Differential DNA methylation patterns between high and low responders to a weight loss intervention in overweight/obese adolescents: The EVASYON study.

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Short title: Epigenetic weight loss biomarkers in obese adolescents

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ABBREVIATIONS:

OB/OW: Obese/Overweight

BMI-SDS: Body mass index- Standard deviation score

MALDI-TOF: Matrix-assisted laser desorption/ionization

AQP9: Aquaporin 9

DUSP22: Dual specificity protein phosphatase 22

HIPK3: Homeodomain-interacting protein kinase 3

TNNI3: Troponin I, cardiac muscle

TNNT1: Troponin T, slow skeletal muscle
ABSTRACT

**Background:** In recent years epigenetic markers emerged as a new tool to understand the influence of lifestyle factors on obesity phenotypes. Adolescence is considered as an important epigenetic window over human being lifetime. **Objective:** To explore baseline changes in DNA methylation that could be associated to a better weight loss response after a multidisciplinary intervention program in Spanish obese/overweight (OB/OW) adolescents. **Design:** One hundred and seven overweight/obese adolescents undergoing 10 weeks of a multidisciplinary intervention for weight loss were assigned as high/low responders to the treatment. A methylation microarray was performed to search for baseline epigenetic differences between the two groups (12 subjects per group), and MALDI-TOF mass spectrometry was used to validate (total n for validation=107) relevant CpG sites and surrounding regions. **Results:** After validation, five regions located in or near AQP9, DUSP22, HIPK3, TNNT1 and TNNI3 genes showed differential methylation levels between high/low responders to the multidisciplinary weight loss intervention. Moreover, a calculated methylation score was significantly associated with changes in weight, BMI-SDS and body fat mass loss after the treatment. **Conclusions:** We have identified five DNA regions differentially methylated depending on the weight loss response. These methylation changes may help to better understand the weight loss response. **Keywords:** Epigenetics, weight loss intervention, biomarkers, dieting response.
INTRODUCTION

Genetic and lifestyle factors as well as gene x environment interactions have been involved in the etiology of obesity (1). In recent years, epigenetics has risen as a new tool to understand the influence of lifestyle factors on obesity (2-5). In this sense, epigenetics refers to the study of changes on heredity patterns of gene expression which occur without changes in the DNA sequence. The most studied epigenetic mechanisms in relation to obesity are DNA methylation at cytosines followed by guanines (CpG regions), and changes in chromatin organization by histone modification (6). Adolescence is considered as an important epigenetic window over human being lifetime (7).

Studies in obese adolescents are important because adverse patterns of obesity-related disease begin in this period (8). In fact, adolescent obesity is seen as an independent risk factor for adult cardiovascular disease (9) and moderate weight loss provides significant metabolic improvements in adult life.

Obesity has been shown to be associated with methylation changes in leukocyte DNA in obese adolescents and young adults (10). However, few data are available on the effect of lifestyle interventions on epigenetic markers in obese teenagers. A recent study has suggested that the individual epigenetic profile could be used as a predicting factor to estimate the response to diets (11). Thus, our aim was to investigate possible changes in epigenetic modifications between high and low responders to an integral weight loss program in obese/overweight adolescents.

SUBJECTS AND METHODS

Study population and experimental design
The study population consisted of 204 overweight or obese adolescents undergoing a 10 week intensive lifestyle intervention, the EVASYON study (www.estudioevasyon.org). This was a lifestyle and nutritional educational weight loss program supported by a multidisciplinary team of nutritionists, physiotherapists, psychologists and paediatricians. Data from these adolescents were collected at the beginning and after 10 weeks of treatment and participants were recruited from five Spanish cities (Granada, Madrid, Pamplona, Santander and Zaragoza). In the present study, firstly, a methylation array was carried out in a subsample of 24 adolescents (42% males). These adolescents were selected from the general population sample and are those with the best/worst response to weight loss intervention. They were considered as “high responders” (losing more than 1.1 BMI-SDS after 10 weeks of intensive lifestyle intervention; n=12) and “low responders” (those not achieving a successful weight loss; less than 0.4 BMI-SDS after treatment; n=12) respectively. Secondly, a validation of the methylation array results was performed in the same 24 adolescents and the sample was increased with 12 more adolescents (6 high responders and 6 low responders). Supplementary Figure 1 shows a diagram of the study sample available in each phase of the study. This study included only 12 to 16 years old overweight or obese adolescents, according to Cole’s criteria (12), who have been raised in Spain and who have not been diagnosed with a disease associated with obesity or pharmacological treatment.

Written consent to participate was requested from both parents and adolescents. The study protocols were performed in accordance with the ethical standards laid down in the 1961 Declaration of Helsinki (as revised in South Korea in 2008), following the European Economic Community (EEC) Good Clinical Practice guidelines (document 111/3976/88 of July 1990) and current Spanish law, which regulates clinical research in humans (Royal
Decree 561/1993 regarding clinical trials). The study was approved by the five local ethics committees.

**Multidisciplinary Intervention**

According to food intake questionnaires, a personalized balanced diet (30% of energy as fat, 15% as proteins and 55% as carbohydrates) and a physical activity programme was handed in to each adolescent. During the 10 week intensive program period, the adolescents attended weekly group sessions where they received nutritional and physical advice, as well as psychological support. The description of the complete EVASYON study design has been previously published elsewhere (13).

**Physical Activity, Energy Intake, Metabolic and Anthropometric Data**

All the adolescents were asked to fill in a series of validated questionnaires in order to determine their physical activity level and estimate their basal metabolism rates (14-15). A semi-quantitative food-frequency questionnaire, previously validated in Spain (16), and containing 132 food items, as well as a 72-hour recall was filled in at the beginning of the follow-up. Weight and height were measured with an electronic scale (Type SECA 861) and a telescopic height measuring instrument (Type SECA 225) respectively. BMI was calculated as weight (kg)/height$^2$ (m$^2$), then, individual BMI values were converted into standard deviation scores (SDS) using age and specific cut-points according to the Spanish children and adolescent growth references (17). Skinfolds were measured with a skinfold calliper (Caliper Holtain; Holtain Ltd., Walles, UK) and waist and hip girths with a flexible non-stretchable measuring tape (Type SECA 200). Pubertal developmental was determined according to Tanner stage (18). Blood pressure was obtained using the left arm after the adolescent had rested quietly for 15 minutes using a blood pressure monitor (Mod. OMRON M6.) by following validated procedures.
**DNA extraction**

Venous blood samples were collected at the beginning of the study to obtain DNA. Genomic DNA was extracted using the MasterPure™ DNA purification Kit for Blood Version II (Epicentre Biotechnologies, Madison, WI) and was stored at -80°C until its processing.

**Methylation Array**

The epigenetic profiling of genomic DNA was analyzed in 24 subjects using the methylation assay HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA) covering 27,578 CpG dinucleotides in 14,495 genes. Infinium technology for genome-wide DNA methylation screening was employed. The methylation assay was performed as described previously (19). For each CpG site, four probes were designed: two allele-specific oligos (ASO) and two locus-specific oligos (LSO). Each ASO-LSO pair corresponded to either the methylated or unmethylated state of the CpG site. Bisulfite conversion of DNA samples was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) on 1000 ng of DNA. The remaining assay steps were identical to those of the HumanMethylation27 BeadChip assay (Illumina, Inc), using reagents and conditions recommended by the manufacturer. The fluorescently stained chip was imaged by the Illumina BeadArray Reader. Illumina's Genome Studio program was used to analyze BeadArray data to assign site-specific DNA methylation values to each CpG site. The proportion of methylation (%) for each subject at each CpG site was computed by first subtracting the background signal intensity of negative controls from both the methylated and unmethylated signals; then dividing the ratio of the methylated signal intensity by the sum of both methylated and unmethylated signals. Thus, the percentage value is a continuous variable ranging between 0 and 1.

**Methylation profile by MALDI-TOF mass spectrometry**
From the Methylation array data seven CpGs from or near seven genes: adiponutrin (*ADPN*), aquaporin 9 (*AQP9*), dual specificity phosphatase 22 (*DUSP22*), coagulation factor XII (*F12*), homeodomain interacting protein kinase 3 (*HIPK3*), troponin I type 3 (*TNNI3*) and troponin T type 1 (*TNNT1*) were selected for validation by using the Sequenom EpiTyper approach (Sequenom, San Diego, CA, USA) consisting of a base-specific cleavage followed by MALDI-TOF mass spectrometry. According to the methylation array results, 7 amplicons (400-500bp) covering the most relevant CpGs were designed and tested in 107 subjects (Table 2). Table 1 shows amplicons for the 5 validated CpGs. The complete methodology has been previously described (11). After the process, microarray results were validated by Sequenom EpiTyper using Pearson’s correlation test. To compare methylation of identical CpG regions among high and low responder groups, we determined the mean methylation index (MMI), a value that describes the average methylation level of CpG residues within a defined region. It is computed by dividing the sum of the methylation levels of all CpG sites present within a region by the number of CpG sites present in that region. The MMI can have a maximum value of 1 (complete methylation) or a minimum of 0 (no methylation).

**Epigenetic Score**

For the epigenetic score, the 97 CpGs with more than a 5% change in DNA methylation between low and high responders at baseline and with a significant differential response effect (p<0.05) were taken into account (Supplementary table 1). Each CpG site methylation value was converted into 0, 1 or 2 (tertiles) according to its baseline methylation rate (0= lowest methylation group, 1= intermediate methylation and 2= highest methylation percentages). A cumulative methylation score was calculated for each adolescent as previously done by other authors (20), by summing the tertile value of these 97 genes (global score ranged between 41 and 182). Finally, due to its wide range, this cumulative methylation score was categorized
into quartiles for further analyses from quartile 1 (lowest methylation score group) to quartile 4 (highest global methylation percentages).

**Statistical analysis**

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software 15.0 (SPSS INC., Chicago, IL). The Kolmogorov-Smirnov and the Shapiro-Wilks tests were used to determine variable distribution.

The differences in clinical parameters after the lifestyle intervention between high and low responders to the diet were tested with the Student t test or the Mann Whitney U test. Differentially methylated CpGs were selected by two-way ANOVA (response to diet-sex effect) using the software Genespring GX 10.0 (Agilent Technologies, Santa Clara, CA, USA). Only DNA methylation levels with more than a 5% change between both groups and with a p<0.05 were considered for analyses as previously observed in other microarray studies (21-22). A volcano plot was calculated using the Array Studio 5.0 program (Omicsoft Corp., Cary, NC, USA).

Differences in DNA methylation levels by using the EpiTyper approach were analyzed with the Mann-Whitney U test (high responders vs. low responders). Pearson’s correlation coefficient was used for comparing the two epigenetic tools in order to focus the analysis on the most reliable CpGs and for comparing cytosine methylation levels of CpGs with changes in anthropometric variables.

**RESULTS**

The general features of obese adolescents after the weight loss response to a 10-week lifestyle intervention are presented in table 2. High responders are adolescents who lost more than 1.1 points of their initial BMI-SDS, while low responders lost less than 0.4 BMI-SDS. This
weight loss was accompanied by a significant improvement in anthropometric measurements among good responders whereas bad responders appeared to get less benefit from the multidisciplinary intervention programme (table 2).

From the results of the HumanMethylation27 BeadChip array, a Volcano plot showing the distribution of the 27,578 analyzed CpG sites, was depicted (Fig. 1). Among these 27,578 CpGs, the microarray study showed 97 CpG sites differentially methylated (fold change ≥ 5%; p<0.05) between low and high responders at the start of the intervention. 34 of them were hypomethylated and 63 hypermethylated in the high responders group compared with the low responders (Supplementary table 1). Supplementary Tables 2 and 3 show genes differentially methylated between high and low responders (fold change ≥ 5%; p<0.05) in males and females respectively.

Interestingly, a calculated baseline epigenetic score (quartiles) was built in obese adolescents (Fig. 2). Subjects with the highest methylation score had a significantly better response (p<0.001) to the treatment programme, with a higher BMI-SDS decrease, than those with a lower methylation score.

To validate findings of the DNA methylation arrays seven CpGs sites (corresponding to AQP9, DUSP22, HIPK3, TNNI3, TNNT1, F12, and PTPRG), were chosen to be further analysed by MALDI-TOF in an extending sample of 107 subjects. The results confirmed significant DNA baseline methylation differences between high and low responders in five of the selected regions in or near these genes (Fig. 3 and Supplementary Table 4).

During the validation process, in addition to the total methylation percentage of the regions, new CpGs from specific regions in each gene were analyzed. Figure 4 shows the methylation percentages of low and high responders before the intervention for the CpGs studied, and their association with anthropometric changes after 10 weeks of treatment. Significant
differences (p<0.05) in total methylation (MMI) between high and low responders to the lifestyle multidisciplinary intervention were observed in the genomic region corresponding to HIPK3 gene. Higher baseline methylation levels of HIPK3 gene led to a greater weight, BMI-SDS, and fat mass loss in these adolescents following the EVASYON program intervention. Moreover, as seen in figure 4, differences in basal DNA methylation of several CpGs in *AQP9, DUSP22, HIPK3, TNNI3* and *TNNT1* genes were significantly associated with changes in body weight, BMI-SDS, waist girth and body fat mass after the weight loss intervention.

DISCUSSION

The term “personalized nutrition” has risen as a new concept in the line with the fields of nutrigenomics, proteomics and metabolomics (23). The advances in nutrigenomics are expected to lead to genome-customized diets for obesity prevention or treatment based on personalized approaches (24). In this sense, genetic and epigenetic markers have been considered as predictive tools to assess individual differences in response capacity after a nutritional intervention (25).

Our analysis revealed that the epigenetic pattern based on an epigenetic score calculated from baseline differentially methylated genes between high and low responders to a weight loss multidisciplinary programme, could help to predict the individual BMI-SDS decrease in adolescents. To our knowledge this is the first time that an epigenetic predisposition score for obesity has been calculated and our data suggest that could be used as a prognostic tool to predict weight loss outcome.

In relation to the weight loss response, we have divided the study sample into high and low responders according to their BMI-SDS reduction. In this sense, a recent study by Ford *et al.*
(2010) reported that reducing BMI-SDS by $\geq 0.5$, achieved significant improvements in body composition measures leading to important reductions in key metabolic risk factors (26).

The EpiTyper validation study showed five regions located in or near five genes ($AQP9, DUSP22, HIPK3, TNNI3$ and $TNNT1$) with a significant methylation change between high and low responders to the EVASYON program (>5%). Two CpGs located in the $AQP9$ gene were differentially methylated between high and low responders to the diet. The hypermethylation of CpG1 was associated with greater weight loss as reported in adult subjects (11). AQP9 acts as a facilitative carrier for glycerol (27); it is expressed in omental and subcutaneous fat depots allowing glycerol exit and in hepatocytes facilitating glycerol entry for gluconeogenesis. Insulin and leptin act as regulators of its activity (28), meaning an elevated expression of the $AQP9$ gene that may lead to increased lipogenesis. The repression of AQP9 expression through an increased methylation could improve weight loss response.

Concerning $DUSP22$ gene, we found an association between total gene methylation at baseline and anthropometric changes after the intervention. High responders had significantly lower methylation percentages before treatment. Dual specificity phosphatase 22 acts as a negative regulator of the IL6/LIF/STAT3-mediated signalling pathway by desphosphorylating STAT3, which is stimulated by leptin (29). It has been suggested that IL6 acts by linking obesity-derived chronic inflammation with insulin resistance through activation of STAT3 (30). As a result, an increased expression of $DUSP22$ gene could inhibit this inflammatory pathway regulating gluconeogenesis and hepatic glucose output (31).

The homeodomain interacting protein kinase 3 ($HIPK3$), is involved in a number of important cellular processes and its effects are mediated by the phosphorylation of several important proteins (32). Up to now, it has not been involved in metabolism related pathways, but a study carried out in American adolescents has shown that this gene is hypermethylated in lean
compared with obese subjects (10). In our study high responders had at baseline a significantly higher percentage of HIPK3 methylation, which suggests that this hypermethylation could somehow protect from obesity leading to a positive response to treatment.

Moreover, we observed that the baseline hypermethylation of TNNT1 and TNNI3 genes (for total methylation as well as individual CpGs methylation) was related to a higher improvement of several anthropometric parameters (body weight, BMI-SDS and body fat mass) after 10 weeks of multidisciplinary treatment. Cardiac troponin I (TNNI3) and slow skeletal muscle troponin T (TNNT1) are both located in chromosome 19 within a 11 kb separated frame from each other (33). TNNT1 has been described as a marker of cardiac damage, its increased expression has been positively correlated with total cholesterol, triglycerides and LDL-cholesterol and negatively correlated with HDL-cholesterol (34). Our results suggest that a higher methylation level of the TNNT1 gene in high responders compared with low responders could lead to a lower gene transcription rate in those adolescents, allowing them to obtain greater benefits from the weight loss intervention. In regard to the TNNI3 gene, minor troponin I elevations appear to occur in a small number of subjects with type 1 diabetes presenting ketoacidosis (35), but its implication in obesity is still not clear.

The fact that our study was conducted in adolescents, allows us to explore one of the most significant postnatal stages susceptible to epigenetic variations (7), not only to search for specific methylation profiles that could help in predicting the response to a weight loss program to treat obese adolescents, but also to develop new individualized therapeutic strategies. The use of whole blood (WB) DNA for the epigenetic study is justified in this adolescent population considering that it is a non-invasive technique and since the use of WB
for methylation studies has been supported in previous studies (36-38). One additional advantage of our analysis in adolescents is the absence of obesity associated comorbidities or pharmacological treatment that could mask the results. Moreover, obesity treatment during adolescence should be a priority study subject, since improvements in obesity at this stage have been demonstrated to lead to maintained changes during adulthood that could decrease the risk of developing obesity related comorbidities such as metabolic syndrome (39), hypertension (40) or even some cancer types (41).

In this study we have confirmed two regions in or near two genes (AQP9 and HIPK3) that have been previously associated with weight loss response or obesity (10-11), suggesting that methylation changes in these regions could act as reliable biomarkers for weight loss. However, new studies with larger populations are needed in order to confirm these results. In summary, in this work, we have found that an epigenetic score could be used to predict body weight changes. We have also identified five DNA regions differentially methylated depending on the weight loss response. These epigenetic changes may help to better understand weight loss response.

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received the award from AESAN (Spanish Agency for Food Security and Nutrition) from the Spanish Ministry of Health and Consumption to the best applied research project in 2009.

Conflict of interest

The authors declare no conflict of interests.

EVASYON study group

Coordinator: Marcos, A


Deceased.


Table 1: Microarray data of differentially methylated CpGs between high and low responders to a multidisciplinary weight loss intervention after the -way ANOVA, and the primers used for their validation with Sequenom Epityper approach. Δβ: change in methylation levels.

<table>
<thead>
<tr>
<th>SYMBOL</th>
<th>Illumina ID</th>
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<th>CpG position</th>
<th>Primers</th>
<th>p-value (Response)</th>
<th>p-value (Response-Sex)</th>
<th>p-Value (sex)</th>
<th>Δβ</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L: aggaagagagTGAAATTTTTTTTGGATTTAGGGTT</td>
<td>0.0155</td>
<td>0.6812</td>
<td>0.6121</td>
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<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td>AQP9</td>
<td>cg11098259</td>
<td>15</td>
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<td>HIPK3</td>
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Differentially methylated regions between high and low responders in males

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<th>p-value (Response-Sex)</th>
<th>p-Value (sex)</th>
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<tr>
<td>DUSP22</td>
<td>cg15383120</td>
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<td>236909</td>
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<td>R: cagtaaatagactcactatagggagaaggctAAATCTCCTAAA</td>
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Differentially methylated regions between high and low responders in females

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<th>p-value (Response-Sex)</th>
<th>p-Value (sex)</th>
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<td>TNNT1</td>
<td>cg19504245</td>
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<td>60352432</td>
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Table 2: Clinical characteristics at baseline and after a 10 week weight loss intervention in obese/overweight adolescents. Mean±SEM

<table>
<thead>
<tr>
<th></th>
<th>High Responders (n=12)</th>
<th>Low Responders (n=12)</th>
<th>p difference between groups</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>After 10 weeks</td>
<td>Baseline</td>
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<tr>
<td>Weight (kg)</td>
<td>92.7± 4.1</td>
<td>83.3±3.7***</td>
<td>80.1±4.7</td>
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<td>BMI-SDS</td>
<td>5.5±0.3</td>
<td>4.0±0.3***</td>
<td>4.2±0.6</td>
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<td>Waist girth (cm)</td>
<td>102.5±3.1</td>
<td>97.4±2.8*</td>
<td>108.1±3.8</td>
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<tr>
<td>Hip girth (cm)</td>
<td>110.4±4.6</td>
<td>105.2±4.3***</td>
<td>80.7±10.0</td>
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<tr>
<td>Waist/Hip ratio</td>
<td>0.95±0.07</td>
<td>0.95±0.07</td>
<td>1.48±0.13</td>
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<td>Waist/Height ratio</td>
<td>0.62±0.02</td>
<td>0.58±0.01*</td>
<td>0.67±0.02</td>
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<td>SBP (mmHg)</td>
<td>127.6±4.2</td>
<td>116.1±4.9**</td>
<td>102.6±5.9</td>
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<tr>
<td>DBP (mmHg)</td>
<td>82.9±4.3</td>
<td>69.2±2.1***</td>
<td>77.6±2.5</td>
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<td>Fat Mass (%)</td>
<td>47.0±2.2</td>
<td>42.6±2.2**</td>
<td>42.3±2.7</td>
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<tr>
<td>Σ7 Skinfolds</td>
<td>190.3±7.3</td>
<td>172.1±9.7**</td>
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<tr>
<td>Physical Activity (counts)</td>
<td>358.4±27.1</td>
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<td>407.1±90.7</td>
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<td>Glucose</td>
<td>80.0±3.3</td>
<td>79.1±4.0</td>
<td>87.6±3.4</td>
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<td>Adiponectin (gr/gr fat mass)</td>
<td>0.29±0.06</td>
<td>0.38±0.06*</td>
<td>0.09±0.05</td>
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<td>Leptin (gr/gr fat mass)</td>
<td>0.16±0.03</td>
<td>0.07±0.02***</td>
<td>0.24±0.10</td>
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<td>Total cholesterol (mg/dl)</td>
<td>149.6±7.0</td>
<td>127.9±6.8***</td>
<td>175.5±12.5</td>
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<td>ApoA1 (mg/dl)</td>
<td>112.1±4.8</td>
<td>99.0±5.1***</td>
<td>104.0±26.0</td>
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<td>ApoB (mg/dl)</td>
<td>67.0±3.9</td>
<td>60.7±3.5*</td>
<td>55.5±6.5</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>2.7±0.5</td>
<td>1.3±0.2**</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>LAP index</td>
<td>47.2±6.7</td>
<td>41.4±5.9*</td>
<td>61.1±19.0</td>
</tr>
</tbody>
</table>

SBP : Systolic Blood Pressure; DBP : Diastolic Blood Pressure; CRP: C-reactive Protein; LAP: Lipid Accumulation Profile
*p<0.05 between baseline and after 10 weeks of intervention; **p<0.01 between baseline and after 10 weeks of intervention
FIGURE LEGENDS:

Figure 1: Volcano plot showing methylation data. The dashed red line shows significantly (p<0.05) differentiated genes between high/low responders to the diet.

Figure 2: Correlation between BMI-SDS change after 10 weeks of a weight loss intervention and a global epigenetic score in overweight/obese adolescents. Different letters (a, b, c) indicate statistical differences (p<0.05) between epigenetic score groups, each group is expressed as mean, SEM; (n for each score group=6)

Figure 3: Percentages of methylation of TNNT1, AQP9, TNNI3, HIPK3 and DUSP22* CpGs in overweight /obese adolescents: strong association between data from the Methylation array and MALDI-TOF mass spectrometry (n=24) measured by Pearson correlation test.*EpiTYPER validation for DUSP22 was only performed in males samples (n=12).

Figure 4: CpGs analyzed in AQP9, DUSP22, HIPK3, TNNI3 and TNNT1. *Stands for a significant difference in methylation percentage between obese adolescents high (n=17) and low (n=18) responders to the intervention programme. Grey boxes represent CpGs, which methylation at the beginning of the study correlates with changes in anthropometric parameters as weight, BMI-SDS, waist girth or fat mass percentage.