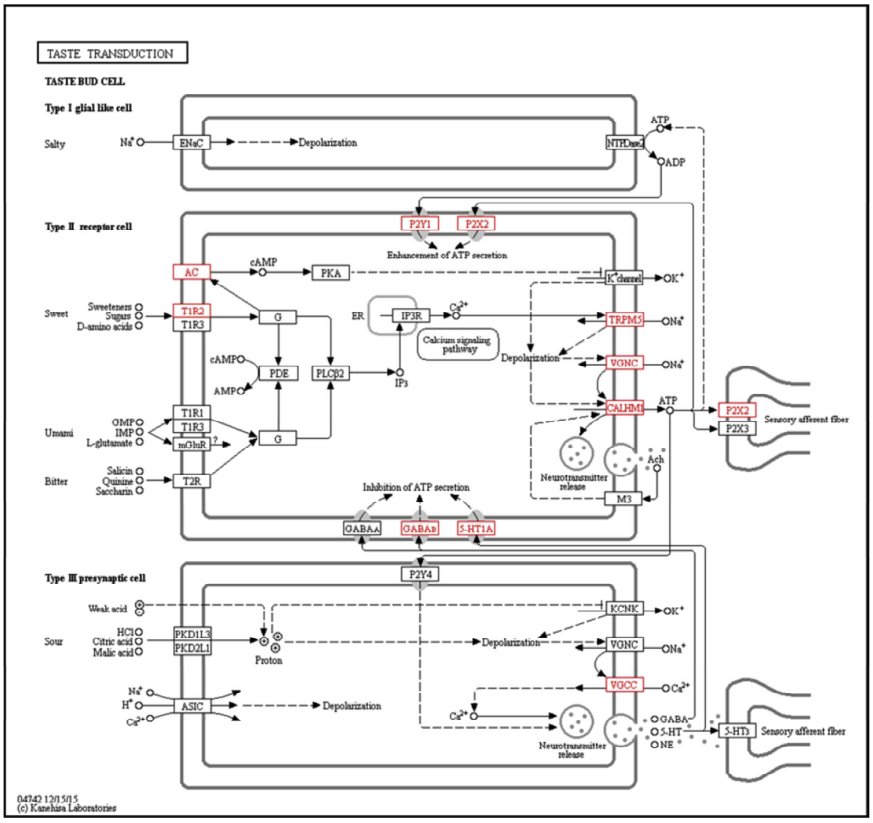

Fig. 3. Taste transducing genes whose methylation levels are associated with BMI (red color). List of genes: *TAS1R2*, *TRPM5*, *SCN9A*, *CALHM1*, *ADCY6*, *P2RY1*, *P2RX2*, *HTR1E*, *GABBR2*, *GABBR1* and *CACNA1C*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

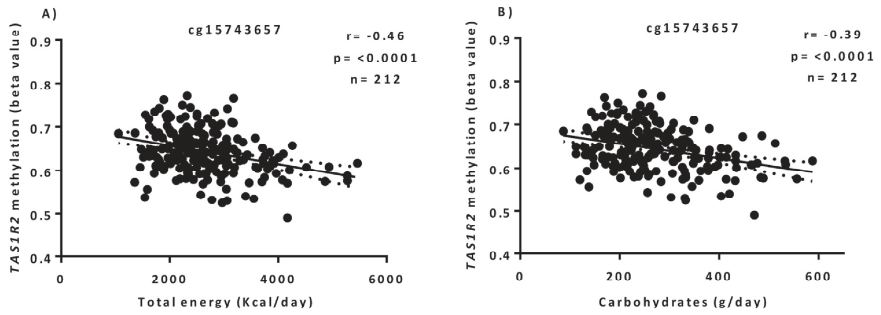

Fig. 4. Correlations between *TAS1R2* methylation at cg15743657 and total energy intake (A) and carbohydrate consumption (B) after adjustments for age and sex. Statistical test: linear regression.

in animal models revealed that 5-HT and GABA reduce taste-evoked ATP secretion from TRC, acting as inhibitory neurotransmitters in taste buds (Dvoryanchikov, Huang, Barro-Soria, Chaudhari, & Roper, 2011; Huang, Dando, & Roper, 2009). Additionally, type III presynaptic cells express voltage-gated calcium channels (VGCC) such as CACNA1C, which is involved in sour taste signaling (Richter, Caicedo, & Roper, 2003).

Another important finding of this study was the selective correlation between *TAS1R2* methylation at cg15743657 and dietary intakes of total energy and carbohydrates, which may explain the correlation with BMI. The T1R2 receptor plays a crucial regulatory function in sweet taste perception (Li et al., 2002). Since cg15743657 site is located in the promoter region of the *TAS1R2* gene, its effect in food consumption might be related to differences in *TAS1R2* expression and receptor activity. Similarly, functional single nucleotide polymorphisms within *TAS1R2* gene have also been associated with sugar taste thresholds (Dias et al., 2015) and carbohydrate intakes in several populations (Eny, Wolever, Corey, & El-Sohemy, 2010; Ramos-Lopez, 2016a, 2016b). Nonetheless, experimental studies using knockout animal models have reported contradictory results related to *Tas1r2* expression and dietary intake. For example, no variation in food intake was reported in *Tas1r2* knockout mice compared to wild type mice (Simon et al., 2014). In contrast, *Tas1r2* knockout mice fed a high-fat/low-carbohydrate diet were hyperphagic as compared with wild type mice (Smith et al., 2016). Thus, further research concerning *TAS1R2* expression, sweet taste perception and food intake in humans is warranted.

A limitation of this research was the analysis of DNA methylation signatures in peripheral white blood cells instead of taste bud cells. However, gene methylation parallelisms between peripheral blood cells and oral mucosa samples have been recently reported (San-Cristobal et al., 2016). Also, a methylation map in circulating leukocytes mirrors subcutaneous adipose tissue methylation patterns in obese and non-obese patients (Crujeiras et al., 2017). However, further methylation analyses in taste receptor cells from the tongue and their association with obesity phenotypes are convenient, as well as to point out the potential connection between blood immune cells and BMI (González-Muniesa et al., 2015). Additionally, despite that relevant statistical outcomes were found, type 1 and type 2 errors cannot be ruled out. Because of using strict FDR values to discriminate those CpGs that showed best correlation with BMI, some "nominal" statistical significances could have disappeared after applying a correction for multiple comparison tests, which should have been taken into account when interpreting the data. We could not also analyze in these subjects taste perception thresholds and gene expression. On the other hand, the strengths of this study include a large sample analyzed, and the statistical adjustments for potential confounding factors such as sex, age, cohorts, DNA methylation chips, and cell composition heterogeneity.

Nowadays, obesity epidemic worldwide is one of the greatest healthcare challenges. This feature is driving an intensive search for the identification of new factors implicated in the onset and progression of obesity and associated complications. This study suggests that epigenetic-environmental interactions might be involved in the regulation of sweet taste and dietary intake, which could contribute to the implementation of genotype intervention strategies for the diagnosis, prognosis and treatment of obesity (Goni et al., 2016; Ramos-Lopez et al., 2017).

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Conflict of interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.appet.2017.09.004.

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