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ORIGINAL ARTICLE

DNA methylation of leptin and adiponectin promoters in children is reduced by the combined presence of obesity and insulin resistance

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OBJECTIVE: Epigenetic alterations have been suggested to be associated with obesity and related metabolic disorders. Here we examined the correlation between obesity and insulin resistance with the methylation frequency of the leptin (*LEP*) and adiponectin (*ADIPOQ*) promoters in obese adolescents with the aim to identify epigenetic markers that might be used as tools to predict and follow up the physiological alterations associated with the development of the metabolic syndrome.

SUBJECTS: One hundred and six adolescents were recruited and classified according to body mass index and homeostasis model of assessment-insulin resistance index. The circulating concentrations of leptin, adiponectin and of several metabolic markers of obesity and insulin resistance were determined by standard methods. The methylation frequency of the *LEP* and *ADIPOQ* promoters was determined by methylation-specific PCR (MS-PCR) in DNA obtained from peripheral blood samples.

RESULTS: Obese adolescents without insulin resistance showed higher and lower circulating levels of, respectively, leptin and adiponectin along with increased plasmatic concentrations of insulin and triglycerides. They also exhibited the same methylation frequency than lean subjects of the CpG sites located at −51 and −31 nt relative to the transcription start site of the *LEP* gene. However, the methylation frequency of these nucleotides dropped markedly in obese adolescents with insulin resistance. We found the same inverse relationship between the combined presence of obesity and insulin resistance and the methylation frequency of the CpG site located at −283 nt relative to the start site of the *ADIPOQ* promoter.

CONCLUSIONS: These observations sustain the hypothesis that epigenetic modifications might underpin the development of obesity and related metabolic disorders. They also validate the use of blood leukocytes and MS-PCR as a reliable and affordable methodology for the identification of epigenetic modifications that could be used as molecular markers to predict and follow up the physiological changes associated with obesity and insulin resistance.

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INTRODUCTION

Obesity and related metabolic diseases are major public health problems of pandemic proportion. In Europe, between 10 and 30% of adults are obese and up to 24% of the 6–9 years old children are overweight or obese,[1,2] whereas in the US, one-third of Americans over the age of 20 are obese.[3] Obesity is also a health problem in developing countries where malnutrition related to micronutrient deficiencies coexists with an industrialized profile of food intake and lifestyle characterised by the ingestion of high-energy food combined with low physical activity.[4,5] As a consequence, the incidence of obesity in adults and children in certain developing countries is similar to that of the US.[6] The prevalence of childhood obesity is of particular concern not only because obese children are at high risk of developing metabolic and psychological disorders, including insulin resistance, lower self-esteem and depression,[7,8] but also because childhood obesity contributes substantially to obesity and disease risk in adulthood.[8] Actually, more than 60% of obese adolescents become obese adults.[9,10] Therefore, the implementation of strategies for the early identification and prevention of childhood obesity is fundamental to combat this epidemic disease and its resultant comorbidities, which are the main causes of death in all parts of the world.

Although reduced energy expenditure and increased consumption of highly caloric foods are important factors contributing to the current epidemic of obesity,[11,12] data from animal models and human epidemiologic studies suggest that the development of obesity and associated metabolic disorders involves complex

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interactions between genetic and environmental factors, which are underpinned by epigenetic mechanisms. Epigenetics refers to biological processes that regulate mitotically or meiotically heritable changes in gene expression without altering the DNA sequence. Eukaryotic DNA is packaged into a chromatin structure consisting of repeating nucleosomes formed by wrapping 146 base pairs of DNA around an octamer of four core histones (H2A, H2B, H3 and H4). DNA can be modified by methylation of cytosine residues in CpG dinucleotides, and the N-terminal tails of histone proteins are subject to a wide range of modifications (acetylation, methylation, phosphorylation and ubiquitylation). DNA methylation and histone modifications may act alone or in concert in a context-dependent manner to facilitate or block the accessibility of transcription factors to the promoters of genes and, therefore, to induce or inhibit their expression. Notably, increased acetylation of histone 3 at the promoter regions of TNF-α and COX-2, two inflammatory genes implicated in obesity and insulin resistance, has been reported in human blood monocytes from diabetic subjects.[13] Similarly, hypermethylation of the pro-opiomelanocortin and serotonin transporter genes has been positively associated with childhood or adult obesity.[14,15] Moreover, differences in weight lost after dietary intervention have been associated with changes in DNA methylation patterns.[16–18] However, to date, the identification of the epigenetic changes associated with obesity has been based on the use of genome-wide analysis tools and sophisticated molecular biology techniques, which have a prohibitive high cost and are difficult to use in current clinical practice. Therefore, in the present study we aimed to develop a simple and reliable method for the identification of epigenetic markers in peripheral blood leukocytes associated with obesity. Our premise is that gene expression and epigenetic profiles of blood cells constitute a fingerprint of disease risk and might shed light on the mechanisms underlying the development of obesity and related metabolic disorders. Specifically, we used methylation-specific PCR (MS-PCR) to evaluate the correlation between the presence of obesity and/or insulin resistance and the methylation profile in selected CpG sites within the promoters of the leptin (*LEP*) and adiponectin (*ADIPOQ*) genes in genomic DNA samples from blood leukocytes of obese adolescents. Leptin is a key hormone released from adipose tissue that regulates energy metabolism and insulin sensitivity, and leptin circulating levels are significantly increased in obese people.[19–21] Conversely, adiponectin improves peripheral insulin sensitivity and blood adiponectin levels are inversely correlated to body fat mass, insulin sensitivity and type 2 diabetes.[22–24]

SUBJECTS AND METHODS

Subjects

Lean and obese adolescents (10–16 years old) from both sexes (40 girls and 66 boys) were recruited from the Hospital Infantíl de México Federico Gómez, Mexico City, Mexico. Children with endocrine disorders, familial hyperlipidemia, hypertension, diagnosed diabetes or pharmacological treatments were excluded from the study. The clinical and molecular studies were approved by the ethics committee of the Hospital Infantil de México Federico Gómez. Parents and children were informed as to the nature and purpose of the study. Parents gave their written informed consent. Children gave their consent verbally.

Overview of the protocol

Body weight was measured in underwear to the nearest 0.1 kg using a calibrated balance. Height was measured to the nearest centimeter using a rigid stadiometer. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in meters and expressed using the percentile charts of the Center for Disease Control and Prevention for the assessment of somatic development in children and adolescents. During the medical interview, two 5 ml samples of venous blood were taken from the left cubital vein, between 08:00 and 09:00 am, after an overnight fast. One of the samples was used to obtain serum that was kept at −70 °C until metabolite analysis and the other one was conserved in EDTA-containing tubes at −20 °C to further purify genomic DNA.

The concentrations of glucose, cholesterol and triglycerides in serum were measured in the central laboratory of the Hospital Infantil de México Federico Gómez using standard enzymatic assays. The concentration of insulin was determined by solid-phase, two-site chemiluminescent immunometric assay (Immulite, Diagnostic Products Corp., Los Angeles, CA, USA). Insulin resistance was determined by the homeostasis model of assessment-insulin resistance (HOMA-IR), using the following formula: HOMA-IR = (insulin (mU l^{-1}) × glucose (mMol l^{-1}))/22.5. A HOMA-IR value of 3.4 was chosen as the cutoff point to define insulin resistance. This value corresponds to the 90th percentile in a population of healthy children. Moreover, a HOMA-IR index higher than 3.4 is a cardiovascular risk factor.[25]

Leptin and adiponectin assays

Circulating levels of leptin and total adiponectin in serum were determined by enzyme-linked immuno sorbent assay (ELISA) using the Human Leptin ELISA and Human Adiponectin ELISA kits (Millipore, St Charles, MO, USA). Leptin and adiponectin concentrations were measured in triplicate and calculated from standard curves generated for each assay using the recombinant human leptin and adiponectin provided in the kits. The intra- and inter-assay coefficients of variation were, respectively, 3.4% and 4.2% for leptin, and 1.8% and 6.2% for adiponectin.

DNA extraction and sodium bisulfite modification

Genomic DNA was isolated from peripheral blood using the FlexiGene DNA kit (Qiagen, Hilden, Germany). Briefly, 300 µl blood were mixed with 750 µl lysis buffer. Cell nuclei and mitochondria were pelleted by centrifugation (10,000 *g* for 20 s) and resuspended in 150 µl denaturing buffer. After protein digestion at 65 °C for 5 min, DNA was precipitated in 150 µl isopropanol, recovered by centrifugation (3 min at 10,000 *g*), washed with 150 µl ethanol, and air dried. Finally, DNA was resuspended in 200 µl hydration buffer and stored at −20 °C.

DNA integrity and concentration were assessed by agarose gel electrophoresis and spectrophotometric analysis. Then, DNA was treated with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen). Briefly, 2 µg DNA were mixed with 85 µl sodium bisulfite, 15 µl DNA protect buffer and RNase-free water up to 140 µl. Bisulfite DNA conversion was performed in a thermal cycler using the following conditions: 5 min at 95 °C, 25 min at 60 °C, 5 min at 95 °C, 85 min at 60 °C, 5 min at 95 °C, 175 min at 60 °C and a final step at 20 °C. For the purification, samples were mixed with 560 µl buffer BL containing 10 µg ml^{-1} carrier RNA and loaded on EpiTech spin columns. Then, the DNA was washed, 20 µl buffer EB was added to each sample and converted DNA was eluted by centrifugation (15,000 *g* for 5 min) and stored at −20 °C.

Methylation-specific PCR

On the basis of previous published studies on the *LEP* promoter,[26–30] we selected CpG sites located at −51 and −31 nt relative to the transcription start site for the methylation analysis. These nucleotides encompass a CCAAT/enhancer binding protein binding site and the TATA box, respectively (Figure 1a). For the methylation analysis of the *ADIPOQ* promoter, we decided to study CpG sites located at −283 and −74 nt relative to the transcription start site, which encompass a CCAAT/enhancer binding protein (C/EBP) binding site and an E-box sequence, respectively (Figure 1b). For each CpG site, a pair of MS-PCR primers was designed using PRIMER 3 (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and OLIGO ANALYZER (www.idtdna.com/analyzer/Applications/OligoAnalyzer/) programs. Bisulfite-treated DNA templates were PCR amplified using HotStarTaq d-Tect Polymerase (Qiagen), which allows the analysis of a single specific CpG site because it is not able to extend DNA strand in the presence of a single-base mismatch at the 3'-end of primers during annealing and extension steps. PCR assays were performed with one-fourth volume of the converted DNA, 25 µl EpiTect Master Mix, 0.4 µM each primer and RNase-free water up to 50 µl. Amplification conditions were: 95 °C for 13 min; 94 °C for 30 s, *Tm* for 30 s and 72 °C for 1 min, for 35 cycles, and 72 °C for 10 min. To validate primer design and to control for the bisulfite treatment efficiency, human methylated and unmethylated DNA (EpiTect PCR Control DNA set, Qiagen), were used as positive and negative controls. PCR products were separated by 10% PAGE, stained with ethidium bromide and visualized by UV irradiation.

a

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-320 CACCTTCCCA AGGGGCTGGT CCTTGCGCCA TAGTCGCGCC GGAGCCTCTG GAGGGACATC
                            C/EBP
-260 AAGGATTTCT CGCTCCTACCAGCCACCCCC AAATTTTTGG GAGGTACCCA AGGGTGCGCG

-200 CGTGGCTCCT GGCGCGCCGA GGCCCTCCCT CGAGGCCCCGCGAGGTGCAC ACTGCGGGCC

-140 CAGGGCTAGC AGCCGCCCGG CACGTCGCTA CCCTGAGGGG CGGGGCGGGA GCTGGCGCTA
                     SP1          ▼                ▼
 -80 GAAATGCGCC GGGGCCTGCG GGGCAGTTGC GCAAGTTGTG ATCGGGCCGC TATAAGAGGG
                                C/EBP                    TATA-box
 -20 GCGGGCAGGC ATGGAGCCCC
     SP1
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b

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                                                  ▼
-320 TTAATTCATT TACATTAGGA AGTTTCTCCG TCAGATGCAG GAAAAAAATC TTGTTTTCCT
                                                C/EBP
-260 GCTGTGGTTT TGACTTTTGC CCCATCTTCT GTTGCTGTTG TAGGAGGCAA AATAAGGGTC
                              LRH/RE
-200 AAGGCCTGGA AACACAAGTG CTTTGACTGA AGCTCCACTT GGCTTCCGAA GCCCAAGCTG

-140 GGTTGTACCA GGTTCCCTAG GGTGCAGGCT GTGGGCAACT GCCAGGGACA TGTGCCTGCC
          ▼                       E-box
 -60 CACCGGCCTC TGGCCCTCAC TGAGTTGGCC AATGGGAAAT GACAATTGTG AGGTGGGGAC
           E-box       E-box
 -20 TGCCTGCCCC CGTGAGTACC
```

c

Gene name	CpG site	Forward (F) and reverse (R) primers	Product size
LEP	- 51 nt	F: 5′-GCTGGTCCTTGCGCCATAGT-3′	275 bp
		R: 5′-CGGCCCGATCACAACTTGCG-3′	
	- 31 nt	F: 5′-GCCTCTGGAGGGACATCAAG-3′	262 bp
		R: 5′-CCTGCCCGCCCCTCTTATAG-3′	
ADIPOQ	- 283 nt	F: 5′-GAAGTTTCTCCGTCAGATGC-3′	181 bp
		R: 5′-CCTAGGGAACCTGGTACAAC-3′	
	- 74 nt	F: 5′-TGCCCCATCTTCTGTTGCTG-3′	186 bp
		R: 5′-AACTCGATGAGGGCCAGAGG-3′	

Figure 1. Selection of CpG sites and primer design for methylation specific PCR (MS-PCR). Human *LEP* (**a**) and *ADIPOQ* (**b**) proximal promoter regions. Binding sites for transcription factors are underlined. CpG sites are in bold. Arrowheads point to the CpG sites analyzed in this study. (**c**) Primer sequences used for methylation-specific PCR (MS-PCR).

Statistical analyses

Statistical analyses were performed using the computer program SPSS 17.0 (IBM Company, Mexico City, Mexico). Data are expressed as means ± s.e.m. Pairwise comparisons of metabolic parameters between girls and boys were assessed by unpaired Student´s *t*-test and the Mann–Whitney *U*-test for, respectively, variables normally distributed or not normally distributed. Statistical differences in metabolic characteristics among patients of the same sex were determined by one-way analysis of variance followed by Dunnett's multiple comparison test. Differences in the proportion of methylation frequency were determined by Fisher's exact test. The Spearman analysis was used to evaluate the correlation between methylation frequency and metabolic variables. Statistical significance was set at $P \leqslant 0.05$.

RESULTS

Morphological and metabolic phenotype of the children

Among the 106 adolescents, recruited from the Hospital Infantíl de México Federico Gómez, 16 exhibited normal weight, 29 were overweight and 61 were obese, including 22 suffering from morbid obesity (Table 1). In addition to high BMI, all overweight and obese adolescents exhibited higher serum levels of triglycerides and insulin, as well as higher HOMA-IR values in comparison with lean subjects. In contrast, obese and normoweight children exhibited the same levels of glucose in serum. Moreover, with the exception of lower body height in overweight girls as compared with overweight boys, there were no differences between girls and boys in any of the parameters (Table 1).

Each individual within the obese and overweight group was then classified as insulin resistant if his HOMA-IR was equal or higher than 3.4. Given that no significant differences between the matched subgroups of girls and boys were observed neither in body weight nor in the levels of serum analytes (Supplementary Table 1), values from both sexes were averaged. Using the HOMA-IR as criterion, it turned out that 24% of overweight adolescents exhibited insulin resistance, whereas 51% of obese and 68% of morbidly obese patients were affected by this pathological condition. Except for their disparities in HOMA-IR and their circulating levels of insulin, no differences in body weight, BMI and serum levels of glucose, total cholesterol and triglycerides were observed between subjects with or without insulin resistance (Table 2). Lean and overweight adolescents exhibited nearly the same concentration of leptin in serum (lean: 9.6 ± 1.9 ng ml^{-1}; overweight: 13.7 ± 1.8 ng ml^{-1}), which are within the normal range (1–15 ng ml^{-1}). In contrast, both obese and morbidly obese children presented significant increased levels of leptin as compared with lean patients (obese: 25.9 ± 3.1 ng ml^{-1}, $P < 0.01$; morbidly obese: 23.1 ± 3.4 ng ml^{-1}, $P < 0.05$). As expected, the levels of adiponectin in obese and morbidly obese subjects were

Table 1. Metabolic phenotype of the studied patients

	Girls				*Boys*			
	Lean	*Overweight*	*Obese*	*Morbidly obese*	*Lean*	*Overweight*	*Obese*	*Morbidly obese*
Number of patients	4	12	16	8	12	17	23	14
Height (cm)	150.5 ± 2.72	156.4 ± 2.7[a]	157.2 ± 1.4	157.5 ± 2.8	157.6 ± 2.7	163.9 ± 2.4	161.7 ± 2.6	160.9 ± 2.7
Weight (kg)	40.25 ± 2.0	63.7 ± 3.0**	71.8 ± 2.0***	93.0 ± 7.2***	48.2 ± 2	66.5 ± 2.5*	74.4 ± 3.2 ***	95.9 ± 7.2***
BMI (kg m^{-2})	17.8 ± 0.4	25.9 ± 0.7***	29.1 ± 0.8***	37.1 ± 1.7***	19.32 ± 0.5	24.6 ± 0.4***	28.1 ± 0.5***	36.5 ± 2***
BMI (percentile)	45.50 ± 10.0	90.9 ± 1.3***	97.25 ± 0.3***	99.1 ± 0.1***	51.92 ± 6.4	90.7 ± 1***	97.1 ± 0.2***	99.36 ± 0.1***
Glucose (mg dl^{-1})	73.8 ± 7.4	87.3 ± 2.8*	87.4 ± 1.7*	85.5 ± 2.3	79.8 ± 4.8	88.6 ± 1.4*	86.2 ± 1.5	87.4 ± 1.9
Insulin (mU l^{-1})	4.75 ± 0.9	10.4 ± 1.2	17.9 ± 1.4***	19.4 ± 2.6***	3.9 ± 0.7	11 ± 1.5	14.7 ± 1.3**	29.3 ± 4.5***
HOMA-IR	0.81 ± 1	2.27 ± 0.3	3.89 ± 0.4***	4.1 ± 0.6***	1.0 ± 0.1	2.4 ± 0.3	3.2 ± 0.3*	6.4 ± 1***
Leptin (ng ml^{-1})	9.4 ± 6.22	15.8 ± 2.9	20.16 ± 4.1	20.4 ± 4.4	9.7 ± 1.8	12.1 ± 2.4	30.2 ± 4.3**	24.7 ± 4.9
Adiponectin (μg ml^{-1})	13.2 ± 1.1	17.4 ± 3.1	4.5 ± 1	6.12 ± 1.8***	16.0 ± 1.1	16.1 ± 2.4	4.8 ± 0.7***	6.6 ± 1.2***
Cholesterol (mg dl^{-1})	144.0 ± 24.6	125.6 ± 4.8	148.0 ± 6.1	146.3 ± 11	118.5 ± 9.6	133.9 ± 4.9	139.4 ± 5.7	146.3 ± 3.4*
Triglycerides (mg dl^{-1})	71.5 ± 7.6	119.0 ± 19.2	127.3 ± 12.7	150.8 ± 22.8	69.8 ± 7.6	123.9 ± 10.3 *	120.7 ± 10.3*	142.2 ± 23.9**

Abbreviation: HOMA-IR, homeostasis model of assessment-insulin resistance. Data are expressed in mean ± s.e.m. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs lean patients of the same sex (one-way analysis of variance). [a]$P < 0.05$ vs overweight boys (Student's *t*-test).

Table 2. Main metabolic characteristics of the subgroups with or without insulin resistance

	Overweight		*Obese*		*Morbidly obese*	
	Without IR	*With IR*	*Without IR*	*With IR*	*Without IR*	*With IR*
N (G/B)	22 (10/12)	7 (2/5)	19 (6/13)	20 (10/10)	7 (3/4)	15 (5/10)
Age (year)	14.8 ± 0.4	13.8 ± 0.5	14.0 ± 0.3	13.9 ± 0.4	13.1 ± 0.6	14.3 ± 0.5
Height (cm)	161.5 ± 2.3	158.5 ± 3.2	159.7 ± 2.7	159.9 ± 2.0	156.7 ± 4.1	161.0 ± 2.2
Weight (kg)	66.4 ± 2.3	61.8 ± 3.3	72.7 ± 3.1	73.9 ± 2.7	84.4 ± 7.6	99.7 ± 6.4
BMI (kg m^{-2})	25.4 ± 0.5	24.5 ± 0.4	28.3 ± 0.6	28.7 ± 0.6	34.0 ± 2.1	37.9 ± 1.7
BMI (percentile)	90.7 ± 0.9	91.1 ± 1.6	97.0 ± 0.2	97.3 ± 0.2	99.1 ± 0.1	99.3 ± 0.1
Glucose (mg dl^{-1})	87.5 ± 1.7	89.9 ± 2.5	83.7 ± 1.6	89.5 ± 1.2	83.1 ± 2.5	83.4 ± 1.7
Insulin (mU l^{-1})	8.4 ± 0.6	18.2 ± 1.1[a]	11.3 ± 0.8	20.4 ± 1.0[a]	11.2 ± 1.0	32.5 ± 3.3[a]
HOMA-IR	1.8 ± 0.1	4.0 ± 0.2[a]	2.3 ± 0.1	4.51 ± 0.2[a]	2.3 ± 0.2	7.1 ± 0.7[a]
Cholesterol (mg dl^{-1})	127.4 ± 3.4	140 0 ± 9.6	141.5 ± 5.4	144.2 ± 6.4	137.6 ± 8.4	150.3 ± 5.1
Leptin (ng ml^{-1})	13.0 ± 2.1	16.0 ± 3.7	26.2 ± 4.2	25.6 ± 4.5	27.4 ± 8.2	20.9 ± 3.2
Adiponectin (μg ml^{-1})	17.0 ± 2.1	14.7 ± 4.4	4.4 ± 0.7	4.9 ± 0.9	5.2 ± 2.0	7.0 ± 1.0
Triglycerides (mg dl^{-1})	117.4 ± 10.7	136.0 ± 23.0	115.2 ± 11.2	131.1 ± 11.1	111.9 ± 12.7	160.9 ± 23.4

Abbreviations: B, boys; BMI, body mass index; G, girls; HOMA-IR, homeostasis model of assessment-insulin resistance. Data are expressed in mean ± s.e.m. [a]$P < 0.01$ vs matched group without IR (Student's *t*-test).

significantly decreased in comparison with lean and overweight subjects (lean: 15.3 ± 0.9 μg ml^{-1}; overweight: 16.6 ± 1.9 μg ml^{-1}; obese: 4.7 ± 0.6 μg ml^{-1}, $P < 0.01$; morbidly obese: 6.4 ± 1.0 μg ml^{-1}, $P < 0.01$). However, no significant differences in serum leptin and adiponectin were observed between subjects with or without insulin resistance in overweight, obese or morbidly obese patients (Table 2).

Changes in *LEP* gene promoter methylation associated with obesity and insulin resistance

To determine the methylation status of the *LEP* and *ADIPOQ* promoters, genomic DNA was obtained from peripheral blood and submitted to sodium bisulfite conversion and MS-PCR. Primer design was first validated using human methylated DNA as control. These preliminary experiments showed that PCR gave a single product of the expected molecular size for both genes. Specifically, PCR using primers targeted to the CpG sites at − 51 and − 31 nt of the *LEP* promoter yield single bands of, respectively, 275 (*T*m = 51 °C) and 262 bp (*T*m = 56 °C), whereas PCR with primers targeted to the CpG sites at − 283 and − 74 nt of the *ADIPOQ* promoter yield products of, respectively, 181 pb (*T*m = 58 °C) and 186 bp (*T*m = 56 °C). In contrast, no product was amplified when human non-methylated DNA was used as negative control (data not shown). Then, all sodium bisulfite-converted DNA samples were analyzed using the four sets of primers. The amplification of a DNA fragment corresponding to the expected molecular size indicates that the corresponding CpG site is methylated, whereas the lack of amplification indicates that the CpG site is unmethylated (data not shown).

As for body weight and serum analytes, there were no significant differences between the different subgroups of girls and boys in the proportion of patients exhibiting DNA methylation of the *LEP* and *ADIPOQ* promoters (Supplementary Table 2). Consequently, data from both sexes were mixed for analysis. The CpG site at − 51 nt of the *LEP* promoter was methylated in all the normoweight adolescents (16 over 16 children). In contrast, it was methylated in only 79% of overweight (23 over 29 children) and 56% of obese (22 over 39 children, $P < 0.001$ vs lean children) adolescents. This percentage decreased to 36% (8 over 22 children, $P < 0.001$ vs lean children) in subjects with morbid obesity. A similar methylation frequency was found in the case of the CpG site at − 31 nt in *LEP* promoter. Thus, although this site was methylated in 100% of control subjects, the proportion of overweight adolescents in whom the *LEP* promoter was methylated was reduced to 86% and to 69% ($P < 0.05$ vs lean children), and 59% ($P < 0.01$ vs lean children) in obese and morbidly obese patients, respectively.

Interestingly, when the methylation data were analyzed according to the HOMA-IR values of the children, we noticed that the proportion of overweight subjects in whom the *LEP* promoter was methylated was the same independently of the presence of insulin resistance and of the site position of the CpG site. However, the methylation status of the *LEP* promoter was clearly dependent on the presence of insulin resistance in obese and morbidly obese patients. Actually, the CpG site at position − 51 nt was methylated in 90% of obese patients without insulin resistance, whereas there were only 25% of insulin-resistant obese patients in whom the same CpG site was methylated. The influence of insulin resistance on the methylation status of the leptin promoter was even greater in morbidly obese subjects, as only 7% of these patients exhibited methylation of the CpG site located at position − 51 nt (Figure 2a). An identical combined effect of obesity and insulin resistance was found in the case of the CpG site at − 31 nt. Thus, the methylation frequency of this site was 86% in both insulin-resistant and non-insulin-resistant overweight children, 50 and 90% in obese children with and without insulin resistance, and 40 and 100% in morbidly obese adolescents with and without insulin resistance (Figure 2b).

Changes in *ADIPOQ* gene promoter methylation associated with obesity and insulin resistance

There were no statistically significant differences in the percentage of children exhibiting methylation of the CpG site at − 283 nt of the *ADIPOQ* promoter between lean and obese and morbidly obese adolescents. Actually, the methylation frequency at this site was 63% (10 over 16 children) in normoweight adolescents and 51% (20 over 39 children) and 46% (10 over 22 children) in obese and morbidly obese adolescents. In contrast, none of the overweight adolescents showed methylation at this promoter site. As for the *LEP* promoter, the methylation status of the CpG site at − 283 nt of the *ADIPOQ* promoter was tightly associated with the presence of insulin resistance such that the proportion of patients in whom the CpG site at this position was methylated decreased from 74% in obese children without insulin resistance to 30% in insulin-resistant obese children and from 86% in morbidly obese adolescents without insulin resistance to 27% in morbidly obese patients with insulin resistance (Figure 2c).

With regard to the CpG site at − 74 nt, its methylation frequency was significantly decreased in obese (31%, 12 over 39 children, $P < 0.001$ vs lean patients) and morbidly obese adolescents (50%, 11 over 22 children, $P < 0.01$ vs lean patients) in relation to lean (94%, 15 over 16 children) or overweight (76%, 22 over 29 children) adolescents. Moreover, the proportion of insulin-resistant patients in whom the CpG site at − 74 nt was methylated was of 57% in overweight children, of 40% in obese children and of 47% in children suffering from morbid obesity. Therefore, the methylation status of the CpG site at − 74 nt of the *ADIPOQ* promoter is directly related to obesity as indicated by the decreased proportion of obese and morbidly obese patients in whom this CpG site was methylated and the lack of difference between insulin- and non-insulin-resistant groups in the proportion of patients exhibiting methylation at this CpG site (Figure 2d).

Correlation between methylation frequency and metabolite profile

To determine to what extent there was a correlation between the methylation status of the leptin and adiponectin promoters and the metabolic phenotype of the adolescents, we further analyzed the data by the Pearson analysis. The results of this study indicated that the methylation frequency of the *LEP* promoter at both CpG sites at − 51 and − 31 nt is negatively associated with BMI, HOMA-IR, and total cholesterol and insulin in serum (Figure 3). Moreover, the methylation frequencies of these two CpG sites were also found to be strongly correlated with each other ($r = 0.998$; $P < 0.01$). However, no significant correlation was observed between the circulating levels of leptin and the methylation frequencies of the two selected CpG sites of the *LEP* promoter. On the other hand, with the exception of a negative correlation between the circulating levels of leptin and the methylation of the CpG site located at position − 74 nt, no significant association was observed between the methylation frequency of the *ADIPOQ* promoter and the anthropometric

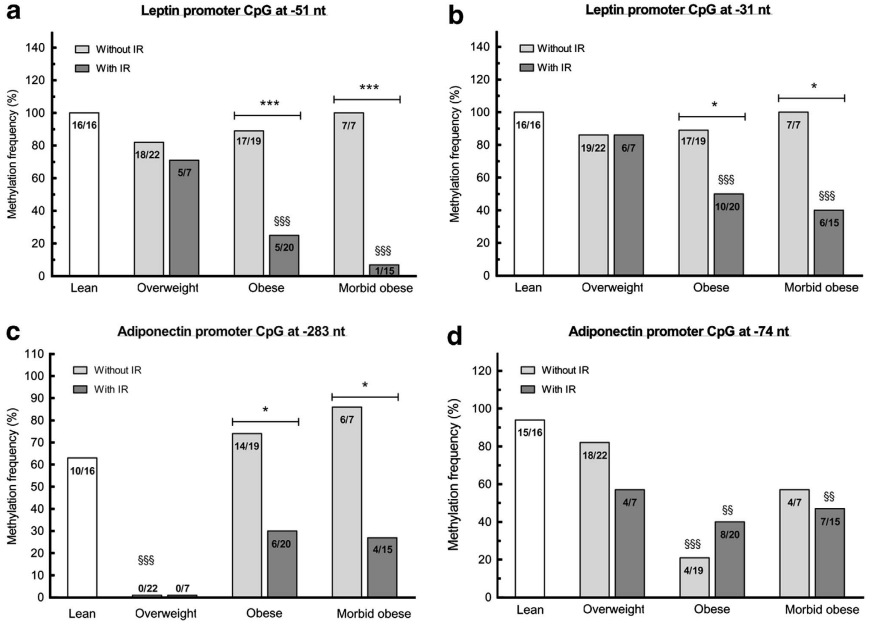

Figure 2. Effects of obesity alone and of obesity plus insulin resistance in the methylation frequency of the *LEP* (**a, b**) and *ADIPOQ* (**c, d**) promoters in children. Values inside the bars indicate the number of adolescents over the total number of children whose genomic DNA gave a positive MS-PCR reaction. $^{*}P < 0.05$, $^{***}P < 0.001$ and $^{§§}P < 0.01$, $^{§§§}P < 0.001$ vs lean subjects (Fisher's exact test).

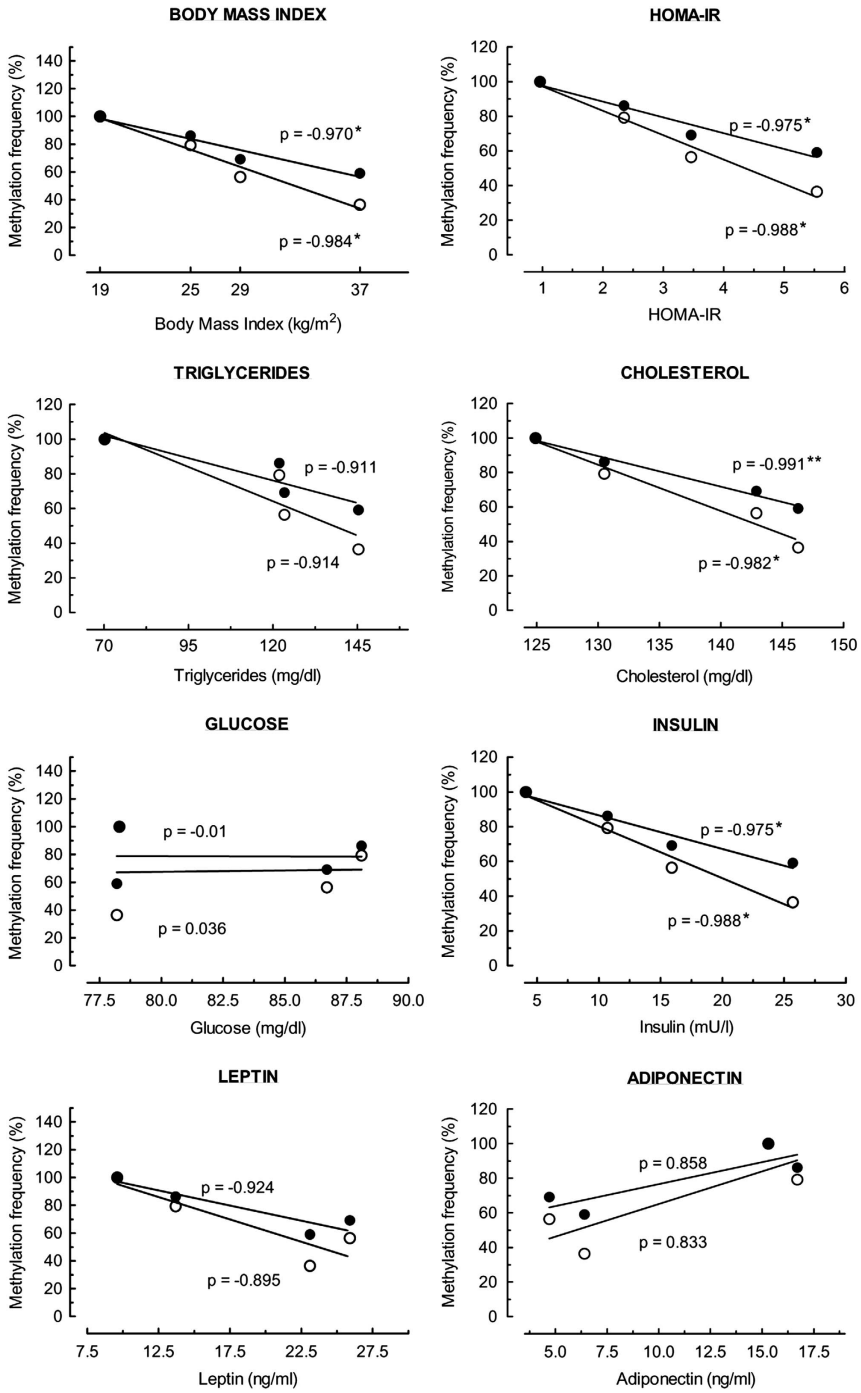

Figure 3. Correlation between the methylation frequency of the *LEP* promoter and the metabolic phenotype of lean and obese adolescents. The curves in the graphs illustrate the correlation between the methylation frequency of the CpG sites located at −51 nt (open circles) and −31 nt (closed circles) relative to the transcription start site of the *LEP* gene and the indicated metabolic parameters. *$P < 0.05$; **$P < 0.01$. *P*, Pearson's correlation coefficient.

characteristics or the concentrations of the different metabolites in serum (Figure 4).

When adolescents were classified according to their HOMA-IR index independently of their BMI, the methylation frequency of both selected CpG sites in the *LEP* promoter was significantly associated with serum glucose ($r = -0.968$, $P < 0.05$ for CpG site at −51 nt and $r = -0.997$, $P < 0.01$ for CpG site at −31 nt) in all subjects without insulin resistance. However, in the case of individuals with insulin resistance, the significant association was observed with total cholesterol ($r = -0.997$, $P < 0.05$ for CpG site at −51 nt and $r = -1.000$, $P < 0.01$ for CpG site at −31 nt, respectively). Moreover, the methylation frequency of CpG site at −283 nt in *ADIPOQ* promoter was significantly associated with serum adiponectin levels ($r = -1.000$, $P < 0.05$) (data not shown).

DISCUSSION

Over the last years there has been an increasing interest in studying the role of epigenetic modifications related to obesity and associated metabolic diseases. This interest stems from the

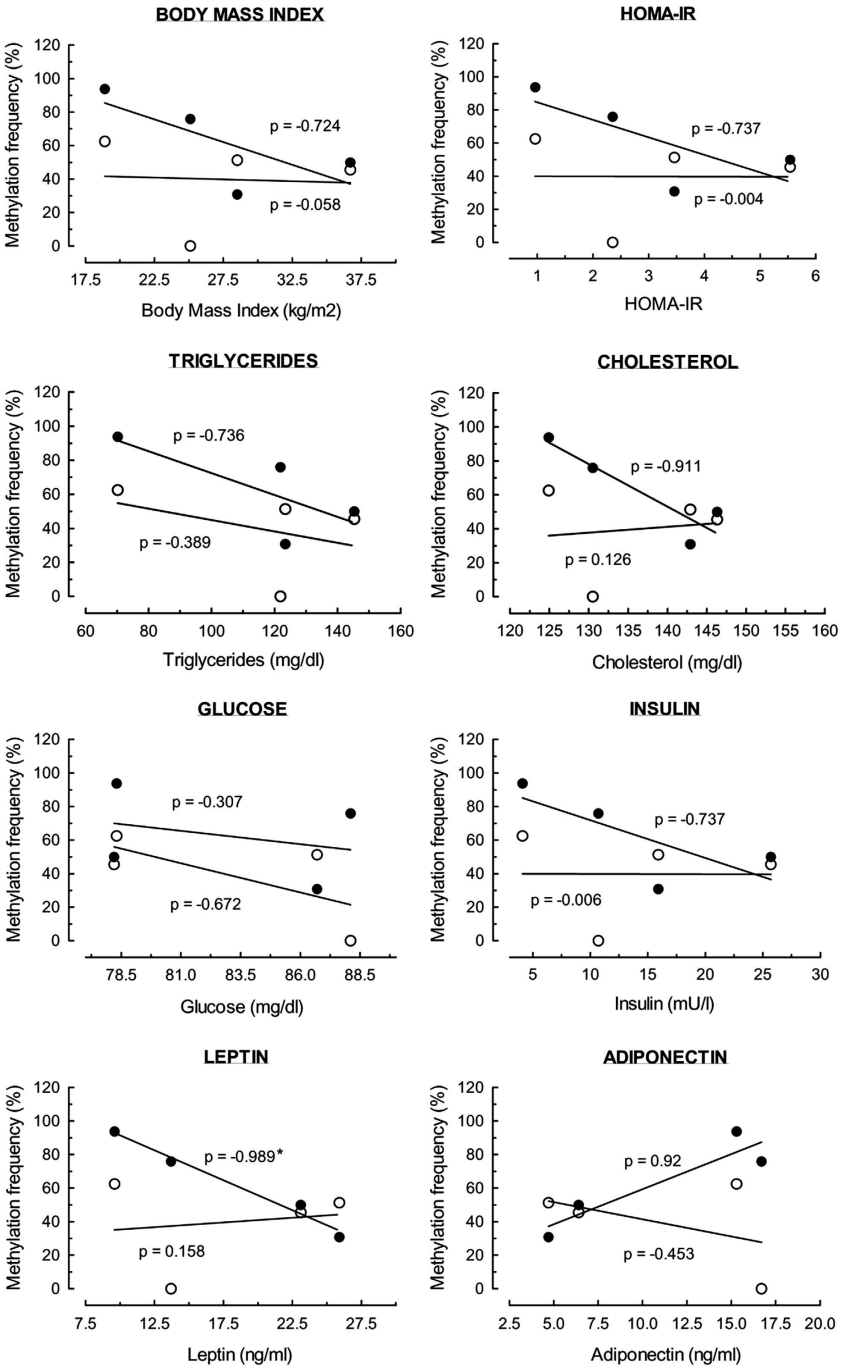

Figure 4. Correlation between the methylation frequency of the *ADIPOQ* promoter and the metabolic phenotype of lean and obese adolescents. The curves in the graphs illustrate the correlation between the methylation frequency of the CpG sites located at − 283 nt (open circles) and − 74 nt (closed circles) relative to the transcription start site of the *ADIPOQ* gene and the indicated metabolic parameters. *$P < 0.05$. *P*, Pearson's correlation coefficient.

recognition that (1) the current obesity epidemic results from the complex interplay between genetic and environmental factors and (2) the epigenetic regulation of gene expression has a key role in the control of genome function by environmental stimuli. Accordingly, using genome-wide methods, a number of epigenetic modifications associated with obesity have been identified.[13–18] However, the translation of these results into clinical practice is limited because of the high cost and the complexity of these molecular biology techniques. Thus, with the aim of developing a simple and reliable epigenetic method for the diagnosis of obesity and insulin resistance affordable to a standard clinical laboratory, here we used MS-PCR to evaluate the methylation status of the *LEP* and *ADIPOQ* genes in blood samples from a cohort of obese adolescents.

Our results show that the methylation status of the *LEP* promoter in genomic DNA from peripheral blood is associated with the morphological and serum-metabolite patterns of obesity and insulin resistance. Specifically, there is a negative relationship between the combined presence of obesity and insulin resistance and the methylation at specific CpG sites of the *LEP* and *ADIPOQ* promoters. There is also a negative correlation between the methylation levels of the *LEP* promoter and the circulating levels

of triglycerides and cholesterol. Given that obesity associated with dyslipidemia and insulin resistance is a defining criteria for metabolic syndrome, our results indicate that DNA methylation from peripheral leukocytes can be used as an epigenetic marker of this pathological entity and support the hypothesis that epigenetic modifications constitute a potential mechanism underlying the development of obesity and related metabolic disorders.

Previous studies on the proximal promoter of the human *LEP* gene have shown that it contains a set of 32 CpG sites. Among them, those located next to the TATA box (−31 nt), and within the C/EBPα binding site (−51 nt), exhibit high frequency of methylation under basal conditions and have a crucial role in the regulation of leptin expression.[26,27] We therefore decided to focus our study on these two CpG sites. Our results show that there is a negative relationship between the methylation of these CpG sites and BMI and insulin resistance. However, the evaluation of data by the Pearson analysis indicated that the methylation of the −31 and −51 CpG sites was correlated to insulin but not to serum glucose levels, suggesting that the methylation status of the *LEP* promoter is rather associated with insulin resistance than to the physiological dysfunctions induced by hyperglycemia. Moreover, the fact that methylation of the *LEP* promoter is negatively associated with BMI in obese adolescents confirms the relevance of the CpG sites analyzed in our study. Actually, although all subjects recruited in this study declared themselves as healthy adolescents, several overweight and obese children already presented insulin resistance and increased serum levels of cholesterol, which are risk factors for the development of diabetes mellitus in adulthood.

It has been shown that CpG sites at −51 nt and −31 nt in the *LEP* promoter are methylated in preadipocytes and unmethylated in mature adipocytes, suggesting that demethylation is required to activate the transcription of the *LEP* gene in mature cells.[26,31] However, we did not find a statistically significant relationship between the methylation frequency of the *LEP* promoter and the circulating levels of leptin in obese subjects. In contrast, in overweight and obese adolescents with insulin resistance, the methylation frequency of the CpG site at −283 nt from the transcription start site of the *ADIPOQ* promoter was significantly associated with serum adiponectin levels but not with insulin resistance. This latter observation is at odds with the results of Bouchard *et al.*[32] who recently reported that reduced methylation of the *ADIPOQ* promoter in the placenta of diabetic mothers is associated with increased insulin resistance and higher levels of adiponectin in blood from both the maternal circulation and the umbilical cord. The discrepancy between our observations and those of Bouchard *et al.* can be explained by the differences in the kind of studied subjects (obese adolescents vs pregnant women), the type of analyzed samples (peripheral blood vs placenta) and the experimental approach used to determine the methylation levels of the *ADIPOQ* promoter (analysis of the methylation at a single CpG nucleotide vs the averaged methylation levels of a stretch of 17 CpG sites).

DNA methylation profiles are cell specific. In the present study, we used DNA from whole blood, which consists of different cell populations that might exhibit distinct epigenetic profiles. The question therefore remains as to what extent the variations in the methylation status of *LEP* and *ADIPOQ* promoters in peripheral blood leukocytes from obese and insulin-resistant children are a reliable marker of dysfunction for less-accessible tissues that are directly involved in the development of these pathological entities, such as adipose tissue. Although further studies are needed to answer this question, it is worth mentioning the striking correspondence between human adipose tissue and peripheral leukocytes in the high methylation frequency of the −51 CpG site of the human leptin promoter.[27] Moreover, epigenetic changes in peripheral blood have already been validated as reliable molecular markers of several forms of cancer in less-accessible tissues including brain,[33] colorectal mucosa[34,35] and prostate.[36] Finally, the epigenetic modifications of the leptin promoter in blood can by themselves contribute to the pathogenesis of the cardiovascular diseases associated with obesity and diabetes. Indeed, leptin favours the development of atherosclerosis by inducing the production of reactive oxygen species as well as the expression of monocyte chemoattractant protein-1 and of several mediators of cellular inflammation in endothelial cells.[37,38] It has also been shown that leptin increases arterial pressure by stimulating sympathetic nerve activity in the kidney.[39]

CONCLUSION

The results of our study show that the methylation of the *LEP* and ADIPOQ promoters in peripheral blood is associated with BMI, dyslipidemia and insulin resistance in obese children. Specifically, our analysis revealed an important reduction in the methylation frequency of the CpG sites at −51 and −31 nt in the *LEP* promoter and of the CpG site at −283 nt in the *ADIPOQ* promoter in obese and morbidly obese with insulin resistance. We also observed that obesity alone is associated with reduced methylation frequency of the CpG site at −74 nt of the *ADIPOQ* gene. These results support the hypothesis that epigenetic modifications might underpin the development of obesity and related complications. They also validate the use of MS-PCR in blood leukocytes as a reliable and affordable tool for the identification of epigenetic modifications that could be used as molecular markers to predict and follow up the physiological changes associated with obesity and insulin resistance in response to different environmental stimuli including interventions on diet and physical activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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