Serum profiling of anorexia nervosa: A $^1$H NMR-based metabolomics study

Alireza Salehi M. a,*, Ida AK Nilsson b,c,d, João Figueira e, Laura M. Thornton f, Israa Abdulkarim a, Erik Pålsson h, Cynthia M Bulik a,f,g, Mikael Landén a,h

a Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden  
b Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden  
c Center for Molecular Medicine, Karolinska University Hospital, Stockholm, Sweden  
d Centre for Eating Disorders Innovation, Karolinska Institutet, Stockholm, Sweden  
e Department of Chemistry, SciLifeLab, Umeå University, Sweden  
f Department of Psychiatry, University of North Carolina at Chapel Hill, NC, United States  
g Department of Nutrition, University of North Carolina at Chapel Hill, NC, United States  
h Institute of Neuroscience and Physiology, Department of Psychiatry, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden

Received 17 September 2020; received in revised form 28 January 2021; accepted 20 February 2021

KEYWORDS
Metabolomics; Serum; Anorexia nervosa; NMR; Classification

Abstract
Our understanding of pathophysiological mechanisms underlying anorexia nervosa (AN) is incomplete. The aim was to conduct a metabolomics profiling of serum samples from women with AN ($n = 65$), women who have recovered from AN (AN-REC, $n = 65$), and age-matched healthy female controls (HC, $n = 65$). Serum concentrations of 21 metabolites were measured using proton nuclear magnetic resonance ($^1$H NMR). We used orthogonal partial least-squares discriminant analysis (OPLS-DA) modeling to assign group classification based on the metabolites. Analysis of variance (ANOVA) was used to test for metabolite concentration differences across groups. The OPLS-DA model could distinguish between the AN and HC groups ($p = 9.05 \times 10^{-11}$, $R^2_Y = 0.36$, $Q^2 = 0.37$) and between the AN-REC and HC groups ($p = 8.47 \times 10^{-6}$, $R^2_Y = 0.36$, $Q^2 = 0.24$), but not between the AN and AN-REC groups ($p = 0.63$). Lower methanol concentration in the AN and AN-REC group explained most of the variance. Likewise, the strongest finding in the univariate analyses was lower serum methanol concentration in both AN and AN-REC compared with HC, which withstood adjustment for body mass index (BMI). We report for the first time lower serum concentrations of methanol in AN. The fact that low methanol was

* Corresponding author.
E-mail address: alireza.salehi@ki.se (A. Salehi M.).

https://doi.org/10.1016/j.euroneuro.2021.02.015
0924-977X/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/)
1. Introduction

Anorexia nervosa (AN) is a serious psychiatric disorder characterized by severe weight reduction or failure to achieve expected weight gains associated with extreme food restriction and/or energy expenditure with or without the presence of binge/purging behavior (Biomarkers Definitions Working, 2001; Treasure et al., 2015; Zipfel et al., 2015). Mortality rate in AN is higher than all other psychiatric disorders (Bulik et al., 2019). AN is more common in women than men (1:8) and the onset of the disorder typically peaks between the ages of 15 and 19 (Lucas et al., 1991). Thus, adolescent girls and young women are at highest risk for developing the disorder (Zipfel et al., 2015), which is often associated with other psychiatric comorbidities such as anxiety or mood disorders (Boraska et al., 2014; Ulfvebrand et al., 2015). Duration of illness is typically protracted with around 30% of affected individuals achieving full recovery and about 25% developing a chronic course (Fichter et al., 2017; Warrier et al., 2018). No medications are effective in treating the illness—none with regulatory approval—and clinical remission and behavioral treatments are the mainstays of intervention, with the evidence base for treatment of adults being particularly weak (Warrier et al., 2018; Zipfel et al., 2015). The etiology of AN is believed to be multifactorial albeit with a considerable genetic component where twin-based heritability estimates range from 50% to 60% (Yilmaz et al., 2015). A recent genome wide association study identified eight genome-wide significant loci and reported significant genetic correlations with both psychiatric traits and metabolic traits, encouraging a rebranding of AN as a metabo-psychiatric disorder (Watson et al., 2019).

Metabolomics is the ability to measure the concentration of many different metabolites in serum simultaneously (Nicholson et al., 1999a). Proton nuclear magnetic resonance (¹H NMR) spectroscopy (Beckonert et al., 2007; Nicholson et al., 1999b) has been used for metabolomics quantification and profiling in various diseases (Baranovicova et al., 2018; Focker et al., 2012; Giskeodegard et al., 2018; Nagana Gowda et al., 2018), including psychiatric disorders (Sethi et al., 2017; Song et al., 2018). Starvation might impact gene transcription (Rennert et al., 2018). Thus, an altered metabolome in AN could reflect traits of the underlying disorder, but could also be a state effect of starvation. One way to distinguish between state and trait effects on the metabolome in AN is to compare acutely ill individuals with low body mass index (BMI) to individuals with AN who are weight recovered, and compare both groups to healthy controls.

Here, we used ¹H NMR spectroscopy to investigate serum metabolites in three groups of females: AN, recovered from AN (AN-REC), and healthy controls (HC).

2. Materials and methods

2.1. Participants

Cases and controls were identified from the Swedish cohort of the international genetic study Anorexia Nervosa Genetics Initiative (ANGI). The case recruitment procedures and study design for ANGI by site have been outlined in detail by Thornton and coworkers (Thornton et al., 2018). The general inclusion criteria were females (at least 18 years old) with a lifetime history of meeting modified (amenorrhea was not required) DSM-IV criteria for AN.

For the purpose of the present investigation, patients were divided into two groups: The AN group (n = 65) met DSM-IV criteria for the disorder. The AN-REC group (n = 65) had previously met DSM-IV criteria for AN, but exhibited no disordered eating behaviors for at least one year and were no longer underweight (i.e., BMI ≥ 18.5) at the time of blood sampling. The ANGI study also recruited population-based female controls (n = 65) matched to cases on age. Identified individuals were asked about their history with regards to height and weight, eating disorder, and other psychiatric disorders. See Table 1 for further information on the study participants. The study was approved by the Stockholm regional Ethics committee, Sweden (2013/2:2). All participants provided oral and written informed consent to participate.

2.2. Sample collection and serum preparation

Consenting cases and controls who were between 18 and 65 years old were sent a phlebotomy kit along with instructions on how to donate blood at a local laboratory. Venous blood was collected...
Table 1 Demographic and clinical characteristics of the study participants.

| Characteristic                  | AN   | AN-REC | HC   |
|---------------------------------|------|--------|------|
| n                               | 65   | 65     | 65   |
| Female, %                       | 100  | 100    | 100  |
| Age, median years (IQR)         | 25.0 (21.0–30.5) | 25.0 (20.0–30.0) | 27.0 (23.0–36.0) |
| BMI, median (IQR)               | 16.9 (16.2–17.7) | 20.7 (19.5–22.6) | 22.2 (20.7–23.9) |
| Lowest participant BMI (kg/m²)  | 11.9 | 18.5   | 18.7 |
| Lowest lifetime BMI, median (IQR)| 13.9 (12.8–14.7) | 15.7 (14.4–16.9) | - |
| Years since AN onset, median (IQR) | 7 (3.0–12.0) | 10.0 (7.0–15.0) | - |

AN = anorexia nervosa, AN-REC = recovered from anorexia nervosa, BMI = body mass index, HC = healthy controls, IQR = interquartile range.

into gel serum tube (8.5 mL) that were sent with overnight mail to Karolinska Institutet Biobank and processed upon arrival. After centrifugation, serum samples were stored at -80 °C.

2.3. Sample preparation and 1H NMR acquisition

For 1H NMR analyses, 195 serum samples were thawed and transferred from tubes to cryovial. The samples were randomized prior to 1H NMR analyses. We did not use methanol serum extraction. 200 μL aliquots of serum were mixed with 400 μL of buffer (PBS with 10% D2O, NaN3 and 5.5 mM trimethylsilylpropaneic acid (TMSP, pH 7.4) directly into 5 mm SampleJet NMR tubes using a Bruker SamplePro liquid handling system. Both the cryovials and the NMR tubes were kept at 4 °C during the sample preparation. Mixing was performed directly in the SampleJet tubes using a 2-step mixing cycle.

The proton spectra were acquired at 37 °C on a Bruker 600 MHz Avance III HD spectrometer equipped with a 5 mm BBO broadband (1H/19F/2D) z-gradient cryo-probe and a SampleJet sample changer. T2-relaxation-edited spectra were recorded with a total cpmp spin-lock time of 100 ms to attenuate broad signals from proteins and lipids. Pre-saturation was used to suppress the water signal. 64 scans were recorded with a spectral width of 14 ppm and a relaxation delay of 1.1 s. After each excitation pulse, we recorded 64k (specifically, 65,536) data-points in the Free Induction Decay (Rosso et al., 1998) that make up the signal from the sample. The FIDs were added together to give higher signal-to-noise ratio. The FID was zero-filled, and an exponential line-broadening function of 0.3 Hz was applied to the FID prior to Fourier transformation. All spectra were manually phase-corrected and calibrated using the TMS resonance at 0 ppm. Processing was performed in Topspin 3.1 software, Bruker Biospin, Rheinstetten, Germany.

2.4. Bucketing procedure, data processing of 1H NMR spectra, and metabolite identification

We used aligned (icoshift) and normalized spectra (totalsum). Further, 500 bins (0.02 ppm width) of the Region of Interest (ROI, 10-0.05 ppm) were collected using Matlab. Using these bins, we performed OPLS-DA modeling on HC vs. AN and HC vs. AN-REC. The selection was done with a cut-off of |w*| ≥ 0.05. These bins were then used to identify and integrate the corresponding regions of the spectra (1H NMR). This process was carried out using Rui Pinto’s (RP) mplot Matlab script and a total of 22 regions were selected. 22 integrals were used for the multivariate analysis modeling. Some of the bins corresponded to regions of noise, which is most probably an artefact of using UV (Unit variance) scaling. Further details of the NMR procedure can be found in the Supplementary material.

The spectra were prepared for statistical analysis by manually dividing the spectra into 98 regions containing one or, in crowded regions, several peaks. These regions were aligned individually using an in-house Matlab script to alleviate problems caused by peak shifts, small variation in pH, salt concentration, and temperature (Stenson et al., 2016). The aligned regions were then calculated, and the peak areas were used in subsequent statistical analyses. Identification of metabolites was accomplished with a combination of spectral line fitting in Chenomx 8.0 NMR software (www.chenomx.com) and reference spectra in the Human Metabolome Database, HMDB (www.hmdb.ca). The serum samples were analyzed for different metabolites that have a crucial role in human metabolism and biochemical mechanism which include amino acids, carbohydrates, and fatty acids (Table 2).

2.5. Statistical analyses

We applied both multivariate analysis and univariate analysis to identify group differences in serum metabolite patterns. For multivariate statistical analysis, we used supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) as implemented in the SIMCA 15 software (Umetrics, Sweden), which is an excellent tool to identify differences between two groups. To evaluate the quality of the OPLS-DA model, R2 and Q2 metrics are calculated using averages of the seven-fold cross-validation. R2 signifies the explained variation and indicates “goodness of fit”. Q2 is an estimate of the predictive ability of the model (Mickiewicz et al., 2014). R2 and Q2 varies between 0 and 1 and the difference between R2 and Q2 should not be too large (≤0.2). OPLS-DA also generates a loading plot, which summarizes how each original variable contribute to the group difference.

For univariate analysis, analysis of variance (ANOVA) and analysis of covariance (ANCOVA) with BMI as covariate were used to determine whether there were any statistically significant differences between the serum concentrations of the three groups. We used the false discovery rate (FDR) method to correct for multiple testing. A threshold q value of 0.1 was used, meaning that 10% of the statistically significant values are possible false positives. The univariate analyses were performed using STATISTICA 13.3 software.

3. Results

Demographic and clinical characteristics of our study population are summarized in Table 1. Age did not differ across
Table 2  Serum metabolite values for AN, AN-REC, and HC groups. The ANOVA compares the three groups. The ANCOVA compares the 3 groups with BMI as covariate. Bonferroni posthoc test values are presented for those markers were the omnibus F-test was significant after FDR-correction (q ≤ 0.1).

| Serum metabolite | Mean (SD) NMR signal peak area [a.u.] | ANCOVA F(2, 192) | ANCOVA* F(2191) |
|------------------|----------------------------------------|------------------|------------------|
|                  | AN          | AN-REC         | HC             | F (p-value) | FDR q-value | p-value post hoc test AN vs HC AN-REC | p-value post hoc test AN vs HC AN vs AN-REC |
| Methanol         | 0.011 (0.003) | 0.010 (0.003) | 0.015 (0.005) | 27.0 | (4.4 × 10⁻¹⁰) | 2.6 × 10⁻⁷ | 24.7 | 5.9 × 10⁻⁹ | 2.6 × 10⁻⁷ |
| Glutamine        | 0.031 (0.006) | 0.028 (0.004) | 0.028 (0.005) | 5.0 | (0.008) | 0.008 | 0.01 0.03 | 2.2 (0.11) | 0.33 |
| Threonine        | 0.009 (0.001) | 0.010 (0.002) | 0.009 (0.002) | 5.0 | (0.097) | 0.068 | 0.07 0.01 | 1.7 (0.17) | 0.36 |
| Glucose          | 0.019 (0.013) | 0.020 (0.013) | 0.026 (0.016) | 4.3 | (0.014) | 0.075 | 0.01 0.11 | 1.4 (0.24) | 0.43 |
| Glycoproteins    | 0.061 (0.009) | 0.065 (0.011) | 0.066 (0.013) | 3.9 | (0.021) | 0.089 | 0.02 0.01 | 0.1 (0.95) | 0.95 |
| Glycolate        | 0.002 (0.001) | 0.003 (0.001) | 0.003 (0.001) | 3.0 | (0.049) | 0.17 | - | - | 0.2 (0.82) | 0.11 |
| Lipids (CH2-C=C) | 0.074 (0.019) | 0.072 (0.022) | 0.072 (0.022) | 2.0 | (0.13) | 0.41 | - | - | 2.6 (0.074) | 0.39 |
| Proline          | 0.003 (0.001) | 0.003 (0.001) | 0.003 (0.001) | 1.6 | (0.21) | 0.54 | - | - | 1.9 (0.16) | 0.37 |
| Glycerol from lipids | 0.001 (0.001) | 0.001 (0.003) | 0.002 (0.004) | 1.1 | (0.31) | 0.73 | - | - | 5.2 (0.006) | 0.046 | 0.29 0.95 |
| Citrate          | 0.004 (0.002) | 0.004 (0.002) | 0.035 (0.248) | 1.0 | (0.37) | 0.77 | - | - | 1.5 (0.23) | 0.45 |
| TMAO             | 0.015 (0.008) | 0.016 (0.007) | 0.017 (0.007) | 0.8 | (0.46) | 0.8 | - | - | 0.8 (0.92) | - |
| Phosphocholine   | 0.117 (0.035) | 0.111 (0.037) | 0.108 (0.043) | 0.8 | (0.46) | 0.81 | - | - | 5.5 (0.005) | 0.050 | 0.57 1 |
| Alanine          | 0.048 (0.012) | 0.047 (0.011) | 0.046 (0.011) | 0.5 | (0.62) | 0.99 | - | - | 1.1 (0.36) | 0.59 |
| Serine           | 0.003 (0.001) | 0.003 (0.001) | 0.003 (0.001) | 0.4 | (0.65) | 0.98 | - | - | 0.1 (0.95) | 1 |
| Glycine          | 0.020 (0.005) | 0.021 (0.005) | 0.021 (0.004) | 0.4 | (0.66) | 0.92 | - | - | 0.1 (0.90) | 1 |
| Lactate          | 0.702 (0.256) | 0.723 (0.242) | 0.743 (0.308) | 0.4 | (0.69) | 0.91 | - | - | 0.1 (0.93) | 1 |
| Valine           | 0.024 (0.009) | 0.024 (0.005) | 0.024 (0.006) | 0.3 | (0.71) | 0.88 | - | - | 2.3 (0.10) | 1 |
| Pyruvate         | 0.020 (0.025) | 0.021 (0.028) | 0.017 (0.021) | 0.3 | (0.74) | 0.87 | - | - | 0.4 (0.68) | 1 |
| LDL/VLDL         | 0.717 (0.147) | 0.701 (0.155) | 0.699 (0.191) | 0.2 | (0.81) | 0.89 | - | - | 2.1 (0.12) | 0.33 |
| Choline          | 0.002 (0.002) | 0.002 (0.002) | 0.003 (0.003) | 0.2 | (0.84) | 0.88 | - | - | 0.6 (0.53) | 0.80 |
| HC=C Lids        | 0.112 (0.056) | 0.108 (0.041) | 0.109 (0.056) | 0.1 | (0.87) | 0.87 | - | - | 3.2 (0.41) | 0.17 |

* BMI added as covariate.
Abbreviations: a.u. = arbitrary unit, AN=anorexia nervosa, AN-REC=recovered from anorexia nervosa, HC=healthy controls, LDL/VLDL = low/very low density lipoproteins, TMAO=Trimethylamine N-oxide, SD=standard deviation.
groups (F (2, 192) = 0.07, p = 0.93). BMI was one of the criteria used to separate the active anorexia group from the recovered group (BMI ≥ 18.5) and thus differs between groups by definition (F (2, 192) = 91.4, p<0.0001).

3.1. Multivariate analyses

The SIMCA score plots are shown in Figs. 1, 2, and 3 along with the loading plots. The loading plots show the explained variation of each metabolite between two groups and makes it possible to discern the most influential factors in the OPLS-DA modeling.

Fig. 1 shows the OPLS-DA score plot generated when comparing AN with HC. This showed statistically significantly fit (p = 9.05 × 10−11), with goodness of fit and predictability metrics of R2Y = 0.36 and Q2 = 0.37, respectively. The results of OPLS-DA indicate that ten metabolites contributed significantly to the separation between AN and HC, of which the serum concentrations of methanol, glucose, glycoproteins, glycolate, and trimethylamine N-oxide (TMAO) were lower in AN than in HC. The serum concentration of the remaining five metabolites (valine, alanine, phosphocholine, lipids (CH2-C=C), and glutamine) were significantly higher in AN than HC.

Fig. 2 shows the OPLS-DA score plot generated when comparing AN-REC with HC. This model also showed a statistically significant fit (p = 8.47 × 10−6), with goodness of fit and predictability metrics of R2Y = 0.36 and Q2 = 0.24, respectively. Eight metabolites had a significant effect on separation between AN-REC and HC participants, whereof which methanol, glucose, trimethylamine N-oxide (TMAO), and choline were lower in AN-REC than HC. Low/very low density lipoproteins (LDL-VLDL), proline, glutamine, and lipids (CH2-C=C) were higher in AN-REC than HC.

The regression coefficient plot of the comparison between AN and AN-REC is shown in Fig. 3. Here, the OPLS-DA modeling did not identify any statistically significant classification (p = 0.632) between the groups.

(O)PLS-Y modeling was attempted using age, BMI, height and weight, and metabolite concentrations. We found a good correlation of the integrals with the weight of the participants and with age (Supplementary material).

3.2. Univariate analysis

We compared serum metabolite concentrations across the three groups in a univariate analysis using ANOVA (Table 2). The omnibus F-tests, F = (2, 192) indicated that 5 of the 21 metabolites differed significantly across the three groups (FDR corrected q-value ≤0.10): methanol, glucose, threonine, glucose, and glycoproteins. For these 5 metabolites, Bonferroni corrected post hoc group comparisons are shown in Table 2. The post-hoc comparison of AN versus HC show that serum concentrations of methanol, glucose, and glycoproteins were lower in AN, whereas glutamine was higher in AN than HC. The post-hoc comparison of AN-REC with HC showed lower concentration of methanol in AN-REC. Finally, the post-hoc comparison of AN with AN-REC showed higher glutamine, but lower threonine and glycoproteins in the ac-
The figure shows the score plot for the OPLS-DA model generated for AN-REC (n = 65) and HC (n = 65). The OPLS-DA score plot of the first principal component t(1) significant classification between the AN-REC and HC groups (p = 8.47 x 10^{-6}). The loadings (pq[1]) plots shows eight significant metabolites that are considered important for the group separation. Negative values of the coefficients (the upper part of the diagram) indicate lower metabolite concentration in AN-REC samples than in HC.

The figure shows the score plot for the OPLS-DA model generated for AN (n = 65) and AN-REC (n = 65). The OPLS-DA score plot of the first principal component t(1) not any significant classification between the AN and AN-REC groups (p = 0.632).

To control for BMI, we compared serum metabolite concentrations across the three groups using ANCOVA (Table 2). The ANCOVA omnibus F-tests, F = (2191) indicated that methanol, glycerol from lipids, and phosphocholine differed significantly across the three groups (FDR corrected q-value ≤0.10). However, the Bonferroni corrected post hoc group comparisons (Table 2) were only significant for lower serum concentrations of methanol.
in both the AN and the AN-REC groups compared with controls.

4. Discussion

We used $^{1}$H NMR spectroscopy to conduct a metabolite screening of serum from women with AN, AN-REC, and age-matched HC. We used a multivariate statistical modeling tool (OPLS-DA) and observed a clear separation between AN and HC, as well as between AN-REC and HC. The loading plots (Figs. 1 and 2) show that lower methanol in AN and AN-REC compared with HC explained most of the variance in the respective model. By contrast, the OPLS-DA multivariate modeling failed to yield a significant classification between AN patients and AN-REC. In the univariate analyses, we found higher concentration of glutamine, and lower serum concentration of methanol, glucose, and glycoproteins in AN compared with HC, but only methanol remained after controlling for BMI. Methanol was also significantly lower in AN-REC compared with HC. Taken together, results suggest that low methanol might be a trait marker for AN, or a sequel remaining after recovery in the AN-REC group whereas other metabolites might be altered in AN due to starvation but return to levels on par with healthy controls upon weight restoration.

The most striking finding in this study was low serum concentration of methanol in AN, which has not previously been reported. The results withheld adjustment for BMI and was also lower in the recovered AN group. This suggests that serum concentration of methanol remains low in individuals with AN even after weight restoration. Methanol in serum is usually discussed in terms of intoxication (Skrzydlewska, 2003), but methanol is a normal component of healthy human blood in concentrations that are 400-1000 times less than toxic levels (Dorokhov et al., 2015). To our knowledge, no studies have investigated the possible effects of reduced serum concentrations of methanol. The sources of methanol in blood are diet and metabolic processes. Dietary methanol is obtained mainly from fruit, vegetables, alcoholic beverages, and artificial sweeteners such as aspartame. It is thus possible that the low serum methanol reflects a specific dietary regimen that women with AN uphold even after weight has been restored (Dellava et al., 2011). Of note, one study found markedly higher serum methanol in severely obese persons compared with controls, which rapidly normalized after bariatric surgery. The authors speculated that this was due to postoperative changes in the composition of the gut microbiota (Gralka et al., 2015). Interestingly, patients with AN have lower gut microbiota diversity than controls, which increases upon weight restoration but remains lower than controls (Kleiman et al., 2015). Altered microbiome metabolism in AN that persists after weight restoration is one potential cause of low serum methanol both in AN and AN-REC groups. Future studies should address whether methanol could be used as an early biomarker in cases where it is uncertain if low BMI is due to incipient AN or other reasons, e.g., constitutional leaness or failure to sustain normal BMI during a teenage growth spurt.

With respect to other metabolites, we found about 10% higher mean serum glutamine in AN compared with HC. This echoes findings in previous studies using other metabolomics techniques such as MS (Focker et al., 2012; Halmi et al., 1987; Moyano et al., 1998; Nakazato et al., 2010). However, the finding was not significant when controlling for BMI, and the recovered AN group did not differ from HC, which replicates a previous study (Nakazato et al., 2010). Hence, the increase of glutamine in active AN is likely due to starvation, tentatively because of gluconeogenesis and muscle breakdown as suggested by Nakazato and coworkers (Nakazato et al., 2010). Glutamine is a non-essential amino acid produced in the body. Endogenous synthesis of glutamine accounts for 90% of the total

Fig. 4 The figure shows the metabolites that differed across group in the ANOVA omnibus F-test ($p \leq 0.05$). The mean is shown as a straight line. The post-hoc Bonferroni corrected values are shown in Table 2.
amount of the circulating concentration and primarily occurs in striated muscles (He et al., 2010). In the central nervous system, glutamine is converted to glutamate in astrocytes (Newsholme et al., 2003) that functions as an excitatory neurotransmitter (Behar and Rothman, 2001). Increased serum glutamine leading to increased glutamate concentration in the brain might therefore contribute to psychological and cognitive symptoms in active AN. Indeed, Nakazato and colleagues found that serum glutamine concentrations and HADS anxiety scores were positively correlated (Nakazato et al., 2010).

With respect to threonine, a previous study reported lower concentrations in AN patients compared with controls (Halmi et al., 1987). This finding was partly replicated in our study where we found significantly lower threonine concentrations in AN compared with AN-REC and no group difference was found when adjusting for BMI. Threonine is an essential amino acid found in, e.g., pork, chicken, fish, cottage cheese, lentils. Diet change is thus one possible explanation for the difference between AN and AN-REC.

Serum glucose concentrations were lower in AN than HC. But the fact that AN-REC did not differ significantly from HC, and that the difference did not survive correction for BMI, suggests that reduced food intake is the cause (Brown and Mehler, 2015). The brain stores little glycogen and is therefore dependent on blood glucose for energy (Duran and Guinovart, 2015). Hypoglycemia might thus contribute to emotional (depression, irritability, anxiety) and cognitive changes that occur during starvation in AN (impaired concentration and reduced alertness).

We conducted multivariate analyses (OPLS-DA) to identify potential class differences based on our metabolomics dataset. The analyses could clearly distinguish between AN patients and HC, as well as between AN-REC and HC, but failed to distinguish between AN and AN-REC. The latter might be surprising given that most of the metabolic changes seen in active AN were not present in recovered AN. But the likely explanation is that methanol explained much of the variance, which was lower also in recovered AN-REC.

4.1 Strengths and limitations

In order to distinguish between state and trait biomarkers, we included not only women with active AN and controls, but also women who had recovered from AN. NMR metabolomics is a robust and reproducible technique. We employed 1H NMR in solution, which is a very sensitive analytical method for detecting protons in biological samples. There are, however, some limitations to consider.

The first is that blood samples were sent via overnight mail before being processed. One study found that metabolites do not to change significantly during 7 h. (Marjani, 2006) but given that centrifuging and freezing could occur up to 24 h. After sampling in our study, we cannot exclude that variation in transport time or other factors might have impacted metabolite concentrations. However, given that sampling was not related to group status, any such variation is more likely to introduce noise and would not explain the group differences that we found. With respect to clinical data, we lacked information on some clinical factors such as drug abuse and medications that potentially could affect the metabolites. Regarding the multivariate analyses, OPLS tend to over-fit models to data (Tribal et al., 2015). Here, we applied these models to investigate the potential of metabolic fingerprinting to classify cases and controls, but it would require further validation to ensure model reliability before these models could be used to predict class from a single sample. Regarding the univariate analyses, we used FDR to correct for multiple comparisons and chose the commonly used threshold of 0.1. Though this means that 10% of the significant results might be false positives, the finding of lower methanol concentration in AN and AN-REC was strong and unlikely to be a false positive.

In conclusion, we report for the first time lower serum concentration of methanol in women with anorexia nervosa and in women recovered from the disorder, suggesting that methanol is a trait or scar biomarker of anorexia nervosa. Prospective studies are warranted to test if serum methanol can be used for early identification of individuals that are at risk of developing anorexia nervosa.

Declaration of Competing Interest

The authors declare no conflict of interest.

Funding

Funding support was provided from the Swedish foundation for Strategic Research (KF10-0039) and the Swedish Research Council (#2018-02653; #538-2013-8864). The Anorexia Nervosa Genetics Initiative (ANGI) is an initiative of the Klarman Family Foundation.

Disclosures

C. Bulik has served on Advisory Boards for Shire, is a consultant for Idorsia, and is an author and royalty recipient from Pearson. A. Juréus is currently employed at the Swedish Medical Products Agency, SE-75103 Uppsala, Sweden, the views expressed in this paper are the personal views of the authors and not necessarily the views of the Government agency.

Acknowledgments

We thank the patients who volunteered to participate in this study. The help of ANGI staff at Karolinska Institutet in recruiting patients is gratefully acknowledged, including data manager Bozenna Iliadou. We also wish to thank the National Quality Registry for Eating Disorders (RIKSÅT) for help with recruiting patients and the Swedish NMR centre at Umeå University, Mattias Hedenström, NMR for Life and Scilife Lab (JF) for support with the NMR time and analysis. We finally wish to thank the BBMRI.se and KI Biobank at Karolinska Institutet for professional biobank service. Funding support was provided from the Swedish foundation for Strategic Research (KF10-0039) and the Swedish Research
The Anorexia Nervosa Genetics Initiative (ANGI) is an initiative of the Klaraan Family Foundation.
