A longitudinal, epigenome-wide study of DNA methylation in anorexia nervosa: results in actively ill, partially weight-restored, long-term remitted and non-eating-disordered women

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Background: This study explored state-related tendencies in DNA methylation in people with anorexia nervosa. **Methods:** We measured genome-wide DNA methylation in 75 women with active anorexia nervosa (active), 31 women showing stable remission of anorexia nervosa (remitted) and 41 women with no eating disorder (NED). We also obtained postintervention methylation data from 52 of the women from the active group. **Results:** Comparisons between members of the active and NED groups showed 58 differentially methylated sites ($Q < 0.01$) that corresponded to genes relevant to metabolic and nutritional status (lipid and glucose metabolism), psychiatric status (serotonin receptor activity) and immune function. Methylation levels in members of the remitted group differed from those in the active group on 265 probes that also involved sites associated with genes for serotonin and insulin activity, glucose metabolism and immunity. Intriguingly, the direction of methylation effects in remitted participants tended to be opposite to those seen in active participants. The chronicity of illness correlated (usually inversely, at $Q < 0.01$) with methylation levels at 64 sites that mapped onto genes regulating glutamate and serotonin activity, insulin function and epigenetic age. In contrast, body mass index increases coincided (at $Q < 0.05$) with generally increased methylation-level changes at 73 probes associated with lipid and glucose metabolism, immune and inflammatory processes, and olfaction. **Limitations:** Sample sizes were modest for this type of inquiry, and findings may have been subject to uncontrolled effects of medication and substance use. **Conclusion:** Findings point to the possibility of reversible epigenetic alterations in anorexia nervosa, and suggest that an adequate pathophysiological model would likely need to include psychiatric, metabolic and immune components.

Introduction

Anorexia nervosa can devastate medical, psychological and social adjustments, and despite treatment advances, only about half of people with the condition achieve full remission after 10 or more years.¹ Such realities point to the need for improved knowledge of the causal and maintaining factors of what can be an insidious disorder.

Anorexia nervosa is understood to have multiple determinants, including genetic factors (shaping such processes as emotion regulation, reward sensitivity and energy metabolism), environmental triggers (including perinatal, develop-

mental and later-life events), state-related effects (owing to nutritional and mental status) and social inducements toward intensive caloric restraint.^{2,3} Although twin studies have shown anorexia nervosa to be strongly heritable, efforts to identify syndrome-relevant genes have been disappointing.^{4,5} However, a recent study has documented a genetic locus that reached genome-wide significance for anorexia nervosa: a site on chromosome 12 associated with type 1 diabetes and autoimmune diseases.⁶ Genetic correlations associated anorexia nervosa with both mental-illness (schizophrenia and neuroticism) and metabolic (glucose and lipid metabolism) phenotypes. In other words, broad

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psychiatric, metabolic and autoimmune spheres have been indicated.

Gene–environment interactions and epigenetics

Complex psychiatric syndromes are almost certain to involve the environmental activation of hereditary potentials, and this likely applies to anorexia nervosa: risk of anorexia nervosa is increased when people who are genetically predisposed experience familial distress.⁷ Likewise, animal data suggest that genetic susceptibility, combined with adolescent social stress and caloric restraint, produces a mouse analog to anorexia nervosa: mice that let themselves starve.⁸ Diverse environmental influences have been postulated to act in anorexia nervosa, including obstetric insults, gestational stress, childhood trauma, familial conflict and social inducement toward caloric restraint.^{9,10}

Epigenetic processes influence gene expression and corresponding phenotypic variations.¹¹ Some epigenetic mechanisms appear to be environmentally responsive and constitute plausible substrates for gene–environment interactions that underlie mental-illness phenotypes, including anorexia nervosa.^{6,10,12} The best-studied epigenetic mechanism is DNA methylation, which generally reduces gene expression.¹¹ Research has shown that DNA methylation responds to perinatal insults¹³ and early-life stresses,^{14–16} and is also influenced by psychotherapeutic, nutritional and pharmacologic interventions.^{17–19} If so, DNA methylation could be a marker of disease staging or therapeutic response.

DNA methylation in anorexia nervosa

An accumulating literature has associated anorexia nervosa with altered DNA methylation.^{5,6,10} Candidate-gene studies have linked anorexia nervosa to hypermethylation of genes for α -synuclein (involved in neurotransmitter release),²⁰ natriuretic peptide (implicated in anxiety, depression and stress responses),²¹ dopamine function (implicated in reward sensitivity and binge-eating)²² and oxytocin (linked to attachment).²³ Other studies in anorexia nervosa have estimated global DNA methylation levels, although they have been limited by small samples or methods that interrogate narrow genomic regions. One study, using DNA from peripheral blood obtained from 10 women with anorexia nervosa and 10 without, found no differences when comparing methylation of LINE1 repetitive elements (a proxy for global methylation).²⁴ Two other studies, 1 involving 22 adults with anorexia nervosa (subtype unspecified)²⁰ and another involving 32 adolescents with anorexia nervosa, restricting type,²⁵ reported lower global methylation; the first used a nonradioactive elongation assay, the second involved incorporation of [³H]dCTP following a cut with *HpaII*.

Because global methylation measures provide a rather blunt indicator of possible epigenetic effects, gene-specific methylation differences assessed using microarray technologies are likely to be more informative. We recently used a high-throughput technology (Infinium Human Methylation 450 BeadChip Kit; Illumina Inc.) and DNA in leukocytes

from 29 women with anorexia nervosa and 15 women without anorexia nervosa and within a healthy weight range to perform a genome-wide examination of methylation levels.²⁶ Case–control comparisons with false discovery rate (FDR) corrections identified 14 differentially methylated CpG probes, corresponding to genes acting in histone acetylation, cholesterol storage, lipid transport and dopamine and glutamate signalling. Findings also linked the chronicity of illness to methylation at 119 genes associated with anxiety, social behaviour, immunity and nervous-system function. In other words, like the genome-wide association study described earlier,⁶ our study indicated altered psychiatric, physiologic and immune functions. A subsequent independent study using the same technology compared DNA methylation in 47 women with anorexia nervosa to 100 population-based controls²⁷ and identified 81 differentially methylated sites. Both studies isolated the *NR1H3* gene (involved in lipid homeostasis and inflammation) and the *TNXB* gene (associated with connective-tissue disorders).

Present study

We extended our investigation of DNA methylation in people with and without anorexia nervosa, increasing sample sizes and adding a sample of women who, when tested, had been free of eating-disorder symptoms and weight-restored for at least 1 year. We also investigated within-subject longitudinal changes in methylation levels (attributable to stabilization of symptoms and weight restoration). Our combined prospective/cross-sectional design was intended to help differentiate transient illness sequelae from stable epigenetic traits.

Methods

Procedures in this study complied with the ethical standards of relevant institutional committees and the Helsinki Declaration, revised in 2008.

Participants

Participants with an active eating disorder (active group), recruited at a specialized eating disorders program, included 75 women with an anorexia nervosa diagnosis according to DSM-5 and a body mass index (BMI) under 18 kg/m². Of these, 34 had anorexia nervosa restrictive type and 41 had anorexia nervosa binge/purge type. Of the total, 29 figured in our earlier report (see Booij and colleagues²⁶). Diagnoses were based on interviews with the Eating Disorders Examination (EDE)²⁸ or clinical interviews conducted by specialist clinicians, complemented by results from the EDE Questionnaire (EDE-Q; 4 cases).²⁹ We obtained follow-up methylation and clinical data on 52 women (28 with anorexia nervosa restrictive type and 24 with anorexia nervosa binge/purge type) after an average of 114 days (range 62 to 182 days) of treatment emphasizing weight restoration.

We also recruited 31 women who had previously fulfilled the DSM-5 criteria for anorexia nervosa but who, upon entry into the study, did not meet criteria for anorexia nervosa or

bulimia nervosa (based on retrospective EDE interviews), and had maintained a self-reported BMI of 18 kg/m² or higher for at least 12 months (remitted group).

Excluding people taking medications was impractical and undesirable on grounds of representativeness, so we retained 51 of 75 women (68.0%) in the active group and 10 of 31 women (32.3%) in the remitted group who were taking psychoactive medication. We also retained participants who had mood, anxiety, substance use or personality disorders, but not conditions that would create interpretive confounds (e.g., endocrinologic, diabetic, neoplastic or cardiovascular diseases; substance dependence, psychosis or bipolar illness).

Details of participants' BMI, age, illness characteristics, medication use and cigarette smoking are provided in Table 1. Groups did not vary according to mean age, but did differ as expected in terms of mean BMI and EDE total scores. The 2 active anorexia nervosa groups showed sig-

nificantly lower BMI and had significantly more eating disorder symptoms than the other 2 groups did. Compatible with known differences with respect to the prevalence of cigarette smoking,³⁰ participants with anorexia nervosa binge/purge type were significantly more likely to smoke than members of other groups were (Table 1). We controlled statistically for effects attributable to age and smoking. However, variations related to comorbidity, substance use and medication type rendered application of meaningful statistical controls unrealistic.

We recruited 41 non-eating-disordered (NED) women through public and university-/college-based announcements. These women showed no eating disorder or other psychiatric illnesses according to structured interviews. None of the women in the NED group used psychoactive medications. Data for 15 of the women in the NED group were included in our earlier report.²⁶

Table 1: Participant characteristics, medication use, psychiatric comorbidity and habits

Parameter	Active, anorexia nervosa restrictor type (<i>n</i> = 34)	Active, anorexia nervosa binge/purge type (<i>n</i> = 41)	NED (<i>n</i> = 41)	Remitted (<i>n</i> = 31)
	Mean ± SD (range)			
Participant characteristics				
BMI*	14.39 ± 1.72 (10.80–17.54)	15.78 ± 1.20 (13.09–17.81)	22.55 ± 2.38 (18.31–29.00)	21.10 ± 2.02 (18.43–25.43)
Age†	24.88 ± 8.39 (18–53)	23.41 ± 5.28 (18–43)	23.93 ± 5.33 (18–40)	26.81 ± 5.41 (19–38)
Chronicity, mo‡	96.00 ± 98.91 (12–456)	78.44 ± 57.68 (12–192)	NA	84.39 ± 44.31 (12–216)
EDE-Q total score§	3.19 ± 1.10 (1.14–5.40)	4.20 ± 1.09 (1.09–5.59)	0.32 ± 0.31 (0.00–1.39)	0.92 ± 0.83 (0.05–2.68)
	No. (%)			
Medication use				
Antidepressants	16 (47.06)	21 (51.22)	0	8 (25.81)
Antipsychotics	9 (26.47)	11 (26.83)	0	2 (6.45)
Hypnotics	1 (2.94)	2 (4.88)	0	0
Anxiolytics	6 (17.65)	4 (9.76)	0	0
Mood stabilizers	0	1 (2.44)	0	0
Psychiatric comorbidity				
Major depressive episode	4 (11.76)	15 (36.59)	0	2 (6.45)
Dysthymia	2 (5.88)	1 (2.44)	0	0
Panic disorder	1 (2.94)	0	0	2 (6.45)
Panic disorder with agoraphobia	2 (5.88)	2 (4.88)	0	1 (3.23)
Agoraphobia without panic disorder	0	1 (2.44)	0	0
Social anxiety disorder	1 (2.94)	5 (12.20)	0	0
Specific phobia	2 (5.88)	4 (9.76)	0	1 (3.23)
Generalized anxiety disorder	9 (26.47)	8 (19.51)	0	3 (9.68)
Obsessive–compulsive disorder	2 (5.88)	2 (4.88)	0	1 (3.23)
Posttraumatic stress disorder	0	2 (4.88)	0	1 (3.23)
Obsessive–compulsive personality disorder	11 (32.35)	9 (21.95)	0	1 (3.23)
Borderline personality disorder	1 (2.94)	2 (4.88)	0	1 (3.23)
Cigarette smoker¶	2 (5.88)	12 (29.27)	5 (12.20)	3 (9.68)

BMI = body mass index; EDE-Q = Eating Disorder Examination Questionnaire; NA = not applicable; NED = non-eating-disordered; NS = not significant; SD = standard deviation.

Means with different superscript letters differed at *p* < 0.05.

**F*_{3,143} = 166.29, *p* < 0.05.

†*F*_{3,143} = 2.0, NS.

‡*F*_{2,101} = 0.57, NS.

§*F*_{3,127} = 148.89, *p* < 0.05.

¶*χ*²₃ = 9.20, *p* < 0.05.

Clinical measures

We assessed eating-disorder symptoms using the EDE interview²⁸ and/or the EDE-Q.²⁹ The EDE is a gold-standard interview for assessing anorexic and bulimic symptoms (Cronbach $\alpha = 0.67$ – 0.90). The EDE-Q is a 41-item self-report questionnaire based on the EDE ($\alpha = 0.78$ – 0.93); EDE-Q results correspond well to those of the EDE.³¹ To estimate chronicity of illness (i.e., time since the onset of clinically significant symptoms of an eating disorder), trained interviewers established a retrospective portrait of each participant's eating-symptom history and, in reference to EDE diagnostic items, estimated the point at which symptoms became severe enough to support a diagnosis based on DSM-5 criteria. We used anthropometric measures to compute participants' BMI.

Because we started this study before the release of structured interviews based on DSM-5, we evaluated psychiatric comorbidity using the Structured Clinical Interview for DSM-IV (SCID-I)³² and the Structured Clinical Interview for DSM-IV Axis II (SCID-II)³³ modules for borderline personality disorder and obsessive-compulsive disorder. However, we updated diagnostic determinations using DSM-5 criteria. During study recruitment, we conducted inter-rater reliability checks on diagnostic determinations in 25 pseudo-randomly chosen interview pairs. Those checks yielded prevalence-adjusted, bias-adjusted κ coefficients (used for imbalanced distribution of marginal cell totals³⁴) of 0.84 to 1, and percent agreements of 92% to 100% (Appendix 1, Table S1, available at jpn.ca/170242).

Genome-wide DNA methylation analyses

We collected whole blood in EDTA tubes and extracted DNA from leukocytes using a DNA extraction kit (Qiagen). Epigenome-wide analyses were conducted at the Genome Quebec Innovation Centre using the Infinium Human Methylation 450 BeadChip Kit (Illumina Inc.; $n = 171$ samples) or the Infinium MethylationEPIC BeadChip Kit (Illumina Inc.; $n = 28$ samples). Analyses were performed according to standard procedures,²⁶ except that single-base extension and staining was automated. Samples from the 2 arrays were merged using the combineArrays function in minfi (Bioconductor). The reason different methylation array kits were used was that Illumina discontinued the 450 BeadChip kit. However, manufacturer information and an independent study suggest that samples can be combined across kits.³⁵ Isolated DNA was first checked for quality with picogreen, and then bisulfite-converted using the EZ-96 DNA Methylation-Gold Kit (Zymo Research). Next, samples were transferred to BCD and then MSA4 plates, and neutralized before overnight amplification. The MSA4 plates were fragmented, precipitated and resuspended before hybridization and transfer to Multi BeadChips. The Multi BeadChips then underwent washing, single-base extension and staining before imaging using the HiScan array scanner (Illumina Inc.).

Data processing and data filtering

We processed raw data using R (Bioconductor). We performed quality control using minfi metrics. Functional normalization was applied and values were background-corrected. Primary outcome measures were β values at each probe, representing the proportion of methylated cells. The specific chromosomal position and associated gene for each probe were defined using manifest files from Illumina (https://support.illumina.com/downloads/humanmethylation450_15017482_v1-2_product_files.ilmn). To correct for cell-type distribution effects, we applied a statistical correction based on the reference methylation profiles of separated peripheral blood mononuclear cells.³⁶

The initial data set consisted of 453 093 probes obtained from 147 participants. Because all participants were female, we ignored probes located on the Y chromosome. To remove random technical variations, we retained only CpGs that were associated with known protein-coding genes and with a $> 5\%$ deviation in methylation. After functional normalization, we observed possible probe-wise batch effects (i.e., β values for some probes on a given plate fell far outside the range of β values on all other plates). To eliminate such cases, we removed probes with at least 1 value below the estimated 0.01st percentile or above the estimated 99.99th percentile of the samples on the remaining plates. The final data set for subgroup comparisons and chronicity analyses included 35 513 probes (7.8% of the original probes). The final data set for pre/post comparisons included 37 507 probes (8.2% of the original probes).

We compared groups based on descriptive and clinical variables using χ^2 or 1-way analysis of variance (ANOVA) as appropriate, performed with SPSS 23 (SPSS Inc.). We conducted methylation analyses using R. We conducted group comparisons at individual probes using linear models, with methylation level as the dependent variable, group as the independent variable and estimated cell proportions³⁶ as covariates. We first tested for overall differences among groups (active versus remitted versus NED; anorexia nervosa restrictive type versus anorexia nervosa binge/purge type versus NED) using ANOVA, followed by pairwise comparisons where overall effects were significant. We repeated group comparisons with covariates that controlled for age variations and the presence or absence of cigarette smoking. Rare changes in significance levels resulting from application of covariates are annotated in the text and in Table 2. To examine associations between chronicity and methylation in the active sample, we used linear regression analysis. We used quantile regression to model the effects of median pre-/post-treatment differences in BMI on pre/post changes in probe-wise methylation levels, with the estimated cell-type proportions as a covariate. Quantile regression allowed for the comparison of findings in participants who had achieved substantial pre/post weight gain to those who had not. We applied FDR corrections within each set of analyses (i.e., within ANOVAs, analyses of covariance, pairwise contrasts, linear regression and quantile regression).

Results

Active versus remitted versus NED

A 1-way ANOVA for 3 groups showed differential methylation (at $Q < 0.01$) on 373 probes (representing 349 genes). For 295 of these (representing 277 genes), we found at least 1 significant pairwise comparison (at $Q < 0.01$). The ANOVA and pairwise comparison results for these 295 probes, ranked in order of statistical significance in the ANOVA, can be found in Appendix 1, Table S2.

Pairwise comparisons between the active and NED groups revealed 58 differentially methylated sites ($Q < 0.01$), corresponding to 55 genes. Some of the identified genes were

linked to nutrition: for example, lipid processing (*PRKAG2* [2 probes], *SCARF1*) and glucose metabolism (*RPTOR* [2 probes], *SORBS1*). Others were relevant to bone health (*MICAL2*) and immune function (*FCGR2A*, *NOD1*). One gene was positively associated with serotonin 2B receptor function (*HTR2B*). According to β values, methylation levels on 53 (91.4%) of the differentially methylated probes were higher in the active group, implying generally reduced expression of implicated genes in anorexia nervosa.

Pairwise comparisons between the active and remitted groups revealed 265 differentially methylated probes ($Q < 0.01$), corresponding to 252 genes (Appendix 1, Table S2). Similar to the active versus NED comparisons, many differentially methylated probes were linked to genes related to

Table 2: Probes and genes that overlapped between active versus NED and active versus remitted comparisons*

CpG	Gene	Description	Q values		
			Overall ANOVA	Active v. NED	Active v. remitted
Neurotransmitter function					
cg04042861	HTR2B	Serotonin system	8.66E-07	0.00760	8.47E-07
cg00968616	CUEDC1	Glutamate concentration in brain	2.94E-05	0.00375	0.000785
Physiologic and immune functions					
cg00129811	DSE	Ehler-Danlos syndrome	0.000310	0.00597	0.00239
cg06350097	FCGR2A	Immune function; ulcerative colitis; rheumatoid arthritis; systemic lupus erythematosus	0.000128	0.00375	0.00653†
cg24101945	HFM1	Ovarian insufficiency; genome integrity in germ line	9.12E-05	0.00941	0.000122
cg14666404	MCF2L2	Polycystic ovary syndrome	6.90E-05	0.00334	0.000488
cg27631389	MICAL2	Bone health	7.43E-05	0.00781	0.000201
cg18734095	NOSIP	Nitric oxide synthesis; enterocolitis	2.94E-05	0.000645	0.00236
cg17272549	EML6	Refractive astigmatism	3.17E-07	1.29E-05	1.11E-05
Nutrient metabolism					
cg01423695	PRKAG2	Lipid metabolism; cholesterol biosynthesis	0.000132	0.00552	0.00128
cg22091236	RPTOR	Response to nutrient and insulin levels	0.000268	0.00707	0.00639
cg23011194	SORBS1	Insulin signalling	1.03E-05	0.00933	3.69E-06
cg08106319	ZFAND3	Insulin resistance; diabetes	1.19E-05	0.00963	3.57E-05
cg00323842	ZNF608	BMI; visceral fat	3.17E-07	8.32E-05	3.52E-05
General cellular processes					
cg10095247	ANKIB1	Cellular activity	0.00201	0.00933†	0.00867†
cg09144769	AP2B1	Protein transport	0.00108	0.00941†	0.00888†
cg20124877	KPNA1	Nuclear protein import	4.76E-05	0.00552	0.000129
cg15278374	GPR133	Cellular activity	0.000852	0.00645	0.00545
cg12872357	RAB32	Mitochondrial fission	0.000397	0.00570	0.00110
cg25202261	SNX27	Protein transport	0.000840	0.00462	0.00647†
Transcriptional activity					
cg08255475	CDT1	DNA replication	2.75E-05	0.00386	0.000292
cg19161559	CUX1	Gene expression; antidepressant response	0.000752	0.00954†	0.00404
cg14422240	FTSJD2	Methyltransferase	2.16E-06	9.33E-05	0.000609
cg03233624	MSI2	Transcriptional regulation	1.98E-05	0.000553	9.12E-05
Unspecified function					
cg25197194	CCDC48	—	2.70E-06	0.00194	0.000109
cg01810593	KIAA1967	—	0.000261	0.00230	0.00649
cg18071202	LOC100292680	—	0.000343	0.00933	0.00235
cg06983052	LRR8D	—	8.66E-07	8.32E-05	0.000141

ANOVA = analysis of variance; BMI = body mass index; NED = non-eating-disordered.

*Gene functions and/or associated phenotypes are obtained using the National Center for Biotechnology Information gene database. Empty cells indicate genes on which clear functions are not listed. (See Appendix 1, Table S2, available at jpn.ca/170242, for full details of effect sizes and significance levels).

† $p < 0.05$ on a probe-wise comparison after adjustment for age and smoking. Significance level is otherwise $p < 0.01$ when the same covariates are applied.

nutrition, including metabolism of glucose and insulin (e.g., *RPTOR*, *LMX1A*, *SORBS1*, *PIK3R1*, *CRYL1*), cholesterol and lipids (*PRKAG2*, *PITPNC1*, *APOC1*), and appetite (*CAMKK2*, *GHRL*). We also observed differential methylation of genes related to bone and tissue health (*MICAL2*, *KRT17*), immune function/inflammation (e.g., *FCGR2A*, *RELT* TNF receptor, *NLRP10*, *CCR1*, *CCR7* [2 probes], *CTPS*) and cardiac function (*PRKG1* [2 probes]). Finally, as before, 1 probe was associated with the serotonin 2B receptor (*HTR2B*). The significance of pairwise effects for *PIK3R1*, *CRYL1*, *GHRL*, *FCGR2A* and *PRKG1* was reduced to $p < 0.05$ when we applied covariates for age and smoking. The direction of differences on 194 of these 265 probes (73.2%) pointed toward lower methylation in the remitted group than in the active group.

Among the 28 probes that were common to active versus NED and active versus remitted comparisons (representing 9.5% of the 295 identified probes and 10.1% of the 277 identified genes), effects across the analyses were always in opposite directions; that is, when methylation was higher in people with active versus NED, it was lower in people with remitted versus active, and vice versa. These findings suggest an average shift toward restored methylation levels in people who are remitted. We found no significant differences between the NED and remitted groups. Table 2 lists the 28 common probes, along with corresponding genes and functions or phenotypes (derived from the National Center for Biotechnology Information gene database). Among these, we noted 2 genes relevant to psychiatric status or neurotransmitter function (*HTR2B*, *CUEDC1*); others associated with physiologic or immune functions (*DSE*, *FCGR2A*, *HFM1*, *MCF2L2*, *MICAL2*, *NOSIP*, *EML6*); others with functions linked to nutritional status, insulin activity or energy metabolism (*PRKAG2*, *RPTOR*, *SORBS1*, *ZFAND3*, *ZNF608*); and a remainder associated with more general cellular or transcriptional processes (*ANKIB1*, *AP2B1*, *KPNA1*, *GPR133*, *RAB32*, *SNX27*, *CDT1*, *CUX1*, *FTSJ2*, *MSI2*). Note the observed connection between *DSE* and *FCR2A* and Ehler-Danlos and autoimmune syndromes, respectively. Associations between anorexia nervosa and Ehler-Danlos,³⁷ rheumatoid arthritis and systemic lupus erythematosus³⁸ have been documented elsewhere.

Anorexia nervosa restrictive type versus anorexia nervosa binge/purge type versus NED

The results from 1-way ANOVAs comparing probe-wise methylation levels for anorexia nervosa restrictive type versus anorexia nervosa binge/purge type versus NED comparisons, along with corresponding pairwise comparisons, can be found in Appendix 1, Table S3. Although reduced n limits power, a 3-group ANOVA identified 9 differentially methylated probes (at $Q < 0.01$). On many of these probes, pairwise comparisons were significant at $Q < 0.01$ and, allowing for effects at $p < 0.05$ (see superscripts in Appendix 1, Table S3), 8 of 9 probes were shared across anorexia nervosa restrictive type and anorexia nervosa binge/purge type. In all but 1 instance, the indicated effect was consistent with increased methylation in anorexia nervosa. Many of the identified genes have been associated with disorder-relevant functions

such as body mass and adiposity (*ZNF608*, *CCDC146*), digestive function (*NOSIP*) and taste and olfaction (*C2orf85*).

Chronicity of illness

Findings (at $Q < 0.01$) that linked chronicity of illness to methylation status identified 64 probes mapping onto 55 genes (Appendix 1, Table S4). Among these were 3 probes related to the glutamate metabotropic receptor 2 (*GRM2* [3 probes]). We also observed links to probes associated with genes that play a role in glia–neuron interactions (*ICAM5*, [5 probes]) and white-matter integrity/myelination (*ASPA*). Chronicity was also associated with the serotonin 2A receptor (*HTR2A*). Finally, the analysis isolated probes related to wound healing (*TNXB*), taste (*TAS1R3*), insulin metabolism (*IRS2*, *RPTOR*), skeletal homeostasis (*LRP5*) and age-related conditions (e.g., *ELOVL2*, *FHL2*).^{39,40} The direction of differences (on 39 of 64 [60.9%] differentially methylated probes) suggested a trend toward lower methylation in people with more chronic anorexia nervosa. Among identified genes, *PRRT1*, *ZNRF2*, *RPTOR*, *NRXN2*, *SPTA1* and *LRP5* were also isolated in the active versus remitted versus NED comparison described earlier.

Pre-/post-treatment changes in methylation

We found 73 probes (corresponding to 72 genes) at which the median change in BMI correlated with change in methylation at $Q < 0.05$ (and at $Q < 0.01$ on 8 probes; Appendix 1, Table S5). Differentially methylated probes included genes acting in cell growth (*RPS6KA2*), immune function (*CLEC7A*, *IRAK3*, *TAP2*, *HHLA2*) and inflammatory responses (*NFKBIA*, *NR1H3*). Other probes were associated with genes implicated in olfaction (*OR4C45*), lipid metabolism (*PEBP4*, *NR1H3*), insulin function (*IGF2R*) and a nicotinic acetylcholine receptor (*CHRNA5*). One of the probes (corresponding to *ASPA*) was identified in the effects of chronicity described above. The direction of difference on 56 of 73 (76.7%) differentially methylated probes in the pre/post analysis showed that people who gained more weight during treatment tended to show larger increases in DNA methylation, implying that the direction of methylation-level changes associated with weight gain was opposite to that associated with chronic illness exposure. Among the identified genes, *ESM1*, *GTDC1*, *RNF39*, *EML6*, *RPS6KA2* and *CUX1* were also identified in the active versus remitted versus NED comparison described earlier. Regressions examining associations with pre/post changes on the EDE-Q total or subscale scores yielded no significant results.

Discussion

We examined genome-wide methylation patterns in women with anorexia nervosa that was active or in stable remission, and in those who had no history of an eating disorder. In the active group, we also investigated methylation patterns as a function of chronicity of illness and treatment-linked changes in BMI and eating symptoms.

Group comparisons

Participants in the active and NED groups showed many differentially methylated sites, with significant between-group differences corresponding to genes implicated in metabolic and nutritional status (e.g., lipid, insulin and glucose metabolism), bone and tissue health, psychiatric status and immune function. At most differentially methylated probes, methylation levels were higher in the active group than the NED group, implying generally reduced expression of affected genes in actively ill participants. Comparisons between the active and remitted groups (the latter group presumed to represent susceptibility to anorexia nervosa in the absence of active illness sequelae) identified a parallel set of genes implicated in lipid, glucose and insulin metabolism; bone and tissue health; serotonin function; and immune function. Notably, the directional tendency of effects complemented that obtained in the active versus NED comparisons; that is, methylation levels on affected probes were generally lower in the remitted group than in the active group. Likewise, effects on probes that were common to active versus NED and active versus remitted comparisons pointed in opposite directions. Together, the pattern of results suggests methylation changes in people who are actively ill with anorexia nervosa — changes that can be “reset” by partial or full remission of illness. Similarly, findings that mainly differentiated people who were actively ill from those who were not currently ill (whether they had no eating disorder or were in remission from anorexia nervosa) leaned again toward the concept of DNA methylation being responsive to illness activity, rather than a stable, trait-like factor.

Correlational analyses

Consistent with the idea that many epigenetic changes constitute illness sequelae, longer exposure to active illness seemed to coincide with more marked methylation-level changes. Notably, the chronicity of illness was associated with methylation levels at genes associated with the functioning of 2 major neurotransmitters: glutamate and serotonin. Glutamate and serotonin systems have been implicated before in anorexia nervosa,^{5,41–43} and they are likely candidates underlying anorexia nervosa’s characteristic psychiatric manifestations. Moreover, findings isolated the serotonin-2A receptor gene (*HTR2A*), a gene modulating affect, cognition and emotion regulation;^{43,44} *HTR2A* has been linked to anorexia nervosa in previous candidate-gene studies,⁵ and in our own previous epigenome-wide study.²⁶

Among the probes most strongly associated with illness duration, we found *ELOVL2* and *FHL2*, both of which have been associated with epigenetic age and age-related conditions.^{39,40,45,46} It is an intuitive point that the length of a person’s exposure to anorexia nervosa is likely to be detrimental to their health prospects, and our findings suggest that several adverse effects of this type may be epigenetically regulated. Consistent with possible effects of the underconsump-

tion of nutrients implicated in DNA methylation, illness chronicity was inversely correlated with methylation levels at most of the implicated probes: greater duration of illness was associated with lower methylation. In contrast, longitudinal data indicated that BMI increases generally coincided with increases in methylation-level changes at genes associated with lipid and glucose metabolism, immune and inflammatory processes, and olfaction. Together, these results suggest that methylation levels may be responsive to short- and long-term alterations in illness status. If so, methylation measures could have promise as markers of illness staging.

We also want to comment on the possible disorder-specific relevance of differential methylation of 4 genes. We found that the presence and chronicity of illness was associated with methylation at probes related to the *RPTOR* gene, which influences cell growth in response to nutrients and insulin, and has been linked to such metabolic disorders as obesity and type 2 diabetes, and to the functioning of the gut microbiota.⁴⁷ We also found active illness to be associated with increased methylation at probes associated with the *PRKAG2* gene. Lower methylation of *PRKAG2* has been associated with higher BMI in a community sample.⁴⁸ In addition, similar to 2 previous studies in anorexia nervosa,^{26,27} we found that *TNXB* methylation was associated with the presence of anorexia nervosa; the 2 previous studies reported that methylation at this gene was altered in people with anorexia nervosa.^{26,27} *TNXB* has been involved in connective-tissue disorders. Furthermore, the observed decreases in *NR1H3* methylation post-treatment was in line with the results of 2 studies showing hypermethylation of this gene in active anorexia nervosa.^{26,27}

Limitations

Given the tissue specificity of DNA methylation,^{49,50} brain tissue is the optimal medium for epigenetic studies on mental health entities. However, in the absence of postmortem brain banks for anorexia nervosa, studies such as ours must rely on biomaterials from peripheral tissues. Despite these limitations, however, epigenetic studies involving peripheral tissues can inform the understanding of processes in the brain. First, DNA methylation in the periphery may reflect a peripheral component of a body-wide somatic response to environmental signals that also alters methylation in the brain.⁵¹ Second, various blood–brain mediators (e.g., glucocorticoids, cytokines such as IL-1 β) may link central and peripheral effects.^{49,52} Third, on certain genes, there are relatively good correspondences between epigenetic markings obtained in blood and brain.^{50–55} Finally, unique disease-relevant epigenetic markings in peripheral tissues, even if only partially representative of the effects in brain, may yet have clinical potential,⁵¹ especially in the case of a disorder such as anorexia nervosa, which has widespread effects throughout the body.

Without measures of gene expression, our results cannot ascertain the functional significance of the altered methylation levels we observed. However, the literature affirms that methylation levels correspond to expression

levels on many of the genes of interest found in this study, including *HTR2A*,⁵⁶ *ICAM5*,⁵⁷ *CAMKK2*,⁵⁸ *CCR7*,⁵⁹ *ELOVL2* and *FHL2*.⁶⁰ Future investigations (including in vitro models to establish direct influences of changes in DNA methylation on gene expression) may help elucidate the actual functional significance of the identified genes. Finally, some of our findings may have been influenced by the effects of medication or substance use. Future replication of findings will, therefore, be a determinant.

Conclusion

Our findings have associated anorexia nervosa with epigenetic alterations bearing upon individuals' mental and physical status, metabolic functions and immunity, and also point to the likelihood (given that they tended to differentiate people with active anorexia nervosa from people in full or partial recovery from anorexia nervosa and from people without an eating disorder) that many of the alterations observed were reversible consequences of active illness. These findings are intriguing, because they may point to biomarkers for disease staging or targets for innovations in nutritional or pharmacological treatments.

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