CLOCK, PER2 and BMAL1 DNA methylation: Association with obesity and metabolic syndrome characteristics and monounsaturated fat intake

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Abbreviated title: CLOCK, BMAL1 and PER2 methylation in obesity

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Abbreviations: MetS, Metabolic syndrome; DOHaD, Developmental origin of health and disease; TEE, Total energy expenditure; BMI, Body mass index; IPAQ, International Physical Activity Questionnaire; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; IDF, International Diabetes Federation; HOMA, Homeostasis model assessment; QUICKI, Quantitative insulin-sensitivity check index; HAT, histone acetyltransferase; FASN, fatty acid synthase; NDUFB6, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6.

Tables: 3; **Figures:** 4. Supplementary tables: 1, Supplementary figures: 2

ABSTRACT

The circadian clock system instructs 24-hour rhythmicity on gene expression in essentially all cells, including adipocytes, and epigenetic mechanisms may participate in this regulation. The aim of this research was to investigate the influence of obesity and metabolic syndrome features in clock gene methylation and the involvement of these epigenetic modifications in the outcomes. Sixty normal-weight, overweight and obese women followed a 16-weeks weight reduction program. DNA methylation levels at different CpG sites of *CLOCK*, *BMAL1* and *PER2* genes were analyzed by Sequenom's MassARRAY in white blood cells obtained before the treatment. Statistical differences between normal-weight and overweight+obese subjects were found in the methylation status of different CpG sites of *CLOCK* (CpGs 1, 5-6, 8 and 11-14) and, with lower statistical significance, in *BMAL1* (CpGs 6-7, 8, 15 and 16-17). The methylation pattern of different CpG sites of the three genes showed significant associations with anthropometric parameters such as BMI and adiposity, and with a metabolic syndrome score. Moreover, the baseline methylation levels of *CLOCK* CpG 1 and *PER2* CpGs 2-3 and 25 correlated with the magnitude of weight loss. Interestingly, the percentage of methylation of *CLOCK* CpGs 1 and 8 showed associations with the intake of monounsaturated and polyunsaturated fatty acids. This study demonstrates for the first time an association between methylation status of CpG sites located in clock genes (*CLOCK, BMAL1* and *PER2*) with obesity, metabolic syndrome and weight loss. Moreover, the methylation status of different CpG sites in *CLOCK* and *PER2* could be used as biomarkers of weight loss success, particularly *CLOCK* CPGs 5-6.

Keywords: Epigenetics, MUFA, cytosine methylation, MassARRAY, biomarker

INTRODUCTION

The interaction between poor dietary habits and lifestyles with genetic traits is crucial in the development of obesity and metabolic syndrome (MetS) (Garaulet et al., 2010a). However, other putative causal factors contributing to excessive fat accumulation have been recently proposed, which includes sleep duration and quality and epigenetics, among others (McAllister et al., 2009).

Concerning the first one, it has been reported that patients with visceral obesity and MetS exhibit disturbances in the circadian rhythm (chronodisruption) that may be associated with higher weight increase and development of diabetes and atherosclerotic disease (Gómez-Santos et al., 2009; Cardinali et al., 2011). Thus, experimental studies have shown that prolonged short sleep durations could increase appetite, compromise insulin sensitivity and raise blood pressure, being probably involved in the development of metabolic disorders, such as obesity, diabetes and hypertension (Gangwisch, 2009).

Regarding the second one, in the last years, epigenetic phenomena and intergenerational effects have been considered as potential contributors to the obesity epidemic, in some cases associated to the developmental origin of health and disease (DOHaD) hypothesis (Wadhwa et al., 2009). Cytosine DNA methylation, the most studied epigenetic mechanism, is usually considered a flexible method for repressing gene expression. Different dietary and lifestyle factors are able to modulate the methylation of specific CpG sites in gene promoters even in the adult age (Campion et al., 2010). In relation to obesity, the percentage of methylation of specific CpG sites in blood cells is being investigated as a potential predictor of weight loss response (Milagro et al., 2011) or as a personalized signature of obesity (Feinberg et al., 2010). In this way, peripheral blood DNA from Italian subjects working in chemical plants has been used to study the effects

of shiftwork exposure on DNA methylation (Bollati et al., 2010). In this study, different factors such as job seniority, length of shiftwork, and morning and evening types were associated with the methylation of the promoters of glucocorticoid receptor, TNF-alpha, and IFN-gamma.

With respect to clock genes, a recent study (Zhu et al., 2011) has reported that long-term shiftwork, previously related to obesity and MetS risk (Manenschijn et al., 2011), induces hypomethylation in the promoter of *CLOCK* gene and hypermethylation of *CRY2* in peripheral blood DNA. In this context, a relationship between clock genes and obesity has been suggested from the analysis of *BMAL1* and *CLOCK* mutant mice. Thus, *CLOCK* mutant mice are hyperphagic and obese, and develop a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia (Turek et al., 2005), whereas *BMAL1* null animals show changes in glucose homeostasis (Rudic et al., 2004) as well as dyslipidemia and ectopic fat accumulation in liver and skeletal muscle (Shimba et al., 2011). In fact, both models show reduced insulin secretion, impaired glucose tolerance, and defects in proliferation and size of pancreatic islets that lead to diabetes (Marcheva et al., 2010). Moreover, genetic variations (SNPs) in some of the clock genes, such as *PER2* (Garaulet et al., 2010b) and *CLOCK* (Garaulet et al., 2011b), have been related with caloric intake, circulating cytokine and ghrelin levels, eating behavior and attrition in weight-loss treatments. These data suggest that a relationship between clock gene dysregulation and obesity and MetS may exist.

Another point of interest is the possible involvement of fatty acids in the methylation process and in the regulation of the expression of clock genes. The latter has been recently demonstrated by Fick et al. (2011) when culturing immortalized, hypothalamic

neurons with palmitate. However, to date, no study in humans (i.e., intervention studies) has obtained evidence for a direct involvement of fatty acids on DNA methylation regulation. Nevertheless, some *in vitro* studies suggest that monounsaturated and polyunsaturated fatty acids could play a role in this epigenetic mechanism, particularly n-3 PUFA (Kulkarni et al., 2011; Ceccarelli et al., 2011).

The aim of this research was to investigate the influence of obesity and MetS features in clock gene methylation and the description of some epigenetic modifications in clock genes that could be used as predictors of weight loss success. Additionally, we look for dietary and life style factors that could be related to the methylation of these genes in white cells and that could interrelate with circadian clock alterations, environmental factors and obesity development.

METHODS

Subjects and Methods

We recruited normal-weight (*n*=20), overweight/obese (*n*=20) and morbidly obese women (*n*=20) (BMI<25 kg/m^2, 29-35 kg/m^2 and >40 kg/m^2, respectively) within the age range of 25–53 years (total *n*=60) who attended five outpatient obesity clinics during 2009-2010 in the city of Murcia, southeastern Spain. Those patients who were out of this range of age, under treatment with thermogenic or lipogenic drugs, on a special diet, or diagnosed with diabetes mellitus, chronic renal failure, hepatic diseases or cancer were excluded from the study (9%). All clinical investigations described in this manuscript have been conducted in accordance with the guidelines in the Declaration of Helsinki. Written consent was obtained from each patient before participation and the study principles were approved by the Research Ethics Committee of the Virgen de la Arrixaca Hospital. Patient data were codified to guarantee anonymity. The experimental protocol is conform to international ethical standards (Portaluppi et al., 2010). Figure 1 shows the intervention protocol carried out.

Characteristics of the Treatment

The characteristics of the weight reduction program (Garaulet method©) have been described elsewhere (Corbalan et al., 2009) and are represented in Figure 1. Briefly, during the initial 4 months, subjects attended a weekly 60-min therapy session in support groups (n=10), followed by a 5-month maintenance period. Sessions were conducted by a nutritionist. Treatment was based on the following issues:

The energy requirements were calculated using the Harris-Benedict formula and, according to the type of physical activity, were decreased to about 2.6MJ/day so as to induce an approximate loss of between 0.5 and 1kg/week. A Mediterranean-type diet was followed, with the distribution of the principal components as recommended by the Spanish Society of Community Nutrition (Serra-Majem et al., 2001): 35% fat (<10% saturated and 20% monounsaturated), 50% carbohydrates and 15% to 20% of proteins. Patients were advised to consume unlimited amount of vegetables with a minimum of 200g per day, at least 250 to 300 g of fruits daily and olive oil as the only cooking fat. They were also encouraged to consume the following foods for lunch: at least 100 g of legumes three times a week, 100 g rice once a week, 100 g wheat and pasta once a week, and at least one day of fish per week. Total cholesterol intake was recommended to be below 300 mg, and fiber intake was higher to 15 g per day. Patients were also rewarded with extra calories (optional calories) and extra food interchanges (floating portions) for special occasions.

During the group therapy, sessions were classified into four types: 1) Nutrition-based sessions, to help choose the food for the dietary record. 2) Physiological-based sessions, to help explain body weight regulatory mechanisms, processes of nutrient absorption and digestion and the physiology of weight loss. 3) Behavioral and cognitive sessions, which help the patient to control stimuli, avoid negative thoughts or detect the main obstacles to weight loss; and 4) Practical ideas, to help to organize outings, shopping lists, alternatives to dinner, the importance of breakfast, etc. Every week the sessions explained one simple recipe.

Individualized long-term goals were established to accumulate at least 30 minutes or more of moderate-intensity physical activity on most, and preferably, all

days of the week, unless medically contraindicated. Patients were stimulated to fulfill these recommendations with the use of a pedometer, incentivizing them to reach at least 10.000 steps per day.

Different behavioral techniques were included: Stimulus control, referring to how the immediate environment can be altered to promote behaviors that help weight loss; Self-monitoring, considered as one of the principal pillars of the treatment and referring to keep a daily record of food consumed and physical exercise; Positive reinforcement, patients being rewarded with incentives not related with food, such as a small gifts, prizes or diplomas when the aimed-for weight goal was reached; and Preventing relapses, by teaching how to identify “slips”.

Habitual Dietary Intake

To evaluate food habits, the initial nutrient intake was determined by a 24-h dietary recall. Interviews were conducted from Monday to Friday, including 24-h recalls of food intake from weekends and weekdays. Total energy intake and macronutrient composition from the initial 24-h recalls were analyzed with the nutritional evaluation software program Grunumur (Perez-Llamas et al., 2004), based on Spanish food composition tables (Moreiras et al., 2009). The intakes of the main saturated fatty acids, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), including linoleic [18:2(n-6)], linolenic [18:3(n-6)], eicosapentaenoic [20:5(n-3)], and docosahexaenoic [22:6(n-3)] acids, were also calculated from Spanish food-composition tables (Moreiras et al., 2009).

Chronodisruptors

Sleep duration: Habitual sleep time was estimated by a questionnaire containing the following questions: 'During week days: How many hours (and minutes) do you usually sleep? and; 'During weekend days: How many hours (and minutes) do you usually sleep? A total weekly sleep score was calculated as: ((min Weekdays x 5) + (min Weekend days x 2))/7 (Garaulet et al. 2011a).

Snacking frequency and different *eating behaviors* were assessed by a behavioral data checklist (Corbalan et al., 2009). Logistic regression models were fitted to estimate the risk of a specific behavior associated with high or low methylation levels in the different clock genes studied, which were dichotomized according to the median value of the population.

Eveningness was assessed by the Horne and Ostberg questionnaire to assess morningness–eveningness (Horne & Ostberg, 1976). This questionnaire establishes five behavioral categories: definitively morning types (score = 70-86), moderately morning types (score = 59-69), neither types (score = 42-58), moderately evening types (score = 31-41), definitively evening types (score = 16-30).

Anthropometric Measurements and Blood Pressure

Subjects were weighed barefoot wearing light clothes, with a digital scale to the nearest 0.1 kg, at the same time each day weekly to assess weight loss during treatment. Height was measured using a Harpenden digital stadiometer (rank 0.70–2.05). The subject was positioned upright, relaxed and with the head in the Frankfurt plane. BMI was calculated as weight (kg)/(height(m))^2. Total body fat was measured by bioelectrical impedance using TANITA TBF-300 (TANITA Corporation of America,

Arlington Heights, IL, USA) equipment. Body fat distribution was assessed by the measurement of different circumferences: waist circumference, at the level of the umbilicus. All measurements were made with a flexible and inextensible tape measure. Blood pressure was measured seated with arm resting on a table following WHO criteria and recorded as millimeters of mercury (mm Hg).

Biochemical and Endocrine Variables

Plasma glucose, cholesterol, triglycerides (TG) and lipoprotein concentrations were determined by an automated chemical analysis. VLDL-cholesterol (VLDL-C) (d< $1.006 \cdot 10^{-3}$ g/L) was prepared by ultracentrifugation (Havel et al., 1955). HDL-cholesterol (HDL-C) was measured after precipitation of apoB-containing lipoproteins with dextran sulfate and magnesium (Warnick et al., 1982). LDL cholesterol (LDL-C) was calculated as total cholesterol (TC) minus HDL-C plus VLDL-C using the Friedewald equation when TG concentration was < 4.52 mmol/L.

Plasma ghrelin levels at baseline were measured by radioimmunoassay (Linco Research, St. Charles, MO). Serum insulin at baseline was measured by radioimmunoassay (Coat A Count Insulin, DCP) (assay precision: CV <10% at 16 µU/mL concentration, cross reactivity with proinsulin=20%). Indexes of insulin resistance for the homeostasis model assessment (HOMA IR = fasting glucose (mmol/L) × fasting insulin (µIU/L)/22.5) and insulin sensitivity (QUICKI = 1/log fasting insulin + log glycemia in mg/dL) were calculated with validated formulae.

We used IDF consensus 2006 definition (Alberti et al., 2005) to classify patients for MetS, which was defined by the presence of central obesity (waist circumference >

88 cm) and 2 or more of the following characteristics: Raised triglycerides (TG ≥ 150 mg/dL, 1.7 mmol/L), low HDL-C (HDL-C < 1.29 mmol/L, 50 mg/dL), hypertension (blood pressure ≥ 130/85 mm Hg or taking medication), and elevated fasting plasma glucose (basal glycemia ≥ 110 mg/dL, 6.1 mmol/L) and a MetS score was calculated.

DNA Methylation Analysis

White blood cells were obtained before the treatment. Genomic DNA was isolated from white blood cells (QIAamp DNA Mini KIT, Qiagen Gmbh, Hilden, Germany) and was sodium bisulfite-converted (EZ-96 DNA Methylation Kit, Zymo Research Co., Orange, CA, USA). Sequenom's MassARRAY platform was used to perform quantitative methylation analysis. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base specific cleavage (MassCLEAVE). PCR primers covering four relevant regions (Figure 2) of the three following genes, *CLOCK* (2 regions selected from the NCBI Reference Sequence NC_000004.11), *BMAL1* (from NC_000011.9) and *PER2* (from NG_012146.1), were designed using Epidesigner software (Bruker–Sequenom, San Diego, CA, USA). The primers used were:

For CLOCK-R1 (271 bp length): Left, TTTTTTAGGAGATGGGAGAAGATG,

Right, CCTAAAAACT CTTTAACTTTCCCCC;

For CLOCK-R2 (399 bp length): Left, GTTTTTGGGTTGGTGGAGGA,

Right, CCAAAAAACCTCTCAAACAAAAC;

For BMAL1 (376 bp length): Left, TGAGATTTTGGTAAATTAGGGATTTT,

Right, ACTACTTTCCTACCACCAATCATTTAAC;

For PER2 (430 bp length): Left, AAACTTCCTTATCCCTCTAATCACC,

Right, GTGTGTTTTTGGTTTTGTTTTAGGT.

Genomic DNA position (Assembly GRCh37/hg19) of targeted CpGs and the sequence of the amplicons (in which the analyzed CpGs are highlighted) are shown in Figure 2. The complete methodology, including PCR and MassCLEAVE biochemistry, has been previously described (Milagro et al., 2011). Mass spectra were collected using a MassARRAY mass spectrometer (Bruker–Sequenom) and subsequently analyzed using proprietary peak picking and signal-to-noise calculations by using Epityper software v1.0 (Bruker–Sequenom).

Statistical Analysis

Clinical and anthropometric data are presented as means ± SD. The results of CpG methylation, expressed as the ratio of methylated *versus* total cytosines, are presented as means ± SEM. When the three groups were compared, one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was used. When the overweight/obese and morbidly obese individuals were grouped (overweight+obese group), Student's t-test was used to analyze the differences with the normal-weight group.

Pearson’s correlation coefficients were used for analyzing associations between the percentages of cytosine methylation in several CpGs of clock genes and obesity characteristics, MetS traits, weight loss and fatty acids in the diet. The analysis of correlation was repeated adjusting for BMI. All statistical analyses were carried out using SPSS for windows 15.0 (SPSS Inc, Chicago, IL). The level of significance for all

statistical tests and hypotheses was set at $P<0.05$, although trends towards significance ($0.1>p>0.05$) have been highlighted in the tables.

RESULTS

Demographic, nutritional, anthropometric and biochemical characteristics of the studied population are shown in Table 1. According to the Horne and Ostberg questionnaire, all women had the same chronotype.

The Sequenom's MassArray analysis of DNA methylation revealed differences between normal-weight, overweight/obese and morbidly obese women in different CpGs of *CLOCK* and *BMAL1* genes. As no differences were noted between both overweight/obese and morbidly obese groups for any CpG analyzed, both groups were combined (overweight+obese group) and together compared with the normal-weight subjects. Our analysis of these two groups showed statistically significant differences in the *CLOCK* gene, especially in CpG 1 (2.4% in obese subjects versus 1.1% in normal-weight), CpGs 5-6 (17.4% versus 12.4%) and CpG8 (10.8% versus 8.7%) with the pair of primers R1 (Figure 3, Supplementary Table 1A), and CpGs 11-14 with the pair of primers R2 (Figure 3, Supplementary Table 1B).

In the case of *BMAL1* cytosine methylation levels, negligible differences were observed between the three groups (Supplementary Table 1D). However, statistically significant differences were found when comparing overweight+obese individuals and normal-weight subjects in CpGs 6-7, 8, 15 and 16-17 (Supplementary Figure 1). Finally, no differences were observed in *PER2* cytosine methylation levels when comparing the two groups, normal-weight and overweight+obese subjects (Supplementary Table 1C).

The methylation patterns of different *CLOCK* CpGs showed close associations among them (Supplementary Figure 2), suggesting that they share a common regulation.

Similar associations were observed when the methylation levels of several CpGs of *BMAL1* (Supplementary Figure 2).

Overall, the three genes studied showed significant and positive associations between obesity characteristics and the CpG methylation levels. This was especially the case of the methylation levels of *CLOCK* CpGs 5-6, *PER2* CpGs 2-3 and CpG 25, and *BMAL1* CpGs 16-17. With respect to *CLOCK* gene methylation, BMI and waist circumference were associated with CpGs 1 and 5-6, whereas body fat was associated with CpGs 5-6, 11, 12-13 and 14 (Table 2A). On the other hand, *BMAL1* CpG 16-17 was associated with body fat whereas *PER2* CpGs 2-3 and 25 showed association with BMI (Tables 2C and 2D). These results suggest that the methylation levels of these CpGs in blood cells could be conjointly regulated by adiposity and fat mass, or that some common factors could influence the methylation levels of all these CpG sites.

Tables 2A, 2B and 2C show also the correlations between DNA methylation at the three clock genes and MetS characteristics. In the case of the first region of *CLOCK* gene promoter (CPGs 1, 5-6 and 8), methylation levels were positively associated with waist circumference, serum glucose and triglyceride levels, blood pressure, HOMA index, and the MetS score, but negatively associated with circulating adiponectin. A similar situation was found for *PER2* and *BMAL1*, whose methylation levels were positively associated with MetS risk. In most cases, the significance of the correlations between MetS variables and cytosine methylation levels was maintained after adjusting for BMI (data not shown).

Of note, different chronodisruptors were related to methylation levels. For example, *sleep duration* was inversely correlated to the methylation levels of *CLOCK* CpG 1 after adjusting for obesity parameters and physical activity (r=-0.45; P=0.020).

Another important circadian synchronizer is *food intake* (Garaulet & Madrid, 2010). In this sense, eating behaviors, such as a high frequency of snacking, eating quickly, eating when bored or eating from big packages, were all positively associated to the methylation levels of *CLOCK* CpG 1 (Table 3), and the same trend was found for the other genes although statistical significance was not reached ($P>0.05$). The orexigenic hormone ghrelin could be influencing these results, since the methylation levels of *CLOCK* CpG 1 were significantly and positively correlated to morning fasting ghrelin plasma levels ($r=0.463$; $P=0.035$).

Morningness-eveningness score also tended to correlate inversely to CLOCK CPG 1 methylation levels, which would indicate that there is a tendency to eveningness with higher methylation levels, although statistical significance was not reached ($r=0.274$ $P=0.072$).

Dietary intake could also be influencing the significant associations between DNA methylation and MetS features. Indeed, in this Mediterranean population characterized by a high intake of olive oil and MUFA, the methylation levels of *CLOCK* CpGs 1 and 8 were negatively associated with MUFA intake ($P<0.05$) while the association was positive with PUFA ($P<0.05$) (Figure 4). These results suggest that fatty acid composition of the diet could influence the methylation pattern of genes involved in circadian rhythm regulation.

Finally, the association between weight loss and the baseline methylation levels of *CLOCK* CpG 1, *PER2* CpG 2-3 and *PER2* CpG 25 (Table 4) suggests that these epigenetic marks could be used as markers of weight loss.

DISCUSSION

This research has identified novel associations between methylation patterns at circadian clock genes and MetS factors. It also suggests that some of these CpGs could be used as biomarkers of weight loss response, although these results should be validated in new and larger populations. Different chronodisruptors such as sleep curtailment, frequent snacking or eating quickly, and endocrine parameters, such as ghrelin plasma levels were also associated to changes in the methylation pattern. However, due to the nature of the experiment performed, it is not possible to elucidate whether these epigenetic changes in the clock genes are cause or effects of obesity. In addition, our data point out, for the first time, that dietary intake of MUFA and PUFA could affect the methylation levels of some cytosines, maybe contributing to modulate clock gene expression.

Recent studies have reported that the methylation status of the promoters at several human clock genes is altered in diseases like cancer (Shih et al., 2006). Also, the methylation status of CpGs located in the promoter of some genes has been related to obesity and its comorbidities. Thus, a recent report has evidenced that the methylation of different gene promoters (RXRA, eNOS) at birth is associated with child's later adiposity (Godfrey et al., 2011), suggesting that prenatal developmental may alter the epigenetic regulation of key components of metabolic disease risk. According to this, a perinatal epigenetic analysis might identify subjects at high risk of developing later obesity (Lillycrop & Burdge, 2011). In relation to the clock genes, Suter *et al.* (2011) have found that *in utero* exposure to a maternal high-fat diet significantly alters *NPAS2* promoter occupancy of acetylation of fetal histone H3 at lysine 14 (H3K14ac), a paralog

of the *CLOCK* transcription factor, affecting thus the peripheral circadian system of the fetus.

One important issue is that not only prenatal developmental, but also several factors during adulthood, may alter the epigenetic regulation of metabolic disease risk factors. Thus, obesity *per se*, but also diet composition, inflammation and oxidative stress, which are usually exacerbated in obese adipose tissue, may contribute to alter the epigenetic pattern (Campión et al., 2010). In the current study, although some of the differences between overweight+obese and normal-weight women could be due to perinatal factors or to life style differences, including diet, it is reasonable to assume that obesity itself could be a causative factor of these differences. In fact, the close direct associations found between methylation of several CpGs of *CLOCK* gene and waist circumference, serum glucose and triglyceride levels, blood pressure, HOMA index and the MetS score, as well as the inverse association with circulating adiponectin, together with other results obtained with *BMAL1* and *PER2* genes, strongly suggest that some of these factors could affect the methylation pattern of clock genes or even that this methylation could be a causative factor of obesity and MetS. As in the current study we did not have enough tissue for performing gene expression analyses, new studies must be designed to demonstrate whether methylation in these areas alter mRNA expression in white blood cells. With the exception of CpGs 11-14, the trend of *CLOCK* gene is to increase its methylation levels. According to the traditional view, an increase in methylation would induce a decrease of clock gene expression in white cells. However, the physiological or metabolic consequences of this lower expression of *CLOCK* in these cells remain unknown. Although usually almost all the CpGs of a near region responds in a similar way to an epigenetic factor, in our experiment, CpGs 11-14, which are located in the first intron of *CLOCK* gene, responded in an opposite way than

the promoter. Sometimes this phenomenon is explained because these CpGs overlap with a transcription factor binding site that inhibits the transcription of the gene, but we have not found in our sequence a clear correspondence with an inhibitory transcription factor close to the CpGs 11-14.

Our group has previously reported significant associations between clock genes and MetS alterations. For example, we have confirmed that *PER2* expression in human visceral AT was negatively correlated with waist circumference in men (Gómez-Abellán et al., 2008) and with MetS risk in women (Hernandez-Morante et al., 2011). We have also demonstrated that a genetic variant in human *PER2* has been linked to abdominal obesity and to alterations in eating behaviors highly associated with obesity (Garaulet et al., 2010b). On the other hand, a number of *CLOCK* SNPs have been correlated with predisposition to MetS (Sookoian et al., 2008). In our study, the methylation levels of *CLOCK* CPGs 5-6 show the strongest correlations with MetS features (including BMI and body fat, HOMA index and systolic pressure). In order to confirm this, more studies are required, including a larger sample size, male subjects and the application of multiple testing corrections. Even, methylation of CPGs 5-6 could be analyzed in other cell types, such as adipose tissue, which are more typically involved in obesity and its metabolic sequellae. As a final objective, RNA should be obtained from the same biological samples to assess whether methylation in these areas alter mRNA expression.

Other interesting result obtained in the current population is the particular association between clock gene methylation levels and weight loss. In this sense, recent studies have reported that the percentage of methylation in different CpGs of genes involved in energy metabolism could be used as biomarkers of weight loss response. Thus, by using

methylation microarray analysis at baseline, Bouchard et al. found differences at 35 loci when comparing subcutaneous adipose tissue biopsy samples from high and low responders to a 6-month caloric restriction intervention (Bouchard et al., 2010), whereas Milagro *et al.* (2011) observed 432 CpG sites hypomethylated (<20%) and 602 hypermethylated (>20%) in the peripheral blood mononuclear cells from high responders to an 8-week caloric restriction intervention when compared with the low responders. These initial findings strongly suggest that baseline DNA methylation patterns in white blood cells may be used as epigenetic markers that might help to predict and personalize weight loss. In our case, the baseline methylation levels of *CLOCK* CpG 1, *PER2* CpG 2-3 and *PER2* CpG 25, which showed a correlation with weight loss during the treatment, could be studied for this purpose. These results reinforce a previous work demonstrating the usefulness of some *CLOCK* gene variants in the prediction of weight loss (Garaulet et al., 2010a).

This report is the first demonstrating that epigenetic mechanisms related to cytosine methylation in clock gene promoters might be involved in the onset of obesity and MetS. However, more studies should be accomplished to determine if the methylation of these cytosines has any functional significance for gene expression. Different chronobiological characteristics, such as sleep deprivation, frequent snacking, nocturnal eating and bright light exposure at night (Garaulet et al., 2010a), are recurrent in obesity and might influence the methylation levels of clock genes and *vice versa*. Our data support this hypothesis taking into account that in the current population, sleep duration was inversely correlated to the methylation levels of *CLOCK* CpG 1, which suggests a functional role of *CLOCK* methylation levels on chornodisruption and, as a consequence, in obesity. In this sense, food intake, which is another important synchronizer particularly for the peripheral clocks, coordinating circadian rhythms of

behavior, peripheral clock gene expression and hormone secretion (Sheward et al., 2007), has been also in our experiment related to methylation levels in *CLOCK* gene. Our data also indicate that patients who tended to snack frequently, to eat when bored or to eat from great packages had higher methylation levels in *CLOCK* CpG1 (P=0.026, P=0.008 and P=0.004, respectively).

Ghrelin, which in our study has been also positively associated with methylation levels in *CLOCK* gene, could be implicated in these results. Ghrelin stimulates food intake and recent evidence suggests that ghrelin promotes sleep (Kotronoulas et al., 2009). All these results together support the notion that *CLOCK* methylation levels influence behaviors related to chronodisruption, such as sleep reduction, constant snacking and evening preference, as well as appetite regulators such as plasma ghrelin levels. Similar results have been previously demonstrated for *CLOCK* SNPs. For example, sleep reduction, changes in ghrelin values, alterations of eating behaviors and evening preference that characterize CLOCK 3111C carriers could be affecting obesity and weight loss (Garaulet et al., 2011b). In this sense, although not yet well known, melatonin, the major hormonal regulator of circadian rhythm, works via epigenetic processes through modulation of histone acetylation and DNA methylation (Korkmaz et al., 2009).

Finally, an outstanding result of the current study is the association between the type of fatty acids in the diet (particularly MUFA and PUFA) and the methylation patterns in adults. Our group had previously reported that high-fat diets alter the methylation status of different gene promoters in rat adipose tissue, such as those of fatty acid synthase (FASN) and NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6 (NDUFB6) (Lomba et al., 2010). However, few studies (all of them *in vitro*) have evidenced the

role of n-3 and n-6 PUFA on DNA methylation, particularly docosahexaenoic (Kulkarni et al., 2011), eicosapentaenoic (Ceccarelli et al., 2011) and arachidonic (Kiec-Wilk et al., 2011) fatty acids. The diet of the women participating in the present study can be ascribed to a Mediterranean pattern, being rich in olive oil and, therefore, in MUFA. However, for the moment there are apparently no publications analyzing the effects of MUFA on DNA methylation. In a study performed in a Mediterranean population, we have recently demonstrated that *CLOCK* polymorphisms interact with fatty acids to modulate MetS traits. In that study, the dietary source and membrane content of MUFAs were implicated in the relations between alterations in the circadian system and MetS (Garaulet et al., 2009).

In summary, our research unveils new epigenetic mechanisms involving clock genes that may contribute to better obesity prevention, as well as better prediction of successful weight reduction. Of note, these new data related to DNA methylation are consistent with previous results related to *CLOCK* genetic variants, which demonstrated that sleep reduction, changes in ghrelin values, alterations of eating behaviors and evening preference are all connecting genetics, chronodisruption and obesity. These results support the hypothesis that the influence of the clock genes may be extended to a broad range of variables linked with human behavior and metabolism. Moreover, our data pave the way to the study of epigenetic mechanisms in the regulation of circadian rhythms in relation to obesity and weight loss.

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Table 1. Characteristics of the study population

Table 2. Associations between the percentage of cytosine methylation in several CpG sites located in *CLOCK* gene and metabolic syndrome characteristics. r= Pearson correlation coefficient; P= Probability value (*P<0.05)

Table 3. Association between eating behavior (high frequency of snacking, eating quickly, eating when bored, and eating from big packages) and presence of high methylation levels (> median) of CLOCK CpG 1.

Table 4. Pearson´s correlations between *CLOCK* (CpG 1) and *PER2* (CpG 2-3 and CpG 25) methylation levels and weight loss. r= Pearson correlation coefficient; P= Probability value

Supplementary table 1. Percentages of CpG methylation in the three groups (normal-weight, overweight/obese and morbidly obese) measured by Sequenom´s MassARRAY®. Two regions of *CLOCK* gene were quantified (with the pairs of primers R1 and R2, Tables 1A and 1B respectively) but only one in the case of *PER2* (Table 1C) and *BMAL1* (Table 1D). The data are the average percentage of CpG methylation ± SEM.

Figure legends

Figure 1. Intervention protocol of the study.

Figure 2. Genomic DNA position of targeted CpGs and sequence of the amplicons. Two regions of *CLOCK* gene were quantified (with the pairs of primers R1 and R2) but only one in the case of *BMAL1* and *PER2*. The vertical lines represent the CpGs present in the sequence. The numbered horizontal lines under the sequence represent the regions amplified by the PCR primers and shown below. Number on the left of each sequence file represents the position with respect to the start of transcription (ATG). CpGs are highlighted in bold and consecutively numbered in each sequence. Underlined CpGs are the sites that were reliably quantified by EpiTYPER; boxes in the sequence indicate that these CpGs could not be discriminated by EpiTYPER.

Figure 3. Differences in the percentage of cytosine methylation in several CpGs of *CLOCK* gene between normal-weight and overweight+obese subjects.

Figure 4. Partial correlations between the percentage of methylation of *CLOCK* CpG 1 and *CLOCK* CpG 8 with the percentage of MUFA (3A) and PUFA (3B) in the diet, after adjusting total fat intake by the body mass index of each subject.

Supplementary Figure 1. Differences in the percentage of cytosine methylation in several CpGs of *BMAL1* gene between normal-weight and overweight+obese subjects.

Supplementary Figure 2. Associations between the percentage of cytosine methylation in several CpGs of *CLOCK* and *BMAL1* genes.