Research Paper

Elevated plasma B_{12} and betaine levels in women with anorexia nervosa: possible role in illness pathophysiology and epigenetic regulation

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Background: Phenomenology in anorexia nervosa (AN) appears to be subject to epigenetic regulation via DNA methylation. The micronutrients B_{12} and betaine contribute directly to DNA methylation and have been shown to be abnormally elevated in blood samples from people with AN. **Methods:** We measured plasma B_{12} and betaine levels, as well as leukocyte DNA methylation levels, among women with active AN (AN-active group), those in 1-year remission from AN (AN-remitted group), and those who had never experienced an eating disorder (NED group). We compared the groups on micronutrient levels and on the strength of association between micronutrients and methylation. **Results:** We included 64 women in the AN-active group, 49 in the AN-remitted group, and 49 in the NED group. Relative to those with NED (B_{12}: mean 339.6 [standard deviation (SD) 224.3] μmol/L; betaine: mean 33.74 [SD 17.10] μmol/L), participants with active AN showed high B_{12} and betaine (B_{12}: mean 571.0 [SD 505.2] μmol/L; betaine: mean 43.73 [SD 22.50] μmol/L); AN-remitted participants had elevated B_{12} alone (B_{12}: mean 588.2 [SD 379.9] μmol/L; betaine: mean 33.50 [SD 19.20] μmol/L). There were also group-based differences in the strength of association between B_{12} and site-specific DNA methylation at genes regulating insulin function, glucose metabolism, cell regulation, and neurotransmitter function. These associations between B_{12} and methylation levels were generally stronger among those without an ED than among those with either active or remitted AN. **Limitations:** The extent to which plasma nutrient levels provide a meaningful proxy to cellular processes affecting DNA methylation is uncertain and the sample size limits the stability of results. We included only biological females in this investigation. **Conclusion:** Elevated B_{12} levels in AN resemble elevations reported among people with autoimmune, neoplastic, or other disorders. Such elevations imply that plasma B_{12} levels may misrepresent nutritional status among people with AN. Observed associations between levels of B_{12} and methylation at certain gene regions have ambiguous importance, but may indicate an influence of nutritional status on epigenetic mechanisms or may be the coincidence of separate processes that independently affect levels of micronutrients and DNA methylation.

Introduction

Epigenetic mechanisms result in site-specific molecular modifications that alter gene expression and corresponding phenotypic variations.[1–3] The best studied epigenetic mechanism, DNA methylation, has been implicated in diverse medical and psychiatric disorders, including eating disorders (EDs).[4–7] Epigenome-wide studies of anorexia nervosa (AN) have documented altered methylation levels in blood-based DNA samples at sites implicated in psychiatric, metabolic, and immune functions.[8–12] Furthermore, results published by our group have associated illness duration with more pronounced methylation alterations, and remission of AN with normalization, which suggests reversible effects corresponding to extent and duration of illness exposure.[8,11,12]

The micronutrients B_{12} and betaine participate in the methyl-transfer reactions upon which DNA methylation depends.[13–15] Predictably, levels of these micronutrients are often reduced in nutritionally deprived individuals.[15] However, some AN studies indicate counterintuitive elevations. One study involving people with active AN reported elevated plasma B_{12} and folate levels.[16] Another indicated that, compared with people without an ED, people with active or remitted AN exhibited elevated levels of B_{12} and, less reliably,

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betaine.[17] A third study reported elevated B_{12} among people with AN who were underweight or whose weight was partly restored.[18] The cause of such paradoxical elevations remains uncertain but has been postulated to implicate factors ranging from liver dysfunction (associated with malnutrition-induced apoptosis),[16] to physiologic compensations related to reduced dietary precursors of betaine and B_{12},[17] to inflammation-linked alterations in synthesis of binding proteins.[19,20]

The only study of which we are aware that directly examined the association between B_{12} and DNA methylation levels in blood from people with AN found no association.[21] We sought to examine associations between plasma levels of B_{12} or betaine, on the one hand, and global and site-specific levels of DNA methylation, on the other, among people with and without active AN. We anticipated that the strength of association between plasma micronutrient and DNA methylation levels would vary as a function of presence or absence of active AN.

Methods

Participants

Participants in the current study were members of a larger cohort (n = 310) of women who provided blood samples for assessment of DNA methylation. Of these, some had active AN according to *Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (*DSM-5) criteria[22] (AN-active), some had previously fulfilled DSM-5 criteria for AN but had maintained a self-reported BMI above 18.4 kg/m^2 for at least 12 months (AN-remitted), and some had never experienced an ED (NED). We previously reported probe-wise methylation findings from this cohort at earlier stages of this investigation.[11,12] The present report addresses a subsample of these individuals from whom we also obtained plasma micronutrient levels.

Diagnoses of AN were primarily based on interviews conducted with the Eating Disorders Examination (EDE)[23] or, when EDE results were not available, clinical interviews conducted by experienced clinicians (e.g., psychiatrists, PhD psychologists, other licensed psychotherapists on staff in a specialized tertiary care eating disorders program), complemented by team consensus and results from the EDE-Questionnaire (EDE-Q).[24]

We refer to our participants as women, but acknowledge that they may not all have identified as such.

Measures

To assess ED symptoms and confirm DSM-5 diagnoses of AN, we used the EDE interview and the EDE-Q[2]. Both are well-validated, widely used measures.[25] We calculated BMI using anthropometric measures.

Assays for micronutrients and DNA methylation were conducted using whole blood collected in tubes containing ethylenediaminetetraacetic acid and centrifuged to isolate plasma. As in other studies (e.g., Barron and colleagues[26]), we used nonfasting blood levels to avoid compromising patient recovery. For methylation analyses, DNA was extracted from leukocytes using a Qiagen kit. A portion of plasma was frozen at –80°C for nutrient analyses.

For practical reasons, we were required to assay micronutrient levels at 2 distinct laboratories for each micronutrient, with some samples assayed at McGill University (laboratory A) and others assayed at the Université de Montréal (laboratory B). Concentrations of B_{12} were measured at laboratory A using AccuBind enzyme-linked immunosorbent assay kits and at laboratory B using chemiluminescence microparticle immunoassays from Abbott Diagnostics. At laboratory A, betaine was analyzed in 1 aliquot by liquid chromatography tandem mass spectrometry. At laboratory B, it was assayed using high-performance liquid chromatography coupled with an accurate-mass quadrupole time-of-flight mass spectrometry. The first 120 samples were assayed at laboratory A and the last 42 samples were assayed at laboratory B. We took several measures to ensure that results based on different assay techniques did not substantially differ. First, we examined interlaboratory correlations for B_{12} (r = 0.88, $p < 0.001$) and betaine (r = 0.89, $p < 0.001$). Next, we examined within-group proportions of assays conducted in laboratory A or B (AN-active: 36.7% v. 47.6%; AN-remitted: 33.3% v. 21.4%; NED: 30% v. 31%) and found no differences (χ^2 = 2.41, df = 1, p = 0.3). Finally, when testing for group differences in mean nutrient levels, we controlled for laboratory effects with a covariate. Because of technical difficulties, B_{12} levels were missing for 6 participants (2 in AN-active group, 2 in AN-remitted group, and 2 in NED group).

We performed epigenome-wide methylation assays using either the Infinium HumanMethylation450 BeadChip Kit or the Infinium MethylationEPIC BeadChip Kit (Illumina Inc.). Reliance on different kits was because of discontinued production of the 450 BeadChip kit during the early phase of our data collection. We note, however, that manufacturer information, our previous research,[11,12] and an independent study[27] suggest that samples can be combined effectively across the 450 BeadChip and EPIC kits. Only probes common to both kits were entered into our analyses. Further technical details are provided by Steiger and colleagues.[11]

We obtained information on participants' use of vitamin supplementation during a medication history interview, conducted by research assistants, that had participants report use of nonprescription medications, vitamins, or herbal or homeopathic products. We supplemented the inquiry with a question on nutritional supplements from a food frequency questionnaire, which was completed by a subset of participants as part of a secondary investigation.

Evaluation of liver function was not an original consideration in the design of this study. However, to provide a post hoc evaluation of the extent to which any B_{12} or betaine anomalies might be attributable to liver dysfunction, we made efforts to obtain alanine aminotransferase (ALT) values through chart review; ALT was often, but not systematically, measured as part of routine medical evaluations of treated patients. We limited our search to ALT values obtained within 3 months of the blood draw used for DNA extraction and nutrient assays.

Statistical analysis

We compared groups on descriptive variables and micronutrient levels using general linear models or χ^2 statistics, as appropriate. For group comparisons on micronutrient levels, we included psychotropic medication use (yes v. no), smoking (yes v. no), age, and laboratory (A v. B) as covariates. We investigated significant group effects by pairwise group comparisons with Bonferroni corrections ($p_{corr} < 0.05$).

We conducted analyses involving DNA methylation using MATLAB 2021. Further details on data preprocessing are provided by Steiger and colleagues[12] and in Appendix 1, available at www.jpn.ca/lookup/doi/10.1503/jpn.240155/tab-related-content. The primary outcomes were β values representing the proportion of methylated cells (scale 0–1) at each probe. To test for group-based differences in the strength of association between micronutrient and methylation levels, we used general linear mixed models. Analyses included methylation β values as outcomes and group (AN-active, AN-remitted, or NED), micronutrient level, and a term representing the interaction of group by micronutrient level as predictors. Estimated cell proportions, age, smoking, and use of psychotropic medication were included as covariates, and array (a control for effects of plating) was included as a random factor. We applied a false discovery rate correction ($q < 0.01$) throughout. We used Cook's distance (Cook's *D*) to test for the presence of outliers.

Ethics approval

The study was approved by the Research Ethics Board of the West Island Integrated University Health and Social Services Centre (no. IUSMD-15–30). Written informed consent was obtained from all participants.

Results

We included 64 women in the AN-active group, 49 in the AN-remitted group and 49 in the NED group. Of those with active AN, 39 (60.9%) had AN restricting type, and 25 (39.1%) had AN binge-eating/purging type. Diagnosis of AN relied on clinical interviews rather than the EDE for 6.2% of patients.

Descriptive data on all participants are shown in Table 1. Groups were comparable in mean age but differed in expected directions on mean BMI and EDE-Q scores, with significantly lower BMI and higher EDE-Q scores in the AN-active group than in the AN-remitted and NED groups ($p < 0.001$). The mean EDE-Q score in the AN-remitted group fell below the clinical cut-off but was still higher than that of the NED group ($p_{corr} = 0.02$). Groups did not differ significantly on self-reported use of B_{12} or multivitamin supplements (Table 1).

Self-reported ancestry data were available for all 64 participants in the AN-active group, 40 (81.6%) participants in the AN-remitted group, and 42 (85.7%) participants in the NED group. Participants in each of the groups were mainly of European descent. Specifically, 59 (92.2%) participants in the AN-active group reported European ancestry, 2 (3.1%) reported Hispanic or Latino ancestry, 1 (1.6%) reported Middle Eastern ancestry, and 2 (3.1%) reported other ancestry. In the remitted group, 35 (87.5%) participants reported European ancestry, 2 (5%) reported Middle Eastern ancestry, 1 (2.5%) reported Asian ancestry, and 2 (5%) reported other ancestry. In the NED group, 26 (61.9%) participants reported European ancestry, 5 (11.9%) reported Asian ancestry, 4 (9.5%) reported Black or Caribbean ancestry, 4 (9.5%) reported Hispanic or Latino ancestry, 1 (2.4%) reported Middle Eastern ancestry, 1 (2.4%) reported Indigenous ancestry, and 1 (2.4%) reported other ancestry. There was, thus, a slight skew toward more ancestral heterogeneity in the NED group.

Table 1: Participant characteristics and nutrient variables

	No. (%) of participants*			
Variable†	AN-active *n* = 64	AN-remitted *n* = 49	NED *n* = 49	Statistic
Age, yr, mean ± SD	25.22 ± 7.8	27.18 ± 6.2	24.84 ± 5.5	$F_{2159} = 1.77$, $p = 0.2$
BMI, mean ± SD	15.03 ± 1.7	21.97 ± 2.8	23.04 ± 2.9	$F_{2159} = 183.75$, $p < 0.001$‡
EDE-Q score, mean ± SD	3.87 ± 1.4	1.11 ± 0.9	0.49 ± 0.5	$F_{2141} = 146.69$, $p < 0.001$§
Psychotropic medication	35 (54.7)	17 (34.7)	0 (0)	$\chi^2 = 38.30$, $p < 0.001$¶
Smokers	10 (15.6)	5 (10.2)	7 (14.3)	$\chi^2 = 0.72$, $p = 0.7$
Supplements				
Multivitamin	4 (6.5)	7 (14.3)	4 (8.2)	$\chi^2 = 2.10$, $p = 0.4$
B_{12}	3 (4.8)	3 (6.1)	2 (4.1)	$\chi^2 = 0.22$, $p = 0.9$
B_{12}, μmol/L, mean ± SD	571.0 ± 505.2	588.2 ± 379.9	339.6 ± 224.3	$F_{2149} = 7.64$, $p < 0.001$¶
Betaine, μmol/L, mean ± SD	43.73 ± 22.50	33.50 ± 19.20	33.74 ± 17.10	$F_{2155} = 5.32$, $p = 0.006$‡

AN = anorexia nervosa; BMI = body mass index; EDE-Q = Eating Disorders Examination Questionnaire; NED = no eating disorder; SD = standard deviation.
*Unless indicated otherwise.
†Data on EDE-Q were missing for 6 participants in AN-active group, in 8 in AC-remitted group, and 4 in NED group. Multivitamin supplement use was missing for 2 participants in AN-active group; information on B_{12} supplement use was missing for 1 participant in the AN-active. Data on B_{12} levels were missing for 2 participants in AN-active group, 2 in AN-remitted group, and 2 in NED group).
‡After Bonferroni correction, AN-active group was significantly different ($p < 0.05$) from AN-remitted and NED groups.
§After Bonferroni correction, significant differences ($p < 0.05$) between all 3 groups.
¶After Bonferroni correction, AN-active and AN-remitted groups were significantly different ($p < 0.05$) from NED group.

Plasma nutrient levels and ALT values

Table 1 shows mean plasma B_{12} and betaine levels in the different groups. We observed a significant group effect ($F_{2149} = 7.64$, $p < 0.001$, $\eta_p^2 = 0.093$), with Bonferroni-corrected group comparisons showing AN-active ($p_{corr} < 0.001$) and AN-remitted participants ($p_{corr} = 0.005$) to have higher B_{12} levels than NED participants. Levels of B_{12} did not differ between AN-active and AN-remitted groups ($p > 0.99$). Likewise, there was no difference ($p = 0.2$) between AN-active participants with restrictive and binge–purge subtypes. There was also a significant group effect on betaine levels ($F_{2155} = 5.32$, $p = 0.006$, $\eta_p^2 = 0.064$). Post hoc comparisons showed AN-active participants to have elevated betaine levels, compared with those in either the AN-remitted ($p = 0.01$) or NED groups ($p = 0.02$). Levels in the AN-remitted and NED groups did not differ significantly ($p > 0.99$). Again, within the AN-active group, there were no differences between participants with restrictive or binge–purge AN subtypes ($p = 0.6$).

Values of ALT were available for 46 (71.9%) of 64 participants with active AN. Values were not available for AN-remitted or NED participants, since they were not under active care in our clinic. Values exceeded the normal range in 7 (15.2%) of 46 AN-active participants for whom ALT values were available.

Associations between plasma micronutrient and DNA methylation levels

Methylation data were available for 62 of 64 of AN-active participants, 48 of 49 AN-remitted participants, and 48 of 49 NED participants, for a total sample size of 158 people. We performed epigenome-wide methylation assays using the 450 BeadChip Kit for our first 47 samples and the EPIC BeadChip Kit for 111 samples. Given missing data for B_{12}, the analyses on the association between methylation and B_{12} levels included 153 participants (60 AN-active, 47 AN-remitted, and 46 NED).

Overall associations between global methylation and B_{12} or betaine were nonsignificant ($q = 0.20$ and 0.70, respectively). Likewise, global methylation was not associated with B_{12} or betaine in any of the 3 groups ($q > 0.08$).

At a probe-wise level, we tested overall associations between nutrient and methylation levels, within each group, and between groups (Table 2).

B_{12}

Thirty-six sites showed overall effects (irrespective of group) associating plasma B_{12} levels with DNA methylation. Tests for group-based differences in the strength of association between methylation and nutrient levels (i.e., group × nutrient-methylation) indicated significant associations with 48 probes. However, most of these effects were driven by statistical outliers (Cook's $D > 1$). After exclusion of probes for which Cook's D exceeded 1, group differences for the methylation–B_{12} association (reflected by group × association interactions at $q < 0.01$) remained for 16 probes. Ten of these probes mapped onto distinct genes (Table 2). Generally, there were significant associations between B_{12} and DNA methylation levels in the NED group (at $q < 0.01$ or, in 1 case, $q < 0.02$), whereas in the AN-active and AN-remitted groups, associations tended to be absent. Findings in the NED group

Table 2: Associations between plasma B_{12} levels and site-specific DNA methylation

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CG site	Gene	Gene involvement*	*F*	AN-active	AN-remitted	NED	Interaction
cg10344477	*B3GNTL1*	Glycosylation	2.53	–3.31	–1.99	4.91†	AN-active v. NED: –4.91†
cg07515565	*GMDS*	Fucosylation; cell regulation	3.40	3.89	2.51	–5.35†	AN-active v. NED: 5.46†
cg08292959	*MGAT5B*	Glycosylation; colitis; irritable bowel	14.09†	3.44	2.83	–5.70†	AN-active v. NED: 5.43 AN-remitted v. NED: 5.04†
cg16033053	*MKX*	Cell adhesion; tendon development	9.85	–3.84	–1.29	4.60‡	AN-active v. NED: –4.85†
cg09689342	*NAGPA*	Stuttering and articulation disorder	10.40	3.33	2.36	–5.23†	AN-active v. NED: 5.09†
cg07743764	*NXF1*	Cell regulation; RNA export	5.94	–3.23	–2.24	4.73†	AN-active v. NED: –4.73†
cg09714852	*PTPRN2*	Insulin secretion; diabetes; BMI; waist-to-hip ratio; accumulation of secretory vesicles in hippocampus (monoamines), pituitary (hormones), and pancreatic islets (insulin)	12.46	3.64	1.85	–5.18†	AN-active v. NED: 5.12†
cg10336025	*PXYLP1*	Ehlers–Danlos syndrome	10.94	2.77	2.93	–4.85†	AN-active v. NED: 4.54
cg25313204	*SLC22A3*	Extraneuronal monoamine transport; elimination of organic cations; diabetes type 1	10.24	–3.12	–2.69	5.30†	AN-active v. NED: –5.03†
cg09197075	*SNN*	Apoptosis; response to neurotoxicity	18.54†	2.25	–5.67*	1.36	AN-active v. AN-remitted: 5.41†

AN = anorexia nervosa; BMI = body mass index; NED = no eating disorder.
*Gene functions and associated phenotypes were obtained using the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/gene), the Genecards database (https://www.genecards.org), and additional scientific literature (cited in Discussion).
†$q \leq 0.01$.
‡$q = 0.02$.

associated higher B_{12} levels with lower methylation at probes corresponding to *GMDS*, *MGA5TB*, *NAGPA*, *PTPRN2*, and *PXYLP1*, and with higher methylation levels at probes linked to *B3GNTL1*, *NXF1*, and *SLC22A3* ($q \leq 0.01$). We also noted a statistical trend toward an association between higher B_{12} levels and higher *MKX* gene methylation in the NED group ($q = 0.02$). Exceptionally, 1 probe located in *SNN* showed an association between lower methylation and higher B_{12} levels in the AN-remitted group, with an absence of association in the other 2 groups. Overall, significant differential effects across groups indicated associations between B_{12} and methylation levels to be stronger in the NED group than in either the AN-active or AN-remitted groups. In the AN groups, B_{12}–methylation associations tended to be weak or fully absent (Table 2). Where differential group effects were observed, they were apparent in genes implicated in metabolism, cell regulation, connective tissue development and neurotransmitter function (Table 2).

We obtained group by B_{12}–methylation association interactions ($q < 0.01$) on 6 additional probes (cg01047586, cg19608003, cg08118140, cg18647259, cg13175120, cg06899226). However, these probes were not associated with specific genes.

Betaine

We found 4 probes to be differentially associated with betaine in the AN-active group, compared with associations observed in the AN-remitted and NED groups. However, no overall, main, or interaction effects were retained after exclusion of effects influenced by outliers (Cook's $D > 1$).

Discussion

We compared plasma B_{12} and betaine levels among women who were actively ill with AN, who had once had AN but were nutritionally restored, or who had never had an ED. We also compared the strength of relationships between micronutrient and DNA methylation levels across the 3 groups. In keeping with previous findings,[16–18] we observed apparently paradoxical elevations in micronutrients in AN groups, relative to levels seen in the NED group. Specifically, compared with the NED group, AN-active participants showed increased B_{12} and betaine, whereas AN-remitted participants showed elevated B_{12} alone. This pattern of results was also documented in a previous report by our group,[17] which involved a substantial subset (116 of 162, 71.6%) of the same participants. Similar elevations of B_{12} among people with AN (whether underweight or weight-restored) were documented by Tam and colleagues.[18]

To rule out effects of dietary supplements on B_{12} and betaine levels, we collected data on participants' use of supplements containing B_{12}. These data indicated nearly identical rates of supplement use across the 3 groups, suggesting that dietary supplementation was unlikely to be a confounding factor.

Alternatively, elevated plasma B_{12} among patients with AN has been attributed to malnutrition-induced liver dysfunction.[16,18] Not having ALT values for our NED or AN-remitted participants, we were unable to fully exclude liver dysfunction as an explanation. However, abnormal ALT values were relatively uncommon among AN-active participants (found in only 7 [15.2%] of 46 cases) and were unlikely to have been present at all among AN-remitted patients. Therefore, we considered other explanations for elevations, namely reports of elevated B_{12} values in populations with autoimmune diseases (lupus, rheumatoid arthritis, Still disease), cancers, alcoholism, and kidney and bronchopulmonary diseases.[19,20] Such elevations have been thought to reflect abnormalities of tissue transport and uptake of B_{12} involving the binding proteins transcobalamin-2 and haptocorrin.[19] Transcobalamin-2, a protein synthesized in most tissues, delivers B_{12} from blood to cells in bodily tissues. Notably, its levels are altered in inflammatory disorders. Haptocorrin, a glycoprotein secreted by the salivary glands, protects B_{12} from stomach acid and also binds circulating B_{12} to block uptake by cells. Insufficient B_{12} clearance through cellular uptake could account for excessive B_{12} accumulation in blood, even in the presence of deficient dietary B_{12}.[19,28] Given that AN has pervasive metabolic effects, as well as known association with autoimmune and inflammatory diseases,[29–31] we speculate that elevated B_{12}, seen here among people with active and remitted AN, may represent problems of B_{12} clearance of this type, rather than a direct consequence of liver dysfunction. Regardless, the phenomenon is noteworthy, given that elevated plasma B_{12} levels among people with AN may not only mask what is an actual B_{12} deficiency, but may also indicate an underlying pathology that needs to be better characterized. We therefore advocate for further investigations into the precise causes and clinical implications of elevated B_{12} among people with AN.

Our findings on DNA methylation showed no significant relationships between levels of plasma micronutrients and global DNA methylation in any of our 3 groups. This echoes a similar result indicating an absence of relationship between plasma B_{12} levels and global DNA methylation in AN,[21] and raises doubt about the pertinence of global methylation measures in studies of epigenetic effects in AN. In contrast, examination of the association between nutrient levels and probe-wise methylation levels revealed that a handful of probes showed differential B_{12}-to-methylation relationships across groups. Given that very few probes were implicated, we interpret these findings cautiously. Nonetheless, where such effects were observed, they tended to indicate B_{12} and methylation levels to be linked in the NED group but disconnected in both AN groups (active or remitted). We speculate that the relative absence of association between B_{12} and methylation among participants with AN could imply a breakdown in a link that, according to results in the NED group, is normative. We consider that the preceding could result through the action of independent processes (e.g., a metabolic effect among participants with AN that had no impact on methylation, but that substantially increased plasma B_{12} levels), or a physiologic mechanism — with possible epigenetic relevance — that disrupted the normal contribution of B_{12} to DNA methylation among participants with AN. In support of the latter possibility, we note that observed differential effects across groups mapped onto genes with credible disorder-relevant functions,

namely *MGAT5B* (involved in glycosylation, colitis, and inflammatory bowel disease),[32] *GMDS* (acting in fucosylation, insulin secretion, and diabetes),[33] *PTRN2* (involved in insulin dependence, obesity, type 2 diabetes, and monoamine accumulation in the brain),[34] *SLC22A3* (linked to monoamine uptake and diabetes),[35] *B3GNTL1* (acting in glycosylation),[36] *MKX* (associated with tendon development),[37] and *PXYLP1* (associated with the connective tissue disorder, Ehlers–Danlos).[38] Disorders implicating connective tissues have been associated with AN.[39]

Although their relevance to AN is unclear, altered B_{12}–methylation relationships were also observed among AN-active participants at sites affecting *SNN* (related to the body's response to toxins),[40] *NXF1* (coding for RNA exports from cell nuclei),[41] and *NAGPA* (associated with articulation and developmental stuttering).[42] In addition, we note that comparisons between AN-active and AN-remitted participants indicated significantly different relationships between B_{12} levels and *SNN* methylation levels, and comparisons between AN-remitted and NED participants showed altered associations between B_{12} and *MGAT5B* methylation.

Limitations

We assessed the association between plasma micronutrient levels and site-specific DNA methylation among people with AN. Aspects related to micronutrient levels were quite well controlled, given attention to liver function and dietary supplement use, although the absence of information ascertaining quantities or frequency of supplement use is a limitation. Given multiple intervening factors in the pathway linking nutrient intake to methylation of genes, it remains uncertain to what extent plasma nutrient levels provide a meaningful proxy to cellular processes that affect DNA methylation. As well, we did not address other factors (e.g., childhood abuse, other developmental stressors) that have known impact on DNA methylation levels.[43] Similarly, as with all DNA methylation studies involving peripheral tissues, implications for brain function remain uncertain.[44] Although it was by design (to accommodate the relatively low frequency of males with anorexia nervosa), we included only biological females in our investigation, which limits the generalizability of findings. Finally, there was a small tendency toward more heterogeneous ancestry in our NED group.

Conclusion

This study corroborates previous results indicating elevated plasma B_{12} levels among people who are actively ill with AN, or who have recovered substantially from it.[17,18] These findings have potentially important clinical implications, since they imply that B_{12} levels obtained in routine clinical assessment may not accurately reflect B_{12} status among people with AN. Rather, elevations may serve as a marker of a more complex metabolic disturbance. We also observed isolated, site-specific effects, which imply differential associations between B_{12} and methylation levels in different groups, pointing to genes involved in glucose metabolism, insulin function, and neurotransmitter function. Given that these differential associations were observed in only a small proportion of assessed sites and, more importantly, that they implicate elevated B_{12} levels that have an uncertain physiologic implication, we offer speculations on our findings cautiously. Ultimately, observed associations between levels of B_{12} and methylation could indicate a direct influence of nutrient status on epigenetic mechanisms, but they could also result from a coincidence of processes that independently affect micronutrient levels and methylation. Either way, elevated B_{12} and betaine levels among people who are actively ill with AN may have clinical ramifications, epigenetic and otherwise, that warrant further clarification.

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