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Differential Methylation of Inflammatory and Insulinotropic Genes after Metabolic Surgery in Women

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Abstract

Context: Biliopancreatic diversion with duodenal switch (BPD-DS), a metabolic bariatric operation, induces durable loss of excess weight and reduced cardiometabolic risk. Altered epigenetic marks are mechanistically associated with environment-driven phenotypic variations.

Objective: The current study aimed to compare gene methylation levels before and after BPD-DS to identify epigenetic marks potentially linked to metabolic improvements induced by BPD-DS.

Design and patients: Metabolic risk factors and gene methylation levels of 20 women studied mean 12 years (range 4-22) after BPD-DS were compared to those of 20 severely obese surgical candidates as controls, matched for pre-surgical age, body mass index and dyslipidemia and hypertension prevalences. Whole-genome blood DNA methylation analysis enabled between-group differential methylation analyses. We calculated correlations between methylation levels of the most differentially methylated CpG sites and plasma glucose and insulin levels and HOMA-IR.

Results: Differential methylation analysis identified 15,343 genes demonstrating at least one differentially methylated CpG site ($p < 1.43 \times 10^{-7}$). "Diabetic" and "inflammation/immune" functions were among the most overrepresented from the 200 genes exhibiting the largest group differences in methylation levels. CpG sites methylation levels of genes related to insulin action correlated significantly with fasting insulin levels and homeostatic model of insulin resistance ($p \leq 0.002$ for all).

Conclusion: These findings suggest that differential methylation levels in obese controls versus treated women may partially explain the durable metabolic improvements after BPD-DS.

Keywords: Epigenetics, Bariatric surgery, Genome-wide methylation, Diabetes, Cardiometabolic risk

Abbreviations: BMI, body mass index; BPD-SD, biliopancreatic diversion with duodenal switch; DBP, diastolic blood pressure; ECM, extracellular matrix; HOMA-IR, homeostatic model of insulin resistance; IGT, impaired glucose tolerance; IPA, Ingenuity Pathway Analysis; RYGB, Roux-en-Y gastric bypass; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides.

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Introduction

Obesity with dyslipidemia, type 2 diabetes mellitus (T2DM) and hypertension contributes to high prevalences of morbidity and excess mortality worldwide. Weight loss is associated with an improved metabolic risk profile in severely obese individuals [1]. Metabolic surgery is the most effective treatment of obesity and its comorbidities, improving glucose and lipid metabolism and preventing hypertension and T2DM [2,3] more than non-surgical methods [4]. Moreover, surgery decreases obesity-related low-grade inflammation [5]. Biliopancreatic diversion with duodenal switch (BPD-DS) is the most effective metabolic bariatric operation [2,3] because it combines sleeve gastrectomy and duodeno-jejunal and proximal ileal bypass-induced reduced absorption predominantly of lipids [6].

Epigenetic marks are traditionally defined as heritable changes in gene expression without alterations in DNA sequence, providing a potential mechanism by which environmental factors influence phenotypic variation [7]. Components of the obesity-related metabolic complications are under the influence of complex interactions between genetic/epigenetic and environmental factors [8], thus warranting assessment of gene methylation in relation to obesity complications and weight loss. Animal studies of diets, weight change and caloric restriction demonstrated changes in gene methylation levels [9,10] similar to those we [11] and others [12] observed in human adipose tissue following caloric restriction. Furthermore, the clinical studies demonstrated pre-intervention differences in methylation levels of several genes including some related to energy balance and insulin secretion between high and low responders to caloric restriction [11,12]. Roux-en-Y gastric bypass (RYGB), a metabolic, gastro-pyloro-duodenal restrictive bypass operation altering nutrient digestion, flow and sequence and magnitude of incretin peptide release, induced more profound epigenetic changes in whole blood than very low calorie diets in an analysis focusing on specific genes [13].

The present study was designed to analyze relationships between gene methylation levels in blood and metabolic risk factors comparing women after pylorus-preserving malabsorptive BPD-DS surgery to non-operated, untreated, severely obese women. Pathway analyses were conducted to identify potential mechanisms associated with metabolic improvements induced by this operation.

Methods

Patients

Twenty unrelated women who had undergone BPD-DS [2] between 1998 and 2005 were recruited. Clinical data and blood samples were obtained at follow-up visits in 2010. Preoperative data were obtained from medical records. Lacking banked pre-surgical blood samples for these 20 operated women primarily identified to assess the impact of maternal bariatric surgery on offspring [14], 20 severely obese unoperated women recruited from the list of candidates for BPD-DS at the Quebec Heart and Lung Institute (Quebec City, Quebec, Canada) were matched for age, BMI, comorbidities (hypertension, dyslipidemia, T2DM),

medication and smoking status (current vs. non-smoker) to operated women.

Cardiometabolic markers

Weight, height, body mass index (BMI) and resting systolic (SBP) and diastolic (DBP) blood pressure were measured according to standard practice. Blood samples were collected after an over-night fast in tubes containing EDTA, measuring plasma lipids [total cholesterol (TC), LDL-C, HDL-C, and triglycerides (TG)] and glucose concentrations as described [15]. Diagnoses of T2DM and prediabetes [impaired glucose tolerance (IGT); fasting glucose >6.0 mmol/l, hypertension (SBP >140 or DBP >90), hypertriglyceridemia (TG >2.3 mmol/l) [16] and/or relevant medication were retrieved from medical records. In unoperated obese controls and post-surgery patients insulin levels were measured by ELISA (ALPCO Diagnostics) and homeostatic model of insulin resistance (HOMA-IR) index was calculated as glucose (mmol/l) x insulin (mU/ml) /22.5.

DNA extraction and genome-wide methylation analysis

Blood genomic DNA was isolated from the blood buffy coat using the GenElute™ Blood Genomic DNA kit (Sigma). Quantification and verification of DNA quality were conducted via both NanoDrop spectrophotometer (Thermo Scientific) and PicoGreen DNA methods. Bisulfite conversion and quantitative DNA methylation analysis from 1 µg of DNA was performed at the McGill University and Genome Quebec Innovation Centre (Montreal, Canada). Samples were loaded on nine different Infinium® HumanMethylation450 BeadChips (Illumina Inc.) to minimize any batch effect and processed following manufacturer instructions. The BeadChip interrogates more than 485,000 methylation sites at a single-nucleotide resolution and can accommodate 12 samples simultaneously.

Methylation data were visualized and analyzed with the GenomeStudio® software, version 2011.1 (Illumina Inc.) and the Methylation Module. None of the samples were excluded following quality control steps assessed by bisulfite conversion, extension, staining, hybridization, target removal and negative and non-polymorphic control probes. Methylation levels (beta values; β) were estimated as the ratio of signal intensity between methylated and sum of methylated and unmethylated alleles (β value = $C/(T+C)$). The β values vary from 0 (no methylation) to 1 (100% methylation). Data correction (background subtraction and normalization) used internal control probe pairs. Probe type-specific normalization was conducted using internal control probes using the GenomeStudio Methylation Module normalization algorithm. CpG sites with a detection p-value >0.05 or located on Y chromosome were not analysed. The presence of SNPs in CpG site surrounding regions may potentially affect methylation levels, we excluded polymorphic CpG sites according to Chen et al. [17] (CpG sites with SNP underneath or with SNP at the position where single-base extension occurs) and to BeadChip annotation leaving 349,740 CpG sites for statistical analyses. Between-group differences in methylation levels (mean β values) were tested using Student t-test in Genome Studio

software. Bonferroni correction of the differential methylation analysis provided a p-value cutoff of $p < 1.43 \times 10^{-7}$ (based on 0.05/349,740) for statistical significance. “Overmethylated” and “undermethylated” probes were defined in the study group relative to obese controls.

Gene expression analysis

Expression levels for the most differentially methylated genes were retrieved from previously obtained microarray data (HumanHT-12 v4 Expression BeadChip, Illumina Inc.) for a subset of the post-surgery study group (N=18). Briefly, microarray experiments were carried out using 250 ng of total RNA isolated and purified from whole blood using PAXgene™ Blood RNA Kit (Qiagen) and processed according to the manufacturer’s instructions at the McGill University and Génome Québec Innovation Centre (Montréal, Canada). Expression data was visualized and analyzed as previously described [14].

Gene functions and pathways analysis

We identified potentially altered pathways from the top 200 differentially methylated genes (top 100 overmethylated + top 100 undermethylated) produced from differentially methylated probes, thus focusing on genes showing differences in methylation levels of more than 10% thus having higher probability of functional impact. Analyses were also conducted separately on the full sets of overmethylated (N=188) and undermethylated (N=3660) genes with differences in methylation levels greater than 10%. Ingenuity Pathway Analysis® (IPA) classified genes according to functions and pathways (calculating p-values with right-tailed Fisher’s exact test for each). Statistically overrepresented functions and pathways ($p \leq 0.05$) were then identified in the lists of differentially methylated genes.

Ethics

The study was approved by the Quebec Heart and Lung Institute and Université Laval ethics committees; all participants provided written informed consent. The study protocol respects the ethical guidelines of the “World Medical Association Declaration of Helsinki”.

Statistics

Clinical data were expressed as mean \pm SD. Differences in anthropometric and clinical data between the pre-operative and control patients and between the controls and the post-surgery patients were tested by t-tests. Within-group paired t-tests compared pre- to post-surgery clinical data. Differences in categorical variables and distribution of CpG sites were assessed using the Chi-square test. Pairwise Pearson correlations were computed for all CpG site methylation levels in the control and post-surgery groups. Similar analyses were conducted for a subset of differentially methylated CpG sites. CpG site methylation levels from the most differentially methylated genes were correlated with corresponding gene expression levels. CpG site methylation levels from differentially methylated genes related to the function termed “Insulin-dependent diabetes mellitus” function, thereafter termed “diabetes”, were correlated with plasma

glucose and insulin levels as well as HOMA-IR using pairwise Pearson correlations. Statistical significance was defined as $p \leq 0.05$. Statistical analyses for clinical data were conducted using the SAS software version 9.2 (SAS Institute Inc.) and descriptive statistics for genome-wide methylation data were calculated in R version 2.14.1 (R Foundation for Statistical Computing; www.r-project.org).

Results

Patient characteristics

Pre-surgical clinical characteristics of women from the control and operated groups were similar (29.2 ± 3.3 years for control and 29.9 ± 3.8 for study groups). BMI, plasma lipid and glucose levels were not significantly different ($p > 0.05$) between the groups (Table 1), 3 patients in each group were dyslipidemic and 6 in each were hypertensive where one control patient was on medication.

Mean postoperative follow-up for the post-surgery study group was 12 years 2 months (range: 4-22 years). BPD-DS induced changes in cardiometabolic risk profile following intervention with significant improvements in plasma lipids ($p = 0.003$ for TG and $p < 0.0001$ for HDL-C, LDL-C, TC and TC/HDL-C ratio), blood pressure (SBP and DBP; $p < 0.0001$), and resolution of hypertension and IGT.

All parameters were statistically significantly different between non-operated controls and the post-surgical group ($p = 0.02$ - < 0.0001) reflecting clinically significant metabolic improvements after BPD-DS surgery, as previously reported [2].

Global methylation levels

Briefly, the call rate for methylation data for both groups was 99.9%. Systematic exclusion of polymorphic CpG sites resulted in 349,740 CpG sites for further analyses. Lower global methylation levels were observed in the post-surgical group compared to controls; 40% of highly methylated probes post-surgery vs. 42% in controls ($p < 0.0001$; Figure S1), representing 136,413 CpG sites with low methylation levels in controls in comparison to 142,612 in post-surgery study patients. These results suggest that there are important differences in gene expression levels between groups. The majority of CpG sites demonstrated similar levels of methylation between control and post-surgery groups evident in a correlation of $r = 0.997$ ($p < 2.2 \times 10^{-16}$; Figure 1, Panel A).

Differential methylation analysis

Differential methylation analysis for non-polymorphic CpG sites identified 67,364 sites exhibiting significant postoperative differences ($p < 1.43 \times 10^{-7}$). Lower correlation was observed for differentially methylated sites compared to correlation obtained considering all sites analyzed (Figure 1, Panel B). Most of the differentially methylated CpG sites (88.5%) showed absolute differences ($< 10\%$) in DNA methylation between groups with 11.5% of the sites demonstrating group differences above 10% (Figure 1, Panel C). Most undermethylated and overmethylated genes are shown in Table S1. Among the differentially methylated CpG sites, 80.8% were located within gene regions (Figure 1, Panel D). Exclusion of age-associated CpG sites (according to

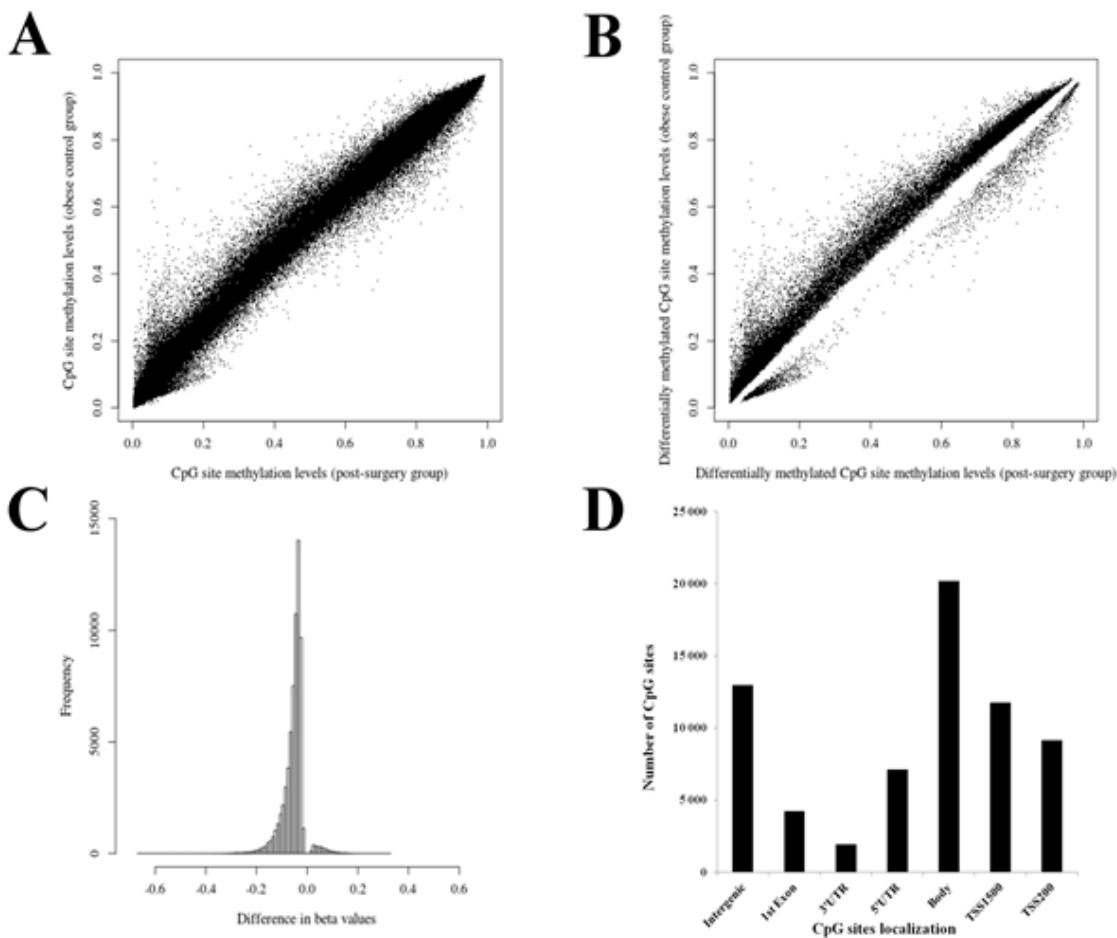


Figure 1 Correlations between global DNA methylation levels in control vs. post-surgery patients for all non-polymorphic CpG sites analyzed (Panel A, $N=349,740$; $r=0.997$, $p<2.2\times 10^{-16}$) and for differentially methylated CpG sites only (Panel B, $N=67,364$; $r=0.992$, $p<2.2\times 10^{-16}$). Frequency distribution of differences in methylation levels between control and post-surgery groups (Panel C) and localization of differentially methylated CpG sites (Panel D).

refs. [18,19]) and their respective genes revealed statistically significant group differences with minor effects on the distribution of CpG site localization in 67,245 CpG sites. From the 15,343 genes demonstrating at least one differentially methylated site, 11,769 (77%) were identified by two or more probes with 179 showing significant differences in methylation levels for all CpG sites assessed. To further assess the potential impact of differences in methylation levels between groups, the correlation between gene methylation and expression levels was assessed in the post-surgery study group for which gene expression levels were available ($N=18$). Significant correlations between gene methylation and expression levels were found for most overmethylated and undermethylated genes with some others demonstrating barely significant correlations (Table S2).

Gene function and pathway analysis

Gene function and pathway analysis, from the list of the 200 differentially methylated genes [top 100 undermethylated + top 100 overmethylated (Table S1)] with the largest between-group differences identified “Systemic autoimmune syndrome”, “Rheumatoid arthritis” and “Diabetes” among the top 10

overrepresented functions. The majority of genes were inflammation- or immune function-related, such as 13 among the 14 genes in the “diabetic” function cluster. Pathway analysis revealed 4 statistically significantly overrepresented pathways (Rac signaling, Actin cytoskeleton signaling, D-myo-inositol (1,4,5)-trisphosphate biosynthesis and Regulation of actin-based motility by Rho). Trends towards overrepresentation ($p<0.10$) were identified for 12 pathways including “Insulin receptor signaling” and “Hypoxia signaling in the cardiovascular system” (Figure S2). Analysis of all overmethylated and undermethylated genes separately confirmed overrepresentation of Rac signaling and Actin cytoskeleton signaling pathways in undermethylated genes. In addition, 159 other pathways were overrepresented from the list of undermethylated genes in the post-surgery study group relative to obese controls among which top pathways were related to intracellular signaling (G-protein coupled receptor signaling, signaling by Rho family GTPases, cAMP-mediated signaling), immunity (IL-8 signaling, leukocyte extravasation signaling) and cancer (molecular mechanisms of cancer, pancreatic adenocarcinoma, non-small cell lung cancer signaling).

Table 1 Characteristics of control and study groups (means \pm SD).

Characteristics	Control group	Study group		P-value (pre-surgery vs. controls)	Paired p-value (pre- vs. post-surgery)	P-value (controls vs. post-surgery)
		Pre-surgery	Post-surgery			
Age at operation (years)	29.2 \pm 3.3	28.9 \pm 3.7	41.0 \pm 5.3	0.800	< 0.0001	< 0.0001
BMI (kg/m ²)	45.8 \pm 5.7	45.0 \pm 7.2	27.6 \pm 4.8	0.695	< 0.0001	< 0.0001
Blood pressure (mm Hg)						
SBP	130.0 \pm 12.4	136.9 \pm 11.1	112.2 \pm 9.5	0.069	< 0.0001	< 0.0001
DBP	81.3 \pm 8.6	87.3 \pm 10.1	68.2 \pm 8.9	0.051	< 0.0001	< 0.005
Hypertension (N)	6	6	0	1.000	0.020	0.020
Lipid profile						
TC (mmol/l) ^{III}	4.68 \pm 0.62	4.93 \pm 0.79	3.52 \pm 0.49	0.283	< 0.0001	< 0.0001
LDL-C (mmol/l) ^{II}	2.79 \pm 0.58	3.00 \pm 0.83	1.68 \pm 0.50	0.379	< 0.0001	< 0.0001
HDL-C (mmol/l) ^I	1.22 \pm 0.21	1.14 \pm 0.26	1.39 \pm 0.25	0.310	< 0.0001	0.023
TC/HDL-C ^I	3.93 \pm 0.75	4.63 \pm 1.69	2.59 \pm 0.59	0.100	< 0.0001	< 0.0001
TG (mmol/l) ^I	1.48 \pm 0.68	1.65 \pm 0.76	0.97 \pm 0.41	0.467	0.003	0.007
Glucose metabolism						
Fasting glucose (mmol/l) ^{IV}	5.2 \pm 0.9	5.7 \pm 2.4	4.7 \pm 0.3	0.346	0.060	0.038
IGT	3	4	0	1.000	0.106	0.231
Insulin (μ U/mL) ^V	26.6 \pm 23.9	N/A	3.1 \pm 1.7	---	---	< 0.0001
HOMA-IR ^V	5.9 \pm 5.0	N/A	0.7 \pm 0.4	---	---	< 0.0001

Abbreviations: SD, standard deviation; N, numbers; N/A, not available; BMI, body mass index; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; IGT, impaired glucose tolerance; HOMA-IR, homeostatic model of insulin resistance.

^I Pre-surgery group N=16. ^{II} Pre-surgery group N=15. ^{III} Pre-surgery group N=18. ^{IV} Pre-surgery group N=19. ^V Pre-surgical insulin levels were not available (N/A) for the study group.

Correlations between CpG site methylation levels and diabetes-related variables

Correlations between methylation levels of the most significantly differentially methylated CpG sites for each of 14 genes related to “diabetes” function, among which the insulin receptor gene, INSR, and fasting insulin levels and HOMA-IR index were statistically significant ($p \leq 0.002$ for all sites; **Table 2**). Among the overmethylated “diabetes” genes, FKBP1A, a regulator of neuronal differentiation and immune responsiveness, and ZNF323, a member of the zinc-finger transcription factor family expressed in the pancreas, exhibited the highest correlations with insulin and HOMA-IR.

Discussion

We found differences in genome-wide CpG site methylation levels between a control group of unoperated severely obese premenopausal surgical candidates matched to pre-operative phenotypes of other women who subsequently underwent BPD-DS, a metabolic gastrointestinal bypass operation. Gene function and pathway analyses identified potentially mechanistic genes

associated with post-surgical metabolic improvements which exhibited correlations between CpG site methylation levels and diabetes-related phenotypes. We show large differences in cardiometabolic parameters associated with differences in DNA methylation levels between the obese control and post-BPD-DS patients. We found a high proportion of CpG sites with high (>75%) or low (<25%) levels of methylation in agreement with other genome-wide methylation studies [20,21] and strong correlations among methylation levels in blood as shown by others in blood, muscle and adipose tissue [18,21].

Our findings of significant long-term differences in global CpG site methylation in blood contrast with one study of blood methylation patterns in post-menopausal women in a diet and exercise program [22], and with a 6-month study of skeletal muscle changes after gastric bypass [23]. In the latter study, differentially methylated CpG sites were almost exclusively undermethylated (397 out of 409) similar to the very long term methylation levels we found. It is tempting to speculate that greater metabolic improvements after BPD-DS [2,3] may cause greater global genome-wide differences in methylation levels via reduction of overnutrition-driven toxicity (nutritoxicity), especially lipotoxicity [9,10,24]. BPD-DS may have both short- and long-term persistent effects on methylation levels explaining the

Table 2 Correlations between diabetes-related phenotypic variables and methylation levels for the most differentially methylated CpG sites from genes related to “diabetes” function.

CpG site (Gene)	Position*	Fasting glucose	Insulin	HOMA-IR
cg14793544 (ABCF1)	Chr6:30538842	0.300 (0.060)	0.597 (<0.0001)	0.614 (<0.0001)
cg16500605 (BAT3)	Chr6:31620861	0.308 (0.053)	0.533 (0.0004)	0.541 (0.0003)
cg07516946 (FKBP1A)	Chr20:1373593	0.293 (0.066)	0.614 (<0.0001)	0.627 (<0.0001)
cg05055326 (HCG9)	Chr6:29945080	0.289 (0.070)	0.611 (<0.0001)	0.627 (<0.0001)
cg17353079 (HSPA1L)	Chr6:31779020	-0.307 (0.054)	-0.536 (0.0004)	-0.565 (0.0001)
cg19280196 (HTT)	Chr4:3234462	-0.336 (0.034)	-0.514 (0.0007)	-0.535 (0.0004)
cg21403543 (IFIT2)	Chr10:91061583	0.378 (0.016)	0.539 (0.0003)	0.569 (0.0001)
cg14900579 (INSR)	Chr19:7294137	0.327 (0.040)	0.604 (<0.0001)	0.623 (<0.0001)
cg21323836 (NCRNA00171)	Chr6:29985506	-0.324 (0.041)	-0.555 (0.0002)	-0.585 (<0.0001)
cg06658129 (OR12D2)	Chr6:29364602	-0.275 (0.086)	-0.576 (<0.0001)	-0.594 (<0.0001)
cg24192846 (PPP1R11)	Chr6:30037773	-0.362 (0.022)	-0.480 (0.002)	-0.524 (0.0005)
cg22772698 (ZBP1)	Chr20:56195991	-0.331 (0.037)	-0.501 (0.001)	-0.526 (0.0005)
cg07369507 (ZNF323)	Chr6:28304287	0.329 (0.038)	0.583 (<0.0001)	0.609 (<0.0001)
cg20325280 (ZNF705A)	Chr12:8324153	-0.338 (0.033)	-0.580 (<0.0001)	-0.597 (<0.0001)

Pearson's *r* coefficients are shown. Associated *p*-values are in parenthesis. Abbreviations: Chr, Chromosome; HOMA-IR, homeostatic model of insulin resistance.

* Genome build 37.

larger differences observed in the present study in comparison to studies evaluating methylation levels 1-2 days, 6 or 12 months after different bariatric operations [13,23].

Overrepresentation of immune and inflammatory genes identified in our study is consistent with reduced low-grade inflammation after bariatric surgery [5], changes in methylation levels in blood following RYGB [13] and expression levels in adipose tissue one year after BPD-DS [25]. Concurrent overrepresentation of immune and inflammatory genes and genes related to cellular assembly and organization (Actin cytoskeleton signaling and Regulation of actin-based motility by Rho pathways) accords with remodeling of adipose tissue extracellular matrix (ECM). These results may represent an interplay between ECM components and inflammatory processes [26] and are in agreement with previously reported similar changes in adipose tissue gene expression levels one year after BPD-DS [25]. Our findings of overrepresentation of the Hypoxia Signaling pathway is concordant with the relative hypoxia of obese patients [27,28] and adipose tissue as well as aberrant pro-inflammatory ECM remodeling [26,29], both of which are reversed postoperatively.

Gene methylation differences in diabetic functions and pathways agree with gene expression profiling in blood before and after bariatric surgery demonstrating changes in genes related to lipid metabolism, obesity and T2DM as well as metabolic pathways related to small molecule biochemistry and gene expression

[30]. Genes related to RNA processing were also overrepresented agreeing with studies of genes related to gene expression and to transcriptional, translational and transport processes (RNA processing, tRNA metabolism, translation, protein transport) in adipose tissue and blood following bariatric surgery [25,30]. Our identification of Rac signaling pathways is in line with the dysmetabolic diathesis of diabetes, Rac1 being activated by insulin, necessary for insulin-stimulated GLUT4 translocation and inducing actin cytoskeleton remodeling of plasma membrane in skeletal muscle [31]. Concurrent overrepresentation of D-myoinositol (1,4,5)-trisphosphate biosynthesis also supports the finding of altered diabetic functions and pathways post-surgery as well as abnormalities in inositol metabolism associated with diabetes and its comorbidities [32]. Supplementation of myoinositol has been shown to improve HOMA-IR in postmenopausal women with the metabolic syndrome [33].

Globally, correlation of methylation levels for selected CpG sites with insulin and HOMA-IR suggests that epigenetic changes after BPD-DS may be determinants of the durable metabolic improvement, although the current study was not designed to analyse differences in gene expression levels between control and post-surgical patients. Nonetheless, our results agree with previous pre- vs. post-surgery reports showing alterations in similar functions [13,25,30,34,35]. Furthermore, specific differentially methylated genes related to those with “diabetic”

functions identified here (ABCF1, FKBP1A, IFIT2, HTT) and correlated with insulin levels and HOMA-IR have also been shown previously to be differentially expressed in adipose tissue and/or skeletal muscle [23,25,34]. FKBP1A participates in neuronal differentiation and plasticity affecting age-related cognitive dysfunction, a comorbidity of diabetes. Furthermore it modulates immune responsiveness in the non-neuronal acetylcholine system mediated by the vagus nerve innervating the foregut. ZNF323 is a member of the zinc-finger transcription factor family and is involved in the development of liver, intestine and pancreas: key organs in nutrient trafficking affecting the dysmetabolic diathesis of diabetes.

Taken together our findings fit conceptually into an ergostatic theory of energy balance postulating a developmentally conserved adaptive framework of energy supplies cyclically fluctuating between surfeit and deficit both of which elicit inflammatory responses and drive neuro-endocrine regulation of pancreatic and intestinal effects on nutrient trafficking contributing to insulin resistance [36].

Limitations of this study

Our patients are cross-sectionally studied without longitudinal sequential samples necessary to fully assess potential temporal postoperative changes in methylation levels. While insulin levels were measured and HOMA-IR index was calculated in control and postoperative women, classical measures of insulin resistance such as oral glucose tolerance tests or hyperinsulinemic euglycemic clamps were not performed. Our study design resulted in age differences between the two groups but we systematically excluded age-associated CpG sites [18,19] to rule out such effects. Our array approach may have led to overrepresentation of CpG sites in gene surrounding regions [37]. Whole-genome bisulfite sequencing may be necessary to fully determine the effects of bariatric surgery on CpG site methylation levels in intergenic regions. Since the presence of SNPs may affect methylation levels of CpG sites we excluded CpG sites with SNPs in surrounding regions. We extracted blood DNA to assess global metabolic changes although analyses of other metabolically relevant tissues might contribute valuable information on postoperative improvements, even though the

overall impact of tissue-specific methylation patterns is likely minor in view of reported similarities between tissues [38]. Our results of long-term effects of BPD-DS might be specific and not applicable to purely restrictive operations or those with other mechanisms.

In summary, BPD-DS associated long-term effects on cardiometabolic risk may be attributable to changes in gene regulation. Differences in blood global methylation levels between obese control and post-surgical patients suggest that CpG site methylation levels are subject to substantial changes following BPD-DS. By reducing inflammation and overnutrition, characteristic of the dysmetabolic syndrome of diabetes, BPD-DS may alter CpG site methylation levels. Although methylation is generally accepted as an epigenetic marker of choice, we must also consider the potential mechanistic importance of histone modification, changes in chromatin structure and/or interfering microRNAs or combinations of these as activators or silencers of specific genes. Future studies utilizing other techniques will be necessary to uncover the upstream mechanisms of the postoperative reversal of diabetes related nutritoxicity.

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