A dual epigenomic approach for the search of obesity biomarkers:

DNA methylation in relation to diet-induced weight loss

Fermin I Milagro1S, Javier Campión1S, Paúl Cordero1, Estibaliz Goyenechea2, Ana M Gómez-Uriz1, Itziar Abete1, Maria A. Zulet1, J. Alfredo Martínez1*

1 Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra, Pamplona, Spain
2 Department of Neurology, Hospital Donostia, San Sebastian, Spain
* Correspondence author
5 Both authors have equally contributed to this work.

Address correspondence to J. Alfredo Martínez; Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra; c/Irunlarrea 1, 31008-Pamplona, Spain.
E-mail: jalfmtz@unav.es Telephone: 0034 948425600 Fax: 0034 948425649

Running head: Epigenetic marker, DNA methylation and weight loss

ABSTRACT

Epigenetics could contribute to explain individual differences in weight loss after an energy restriction intervention. Here, we identify novel potential epigenetic biomarkers of weight-loss comparing DNA methylation patterns of high and low responders to a
hypocaloric diet. Twenty-five overweight/obese men followed an 8-week caloric restriction intervention. DNA was isolated from peripheral blood mononuclear cells and treated with bisulfite. The basal and endpoint epigenetic differences between high and low responders were analysed by methylation microarray, which was also useful to compare the epigenetic changes due to the nutrition intervention. Subsequently, Sequenom EpiTYPER technology was used to validate several relevant CpGs and the surrounding regions.

DNA methylation levels in several CpGs located in ATP10A and CD44 genes showed statistical baseline differences depending on the weight-loss outcome. At the treatment endpoint, DNA methylation levels of several CpGs on WT1 promoter were statistically more methylated in the high than in the low responders. Finally, different CpG sites from WT1 and ATP10A were significantly modified as a result of the intervention.

In summary, hypocaloric diet-induced weight loss in humans could alter DNA methylation status of specific genes. Moreover, baseline DNA methylation patterns may be used as epigenetic markers that could help to predict weight loss.

**Keywords**: CD44, ATP10A, WT1, PBMC, microarray

**INTRODUCTION**

Obesity is defined as an excessive adiposity in relation to lean body mass, which may involve both an altered body fat distribution throughout the body and an enlarged adipose depot size (1). This condition is the result of an imbalance in energy homeostasis, whereby excessive food intake is not balanced by energy expenditure (1). The etiology of obesity involves complex interactions among inadequate dietary habits (excessive caloric supply, high-fat and high-sucrose regimes) and scarce physical exercise associated to sedentary lifestyles, with the genetic background, which may account to more than 40% of the predisposition to obesity (2). Other factors that are related to the recent obesity pandemics are the neuroendocrine status and the
fetal and perinatal programming, in which maternal nutrition, stress and maternal care of the offspring are concerned (3).

One of the most challenging problems concerning obesity management is weight regain after the weight loss (4). Indeed, it is thought that a number of nutrient, neural, and endocrine mediators convey an “energy deficit” signal to the brain that results in an increase in appetite and a decrease in energy (5). Among these signals, weight loss can be further associated to an increase in the number of adipocytes that facilitate the repletion and expansion of fat depots (6). Weight reduction also ameliorates insulin sensitivity, helping thus to store and utilize the caloric excess in an energetically efficient manner (7). In other words, weight loss induces metabolic adaptations that may contribute to rapid weight regain after the cessation of the weight loss program (4). However, strong interindividual differences are involved in the magnitude and ease to regain weight (8).

In this context, epigenetic marks have been described as good indicators of susceptibility or resistance to this weight gain (9, 10). Indeed, the interindividual differences in obesity predisposition, lipid profile and blood pressure may underlie also epigenetic factors, as it has been hypothesized (11, 12). Epigenetic mechanisms include DNA methylation, covalent histone modifications, as well as several types of regulatory RNAs such as microRNAs (miRNA) and large non-coding RNAs (lncRNA), which are affected by the environment, including dietary factors such as methyl donors, polyphenols or some minerals (13, 14). Other obesity-related features that could alter the epigenetic regulatory patterns are oxidative stress (15), a proinflammatory milieu, such as TNF-α (16), oxygen tension including hypoxia (17) and maternal behaviour and the offspring experience of pain and stress (18). In particular, the biological effects of calorie restriction are closely related to epigenetic mechanisms, including chromatin remodelling and DNA methylation (9). Among the metabolic processes that can be epigenetically-regulated by calorie restriction, the slowing of the aging process, the extension of life-span and the resistance to different forms of stress have been highlighted (19).

In this context, cytosine methylation is localized almost exclusively to CG dinucleotides (3–8% of all cytosines in the genome) and is mediated by two types of DNA methyltransferases:
DNMT1 is responsible of methylation during DNA synthesis, whereas DNMT3a and DNMT3b are responsible for de novo methylation (20). These methyl groups are recognized by methylation-sensitive transcription factors and methyl-binding proteins that are associated with gene silencing (21). In this context, different reports suggest that the epigenetic background of each individual, which may depend on a number of environmental factors all along the life course or even be inherited from the parents, may be an important determinant of body weight regulation (11-13).

We had three main goals in this study. First, the description of differences between low and high responders to caloric restriction in the DNA methylation patterns of several genes. This study may allow the identification of epigenetic marks that could be used as predictive markers of weight loss in the design of personalized obesity prevention and management. Second, the description of the effects of a hypocaloric diet treatment on the DNA methylation levels of different genes related to adiposity, inflammation and weight regulation. This study would help to search new mechanisms of metabolic reprogramming owing to energy deprivation. And third, the study of the differences between the DNA methylation patterns of high and low responders to the hypocaloric diet at the endpoint of the treatment, in order to associate them to the metabolic features of each subject. All these measurements were performed in peripheral blood mononuclear cells (PBMC), which can be easily obtained from blood donor buffy coats, do not involve any surgical or invasive intervention, and has been previously used as a reliable source of epigenetic biomarkers for prognosis and treatment of obesity (22).

**MATERIALS AND METHODS**

**Study population and experimental design**

Twenty-five men with excess body weight (BMI: 30.5±0.45 kg/m2), followed an 8-week energy-restricted diet with a 30% energy reduction. Total energy expenditure was calculated from resting energy expenditure measured by indirect calorimetry (Deltatrac; Datex-Ohmeda, Helsinki, Finland) and corrected by physical activity levels (23). The low-calorie, macronutrient balanced diet provided 30% of total energy as fats, 53% as carbohydrates and 17% as proteins. Weight loss was considered successful when a patient lost ≥ 5% of initial body weight (10).
Based on this criterion, the studied population was categorized into two groups: high or low responders to the dietary treatment.

Obese/overweight subjects were recruited among the Endocrinology outpatients of the Hospital of Navarra. To exclude subjects with clinical evidence of diabetes, hypertension, liver, renal or haematological disease, or other clinical disorders, initial screening evaluations included medical history, physical examination and a fasting blood profile (24). Other exclusion criteria included weight changes of over 3 kg within the 3 months prior to the start of the study, recent participation in other scientific trials (90-day period), pharmacological treatments for chronic diseases, drug or surgical obesity treatments, drugs or alcohol abuse. All subjects provided an informed consent prior to participate in the study, whose design was previously approved by the Ethics Committee of the University of Navarra (54/2006).

Data about anthropometry, body composition, energy expenditure, and blood pressure were collected at baseline (day 0) and at the endpoint (day 56). The nutrient/energy intake was controlled by means of 3-day weighed food records (2 weekdays and 1 weekend day), which were performed during the week before the beginning of the intervention (week 1) and during the week before the end of the nutritional trial (week 7). Weight loss was monitored weekly by a trained dietitian. In the weekly visits, reinforcement messages were also made to ensure compliance. Diet records were analysed and quantified using the Medisystem program (Sanocare, Madrid, Spain).

**Anthropometric and metabolic measurements**

Determinations of body weight (BW), height, body mass index (BMI), and waist circumference (WC) were measured as previously described (23). Fat mass and body composition were measured by bioelectric impedance (Quadscan 4000; Bodystat, Douglas, Isle of Man, UK).

Blood pressure was assessed with a standard mercury sphygmomanometer after at least 5 minutes of rest in a sitting position (Heinne Gamma G5, Germany) according to WHO criteria. The mean of 3 measurements of systolic (SBP) and diastolic (DBP) blood pressures was calculated and used in the analysis.
Blood samples were collected after overnight fasting through a venous catheter from an antecubital vein. Plasma and serum were separated from whole blood by centrifugation (1400 g, 15 min at 5°C) and stored at -80°C until assay. Plasma levels of glucose, triacylglycerol, total and HDL cholesterol were assayed on a Cobas-Mira equipment (Roche, Basel, Switzerland). Low-density lipoprotein (LDL) cholesterol data were estimated by the Friedewald equation. Plasma levels of insulin were assessed by radioimmunoassay kits (DPC, Los Angeles, CA). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting insulin (µU/mL) x fasting glucose (mmol/L) divided by 22.5, as previously described (24).

Serum leptin and adiponectin levels were measured by commercially available immunoassays (DPC, Los Angeles, CA). Ghrelin was quantified by a radioimmunoassay kit developed by Linco Research (St Charles, MO, USA). Fasting serum levels of IL-6 were determined by the Quantikine High-Sensitivity Human IL-6 enzymatic immunoassay (R&D Systems, Minneapolis, MN). PAI-1 was measured using an ELISA kit (Hyphen Biomed, Neuville-Sur-Oise, France). Circulating TNF-α was determined by an enzymatic immunoassay (R&D Systems, MN, USA). Serum malondialdehyde was evaluated by a colorimetric assay (OXIS International, Foster City, CA).

Peripheral blood mononuclear cells (PBMCs) were isolated before and after the intervention from total blood by differential centrifugation using the PMN medium (Axis Shield PoC AS, Oslo, Norway), as described elsewhere (22).

**DNA isolation and bisulfite conversion**

DNA from PBMCs was isolated by using the MasterPureTM kit (Epicentre, Madison, WI) and its quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA). Genomic DNA (500 ng) was bisulfite-converted by using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA).

**Methylation microarray**
From the 25 subjects included in the study, twelve of them were chosen for the microarray study. Those individuals that lost more than 10% of initial body weight (n=6) were considered as “high responders”, whereas those that lost less than 5% of initial body weight (n=6) were termed “low responders”.

DNA methylation levels were assessed using Illumina Methylation Assay (HumanMethylation27 BeadChip), covering 27,578 CpG dinucleotides in 14,495 genes. About 200 ng of DNA was bisulfite treated and then the converted DNA was applied to the microarray chip according to the manufacturer’s instructions (Illumina, San Diego, CA). Briefly, the DNA molecules were annealed to two different bead types with locus-specific DNA oligomers, one corresponding to the unmethylated and the other to the methylated state (25). A single-base extension step was performed using DNP- and Biotin-labeled ddNTPs. Thereafter, the array was fluorescently stained, scanned, and analysed.

**Methylation profile by Sequenom EpiTYPER**

From microarray data analysis, thirteen CpG sites from 9 genes (AQP9, ATP10A, CD44, IFNG, MEG3, POR, TNFRS9, and WT1) were selected and validated by using the Sequenom EpiTYPER approach, which relies upon base specific cleavage followed by MALDI-TOF mass spectrometry. Briefly, nine amplicons (400-500 bp) covering the relevant 13 CpGs were designed (Table 1) and tested, in a first step, in the same 12 subjects (6 high responders and 6 low responders to the hypocaloric diet) that were studied by microarray. PCR products were excised from 2% agarose gels, purified by Qiagen Gel Extraction Kit (Qiagen, Valencia, CA), and eluted with 1X Roche FastStart High Fidelity Reaction Buffer (Roche, Indianapolis, IN). In microtiter plates, five μl of the PCR products were treated with 2μl Shrimp Alkaline Phosphatase (SAP) mix (37°C, 20 min) to dephosphorylate unincorporated dNTPs, which were later processed by MassARRAY Matrix Liquid Handler. A 2μl volume of each SAP-treated sample was heat-inactivated (85°C, 5 min) and subsequently incubated for 3 h at 37°C with 5μl of Transcleave mix for concurrent in vitro transcription and base-specific cleavage as described elsewhere (26). After deionization with 6 mg of Sequenom resin and 20 μl of deionized water,
samples (10-15 nl) were transferred onto the spectroCHIP array by nanodispensation. Analysis with the Sequenom MALDI-TOF MS Compact Unit was performed following a 4-point calibration with oligonucleotides of different mass provided in the Sequenom kit. Matched peak data were exported using EpiTYPER software and analyzed. The application of this technique with the selected primers also revealed DNA methylation levels of other built-in 113 CpGs included in these nine regions that were not previously quantified by the microarray. Due to the special nature of the technique, DNA methylation values of some CpGs could not be measured independently (Table 1, right column), which is the case of nearby CpGs and CpGs included in similar chemical fragments after the enzymatic cut. These CpGs were conjointly quantified and an average value of them was reported.

In this first step (n=12), only about half of the CpGs selected from Illumina Microarray (6 of 13) were validated by EpiTYPER using Spearman's rank correlation test: CpG1 and CpG7 of AQP9, CpG26 of CD44, CpG8 of POR, and CpG9-10 and CpG 21 of WT1. In a second phase, in order to validate the results in a larger population, the promoters of CD44, ATP10A and WT1 were studied in the whole sample (n=25).

**Statistical analysis**

The Kolmogorov–Smirnov and the Shapiro-Wilk tests were used to determine variable distribution. Anthropometric and metabolic differences between low and high responders to the nutritional intervention were analyzed by using Mann–Whitney U test at baseline and end-point. To detect differential methylation changes by microarray between high and low responders groups, statistical analyses were made by using the Illumina model (computing false discovery rate or FDR). The calculation of FDR is based on the multiple testing corrections using Benjamini and Hochberg False Discovery Rate (27). This statistical approach allows taking advantage of the standard deviation from bead-level replicates to enable a better estimate of the biological noise (Personal communication from Illumina Inc.). A paired-t test was also used to analyze the effect of the diet when comparing before and after the hypocaloric dietary treatment.
Moreover, for being stringent, only those data that differed by more or less than 20% from the baseline value were considered for both analyses.

Differences in DNA Methylation levels by using EpiTYPER approach were analyzed with the Mann–Whitney U test (high responders vs low responders subjects) and paired-t test (before vs after diet). Spearman's rank correlation coefficient was used for comparing the two epigenetic tools, microarray and EpiTYPER, in order to focus the analysis on the most reliable CpGs. Pearson correlation test was used for comparing cytosine methylation levels of CpG sites (by EpiTYPER) with anthropometric and metabolic variables. For all these statistical analysis, SPSS for Windows was used (release 15.0; SPSS Inc., Chicago, IL).

RESULTS

Anthropometric and biochemical analysis

The results presented here concern an epigenomic study taking into account the baseline and final DNA methylation patterns of 25 overweight/obese men that followed an energy-restricted diet for 8 weeks. Twelve of them, those who lose more weight (high responders, n=6) and those who lose less weight (low responders, n=6) were chosen to perform a DNA methylation microarray study. Baseline values concerning the anthropometric and biochemical measurements were similar in those subjects that successfully achieved a weight loss of more than 5% as compared to those that did not respond to the treatment (Table 2). In addition, this table shows the changes observed in each parameter as a result of the dietary treatment. By design, the high responders were successful in the decrease of BMI, fat mass and waist circumference, contrary to the low responders, who lose very little weight (≤ 5% of initial body weight). The dietary treatment also induced an improvement of the lipid profile (especially total and LDL cholesterol) in the high responders, and decreased the circulating levels of leptin and, unexpectedly, adiponectin, in the same group. A small and non-significant amelioration of fasting insulin and HOMA index was found in the high responders as compared to the low responders (Table 2). However, the diastolic blood pressure decreased in the low responders in a bigger proportion than in the high responders, probably because this last group already had a normal level of this parameter before the treatment. Finally, the nutrition intervention did not
produce an improvement of blood inflammatory (TNF-α, IL-6, PAI) and oxidative (MDA) markers (Table 2).

**Epigenomic analysis**

*Baseline differences in DNA methylation between high and low responders*

Before dietary intervention, the microarray showed 1034 CpGs differentially methylated between high and low responders. Thus, 432 CpGs sites were relatively hypomethylated (>20% of change in the methylation levels) in the high-responder group compared with the low-responders, whereas 602 CpGs sites were relatively hypermethylated (>20% of change) in the high-responder group.

Some metabolically relevant genes were hypomethylated in at least one CpG before the intervention: ATPase class V type 10A (*ATP10a*; Δbeta -0.25), maternally expressed 3 (*MEG3*; Δbeta -0.25), sex hormone-binding globulin (*SHBG*; Δbeta -0.29), and CCAAT/enhancer binding protein γ (*CEBPγ*; Δbeta -0.28). Other relevant genes were hypermethylated in at least one site, such as the CD44 molecule (Indian blood group) (*CD44*; Δbeta 0.22), tumor necrosis factor receptor superfamily member 9 (*TNFRSF9*; Δbeta 0.22), B-cell CLL/lymphoma 2 (*BCL2*; Δbeta 0.28), phosphatase tensin homolog (*PTEN*; Δbeta 0.22), acetyl-Coenzyme A carboxylase α (*ACACA*; Δbeta 0.25), and S-phase kinase-associated protein 2 (*SKP2*, Δbeta 0.33).

From this analysis, four regions of genes related to obesity (*ATP10A, CD44, MEG3, and TNFRSF9*) were selected as putative predictive biomarkers and were analyzed by EpiTYPER (n=12). The primers that covered the genomic region surrounding these CpGs were designed to encompass 57 new CpGs that were not previously screened by the microarray. While the CpG methylation levels of *MEG3* and *TNFRSF9* regions showed no differences between high and low responders before dietary treatment, those concerning *ATP10A* and *CD44* showed promising results as candidate biomarkers. For *ATP10A*, significant differences between low and high responders were observed before the intervention (baseline) for CpG5-10-16, CpG18 and, although not statistically significant, for CpG3-4 (Figure 1A, n=12). When the sample size
was increased to 25 subjects, most of these CpGs showed a statistically significant correlation with some endpoint parameters, such as final leptin values (an endocrine marker of adipose mass) or with the treatment-induced changes in fat mass, body mass index (BMI), or waist circumference (Figure 1B, n=25), confirming that they could be reliable markers of successful response to the hypocaloric diet. In fact, the baseline methylation levels of some of the CpGs analyzed, especially CpG18, strongly predicted the loss of weight during the nutrition intervention program (Figure 1C, n=25) and, in a similar manner, the decreases in waist circumference, as an indicator of visceral adiposity (Figure 1D, n=25). Figure 1 illustrates the validity of CpG18 from *ATP10A* as a reliable marker of response to the diet.

Similarly, several CpGs located in *CD44* region (especially 14 and 29, but also, although not reaching statistical significance, CpGs 9-10, 16 and 30) were more methylated in the responders group, showing a similar trend of co-methylation in the genomic region and pointing out that the analysis of this region could be a good predictor of the treatment-induced changes in fat mass and waist girth, the most widely used marker of visceral obesity (Figure 2A, n=12). When the sample size was increased to 25 subjects, some of these CpGs showed a statistically significant correlation with some endpoint parameters, such as final TNF-alpha values (a pro-inflammatory cytokine) or with the treatment-induced changes in fat mass, body mass index (BMI), or waist circumference (Figure 2B, n=25). Figures 2C, 2D and 2E (n=25) show the correlations of three of these CpGs (CpGs 9-10, 14 and 30) with the decrease in body weight, which was higher in those individuals with a higher percentage of methylation in this CpG. These results suggest that the baseline methylation levels of these CpGs of *CD44* could be used as predictive markers of response to the diet.

**Endpoint differences in DNA methylation between high and low responders**

After the dietary intervention, the microarray showed only 15 CpG sites differentially methylated between high and low responders. The number of CpGs differentially methylated between high and low responders was 69-fold less than before the diet, suggesting that the nutritional intervention reduced the methylation differences between both groups of subjects.
Fourteen CpG sites were relatively hypomethylated (>20% of variation) in the high-responder group compared with the low-responders, including the docking protein 5 isoform A (DOK5; Δbeta -0.21) and B-cell CLL/lymphoma 2 (BCL2; Δbeta -0.20). Meanwhile, only 1 CpG site was relatively hypermethylated (>20%) at the end of the intervention in the high responder group: the LAG1 homolog ceramide synthase 3 gene (LASS3; Δbeta 0.28). Other obesity-related genes, though, were significantly hypermethylated although with a variation lower than 20%, such as interferon γ (IFNG; Δbeta 0.11) and a 800 bp region including four CpGs sites of the Wilms tumor 1 gene (WT1; Δbeta 0.11 to 0.16).

From these results, two genomic regions encompassing relevant CpGs (in WT1 and IFNG) were selected for analyzing endpoint differences in DNA methylation between high and low responders by EpiTYPER. The primers that covered the genomic region surrounding these CpGs were designed to comprise new CpGs that were not previously analyzed by the microarray. Regarding IFNG, no differences were found when comparing high and low responders after dietary treatment. However, EpiTYPER study of WT1 region showed several CpGs statistically hypermethylated in the high responders at the end of the dietary treatment (CpGs 2-3, 4, 7-8, 9-10, 13-32, 14, 15, 16-17, and 21) whereas other CpGs (12, 22-23, 29, and 30-31) tended to significance (Figure 3A, n=12). When the sample size was increased to 25 subjects, the methylation levels of several CpGs of WT1 were highly associated with the intervention-induced changes in diastolic blood pressure (Figures 3B-3E, n=25), suggesting a relationship between the epigenetic modifications of WT1 and blood pressure regulation.

Effects of the dietary treatment on DNA methylation

Taken into account all the microarray-studied subjects (high and low responders, n=12), 170 CpG sites were differentially methylated as a result of the energy restricted dietary intervention. From them, 70 CpGs sites were relatively demethylated (decrease of more than a 20%) by the dietary intervention and 100 CpGs were more methylated (increase of a 20%). Interestingly, the high responder group was more affected by the intervention, modifying about 8-fold more of
CpG sites compared to the low responder group (1570 CpG sites in high responders vs. 194 in low responders).

According to the microarray results, some metabolically relevant genes were hypomethylated in at least one CpG as a result of the intervention study, including aquaporin 9 (AQP9; Δbeta -0.22), tumor necrosis factor receptor superfamily member 9 (TNFRSF9; Δbeta -0.21) again, P450 cytochrome oxidoreductase (POR; Δbeta -0.21), and glucosamine-6-phosphate deaminase 2 (GNPDA2; Δbeta -0.36). The methylation of other genes was increased by calorie restriction, including the H19 imprinted maternally expressed transcript (H19; Δbeta 0.21), apolipoprotein A-II (APOA2; Δbeta 0.24), neurotrophin 3 (NTF3; Δbeta 0.23), and transforming growth factor beta 1 (TGFB1, Δbeta 0.26).

From this statistical analysis, the six genomic regions studied previously (ATP10A, WT1, CD44, IFNG, MEG3, and TNFRSF9) and three new regions (AQP9, NTF3, and POR) were selected as potential regions whose methylation levels were affected by the hypocaloric diet, and they were subsequently analyzed by EpiTYPER. When the analysis was performed in the microarray-reduced sample of 12 subjects, two genomic regions (ATP10A and WT1) showed differential methylation. When tested in the whole sample (n=25), two CpG sites, one from ATP10A (CpG18) and the other from WT1 (CpG21), resulted hypermethylated (p<0.05) due to the dietary intervention (Figure 4), suggesting that the nutrition intervention was able to alter the methylation profile of different genes in PBMCs.

DISCUSSION

The need for personalizing and improving weight loss management is one important factor that has driven the search for new predictive/prognostic biomarkers (28). Biomarkers are used to identify the risk, presence or susceptibility to disease, but also to guide diagnostic and therapeutic interventions and to individualize the treatment of distinct pathophysiological conditions within clinically similar populations (29), including obesity. A big breakthrough of this study is the use of PBMC cells instead of fat biopsies as a non-invasive source of DNA, following a previous finding from our group that described lower methylation of the TNF-α promoter in these cells after a hypocaloric diet (10). As previously stated for transcriptomics
epigenetic analysis of peripheral blood cells is a promising approach for diagnosing
disease, determining the treatment outcome and searching for biomarkers.

Different reports have described that changes in epigenetic marks could be related to the risk of
developing obesity and associated diseases. For example, the maternal diet during gestation and
lactation may alter the offspring’s epigenome, affecting their propensity to develop obesity at a
later age (31, 32). On the other hand, many environmental and nutritional factors have the
potential to modify the epigenome, even during the adult stage, which can also be behind the
increase in obesity prevalence (11). For instance, high fat-induced obesity in rats has been
associated to changes in leptin promoter DNA methylation (33). Also, TNF-α promoter
methylation pattern could be a good biomarker predicting hypocaloric diet-induced weight-loss
(10). In this context, one of the most promising issues is the possibility to personalize the weight
loss program by characterizing the epigenetic marks of the population, which could be inherited
from the ancestors or acquired along the life, with special emphasis on the perinatal period. In
this regard, as other authors have reported (9), the epigenetic marks could be useful to
compartmentalize the population into susceptible and reluctant to lose weight following a given
dietary or therapeutic program.

One of the most successful strategies in the field of biomarker research is the use of microarray-
based approaches comparing a wide range of DNA methylation patterns, which allows a high-
throughput discovery of epigenetic biomarkers. Our hypothesis was that the successful response
to a hypocaloric diet could be accounted for by epigenomic differences among the individuals.
Contrary to transcriptomic profiling, which do not usually show differences between responders
and non-responders before the dietary intervention (34), epigenetic differences characterizing
each group at baseline have been reported (9). With this focus, we have performed a microarray
study in PBMC cells in order to study DNA methylation differences between high and low
responders, as well as the possible changes induced by the diet.

Regarding the first and most important objective of the current work, the description of
epigenetic biomarkers predicting the response to a weight loss intervention, the microarray
analysis pointed out differential methylation in more than a thousand CpGs by using the
Illumina model. Due to the large number of genes showing differential methylation, we decided to employ a dual approach and analyzed some selected genomic regions by Sequenom MassARRAY EpiTYPER technology, which added neighbouring CpGs to the CpG sites selected in the microarray analysis. Using Spearman's rank correlation test, only around a half of the microarray-selected CpGs (6 of 13) were validated with this second technique, which instead of using a sequence-specific oligonucleotide is based on base specific cleavage followed by MALDI-TOF mass spectrometry. In this sense, admitting that methylation microarray generates a high number of false positives, array technology is a useful tool for screening genome-wide DNA methylation status, although with limitations that researchers have to evaluate in a cautious and appropriate manner (35). The results must be always validated by a complementary technique that should be applied to a larger sample size. The dual approach of validating some of the most promising results of the array (in our case not only those results that were statistically significant, but that differed in more than 20% of methylation between the two groups) with a second and more sensitive technique, in our case Sequenom's MassARRAY Epityper, allowed also to increase the number of CpGs assessed per gene. Another advantage of this validation methodology is the possibility of analysing the methylation patterns of contiguous CpG sites and detecting changes in the methylation of not only an isolated CpG (as in the microarray), but of several CpGs (a region of 400-600 pbs) with a common trend in epigenetic regulation.

The EpiTYPER results showed several CpGs located in the promoter of ATP10A (CpGs 5-10-16 and CpG18) whose methylation levels were statistically lower in the high responder group at the baseline (before the dietary intervention). Along with CpGs 3-4, they strongly correlated with the changes in fat mass, BMI, and waist circumference induced by the nutrition intervention in the whole population. ATP10A encodes an aminophospholipid translocase that transports phosphatidylserine and phosphatidylethanolamine from one side of a bilayer to another (36). It is a type IV P-type ATPase related to lipid trafficking and maintenance of the phospholipid asymmetry and fluidity of the plasma membrane, and seems to be involved in modulating body fat (37). Thus, mice inheriting a maternal deletion of ATP10A (also known as
ATP10C) are considered a model of obesity and type 2 diabetes, since they develop more hyperinsulinemia, insulin-resistance and non-alcoholic fatty liver than the mice inheriting the same deletion paternally (38). Similarly, simulations of maternal deletion of ATP10A indicated an anabolic metabolism consistent with the known clinical phenotypes of obesity (39). Although these results suggested the possibility of imprinting, it has been recently discarded in mice (37). This gene has a CpG island encompassing most of exon 1 and extending 5′ of the gene (37), although very little is known about its methylation regulation.

The Sequenom analysis revealed another two CpGs, located in the CD44 promoter (CpGs 14 and 29), that behave as reliable biomarkers of weight loss. When analysing the whole population, these CpGs, but also CpGs 9-10, showed very good correlations with body weight loss and changes in waist circumference and fat mass. CD44 is a cell-surface glycoprotein that acts as a receptor for hyaluronic acid. It is commonly expressed on hepatic Kupffer cells and infiltrating lymphocytes in liver and adipose tissue, being considered as an indirect marker of inflammation and early fibrosis (40). The expression of this gene is enhanced in the liver of patients with obesity and steatohepatitis (41). In subcutaneous adipose tissue, weight loss has been associated with a strong decrease in CD44 gene expression (41). In rodents, the same authors have described that high-fat-diet-induced obesity resulted in increased CD44 and osteopontin (a protein ligand of CD44) gene expressions in both fatty liver and epididymal fat, with both expressions positively correlated with steatosis. Our results, linking the epigenetic regulation of this proinflammatory gene with weight loss, reinforce the importance of the inflammatory milieu in the response to hypocaloric diets.

At the end of the dietary treatment, only fifteen CpGs resulted differentially methylated between high and low responders by using the microarray. This finding suggests that the weight loss regime exerted such a strong effect on DNA methylation that most of the baseline individual differences disappeared and were masked by the effects of the treatment. An interesting gene that maintained the differences after the treatment was WT1. This gene encodes a Kruppel-like zinc-finger protein that behaves as a transcription factor that can act as a tumor suppressor or an oncogene depending upon the cell type in which it is expressed (42). It is also expressed in
hematopoietic tissues, such as the bone marrow and lymph nodes, being especially upregulated in peripheral blood of a variety of leukemias (43). Regarding energy metabolism, this gene is located very close to the brain-derived neurotrophic factor (BDNF), whose chronic administration in the hypothalamic paraventricular nucleus reverses high-fat diet-induced obesity by regulating energy intake downstream of the leptin–proopiomelanocortin signaling pathway (44) and by increasing energy expenditure (45). A rare case of genetic haploinsufficiency in humans that affects on BDNF, PAX6 and WT1 is called the WAGR syndrome (Wilms’ tumour, Aniridia, Genitourinary, and mental Retardation), which in many cases is accompanied by obesity development (46). WT1 methylation has been extensively tackled in cancer research, but this is apparently the first time whose methylation pattern has been studied in obesity. In our study, it is intriguing that the methylation status of several CpGs of WT1 promoter is associated to the diet-induced change in diastolic blood pressure. In this sense, Steege et al. (47) have reported that Wilms’ tumour patients with WT1 gene mutations showed increased plasma renin levels and arterial hypertension, suggesting that the lack of inhibition of renin gene transcription by the mutant WT1 protein could be the cause of hypertension in these patients. Our results suggest that WT1 methylation levels could be affected by blood pressure, although methylation changes due to the nutrition intervention study could also be a causative factor of decreased diastolic blood pressure. Indeed, more studies are needed to unveil the importance of WT1 epigenetic regulation in body weight management and blood pressure regulation.

Concerning the last objective of this work, and taking into account only the high responders in the microarray assay, a 5.8% of the CpGs (1570) were modified by the dietary treatment, which was much more than the CpGs modified in the low responder group (194, a 0.7%). This outcome clearly points out that the high responder group is apparently more sensitive to diet-induced epigenetic modifications, probably as a result of a bigger DNA methylation plasticity, which is defined as the methylation/demethylation changes that occur during growth and development (48). Our results suggest the importance of individual DNA methylation plasticity in the susceptibility to obesity development and, especially, in the response to a weight loss
regime. In other words, higher DNA methylation adaptation seems to be linked to a higher phenotypical plasticity. It is likely that this plasticity could be related to different regulation of the expression or activity of the methylating and demethylating enzymes. Thus, other diets such as the lipogenic methyl-deficient diet, are able to cause non-alcoholic steatohepatitis in mice by inhibiting DNA methylation and by altering the liver expression of the maintenance DNMT1 and de novo DNMT3A proteins (49). Anyway, this finding supports the novel and challenging hypothesis that DNA methylation is responsive to energy-restrictive diet.

Among the CpG sites whose DNA methylation patterns were modified by the hypocaloric treatment and were validated by EpiTYPER in the whole population, we found CpG18 of ATP10A and CpG 21 of WT1. These results show that changes in dietary patterns, including hypocaloric diets and weight loss, are able to alter the methylation profile of different genes in PBMCs and, probably, in other cell types, as we have observed in adipose tissue from rats fed a high-fat diet (50). It is possible that some of these epigenetic modifications could be related to changes in the cell populations within PBMC pool as a result of the dietary treatment, in both composition and activation. However, this fact does not affect the main finding of the current work, which is the use of epigenetic markers in PBMC as predictors of weight loss.

As a conclusion, it is noteworthy that the current experimental design is a reliable way to identify epigenetic markers of the response to a given dietary pattern. The combination of a high-throughput microarray-based assay with a second more specific technique, such as EpiTYPER, including more individuals than in the first technique, allowed to find new putative biomarkers and to reliably validate them. With this dual approach, it has been proven that hypocaloric diets induce changes in the DNA methylation pattern in PBMC cells (CpG18 of ATP10A and CpG 21 of WT1), and that some of this marks could be used as early indicators of response to the metabolic effects of the weight loss program (i.e., CpG18 from ATP10A). Indeed, the interindividual variability of the epigenetic background seems to play a role in the success of weight management programs.

Acknowledgements
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REFERENCES


TABLE 1. Differentially methylated regions in several obesity-related genes by Illumina microarray, and the primers used for their correspondent analyses applying a Sequenom EpiTYPER approach.

<table>
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<tr>
<th>Symbol</th>
<th>Chrom.</th>
<th>Chromosomic CpG position</th>
<th>Illumina ID</th>
<th>PRIMERS</th>
<th>CpGs present in the region</th>
<th>CpG dinucleotide equivalent to Illumina ID</th>
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<td></td>
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<td>SEM</td>
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<td>0.4</td>
<td>2.1</td>
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<td>-9.2</td>
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FIGURE LEGENDS

Figure 1. A: Baseline DNA methylation status (%) of 22 CpG sites of \textit{ATP10A} in high and low responders to the hypocaloric diet by EpiTYPER approach (*; p<0.05 by Mann–Whitney U test). Circle on the pole reveals that the site was quantified. Dark circles and boxes indicate CpGs whose methylation levels correlate with other measurements. A vertical line over the DNA indicates those CpGs whose methylation levels were not reliably detected by EpiTYPER. B: Probability of association between baseline methylation levels of the indicated CpGs and the intervention-induced changes in different anthropometric and metabolic measurements (Pearson’s correlation test). C: Correlation analysis between baseline methylation levels of one CpG from \textit{ATP10A} (CpG18) and weight loss during the intervention period (Pearson’s correlation test). D: Correlation analysis between baseline methylation levels of CpG18 and waist circumference variation during the intervention period (Pearson’s correlation test).

Figure 2. A: Baseline DNA methylation status (%) of 31 CpG sites of \textit{CD44} in high and low responders to the hypocaloric diet by EpiTYPER approach (*; p<0.05 by Mann–Whitney U test). Circle on the pole reveals that the site was quantified. Dark circles and boxes indicate CpGs whose methylation levels correlate with other measurements. B: Probability of association between baseline methylation levels of the indicated CpGs and the intervention-induced changes in different anthropometric and metabolic measurements (Pearson’s correlation test). C: Correlation analysis between baseline methylation levels of one CpG located in \textit{CD44} (CpG9-10) and weight loss during the intervention period (Pearson’s correlation test). D: Correlation analysis between baseline methylation levels of CpG14 and weight loss during the intervention period (Pearson’s correlation test). E: Correlation analysis between baseline methylation levels of CpG30 and weight loss during the intervention period (Pearson’s correlation test).

Figure 3. A: Endpoint DNA methylation status (%) of 34 CpG sites of \textit{WT1} in high and low responders to the hypocaloric diet by EpiTYPER approach (*; p<0.05 by Mann–Whitney U test).
Circle on the pole reveals that the site was quantified. Dark circles and boxes indicate CpGs whose methylation levels correlate with other measurements. A vertical line over the DNA indicates those CpGs whose methylation levels were not reliably detected by EpiTYPER. B: Correlation analysis between endpoint methylation levels of one CpG located in WT1 (CpG4) and changes in the diastolic blood pressure during the intervention period (Pearson’s correlation test). C: Correlation analysis between endpoint methylation levels of CpG9-10 and changes in the diastolic blood pressure during the intervention period (Pearson’s correlation test). D: Correlation analysis between endpoint methylation levels of CpG22-23 and the changes in the diastolic blood pressure during the intervention period (Pearson’s correlation test). E: Correlation analysis between endpoint methylation levels of CpG29 and the changes in the diastolic blood pressure during the intervention period (Pearson’s correlation test). F: Correlation analysis between endpoint methylation levels of CpG30-31 and the changes in the diastolic blood pressure during the intervention period (Pearson’s correlation test).

**Figure 4.** Variation of the methylation patterns of two CpG sites located in ATP10A (CpG18) and WT1 (CpG 21) as a result of the nutrition intervention (comparing before and after the treatment) by EpiTYPER approach (*; p<0.05 when comparing all subjects before and after diet by the paired t-test).