Maternal Pregestational BMI Is Associated With Methylation of the *PPARGC1A* Promoter in Newborns

Carolina Gemma[1], Silvia Sookoian[1], Jorge Alvariñas[2], Silvia I. García[1], Laura Quintana[2], Diego Kanevsky[1], Claudio D. González[3] and Carlos J. Pirola[1]

We explored peroxisome proliferator-activated receptor-γ co-activator 1α gene (PPARGC1A), peroxisome proliferator-activated receptor-γ gene (PPARG), and transcription factor A mitochondrial gene (*Tfam*) promoter DNA methylation in newborns between both extremes of abnormal fetal growth: Small (SGA) and large for gestational age (LGA) in relation to the mother's characteristics. We further sought for the association of rs9930506 variant at *FTO* gene and the promoter patterns of DNA methylation in the aforementioned genes, in relation to the offspring's birth weight. In a cross-sectional study, 88 healthy pregnant women and their babies were included. According to the offspring birth weight, there were 57 newborns with appropriate weight for gestational age (AGA), 17 SGA, and 14 LGA. After bisulphite treatment of umbilical cord genomic DNA, a real-time methylation-specific PCR was used to determine the promoter methylation status in selected CpGs. Promoter methylated DNA/unmethylated DNA ratio, expressed as mean ± s.e., was 0.82 ± 0.15 (45% of alleles) for *PPARGC1A*, and 0.0044 ± 0.0006 (0.4% of alleles) for *Tfam*. *PPARG* promoter was almost 100% methylated in all samples. In univariate analysis, there was no association among characteristics of the newborn and gene promoter methylation. None of the maternal features were related with the status of promoter methylation, except for a positive correlation between maternal BMI and *PPARGC1A* promoter methylation in umbilical cord (Pearson correlation coefficient $r = 0.41$, $P = 0.0007$). Finally, *FTO* rs9930506 AA homozygous in the LGA group showed decreased levels of methylated *PPARGC1A* in comparison with AG + GG genotypes and AGA and SGA infants. In conclusion, our findings suggest a potential role of promoter *PPARGC1A* methylation in metabolic programming.

Obesity (2009) doi:10.1038/oby.2008.605

INTRODUCTION

Data from animal models (1) and human epidemiologic studies strongly suggest that low birth weight (small for gestational age, SGA) is related to an increased risk of developing adult diseases, including type 2 diabetes, hypertension, and cardiovascular disease (2–4).

Likewise, infants who are born large for gestational age (LGA) after being exposed to an intrauterine environment of either diabetes or maternal obesity are also at an increased risk of developing metabolic syndrome (5).

This association between an adverse intrauterine environment and adult metabolic and cardiovascular disorders led to the concept of fetal programming and the "fetal origins" hypothesis that proposes early developmental adaptations to ensure fetal survival (6). Such adaptations, a process also known as "metabolic programming" (7), change "permanently" the metabolism of the newborns and continue to be expressed even without the original stimulus. Thus, an important consequence of the metabolic programming is the transmission of the phenotype from mother to the progeny (7) and even through generations (8).

To date, several pathways have been proposed to explain the development of metabolic alterations following abnormal fetal growth, but no precise mechanisms have been demonstrated; the hypothesis suggesting that this association could be the consequence of genetic–environmental interactions is currently being explored. For instance, we recently reported that mitochondrial DNA content was decreased in newborns with

[1]Molecular Genetics and Biology of Complex Diseases Department, Institute of Medical Research, A. Lanari, University of Buenos Aires-CONICET, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina; [2]Nutrition Unit, Policlinico Bancario, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina; [3]Department of Pharmacology, School of Medicine, University of Buenos Aires, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina.
Correspondence: Carlos J. Pirola (pirola.carlos@lanari.fmed.uba.ar)

Received 20 May 2008; accepted 10 October 2008; advance online publication 15 January 2009. doi:10.1038/oby.2008.605

abnormal weight (either SGA or LGA) in comparison with babies with appropriate weight for gestational age (AGA) (9). We also observed that maternal pregestational BMI was associated with the weight of their offspring.

The idea that epigenetic alterations such as DNA methylation and histone acetylation, which mediate phenomena such as genomic imprinting, may contribute to metabolic programming is gaining acceptance (8–11).

Nevertheless, little is known about the role of differential DNA methylation in master genes that control either mitochondrial number—such as mitochondrial transcription factor A (*Tfam*)—or mitochondrial function, adaptive thermogenesis, and glucose and fat oxidation in muscle and fat tissue, and gluconeogenesis in liver—such as proliferator-activated receptor-γ co-activator 1α gene (*PPARGC1A*)—or adipogenesis and insulin signaling, such as proliferator-activated receptor-γ (*PPARG*).

Based on two lines of reasoning: the high heritability of the birth weight (mothers' birth weight is a good predictor of their own offspring's birth weight (12)) and the well-established permanent effect of the birth weight on the newborn's metabolism leading to adult chronic morbidity and mortality, we hypothesized that promoter DNA methylation of *PPARGC1A*, *PPARG*, and *Tfam* genes may be associated with newborns' anthropometric and laboratory variables, and with their mothers' pre-pregnant BMI. Hence, we evaluated a sample of newborns, including both extremes of abnormal fetal growth (SGA and LGA), in relation to the mother's characteristics. As a secondary aim, we sought for the association between the rs9930506 variant of the *FTO* (fat mass and obesity associated gene) and DNA methylation of the promoter regions in the aforementioned genes. The main reason for focusing on the *FTO* is because genome-wide association scan studies showed that within the *FTO* gene a cluster of 10 single-nucleotide polymorphism in the first intron showed a strong association with BMI (13,14), a finding that was replicated in several populations.

Furthermore, the recombinant FTO protein catalyzes demethylation of 3-methylthymine in single-stranded DNA (15), and the *FTO* is widely expressed in both fetal and adult tissue (13).

METHODS AND PROCEDURES

Eighty-eight consecutive healthy pregnant women and their babies were included in this study. According to the newborn birth weight normalized by sex and gestational age, there were 57 newborns with AGA and 31 with abnormal weight for gestational age: 17 SGA and 14 LGA. There were no multiple pregnancies in the study population. None of the mothers had preeclampsia/eclampsia, hypertension in pregnancy, or gestational diabetes. Besides, none of them was taking any medication.

At the time of delivery, a sample of umbilical cord was stored for further analysis.

All the investigations performed in this study were conducted in accordance with the Guidelines of the Declaration of Helsinki. Written consent from the patients had been obtained, in accordance with the procedures approved by the Ethical Committee of our institution.

Complete medical, obstetrical, and perinatal data were recorded as shown below.

Obstetric variables

Gestational age was defined based on the last menstrual period, physical examination, and ultrasonographic biometry. Length of gestation at delivery was measured as the number of weeks of gestation. The ultrasound measure during the first trimester took precedence when these measures did not agree in terms of dating gestational age at birth.

Maternity data, including age, geographical origin and ancestry, pregestational BMI, education, smoking (number of cigarettes/day), alcohol consumption (ml/day), parity, history of abortion, history of preterm birth and previous abnormal fetal weight, preeclampsia/eclampsia, hypertension in pregnancy, hyperlipidemia or diabetes, pregnancy weight gain, weight of the previous offspring and gender of the newborn, were evaluated. Besides, family history of disease was also recorded.

Resting systolic and diastolic arterial blood pressure was measured in all the mothers after they had been sitting for at least 30 min. A mercury sphygmomanometer was used to measure blood pressure three times at the right arm.

Birth outcomes

Infants were classified as SGA, LGA, or AGA according to the following criteria: SGA was defined as birth weight for gestational age less than the specific 10th percentile cutoff of a published Argentinean fetal growth reference, (16) and oversized infants (LGA) were defined as those whose birth weight was above the 90th percentile for gestational age and sex (17,18). The rest of the newborns were considered AGA.

All babies were weighed at birth at the maternity ward. To ensure reliability and avoid interobserver bias, the same person weighed all the newborns and carried out all the anthropometric measurements. All the newborns were weighed naked on a digital baby balance, regularly calibrated with a measurement accuracy of 10 g. The balance was checked daily by known standard weight before weighing.

Evaluation of the newborn also included physical examination, search for any features suggestive of a malformation syndrome, supine lengths, Apgar score evaluation, and type of delivery.

Biochemical measurements

Blood was drawn from pregnant women who were in a supine resting position for at least 30 min and from the umbilical cord at delivery. Plasma glucose was measured using standard clinical laboratory technique by the time of the delivery. In addition, we analyzed leptin in cord and mother's blood. A commercial enzyme-linked immunosorbent assay kit was used to measure plasma leptin concentrations (Assay Designs, Ann Arbor, MI) in blood samples collected with sodium EDTA.

DNA extraction

Nucleic acids were extracted from a 2 cm piece of umbilical cord and white blood cells from a blood sample of the mothers by a standard method as described previously (19).

Bisulphite treatment and methylation-specific PCR

Genomic DNA was treated with sodium bisulphite using the EZ DNA Methylation Kit according to the manufacturer's protocol (Zymo Research Corporation, Orange, CA). Briefly, this technique is based on bisulphite treatment of genomic DNA from the samples, thereby converting all unmethylated cytosines to uracil, whereas methylated cytosines are conserved.

The chemically modified DNA was then used as a template for a methylation-specific PCR to determine the promoter methylation status of selected CpG dinucleotides in three target genes (*PPARGC1A*, *PPARG*, and *Tfam*) in genomic DNA from the newborn umbilical cord. An assay based on real-time quantitative PCR on an iCycler thermocycler (BioRad Hercules, CA) was used for DNA methylation quantification using SYBR Green (Invitrogen, Buenos Aires, Argentina) as a fluorescent dye.

For methylation-specific PCR experiments, two pairs of primers were used for each gene, one of them was specific for modified and methylated DNA (M primers), and the other pair for modified and unmethylated

Table 1 PCR primers used for methylation-specific PCR (MS-PCR)

Gene	Forward primer 5′→3′	Reverse primer 5′→3′	Size, bp
PPARGC1A-M	ATTTTTTATTGTTATGGGGGTAGTC	AAAAATATTTAAAAACGCAAACGAA	143
PPARGC1A-U	TTTTATTGTTATGGGGGTAGTTGA	AAAAAATATTTAAAAACACAAACAAA	141
TFAM-M	TTAATGGGTTTTATATAGATATACGG	AAAAATAATAACGAAAAAAACGAA	102
TFAM-U	TAATGGGTTTTATATAGATATATGG	CAAAAATAATAACAAAAAAAACAAA	102
PPAR-γ-M	TTGGATAGGTTACGATGGATAGC	AAACGAAATAAAAACGTAAAACACG	101
PPAR-γ-U	TTGGATAGGTTATGATGGATAGTGT	AACAAAATAAAAACATAAAACACAAA	100

M, methylated-specific primers; U, unmethylated-specific primers.

DNA (U primers). Thus, for each sample studied, two PCRs were performed simultaneously using the M primer pair and U primer pair. Successful amplification from M pair and U pair indicated methylation and unmethylation, respectively. A sequence starting 2,000 bp upstream from transcriptional start site (TSS) of *PPARGC1A*, *PPARG*, and *Tfam* was used in MethPrimer program (http://www.urogene.org//methprimer/index1.html) (20) to search for regions with potentially methylated CpG sites and PCR designs. Sequences were retrieved from the Database of Transcriptional Start Sites at http://dbtss.hgc.jp/, with the following ID numbers: NM_013261, chromosome: NCBI36: 4: 23402742..23500798, TSS: 23500789 for *PPARGC1A*, NM_00138712, chromosome: NCBI36: 3: 12304349..12450855, TSS: 12304349 for *PPARG*, and NM_003201, chromosome: NCBI36: 10: 59815182..59825903, TSS: 59815182 for *Tfam*.

For maximal discrimination between methylated and unmethylated alleles, M and U primers were designed to contain at least one CpG site at the most 3′ (20). Primer sequences and resulting PCR products are listed in Table 1. General PCR conditions are available from the authors upon request.

Levels of methylated DNA for each gene are expressed as the ratio of the estimated amount for methylated DNA relative to unmethylated DNA levels calculated for each sample according to as reported previously (21) using the fluorescence threshold cycle (Ct) values for a previously estimated efficiency of 2. In some instances, we expressed DNA methylation as a percentage (methylated DNA/(methylated DNA + unmethylated DNA) × 100.

Specificity of amplification and the absence of primer dimers were confirmed by melting curve analysis at the end of each run and agarose electrophoresis.

AliBaba2 program, available at http://www.gene-regulation.com/pub/programs/alibaba2, was used for *in silico* prediction of transcription factor binding sites in the DNA studied sequences (22).

FTO genotyping

The genetic analysis was done on genomic DNA extracted from umbilical cord. Genotyping for the rs9930506 A/G variant of the *FTO* gene was performed by hot-start PCR-based restriction fragment length polymorphism analysis using Molecular Biology grade reagents unless indicated, and a Robocycler 96 thermal cycler, (Stratagene, La Jolla, CA).

A 237-bp product was amplified using the following forward and reverse primers: 5′-TGATGAGAATGTAAGAAGGGAGA-3′ and 5′-TCATTTGACAGATGGACTTTTCA-3′, respectively; genotyping of the variant used digestion with Hin1II (Fermentas Life Sciences, Hanover, MD). Visualization of PCR product was performed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Statistical analysis

Quantitative data were expressed as mean ± s.e. unless indicated. Because methylated DNA/unmethylated DNA ratio was not normally distributed, we assessed differences in this variable after a log transformation. Differences between groups were assessed by ANOVA or analysis of covariance using mothers' age and BMI as covariates and contrast vectors for individual mean pairs. Correlation between two variables was performed by Pearson correlation test. To perform these analyses, we used the CSS/Statistica program package, StatSoft V 6.0 (Tulsa, OK).

Table 2 Clinical features, anthropometric variables, and laboratory findings of pregnant women at the time of delivery and newborns

Maternal characteristics	
Age, years	29.75 ± 1.01
Pregestational BMI, kg/m^2	23.31 ± 0.50
Body weight gain during pregnancy, kg	12.22 ± 0.60
Smoking (number of cigarettes/day)	2.9 ± 0.7
Alcohol consumption (ml/week)	18.1 ± 13.8
DABP, mm Hg	70.57 ± 1.01
SABP, mm Hg	115.58 ± 1.23
Heart rate, beats/min	76.73 ± 0.59
Fasting glucose, mmol/l	4.46 ± 0.08
Leptin, ng/ml	31.94 ± 2.57
Last offspring birth weight, g	3223 ± 133
Newborn characteristics	
Birth weight adjusted for gestational age and sex, *Z* score	−0.05 ± 0.13
Neonate birth weight, g	3224.27 ± 96.33
Head circumference, cm	34.05 ± 0.26
Neonate length, cm	48.03 ± 0.46
Apgar score	8.36 ± 0.19
Mother's gestational age, weeks	38.12 ± 0.27
Leptin, ng/ml	15.37 ± 1.33

Results are expressed as mean ± s.e. All measurements are in SI units.
DABP, diastolic arterial blood pressure; SABP, systolic arterial blood pressure.

RESULTS

Clinical features, anthropometric variables, and laboratory findings of pregnant women (at the time of delivery), and newborns are summarized in Table 2. Features according to offspring's birth weight were reported previously (9).

We used methylation-specific PCR to assess DNA methylation of three putative methylation target sites (CpG) in the promoter of the three previously mentioned candidate genes located at positions relative to transcription star sites: −513, −519, and −615 in *PPARGC1A*, −608, −627, and −681 in

PPARGC1A
–688–5′
CCTTATATGGAGCAAAGAAAACTGCAGCAACTCTTCGGGAGCTGGT**ATTCCCTACTGCCA**
TGGGGGCAGCCGAATTCTGGGTGGAGGAGTTTGTTTATACCTTAACACATACAGGCTA
–615
TTTTGTTGATTAAACAAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAGC**CCCGTTTGCGCT**
HNF-1 C/EBpalp –519 –513
TTCAAACACTCCCTCAATGAGAAAATGTCTCATAAAAATGCATCATGTGATAAGCTCTT
GATA-1 C/EBpalp
GCTTTAGTCCCAAACTGAGCTTGAGTCCACTTGGAGATCTTAGAATTAAAGAGTTCTTAGGGAAT
ACACGTTTTAGCTAAGAATATAGTTACTCTGTCATGAAACAGGGAGCTTTGCCACTTGCTTGTTT
TGGAAGGAAAATAAATTAAAAAAAGATTGCAGGGGATTTTGGTTATTATATGGCCAGGGCTCCG
TTTAGAGTCTGTGGCATTCAAAGCTGGCTTTAATCACAGCATGATGCTTGAAGCCTCCAAAAGTC
TAAGTGTTTCCTTTCTTTCTTTCTTTTCTTTTTCTTTCTTTTTTTTTTTTTTTAAAGCGTTACTTCACT
GAAGCAGAGGGCTGCCTTTGAGTGACGTCACGAGTTAGAGCAGCAAGCTGCACAGGGGAAGGG
AGGCTGGGTGAGTGACAGCCCAGCCTACTTTTTAATAGCTTTGTCATGTGACTGGGGACTGT-3′.
Transcriptional start site

TFAM
–637–5′
AGACTGCAGTAGATGGTAGAATAAACAGTCTAAAGCTATATTGCTTTAAATGTGGTCTCAAG
HNF-1
ACCACCGACATTAGAACTTGTTAAATTCTGGGGAACTCGCTAAAATGAAG**ATTAATGGGCC**
CCACATAGACACACGGAATTAAGCTCTGCGGTAAGGCCTTGGAATCTGCATTTTTAG
–499
GTTTGCGAAT**CCCCGCCTCTCCCGTTACTATTTCTG**AACTCCGAGCTCCAGCCCTGG
–442 **–433**
CTTGAACTGAGACGCTCCGCTGGGCGCGCAGCAGCCGCCGATCGGACCTCGGGGTCCTGGATGC
AGGACTGTCTGTTACGTACAGCCCTTGTGACCGTCACGGGCGGACACCGGCCAACGCCGGGTTG
GGGTGAGGCCGCCGCCGCGGTCCCTCCATCACCCTCCTGGCCCGGCAGAGGAACCCACTGCTCC
GGGCGGCCGGGGACAGAGGTGGCTCAACAGCGCCGCCTCGAAGCCAGAGCCCTCCGCAGGCTA
GAGGATTGCGGTTTCCCTTCATCTCCGCGGCTCTTATTCCTCCCCCGCAAGGCCGCCCACCGGGG
TACGCTCTCCCGCGCCTGCGCCAATTCCGCCCCGCCCCGCCCCCATCTACCGACCGGATGTTAGC
AGATTTCCCATAGTGC-3′. **Transcriptional start site**

PPARG
–844–5′

GGGCCTCAGGCATCGCTGCCTCCCCAAAGAGGATCAGGCCCAGAACAGTATGCTCCAGAAAT
GATA-1
AAGACTGGAAAAAGGGAAAGAGGGGCCTCAAGTCCAGGAGACCAGCGGCTTTCTGAACGCG
T3R-alpha
CACCTGCCAACCCACTT**TGGACAGGTCACGATGGACAGCGT**GGCAGGAAAAGAAA
–681
AGGTCACTGTCTACCCAACACATGAGAAACTGTTTCT**CGTGCCTCACGTCCCCACTCC**
–627 –608
GTCCCCACCCATGTTGTCTGAGTCCCTCGGTGTCAGAAACACTGCTAAGAAATTTAAGAAAT
NF-kappaB
TCTGTTTAATGAGTTTAAGAAATGTTTTTAATGATTAAAAGTCAGTGACTTGTGAATAACCATG
HNF-1
TAACTTACAAACGCAAGGAACTCTGAAAGTGTGCAGCACCACCGATCAGAAGAGAAAACCAAG
GGACCCGAAATATGCTTTAATTAAATTTTCTTTTAAAATGTCACTGGAAAGAACATCTTGGGAAG
ACGGCCTGGCCGATCGCCGTGTGAAGGGCAAGCCACTCTGGCCGAGAGGGAGCCCCACACCTCG
GTCTCCCCAGACCGGCCCTGGCCGGGGGCATCCCCCTAAACTTCGGATCCCTCCTCGGAAATGG
GACCCTCTCTGGGCCGCCTCCCAGCGGTGGTGGCGAGGAGCAAACGACACCAGGTAGCCTGCCG
CGGGGCAGAGAGTGGACGCGGGAAAGCCGGTGGCTCCCGCCGTGGGCCCTACTGTGCGCGGGC
GGCGGCCGAGCCCGGGCCGCTCCCTCCCAGTCGCGCGCCGCCGCCCCCGCCCCCGCCCCCGCCC
CCGCCCCACCCCCACCCCCACCCCCACCCCCAGCCG-3′. **Transcriptional start site**

Figure 1 The DNA sequence of the *PPARGC1A*, *PPARG*, and *Tfam* showing the methylation target sites in the promoter assessed in this study (–513, –519, and –615 in *PPARGC1A*, –609, –627, and –681 in *PPARG*, and –433, –442, and –499 in *Tfam*), and also the putative transcription factor binding sites. Primer forward and reverse are shown in bold. *PPARG*, peroxisome proliferator-activated receptor-γ; *PPARGC1A*, peroxisome proliferator-activated receptor-γ co-activator 1α gene; *Tfam*, mitochondrial transcription factor A.

PPARG, and −433, −442, and −499 in *Tfam* (**Figure 1**). These CpG dinucleotides and putative transcription factor binding sites in the promoter region evaluated for DNA methylation in each gene are also shown in **Figure 1**.

Promoter-methylated DNA/unmethylated DNA ratio was 0.82 ± 0.15 (45% of alleles) for *PPARGC1A*, and 0.0044 ± 0.0006 (0.4% of alleles) for *Tfam*. Methylation of *PPARG* promoter was almost complete in all samples studied, thus *PPARG* promoter methylation was not further analyzed.

To ensure the specificity of the method and to avoid variability in the results because of the presence of two CpG dinucleotides in the reverse primer, we designed for each gene (*PPARGC1A*, *PPARG*, and *Tfam*) a degenerate reverse primer that introduced a mismatch in the second CG site, and we observed lack of amplification regardless of the target DNA (data not shown), indicating that primers recognize the status of both CpG simultaneously.

In univariate analysis, there was no association among clinical, biochemical, and anthropometrical characteristics of the newborns and the status of promoter methylation of *PPARGC1A* and *Tfam*. In the same way, none of the maternal features were related to the status of promoter methylation of the two genes, except for a positive correlation between maternal BMI and promoter *PPARGC1A* methylation in umbilical cord (Pearson correlation coefficient $r = 0.41$, $P = 0.0007$), **Figure 2**.

Although a high correlation between mother's BMI and newborn sex and gestational age–adjusted body weight was observed ($r = 0.425$, $P < 0.0001$), we found no significant correlation between *PPARGC1A* promoter methylation and the sex and gestational age–adjusted newborn's weight ($r = 0.109$, $P < 0.37$). This result should be taken with caution because the statistical power for this analysis is low (around 17%). This lack of association persisted even after adjusting by age and maternal BMI: SGA (−0.45 ± 0.10), AGA (−0.32 ± 0.05), and LGA (−0.37 ± 0.21). Finally, we did not observe any correlation between *PPARGC1A* promoter methylation from mothers' leukocytes and any mothers' and newborns' clinical and biochemical features (data not shown).

FTO rs9930506 A/G variant was evaluated in newborn genomic DNA and genotype frequencies were in Hardy-Weinberg equilibrium. Genotype counts according to birth weight are shown in **Table 3**.

Interestingly, AA homozygous newborns show a decreased gestational sex and gestational age–adjusted body weight in comparison with G allele carriers (−0.50 ± 0.23, $n = 34$ vs. 0.11 ± 0.16, $n = 45$, $P < 0.044$, respectively).

In univariate analysis, the *FTO* rs9930506 A/G variant seems not to be associated with DNA methylation of the studied promoters (data not shown). However, we studied the *PPARGC1A* promoter methylation associated with the aforementioned *FTO* variant in relation with the offspring's birth weight and we observed that AA homozygous in the LGA group showed decreased levels of methylated DNA (**Figure 3**) in comparison

Table 3 Genotype counts of *FTO* rs9930506 A/G variant according to birth weight

	SGA	AGA	LGA
AA	11	18	5
AG	5	24	5
GG	2	10	2

Note that the subject number included in this analysis is slightly lower for some genotypes owing to failure of genotyping in few subjects.
AGA, appropriate weight for gestational age; LGA, large for gestational age; SGA, small for gestational age.

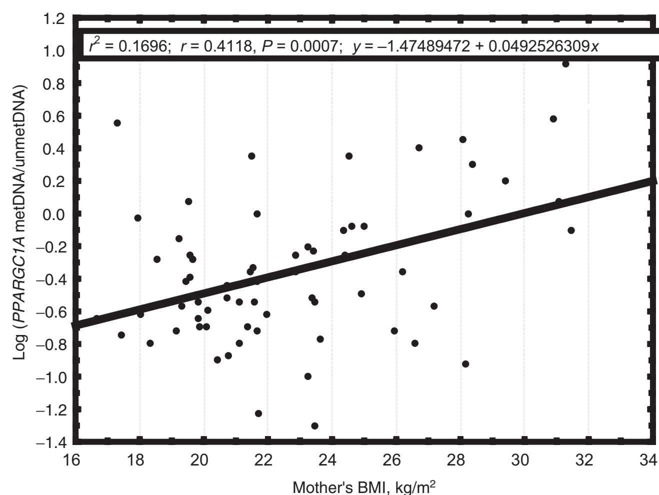

Figure 2 Correlation between log-transformed *PPARGC1A* promoter methylated DNA/unmethylated DNA ratio and mothers' BMI. *PPARGC1A*, peroxisome proliferator-activated receptor-γ co-activator 1α gene.

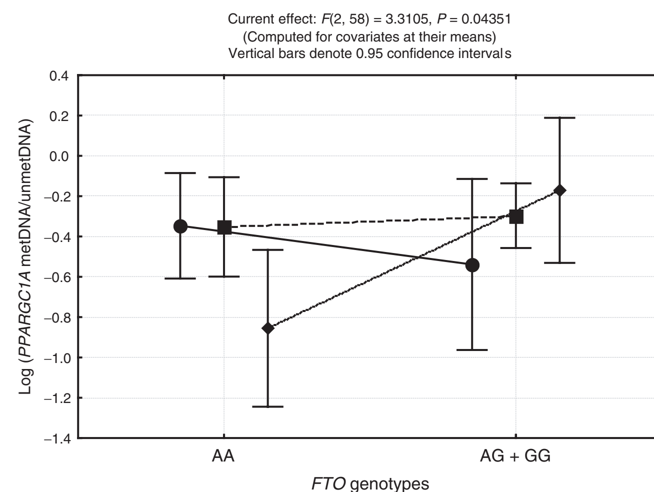

Figure 3 *PPARGC1A* promoter methylation according to the *FTO* rs9930506 A/G variant genotypes (homozygous AA in comparison with G allele carriers) in relation with the offspring's birth weight. By ANCOVA with mothers' age and BMI as covariates, AA homozygous in the large for gestational age group (diamonds) showed decreased ($P < 0.02$, $n = 5$) levels of methylated DNA in comparison with G carriers in the same group ($n = 7$). No differences were observed in the appropriate weight for gestational age (squares) and small for gestational age (circles) infants. ANCOVA, analysis of covariance; *PPARGC1A*, peroxisome proliferator-activated receptor-γ co-activator 1α gene.

with G allele carriers (AG + GG genotypes) in the same group and AGA and SGA infants.

DISCUSSION

Epigenetic alterations provide an emerging hypothesis to explain the possible biological phenomena through which an adverse intrauterine environment initiate a "metabolic imprinting" in the neonate, and further contribute to the pathogenesis of adult complications such as metabolic, cardiovascular, and endocrine disorders. The relevance of epigenetics to the fetal programming and its relation with the thrifty phenotype hypothesis is well documented in animal models (23–26). Conversely, few human studies examined the interplay between growth restriction *in utero* and epigenetic modifications as a potential mechanism to explain the later development of metabolic syndrome–related phenotypes.

In this study, we explored for the first time, although restricted to a few CpG sites, the status of promoter DNA methylation of three key genes related with metabolic syndrome (*PPARGC1A*, *PPARG*, and *Tfam*) in newborns between both extremes of abnormal fetal growth, and their relationship to the mother's pre-pregnant characteristics. *PPARGC1A* and *Tfam* showed 45 and 0.4%, respectively, of the alleles methylated at the particular CpGs analyzed, which were located at CG rich promoter regions. *PPARG* promoter was methylated in almost 100% of alleles.

Interestingly, we observed that promoter methylation of *PPARGC1A* in umbilical cord of newborns was positively correlated with maternal BMI. Based on our findings, we might speculate that maternal obesity may influence her offspring's metabolism throughout several mechanisms, among them epigenetic regulation of many genes, for instance *PPARGC1A* promoter methylation, and so her baby might be at risk of becoming obese in later life.

Despite novel, several lines of evidence may support this finding. First, the biological plausibility of this relationship, as *PPARGC1A* is a transcriptional co-activator involved in mitochondrial biogenesis and function, and has emerged as a potential candidate gene for diabetes-related metabolic phenotypes in adults and adolescents (27,28). With the same rational, *PPARGC1A* is a potential candidate gene participating in metabolic programming by epigenetic regulation.

Many studies have established that the *PPARG* co-activators are regulated at the transcriptional level in response to a variety of nutritional and environmental stimuli and also to different nutritional states (29). For instance, obesity and experimental high-fat feeding in healthy humans decrease *PPARGC1A* gene expression (30,31). In contrast, weight loss upregulates *PPARGC1A* (32).

In addition, recent evidence from a human study showed changes in DNA methylation of the *PPARGC1A* promoter in type 2 diabetic human islets (33). Ling *et al.* reported that four methylation sites (772, −903, −936, and −961) in the *PPARGC1A* promoter significantly correlate with gene expression in pancreatic islets from the type 2 diabetic (33).

Second, transgenerational effects through the inheritance of epigenetic changes have been demonstrated in animal models of intrauterine growth retardation (34,35).

Finally, the methylated CpG sites in *PPARGC1A* promoter (−513, −519, and −615) were located near a putative hepatic nuclear factor 1 and C/EBPα-binding motif. It is noteworthy to mention that hepatic nuclear factor 1 has a synergistic interaction with C/EBP-α (36), and both transcription factors are strongly related with the metabolic syndrome and related diseases. Transcriptional activity of C/EBPα is critical for the establishment and maintenance of energy homeostasis in neonates (37), and is also involved in the regulation of genes whose products are necessary for energy metabolism and adipocyte differentiation (38).

Methylation of CpG dinucleotides in CpG islands is believed to inhibit promoter activity and is a mark of silencing in human cells. One caveat of this study could be that we did not measure the protein level of *PPARGC1A* in umbilical cord. Therefore, we cannot assure that methylation level of the *PPARGC1A* promoter is related with altered gene expression. Besides, a note of caution should be added as different tissue and cell types show distinct patterns of promoter methylation, and umbilical cord may be not a good representative of other fetal tissues. Nevertheless, because of the nature of the study, umbilical cord is the only available source of babies' tissue. In addition, some arguments are worthy to emphasize regarding the biological implications of carrying out studies based on umbilical cord as the object of analysis. The umbilical cord represents the link between mother and fetus during pregnancy. In addition, several experiments carried out in rodents demonstrated that the *in vitro* and *in vivo* differentiation competence of the human umbilical cord stromal cells is highly related with adipogenic cell lineages (39). Karahuseyinoglu *et al.* demonstrated that umbilical cord stromal cells are capable of differentiating into premature adipocytes bearing smaller multilocular lipid droplets (40). Finally, adipogenically induced cells from umbilical cord specifically express adipocyte-specific genes (41). Hence, we consider that examining umbilical cord in our study is a good approximation to as examining the ideal tissue (adipose tissue, for instance) but future studies using cord-derived cells in culture are warranted.

Other study limitation is the cross-sectional design of this study that does not allow us to evaluate the long-term metabolic and cardiovascular complications.

Given the established relation of the rs9930506 A/G variant located at the first intron of the *FTO* gene to adiposity, BMI and related phenotypes in previous studies (14), we further evaluated the association of this variant with the children and mothers' features, and also with the status of *PPARGC1A* promoter methylation. When we evaluated the genotype frequencies of the rs9930506 according to the babies' birth weight, we observed a significant association between the *PPARGC1A* promoter methylation in LGA babies and rs9930506 genotypes, showing that in this group, homozygous AA carriers had lower level of *PPARGC1A* promoter methylation in comparison with babies with AG and GG genotypes. Although

preliminary, this finding suggests that the *FTO* rs9930506 A allele may affect *PPARGC1A* promoter methylation depending on a particular fetal environment. Bioinformatics analyses predict that the protein product of *FTO* shares sequence motifs with Fe(II)- and 2-oxoglutarate-dependent oxygenases, and these enzymes are involved in diverse processes, including DNA repair, fatty acid metabolism, and posttranslational modifications, for example, proline hydroxylation and histone lysine demethylation (15), which makes the FTO protein a good candidate for an epigenetic mark modifier. Given that the role of the encoded proteins of both *FTO* and *PPARGC1A,* it will be of interest to explore the molecular mechanisms by which the genetic rs9930506 A/G variant of *FTO* affects the level of *PPARGC1A* promoter methylation in newborns who are born LGA. Moreover, despite that *FTO* mRNA is regulated in the arcuate nucleus by feeding and fasting (15), it was shown that environmental factors may also regulate *FTO* expression as it was recently reported that low physical activity seems to accentuate the effect of *FTO* rs9939609 (a single-nucleotide polymorphism from the cluster of 10 single-nucleotide polymorphisms in the first intron of FTO also associated with BMI) on body fat accumulation (42). Interestingly, newborns homozygous for A allele seems to be smaller than the G allele carriers.

To summarize, the association between birth weight and adult chronic disease morbidity and mortality suggests that the *in utero* exposure to an altered metabolic environment may permanently affect the offspring metabolism, with further impact on adult health status. The change in the physiology and metabolism of the offspring, which persist to be expressed even without the stimuli that initiated them, requires several complex processes currently acknowledge as metabolic programming. In this study, we explored the potential role of epigenetics in metabolic programming through the analysis of promoter DNA methylation in three master genes that control several metabolic pathways. We have found, in our knowledge for the first time, that the *PPARGC1A* promoter methylation in newborns was significantly associated with maternal BMI, hypothesizing that this pattern of DNA methylation may potentially led to, for instance, either altered gene expression in specific tissues or induce transcriptional silencing by blocking the binding of transcription factors.

Our results do not allow us to ascertain whether this is an epiphenomenon or an epigenetic risk factor for the early onset of adult metabolic syndrome. We hope that future experiments may solve this central question for the public health.

ACKNOWLEDGMENTS

This study was supported partially by grants M055 (Universidad de Buenos Aires), PICT 2006-124 and PICT 25920 (Agencia Nacional de Promoción Científica y Tecnológica) and funds provided by Fundación Alfredo Lanari. S.S., C.G., S.G., and C.J.P. belong to Consejo Nacional de Investigaciones Científicas y Técnicas.

DISCLOSURE

The authors declared no conflict of interest.

© 2009 The Obesity Society

REFERENCES

1. Simmons RA, Templeton LJ, Gertz SJ. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. *Diabetes* 2001;50: 2279–2286.
2. Barker DJ, Hales CN, Fall CH *et al*. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 1993;36:62–67.
3. Barker DJ. Fetal origins of coronary heart disease. *BMJ* 1995;311:171–174.
4. Curhan GC, Willett WC, Rimm EB *et al*. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation* 1996;94:3246–3250.
5. Boney CM, Verma A, Tucker R, Vohr BR. Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus. *Pediatrics* 2005;115:e290–e296.
6. Barker DJ. Intrauterine programming of adult disease. *Mol Med Today* 1995;1:418–423.
7. Patel MS, Srinivasan M. Metabolic programming: causes and consequences. *J Biol Chem* 2002;277:1629–1632.
8. Junien C, Gallou-Kabani C, Vige A, Gross MS. [Nutritionnal epigenomics: consequences of unbalanced diets on epigenetics processes of programming during lifespan and between generations]. *Ann Endocrinol (Paris)* 2005;66:2S19-2S28.
9. Gemma C, Sookoian S, Alvarinas J *et al*. Mitochondrial DNA depletion in small- and large-for-gestational-age newborns. *Obesity (Silver Spring)* 2006;14:2193–2199.
10. Thorvaldsen JL, Bartolomei MS. Molecular biology. Mothers setting boundaries. *Science* 2000;288:2145–2146.
11. Park JH, Stoffers DA, Nicholls RD, Simmons RA. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. *J Clin Invest* 2008;118:2316–2324.
12. Magnus P, Bakketeig LS, Skjaerven R. Correlations of birth weight and gestational age across generations. *Ann Hum Biol* 1993;20:231–238.
13. Frayling TM, Timpson NJ, Weedon MN *et al*. A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. *Science* 2007;316:889–894.
14. Scuteri A, Sanna S, Chen WM *et al*. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet* 2007;3:e115.
15. Gerken T, Girard CA, Tung YC *et al*. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. *Science* 2007;318:1469–1472.
16. San PM, Grandi C, Larguia M, Solana C. [Standard of birth weight for gestational age in 55706 healthy newborns in a public maternity of Buenos Aires]. *Medicina (B Aires)* 2001;61:15–22.
17. Das UG, Sysyn GD. Abnormal fetal growth: intrauterine growth retardation, small for gestational age, large for gestational age. *Pediatr Clin North Am* 2004;51:639–654, *viii*.
18. Kitzmiller JL, Cloherty JP, Younger MD *et al*. Diabetic pregnancy and perinatal morbidity. *Am J Obstet Gynecol* 1978;131:560–580.
19. Kawasaki ES. Sample preparation from blood, cells, and other fluids. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). *PCR Protocols. A Guide to Methods and Applications*, vol 18. Academic Press: San Diego, 1990, pp. 146–152.
20. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* 2002;18:1427–1431.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408.
22. Grabe N. AliBaba2: context specific identification of transcription factor binding sites. *In Silico Biol* 2002;2:S1–S15.
23. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? *J Physiol* 2004;561:355–377.
24. Gallou-Kabani C, Junien C. Nutritional epigenomics of metabolic syndrome: new perspective against the epidemic. *Diabetes* 2005;54:1899–1906.
25. Issa JP. Epigenetic variation and human disease. *J Nutr* 2002;132: 2388S–2392S.
26. Holness MJ, Sugden MC. Epigenetic regulation of metabolism in children born small for gestational age. *Curr Opin Clin Nutr Metab Care* 2006;9: 482–488.

27. Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC-1 α): transcriptional coactivator and metabolic regulator. *Endocr Rev* 2003;24:78–90.
28. Sookoian S, Garcia SI, Porto PI *et al*. Peroxisome proliferator-activated receptor γ and its coactivator-1 α may be associated with features of the metabolic syndrome in adolescents. *J Mol Endocrinol* 2005;35:373–380.
29. Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. *Cell Metab* 2005;1:361–370.
30. Crunkhorn S, Dearie F, Mantzoros C *et al*. Peroxisome proliferator activator receptor γ coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. *J Biol Chem* 2007;282:15439–15450.
31. Sparks LM, Xie H, Koza RA *et al*. A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle. *Diabetes* 2005;54:1926–1933.
32. Gastaldi G, Russell A, Golay A *et al*. Upregulation of peroxisome proliferator-activated receptor γ coactivator gene (PGC1A) during weight loss is related to insulin sensitivity but not to energy expenditure. *Diabetologia* 2007;50:2348–2355.
33. Ling C, Del GS, Lupi R *et al*. Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion. *Diabetologia* 2008;51:615–622.
34. Maclennan NK, James SJ, Melnyk S *et al*. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. *Physiol Genomics* 2004;18:43–50.
35. Burdge GC, Hanson MA, Slater-Jefferies JL, Lillycrop KA. Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life? *Br J Nutr* 2007;97:1036–1046.
36. Wu KJ, Wilson DR, Shih C, Darlington GJ. The transcription factor HNF1 acts with C/EBP α to synergistically activate the human albumin promoter through a novel domain. *J Biol Chem* 1994;269: 1177–1182.
37. Wang ND, Finegold MJ, Bradley A *et al*. Impaired energy homeostasis in C/EBP α knockout mice. *Science* 1995;269:1108–1112.
38. Rosen ED, Hsu CH, Wang X *et al*. C/EBPα induces adipogenesis through PPARγ: a unified pathway. *Genes Dev* 2002;16:22–26.
39. Can A, Karahuseyinoglu S. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells* 2007;25:2886–2895.
40. Karahuseyinoglu S, Kocaefe C, Balci D, Erdemli E, Can A. Functional structure of adipocytes differentiated from human umbilical cord stroma-derived stem cells. *Stem Cells* 2008;26: 682–691.
41. Akerman F, Lei ZM, Rao CV. Human umbilical cord and fetal membranes co-express leptin and its receptor genes. *Gynecol Endocrinol* 2002;16: 299–306.
42. Andreasen CH, Stender-Petersen KL, Mogensen MS *et al*. Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation. *Diabetes* 2008;57:95–101.