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A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss

Fermin I. Milagro,*,[1] Javier Campión,*,[1] Paúl Cordero,* Estíbaliz Goyenechea,† Ana M. Gómez-Uriz,* Itziar Abete,* Maria A. Zulet,* and J. Alfredo Martínez*,[2]

*Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra, Pamplona, Spain; and †Department of Neurology, Hospital Donostia, San Sebastian, Spain

ABSTRACT **Epigenetics could help 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 or obese men participated in an 8-wk 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 analyzed by methylation microarray, which was also useful in comparing epigenetic changes due to the nutrition intervention. Subsequently, MALDI-TOF mass spectrometry was used to validate several relevant CpGs and the surrounding regions. DNA methylation levels in several CpGs located in the *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 the 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.—Milagro, F. I., Campión, J., Cordero, P., Goyenechea, E., Gómez-Uriz, A. M., Abete, I., Zulet, M. A., Martínez, J. A. A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. *FASEB J.* 25, 000–000 (2011). www.fasebj.org**

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Obesity is defined as excessive adiposity in relation to lean body mass, which may involve both altered body fat distribution throughout the body and 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), scarce physical exercise (associated with sedentary lifestyles), and genetic background (which may account for >40% of the predisposition to obesity; ref. 2). Other factors that are related to the recent obesity pandemics are neuroendocrine status and fetal and perinatal programming, which involve maternal nutrition, stress, and maternal care of the offspring (3).

One of the most challenging problems concerning obesity management is weight regain after 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 with an increase in the number of adipocytes that facilitate the repletion and expansion of fat depots (6). Weight reduction also ameliorates insulin sensitivity, thus helping 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 of weight regain (8).

In this context, epigenetic marks have been described as good indicators of susceptibility or resistance to this weight gain (9, 10). Indeed, it has been hypothesized that interindividual differences in obesity predisposition, lipid profile, and blood pressure may also underlie epigenetic factors, (11, 12). Epigenetic mechanisms include DNA methylation, covalent histone modifications, and several types of regulatory RNAs, such as microRNAs (miRNAs) and large noncoding RNAs (lncRNAs), which are affected by the environment, including dietary factors such as methyl donors,

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra; c/Irunlarrea 1, 31008-Pamplona, Spain., E-mail: jalfmtz@unav.es

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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 behavior 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 remodeling and DNA methylation (9). Among the metabolic processes that can be epigenetically regulated by calorie restriction, slowing of the aging process, extension of life span, and 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 (BW) 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 with the metabolic features of each subject. All these measurements were performed in peripheral blood mononuclear cells (PBMCs), which can be easily obtained from blood donor buffy coats, do not involve any surgical or invasive intervention, and have 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 BW [body mass index (BMI): 30.5 ± 0.45 kg/m^2] participated in an 8-wk energy-restricted diet treatment 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 BW (10). On the basis of this criterion, the studied population was categorized into two groups: high or low responders to the dietary treatment.

Obese or overweight subjects were recruited among the endocrinology outpatients of the Hospital of Navarra (Pamplona, Spain). To exclude subjects with clinical evidence of diabetes, hypertension, liver, renal or hematological 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 mo prior to the start of the study, recent participation in other scientific trials (90-d period), pharmacological treatments for chronic diseases, drug or surgical obesity treatments, and drugs or alcohol abuse. All subjects provided a signed informed consent form prior to participation in the study. The study design was previously approved by the Ethics Committee of the University of Navarra (54/2006).

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

Anthropometric and metabolic measurements

Determinations of BW, height, BMI, and waist circumference (WC) were measured as described previously (23). Fat mass and body composition were measured by bioelectric impedance (Quadscan 4000; Bodystat, Douglas, UK). Blood pressure was assessed with a standard mercury sphygmomanometer after ≥5 min of rest in a sitting position (Heine Gamma G5; Heine Optotechnik, Herrsching, Germany) according to World Health Organization criteria. The means of 3 measurements of systolic blood pressure (SBP) and diastolic blood pressure (DBP) were 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, and total and high-density lipoprotein (HDL) cholesterol were assayed on 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, USA). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as [fasting insulin (μU/ml) × fasting glucose (mM)]/22.5, as described previously (24).

Serum leptin and adiponectin levels were measured by commercially available immunoassays (DPC). 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, USA). 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). Serum malondialdehyde (MDA) was evaluated by a colorimetric assay (Oxis International, Foster City, CA, USA).

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 MasterPure kit (Epicenter, Madison, WI, USA), and its quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA (500 ng) was bisulfite converted by using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA).

Methylation microarray

From the 25 subjects included in the study, 12 were chosen for the microarray study. Those individuals that lost >10% of initial BW (n=6) were considered to be high responders, whereas those that lost <5% of initial BW (n=6) were termed "low responders."

DNA methylation levels were assessed using a methylation assay (HumanMethylation27 BeadChip; Illumina, San Diego, CA, USA), 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). 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 analyzed.

Methylation profile by MALDI-TOF mass spectrometry

From microarray data analysis, 13 CpG sites from 9 genes [aquaporin 9 (*AQP9*), ATPase class V type 10A (*ATP10A*), CD44 molecule (Indian blood group; *CD44*), interferon-γ (*IFNG*), maternally expressed 3 (*MEG3*), neurotrophin 3 (*NTF3*), *P*-450 cytochrome oxidoreductase (*POR*), tumor necrosis factor receptor superfamily member 9 (*TNFRSF9*), and Wilms tumor 1 (*WT1*)] were selected and validated by using the Sequenom EpiTyper approach (Sequenom, San Diego, CA, USA), which relies on base-specific cleavage followed by MALDI-TOF mass spectrometry. Briefly, 9 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 prod-

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*

Microarray				EpiTyper		
Symbol	Chr.	Chr. CpG position	Illumina ID	Primers	CpGs in region	CpG equivalent to Illumina ID
AQP9	15	56217683	cg11098259	L: aggaagagagTGAAAATTTTTTTTGGATTAGGGTT	7	CpG1
		56217974	cg11577097	R: cagtaatacgactcactatagggagaaggctAATCCTCACTTT CACAACCAAATAA		CpG7
ATP10A	15	23577342	cg11015241	L: aggaagagagAGGTTGGTTTTTTTATTTAGGTTGG	22	CpG3, CpG4
		23577440	cg17260954	R: cagtaatacgactcactatagggagaaggctCTCCCAAATTCA AATAATTCTCCTA		CpG5, CpG10, CpG16
CD44	11	35117468	cg18652941	L: aggaagagagGGATATTATGGATAAGTTTTGGTGG	31	CpG2, CpG3
		35117805	cg04125208	R: cagtaatacgactcactatagggagaaggctCCTTTCTAAAAA ACCCATTACCAAC		CpG26
IFNG	12	66839844	cg26227465	L: aggaagagagTGTGTTGTATTTTTTTTGGTTGTTG	5	CpG1
				R: cagtaatacgactcactatagggagaaggctAAAAAACTTCCT CACCAAATTATTC		
MEG3	14	100362433	cg05711886	L: aggaagagagATGGGTTTTGTTTTTTTGGATATGT	6	CpG2
				R: cagtaatacgactcactatagggagaaggctTAAACTAAAAT CCCTACCACCCAAC		
NTF3	12	5473803	cg04740359	L: aggaagagagTTTTTTTAGAATGTTTAGAGGGGAG	6	CpG6
				R: cagtaatacgactcactatagggagaaggctAAAAACCTCA ACTTTAAACAAAATACTCT		
POR	7	75421357	cg20748065	L: aggaagagagGGGGTAAGGTTTAGTATTTAGGTGG	11	CpG8
				R: cagtaatacgactcactatagggagaaggctTCTAACAAAAAA ACAAAACCCAAAA		
TNFRS9	1	7922901	cg08840010	L: aggaagagagTATAAGAGGTTGAATGATTTTGTT GTG	4	CpG3
				R: cagtaatacgactcactatagggagaaggctAAAAAATACACC CTCAAACTTTAACAA		
WT1	11	32406026	cg04096767	L: aggaagagagGGGAGATTAGTTTTAATTTTTTTT AAG	34	CpG9, CpG10
		32406214	cg12006284	R: cagtaatacgactcactatagggagaaggctCTAAATCTCCCT CCATCCCAAATAC		CpG21

Chr., chromosome; L, left; R, right.

ucts were excised from 2% agarose gels, purified by Qiagen Gel Extraction Kit (Qiagen), and eluted with 1X Roche FastStart high-fidelity reaction buffer (Roche). In microtiter plates, 5 μl of the PCR products were treated with 2 μl shrimp alkaline phosphatase (SAP) mix (37°C, 20 min) to dephosphorylate unincorporated dNTPs, which were later processed by MassArray Matrix Liquid Handler (Sequenom). A 2-μl volume of each SAP-treated sample was heat-inactivated (85°C, 5 min) and subsequently incubated for 3 h at 37°C with 5 μl of Transcleave mix (Sequenom) 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 (Sequenom) 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 113 other built-in CpGs included in these 9 regions that were not previously quantified by the microarray. Because of the special nature of the technique, DNA methylation values of some CpGs could not be measured independently (Table 1, rightmost 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 was reported.

In this first step (n=12), only ~50% of the CpGs selected from the 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 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 endpoint. To detect differential methylation changes by microarray between high- and low-responder groups, statistical analyses were made by using the Illumina model to compute the false discovery rate (FDR). The calculation of FDR is based on multiple testing corrections using the Benjamini and Hochberg method (27). This statistical approach allows taking advantage of the standard deviation from bead-level replicates to enable a better estimate of the biological noise (Illumina Inc.; personal communication, August 9, 2010). 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 stringency, only those data that differed by ≥20% from the baseline value were considered for both analyses.

Differences in DNA methylation levels by using the EpiTyper approach were analyzed with the Mann-Whitney *U* test (high responders *vs.* low responders) 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's correlation test was used for comparing cytosine methylation levels of CpG sites (by EpiTyper) with anthropometric and metabolic variables. For all of these statistical analysis, SPSS 15.0 for Windows was used (SPSS, Chicago, IL, USA).

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 or obese men that consumed an energy-restricted diet for 8 wk. Twelve participants, including both individuals who lost more weight (high responders, n=6) and individuals who lost less weight (low responders, n=6) were chosen for a DNA methylation microarray study. Baseline values concerning the anthropometric and biochemical measurements were similar in subjects that successfully achieved a weight loss of >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 reducing BMI, fat mass, and WC, contrary to the low responders, who lost very little weight (≤5% of initial BW). 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 nonsignificant amelioration of fasting insulin and HOMA index was found in the high responders as compared to the low responders (Table 2). However, proportional DBP decrease was greater in the low responders than in the high responders, probably because the latter group already had a normal level of this parameter before the treatment. Finally, the nutrition intervention did not produce an improvement in blood inflammatory (TNF-α, IL-6, PAI-1) 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 CpG sites were relatively hypomethylated (>20% change in methylation levels) in the high-responder group compared with the low responders, whereas 602 CpG sites were relatively hypermethylated (>20% change) in the high-responder group.

Some metabolically relevant genes were hypomethylated in ≥1 CpG before the intervention: *ATP10A* (Δβ −0.25), *MEG3* (Δβ −0.25), sex hormone-binding globulin (*SHBG*; Δβ −0.29), and CCAAT/enhancer binding protein γ (*CEBPG*; Δβ −0.28). Other relevant genes were hypermethylated in ≥1 site, such as *CD44* (Δβ 0.22), *TNFRSF9* (Δβ −0.22), B-cell CLL/lymphoma 2 (*BCL2*; Δβ 0.28), phosphatase tensin homologue (*PTEN*; Δβ 0.22), acetyl-coenzyme A carboxylase α (*ACACA*; Δβ 0.25), and S-phase kinase-associated protein 2 (*SKP2*, Δβ 0.33).

From this analysis, 4 regions of genes related to obesity

TABLE 2. *Anthropometric and biochemical differences between high and low responders to the diet before intervention (baseline) and variation due to the weight-loss program*

	Baseline		Intervention-induced variation (%)	
Parameter	High responders, $n = 6$	Low responders, $n = 6$	High responders, $n = 5$	Low responders, $n = 5$
BW (kg)	96.7 ± 2.7	93.8 ± 4.2	−12.4 ± 0.6	−3.6 ± 0.5***
BMI (kg/m^2)	31.0 ± 0.7	30.2 ± 0.6	−12.6 ± 0.5	−3.6 ± 0.4***
Fat mass (kg)	26.7 ± 1.3	26.6 ± 2.0	−24.5 ± 1.2	−6.3 ± 1.8***
WC (cm)	102 ± 3	100 ± 2	−10.2 ± 0.6	−4.4 ± 0.8***
SBP (mmHg)	127 ± 5	131 ± 2	−3.2 ± 2.1	−4.9 ± 1.5
DBP (mmHg)	67 ± 3	77 ± 6	−3.8 ± 3.3	−17.0 ± 5.8*
Total cholesterol (mg/dl)	196 ± 21	237 ± 22	−24.9 ± 5.2	−1.2 ± 4.2***
HDL cholesterol (mg/dl)	47 ± 3	46 ± 2	−15.1 ± 5.1	−0.5 ± 6.6
LDL cholesterol (mg/dl)	127 ± 19	168 ± 17	−28.4 ± 7.9	−1.8 ± 4.2*
Triglycerides (mg/dl)	108 ± 17	117 ± 20	−23.0 ± 7.5	8.2 ± 17.9
Glucose (mg/dl)	92 ± 5	95 ± 3	3.0 ± 5.9	10.8 ± 3.4
Insulin (μU/ml)	17.9 ± 5.6	11.2 ± 1.8	−46.1 ± 21.2	−19.4 ± 6.7
HOMA	4.1 ± 1.4	2.6 ± 0.4	−38.6 ± 29.3	−10.3 ± 9.4
Leptin (ng/ml)	13.2 ± 2.9	13.8 ± 2.1	−66.2 ± 3.1	−21.8 ± 5.5***
Adiponectin (μg/ml)	8.6 ± 1.6	7.5 ± 0.7	−7.2 ± 11.2	11.9 ± 13.1***
Ghrelin (pg/ml)	982 ± 120	941 ± 55	6.6 ± 7.1	7.4 ± 5.8
PAI-1 (ng/ml)	172 ± 16	139 ± 19	−10.4 ± 12.0	−11.0 ± 10.2
IL-6 (pg/ml)	1.3 ± 0.1	3.1 ± 1.0	9.1 ± 21.1	−27.3 ± 20.8
TNF-α (pg/ml)	0.9 ± 0.2	1.5 ± 0.3	2.9 ± 35.1	−21.2 ± 14.5
Plasma MDA (μM)	2.1 ± 0.4	2.1 ± 0.3	−9.2 ± 7.5	2.0 ± 3.4

Values are means ± SE. Differences are nonsignificant except as noted; calculated by Mann-Whitney U test. *$P < 0.05$, ***$P < 0.001$ *vs.* high responders.

(*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 CpGs 5–10-16, 18, and, although not statistically significant, 3–4 (**Fig. 1*A***, 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, BMI, or WC (Fig. 1*B*, 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 (Fig. 1*C*, n=25) and, in a similar manner, the decreases in WC, as an indicator of visceral adiposity (Fig. 1*D*, 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 the *CD44* region (especially CpGs 14 and 29, but also, although not reaching statistical significance, CpGs 9–10, 16, and 30) were more methylated in the responder group, showing a similar trend of comethylation 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 WC, the most widely used markers of visceral obesity (**Fig. 2*A***, 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-α values (a proinflammatory cytokine) or with the treatment-induced changes in fat mass, BMI, or WC (Fig. 2*B*, n=25). Figure 2*C–E* (n=25) shows the correlations of three of these CpGs (CpGs 9–10, 14, and 30) with the decrease in BW, 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*; Δβ −0.21) and *BCL2* (Δβ −0.20). Meanwhile, only 1 CpG site was relatively hypermethylated (>20%) at the end of the intervention in the

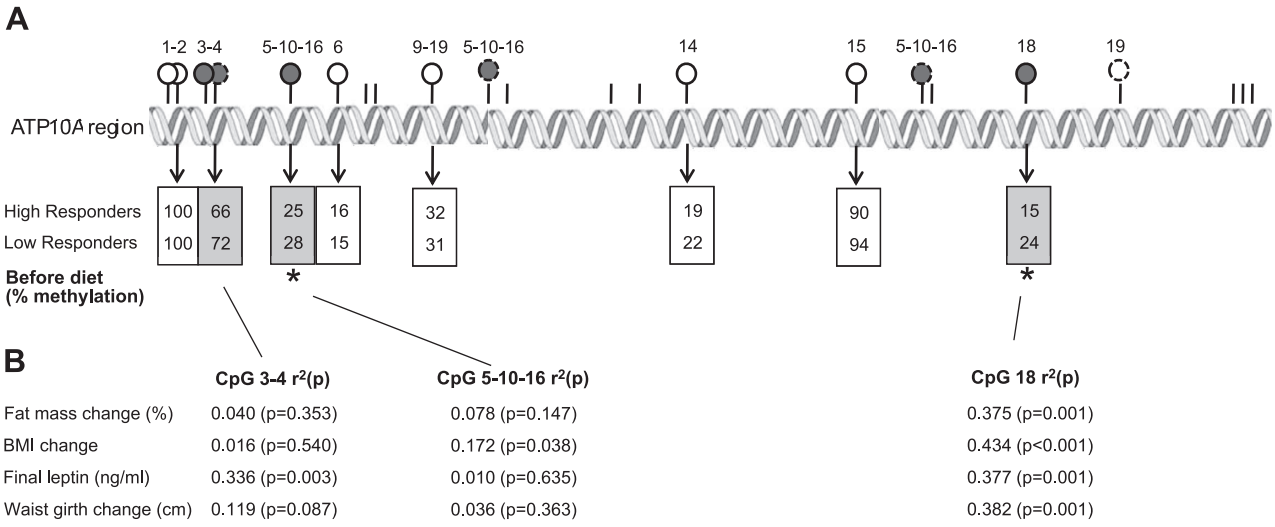

Figure 1. *A*) Baseline DNA methylation status (%) of 22 CpG sites of *ATP10A* in high and low responders to the hypocaloric diet by EpiTyper approach. Circle on a pole indicates that the site was quantified. Solid circles and shaded boxes indicate CpGs whose methylation levels correlated with other measurements. Vertical lines over the DNA indicate CpGs whose methylation levels were not reliably detected by EpiTyper. *$P<0.05$; Mann-Whitney *U* test. *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 *ATP10A* (CpG18) and weight loss during the intervention period (Pearson's correlation test). *D*) Correlation analysis between baseline methylation levels of CpG18 and WC variation during the intervention period (Pearson's correlation test).

high-responder group: the LAG1 homologue ceramide synthase 3 gene (*LASS3*; Δβ 0.28). However, other obesity-related genes were significantly hypermethylated, although with a variation <20%, such as *IFNG* (Δβ 0.11), and a 800-bp region, including 4 CpG sites of *WT1* (Δβ 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 the *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 others (CpGs 12, 22–23, 29, and 30–31) trended toward significance (**Fig. 3*A***, $n=12$). 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 DBP (Fig. 3*B–E*, $n=25$), suggesting a relationship between the epigenetic modifications of *WT1* and blood pressure regulation.

Effects of the dietary treatment on DNA methylation

Taking 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. Of these, 70 CpG sites were relatively demethylated (decrease of >20%) by the dietary intervention and 100 CpGs were more methylated (increase of 20%). Interestingly, the high-responder group was more affected by the intervention, modifying ~8-fold more 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 ≥1 CpG as a result of the intervention study, including *AQP9* (Δβ −0.22), *TNFRSF9* (Δβ −0.21) again, *POR* (Δβ −0.21), and glucosamine-6-phosphate deaminase 2 (*GNPDA2*; Δβ −0.36). The methylation of other genes was increased by calorie restriction, including the H19 imprinted maternally expressed transcript (*H19*; Δβ 0.21), apolipoprotein A-II (*APOA2*; Δβ 0.24), *NTF3*

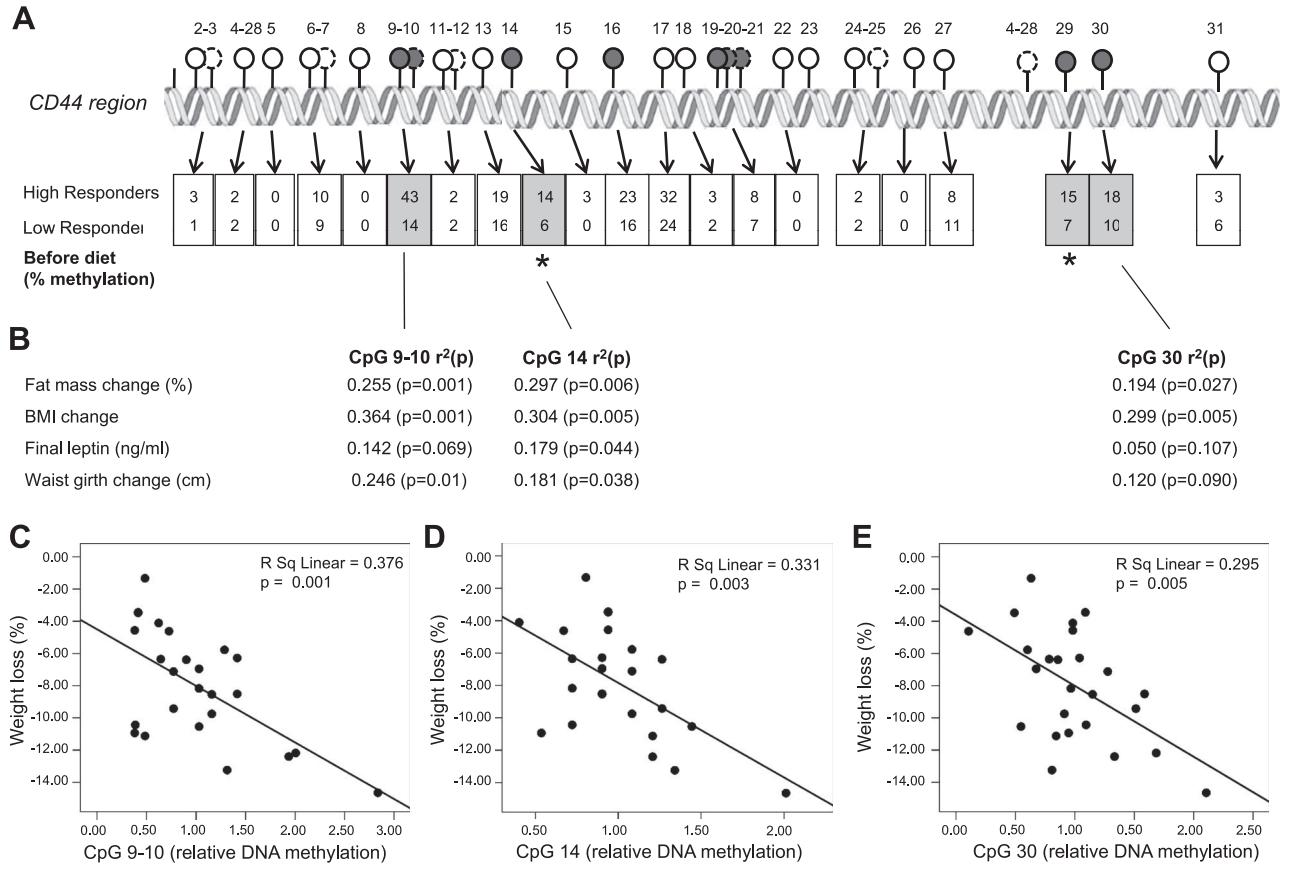

Figure 2. *A*) Baseline DNA methylation status (%) of 31 CpG sites of *CD44* in high and low responders to the hypocaloric diet by EpiTyper approach. Circle on a pole indicates that the site was quantified. Solid circles and shaded boxes indicate CpGs whose methylation levels correlated with other measurements. *$P < 0.05$; Mann-Whitney *U* test. *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 *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).

(Δβ 0.23), and transforming growth factor β 1 (*TGFB1*, Δβ 0.26).

From this statistical analysis, the 6 genomic regions studied previously (*ATP10A*, *WT1*, *CD44*, *IFNG*, *MEG3*, and *TNFRSF9*) and 3 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, 2 genomic regions (*ATP10A* and *WT1*) showed differential methylation. When tested in the whole sample (n=25), 2 CpG sites, one from *ATP10A* (CpG18) and the other from *WT1* (CpG21), became hypermethylated (P<0.05) due to the dietary intervention (**Fig. 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 or 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 PBMCs instead of fat biopsies as a noninvasive 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 (22, 30), epigenetic analysis of peripheral blood cells is a promising approach for diagnosing disease, determining the treatment outcome, and searching for biomarkers.

Different studies have reported 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 epigenomes, affecting their propensity to develop obesity at a later age (31, 32). On the other hand, many environmental and nutritional factors have

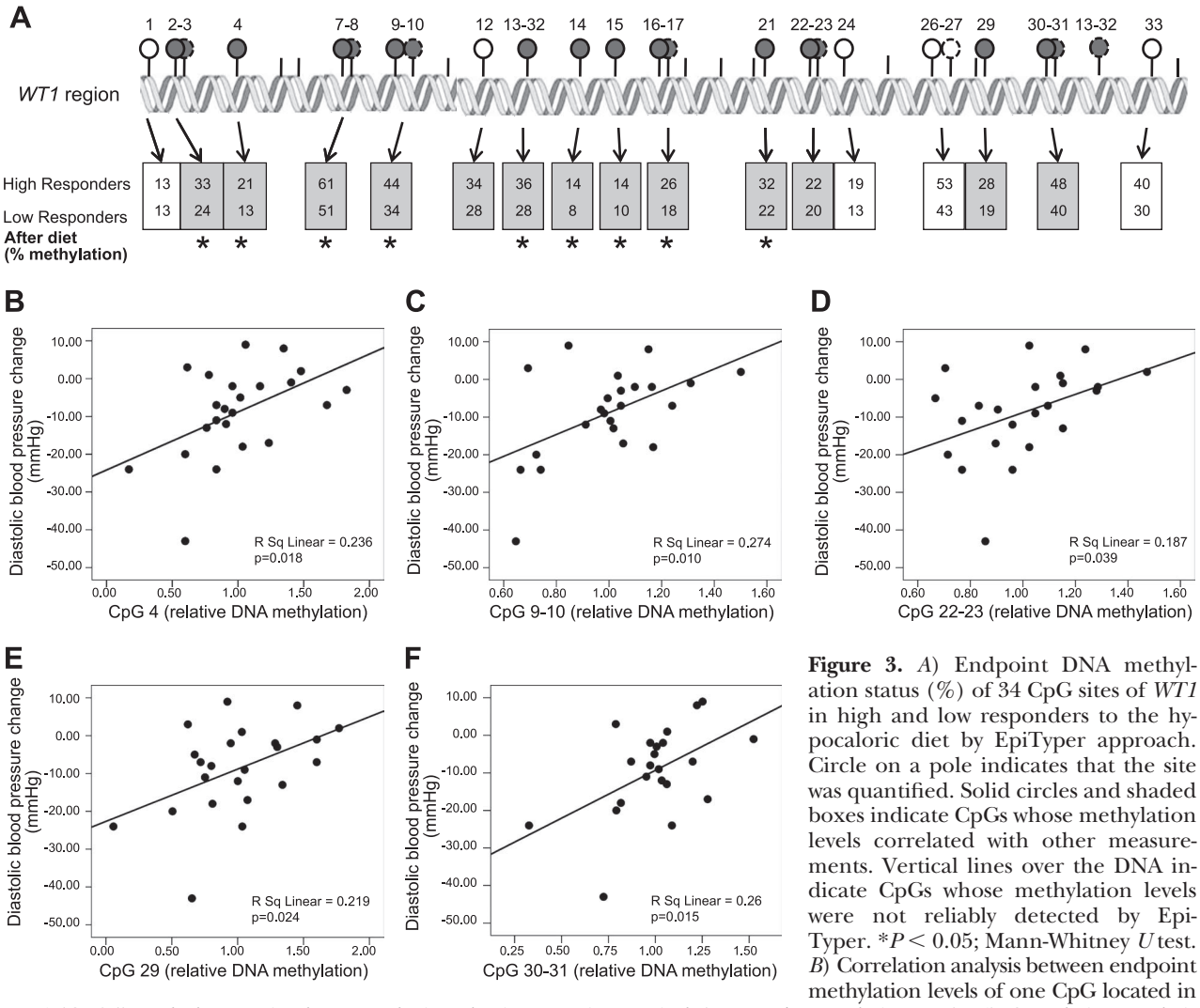

Figure 3. *A*) Endpoint DNA methylation status (%) of 34 CpG sites of *WT1* in high and low responders to the hypocaloric diet by EpiTyper approach. Circle on a pole indicates that the site was quantified. Solid circles and shaded boxes indicate CpGs whose methylation levels correlated with other measurements. Vertical lines over the DNA indicate CpGs whose methylation levels were not reliably detected by EpiTyper. $*P < 0.05$; Mann-Whitney *U* test. *B*) Correlation analysis between endpoint methylation levels of one CpG located in *WT1* (CpG4) and changes in the DBP during the intervention period (Pearson's correlation test). *C*) Correlation analysis between endpoint methylation levels of CpG9–10 and changes in DBP during the intervention period (Pearson's correlation test). *D*) Correlation analysis between endpoint methylation levels of CpG22–23 and the changes in the DBP during the intervention period (Pearson's correlation test). *E*) Correlation analysis between endpoint methylation levels of CpG29 and the changes in the DBP during the intervention period (Pearson's correlation test). *F*) Correlation analysis between endpoint methylation levels of CpG30–31 and the changes in the DBP during the intervention period (Pearson's correlation test).

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 diet-induced obesity in rats has been associated with changes in leptin promoter DNA methylation (33). Also, TNF-α-promoter methylation pattern could be a good biomarker for 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 during the life span, with special emphasis on the perinatal period. In this regard, as other researchers 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 does not usually show differences between responders and nonresponders 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 PBMCs 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 differen-

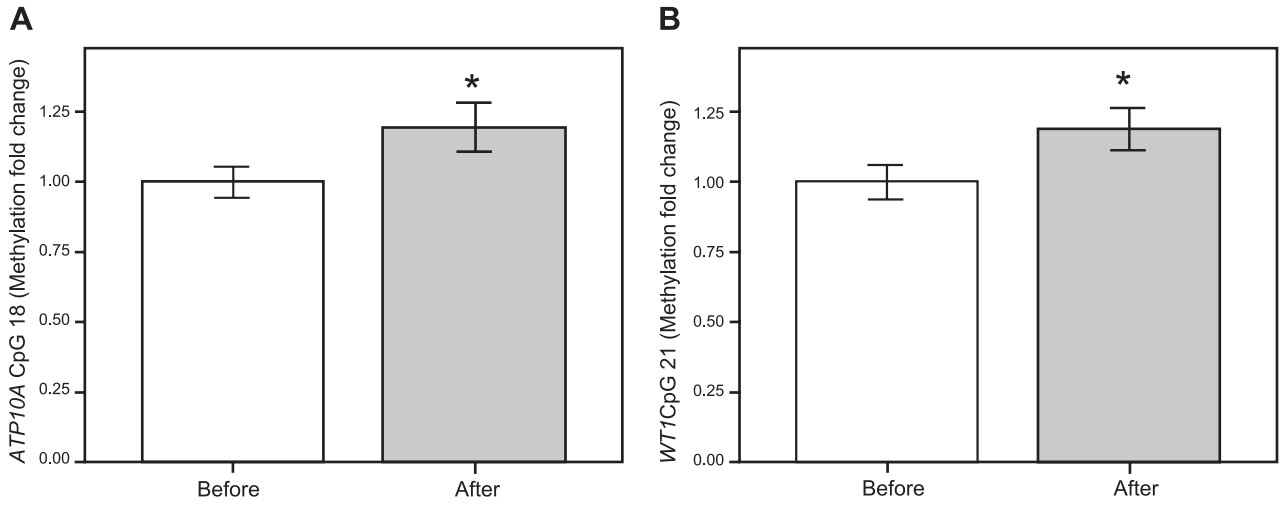

Figure 4. Variation of the methylation patterns of 2 CpG sites located in *ATP10A* (CpG18; *A*) and *WT1* (CpG 21; *B*) as a result of the nutrition intervention (comparing before *vs.* after treatment) by EpiTyper approach. $*P < 0.05$; paired *t* test.

tial methylation in >1000 CpGs by using the Illumina model. Because of 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 neighboring CpGs to the CpG sites selected in the microarray analysis. Using Spearman's rank correlation test, only ~50% 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 always be 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 also those that differed >20% in methylation between the two groups) with a second and more sensitive technique, in our case Sequenom's MassArray EpiTyper, also allowed us to increase the number of CpGs assessed per gene. Another advantage of this validation methodology is the possibility of analyzing the methylation patterns of contiguous CpG sites and detecting changes in methylation of not only an isolated CpG (as in the microarray), but of several CpGs (a region of 400–600 bp) with a common trend in epigenetic regulation.

The EpiTyper results showed several CpGs located in the promoter of *ATP10A* (CpGs 5–10-16 and 18) whose methylation levels were statistically lower in the high-responder group at baseline (before the dietary intervention). Along with CpGs 3–4, they strongly correlated with the changes in fat mass, BMI, and WC 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 nonalcoholic fatty liver tissue than 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, this hypothesis 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 analyzing the whole population, these CpGs, but also CpGs 9–10, showed very good correlations with BW loss and changes in WC 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, and is considered 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* expression (41). In rodents, the same researchers have reported that high-fat diet-induced obesity resulted in increased CD44 and osteopontin (a protein ligand of CD44) gene expression in both fatty liver and epididymal fat, with both expressions positively correlated with steatosis. Our results, linking the epigenetic regulation of this proinflam-

matory 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 15 CpGs resulted in differential methylation between high and low responders, as determined 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 on 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 up-regulated 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*), chronic administration of which 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 *BDNF, PAX6,* and *WT1* is the Wilms' tumor, aniridia, genitourinary, and mental retardation (WAGR) syndrome, which in many cases is accompanied by obesity development (46). *WT1* methylation has been extensively tackled in cancer research, but the present report is apparently the first in which this methylation pattern has been studied in obesity. In our results, it is intriguing that the methylation status of several CpGs of *WT1* promoter is associated with the diet-induced change in DBP. In this sense, Steege *et al.* (47) have reported that Wilms' tumor 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 DBP. Indeed, more studies are needed to unveil the importance of *WT1* epigenetic regulation in BW management and blood pressure regulation.

Concerning the last objective of this work, and taking into account only the high responders in the microarray assay, 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; 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 greater 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 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 nonalcoholic 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 dietary treatment.

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 the 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 PBMCs as predictors of weight loss.

In summary, 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, including more individuals than in the first technique, allowed us 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 PBMCs (CpG18 of *ATP10A* and CpG 21 of *WT1*) and that some of these markers 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. FJ

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