Differential methylation in glucoregulatory genes of offspring born before vs. after maternal gastrointestinal bypass surgery

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Obesity and overnutrition during pregnancy affect fetal programming of adult disease. Children born after maternal bariatric gastrointestinal bypass surgery (AMS) are less obese and exhibit improved cardiometabolic risk profiles carried into adulthood compared with siblings born before maternal surgery (BMS). This study was designed to analyze the impact of maternal weight loss surgery on methylation levels of genes involved in cardiometabolic pathways in BMS and AMS offspring. Differential methylation analysis between a sibling cohort of 25 BMS and 25 AMS (2–25 y-old) offspring from 20 mothers was conducted to identify biological functions and pathways potentially involved in the improved cardiometabolic profile found in AMS compared with BMS offspring. Links between gene methylation and expression levels were assessed by correlating genomic findings with plasma markers of insulin resistance (fasting insulin and homeostatic model of insulin resistance). A total of 5,698 genes were differentially methylated between BMS and AMS siblings, exhibiting a preponderance of glucoregulatory, inflammatory, and vascular disease genes. Statistically significant correlations between gene methylation levels and gene expression and plasma markers of insulin resistance were consistent with metabolic improvements in AMS offspring, reflected in genes involved in diabetes-related cardiometabolic pathways. This unique clinical study demonstrates that effective treatment of a maternal phenotype is durably detectable in the methylome and transcriptome of subsequent offspring.

developmental origins | epigenetics | intrauterine environment | glucose metabolism | adiposity

Childhood overweight and obesity have increased dramatically in recent decades (1). Parental obesity increases the risk of obesity in offspring through genetic, biological, and environmental influences evident in associations between maternal body mass index (BMI), offspring adiposity, and cardiovascular disease (CVD) risk factors (2–4). Maternal obesity, weight gain, increased interpregnancy BMI, and gestational diabetes all increase risks of offspring obesity and type 2 diabetes mellitus (T2DM) (5, 6). Several genetic studies of nutritional response and metabolic control support the hypothesis that specific epigenetic changes contribute to early nutritional fetal programming, increasing the risk of metabolic disorders later in life (7–9).

The intrauterine environment including nutritional factors, toxic exposures, and maternal stress participates in fetal programming (10). Maternal diet and adiposity impact methylation levels affecting specific gene functions. Prenatal exposure to famine during the Dutch hunger winter of 1944 is associated with obesity with less DNA methylation ("undermethylation") of the imprinted insulin-like growth factor 2 (*IGF2*) gene in exposed offspring relative to their unexposed siblings (11). Recently, retinoid X receptor alpha (*RXRA*) promoter methylation was demonstrated to correlate with increased adiposity in two independent cohorts of children of mothers with low carbohydrate intake (12).

Weight loss surgery is the most effective treatment for severe obesity, improving glucose and lipid metabolism (13, 14) and preventing arterial hypertension and T2DM (15, 16). Several studies have demonstrated changes in genes associated with insulin action after bariatric operations (17, 18). In previous studies, we demonstrated that the prevalence of obesity in children born after maternal bypass surgery (AMS) was significantly lower than in siblings born before maternal surgery (BMS) (19) and was associated with greater insulin sensitivity, less adiposity, hypertension, and dyslipidemia compared with BMS offspring, suggesting that these improvements in cardiometabolic markers may be attributable to an improved intrauterine environment (20).

This study was designed to analyze the impact of maternal gastrointestinal bypass surgery on methylation levels of genes of cardiometabolic pathways in AMS compared with BMS offspring. Differential methylation analyses of candidate genes and pathway analyses identified links between gene methylation, gene expression, and insulin resistance in this unique sibling cohort.

Results

Characteristics of Mothers and Offspring. Mean postoperative follow-up was 12 y and 2 mo at the time of the study. Table 1 exhibits significant improvements in plasma lipids ($P \leq 0.01$ for all) and reductions in insulin resistance ($P \leq 0.001$) and blood pressure [systolic blood pressure (SBP) and diastolic blood pressure (DBP); $P \leq 0.001$] with a trend toward lower plasma glucose levels ($P = 0.06$).

Offspring ages ranged between 32 mo and 24 y and 11 mo, with similar sex distribution in the two groups (60% female; Table 2). BMS offspring were older in comparison with AMS siblings (mean ages of 14.9 y and 9.6 y, respectively; $P = 0.002$) and 40% of BMS offspring were prepubertal whereas 80% of AMS siblings were prepubertal. Prepubertal offspring were 7.9 ± 3.1 (mean ± SD) y old, with a BMI percentile of 65.7 ± 34.9, *Z*-score of 1.07 ± 1.61, and 22.2% ± 11.4% body fat. Postpubertal male offspring mean age was 19.6 ± 3.1 y, with a BMI percentile of 98.2 ± 4.0, whereas postpubertal female offspring were 18.3 ± 4.3 y old with a lower BMI percentile of 58.2 ± 42.3. Following adjustments for the effect of sex and puberty, BMS offspring demonstrated higher weight, height, and waist and hip

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo [accession nos. GSE44798 (gene methylation arrays) and GSE44407 (gene expression arrays)].

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Table 1. Mothers' characteristics before (BS) and after surgery (AS; $n = 20$)

Characteristics	BS	AS
Age, y**	29.5 ± 4.1	41.0 ± 5.3
Anthropometric data		
Weight, kg**	121.5 ± 18.2	74.8 ± 11.9
Height, m	164.5 ± 5.1	164.9 ± 6.6
BMI**	45.0 ± 7.2	27.6 ± 4.8
Glucose metabolism		
Fasting glucose, mmol/L***	5.72 ± 2.37	4.70 ± 0.32
Insulin, microunits/mL†,**	39.9 ± 25.6	9.0 ± 2.4
HOMA-IR†,**	14.6 ± 13.1	1.9 ± 0.6
Lipidemia		
TG, mmol/L*	1.65 ± 0.76	0.97 ± 0.41
HDL-C, mmol/L**	1.14 ± 0.26	1.39 ± 0.25
LDL-C, mmol/L**	3.00 ± 0.83	1.68 ± 0.50
Total-C, mmol/L**	4.93 ± 0.79	3.52 ± 0.49
Total-C/HDL-C**	4.63 ± 1.69	2.59 ± 0.59
Blood pressure		
SBP, mm Hg**	137.4 ± 11.4	112.2 ± 9.5
DBP, mm Hg**	88.8 ± 10.8	70.2 ± 14.3

Mean ± SD of mothers BS vs. AS and paired *t* test: *$P \leq 0.01$; **$P \leq 0.001$; ***$P \leq 0.10$. Abbreviations: AS, after surgery; BMI, body mass index; BS, before surgery; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C; low-density lipoprotein cholesterol; SBP, systolic blood pressure; plasma TG, triglycerides; Total-C, total cholesterol.
†Insulin levels and HOMA-IR index were available for five women BS.

girth ($P < 0.05$) than AMS offspring who showed a trend toward lower percentage of body fat ($P = 0.07$). With respect to cardiometabolic risk factors, AMS offspring demonstrated improved fasting insulin levels ($P = 0.03$) and homeostatic model of insulin resistance (HOMA-IR) index ($P = 0.03$) and had lower blood pressure compared with BMS offspring ($P < 0.05$). Offspring characteristics according to BMS and AMS groups are presented in Table 2 (unadjusted values) and in Table S1 (adjusted values).

Offspring Methylation Profiles. We detected 485,294 (99.95%) of 485,557 probes on the array. Methylation levels (β-values) between BMS and AMS were analyzed and 14,466 probes exhibited significant differences [false discovery rate (FDR)-corrected DiffScore $\geq |13| \sim P \leq 0.05$], corresponding to 5,698 unique genes with available accession numbers. The most significant differentially methylated probes between groups with differences in β-values (delta β) are presented in Table S2. Methylation levels of 3 overmethylated [cg14018024, laminin, gamma 3 (*LAMC3*); cg15012662, Ca2+-dependent activator protein for secretion 2 (*CADPS2*); and cg16312514, SH3 and multiple ankyrin repeat domains 2 (*SHANK2*)] and 2 undermethylated [cg04850148, chemokine (C-C motif) ligand 4-like 2 (*CCL4L2*); and cg20017683, Rh blood group, D antigen (*RHD*)] CpG sites from the list of most significant differentially methylated probes (Table S2) were validated. Significant correlations between array and EpiTYPER data were observed for probes located in *CADPS2*, *LAMC3*, *RHD*, and *SHANK* (r ranged from 0.643 to 0.916, $P < 0.0001$ for all) whereas no correlation for CpG site located in *CCL4L2* ($r = -0.031$, $P = 0.84$) was found (Table S3). Despite slight differences in group composition between EpiTYPER ($n = 48$) and array ($n = 50$) data, significant differences between BMS and AMS offspring were confirmed for *CADPS2*, *LAMC3* and *SHANK2*. Similar delta β-values were also observed using both methods (array vs. EpiTYPER: *CADPS2*, 0.13 vs. 0.09; *LAMC3*, 0.12 vs. 0.07; *SHANK2*, 0.14 vs. 0.06).

Identification of Altered Functions and Pathways. The ingenuity pathway analysis (IPA) system mapped 5,607 of the 5,698 genes differentially methylated between AMS and BMS siblings, classified them according to functions and pathways, and calculated *P* values for function or pathway overrepresentation among datasets. The most statistically significant biological functions according to IPA were those related to autoimmune disease, pancreas disorders, diabetes mellitus, and disorders of glucose metabolism. Most represented functions were in three categories listed in Table 3. The genes from the most represented functions identified from differential methylation are presented in Table S4. IPA revealed 160 canonical pathways significantly overrepresented in the list of submitted probes ($P < 0.05$), among which immune response (antigen presentation, dendritic cell maturation, and role of nuclear factor of activated T cells in regulation of the immune response) and diabetes signaling pathways can be found. Fig. S1 shows the top 20 differentially methylated pathways with numbers of differentially methylated genes and associated *P* values. The differentially methylated genes from these pathways are listed in Table S4.

Gene Expression Analysis. Among the 47,323 probes, 33.9% showed significant gene expression. Differential expression analysis revealed a total of 862 probes differentially expressed ($P \leq 0.05$; Welch's unequal-variance *t*-statistic) with a symmetrical fold change $\geq |1.2|$ (fold change <0.83 or >1.2). A list of the most up- and down-regulated genes is found in Table S5. Five genes with immune-, inflammatory-, or glucose metabolism-related functions from the list of most up- and down-regulated genes [G protein-coupled receptor 44 (*GPR44*), immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides (*IGJ*), orosomucoid 1 (*ORM1*), solute carrier family 2 (facilitated glucose transporter), member 11 (*SLC2A11*), and triggering

Table 2. Offspring characteristics

Characteristics	BMS†	AMS†
N (male %)	25 (40%)	25 (40%)
Age, y**	14.9 ± 6.2	9.6 ± 5.3
Anthropometric data		
BMI percentile	69.2 ± 40.7	65.7 ± 32.6
BMI *Z*-score	1.84 ± 2.08	0.85 ± 1.47
Waist girth, cm*	88.5 ± 29.5	66.7 ± 16.0
Hip girth, cm**	92.6 ± 27.6	69.2 ± 15.6
Waist-to-hip ratio	0.95 ± 0.07	0.97 ± 0.08
Fat percent‡,***	29.9 ± 13.9	21.4 ± 10.3
Glucose metabolism§		
Fasting glucose, mmol/L	4.92 ± 0.43	4.71 ± 0.43
Insulin, microunits/mL; log10*	18.8 ± 12.2	11.3 ± 7.4
HOMA-IR, log10*	4.27 ± 3.23	2.43 ± 1.72
Lipidemia§		
TG, mmol/L	1.04 ± 0.43	0.82 ± 0.37
HDL-C, mmol/L	1.30 ± 0.29	1.30 ± 0.25
LDL-C, mmol/L	2.65 ± 0.55	2.53 ± 0.58
Total-C, mmol/L	4.43 ± 0.66	4.21 ± 0.58
Total-C/HDL-C	3.56 ± 0.93	3.37 ± 0.85
Blood pressure		
SBP, mm Hg*	111.3 ± 14.7	97.4 ± 14.6
DBP, mm Hg**	64.6 ± 9.8	53.4 ± 12.8

P values adjusted for sex and puberty status comparing BMS to AMS offspring: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.10$. Abbreviations: AMS, after maternal surgery; BMI, body mass index; BMS, before maternal surgery; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; plasma TG, triglycerides; Total-C, total cholesterol.
†Values presented (mean ± SD) are untransformed and unadjusted values. Adjusted values (adjusted means ± SD) are presented in Table S1.
‡Subjects 6 y or older (BMS, $N = 23$; AMS, $N = 17$).
§Values from $N = 25$ BMS and $N = 21$ AMS.

Table 3. Most frequently represented functions resulting from analyses of differential methylation and IPA

Categories	IPA biological functions	*P* value* (*N*†)
Metabolic and gastrointestinal diseases/endocrine system disorders	Pancreas disorder	1.10×10^{-75} (1,152)
	Diabetes mellitus	9.98×10^{-73} (1,066)
	Disorder of glucose metabolism	1.13×10^{-71} (1,114)
	Non-insulin-dependent diabetes mellitus	1.61×10^{-54} (651)
	Insulin-dependent diabetes mellitus	4.74×10^{-38} (489)
Immunological, inflammatory, connective tissue, skeletal and muscular disorders/gastrointestinal diseases	Autoimmune disease	6.50×10^{-76} (1,073)
	Rheumatic disease	8.97×10^{-59} (855)
	Arthritis	2.39×10^{-57} (811)
	Rheumatoid arthritis	3.80×10^{-54} (721)
	Inflammatory bowel disease	1.79×10^{-48} (565)
	Crohn's disease	3.11×10^{-45} (522)
Cardiovascular diseases	Vascular disease	2.92×10^{-59} (696)
	Arteriosclerosis	9.39×10^{-59} (636)
	Atherosclerosis	1.46×10^{-58} (633)
	Arterial occlusive disease	1.67×10^{-58} (636)
	Ischemia	2.62×10^{-58} (627)
	Coronary artery disease	2.72×10^{-57} (580)
	Coronary disease	7.59×10^{-57} (592)
	Myocardial ischemia	2.38×10^{-56} (593)
	Heart disease	3.59×10^{-53} (727)

*P values obtained using a right-tailed Fisher's exact test for the measurement of the likelihood that differentially methylated genes participate in each function.
†*N*, number of differentially methylated genes.

receptor expressed on myeloid cells 1 (*TREM1*)] were validated by real-time PCR (RT-PCR). Similar mean fold expression difference (MFED) and significant correlations (r ranged from 0.635 to 0.891, $P < 0.0001$ for all) between microarray and RT-PCR data were obtained for four genes (array MFED vs. RT-PCR MFED: *GPR44*, 1.71 vs. 1.41; *IGJ*, 1.93 vs. 1.94; *ORM1*, −2.37 vs. −2.37; *TREM1*, −2.27 vs. −1.46) whereas a trend toward significant correlation ($r = 0.272$, $P = 0.07$) was observed for *SLC2A11*.

Integration of Methylation and Expression Data. For relevant biological functions listed in Table 3 (diabetes mellitus, autoimmune disease, and vascular disease functions), correlations between gene methylation and expression levels were computed. After merging, probe pairs with both expression data and differential methylation were kept. Correlation coefficients were calculated for 1,129, 1,414 and 649 probe pairs and 319, 377, and 164 were significant ($P \leq 0.05$), respectively, representing 25–28% of the pairs. Stratification according to methylation state (under- or overmethylated in AMS) resulted in higher mean correlations (Table 4).

Correlations Between Gene Methylation, Gene Expression, and Insulin Resistance. We selected the most significant overrepresented glucoregulatory pathway (P value $= 3.95 \times 10^{-5}$; −log P value = 5.40), the type 1 diabetes mellitus (T1DM) signaling pathway, as representative of glucoregulatory pathways; T2DM and insulin-like growth factor 1 (IGF1) signaling pathways were also identified as overrepresented among differentially methylated genes. Among the 121 genes assigned to the T1DM pathway by IPA, 48 genes (140 probes) were found to be differentially methylated (Fig. S1 and Table S4). Correspondence between methylation and expression probes was found for 126 of the differentially methylated probes (35 genes), where correlation analysis identified 62 significant correlations representing 17 different genes (Table S6). Correlations found between gene methylation and expression according to CpG site localization (promoter or gene body) are provided in Table S6.

Comparisons between gene methylation and insulin levels led to the identification of significant correlations for 12 different genes (42 CpG sites; Table S7). Among the 17 genes analyzed, correlations between gene expression and insulin levels were significant for 8 (9 transcripts; Table S7). Six among the 17 genes with significant methylation-expression correlations demonstrated correlations between gene methylation, gene expression, and fasting insulin levels [*CD28*, *CD247*, *CD3E*, *HLA*-DM beta (*DMB*), *HLA*-DQ beta 1 (*DQB1*), and signal transducer and activator of transcription 1, 91kDa (*STAT1*)]. Validation of gene

Table 4. Correlations between gene methylation and expression for differentially methylated genes according to biological functions

Biological functions	*N* genes	Meth probes*	Meth probes with express†	All probes (*N*)‡	Undermeth in AMS (*N*)‡	Overmeth in AMS (*N*)‡
Diabetes mellitus	1,066	2,550	1,129	0.127 (319)	−0.334 (88)	0.302 (231)
Autoimmune disease	1,073	2,722	1,414	0.078 (377)	−0.316 (126)	0.277 (251)
Vascular disease	696	1,758	649	0.160 (164)	−0.348 (40)	0.323 (124)

Abbreviations: AMS, after maternal surgery; Express, expression; Meth, methylation; *N*, number; Overmeth, overmethylated; Undermeth, undermethylated.
*Number of genes with differential methylation.
†Number of genes with differential methylation and expression data.
‡Mean correlation for significantly correlated probe pairs ($P \leq 0.05$) with both detected expression and significant differential methylation.

expression data was conducted for *CD247*, *CD28*, *CD3E*, *HLA-DMB*, and *STAT1*. For all 5 genes significant correlations (*CD247* $r = 0.677$, $P < 0.0001$; *CD28* $r = 0.311$, $P = 0.04$; *CD3E* $r = 0.556$, $P < 0.0001$; *HLA-DMB* $r = 0.658$, $P < 0.0001$; *STAT1* $r = 0.677$, $P < 0.0001$) between gene array and RT-PCR data were observed. Gene methylation levels were obtained for *CD247*, *CD3E*, and *HLA-DMB* (2 CpG sites each), using Sequenom EpiTYPER technology. For all 6 CpG sites significant correlations (r ranged from 0.791 to 0.924, $P < 0.0001$ for all) between gene array and EpiTYPER data were observed (Table S3).

Discussion

This unique clinical study demonstrates that sustained amelioration of a pregravid maternal disease phenotype following successful treatment is durably detectable in subsequent offspring's phenotype, methylome, and transcriptome. Comparing methylation profiles in siblings born after to those of siblings born before maternal bypass surgery, we found that 3.0% of probes were differentially methylated and were correlated with functionally relevant gene expression. Through the analysis of gene functions and pathways, we identified differences in gene methylation potentially responsible for the improved cardiometabolic risk profile with greater insulin sensitivity in AMS compared with BMS siblings.

Although our findings are unique, they are supported by other studies. Altered functions of differentially methylated genes involved in glucose-related disorders in offspring born after maternal surgery are consistent with findings of maternal diet-induced epigenetic changes in glucose homeostasis in the offspring (12). Furthermore, a recent pilot study in patients after gastric bypass revealed gene expression changes of diabetes- and obesity-related pathways (17).

Strong overrepresentation of glucose-related functions and differences in fasting insulin levels and HOMA-IR index found between BMS and AMS siblings suggested analyzing glucoregulatory pathways. Glucose metabolism pathways including those termed T1DM, T2DM, and IGF1 in IPA were differentially altered in BMS and AMS siblings. The most significant overrepresented glucoregulatory pathway, the T1DM signaling pathway, was selected as representative of glucoregulatory pathways and demonstrated general overmethylation with several differentially methylated genes known to be involved in immune response, encoding cytokines, T-cell receptor, or major histocompatibility complex (MHC) class I or class II subunits (Table S6). Regulation of immune response genes by differential CpG methylation in offspring is coherent with the pathogenesis of diabetes. Tumor necrosis factor-α and interleukin-1 (IL-1) signaling pathways are known to be involved in pancreatic β-cell death (21). Accordingly, *IL-1* was identified among genes with differential methylation (Table S4) and an overrepresentation of the IL-1 signaling pathway was found. Alterations of the T1DM signaling pathway are supported by the strong correlations observed between gene methylation, expression, and insulin resistance for six genes: Two are part of the MHC class II (*HLA-DMB* and *HLA-DQB1*); three are related to T-cell proliferation, survival, cytokine production, and coupling of antigen recognition to intracellular signal-transduction pathways (*CD247*, *CD28*, and *CD3E*) (22); and another (*STAT1*) is a transcriptional activator acting in response to cytokines and growth factors known to be involved in β-cell death (21). Although speculative, regulation of glucoregulatory pathways mediated through the regulation of immune and inflammatory genes may explain the improvements observed in insulin resistance.

Potential mechanisms for the acquisition of increased risks of offspring obesity and T2DM have been proposed. Epigenetic mechanisms may be involved through the control of placental and embryonic epigenetic machinery and regulated by the in utero environment including hormonal factors, inflammatory cytokines, and nutrients availability. Hyperglycemia and oxydative stress interfere with DNA methylation and may affect long-term gene expression programming (23). Food intake and energy balance may lead to hypothalamic programming and hormonal changes in the offspring (24, 25). Inversely, the hypothalamus plays an important role in the regulation of energy and glucose homeostasis (26, 27).

The variability in correlation coefficients of functions found in the present study is concordant with results in studies from HapMap cell lines (28). Most of the genes with more than one significant correlation between CpG site methylation and expression demonstrate the same directionality and are in general agreement with studies reporting CpG methylation correlated across genomic regions of 1–2 kb (29, 30). However, we also observed opposite correlations for CpG sites within the same genomic region, demonstrating the need for complementary gene-based approaches with a higher coverage of specific gene regions such as methylation-specific sequencing, rather than a genome-wide approach with lower coverage.

This study has limitations, some real and some perceived. It was performed in a racially and ethnically homogeneous, tight-knit population sharing a strong sense of community, family values, politics, and religion, potentially limiting its applicability to more diverse populations. However, this limitation is also a strength, enabling us to achieve remarkable follow-up: 90% of all children born AMS were located (by P.M.). Furthermore the Canadian healthcare system provided an organizational and financial basis for high patient retention.

The size of our population may appear small, but the rarity of gastrointestinal bypass surgery, especially biliopancreatic bypass, and the relatively low pregnancy rates after maternal surgery limit the pool of offspring. Thus, we were unable to achieve statistically significant differences in several parameters comparing BMS to AMS siblings. Nevertheless, this smaller cohort [66% of which was part of the earlier larger cohort (20)] did not exhibit any statistically significant differences compared with the respective larger BMS and AMS cohorts. It is necessary to keep in mind that the offspring, several of whom were emancipated, were relatively young, seeing no need for doctor visits or testing. Thus, we were limited to blood sampling yielding monocytes in lieu of biopsies of target tissues (e.g., fat, muscle, or liver) for gene methylation studies.

Tanner classifications for precise ascertainment of puberty according to sex and age were not available. Sex-specific autosomal methylation patterns have not been found (28, 30) and genome-wide methylation analysis demonstrates that age-related differential methylation is site and location dependent (31, 32) and concerns only a very small proportion of CpG sites (1.3%; 360 sites on 27,578 CpGs analyzed) (33). A recent study aiming to build a prediction model for the aging methylome combining genome-wide methylation analysis with a data mining tool revealed an overenrichment of associations near genes with functions related to cell communication, locomotion, proliferation, and growth (34). To our knowledge, the impact of puberty on blood methylome has not been assessed on a genome-wide scale. According to the lack of sex-specific autosomal methylation patterns and to the limited impact of age on gene methylation, it is reasonable to speculate on the low effect of puberty on gene methylation in the present study, especially in regard to glucoregulatory pathways. We chose to analyze CpG site methylation as epigenetic marks owing to their overall stability although the present study revealed discordant or complex patterns relating gene expression and insulin resistance, thus requiring further studies to elucidate the impact and mechanisms of methylation changes. This is not a mechanistic study; however, several mechanisms are well studied in laboratory animals, associating gene modifications, engineered or environmental, affecting relevant conserved phenotypic traits (8–12, 37). Last but not least it is important to keep in mind that the excellent metabolic results we achieved with biliopancreatic bypass are likely not applicable to purely gastric restrictive bariatric banding operations.

In conclusion, our study as well as others has demonstrated health benefits for offspring after maternal bypass surgery (20, 35). We demonstrate unique altered methylation and gene expression profiles, comparing offspring born before to those born after maternal bypass surgery. In agreement with our previous study demonstrating simultaneous improvements in insulin resistance and lower C-reactive protein levels (20), we observed an overrepresentation of genes with glucose-metabolic and inflammation-related functions. Although the relative contributions of parental phenotype and genotype, fetal genotype, and intrauterine environmental factors remain to be determined, this study demonstrates powerful effects of maternal bypass surgery on offspring at both epigenetic and transcriptional levels.

Materials and Methods

Subjects. Women from the eastern part of the province of Quebec (Canada) who had given birth before and after biliopancreatic bypass with duodenal switch for severe obesity performed at the Québec Heart and Lung Institute were eligible for the current study (36). We studied a subset of 20 unrelated mothers aged 34–51 y, residing in Quebec City and suburbs (administrative regions of Capitale-Nationale, Mauricie, and Chaudière-Appalaches) and having offspring currently aged 2–25 y, 25 born before and 25 born after surgery. Mothers and offspring attended the Québec Heart and Lung Institute or a regional hospital for assessment between July and October 2010. There were 17 mothers with siblings born before and after surgery (23 BMS and 24 AMS), 2 with BMS offspring only (2 BMS), and 1mother with only 1 AMS offspring. This study was approved by the Québec Heart and Lung Institute Ethics Committee. Written informed consent was obtained from adults and parents and assent from minor offspring before inclusion in the study.

Maternal presurgical data were obtained from medical records. At the interview, weight and body fat percentage were measured using bioelectrical impedance (Tanita Corporation of America). Standardized procedures were used to measure body height, waist and hip girth, and resting SBP and DBP. BMI was calculated for mothers and BMI percentile for children was obtained from the National Health and Nutrition Examination Survey 2000 chart (37). BMI *Z*-score was calculated for children, using charts from the Centers for Disease Control and Prevention (38). Blood samples were collected after an overnight fast from an antecubital vein into tubes containing EDTA and PAXgene Blood RNA collection tubes (Qiagen). Plasma lipid, glucose, and insulin concentrations were measured, as previously described (39). The HOMA-IR index was calculated as glucose × insulin/22.5.

DNA Methylation Analysis. Genomic DNA was isolated from buffy coat, using the GenElute Blood Genomic DNA kit (Sigma). Quantification was conducted using NanoDrop Spectrophotometer (Thermo Scientific) and PicoGreen DNA methods. DNA (1 μg) was bisulfite converted and quantitative DNA methylation analysis was carried out at the McGill University and Génome Québec Innovation Centre (Montreal), using the Infinium HumanMethylation450 BeadChip (Illumina) according to the manufacturer's instructions.

For visualization, analysis, and extraction of methylation data, the GenomeStudio software version 2011.1 (Illumina) and the Methylation Module were used. Methylation levels (β-values) were estimated as the ratio of signal intensity of the methylated alleles to the sum of methylated and unmethylated intensity signals of the alleles: $\beta = M/(U + M)$, where M was the fluorescence level of the methylation probe and U was the fluorescence level of the unmethylated probe. The β-values vary from 0 (no methylation) to 1 (100% methylation). Data correction (background subtraction and normalization) was applied using internal control probe pairs. Differential methylation analysis between BMS and AMS groups (mean β-values) was conducted for detected CpG sites (detection P value < 0.05), using the Illumina Custom model. FDR-corrected P values and DiffScore were computed: DiffScore is a differential methylation score calculated from P values and delta β-values between the two groups. It was used here as the main statistical results for comparison of BMS and AMS siblings.

A mass spectrometry-based approach (EpiTYPER; Sequenom) was used to validate methylation levels of CpG sites (*i*) showing differential methylation and (*ii*) located in nonpolymorphic regions [cg15012662, *CADPS2*; cg04850148, *CCL4L2*; cg14018024, *LAMC3*; cg20017683, *RHD*; cg16342514, *SHANK2*; and cg21966345, transcription factor Dp-1 (*TFDP1*)]. Genomic DNA (500 ng) was bisulfite converted using the EZ DNA Methylation kit (Zymo Research) and assays were conducted according to manufacturer's recommendations at the McGill University and Génome Québec Innovation Centre. *TFDP1* was excluded from methylation data validation due to CpG site-containing fragments with equal or overlapping mass, making them irresolvable by mass spectrometry. Similar analyses were also conducted to validate methylation levels of three genes with significant correlations between gene methylation, gene expression, and fasting insulin levels (*CD247*, *CD3E*, and *HLA-DMB*). EpiTYPER assays and methylation levels are presented in Tables S8 and S9, respectively.

Function and Pathway Analysis. Analysis of potentially altered functions and pathways was conducted using the knowledge base of IPA. The differential methylation analysis enabled production of a list of probes with significant DiffScore and corresponding gene accession numbers. When several statistically significant probes were identified for a single gene accession number, the most significant probe was kept. The list of differentially methylated probes with corresponding unique gene accession number was thereafter submitted to IPA. IPA measures the likelihood that these genes participate in a particular function or pathway and calculates P values using a right-tailed Fisher's exact test for each pathway and function. Specific pathway analysis was conducted for the T1DM signaling pathway according to differences identified in insulin levels and HOMA-IR between BMS and AMS siblings.

Gene Expression Analysis. Total RNA was isolated and purified from whole blood, using the PAXgene Blood RNA Kit (Qiagen) following the manufacturer's recommendations. The integrity of the purified RNA was analyzed using both the NanoDrop (Thermo Scientific) and the 2100 Bioanalyzer (Agilent Technologies). Expression levels were measured using the HumanHT-12 v4 Expression BeadChip (Illumina). Microarray experiments were carried out using 250 ng of total RNA and processed according to the manufacturer's instructions at the McGill University and Génome Québec Innovation Centre. Expression data were visualized and analyzed using the FlexArray software (version 1.6) (40) and the Lumi algorithm was used for expression data analysis and normalization. Probe detection analysis was conducted using the FlexArray filter algorithm. To be considered as expressed, a probe had to show a detection P value ≤ 0.05 in at least 25% of samples of a group. The "Significance Analysis of Microarrays" (SAM) algorithm (41) with unequal-variance (Welch's) *t*-statistic was used to assess differences between AMS and BMS siblings for significantly expressed probes. A cutoff of $P \leq 0.05$ was used to detect differentially expressed probes. In addition, fold change and symmetrical fold change were calculated. A cutoff of fold change ≥ 1.2 or ≤ 0.83 (symmetrical fold change ≥ 1.2 or ≤ -1.2) was applied.

Gene expression microarray results were validated using RT-PCR (Applied Biosystems Gene Expression Assays) for five genes from the list of top differentially expressed genes (*GPR44*, NM_004778; *IGJ*, NM_144646; *ORM1*, NM_000607; *SLC2A11*, NM_001024938; and *TREM1*, NM_018643) with immune-, inflammatory- or glucose metabolism-related function. A subset of genes with correlation between gene expression and methylation levels (*CD247*, NM_198053; *CD28*, NM_006139; *CD3E*, NM_000733; *HLA-DMB*, NM_002118; *HLA-DQB1*, NM_002123; and *STAT1*, NM_007315) was also tested using RT-PCR. Expression data were obtained for five of these genes whereas *HLA-DQB1* was excluded from the analysis due to the presence of polymorphic sites in RT-PCR probes. Samples were analyzed in duplicate, using predesigned probes (Hs00173717_m1, Hs00376160_m1, Hs01590790_g1, Hs00368843_m1, Hs00218624_m1, Hs00609515_m1, Hs01007422_m1, Hs01062241_m1, Hs00157943_m1, Hs03054971_m1, and Hs01013996_m1), and calibrated to the *GAPDH* housekeeping gene (endogenous control; Hs99999905_m1), which was measured in triplicate. Relative quantification estimations were performed on an Applied Biosystems 7500 Real Time PCR System.

Statistical Analysis. Data were expressed as mean ± SD for unadjusted values whereas sex and puberty adjusted data were produced using least-squares means and expressed as adjusted means ± SD. The effect of bariatric surgery in mothers was assessed using a within-subject, paired *t* test. Differences in anthropometric data, fasting plasma glucose and insulin levels, lipid profiles, and blood pressure between BMS and AMS siblings were tested using analysis of variance (general linear model, type III sum of squares). Transformations were applied to nonnormally distributed variables (log10 transformed: insulin and HOMA-IR). Results were adjusted for the effect of sex and puberty. In the absence of Tanner scores, we arbitrarily defined puberty as 12 y for female and 14 y for male offspring. Pairwise Pearson correlations between methylation, expression, and insulin levels were computed. Correlations of gene methylation and expression microarray data with Sequenom EpiTYPER methylation and RT-PCR data, respectively, were also computed using pairwise Pearson correlations. All statistical analyses were done using the SAS software version 9.2 (SAS Institute). Statistical significance was defined as $P \leq 0.05$.

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