Methylation Status of GLP2R, LEP and IRS2 in Small for Gestational Age Children with and without Catch-up Growth

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

What this study adds?
This study presents simultaneous analysis of promoter methylation status of multiple genes that are not related to parental imprinting and that may play a role in the development of metabolic diseases in children with SGA. Investigation of the methylation status of leptin (LEP), glucagon-like peptide-2 receptor (GLP2R), insulin receptor substrate-2 (IRS2) in SGA patients showed no association between IRS2 promoter methylation and the catch-up growth phenotype in this population. In addition, GLP2R and LEP were methylated in all samples. Children with catch-up should be routinely followed to perform timely diagnosis of possible metabolic impairments.

Abstract
Objective: In small for gestational age (SGA) children, catch-up growth could be influenced by methylation of several genes involved in metabolism. Epigenetics may influence the development of metabolic diseases in adulthood. 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 of SGA children. 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: Forty-eight SGA patients were included. Twenty-six (54.2%) had catch-up growth phenotype and 22 (45.8%) 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 55 cm [RIC 52-58] versus median 49.5 cm [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
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). In 2012, in low- and middle-income countries, approximately 23.3 million infants were born SGA (2). In Colombia in the last decade, the SGA rate increased from 70 to 90 SGA newborns per 1,000 live births (3).

SGA is associated with decreased lean mass, muscular mass, and bone mineral content. An SGA infant could have reduced protein, nitrogen and glycogen content in skeletal muscle and liver, due to decreased plasma glucose and insulin concentrations (4). These factors are associated with increased risk of hypoglycemia, hypocalcemia, polycythemia, hyper-viscosity, impaired thermoregulation, and immune dysfunction (5). SGA infants have a 20 times greater risk of mortality than healthy controls during the neonatal period (6). SGA also has poor long-term outcomes, including significantly increased risk of chronic cardiovascular and metabolic diseases compared with Appropriate for Gestational Age infants (7,8,9,10).

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 with low weight and height 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).

The term “epigenetics” 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 well-researched 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 diseases 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 insulin receptor substrate-2 (IRS2), glucagon-like peptide 2 receptor (GLP2R), and leptin (LEP) genes 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. LEP, GLP2R, and IRS2 were categorized according to whether the patient presented with a catch-up growth or non-catch-up growth phenotype during early childhood (before five 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 ethics review board of Fundación Valle del Lili approved the study (Act 084-2014).

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. The natality rate in Cali 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

Demographic 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 of a parental 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 takes time to eat 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 on finishing 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 the classification of patients into two groups: children who presented with the catch-up growth phenotype; and children who presented with 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 Genotek Inc. Ottawa, ON, Canada), and stored at 4 °C until DNA extraction and analysis. Catch-up growth was defined as the height velocity above the limits of normal for age for at least one 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 Waltham, MA, USA). 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 (Supplemental 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; and CpG site 3, *GLP2R* promoter: 1108-1507.

Qualitative methylation analysis of genomic DNA was performed by real-time polymerase chain reaction (PCR) assay, using an EpiTect MethyLight PCR kit (Qiagen Inc., Germantown, MD, USA). This methylation-specific assay is comprised of two 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, USA), 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.

| Variable                              | Non-catch-up growth | Catch-up growth | p value |
|---------------------------------------|---------------------|-----------------|---------|
| 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 (%)                       | 10 (45.5)           | 3 (11.5)        | 0.008   |
| Slow eater                            | 10 (45.5)           | 3 (11.5)        | 0.008   |
| Voracious eater                       | 2 (9.1)             | 11 (42.5)       | 0.555   |
| Lack of appetite                      | 10 (45.5)           | 12 (46.2)       | 0.555   |
| Hours of physical activity/per week*  | 9 (7-10)            | 7.5 (5-10)      | 0.555   |
| Family history, n (%)                 |                     |                 |         |
| Dyslipidemia                          | 6 (27.3)            | 11 (42.5)       | 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)        | 0.52*   |
| Negative                              | 7 (31.8)            | 7 (26.9)        |         |
| Positive                              | 14 (63.6)           | 15 (57.7)       |         |
| Waist circumference*                  | 49.5 (46-52)        | 55 (52-58)      | <0.001  |

*Median (interquartile range), *Chi-square test performed with inconclusive IRS2 gene methylation status.
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 (19) 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 range (IQR), or mean and standard deviation (SD) if normally distributed. Comparisons were made using the χ² 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 infancy 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 four children with catch-up growth group children and one non-catch-up growth child, the status of promoter methylation could not be determined (Supplemental Figure 1 and Supplemental 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 (12,20). We hypothesized that IRS2 promoter methylation status may play a role in catch-up growth. However, no association was identified 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 Liu et al (21), 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. 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 (22,23). 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, has been demonstrated (23). The similar composition and function of mucosa between the oral mucosa and intestinal mucosa suggests that comparable methylation profiles between saliva and intestinal tissue might exist, and strengthens the idea that
saliva has the potential to be used as an alternative for more difficult to sample tissues (22).

Promoter methylation of GLP2R and LEP was observed in all samples, suggesting the population-wide down-regulation of GLP2 and LEP. These genes have been physiologically associated with appetite control, satiety, and glucose homeostasis (24). 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 an increased methylation status among SGA children (25). Reynolds et al (26), in 2017, suggested that high-birth-weight babies showed an increased expression levels of obesity-related genes including lipoprotein lipase (LPL) and LEP receptor (LEPR). 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 (27), 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. IRS1 and IRS2 proteins play roles during the regulation of the insulin signal transduction pathway, through phosphorylation and binding with the insulin receptor. IRS1 is expressed predominately in skeletal muscle, whereas IRS2 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 (28). A recent study showed that the downregulation of IRS2 in an SGA murine model was associated with abnormal glucose metabolism (29). 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 (26).

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 should be examined further but 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 of 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.

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Ethics
Ethics Committee Approval: The institutional ethics review board of Fundación Valle del Lili approved the study (Act 084-2014, date: 21.04.2014).

Informed Consent: Written informed consent was obtained from all patients’ parents before study participation.

Peer-review: Externally peer-reviewed.

Authorship Contributions
Surgical and Medical Practitioners: Mario Angulo, Ximena García, Rodrigo Lemus, Concept: Mario Angulo, Design: Mario Angulo, Diana M. Dávalos, Harry Pachajoa, Data Collection or Processing: Ximena García, Rodrigo Lemus, Danielle Floyd-Aristizábal, Ana M. Aristizabal, Lorena Díaz-Ordoñez, Diana Ramírez-Montaño, Analysis or Interpretation: Mario Angulo, Lorena Díaz-Ordoñez, Harry Pachajoa, Diana Ramírez-Montaño, Laura Torres-Canchala, Literature Search: Diana Ramírez-Montaño, Laura Torres-Canchala, Writing: Diana Ramírez-Montaño, Laura Torres-Canchala.

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**Supplemental Figure 1. Examples of quantitative polymerase chain reaction graphs obtained during the methylation assay**

| Gene  | Forward | Result |
|-------|---------|--------|
| LEP   | ![LEP](image1) | Optimal |
| IRS2  | ![IRS2](image2) |        |
| GLP2R | ![GLP2R](image3) |        |
### 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 | CGCACTACGAAACCCTCCTCTAACC | GGTTTGGACGTTAGGGAAGTTTA |
| IRS2 | AAGTTTAATTGCGAGTAGTCTGCG | ACCGAATCTGCCGCTACATCCCA | TCCAAATAATCTGAAAATPTCTACGC |
| GIP | CGCCCAAACTAACAACACAATAACG | CTCTACAAACCTGCCCTTACGAATT | CGCCCAAATCAAACAAAATAACCC |
| GLP2R | GAATTTTGAAAGATTTCCGTAATTTTAG | ACACCGCAAACAACCTCCTCTACATTCC | AAATACATCTCTCTACCGTCCAAA |
| IG2F | TTTGCGTGGTTTGGCTGTAATTTCG | TAAACCCTCTACGAAACCTCTCTACCA | TACTACGTACGCAAACCGAACAA |

### Supplemental Table 2. Relative quantification for LEP in samples according to ΔΔCT

| Well | Sample name | Target name | Reporter | Quencher | C_T | CT mean | CT SD | ΔC_T | ΔΔC_T |
|------|-------------|-------------|----------|----------|-----|---------|------|------|-------|
| A1   | 017         | LEP         | FAM      | NFQ-MGB  | 32.48601 | 32.73549 | 0.309419 | -0.86849 | -34.2468 |
| A2   | 017         | LEP         | FAM      | NFQ-MGB  | 33.0804  | 32.73549 | 0.309419 | -0.2741 | -33.6524 |
| A3   | 017         | LEP         | FAM      | NFQ-MGB  | 32.63406 | 32.73549 | 0.309419 | -0.72044 | -34.0987 |
| A4   | 017P        | LEP         | FAM      | NFQ-MGB  | 31.29929 | 31.27273 | 0.110781 | -0.05521 | -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 | -2.20542 | -35.5817 |
| A7   | 018         | LEP         | FAM      | NFQ-MGB  | 32.19761 | 31.92159 | 0.379013 | -1.15689 | -35.4335 |
| A8   | 018         | LEP         | FAM      | NFQ-MGB  | 32.0777  | 31.92159 | 0.379013 | -1.2768 | -35.6651 |
| 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  | 32.25765 | 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 | -35.0063 |
| 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 |
| Sample | Type     | Primer | Control | ΔC<sub>T</sub> Mean | 95% CI Lower | 95% CI Upper |
|--------|----------|--------|---------|---------------------|--------------|--------------|
| G6     | 005      | LEP    | FAM     | 32,45337            | 32,604       | -0,9013      | -34,2794     |
| G7     | 005P     | LEP    | FAM     | 31,71308            | 31,54452     | 0,22419      | -1,64142     | -35,0197     |
| G8     | 005P     | LEP    | FAM     | 31,29009            | 31,54452     | 0,22419      | -2,06441     | -35,4427     |
| G9     | 005P     | LEP    | FAM     | 31,63039            | 31,54452     | 0,22419      | -1,72411     | -35,1024     |
| G10    | 016P     | LEP    | FAM     | 30,58578            | 30,45538     | 0,382092     | -2,76872     | -36,147      |
| G11    | 016P     | LEP    | FAM     | 30,75513            | 30,45538     | 0,382092     | -2,59937     | -35,9777     |
| G12    | 016P     | LEP    | FAM     | 30,02522            | 30,45538     | 0,382092     | -3,32928     | -36,7076     |
| H1     | 002P     | LEP    | FAM     | 31,24798            | 31,14372     | 0,098846     | -2,10652     | -35,4848     |
| H2     | 002P     | LEP    | FAM     | 31,13182            | 31,14372     | 0,098846     | -2,22268     | -35,601      |
| H3     | 002P     | LEP    | FAM     | 31,05136            | 31,14372     | 0,098846     | -2,30314     | -35,6814     |
| H4     | 003      | LEP    | FAM     | 31,08652            | 31,20967     | 0,185724     | -2,26798     | -35,6463     |
| H5     | 003      | LEP    | FAM     | 31,11919            | 31,20967     | 0,185724     | -2,23531     | -35,6136     |
| H6     | 003      | LEP    | FAM     | 31,42329            | 31,20967     | 0,185724     | -1,93121     | -35,3095     |
| H7     | 003P     | LEP    | FAM     | 31,99791            | 31,84208     | 0,198175     | -1,35659     | -34,7349     |
| H8     | 003P     | LEP    | FAM     | 31,90929            | 31,84208     | 0,198175     | -1,44521     | -34,8235     |
| H9     | 003P     | LEP    | FAM     | 31,61904            | 31,84208     | 0,198175     | -1,73546     | -35,1138     |
| H10    | Control DNA metilado | LEP    | FAM     | 33,3545             | 33,3783      | 0,033653     | 0           | -33,3783     |
| H11    | Control DNA metilado | LEP    | FAM     | 33,40209            | 33,3783      | 0,033653     | 0,047592     | -33,3307     |
| H12    | Blanco   | LEP    | FAM     | Undetermined        |             |             |             |             |

**Analysis Type**: Singleplex  
**Endogenous Control**: LEP  
**RQ Min/Max Confidence Level**: 95.0  
**Reference Sample**: 002P  
**SD**: Standard deviation  

**ΔC_{T} Mean**: 33.3782959