Plasma Levels of One-Carbon Metabolism Nutrients in Women with Anorexia Nervosa

Short Running Title: One-Carbon Metabolism Nutrients in Anorexia Nervosa

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Abstract

Objective: People who are ill with Anorexia Nervosa (AN) show altered availability of key plasma nutrients. However, little is known about the patterning of alterations that occurs across diverse nutrients during active phases of illness or about the persistence of any such alterations following remission of illness. Method. We compared plasma levels of one-carbon metabolism nutrients across women with active AN (AN-Active: *n*=53), in remission from AN (AN-Remitted: *n*=40), or who had no eating-disorder history (NED: *n*=36). We also tested associations between Body Mass Index (BMI) changes and changes in pre-to post-treatment nutrient levels, and explored the association between nutrient levels, on the one hand, and BMI and eating symptoms, on the other. Choline, betaine, and methionine were analyzed using mass spectrometry. Folate and B12 were analyzed using the AccuBind® ELISA kit. Eating-disorder symptoms were assessed by interview and self-report. Results: Compared to NED individuals, AN-Active individuals exhibited significantly elevated B12 and (less-reliably) betaine. In AN-Active individuals, lower BMI was associated with higher B12. Discussion: The observed alterations run contrary to the intuition that plasma nutrient levels should be directly responsive to nutritional status and suggest, instead, the existence of compensatory adaptations to malnutrition in individuals with active AN. Further study is required to clarify mechanisms that underlie such effects.

Keywords: Anorexia Nervosa, micronutrients, one-carbon metabolism, malnutrition, nutrigenomics, choline, B12

As part of an ongoing study on DNA methylation in people with Anorexia Nervosa (AN), we have been measuring plasma levels of micronutrients involved in the one-carbon metabolism (OCM) pathway. Nutrients in this pathway allow for methionine production, which contributes directly to DNA methylation (Anderson et al., 2012). Few studies have examined plasma levels of OCM nutrients in AN. However, several that do indicate counter-intuitive elevations (or absence of deficits) on nutrient levels measured in actively ill, nutritionally deprived individuals. For example, one study reported elevated plasma B12 and folate levels (Corbetta et al., 2015), another reported elevated methionine (Moyano, Vilaseca, Artuch, & Lambruschini, 1998), and another noted that B12 and folate deficiencies are surprisingly rare (Hanachi et al., 2019). We sought to extend the existing literature by examining levels of selected OCM nutrients in people who were currently or formerly ill with AN.

We compared plasma OCM nutrient levels across three groups: Women with active AN (AN-Active), women in remission from AN (AN-Remitted), or women with no eating-disorder (NED) history. We also tested associations between pre-to post-treatment body mass index (BMI) changes and pre-to post-treatment changes in nutrient levels, and examined whether or not plasma nutrient levels differed between individuals with binge/purge and restrictive types of AN. Finally, we explored the association between plasma nutrient levels, on the one hand, and BMI and eating-disorder symptoms, on the other.

Methods

Participants

This research ethics board approved study was conducted with informed consent. For our AN-Active group, we recruited 53 women meeting DSM-5 criteria for AN and having a Body Mass Index (BMI) ≤17.5. Of these women, 30 had AN-restrictive type and 23 had AN-

binge/purge type. We also recruited 40 women who once met full DSM-5 criteria for AN (AN-Remitted group) according to retrospective EDE interviews, but who no longer met criteria for an ED and who had maintained a BMI of at least 18.5 (normal weight; World Health Organization) for at least 1 year – except for one individual with BMI= 18.43 who was included because she exhibited no ED-symptoms upon interview. Of these women, 31 had a bingeing or purging history upon interview (and were classified as in the binge/purge type spectrum). Conversely, 9 showed no bingeing or purging history, and were classified as restrictive type. Excluding medicated individuals was impractical and would have compromised representativeness. We thus included 31 AN-Active individuals (58.69% of the sample) and 14 AN-Remitted individuals (35% of the sample) who were taking a psychotropic medication. We recruited 36 non-eating disordered women (NED group) through public and university-based announcements. NED participants had no EDs or other psychiatric illnesses according to structured interview, and were not taking any psychotropic medications. In a subgroup of 28 AN-Active participants, we obtained a second measurement of nutrients following an interval in treatment focused on weight restoration (*M* = 107 days, range = 66-143 days).

Measures

ED-symptoms were assessed using the Eating Disorders Examination (EDE) interview (Fairburn et al., 2008) and the Eating Disorder Examination Questionnaire (EDE-Q; Fairburn et al., 2008). BMI (Kg/m^2) was calculated using anthropometric measures.

Nutrient Assays. As in other studies (see Barron et al., 2017), we obtained non-fasting blood levels to avoid compromising patient recovery. Whole blood was collected in EDTA tubes, centrifuged to isolate plasma, which was frozen at -80 °C. Folate and B12 concentrations were measured using AccuBind® ELISA kits following manufacturer's protocol. Choline,

betaine, and methionine were analyzed in one aliquot by tandem mass spectrometry (LC-MS/MS). For each participant, metabolite concentrations were measured in duplicates. The mean for each duplicate pair was used in statistical analyses. In cases where one of two sample dilutions yielded concentrations that were outside of the assay's sensitivity range (i.e., in seven folate assays, four B12 assays, and one methionine assay), we retained only the viable concentration value.

Data Analyses

Groups were compared on descriptive variables using one-way ANOVAs. Nutrient levels were compared across groups using a one-way MANOVA. Significant overall MANOVA results ($p<.05$) were followed by ANOVA with Bonferroni multiple-comparisons corrections. A linear mixed model analysis was performed to examine whether changes in BMI were associated with pre- to post-treatment changes in nutrient levels in the AN-active group. Nutrient variables on which pre-treatment levels in the AN-active group differed from those in the NED group were entered as time-varying covariates. We then tested for associations between nutrient levels and BMI, as well as ED-symptoms using separate linear regression analyses within each group. BMI and EDE-Q Global scores were used as separate criterion variables with the five nutrient variables entered as predictor variables. Secondary analyses comparing nutrient levels between participants with restrictive or binge/purge variants were conducted separately for AN-Active and AN-Remitted groups using independent *t*-tests.

Results

Data on participants' clinical and demographic characteristics are presented in Table 1. Predictably, AN-Active individuals had lower BMIs and more-pronounced ED-symptoms than

did AN-Remitted or NED individuals. AN-Active individuals also showed tendencies towards more medication use and more psychiatric comorbidity.

Results from the one-way MANOVA revealed a significant multivariate effect, $F(10, 226) = 3.97$, $p < .001$; Wilk's $\Lambda = 0.724$, $\eta_p^2 = .149$. Univariate tests isolated significant differences on levels of B12 ($F(2, 117) = 6.46$; $p = .002$; $\eta_p^2 = .099$) and betaine ($F(2, 117) = 3.55$; $p = .032$; partial $\eta^2 = .057$). Post-hoc tests using Bonferroni corrections at $p < .05$ suggested that AN-Active ($M = 702.67$, $SD = 538.3$, $p < .002$) and AN-Remitted individuals ($M = 641.50$, $SD = 381.2$, $p = .03$) had higher B12 levels than did NED individuals ($M = 371.53$, $SD = 254.5$). Betaine results did not survive Bonferroni correction, but uncorrected comparisons suggested that AN-Active individuals had higher betaine levels ($M = 42.04$, $SD = 22.1$) than did AN-Remitted individuals ($M = 32.33$, $SD = 20.1$, $p = .03$) and NED individuals ($M = 31.63$, $SD = 17.5$, $p = .02$). We tested each nutrient for differences owing to the presence of psychotropic medication-use with independent *t*-tests and found no differences. Among all comparisons between restrictive and binge/purge subtypes on nutrient levels, we observed only one significant difference, on folate levels when comparing remitted individuals showing restrictive versus binge/purge subtypes, $t(36) = 2.12$, $p = .04$ (binge/purge type $M = 13.67$, restrictive type $M = 7.81$). Given the number of comparisons involved and a limited sample size, we regard stability of the latter difference to be questionable.

General linear mixed models showed that BMI increased following treatment ($\beta = 2.01$, $SE = 0.43$, $t = 4.74$, $df = 29.98$, $p < .001$). However, BMI change was not associated with change in B12 ($p = .86$) or betaine ($p = .51$) levels.

Supporting Information Table 1 presents results from linear regression analyses within AN-Active individuals where BMI was the dependent variable and nutrient levels were the

predictors. The overall association between BMI and all plasma nutrients showed as a statistical trend in the AN-active group ($F(5,32) = 2.39$, $p = .054$), and we noted that higher B12 was significantly associated with lower BMI ($\beta = -.48$, $p = .01$) (See Supporting Information Figure 1). Separate analyses indicated that BMI was not associated with nutrient levels in AN-Remitted or NED individuals. Likewise, significant associations were never observed between nutrient levels and EDE-Q global or subscale scores in any groups.

Discussion

We compared plasma levels of nutrients involved in one-carbon metabolism across groups of AN-Active, AN-Remitted and NED individuals. In a subset of actively ill participants we obtained longitudinal data to examine associations between changes in BMI and nutrient levels following treatment.

Assuming that plasma nutrient levels should correspond to an individual's nutritional intake, we expected that AN-Active individuals would display decreased nutrient levels compared to individuals who were not restricting food intake. Instead, our findings showed that AN-Active individuals had unexpected elevations of B12 and, less certainly, of betaine.

Plasma B12 elevations have previously been observed in adolescents with AN (Corbetta et al., 2015), and the authors presumed that such elevations were associated with liver dysfunction. While the preceding provides a plausible explanation for our elevated B12 finding, we verified liver function in our AN-Active individuals with alanine aminotransferase (ALT) levels and found anomalies in only 3 cases. Furthermore, liver dysfunction would not be likely to explain elevations on folate or methionine that have been reported elsewhere in actively ill people (Moyano et al., 1998). We therefore consider an alternative explanation, centered on choline regulation.

Based on the logic that nutrient levels should be reduced in malnourished individuals, we had expected to find reduced choline levels in AN-Active individuals. Instead, we found levels in ill individuals to be comparable to those in AN-remitted and NED individuals. This being so, we believe it necessary to consider the possibility that some regulatory process, acting to compensate for malnutrition effects, may have been at work. Previous studies suggest that the body will protect choline levels during malnourished states by increasing choline flux—i.e., finding alternatives to dietary choline sources. For instance, one study observed that mice fed a choline-deficient diet still exhibited normal choline levels in both plasma and brain samples (Li et al., 2007). The authors suggested that when choline levels are depleted due to decreased dietary intake, peripheral organs mobilize choline to replenish supplies for essential functions. Assuming the preceding to be so, we propose that choline flux may have been increased in our AN-Active participants in compensation for their extreme malnutrition. Increased choline flux could have raised levels of nutrients occurring later in the one-carbon metabolism pathway—namely betaine and B12 (Anderson et al., 2012) (See Figure 1). Supporting our proposal, we note that betaine is metabolized from choline (so that choline levels would directly impact those of betaine), and that studies have suggested choline and B12 levels are linked (e.g., Compher, Kinosian, Stoner, Lentine, & Buzby, 2002).

Consistent with previous studies (e.g., Levine et al., 2007), we observed no differences between nutrient levels observed in AN-restrictive or AN-binge/purge individuals—perhaps suggesting that alterations in nutrient levels observed in AN-Active individuals were more attributable to malnutrition than to consequences of binging and/or purging. In AN-Active individuals, we observed BMI increases following treatment. However, BMI changes were not associated with changes in nutrient levels. Non-significant findings may be attributable to the

small sample size, or to the fact that short-term changes in BMI do not rapidly register as changes in plasma nutrient levels.

Although associations between plasma nutrient levels and eating-disorder symptoms were not observed, plasma B12 levels were negatively associated with BMI in AN-Active individuals. An association between B12 levels and BMI has been observed in studies examining obesity (Allin et al., 2017)—in which case *low* B12 levels have been associated with high BMI and obesity rates (e.g., Baltaci et al., 2013; Knight et al., 2015). The present findings are intriguing in that they may suggest the corollary on a continuum of effects—that elevated B12 levels are associated with low BMI (and hence with AN).

The present study has several limitations: First, we included medicated individuals. Although medication effects could potentially confound nutrient findings, we note that tests comparing nutrient levels between medicated and non-medicated individuals revealed no such differences. Second, to protect the safety of actively ill participants, we measured nutrient levels in a non-fasting state. As such, we cannot rule out the possibility that observed effects on nutrient levels may have been "contaminated" by effects of recent nutrient intake. Finally, we did not control for usage of vitamin supplements. However, our treatment protocol aims for nutritional rehabilitation without vitamin supplementation, and we are under the impression that few of our patients use such supplements.

Conclusions

Findings suggest that despite being malnourished, individuals with active AN exhibit normal or elevated plasma levels of nutrients involved in one-carbon metabolism. We have interpreted the finding as implicating the actions of one or both of two distinct processes: A compensatory adaptation to malnutrition, in which there is increased choline-flux from bodily

tissues to the bloodstream, or a result of altered liver function in highly malnourished individuals. Clarification of specific mechanisms that underlie such effects will inform future studies on the pathways linking nutrient intake to metabolic processes in individuals with AN-induced malnutrition.

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Figure 1 Caption

Figure 1. Nutrients within the one-carbon metabolism pathway. Folate is converted into its reduced form, 5-methyl-tetrahydrofolate (THF). 5- methyl-THF helps convert homocysteine into methionine with the support of vitamin B12. Choline is oxidized into betaine, which independently of folate, can generate methionine from homocysteine via enzymatic reaction.

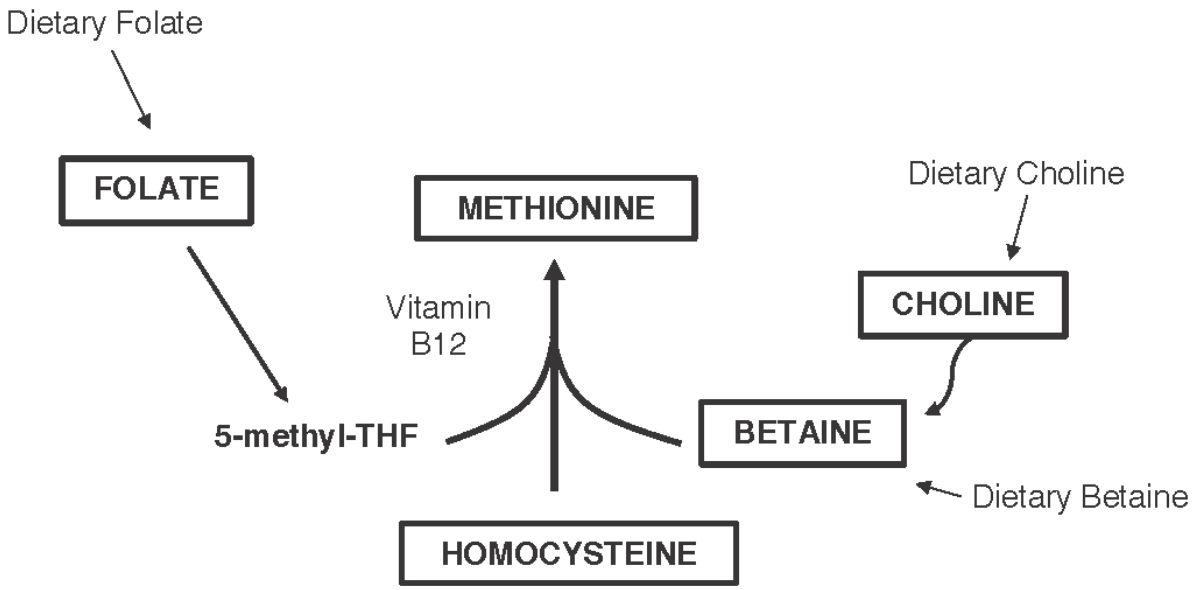

Table 1

Participant characteristics, medication use, and concurrent psychiatric comorbidity

	AN-R (*n*=30)	AN-BP (*n*=23)	AN-Remitted (*n*=40)
		Mean ± SD (Range)	
Characteristics			
Age[†]	26.57 ± 9.5 (18-53)	23.83 ± 5.9 (18-37)	26.55 ± 5.6 (19-38)
BMI[‡]	14.53 ± 1.4 [a] (11.60-17.12)	16.08 ± 1.1 [b] (13.10-17.50)	21.73 ± 2.8 [d] (18.43-30.99)
EDE-Q Score[§]	3.49±1.3 [a] (1.14-5.60)	4.37±1.3 [b] (1.69-5.80)	1.16±1.1 [d] (0.00-5.80)
Chronicity, months[¶]	101.47± 104.4 (12-456)	100.43± 65.6 (12-264)	89.70± 53.6 (12-288)
		Frequency (% of group)[1]	
Any Medication	17 (56.67)	14 (60.87)	14 (35)
Antidepressants	17 (56.67)	11 (47.83)	12 (30)
Antipsychotics	5 (16.67)	9 (39.13)	3 (7.5)
Hypnotics	1(3.33)	0	0
Anxiolytics	5 (16.67)	0	0
Mood stabilizers	0	1 (4.35)	0
Psychiatric comorbidity			
MDE	5 (16.67)	5 (21.74)	2 (5)
Dysthymia	1 (3.33)	1 (4.35)	1 (2.5)
Panic disorder	2 (6.67)	1 (4.35)	1 (2.5)

Panic disorder w/agoraphobia	1 (3.33)	1 (4.35)	0
Agoraphobia w/o panic disorder	1 (3.33)	0	0
Social anxiety disorder	1 (3.33)	2 (8.7)	2 (5)
Specific phobia	2 (6.67)	2 (8.7)	1 (2.5)
GAD	4 (13.33)	6 (26.09)	3 (7.5)
OCD	3 (10)	3 (13.04)	1 (2.5)
PTSD	0	2 (8.7)	1 (2.5)
OCPD	4 (13.33)	8 (24.78)	2 (5)
BPD	0	3 (13.04)	1 (2.5)

[1] Due to low frequencies in some cells, we did not subject frequency data to statistical tests.
AN-R = Anorexia Nervosa Restrictive Type; AN-BP = Anorexia Nervosa Binge/Purge Type; AN-Remitted: Anorexia Nervosa Remitted; BMI= body mass index; BPD= Borderline personality disorder; Chronicity, months: number of months since the onset of clinically significant eating-disorder symptoms; NA= not applicable; EDE-Q Score: Eating Disorder Examination-Questionnaire Global Score; GAD= Generalized anxiety disorder; MDE= Major depressive episode ; NED = Non-eating disordered; n.s.= not significant; OCD= Obsessive-compulsive disorder; OCPD= Obsessive-compulsive personality disorder; PTSD= Posttraumatic stress disorder; SD= standard deviation; w/= with; w/o= without;
Means with different superscript letters differed at $p < 0.05$.
† F (3, 125) = .42, n.s.
‡ F (3, 125) = 105.56, p <.001
§ F (3,106) = 64.77, p <.001
¶ F (2,90) = .25, n.s.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.