Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a Low Calorie Diet

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ABSTRACT

Obesity-associated adipose tissue enlargement is characterized by an enhanced proinflammatory status and an elevated secretion of adipokines such as leptin and cytokines such as TNF-alpha. Among the different mechanisms that could underlie the interindividual differences in obesity, epigenetic regulation of gene expression has emerged as a potentially important determinant. Therefore, twenty-seven obese women (age: 32-50 years; baseline Body Mass Index, BMI: 34.4±4.2 Kg/m^2) were prescribed an eight-week Low-Calorie-Diet and epigenetic marks were assessed. Baseline and endpoint anthropometric parameters were measured and blood samples were drawn. Genomic DNA and RNA from adipose tissue biopsies were isolated before and after the dietary intervention. Leptin and TNF-alpha promoter methylation were measured by MSP after bisulfite treatment and gene expression was also analyzed. Obese women with a successful weight loss (≥5% of initial body weight, n=21) improved the lipid profile and fat mass percentage (-12%, $p<0.05$). Both systolic (-5%, $p<0.05$) and diastolic (-8%, $p<0.01$) blood pressures significantly decreased. At baseline women with better response to the dietary intervention showed lower promoter methylation levels of leptin (-47%, $p<0.05$) and TNF-alpha (-39%, $p=0.071$) than the non-responder group (n=6), while no differences were found between responder and non-responder group in leptin and TNF-alpha gene expression analysis. These data suggest that leptin and TNF-alpha methylation levels could be used as epigenetic biomarkers concerning the response to a Low-Calorie-Diet. Indeed, methylation profile could help to predict the susceptibility to weight loss as well as some comorbidities such as hypertension or type 2 diabetes.

Key words: DNA methylation, epigenetic biomarker, obesity, hypocaloric diet, TNF-alpha, leptin

INTRODUCTION

Obesity is a multifactorial chronic disease characterized by an increase of body weight associated with an excesive fat mass deposition [29] and changes in adipocyte cellularity [6]. Up to now, obesity has been studied as a polygenic disease involving complex gene-gene and gene-environment interactions [28]. However, epigenetics is emerging as a new framework for researching etiological factors and gene expression regulation in environment-related disease [10, 12]. Indeed, epigenetics is the study of the changes in gene expression that are not directly due to variations in the DNA nucleotide sequence and can potentially be transmitted to the offspring [17]. Two major epigenetic mechanisms are the modifications in amino acids in the terminal tails of histones affecting the chromatin folding, and DNA methylation at cytosines followed by guanines (CpG sites). The CpG-rich regions are called CpG islands and are usually located in or near the promoters of 50% of the human genes [9, 12]. When these islands are highly methylated they are usually associated with an inhibition of gene expression, suggesting a steric impediment for the transcription factor binding to their target sites [13].

There are dynamic changes in the DNA methylation pattern during the lifetime and several environmental factors such as dietary compounds [12], oxidative stress [15], hypoxia [36], inflammation [37] or ageing [35] may affect methylation status and modify gene expression. Thus, changes in the DNA methylation pattern as a result of dietary treatments or conditions have been studied in the last years, comparing vegetarians with omnivores [39], the effects of different obesogenic diets [26, 31] or different dietary treatments to lose weight [7]. Interventional studies are showing that the DNA methylation profile previous to the treatment might be able to predict the response to the low calorie diet.

In this context, leptin is an adipokine overexpressed in obese subjects that regulates appetite, energy balance and metabolism [3, 14]. Previous studies by bisulfite sequencing PCR (BSP, a time-consuming technique) showed that leptin promoter methylation in rat adipocytes was modified by a high fat diet [31]. On the other hand, the cytokine TNF-alpha is a recognized marker of the obesity-related inflammatory state [33], and DNA methylation pattern of its promoter in blood cells is able to predict the response to a hypocaloric treatment in humans [12].

Our objective was to investigate whether the basal methylation profiles of specific CpGs located in leptin and TNF-alpha promoters, as measured by methyl specific PCR (MSP, a rapid and cost-effective technique) in subcutaneous adipose tissue from obese and overweight human subjects could predict the response to a low-calorie-diet treatment.

MATERIAL AND METHODS

Subjects and study design

A group of twenty seven obese women (age: 32-50 years; Body Mass Index: 34.4±4.2 Kg/m^2) were enrolled in an 8-week low-calorie-diet (30% energy-restricted). The dietary approach was designed to supply 55% of energy as carbohydrates, 15% as proteins and 30% as fat [23]. At the end of dietary treatment, twenty one patients presented a positive response to the diet (>5% weight loss, n=21) whereas the other group was considered as non-responders (<5% weight loss, n=6). Baseline anthropometric and clinical parameters were measured and blood and subcutaneous adipose tissue samples were obtained at the beginning and at the end of the treatment. Total fat mass was also measured by electric bioimpedance with Quadscan 4000 (Bodystat, UK).

Initial screening evaluation included medical history and fasting blood profile to exclude subjects with clinical disorders that could affect the weight loss process. Other exclusion criteria were weight change > 3 kg within the 3 months before the start of the study, drug prescription, pregnancy, previous surgically or drug treated obesity, alcohol or drug abuse. All the procedures were performed in accordance with the Ethical Committee of the University Clinic of Navarra.

Serum measurements

General biochemical parameters were measured by specific colorimetric assays (Horiba ABX Diagnostics, Montpellier, France) using an automated analysis system (COBAS MIRA, Roche, Basel, Switzerland). Glucose was measured by the HK-CP kit (ABX Pentra, Montpellier, France), serum triglycerides were determined with the RANDOX kit for the in vitro diagnostic of triglycerides (Randox LTD Laboratories, Ardmore Road, UK), circulating total cholesterol was measured using the Cholesterol-CP kit (ABX Pentra, Montpellier, France) and high-density lipoprotein cholesterol with the HDL direct-CP kit (ABX Pentra, Montpellier, France). Low-density lipoprotein cholesterol (LDL-c) data were calculated by the Friedewald equation. C-reactive protein was measured by an ELISA assay as described by the manufacturer (Immundiagnostics, MA, USA).

MSP (Methyl Specific PCR)

Genomic DNA was extracted from subcutaneous adipose tissue samples by using the QIAamp DNA Mini KIT (Qiagen Gmbh, Hilden, Germany). A total of 500 ng of DNA was modified by using EpiTect Bisulfite kit (Qiagen Gmbh, Hilden, Germany), converting thus cytosine into uracil. DNA was amplified with a methyl-specific primer set and with another unmethyl-specific primer set (figure 1) for TNF-alpha and leptin promoters that were designed with MethPrimer [24]. Primers included CpG site -454 for leptin (equivalent to -443 CpG in rats) [31] and -245 and -239 CpG sites for TNF-alpha [11] and amplicons were 108 and 120 bp lenght respectively. PCR conditions were as follows: initial denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 1 min for 38 cycles, followed by stabilization for 7 min at 72°C. To ensure the linearity of PCR reactions and to validate the DNA quantification, adequate controls and standard curves were performed.

The results of the MSP reaction were visualized in a 1.5% agarose gel with a 100bp ladder and the intensity of the bands was measured by triplicate using Adobe Photoshop software (Adobe, San José, CA) and promoter methylation levels were expressed as described elsewhere [20].

RT-PCR

Total RNA was isolated from subcutaneous adipose tissue according to Trizol manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). DNase treatment was performed with the DNA-free™ kit (Applied Biosystems, Austin, TX, USA), and cDNA was synthesized using MMLV reverse transcriptase as described by the suppliers (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time PCR assays were performed using an ABI PRISM 7000 HT Sequence Detection System and Taqman probes for human Leptin (Hs00174877_m1), TNF-alpha (Hs00173377_m1) and PP1a (Hs99999904_m1) mRNA as internal control following the manufacturer's recommendations (Applied Biosystems, Austin, TX, USA). Fold change between responders and non-responder subjets was calculated using the $2^{-\Delta\Delta Ct}$ method [22].

Statistical analysis

All results are expressed as means ± standard deviations. A level of probability up at $p<0.05$ was set up as statistically significant and $p<0.01$ as very statistically significant. Analysis of comparisons between

groups was performed by student t test after normality assessment. Pearson´s correlation coefficient was calculated to determine statistical relationships between variables. Statistical analyses were carried out using SPSS 15.0 package for Windows (Chicago, IL, USA).

RESULTS

Before starting the dietary treatment, both groups, responders and non-responders, did not present phenotypical differences in any anthropometric and clinical variables analyzed; BMI, weight, waist and hip circunference, sagital fold, systolic and diastolic blood preasure and fat mass percentage.

By design, there were differences in the weight loss achieved by the responder and non-responder groups of women after following the low-calorie diet. Those that responded positively to the dietary treatment lost 9.4 % of their initial body weight and showed the usual pattern of improvements associated with weight loss (table 1). Thus, the subjects showed a decrease in blood pleasure levels (5.2% in systolic blood pressure, $p<0.05$, and 8.5% in diastolic blood pressure, $p<0.01$), total plasma cholesterol (13.7%, $p<0.01$) and waist and hip circumferences ($p<0.01$ and $p<0.05$, respectivelly). No differences were observed in circulating glucose, triglycerides and C-reactive protein levels when comparing both intervention groups.

Concerning to the methylation levels before dietary intervention and measured by MSP, the responder group showed lower leptin (-454 CpG, 42%, $p<0.05$) and TNF-alpha (-245 and -239 CpGs, 39%, $p=0.071$) methylation levels than non-responders (figure 2A). This outcome allows to discriminate both groups according to their epigenetic basal status and to predict the future response to the dietary treatment.

On the other hand, nor leptin neither TNF-alpha analyzed CpGs were modified as a result of the dietary intervention study in the responder group (figure 2B); showing that the weight loss program did not apparently induce epigenetic changes in these specific regions.

Interestingly, TNF-alpha promoter methylation levels before the dietary treatment showed a correlation with baseline systolic ($r=0.421$, $p<0.05$) and diastolic ($r=0.471$, $p<0.05$) blood pressure values (figure 3), suggesting that there may be a relationship between both phenomena.

Finally, mRNA expression levels of leptin and TNF-alpha in subcutaneous adipose tissue were analyzed. At baseline, there were no differences between responder and non-responder groups, being no possible to distinguish the future response with these transcriptomic data. However, the dietary treatment decreased

leptin expression levels in the responder group (39%, $p<0.01$) but did not affect TNF-alpha gene expression in subcutaneous fat.

DISCUSSION

The aim of this study was to identify epigenetic methylation patterns in subcutaneous adipose tissue that could predict the response to a low-calorie diet treatment. The epigenetic biomarkers could be used to identify susceptibility or risk to suffer diseases, as obesity, and also, in this way, to guide personalized nutritional interventions to lose weight [1, [2].

There are a number of techniques to study the epigenetic DNA methylation pattern, such as BSP [16], pyrosequencing [4], Combined Bisulfite Analysis (COBRA) [41], methylation microarrays [7], MSP [19] or Maldi-TOF mass spectrometry [25], and they have differences in their specificity, sensibility, accuracy and cost. We have recently published two reports identifying by BSP different diet-related epigenetic marks in leptin [29] and TNF-alpha [11] that could be used as biomarkers of weight loss. BSP is a highly accurate but time-consuming technique that implies amplification of the selected region, purification of the PCR products, subcloning into a plasmid and sequencing of the amplified region [16]. In this new approach we sought to apply the MSP technique, more adequate for the diagnosis of a long number of samples, to identify new biomarkers of response to a hypocaloric diet [30].

Leptin is an adipokine that involved in the regulation of metabolism and energy homeostasis [27]. It is overexpressed in obese subjects and its plasma levels are related with appetite and energy metabolism [21]. The epigenetic control of this molecule has been studied previously and differences in promoter specific CpG methylation levels associated with diet-induced obesity in rats were found, together with increased plasma and mRNA leptin expression levels [31]. Contrariwise, Okada et al did not found a relationship between increased mRNA leptin expression in white adipose tissue from obese mice and methylation levels of this gene in 3T3-L1 cells [34]. According to the perinatal epigenetic control of leptin, it has been also demonstrated that an inadequate glucose metabolism during pregnancy is able to change leptin methylation levels in humans [8]. The other gene studied in this research involved in obesity development is the proinflammatory cytokine TNF-alpha, which is overexpressed in obesity and related metabolic disorders. Changes in its secretion by macrophages are mediated by epigenetic modifications that occur during the monocyte differentiation [38]. Furthermore methylation differences in TNF-alpha promoter region have been associated with distinct responses to a low-calorie diet in blood [11].

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 [12]. Interestingly, in this study we found differences in the baseline methylation levels of leptin and TNF-alpha gene promoters between the responder and non-responder groups. These findings suggest that DNA methylation differences in both genes could be a useful key to categorize the population into susceptible and reluctant to lose weight following a prescribed dietary or therapeutic program.

Otherwise, no changes were induced in leptin and TNF-alpha promoter methylation by the eight week hypocaloric treatment. However, a previous study of our group [30] analyzed the DNA methylation changes induced by a hypocaloric diet using a methylation microarray assay and a subsequent validation by MALDI-TOF mass spectrometry. This study identified two CpGs in ATP10A and WT1 genes whose methylation profile was modified by the hypocaloric treatment.

Moreover, a direct and statistical significant correlation between baseline TNF-alpha promoter methylation levels in subcutaneous adipose tissue and blood pressure values before the dietary treatment. TNF-alpha is involved in the control of angiotensin activity and, thus, in the regulation of blood pressure [18]. It has also been reported in healthy subjects that plasma levels of inflammatory cytokines, such as TNF-alpha, C-reactive protein and IL-6, are correlated with elevated blood pressure, marking them as possible risk factors in the development of hypertension [5].

On the other hand, the analysis of mRNA gene expression does not fit with DNA methylation changes, contrarily to expectatives [12, 13]. However, our results related to mRNA expression of leptin and TNF-alpha on subcutaneous adipose tissue, agree with those reported by Bouchard et al [7] and Mutch et al [32], who did not find clear differences in the transcriptomic profile at the beginning of the diet that could help to predict the future weight loss response. In our work, leptin mRNA expression levels were lower in the responder group after dietary treatment, which has been previously described and related with a decrease in leptin plasma levels [40].

As a conclusion, we have evidenced that it could be possible to predict the response to a low calorie diet according to the initial methylation profile of leptin and TNF-alpha promoters in subcutaneous adipose tissue by MSP technique. Thus, the specific profile of epigenetic marks may contribute to discriminate responder and non-responder patients to a given treatment at the beginning of the intervention, a task that was not possible to ascertain by assessing anthropometric, clinical, biochemical and transcriptomic

features. After this initial successful approach on epigenetic biomarkers search, further studies are warranted to transfer these results to other kind of samples, such as peripheral blood mononuclear cells (PBMC) instead of fat biopsies as a noninvasive source of DNA. Future research in obesity could add new methylation biomarkers that might be used as an initial MSP array screening and combine the results in a predictive score for identifying the most adequate personalized nutrition to lose weight.

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LEGENDS TO THE FIGURES

Figure 1. MSP primers for the PCR amplification of leptin and TNF-alpha promoter regions (bp; base pair, M; methylated, U; unmethylated).

Figure 2. Leptin and TNF-alpha promoter methylation levels expressed as percentage, measured by MSP in subcutaneous adipose tissue samples and a representative profile of amplicated region with U and M primer combination in agarose gel. 2A- Comparison of the DNA methylation levels between responders and non-responders before following the low-calorie diet. 2B- Comparison between the promoter methylation levels before and after dietary treatment in those patients that responded successfully to the low-calorie diet. (U; unmethylated, M; methylated, R; responder, no-R; non-responder).

Figure 3. Correlation analysis between baseline TNF-alpha methylation levels and the systolic and diastolic blood pressures before the dietary intervention.

Table 1. *Anthropometric, clinical and biochemical measurements before (baseline) and after a low calorie diet intervention in individuals with successful weight loss (≥5% of initial body weight, n=21). (*; $p<0.05$; **; $p<0.01$; n.s.; non significant).*