Research

Methylation Status of *GLP2R, LEP* and *IRS2* in Small for Gestational Age Children with and Without Catch-Up Growth

Angulo M et al. Epigenetic Analysis in SGA Children

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What is already known on this topic?
In the last two decades, significant advances have been made for understanding the epigenetic impact in human growth and development. However, the different approaches and methodologies used do not clearly estimate the genes potentially related to the changes and phenotypes observed in small for gestational age children.

What this study adds?
This study proposes the simultaneal analysis of promoter methylation status of multiple genes not related to parental imprinting and that may play a role in the development of metabolic diseases in children with SGA. The catch-up children should be routinely followed to perform timely diagnosis of possible metabolic impairments.

ABSTRACT
Introduction: In small for gestational age (SGA) children, catch-up growth could be influenced by several gene methylation involved in metabolic process. Epigenetic may influence the development of metabolic diseases in adulthood.
Objective: To compare the methylation of leptin (*LEP)*, glucagon-like peptide-2 receptor (*GLP2R)*, insulin receptor substrate-2 (*IRS2*) in SGA patients with and without catch-up growth.
Methods: Observational prospective study. We included 48 SGA patients. Demographical and clinical variables were collected from clinical records and parents' questionnaire. Methylation status of *LEP, IRS2,* and *GLP2R* promoters was evaluated in DNA extracted from patient and one parent saliva samples.
Results: Twenty-six (54.2%) patients had catch-up growth phenotype and 22 (45.8%) patients did not. The median age was 5.2 years [RIC 4.1-6.8] without difference between groups (p=0.306). The catch-up group had increased appetite (42.3% vs 9.1%, *p*=0.008), family history of dyslipidemia (42.3% vs. 27.3%) and diabetes (34.6% vs. 22.7%) compared to non-catch-up group. Catch-up patients had significantly larger waist circumference compared to non-catch-up group (median 55cm [RIC52–58] versus median 49.5cm, [RIC46-52] p<0.001). *LEP* and *GLP2R* were methylated in all samples. IRS2 was methylated in 60% of SGA patients without difference between groups (p=0.520).
Conclusion: There is no association between *IRS2* methylation and catch-up growth among SGA patients. *LEP* and *GLP2R* were methylated in all SGA patients. Gene methylation may be implicated in metabolic disease later in life. More studies should be performed to confirm this hypothesis.
Keywords: Low birth weight; Infant, Small for gestational age; Epigenetics; Methylation, DNA; Insulin resistance

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Introduction
Small for Gestational Age (SGA) is defined as a newborn with weight, length, and/or head circumference below the 10th percentile according to sex and gestational age[1]. For 2012, in low- and middle-income countries approximately 23.3 million infants were born SGA in low- and middle-income countries[2]. In the last decade, Colombia increased their SGA rate from 70 to 90 SGA newborns per 1,000 live births[3].
SGA is related to decreasing lean mass, muscular mass, and bone mineral content. A SGA infant could have reduced protein, nitrogen and glycogen contents in the skeletal muscle and liver, due to decreased plasma glucose and insulin

concentrations[4]. These factors are associated with increased risks of hypoglycemia, hypocalcemia, polycythemia, hyper-viscosity, impaired thermoregulation, and immune dysfunction[5]. SGA infants has 20 times the probability of death compared with healthy controls during neonatal period[6]. SGA also has poor long-term outcomes. including significantly increased risk of chronic cardiovascular and metabolic diseases compared with Appropriate Gestational Age (AGA) infants [7]–[10].

Additionally, the physiological response to SGA is evident in the growth pattern during the first two years of life, especially during the first 2–6 months. Approximately 15% of children with a history of SGA continue to present low weights and heights for their ages, associated with a non-catch-up growth phenotype[11]. The remaining 85% show excessive, catch-up growth, with height velocities that exceed the normal statistical limits for their age and/or maturity during the first three years of life following the prenatal period of growth inhibition[12].

Epigenetic refers to heritable changes in gene expression that occur without requiring alterations in DNA sequences, including the expression of non-coding RNAs, DNA methylation, and histone modifications. Genomic imprinting is one of the most important and studied forms of epigenetic inheritance, during which the regulation of a gene or chromosomal region is dependent on the sex of the transmitting parent[13]. Imprinted regions play vital roles during embryonic development and have been associated with low birth weight (LBW) and other phenotypes associated with abnormal weight, such as overgrowth syndromes[14]. The epigenetic regulation of the genome is a critical facet of development. Several genes located in imprinted regions are associated with the control of embryonic growth, such as *IGF2, H19,* and *MEST*[15]. However, most studies that have examined the genes in imprinted regions[15], [16] have not been able to consistently or conclusively determine their associations with LBW[11].

The embryonic environmental characteristics of patients with SGA could induce changes at the epigenetic level that affect gene transcription and would be stable throughout life[5], [14]. Because of the importance of epigenetic regulations during human development, these changes could be associated with the disease and pathological phenotypes that present during childhood and adulthood in patients with a history of SGA. The aim of this study was to compare the methylation status of *IRS2, GLP2R*, and *LEP* in SGA patients with and without catch-up growth. These genes were chosen due to their importance in appetite control and their role in regulation of carbohydrate metabolism.

Experimental Subjects

This is an observational prospective study. SGA patients were selected from the pediatric endocrinology outpatient clinic. Patients with metabolic comorbidities or whose parents did not agree to enter the study were excluded. Leptin (*LEP)*, glucagon-like peptide 2 receptor (*GLP2R)*, insulin receptor substrate 2 (*IRS2*) were categorized according to whether the patient presented with a catch-up growth or non-catch-up growth phenotype during early childhood (before 5 years of age). This study was performed in accordance with the Declaration of Helsinki Good Clinical Guidelines. Written informed consent was obtained from all patients' parents before study participation. The institutional IRB of Fundación Valle del Lili approved the study (Act 084-2014).

Materials and Methods

Data and Setting

Cali is a city of 2.3 million inhabitants, and it is the capital of the Valle del Cauca Department in Southwestern Colombia. Cali's natality rate was 12 per 1000 inhabitants in 2017 with an infant mortality rate of 9 per 1000 life newborns, and 11% LBW [17]. Fundación Valle del Lili is a tertiary care university hospital with a catchment area of approximately 10 million people.

Exposure Variables

Demographical and clinical variables were obtained from clinical records. The SGA criterion was defined as birth weight and birth height below the 10th percentile for gestational age [1]. The parents or legal guardians of the patient were asked through a survey for information regarding demographic characteristics, lifestyle, and other variables such as a lack of appetite or a voracious appetite, hours per week of physical activity and abdominal circumference. In addition, we asked for self-report clinical diagnosis of dyslipidemia, diabetes mellitus, arterial hypertension and cardiovascular disease.

To assess appetite, parents were asked to determine their child´s appetite by choosing among three options: a) the child has very good appetite, eats everything on the plate and constantly asks for additional food (voracious appetite); b) The child spends many time for taking his/her meals or snacks but eats what is recommended for his/her age (slow eater); and c) The child has low appetite, parents must insist to finish eating or remain many hours without eating (lack of appetite). Weight in kilograms and height in centimeters were measured at the time of evaluation, which allowed us to classify patients into two groups: children who present the catch-up growth phenotype and children who present the non-catch-up growth phenotype.

Outcome Variables

Saliva samples of children and their parents were collected during clinical assessment using the Oragene-DNA OG500 saliva self-collection kit (DNA Genotex, Canada), and stored at 4 °C until its extraction and analysis. Catch-up growth was defined as the height velocity above the limits of normal for age for at least 1 year after a transient period of growth inhibition [18]. The examined genes were selected based on their significant contributions to the anabolic metabolism of the pediatric population: *LEP, GLP2R* and *IRS2*.

DNA Methylation Analysis

DNA extraction was performed from oral fluid samples using the prepIT-L2P kit® (DNA Genotek Inc. Ottawa, ON, Canada), according to the manufacturer's instructions, at the Genomic Medicine Laboratory at Universidad Icesi. Extracted DNA was quantified using a NanoDrop 2000 (Thermo Scientifics). The bisulfite conversion of DNA was

performed using the Epitect kit® Fast Bisulfite Conversion (Qiagen Inc: Germantown, MD, USA). All samples were processed and analyzed in an anonymous manner.

Primers were designed, using the freely available, web-based software program Beacon Designer v.8.14 (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1), to cover the CG-rich regions, with amplifications in the target range of 400–600 bp (Suppl. Table 1). The promoter regions for *LEP, GLP2R* and *IRS2,* were examined, and 1 CpG site for each gene was selected for methylation measurements: CpG site 1, *LEP* promoter: 1120-1718; CpG site 2, *IRS2* promoter: 4256-4600; CpG site 3; CpG site 4, *GLP2R* promoter: 1108-1507; and CpG site 5.

Qualitative methylation analysis of genomic DNA was performed by real-time PCR assay, using an EpiTect MethyLight PCR kit (Qiagen Inc: Germantown, MD, USA). This methylation-specific assay is comprised of 2 non-specific methylation primers and a Taqman probe, which specifically amplified methylated DNA within the gene locus. PCR was performed in a total volume of 25 µl, containing 12.5 µl EpiTect MethyLight PCR Master Mix (Qiagen Inc: Germantown, MD, USA), 1 µl (20 ng) bisulfite-converted genomic DNA, 2.5 µl 10× Primer-probe mix, and 9 µl water. Real-time PCR was performed using a 7500 fast real-time PCR instrument (Applied Biosystems, CA), with the following temperature profile: 20 min at 95°C and 55 cycles of 15 sec at 95°C and 30 sec at 60°C. EpiTect Control DNA (human), which was methylated and bisulfite-converted (Qiagen), was used as the positive control for methylation assays. CpG units that yielded data in more than 90% of samples passed the initial quality control step. Poor-quality data for each CpG site were excluded during the qualitative evaluation of methylation.

For each sample, a relative methylation value was determined using the ΔCT method and ΔΔCT method and normalized against the ΔCT mean of EpiTect Control DNA. The ΔCT values for each sample were measured in triplicate. Samples were considered negative (non-methylated) in the study when more than two replicates showed cycle threshold (CT) values greater than 35 during the total DNA quantification assay. The area under the curve of the receiver operating characteristic was computed, using the trapezoidal rule.

Statistical Analysis

Dichotomous variables were reported as percentages and continuous data were reported as the median and interquartile ratio (IQR) or mean and standard deviation (SD) if normally distributed. Comparisons were made using the χ^2 or Fisher's exact test, for dichotomous variables, as appropriate. the Mann-Whitney U-test was used for comparisons of continuous data. P-values were considered significant at $p < 0.1$. The statistical analysis was performed using STATA® 14.0 (StataCorp, College Station, TX, USA) registered to Fundación Valle de Lili.

Results

Between November 2013 to January 2015, 48 children with a history of SGA were treated at the pediatric endocrinology clinic. None of the patients were excluded. Of these, 45 (93.7%) were born at term (37 weeks of gestational age or more), 28 (58.3%) were girls, and the median age at medical assessment was 5.1 years old (IQR 4.1–6.8). The demographic and clinical characteristics of these patients during infanthood are shown in Table 1.

Twenty-six patients had catch-up growth phenotype and 22 children did not. Catch-up patients were characterized as voracious eaters (42.3% vs 9.1%) and had higher waist circumferences (median 55 cm vs 49.5 cm) than patients presenting with the non-catch-up growth phenotype. No differences in the hours per week of physical activity were observed between groups. Family history of dyslipidemia, diabetes mellitus, and arterial hypertension were more common in the catch-up growth group than in the non-catch-up growth group.

The qualitative methylation-specific assay found that CpG sites associated with *GLP2R* and *LEP* were methylated in all samples. Methylation of the *IRS2* promoter was observed in 57.7% of the catch-up growth group and in 63.6% of the non-catch-up growth group ($p = 0.52$). In 4 catch-up growth group children and 1 non-catch-up growth child, the status of promoter methylation could not be determined (Suppl. Figure 1 and Suppl. Table 2).

Discussion

Catch-up growth acts as a compensatory mechanism for perinatal age, reducing morbidity. However, catch-up growth is also associated with adverse outcomes, including obesity, insulin resistance, glucose intolerance, type 2 diabetes mellitus, and cardiovascular disorders, in adulthood.[12], [19] We hypothesize that *IRS2* promoter methylation status may play a role in catch-up growth; however, we did not identify an association between *IRS2* promoter methylation and the catch-up growth phenotype in this population. In addition, *GLP2R* and *LEP* were methylated in all samples.

Few studies have examined the methylation status of genes in SGA infants. The largest study was conducted by Ying Liu et al., in 2012, who measured the methylation status of *IGF2/H19*, *MEST,* and other imprinted genes, using a bisulfite pyrosequencing method on cord blood DNA from 508 infants, and found no significant differences in the methylation levels of the *MEST* differentially methylated region between LBW neonates and normal-weight neonates[20]. No study reported in the literature included all of the genes that were examined in the present study and saliva samples used here are another differential factor.

Saliva is composed of more than 99% water, and also contains white blood cells and epithelial cells, which represent the cell types of the oral mucosa. Previous DNA methylation studies comparing profiles between tissue types within individuals have shown that regions of tissue-specific differential methylation mainly map to CpG poor regions and demonstrated that methylation profiles correlating positively between saliva and diverse tissue in question [21], [22]. The viability of saliva as an alternative for less accessible tissues; including brain, lung/bronchial epithelium, and peripheral blood mononuclear cells, and in a recent study, intestinal mucosa [22]. The similar composition and function of mucosa between the oral mucosa and intestinal mucosa suggest that exits comparable methylation profiles between saliva and that

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tissue, and strengthens the idea that saliva has the potential to be used as an alternative for more invasive tissues, like was our purpose [21].

The promoters methylation of *GLP2R* and *LEP* was observed in all samples, suggesting the population-wide downregulation of *GLP2* and *LEP*. These genes have been physiologically associated with appetite control, satiety, and glucose homeostasis[23]. Whether the methylation of these gene promoters reflects an adaptation response associated specifically with a history of SGA or whether methylation reflects a general tendency in all populations is currently unknown because the methylation status of these promoters has not been previously studied. Other studies have reported a reduction in the methylation status of the *LEP* promoter among obese patients and the increased methylation status among SGA children[24]. Reynolds et al., in 2017, suggested that high-birth-weight babies showed an increased expression levels of obesity-related genes lipoprotein lipase [*LPL*] and LEP receptor [*LEPR*] [25]. Studies in rodents have shown that treatment with *LEP* during late developmental stages in offspring, slows neonatal weight gain and reverses prenatal adaptations caused by stimuli that promote adulthood obesity[26], and *LEPR* expression increases in response to *LEP* insensitivity, as a compensatory mechanism to defend against obesity. *LEP* promoter methylation status approached 100% among the parental control samples, suggesting that *LEP* downregulation in the digestive tract is a generalized adaptation during adulthood.

We did not identify an association between *IRS2* promoter methylation and phenotype that could explain the trend toward insulin resistance that is commonly associated with the catch-up growth phenotype. IRS-1 and IRS-2 proteins play roles during the regulation of the insulin signal transduction pathway, through phosphorylation and binding with the insulin receptor. IRS-1 is expressed predominately in skeletal muscle, whereas IRS-2 is expressed in the liver, fat tissue, and skeletal muscle. The downregulation of these substrates has been associated with insulin resistance, altered secretion patterns in pancreatic β cells, and the development of diabetes mellitus[27]. A recent study showed that the downregulation of IRS-2 in an SGA murine model was associated with abnormal glucose metabolism[28]. In a previous study of high-birth-weight babies, significantly elevated expression levels of *GLUT4* and *IRS2* mRNA were observed and were correlated with insulin resistance, as both proteins are stimulated by insulin and are associated with cellular glucose uptake[25].

However, our findings were not completely consistent with the results reported by previous studies examining the epigenetic dysregulation associated with SGA. Differences between study findings may be related to the methods used to assess methylation, the classification standards used to define SGA in different countries or districts, or differences in population characteristics. The limited sample sizes associated with studies of SGA populations also likely results in increased variation. Studies with larger sample sizes are necessary to replicate our findings. The causal relationship between gene methylation status and SGA must be examined further, and our findings add to the emerging evidence that the methylation of genes associated with metabolic regulation may adversely impact fetal growth and development. Compounds that act as methyl group donors may influence the epigenetic regulation of specific genes, although the implications of these alterations remain unclear.

Study Limitations

Our study has a few limitations. Given the small sample size of our study, our results have low statistical power. Second, the use of saliva samples does not allow for comparisons with other published studies about epigenetics analysis in SGA. Lastly, this study was performed with patients followed in a pediatric endocrinology outpatient clinic. Therefore, the results could not be extrapolated to the general pediatric population.

Conclusion

Understanding potential epigenetic factors associated with the development of metabolic diseases could facilitate the early identification of at-risk populations, which could then be treated with early and adequate preventive intervention methods. The association between SGA and the development of metabolic diseases during adult life represents an opportunity to identify potential epigenetic characteristics and to establish targets for the prevention and treatment of metabolic diseases, which could benefit both these children and the general population. Our findings showed the widespread methylation of genes related to metabolic control, suggesting that the high metabolic risks that have been previously identified in multiple studies of SGA children may be due to epigenetic adaptations that occur *in utero,* and the epigenetic adaptations identified during postnatal life may represent non-specific events.

Further studies are necessary to better understand the development of metabolic pathologies among SGA children and whether the methylation patterns of key genes are associated with the development of short stature and metabolic syndromes among this population, and both comparisons among SGA children with different response phenotypes and comparisons of both populations with the general population should be performed.

Conflict Of Interest: No conflict of interest

Authorship Contribution:

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Table 1. Demographic and clinical characteristics of the study population.

Variable	Non-catch-up growth	Catch-up growth	*p* value
	n=22	n=26	
Male, n(%)	8 (36.4)	12 (46.2)	0.493
Age at recruitment [years]*	6.1 (4.4 - 6.8)	4.7 (4.0 - 6.7)	0.306
Gestational weeks at birth*	37 (37-38)	37 (37-39)	0.565
Appetite, n(%)			
Slow eater	10 (45.5)	3 (11.5)	
Voracious eater	2 (9.1)	11 (42.3)	0.008
Lack of appetite	10 (45.5)	12 (46.2)	
Hours of physical activity/per week*	9 (7-10)	7.5 (5 - 10)	0.555
Family history, n(%)			
Dyslipidemia	6 (27.3)	11 (42.3)	0.278
Diabetes Mellitus	5 (22.7)	9 (34.6)	0.281
Arterial Hypertension	8 (36.4)	12 (46.2)	0.348
Cardiovascular Disease	6 (27.3)	7 (26.9)	0.615
Overweight	5 (22.7)	9 (34.6)	0.670
Obesity	3 (13.6)	4 (15.4)	0.608
IRS2 gene methylation status, n(%)			
Inconclusive	1 (4.5)	4 (15.4)	
Negative	7 (31.8)	7 (26.9)	0.52[a]
Positive	14 (63.6)	15 (57.7)	
Waist circumference*	49.5 (46-52)	55 (52-58)	<0.001

*Median (IQR), [a]Chi2 test performed with inconclusive IRS2 Gene methylation status

Supplemental Table 1. Designed primers and probes for the evaluation of genomic DNA methylation.

GEN	DESIGNED PRIMERS AND PROBE		
	FORWARD PRIMERS	PROBES	REVERSE PRIMERS
LEP	CAACCCCGCAATCTAAATCGAAAA	CGCACTACGAACCGCTCCCTCTAACC	GGTTTTGGACGTTAGGGAAGTTTA
IRS2	AAGTTTAATTGCGAGTAGTCGTCG	ACCGAATCGTCCGCCTACATCCACA	TCCAAAATAATCTCGTAAATATTCTACGC
GIP	CGCCCAAACTAACAAACAATAACG	CTCACTACAACCTCCGCCTACCGAATT	CGCCCAAACTAACAAACAATAACG
GLP2R	GAATTTTGAAGATTTCGTAGATTGTTTTAG	ACACCGCAAACAACCTCCTCTTACATTCC	AAATACATCTCTCTAACCGTCCAAA
IGF2	TTTTCGTTTTGTTTCGTCGTATATTCG	TAACCCTCCTACCGAACACTCCTCTACCA	TACTACGTATCGCAAACCGAACAA

Supplemental Table 2. Relative quantification for *LEP* in samples according to ΔΔCт

Block Type   96fast
Chemistry   TAQMAN
Experiment File Name   D:\Users\INSTR-ADMIN\Desktop\Epigenetica\First run.eds
Experiment Run End Time   2017-03-13 17:07:21 PM COT
Instrument Type   sds7500fast

Well	Sample Name	Target Name	Reporter	Quencher	Cт	Cт Mean	Cт SD	ΔCт	ΔΔCт
A1	017	LEP	FAM	NFQ-MGB	32,48601	32,73349	0,309419	-0,86849	-34,2468
A2	017	LEP	FAM	NFQ-MGB	33,0804	32,73349	0,309419	-0,2741	-33,6524
A3	017	LEP	FAM	NFQ-MGB	32,63406	32,73349	0,309419	-0,72044	-34,0987
A4	017P	LEP	FAM	NFQ-MGB	31,29929	31,27273	0,110781	-2,05521	-35,4335
A5	017P	LEP	FAM	NFQ-MGB	31,36781	31,27273	0,110781	-1,98669	-35,365
A6	017P	LEP	FAM	NFQ-MGB	31,15108	31,27273	0,110781	-2,20342	-35,5817
A7	018	LEP	FAM	NFQ-MGB	32,19761	31,92159	0,379013	-1,15689	-34,5352
A8	018	LEP	FAM	NFQ-MGB	32,0777	31,92159	0,379013	-1,2768	-34,6551
A9	018	LEP	FAM	NFQ-MGB	31,48945	31,92159	0,379013	-1,86505	-35,2433
A10	004	LEP	FAM	NFQ-MGB	31,24569	31,59458	0,356612	-2,10881	-35,4871
A11	004	LEP	FAM	NFQ-MGB	31,95844	31,59458	0,356612	-1,39606	-34,7744
A12	004	LEP	FAM	NFQ-MGB	31,57961	31,59458	0,356612	-1,77489	-35,1532
B1	015	LEP	FAM	NFQ-MGB	32,59671	32,70707	0,100448	-0,75779	-34,1361
B2	015	LEP	FAM	NFQ-MGB	32,73132	32,70707	0,100448	-0,62318	-34,0015

B3	015	LEP	FAM	NFQ-MGB	32,79316	32,70707	0,100448	-0,56134	-33,9396
B4	015P	LEP	FAM	NFQ-MGB	30,79715	31,22849	0,37471	-2,55735	-35,9356
B5	015P	LEP	FAM	NFQ-MGB	31,41473	31,22849	0,37471	-1,93977	-35,3181
B6	015P	LEP	FAM	NFQ-MGB	31,4736	31,22849	0,37471	-1,8809	-35,2592
B7	016	LEP	FAM	NFQ-MGB	32,31133	32,43078	0,166969	-1,04317	-34,4215
B8	016	LEP	FAM	NFQ-MGB	32,35945	32,43078	0,166969	-0,99505	-34,3733
B9	016	LEP	FAM	NFQ-MGB	32,62157	32,43078	0,166969	-0,73293	-34,1112
B10	006	LEP	FAM	NFQ-MGB	31,14703	31,75572	0,527453	-2,20747	-35,5858
B11	006	LEP	FAM	NFQ-MGB	32,07829	31,75572	0,527453	-1,27621	-34,6545
B12	006	LEP	FAM	NFQ-MGB	32,04184	31,75572	0,527453	-1,31266	-34,691
C1	013	LEP	FAM	NFQ-MGB	32,23765	32,20446	0,058418	-1,11685	-34,4951
C2	013	LEP	FAM	NFQ-MGB	32,137	32,20446	0,058418	-1,21749	-34,5958
C3	013	LEP	FAM	NFQ-MGB	32,23872	32,20446	0,058418	-1,11578	-34,4941
C4	013P	LEP	FAM	NFQ-MGB	30,72647	31,1143	0,350237	-2,62803	-36,0063
C5	013P	LEP	FAM	NFQ-MGB	31,20893	31,1143	0,350237	-2,14557	-35,5239
C6	013P	LEP	FAM	NFQ-MGB	31,4075	31,1143	0,350237	-1,947	-35,3253
C7	014	LEP	FAM	NFQ-MGB	31,75308	31,81729	0,174822	-1,60142	-34,9797
C8	014	LEP	FAM	NFQ-MGB	32,01514	31,81729	0,174822	-1,33936	-34,7177
C9	014	LEP	FAM	NFQ-MGB	31,68365	31,81729	0,174822	-1,67085	-35,0491
C10	008	LEP	FAM	NFQ-MGB	30,90038	30,78209	0,248279	-2,45411	-35,8324
C11	008	LEP	FAM	NFQ-MGB	30,94911	30,78209	0,248279	-2,40539	-35,7837
C12	008	LEP	FAM	NFQ-MGB	30,49679	30,78209	0,248279	-2,85771	-36,236
D1	011	LEP	FAM	NFQ-MGB	32,95736	32,91446	0,096077	-0,39714	-33,7754
D2	011	LEP	FAM	NFQ-MGB	32,9816	32,91446	0,096077	-0,3729	-33,7512
D3	011	LEP	FAM	NFQ-MGB	32,8044	32,91446	0,096077	-0,5501	-33,9284
D4	011P	LEP	FAM	NFQ-MGB	31,99075	32,03038	0,114877	-1,36375	-34,742
D5	011P	LEP	FAM	NFQ-MGB	31,94057	32,03038	0,114877	-1,41393	-34,7922
D6	011P	LEP	FAM	NFQ-MGB	32,15983	32,03038	0,114877	-1,19467	-34,573
D7	012	LEP	FAM	NFQ-MGB	32,60457	32,40089	0,182291	-0,74993	-34,1282
D8	012	LEP	FAM	NFQ-MGB	32,25306	32,40089	0,182291	-1,10144	-34,4797
D9	012	LEP	FAM	NFQ-MGB	32,34504	32,40089	0,182291	-1,00946	-34,3878
D10	010	LEP	FAM	NFQ-MGB	31,85977	31,62498	0,29803	-1,49473	-34,873

D11	010	LEP	FAM	NFQ-MGB	31,28969	31,62498	0,29803	-2,06481	-35,4431
D12	010	LEP	FAM	NFQ-MGB	31,72548	31,62498	0,29803	-1,62902	-35,0073
E1	008P	LEP	FAM	NFQ-MGB	31,40598	31,49476	0,08228	-1,94852	-35,3268
E2	008P	LEP	FAM	NFQ-MGB	31,56846	31,49476	0,08228	-1,78604	-35,1643
E3	008P	LEP	FAM	NFQ-MGB	31,50984	31,49476	0,08228	-1,84466	-35,223
E4	009	LEP	FAM	NFQ-MGB	32,8835	32,91939	0,060303	-0,471	-33,8493
E5	009	LEP	FAM	NFQ-MGB	32,88566	32,91939	0,060303	-0,46884	-33,8471
E6	009	LEP	FAM	NFQ-MGB	32,98901	32,91939	0,060303	-0,36549	-33,7438
E7	009P	LEP	FAM	NFQ-MGB	31,8208	30,97832	0,732797	-1,5337	-34,912
E8	009P	LEP	FAM	NFQ-MGB	30,62545	30,97832	0,732797	-2,72905	-36,1073
E9	009P	LEP	FAM	NFQ-MGB	30,48873	30,97832	0,732797	-2,86577	-36,2441
E10	012P	LEP	FAM	NFQ-MGB	31,20582	31,18705	0,061365	-2,14868	-35,527
E11	012P	LEP	FAM	NFQ-MGB	31,23683	31,18705	0,061365	-2,11767	-35,496
E12	012P	LEP	FAM	NFQ-MGB	31,11849	31,18705	0,061365	-2,23601	-35,6143
F1	006P	LEP	FAM	NFQ-MGB	31,27403	31,15672	0,165998	-2,08047	-35,4588
F2	006P	LEP	FAM	NFQ-MGB	31,22935	31,15672	0,165998	-2,12515	-35,5034
F3	006P	LEP	FAM	NFQ-MGB	30,96679	31,15672	0,165998	-2,38771	-35,766
F4	007	LEP	FAM	NFQ-MGB	32,72506	32,83705	0,244365	-0,62944	-34,0077
F5	007	LEP	FAM	NFQ-MGB	32,66875	32,83705	0,244365	-0,68575	-34,064
F6	007	LEP	FAM	NFQ-MGB	33,11734	32,83705	0,244365	-0,23716	-33,6155
F7	007P	LEP	FAM	NFQ-MGB	31,28529	30,71506	0,499296	-2,06921	-35,4475
F8	007P	LEP	FAM	NFQ-MGB	30,3563	30,71506	0,499296	-2,9982	-36,3765
F9	007P	LEP	FAM	NFQ-MGB	30,50359	30,71506	0,499296	-2,85091	-36,2292
F10	014P	LEP	FAM	NFQ-MGB	32,07011	32,06537	0,17548	-1,28439	-34,6627
F11	014P	LEP	FAM	NFQ-MGB	31,88756	32,06537	0,17548	-1,46694	-34,8452
F12	014P	LEP	FAM	NFQ-MGB	32,23843	32,06537	0,17548	-1,11607	-34,4944
G1	004P	LEP	FAM	NFQ-MGB	31,11732	31,35745	0,237773	-2,23718	-35,6155
G2	004P	LEP	FAM	NFQ-MGB	31,36222	31,35745	0,237773	-1,99228	-35,3706
G3	004P	LEP	FAM	NFQ-MGB	31,5928	31,35745	0,237773	-1,7617	-35,14
G4	005	LEP	FAM	NFQ-MGB	32,95717	32,6046	0,306358	-0,39733	-33,7756
G5	005	LEP	FAM	NFQ-MGB	32,40327	32,6046	0,306358	-0,95123	-34,3295
G6	005	LEP	FAM	NFQ-MGB	32,45337	32,6046	0,306358	-0,90113	-34,2794

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G7	005P	LEP	FAM	NFQ-MGB	31,71308	31,54452	0,22419	-1,64142	-35,0197
G8	005P	LEP	FAM	NFQ-MGB	31,29009	31,54452	0,22419	-2,06441	-35,4427
G9	005P	LEP	FAM	NFQ-MGB	31,63039	31,54452	0,22419	-1,72411	-35,1024
G10	016P	LEP	FAM	NFQ-MGB	30,58578	30,45538	0,382029	-2,76872	-36,147
G11	016P	LEP	FAM	NFQ-MGB	30,75513	30,45538	0,382029	-2,59937	-35,9777
G12	016P	LEP	FAM	NFQ-MGB	30,02522	30,45538	0,382029	-3,32928	-36,7076
H1	002P	LEP	FAM	NFQ-MGB	31,24798	31,14372	0,098846	-2,10652	-35,4848
H2	002P	LEP	FAM	NFQ-MGB	31,13182	31,14372	0,098846	-2,22268	-35,601
H3	002P	LEP	FAM	NFQ-MGB	31,05136	31,14372	0,098846	-2,30314	-35,6814
H4	003	LEP	FAM	NFQ-MGB	31,08652	31,20967	0,185724	-2,26798	-35,6463
H5	003	LEP	FAM	NFQ-MGB	31,11919	31,20967	0,185724	-2,23531	-35,6136
H6	003	LEP	FAM	NFQ-MGB	31,42329	31,20967	0,185724	-1,93121	-35,3095
H7	003P	LEP	FAM	NFQ-MGB	31,99791	31,84208	0,198175	-1,35659	-34,7349
H8	003P	LEP	FAM	NFQ-MGB	31,90929	31,84208	0,198175	-1,44521	-34,8235
H9	003P	LEP	FAM	NFQ-MGB	31,61904	31,84208	0,198175	-1,73546	-35,1138
H10	Control DNA metilado	LEP	FAM	NFQ-MGB	33,3545	33,3783	0,033653	0	-33,3783
H11	Control DNA metilado	LEP	FAM	NFQ-MGB	33,40209	33,3783	0,033653	0,047592	-33,3307
H12	Blanco	LEP	FAM	NFQ-MGB	Undetermined				

Analysis Type   Singleplex   **ΔCт Mean**   33,3782959
Endogenous Control   LEP
RQ Min/Max Confidence Level   95.0
Reference Sample   002P

Supplemental figure 1. Examples of qPCR graphs obtained during the methylation assay.

GENE	FORWARD	RESULT
LEP		
IRS2		Optimal
GLP2R		