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Pre-illness data reveals differences in multiple metabolites and metabolic pathways in those who do and do not recover from infectious mononucleosis†

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Abstract

Metabolic pathways related to energy production, amino acids, nucleotides, nitrogen, lipids, and neurotransmitters in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) may contribute to the pathophysiology of ME/CFS. 4501 Northwestern University college students were enrolled in a prospective, longitudinal study. We collected data before illness, during infectious mononucleosis (IM), and at a 6 month follow-up for those who recovered ($N = 18$) *versus* those who went on to develop ME/CFS 6 months later ($N = 18$). Examining pre-illness blood samples, we found significant detectable metabolite differences between participants fated to develop severe ME/CFS following IM *versus* recovered controls. We identified glutathione metabolism, nucleotide metabolism, and the TCA cycle (among others) as potentially dysregulated pathways. The pathways that differed between cases and controls are essential for proliferating cells, particularly during a pro-inflammatory immune response. Performing a series of binary logistic regressions using a leave-one-out cross-validation (LOOCV), our models correctly classified the severe ME/CFS group and recovered controls with an accuracy of 97.2%, sensitivity of 94.4%, and specificity of 100.0%. These changes are consistent with the elevations in pro-inflammatory cytokines that we have reported for patients fated to develop severe ME/CFS 6 months after IM.

Introduction

Metabolomics has allowed investigators to better understand which metabolic pathways are dysregulated in a number of different diseases. For example, four metabolites may signal metabolic pathways that contribute to breast cancer carcinogenesis,[1] and abnormal metabolism of branched-chain amino acids may explain a substantial fraction of the increased risk of breast cancer experienced by women with an elevated body mass index. Large-scale collaborative research on the human metabolome is now investigating

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Conflicts of interest

There are no conflicts to declare.

relationships of disease etiology, diagnosis, and prognosis.[2] Metabolomic predictors might also therefore be associated with the subsequent development of post-viral fatigue following infectious mononucleosis (IM).

Older studies in adult patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) have implicated changes in the serotonin and melatonin systems.[3] Multiple studies in recent years have reported detectable changes in metabolic pathways related to energy production, amino acids, nucleotides, nitrogen, lipids, and neurotransmitters in patients with ME/CFS.[4–9] These findings suggest changes in metabolic pathways that may plausibly contribute to the pathophysiology of ME/CFS.

We have previously shown that college students fated to develop severe ME/CFS following mono have more severe mono[10] and more complaints of fatigue and certain cytokine abnormalities prior to the development of IM.[11] In our current study, we used metabolomic analysis as part of an integrative approach to study ME/CFS in young adults following a known trigger (IM). Metabolites were examined in college students before illness onset for students who ultimately recovered following IM ($N = 18$) *versus* matched participants who developed severe ME/CFS 6 months following IM ($N = 18$).

Method

Jason *et al.*[11] provide demographic characteristics and details of the 4501 Northwestern University students enrolled in our recent, prospective, longitudinal study. Briefly, the students were consented, completed seven questionnaires and had blood taken at least 6 weeks prior to the development of IM (baseline). Students who then developed IM were diagnosed and tracked by the Northwestern University Health Service and other medical providers. IM was defined as a positive monospot or specific Epstein-Barr virus serologies (a positive viral capsid antigen [VCA] IgM or a positive VCA IgG with a negative EB nuclear antigen antibody) in the appropriate clinical setting, in which case the diagnostic specificity is very high.[12] Those who developed IM at least six weeks after enrollment again provided online consent, completed the same questionnaires as during the baseline phase, and had blood taken. Five months after the IM diagnosis, students deemed not recovered and an approximately equal number deemed recovered during a phone screen were invited to participate in a six-month assessment following the onset of IM; these participants completed the same measures again, and in addition underwent a comprehensive medical examination. Institutional Review Board approval was obtained. Of the 4501 students enrolled at baseline in the study, 238 (5.3%) developed IM over the study period (3/5/14-6/12/19).

ME/CFS diagnosis

Participants' six-month medical examination, and the results from their DePaul Symptom Questionnaire[13] and MOS 36-item Short-Form Health Survey[14] were used to make the diagnosis of ME/CFS. While there are multiple ME/CFS case definitions, we selected three major ones including the Fukuda *et al.* criteria,[15] the Canadian Consensus Criteria,[16] and the Institute of Medicine criteria.[17] Those who met more than one case definition (*i.e.,* the Fukuda and either the Canadian and/or Institute of Medicine criteria) were defined as

having severe ME/CFS. The current study involved data on 18 study participants with severe ME/CFS as well as 18 age, gender, and racially/ethnically matched controls. The severe ME/CFS group was majority female (61.1%; male = 38.9%), majority White/Caucasian and Latinx (52.9%; 29.4%; Black/African American = 11.8%; Asian or Pacific Islander = 5.9%), and had a mean age of 18.8 years (SD = 0.45). We examined samples from both sets of participants (those who recovered *vs.* those who met criteria for severe ME/CFS six months following IM) at baseline, prior to development of IM, to determine if there were metabolomic differences between those who were fated to recover from IM *vs.* those who went on to develop severe ME/CFS following IM.

Metabolomic analysis

500 μL of 80% methanol was added to 50 μL plasma, vortexed and protein was precipitated by incubation at −80 °C overnight. Debris were pelleted by centrifugation at 18 000 × *g* for 15 min at 4 °C. The supernatant was transferred to a new tube and evaporated to dryness using a SpeedVac concentrator (Thermo Savant). Metabolites were reconstituted in 50% acetonitrile in analytical-grade water, vortex-mixed, and centrifuged to remove debris. Samples were analyzed by Ultra-High-Performance Liquid Chromatography and High-Resolution Mass Spectrometry and Tandem Mass Spectrometry (UHPLC-MS/MS).

Specifically, the system consisted of a Thermo Q-Exactive in line with an electrospray source and an Ultimate3000 (Thermo) series HPLC consisting of a binary pump, degasser, and auto-sampler outfitted with an Xbridge Amide column (waters; dimensions of 4.6 mm × 100 mm and a 3.5 μm particle size). Mobile phase A contained 95% (vol/vol) water, 5% (vol/vol) acetonitrile, 10 mM ammonium hydroxide, 10 mM ammonium acetate, pH = 9.0; and mobile phase B was 100% Acetonitrile. The gradient was as follows: 0 min, 15% A; 2.5 min, 30% A; 7 min, 43% A; 16 min, 62% A; 16.1–18 min, 75% A; 18–25 min, 15% A with a flow rate of 400 μL min^{-1}. The capillary of the ESI† source was set to 275 °C, with sheath gas at 45 arbitrary units, auxiliary gas at 5 arbitrary units, and the spray voltage at 4.0 kV. In positive/negative polarity switching mode, an *m/z* scan range from 70 to 850 was chosen and MS1 data was collected at a resolution of 70 000. The automatic gain control (AGC) target was set at 1×10^6 and the maximum injection time was 200 ms. The top 5 precursor ions were subsequently fragmented, in a data-dependent manner, using the higher energy collisional dissociation (HCD) cell set to 30% normalized collision energy in MS2 at a resolution power of 17 500. Data acquisition and analysis were carried out by Xcalibur 4.1 software and Tracefinder 4.1 software, respectively (both from Thermo Fisher Scientific). The peak area for each detected metabolite was normalized by the total ion current which was determined by integration of all of the recorded peaks within the acquisition window. Metabolomic analysis was performed blinded as to the outcome of each participant (*i.e.*, recovered *vs.* severe ME/CFS).

Statistical analyses

All statistical analyses were conducted using R 4.1.0 statistical software. Due to the large number of missing values resulting from metabolites being below instrument detection

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limits, values of zero were replaced with half of the lowest non-zero value for each metabolite.[18] Each metabolite was then normalized using logarithmic transformation. As non-normal distributions were present after normalization, non-parametric significance tests (Mann–Whitney *U*) were used to identify statistical differences in peak area values between the severe ME/CFS and recovered groups. Adjusted significance levels were established using the Bonferroni correction. The severe ME/CFS and recovered groups were then classified using binary logistic regression with significant features (*i.e.*, metabolites identified *via* significance testing) imputed into a series of models with a leave-one-out cross-validation (LOOCV) technique. Classification models were assessed according to model accuracy, sensitivity, and specificity.

Results

Analyses included peak area values for 265 metabolites. After values below instrument detection limits were replaced with half of the lowest non-zero value for each metabolite, a logarithmic transformation was performed to normalize the data. Non-parametric significance tests (Mann–Whitney *U*) were conducted for all 265 metabolites with significance levels adjusted using the Bonferroni correction (0.01/265 = 0.000038). Significant differences in peak area value between the severe ME/CFS and recovered groups were observed for eight metabolites (see Table 1). For spermine and carbomyl phosphate, participants who went on to develop severe ME/CFS 6 months following IM had lower values at baseline than controls who recovered from IM; for fructose-1,6/2,6-biphosphate (F-1,6/2,6-DP), spermidine, ATP-dGTP, glutathione disulfide, citrate, and cytidine 5′-diphosphate (CDP), participants who went on to develop severe ME/CFS 6 months following IM had higher values at baseline than controls who recovered from IM.

Utilizing the eight metabolites found in Table 1, a series of binary logistic regressions were conducted to classify the severe ME/CFS and recovered groups using a LOOCV technique. The models produced correctly classified those with severe ME/CFS from recovered controls with an accuracy of 97.2%, sensitivity of 94.4%, and specificity of 100.0%.

Discussion

Our study analyzed pre-illness data from college students who went on to develop severe post-viral fatigue and recovered controls, matched by year of birth, race/ethnicity, and gender. Our study found detectable significant differences between participants fated to develop severe ME/CFS following IM and recovered controls at baseline (prior to onset of IM) in multiple metabolites.

We identified glutathione metabolism, nucleotide metabolism, the TCA cycle, polyamine metabolism (spermine, spermidine), glycolysis (F-1,6/2,6-DP), and urea cycle (carbamoyl phosphate) as potentially dysregulated pathways. These pathways are essential for proliferating cells, particularly during a pro-inflammatory immune response, and are thus consistent with irregularities in cytokines that we have reported in this[10] and a previous[19] cohort. Alterations in these pathways are also potentially consistent with previous reports

of changes in energy production, nucleotide metabolism, TCA metabolism, and reactive oxygen species pathways in adults with ME/CFS.[4–7] The latter correlate with literature supporting oxidative stress in the pathophysiology of ME/CFS[4] and may be reflected in the differing concentrations of spermine, spermidine, glutathione, citrate, and CDP seen in our participants at baseline who did and did not develop severe ME/CFS 6 months following IM, as these metabolites also play a role in mediating or preventing oxidative stress.[20–23]

S-Adenosyl-L-methionine (SAM) is part of one carbon metabolism and is a methyl donor for epigenetic regulation; it can also feed into glutathione production. Glutathione is part of glutathione metabolism. Cysteine is an amino acid that participates in a variety of pathways, including glutathione metabolism. Thiamine is modified and used as a cofactor in several TCA cycle enzymes. *N*-Acetyl-alanine may have a role in protein signaling and post-translational modifications.[24]

These metabolites are thus candidate biomarkers for predicting the development of severe ME/CFS 6 months following IM in college students and suggest changes in metabolic pathways that may plausibly contribute to the pathophysiology of ME/CFS in general. These findings and others[10,25–27] reinforce biological differences between those who fully recover following IM and those who go on to develop severe ME/CFS 6 months later.

This study only looked at plasma obtained from participants prior to the development of IM, so Epstein-Barr virus (EBV) could not be playing a role in any of the current findings. However, in the course of IM, it is possible that EBV affects individuals with different metabolomes differently, leading to ME/CFS in certain individuals but not others; this could be a productive avenue to explore in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Significant metabolomic results at baseline (prior to IM) in controls who recovered from IM *vs.* participants who went on to develop severe ME/CFS 6 months following IM

	KEEG	Metabolite	S-CFS M (SD)	Controls M (SD)	*U*	*p*
a	C00750	Spermine	18.79 (0.13)	19.54 (0.16)	0	0.0000000002
c	C00354···C00665	F-1,6/2,6-DP	14.94 (0.43)	13.90 (0.24)	321	0.0000000015
a	C00315	Spermidine	19.18 (0.66)	17.84 (0.33)	311	0.0000000822
c	C00002···C00286	ATP/dGTP	14.57 (1.02)	13.16 (0.54)	301	0.0000012586
b	C00169	Carbamoyl phosphate	15.67 (0.45)	16.65 (0.12)	23	0.0000012586
a	C00127	Glutathione disulfide	13.63 (1.00)	11.64 (1.11)	300	0.0000015973
c	C00158···C00311	Citrate/citrate(iso)	22.90 (0.28)	22.40 (0.30)	290	0.0000135233
a	C00112	CDP	10.84 (1.29)	8.88 (0.85)	297	0.0000169794

Note:

a = identity confirmed,

b = identity not confirmed,

c = cannot separate. Bonferroni correction ($p < 0.000038$).