



OPEN Cerebrospinal fluid metabolomics, lipidomics and serine pathway dysfunction in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS)

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We proposed that cerebrospinal fluid would provide objective evidence for disrupted brain metabolism in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). The concept of postexertional malaise (PEM) with disabling symptom exacerbation after limited exertion that does not respond to rest is a diagnostic criterion for ME/CFS. We proposed that submaximal exercise provocation would cause additional metabolic perturbations. The metabolomic and lipidomic constituents of cerebrospinal fluid from separate nonexercise and postexercise cohorts of ME/CFS and sedentary control subjects were contrasted using targeted mass spectrometry (Biocrates) and frequentist multivariate general linear regression analysis with diagnosis, exercise, gender, age and body mass index as independent variables. ME/CFS diagnosis was associated with elevated serine but reduced 5-methyltetrahydrofolate (5MTHF). One carbon pathways were disrupted. Methylation of glycine led to elevated sarcosine but further methylation to dimethylglycine and choline was decreased. Creatine and purine intermediates were elevated. Transaconitate from the tricarboxylic acid cycle was elevated in ME/CFS along with essential aromatic amino acids, lysine, purine, pyrimidine and microbiome metabolites. Serine is a precursor of phospholipids and sphingomyelins that were also elevated in ME/CFS. Exercise led to consumption of lipids in ME/CFS and controls while metabolites were consumed in ME/CFS but generated in controls. The findings differ from prior hypometabolic findings in ME/CFS plasma. The novel findings generate new hypotheses regarding serine-folate-glycine one carbon and serine-phospholipid metabolism, elevation of end products of catabolic pathways, shifts in folate, thiamine and other vitamins with exercise, and changes in sphingomyelins that may indicate myelin and white matter dysfunction in ME/CFS.

Keywords Myalgic encephalomyelitis/chronic fatigue syndrome, Cerebrospinal fluid, Metabolomics, Lipidomics, Exercise, Serine pathway, Postexertional malaise, PEM

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic disease with disability, fatigue, post-exertional malaise (PEM), cognitive lapses, nonrefreshing sleep, interoceptive distress, pain and orthostatic complaints^{1–4}. ME/CFS affects all age, sex, and racial and ethnic groups and costs the U.S. economy about \$18–\$51 billion annually^{5–8}. Prevalence is 0.2–1.3%⁹. Females are affected with a 2:1 to 4:1 prevalence. Recovery over time is poor with only 0–8% having full recovery and 17–64% some improvement¹⁰. There are no objective diagnostic tests or FDA approved drug therapies. There is extensive^{8–10} overlap between ME/CFS and Long COVID¹¹.

Postexertional malaise (PEM) is a criterion for ME/CFS. PEM is the severe, prolonged disabling exacerbation of symptoms that follows small increments in cognitive, emotional or physical exertion. The onset may be delayed by hours. Unlike controls, sleep is not refreshing and rest does not lead to recovery of function. The exertional exhaustion can be prolonged for days and subjects may become bed bound or inactive because of fatigue, pain or cognitive inability to plan activities. PEM is a required, discriminating diagnostic criterion for ME/CFS^{2–4}. The subjective and often uniquely personal nature of PEM makes it challenging to define and quantify^{12,13}. Therefore,

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it is logical to objectively assess the consequences of an exercise provocation to understand mechanisms of exertional exhaustion.

Metabolic status in ME/CFS is significantly altered compared to controls in studies of cerebrospinal fluid, plasma, urine and fecal microbiome at baseline. ME/CFS have a wide range of alterations affecting glycolysis, TCA cycle, diverse amino acid pathways, purine, pyrimidine, sphingolipid and phospholipid pathways that imply dysfunction of mitochondrial and microbiome metabolism^{14–18}. Two reports in cerebrospinal fluid analysis found equivalence between ME/CFS and control except for reduced mannose and acetylcarnitine in ME/CFS¹⁹ and a hypometabolic profile with decreases in tryptophan, glutamate, butyrate, polyamine and tricarboxylic acid (TCA) cycle pathways in ME/CFS²⁰. The only report of the effects of exercise on cerebrospinal fluid studied control subjects who had lumbar puncture 1 h after running for 90 min²¹. Postexercise levels were increased for lactate, purines, pyrimidines, dopamine, amino acid neurotransmitters, riboflavin and nicotinamide-ribose. The changes overwhelmingly implicate mitochondrial pathways.

We hypothesized that cerebrospinal fluid constituents at baseline would be significantly different between ME/CFS and controls and would correlate with symptoms such as fatigue, pain and quality of life (disability), semiobjective measures of central sensitization (dolorimetry) that may account for the tenderness and interoceptive complaints, and psychological constructs of disease. We predicted that exercise would induce mechanisms of PEM and cause alterations of metabolite and lipid constituents in ME/CFS. Metabolites and lipids were examined in two independent cohorts of subjects who had lumbar puncture either without exercise (nonexercise group) or after the second of two bouts of submaximal exercise performed on two consecutive days (postexercise)^{22–25}. Data were analyzed by multivariate general linear regression to account for the effects of nonexercise vs. postexercise status, age, body mass index and gender^{17,26,27}. Significant findings were matched to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways to define metabolic modules and then integrated into a metabolomic map to compare to other studies.

Results

Data was obtained for cohorts of nonexercise and postexercise ME/CFS and sedentary control (SC) subjects (Table 1). A uniform protocol of recruitment, online and telephone screening was followed by in-person written informed consent, thorough medical history and physical examination for exclusionary conditions, supervision throughout study participation in the Clinical Research Unit and followup after discharge.

ME/CFS had higher scores for the CFS questionnaire²⁸ and higher rates of fibromyalgia by 1990 pain and tenderness²⁹ and 2010 symptom criteria³⁰ (Table 2). The average of SF36 Vitality, Role Physical and Social Function domains was optimal for showing differences from control. Age and BMI were equivalent. Cerebrospinal fluid was clear, colorless, with normal total protein, albumin, IgG, erythrocytes, leukocytes and differential counts (Table 3).

As a first approximation, log transformed, normalized and standardized data were analyzed by two-tailed unpaired t-test for differences between ME/CFS (*n* = 59) vs. SC (*n* = 36) (Table 4).

ME/CFS had higher levels of serine (Hedges’ *g* = 0.962), five sphingomyelins and five other phospholipids than SC with elevated 1-methylhistidine (*g* = -0.531) in SC.

Next, logarithmically transformed and autocentered abundances for metabolites and lipids were analyzed by multivariate general linear models to test the frequentist hypothesis that point estimates for metabolites and lipids were significantly different between ME/CFS and SC when corrected for gender, exercise (nonexercise vs. postexercise), age and body mass index (BMI). The null hypothesis was equivalence between ME/CFS and SC. The 105 metabolites and 84 lipids that were significantly altered were tabulated in SOM Table S1. Questionnaire and metabolite and lipid abundances are in SOM Table S2.

The univariate contrast for disease (ME/CFS vs. SC) identified higher serine, sarcosine, cysteamine, citrate, five sphingomyelins and five other phospholipids in ME/CFS than SC, with higher 1-methylhistidine and phenylacetylglutamine in SC than ME/CFS (*p* < 0.05 by Sidak test).

The multivariate triple cross-product Disease * Gender * Exercise revealed that the nonexercise cohort had 25 metabolites and 26 lipids that were higher in ME/CFS than SC for female and male subjects. ME/CFS > SC female nonexercise had higher purine metabolites (7-methylguanosine, 1-methyladenosine, xanthosine, AMP), pantothenate, indoleacrylic acid and ureidopropionate. ME/CFS > SC male nonexercise subjects had higher creatine, dopamine, glucose-1-phosphate, glucose-6-phosphate, amino acids (glutamine, lysine, phenylalanine,

Study protocol	Nonexercise protocol with lumbar puncture	Exercise followed by lumbar puncture
Contacted	271	148
Screened	100	73
Medical exclusions	13	11
Declined to participate	22	35
Lumbar puncture	65 (45 female)	27 (11 female)
Groups and N (N females)		
Sedentary Control (SC)	20 (9 female)	12 (2 female)
ME/CFS	45 (36 female)	15 (9 female)
Total	92 (56 female)	

Table 1. CONSORT information.

Mean SD	Control	Control	ME/CFS	ME/CFS
	Nonexercise	Postexercise	Nonexercise	Postexercise
N	20	12	45	15
Female	9	2	36	9
Age	43.3 ± 12.5	46.7 ± 9.9	45.7 ± 11.0	45.0 ± 10.2
BMI	27.2 ± 5.5	30.5 ± 4.8	28.7 ± 7.2	27.3 ± 5.8
FM 1990	15%	8%	44%	13%
FM 2010	5%	0%	50%	60%
CFS Questionnaire (range 0 to 4)				
Fatigue	1.3 ± 1.3	1.2 ± 1.0	3.7 ± 0.5	3.7 ± 0.6
PEM	1.1 ± 1.6	0.4 ± 0.8	3.4 ± 0.9	3.5 ± 0.5
Cognition	1.0 ± 1.2	1.0 ± 1.3	3.1 ± 0.8	2.8 ± 0.7
Sleep	0.7 ± 1.1	1.1 ± 1.2	2.5 ± 1.2	1.6 ± 1.3
Myalgia	1.1 ± 1.4	0.9 ± 1.0	2.9 ± 1.3	2.5 ± 1.3
Arthralgia	0.7 ± 1.0	1.1 ± 1.0	2.5 ± 1.2	2.2 ± 1.4
Headache	1.3 ± 1.5	1.8 ± 1.3	3.5 ± 0.9	3.4 ± 0.6
Sore Throat	0.4 ± 0.7	0.3 ± 0.6	1.5 ± 1.2	1.6 ± 1.0
Sore Nodes	0.3 ± 0.6	0.1 ± 0.3	1.3 ± 1.2	1.1 ± 1.1
Chalder Fatigue (0 to 33)	12.7 ± 6.4	11.5 ± 2.6	22.9 ± 6.2	23.2 ± 6.4
SF-36 Quality of Life (0 to 100) (Tukey HSD test < 0.008 for SC vs. ME/CFS)				
SF36 Physical Functioning	87.0 ± 23.1	88.0 ± 20.8	40.8 ± 22.1	42.9 ± 27.7
SF36 Role Physical	68.8 ± 44.3	81.7 ± 35.9	5.9 ± 18.8	8.9 ± 27.0
SF36 General Health	71.0 ± 18.7	69.7 ± 17.8	34.1 ± 19.1	22.9 ± 12.8
SF36 Bodily Pain	83.3 ± 28.1	80.2 ± 16.5	33.9 ± 21.9	44.3 ± 29.2
SF36 Vitality	59.0 ± 28.6	57.3 ± 21.5	12.6 ± 11.5	14.3 ± 14.3
SF36 Social Functioning	82.5 ± 29.1	76.7 ± 24.5	26.0 ± 21.6	28.6 ± 27.0
SF36 Role Emotional	81.7 ± 35.0	84.4 ± 33.0	58.8 ± 46.1	76.2 ± 42.2
SF36 Mental Health	78.6 ± 11.0	76.8 ± 13.2	64.4 ± 17.7	66.0 ± 17.4
SF36 average RP, V,SF	70.1 ± 31.8	71.9 ± 22.9	14.8 ± 12.6	17.3 ± 20.8
CMSI (0 to 172)	18.6 ± 28.1	17.5 ± 14.9	57.4 ± 25.0	45.6 ± 31.2
CMSI no Pain (interoception)	13.4 ± 19.8	11.4 ± 9.4	38.4 ± 19.7	30.6 ± 23.2
Chemical Symptoms	11.2 ± 17.4	10.5 ± 12.9	46.0 ± 21.8	30.8 ± 22.5

Table 2. Demographics. Mean ± SD. ME/CFS had significantly higher scores than SC for fibromyalgia diagnosis, CFS questionnaire, Chalder fatigue, SF36 domains, chronic multisymptom illness (CMSI) total score and “no pain” domain for interoceptive complaints and chemical symptoms questionnaire (Tukey HSD < 0.001).

	SC Mean ± SD	ME/CFS Mean ± SD	Range
N	32	60	
Total Protein	35.8 ± 11.5	34.4 ± 11.1	16 to 58 mg/dL
Albumin	23.3 ± 8.6	20.2 ± 7.9	8 to 37 mg/dL
Alb CSF/Serum	6.2 ± 2.1	5.1 ± 1.9	< 9
IgG	2.6 ± 1.1	2.4 ± 1.0	1 to 4 mg/dL
Glucose	63.0 ± 8.4	61.9 ± 8.8	50 to 75 mg/dL

Table 3. Cerebrospinal fluid chemistries. There were no erythrocytes or leukocytosis.

tyrosine), transaconitate, O-acetylserine, seven phosphatidylglycerides, three phosphocholines and three phosphoethanolamines.

SC had higher levels than ME/CFS for 11 metabolites and five lipids particularly for females postexercise (SC > ME/CFS female postexercise). SC had higher levels of 5MTHF, riboflavin, flavin mononucleotide, choline and dimethylglycine.

The metabolites and lipids that were significantly different between ME/CFS and SC were tabulated (SOM Table S1), evaluated by KEGG pathways, and mapped to metabolic modules (Fig. 1). Metabolites that were higher in ME/CFS > SC were highlighted in yellow, and SC > ME/CFS in pink. A larger number of significantly different analytes were found using the multivariate approach with correction for the independent variables

Analyte	CFS Average ± SD	SC Average ± SD	2-tailed unpaired t-test	Hedges' g [95%CI]
SERINE	0.328 ± 0.903	-0.553 ± 0.919	0.000017	0.962 [1.412–0.527]
SM(d18:1/22:5)	0.266 ± 0.959	-0.448 ± 0.914	0.00061	0.751 [1.192–0.323]
SM(d18:1/20:5)	0.254 ± 0.986	-0.427 ± 0.882	0.0011	0.712 [1.150–0.285]
SM(d18:1/20:4)	0.205 ± 1.055	-0.346 ± 0.801	0.0090	0.565 [0.998–0.141]
SM(d18:1/18:4)	0.194 ± 1.080	-0.327 ± 0.755	0.014	0.531 [0.963–0.108]
PG(18:0/18:2)	0.215 ± 0.943	-0.362 ± 1.002	0.0063	0.592 [1.026–0.168]
PG(18:1/18:2)	0.206 ± 0.954	-0.347 ± 0.992	0.0088	0.567 [1.000–0.143]
PE(18:0/22:5)	0.198 ± 0.905	-0.333 ± 1.076	0.012	0.542 [0.974–0.119]
PE(P-18:0/20:4)	0.166 ± 0.953	-0.280 ± 1.029	0.036	0.450 [0.879–0.028]
SM(d18:1/20:0)	0.171 ± 1.001	-0.288 ± 0.944	0.031	0.464 [0.894–0.042]
PG(18:2/18:2)	0.175 ± 0.931	-0.295 ± 1.055	0.027	0.477 [0.907–0.055]
1-METHYL-L-HISTIDINE	-0.194 ± 1.094	0.327 ± 0.719	0.014	-0.531 [-0.108–0.963]

Table 4. Hedges' g for logarithmically transformed, normalized and standardized ME/CFS (*n* = 59) vs. SC (*n* = 35) by two-tailed unpaired t-test.

(representative results in Fig. 2) than the simple t-test contrast of ME/CFS vs. SC (Table 4). For example, sarcosine was significant only after correcting for independent variables.

The significantly different metabolites (Fig. 1) were assessed by pathway enrichment in MetaboAnalyst 6.0 using the Small Molecule Pathway Database (SMPDB) with 99 metabolite sets from normal human metabolic pathways and was enriched for Methionine Metabolism, Phenylacetate Metabolism, Pantothenate and CoA Biosynthesis, Pyrimidine Metabolism, Glycine and Serine Metabolism, Arginine and Proline Metabolism, Riboflavin Metabolism and Betaine Metabolism (Fig. 3). Using the 80 metabolite sets based on KEGG human metabolic pathways (Dec. 2023) identified a similar set of Glycine, serine and threonine metabolism, Pyrimidine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Riboflavin metabolism, Purine metabolism, Phenylalanine metabolism, Glyoxylate and dicarboxylate metabolism, Arginine biosynthesis, and Arginine and proline biosynthesis. The lipids were enriched in nervous tissue, myelin sheath and axon. When assessed using Relational database of Metabolomic Pathways (RaMP-DB) that has 3694 metabolite and lipid pathways integrated from KEGG, HMDB, Reactome and WikiPathways, the significant pathways were Oligodendrocyte specification and differentiation leading to myelin components for CNS, Immunoregulatory interactions between lymphoid and non-lymphoid cells, Kennedy pathway from sphingolipids, MTHFR deficiency, Sphingolipid pathway and Glycerolipids and glycerophospholipids.

Univariate analysis for gender identified 57 analytes with 51 elevated in males vs. females. The multivariate triple cross-product added 55 more compounds with 39 elevated in males. Females had higher levels for 12 phosphatidylglycerides and four phosphocholines, homocysteic acid and thymidine derivatives with the nonexercise group having a significant impact. Males had higher levels of amino acids, glycolysis and TCA intermediates, pyrimidine and butyrate derivatives, dimethylcholine, choline and riboflavin.

Univariate analysis for exercise found 56 analytes elevated in nonexercise > postexercise and 11 higher in postexercise. The multivariate cross-products added 28 more that were higher in nonexercise than postexercise and 10 higher in postexercise. The nonexercise > postexercise set included 5MTHF, hexoses (glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate, myoinositol), twelve phosphocholines, twelve phosphoglycerides, six triacylglycerides, four phosphoethanolamines and three sphingomyelins. The multivariate set added more phospholipids, choline, creatinine, dopamine and purine metabolites. This indicated exercise consumed folate, sugars and lipids. Compounds that were higher after exercise included glutathione (reduced), thiamine, riboflavin, butyrate metabolites, and glycine, dimethylglycine and phosphorylcholine that are related to serine and phosphocholine synthesis.

Effect sizes for gender and exercise were reported as Partial Eta Squared in SOM Table S3. ANOVA Simultaneous Component Analysis for metabolites, disease, gender and exercise status in MetaboAnalyst 6.0 identified a different pattern of metabolite relationships (Fig. 4). Disease status was associated with amino adipate, creatinine and dopamine. Exercise status was associated with glutathione (reduced), glucose-1-phosphate, alanine and citrate and gender with glucuronate, 3-(4-hydroxyphenyl)-pyruvate, valine, isoleucine and N-acetylalanine. N-acetylalanine was significant for each of the cross-products. Glutathione (reduced) was significant for the Gender x Disease and Gender x Exercise cross-products. Spermidine, indoleacrylic acid and homocysteic acid were also significant for cross-products.

Analyte abundance was correlated with questionnaire items to find associations between potential markers of disease and disease manifestations. The nonparametric Spearman's correlation matrix was inspected and the quadrant of analytes vs. items excised. Features were sorted to find the metabolites and lipids with the most relationships with questionnaire items. Significance of this process was defined by $|R| > 0.3$ ($p < 0.015$). Three general patterns were found (Fig. 5, SOM Table S4). Serine had the most correlations³⁴, followed by 7-methylguanosine¹³, creatine¹³, 1-methyladenosine¹⁰, and indoleacrylic acid¹⁸. This set was correlated with Multidimensional Fatigue Index Reduced Activity (11 analytes), Physical Fatigue⁸ and sum⁸, Mystery⁹, Permanence⁷ and CPSS⁷. Elevated serine and other metabolites in ME/CFS > SC were correlated with fatigue which was consistent with the diagnostic criteria of ME/CFS.

Metabolic Module Framework for Disease (CFS vs SC) based on Multivariate Analysis

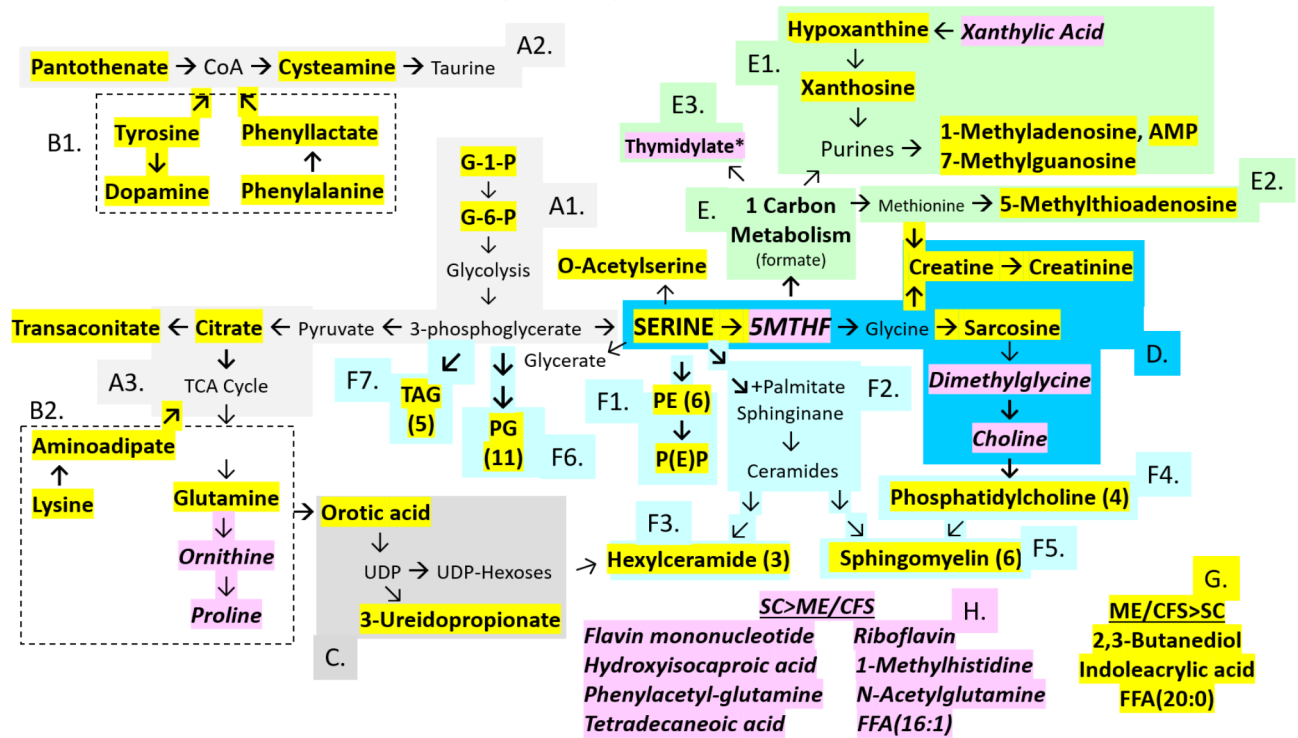


Fig. 1. Metabolic modules and imbalances in nonexercise ME/CFS. The map provides an overview of cerebrospinal fluid metabolic pathways that differed between ME/CFS and SC at baseline and were influenced by exercise in multivariate analysis. Metabolic pathways were summarized (indicated by arrows connecting strings of intermediate compounds) and organized into color coded modules. Metabolites that were significantly elevated in ME/CFS (ME/CFS > SC) were in bold with yellow highlighting while the converse (SC > ME/CFS) were in italics with pink highlighting. Central energy metabolism was shown in grey with A1. Glycolysis, A2. Coenzyme A (CoA) metabolism, and A3. TCA (tricarboxylic acid) cycle. Amino acid metabolism (beige) had two components: B1. Essential aromatic amino acid metabolism that leads to acetylCoA and B2 (boxes with dashed lines) anaplerotic amino acid metabolism related to the TCA cycle. Glutamine played a central role in these pathways and C. pyrimidine metabolism (dark grey). A novel finding was the persistent finding of serine (bold, upper case, yellow highlighting) in ME/CFS and the number of metabolites in the D. Serine – Glycine – 5-methyltetrahydrofolate (5MTHF) – One Carbon pathways (dark blue). E. Methyl transfer (green) is essential for E1. Purine synthesis, E2. Methionine pathway, and E3. Thymidylate synthesis. Serine provides a direct link to phospholipid synthesis (F. blue) as a source material for phosphatidylethanolamine (PE) and phosphatidylethanolamine plasmalogens (P(E)P) (F1). F2. Serine and palmitate combine to form sphinganine that is the precursor of ceramides. F3. UDP-hexoses are attached to form hexylceramides. F4. Glycine is metabolized by N-methylation to form sarcosine, dimethylglycine and choline (trimethylglycine) for synthesis of phosphatidylcholines (PC). F5. Phosphatidylcholines exchange headgroups with ceramides to form sphingomyelins (SM). F6. 3-Phosphoglycerate and dihydroxyacetone phosphate are source materials for the formation of phosphatidylglycerols (PG) and F7. triacylglycerides (TAG). Numbers in parentheses after lipids indicate the number that were significantly altered ($p < 0.05$, Sidak correction for multiple comparisons). Metabolites with other physiologies or microbial origins were tabulated for ME/CFS > SC (G., yellow) and SC > ME/CFS (H., pink). Other abbreviations were G-1-P, glucose-1-phosphate, G-6-P, glucose-6-phosphate and AMP, adenosine monophosphate. Nonsignificant intermediates were in smaller font (not bold). *Thymidylate was a special case because different derivatives from the mass spectrometry analysis were altered in different directions.

The second pattern of correlations linked exercise, gender and Internal locus of control with 5MTHF and seven phosphatidylglycerides.

The third pattern correlated age with five sphingomyelins, two hexylceramides, transaconitate, citrate, dimethylglycine, proline, lysine, tyrosine, riboflavin, glucose-1-phosphate and glucose-6-phosphate. The glucose derivatives may be related to Type II diabetes and older age. The relationships with age, gender and exercise provided rationale for the multivariate regression analysis.

Discussion

Serine was elevated and 5-methyltetrahydrofolate (5MTHF) reduced in cerebrospinal fluid from ME/CFS subjects. This inverse relationship had significant impact on cerebrospinal fluid metabolism. Serine is identified

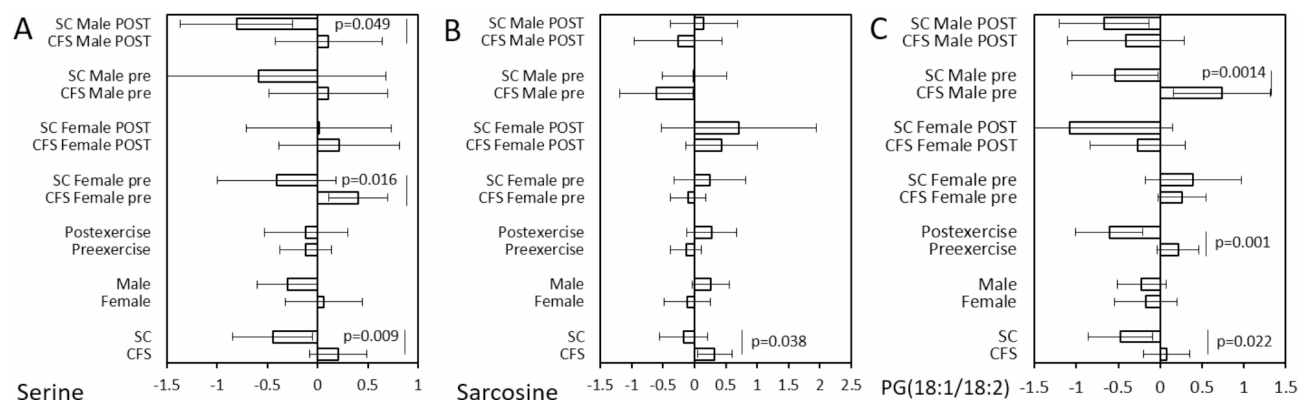


Fig. 2. Metabolite comparison by frequentist multivariate general linear model. Serine, sarcosine and PG(18:1/18:2) were significantly higher in ME/CFS than SC in the multivariate general linear model. Lines between bars indicate Sidak p values. Mean \pm 95% CI.

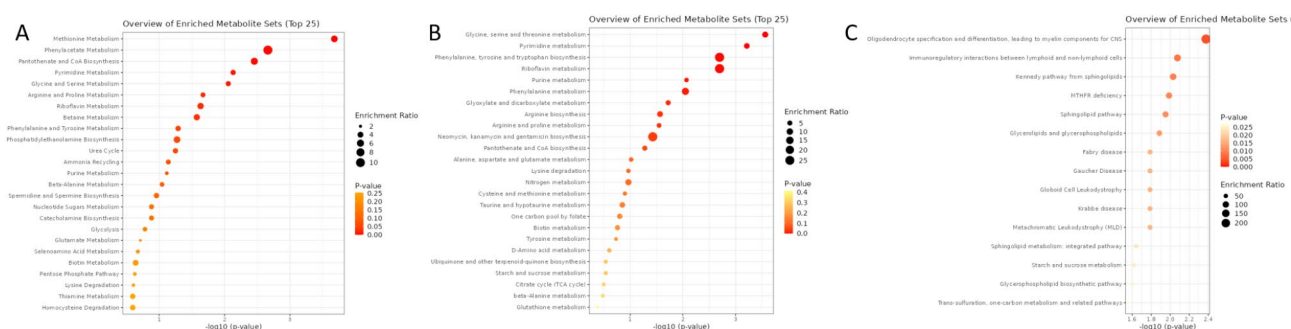


Fig. 3. Pathway enrichment for ME/CFS vs. SC. All of the significantly different metabolites and lipids from the multivariate analysis were pooled then assessed for pathway enrichment in MetaboAnalyst using (A) SMPDB, (B) KEGG and (C) RaMP-DB.

as a central hub in the metabolomic network (Fig. 1) because it linked glycolysis to glycine, 1-carbon metabolism via 5-methyltetrahydrofolate 5MTHF and phospholipid synthesis^{31–33}. This pathway is dysfunctional in cancer and other syndromes and is known to short circuit glycolytic intermediates towards formate in a manner similar to the Warburg pathway that diverts glycolytic intermediates to lactate instead of feeding the tricarboxylic acid (TCA) cycle³¹. Formate was not measured in our assay. Disruption of methylation was suggested by several lines of evidence. Serine is metabolized to glycine with subsequent methylation to form sarcosine (methylglycine) which was elevated in ME/CFS. The processes continues to dimethylglycine and choline that were reduced in ME/CFS. Reduced folate methylation may account for this imbalance. Gender and exercise complicate this balance as male nonexercise ME/CFS subjects had higher dimethylglycine and choline levels than their female counterparts and choline was consumed during exercise while glycine, dimethylglycine and phosphorylcholine were elevated postexercise. Diversion of methylation may have contributed to increased nitrogen metabolism and higher levels of creatine and creatinine in ME/CFS and consumption during exercise. The methionine pathway of methylation may also be impacted because 5-methylthioadenosine, an intermediate in that pathway, was elevated in ME/CFS. Purine and thymidylate synthesis and degradation were disrupted which may be due to folate consumption or insufficiency. 1-Methyladenosine and 7-methylguanosine may be derived from epigenetic methylation of DNA and RNA followed by polymer digestion.

Serine is an essential precursor for lipid biosynthesis (Fig. 1)³⁴. Serine is a direct precursor of phosphatidylethanolamine (PE) and combines with palmitate to form ceramides and subsequently hexylceramides (HCER). 3-Phosphoglycerate is the precursor of serine as well as phosphoglycerides (PG). Choline is the precursor for phosphatidylcholines (PC). ME/CFS had elevated numbers of PG (11 of 17 mass spectrometry targets, $p = 0.000085$ by two-tailed Fisher Exact Test vs. SC), PE (6 of 11, $p = 0.012$), sphingomyelins (6 of 14, $p = 0.016$), HCER (3 of 11) and PC (3 of 13).

In contrast to the current findings, serine, tyrosine and choline were reduced in multiple sclerosis³⁵. Serine may have an anti-inflammatory effect that protects myelin and supports remyelination. Tyrosine is important for neuron development, proliferation and regeneration. Levels of choline and free fatty acids have been proposed to be biomarkers of demyelination or remyelination but these are dynamic processes and serial changes may be more instructive. Phospholipids and sphingomyelins were elevated in ME/CFS which may be compatible with white matter injury.

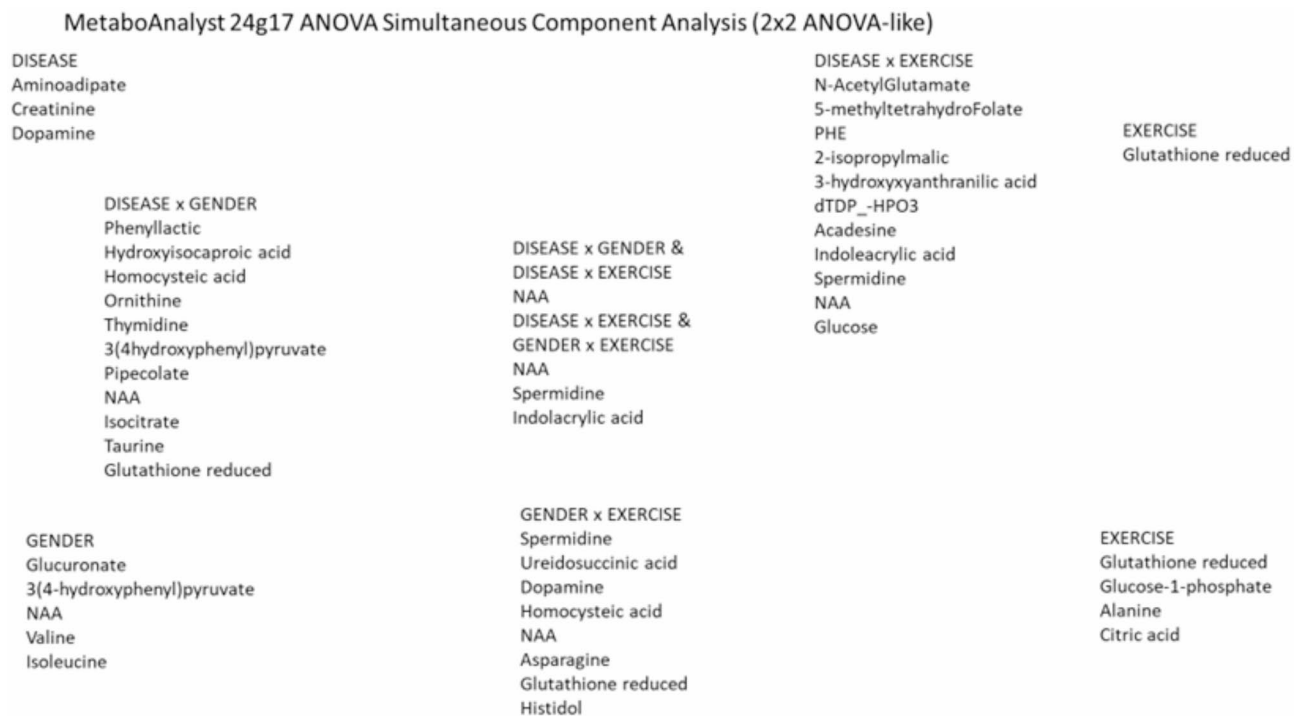


Fig. 4. ANOVA simultaneous component analysis for metabolites, disease, gender and exercise status in MetaboAnalyst 6.0.

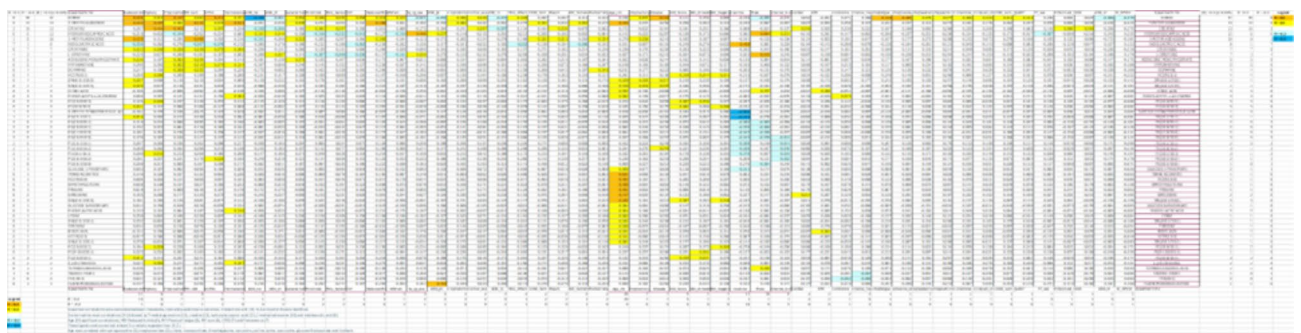


Fig. 5. Spearman correlation matrix for analytes and questionnaire items. Spearman correlation was performed using all metabolites, lipids and questionnaire items. The quadrant comparing analytes and items was excised then sorted to show correlated features with $|R| > 0.3$ ($p < 0.015$). Serine had 34 such interactions. Three patterns emerged: serine and other metabolites correlated with measures of fatigue (upper left corner), age with sphingomyelins and metabolites (center, yellow and orange), and gender, exercise and internal locus of control with 5MTHF and seven phosphatidylglycerides (center, blue). Spearman correlations were > 0.3 (yellow), > 0.4 (orange), < -0.3 (light blue) and < -0.4 (dark blue). The full size figure is in SOM Table S4.

Energy metabolism was disrupted in ME/CFS with elevated glucose-1-phosphate and glucose-6-phosphate and pantothenate and cysteamine that are involved in Coenzyme A (CoA) metabolism. Citrate was elevated which may divert TCA intermediates towards transaconitate and short circuit the TCA cycle in ME/CFS³⁶.

Amino acid metabolism was dysfunctional with elevated phenylalanine, tyrosine and dopamine that are metabolized to acetylCoA. Elevated dopamine in ME/CFS was unanticipated. Citrate and glutamine were elevated suggesting an impact on anaplerotic metabolism of TCA intermediates to amino acid backbones and other pathways³⁷.

SC had elevated 5MTHF, riboflavin and flavin monophosphate suggesting a relative deficiency of these vitamins in ME/CFS which would lead to disruption of enzymatic pathways where they are cofactors. This finding provides the rationale to search for vitamin dysfunction in ME/CFS that may respond to appropriate supplementation. 1-Methylhistidine, ornithine, proline, hydroxyisocaproic acid, N-acetylglutamine and phenylacetylglutamine were elevated in SC which added further evidence for amino acid imbalances in ME/CFS.

Gender has a large impact on plasma²⁶ and cerebrospinal fluid constituents. ME/CFS males had higher levels of metabolites than females with and without ME/CFS (SOM Table S1). Pathway enrichment in males selected Pyrimidine Metabolism, Gluconeogenesis, Warburg Effect, Methionine Metabolism and Spermidine and Spermine Biosynthesis. Males had more hexylceramides (HCER) than females (7 of 11 mass spectrometry targets, $p = 0.0039$ by Fisher Exact Test) while females had a trend for more phosphatidylglycerides (not significant) in the multivariate analysis.

The effects of exercise were compared between ME/CFS and SC. ME/CFS and SC shared the consumption of 5MTHF, hexoses and lipids during exercise. 5MTHF may have been absorbed into the brain for metabolic utilization. Glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate and myoinositol were likely consumed by glycolysis, pentose phosphate shunt or other energy pathways or used for amino acid and other metabolite synthesis during exercise. Phosphatidylglycerols, phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and triacylglycerides were higher in nonexercise than postexercise, and may have been incorporated into lipid membranes or consumed via peroxisomes and mitochondria for energy metabolism (SOM Table S1). Significant changes in carnitines were not found. The nonexercise group had higher levels of choline, creatinine, dopamine and purine metabolites. Conversely, compounds that were generated or released from the brain parenchyma after exercise included glutathione (reduced), thiamine, riboflavin, butyrate metabolites and glycine, dimethylglycine and phosphorylcholine that are related to serine and phosphocholine synthesis.

The interactions of disease, exercise and gender make it a challenge to understand effects related to PEM. The submaximal exercise in this study increased orthostatic complaints modestly in ME/CFS but did not cause severe symptom exacerbation³⁸. Effects caused by one and two day maximal cardiopulmonary exercise stress tests followed by lumbar punctures may be needed to study this problem in order to see if changes during exercise and recovery follow previously reported trends in plasma and urine^{18,39,40}.

The specificity of our results can be placed into perspective by comparison to other studies of cerebrospinal fluid. Walitt et al. reported a hypometabolic state in ME/CFS²⁰ while Lipkin and Fiehn found only reduced mannose and acetylcarnitine in ME/CFS¹⁹. It is not clear if the differences between the three studies reflect the range of possibilities within ME/CFS, subject heterogeneity, differences in disease or physical activity levels prior to lumbar puncture, analytical or statistical techniques. One step towards resolution would be to combine the cerebrospinal fluid specimens for standardized targeted mass spectrometry analysis followed by multivariate analysis of age, gender, exercise status and levels of severity such as poor quality of life, pain, fatigue and interoceptive complaints. Identification of the serine alteration was by recognition of the role of serine in one carbon metabolism and its precursor status for phospholipids that was not derived from KEGG or other pathway analyses. Therefore, reanalysis of the previous reports focusing on serine, folate and phospholipids may reveal relationships despite the suggested consensus that they represent hypometabolic and normal metabolic studies. It is also possible that the brains of ME/CFS subjects may flip between metabolic states rather than being fixed at one end of a spectrum.

The findings can also be placed into the context of other cerebrospinal fluid findings in other diseases. A metabolome-wide, genome-wide association analysis with 338 cerebrospinal fluid metabolites identified 16 genotype-metabolite associations and 19 significant associations with neurological and psychiatric phenotypes⁴¹. Single nucleotide polymorphism - metabolite associations were confirmed for guanosine, ethylmalonate, 3-ureidopropionate, N-acetylhistidine, tryptophan betaine, N-acetyl-beta-alanine, N-delta-acetylornithine, bilirubin, 2'-O-methylcytidine, and methionine sulfone). New associations were found for schizophrenia N-delta-acetylornithine, alphetocopherol, ethylmalonate, N6-methyllysine, guanosine, malate, unknown metabolite X-24699, 2-hydroxy-3-methylvalerate), cognitive performance (N-delta-acetylornithine, glutaroylcarnitine [C5], benzoate), alcoholic drinks per week (N-delta-acetylornithine, glycerol, cysteinylglycine), smoking behavior (ethylmalonate), sleep duration (cysteinylglycine disulfide), post-traumatic stress disorder (PTSD) (unknown metabolite X-24295), and attention deficit hyperactivity disorder (ADHD) (orotate and malate). Our study was limited to the Biocrates target list⁴² and could not detect many of these metabolites.

Ageing has been associated with various metabolomic profiles including elevated isoleucine, acetylcarnitine, pipicolate, methionine, glutaryl carnitine, 5-hydroxytryptophan, ketoleucine, and hippurate with decreased methylthioadenosine and 3-methyladenine⁴³, markers of the carnitine shuttle, sucrose, biopterin, vitamin E metabolism, tryptophan, and tyrosine⁴⁴ and elevated cysteine, pantothenic acid, 5-hydroxyindoleacetic acid (5-HIAA), aspartic acid, and glutamate and reduced asparagine and glycerophosphocholine⁴⁵. Another study implicated disruption of cytochrome P450, tryptophan and kynurenine pathways, cellular energy (NAD⁺, ADP), mitochondrial and ribosomal metabolisms, neurological dysfunction, and an increase of adverse microbial metabolites⁴⁶. Gender affected tryptophan, tyrosine and purine pathways. Females had higher levels of taurine and 5-HIAA⁴⁵ while males had higher cysteine, uric acid and N-acetylserotonin⁴⁷. African Americans had significantly higher concentrations of 3-hydroxykynurenine while white subjects had significantly higher kynurenine, indoleacetic acid, xanthine, alpha-tocopherol, cysteine, melatonin and 7-methylxanthine. Metabolomic - proteomic studies may have greater impact than either alone⁴⁸.

Many inflammatory states in the central nervous system are generalized to be neuroinflammation. This is a considerable oversimplification because there are diverse mechanisms of brain inflammation⁴⁹. Microglia, astrocytes, oligodendroglia, neurons and infiltrating innate and adaptive immune cells, cytokines, immunoglobulin and complement may contribute to inherently negative and maladaptive destructive processes. However, on a more limited basis, some of the mechanisms of neuroinflammation contribute to synaptic pruning and learning, neuroplasticity, and the balance of inflammatory and intrinsic repair processes that contribute to functional recovery. As a result, it is prudent to consider specific disease processes and to try to elucidate the specific inflammatory phenotype in order to classify neuroinflammatory pathologies.

Neuro-post acute sequelae of COVID was of particular interest to ME/CFS and was identified by 69 differentially expressed metabolites including elevated sphinganine and ST1A1, disordered sphingolipid metabolism, and attenuated inflammatory responses⁵⁰. Repair may be indicated by decreased levels of 7,8-dihydropterin and activation of steroid hormone biosynthesis. Another profile showed elevated citrulline, ceramide (d18:1/18:0), and methionine with reduced triglyceride TG(20:1_32:3) in neuro-COVID⁵¹. Levels were different from viral CNS infections and aseptic neuroinflammation. Phospholipids, carnitine and tryptophan are elevated in acute bacterial and viral infections⁵². Metabolomics of cerebrospinal fluid differentiated Lyme disease, West Nile Virus, multiple sclerosis, rabies, and *Histoplasma meningitis* from each other and controls⁵³. Acute enteroviral meningitis was inferred by 91 metabolites and in particular phosphatidylcholine PC.ae.C36.3, PC.ae.C36.5, and PC.ae.C38.5, asparagine and glycine⁵⁴. Metabolites in HIV-associated neurocognitive disorders (HAND) were similar to those of age-associated cognitive decline in the absence of HIV with elevated neurotransmitters (glutamate, N-acetylaspartate), markers of glial activation (myo-inositol), and ketone bodies (beta-hydroxybutyric acid, 1,2-propanediol)⁵⁵.

Acute neuroinflammation is associated with elevated metabolites in the tryptophan-kynurenine pathway, nitric oxide pathway, neopterin and major lipids⁵⁶. Acute encephalitis showed elevated kynurenine, quinolinic acid, and anthranilic acid but decreased tryptophan, 3-hydroxyanthranilic acid and kynurenic acid⁵⁶. Acute viral encephalitis had activation of nitric oxide pathway with elevated asymmetric dimethylarginine and argininosuccinic acid and decreased arginine and citrulline⁵⁷. Proline was elevated in autoimmune neuroinflammation. Higher concentrations of free phospholipids in viral infections were consistent with disruption of host cell membranes, whereas the elevated short-chain acylcarnitines may reflect compromised mitochondrial homeostasis and energy generation.

Patients with status epilepticus (SE) had 37 elevated metabolites including pyruvate, quinolinic acid, and keto butyric acid levels with decreased levels of arginine, N-acetylaspartylglutamate (NAAG), tryptophan, uracil, and uridine⁵⁸. The tryptophan kynurenine pathway was significantly altered resulting in the overproduction of quinolinic acid, an N-methyl-d-aspartate (NMDA) receptor agonist with pro-inflammatory properties.

Disorders of consciousness were associated with lower levels of acylcarnitines in trauma patients, metabolites of glutamate and GABA metabolism in the minimally conscious state and the vegetative state, and a set of eight phospholipids as a biomarker to predict the recovery of consciousness⁵⁹.

Pain may not have a specific biosignature as the most commonly found analytes were metabolites of acetaminophen rather than endogenous metabolites⁶⁰. Dysfunction of tryptophan – indole – kynurenine – niacin and tryptophan – serotonin – melatonin pathways may contribute to migraine and other severe headaches⁶¹.

Chronic brain dysfunction is associated with multiple patterns of metabolomic dysfunction. Patients with mild cognitive impairment (MCI) and Alzheimer disease (AD) had decreased monohexosylceramides⁶². Parkinson's disease (PD) had disrupted purine and tryptophan metabolism, oligomeric alpha-synuclein and neurofilament light chain expression compared to neurodegenerative diseases⁶³. Multiple sclerosis (MS) was associated with significant changes in concentrations of arginine, histidine, spermidine, glutamate, choline, tyrosine, serine, oleic acid, stearic acid and linoleic acid³⁵. Reduced histidine was correlated with Expanded Disability Status Scale values. Metabolomic profiles distinguish multiple sclerosis, Parkinson disease and neurodegenerative disease suggesting future diagnostic utility⁶⁴. Secondary progressive and relapsing-remitting Multiple Sclerosis were differentiated by perturbed tryptophan and pyrimidine metabolism with altered kynurenate, 5-hydroxytryptophan, 5-hydroxyindoleacetate, N-acetylserotonin, uridine, deoxyuridine, thymine and glutamine⁶⁵. Autism was associated with hippuric acid and salicyluric acid, glycolysis/gluconeogenesis, carbon and glutathione pathways⁶⁶.

Depression was associated with decreased ethanolamine⁶⁷ or reductions in metabolites associated with tryptophan and tyrosine pathways⁶⁸. Treatment refractory depression was associated with low folate and tetrahydrobiopterin⁶⁹. 5-methyltetrahydrofolate, homocarnosine, and depression and anxiety scores were correlated⁷⁰. Serotonin is associated with first episode psychosis⁷¹.

Peripheral diseases can interfere with cerebrospinal fluid metabolomes. Type 2 Diabetes Mellitus had significantly increased levels of alanine, leucine, valine, tyrosine, lactate, pyruvate, and decreased levels of histidine⁷² but these changes were not reflected in the current study. Liver disease leads to hepatic encephalopathy with alteration of 73 cerebrospinal fluid metabolites including amino acids, acylcarnitines, bile acids, nucleosides and acetylated compounds possibly due to defects of the Krebs cycle and alterations of ammonia, amino-acid and energy metabolism⁷³. Obstructive sleep apnea in Alzheimer's was associated with oxidized triglyceride OxTG(57:2), oxidized ceramide OxCer(40:6) and unknown lipids⁷⁴.

Overall, the metabolomics results of these published cerebrospinal fluid investigations of a wide range of disease conditions did not match our ME/CFS cerebrospinal fluid pattern of disordered serine pathways with sarcosine and phospholipid production, folate and one carbon exchange, purine, pyrimidine, aromatic and anaplerotic amino acid metabolism.

This cursory review of cerebrospinal fluid metabolomics results is limited because of the diversity of targeted and untargeted mass spectrometry results that cannot be directly compared between studies or to the Biocrates method used here. Sample sizes were small which can lead to misleading conclusions especially regarding correlations with subjective sensations and questionnaire results that have small effect sizes. The diversity of methods makes meta-analyses challenging to perform. Effects of age, gender, race, comorbid conditions such as diabetes are often not assessed in the literature; we used multivariate frequentist approach here to include the potential impacts of these covariates. Our postexercise group was small, but indicated consumption of metabolites after exercise by ME/CFS but relative production or accumulation in cerebrospinal fluid for controls. The comparison of nonexercise to postexercise results should be viewed with caution because samples were collected from a different cohort at a later date and may suffer from a batch effect. Batch effects were addressed by using MetaboAnalyst reconciliation tools (e.g. COMBAT) but should still be kept in consideration. Our findings

were at variance with prior hypometabolic findings in baseline studies in ME/CFS. This may reflect our patient population and intensive testing paradigm.

Thiamine was elevated postexercise which may be a false positive result if bleeding had been induced by lumbar puncture. Thiamine can be elevated even if erythrocytes are centrifuged from the specimens⁷⁵. However, bleeding with lysis of erythrocytes would have caused elevation of aspartate, glutamate, taurine, ornithine, glycine, citrulline, pyridoxal 5'-phosphate, 5-methyltetrahydrofolate, and thiamine and reductions for arginine, 5-hydroxyindoleacetic and homovanillic acids which was not observed.

Sample size was an important limitation of this study especially when assessing the results of the triple crossproduct analysis. To avoid the effects of small subgroups, effects sizes were calculated in two ways. The comparison of ME/CFS vs. SC was performed after Student's t-test to estimate Hedges' g (Table 4) and Partial Eta Squared for the univariate assessment of disease in the multivariate analysis (SOM Table S3). The latter were estimated for gender and exercise as well. The smallest subgroup had 30 subjects suggesting the effects sizes may be used to estimate sample sizes for future studies to confirm these preliminary findings. The findings of the triple cross-products should not be overinterpreted because of the have small sample sizes of subgroups.

The novel conclusions generate new hypotheses regarding serine-folate-glycine one carbon and serine-phospholipid metabolic pathways, elevation of end products of catabolic pathways, shifts in folate, thiamine and other vitamins with exercise, and changes in sphingomyelins that may indicate myelin and white matter dysfunction in ME/CFS. The current results can be used to power future confirmatory investigations of the cerebrospinal fluid metabolome in ME/CFS.

Methods

Subjects

Protocols were approved by the Georgetown University Institutional Review Board (2006–481, 2009–229, 2013–0943, 2015–0579) in accordance with the Declaration of Helsinki and listed in clinicaltrials.gov (NCT03567811, NCT00810329).

Sedentary ME/CFS, and healthy control subjects were recruited from websites, word of mouth, fliers, newspaper and online advertisements, and personal contacts in clinics and support groups. Interested participants responded via telephone or email. After obtaining verbal informed consent, each volunteer had an initial telephone screening with a clinical research associate who read a scripted outline of the study to assess inclusion and exclusion criteria. Candidates were screened for Center for Disease Control (CDC) criteria for Chronic Fatigue Syndrome (CFS)¹; current medications; chronic medical and psychiatric illnesses^{76–78}. Eligible subjects came to the Georgetown Howard Universities Clinical and Translational Science Clinical Research Unit where diagnosis and study inclusion were confirmed by history and physical examination and subjects provided in person written informed consent.

ME/CFS was defined using 1994 CDC “Fukuda” criteria¹ plus Canadian Consensus Criteria^{2,3}. The CDC criteria require disabling fatigue lasting more than 6 months that cannot be explained by exclusionary medical or psychiatric diagnoses^{8–10} plus at least 4 of 8 ancillary symptoms: short term memory or concentration problems, sore throat, sore lymph nodes, myalgia, arthralgia, headache, sleep disturbance, and post-exertional malaise (exertional exhaustion). The Carruthers Canadian Consensus Criteria emphasizes fatigue, postexertional malaise, sleep, pain, cognition, and an array of flu-like, autonomic and interoceptive symptoms^{2,3}. Subjects were not retrospectively assessed for 2015 Institute of Medicine criteria for Systemic Exertion Intolerance Disease (SEID) with fatigue, postexertional malaise, sleep and either cognitive or orthostatic problems⁴.

Sedentary controls in the nonexercise and postexercise groups had a sedentary lifestyle with less than 40 min of aerobic exercise per week and did not meet ME/CFS criteria.

Exclusion criteria included substance abuse, hospitalization for a psychiatric disorder in the past 5 years, or a chronic medical or psychiatric condition^{76–78}.

Two cohorts were studied^{22–25}. The first day of the 2 protocols was considered an adjustment period, and included the patient's history and physical, blood work, and baseline studies. The nonexercise cohort rested before having lumbar puncture and did not have exercise. The postexercise cohort had magnetic resonance imaging (MRI), submaximal bicycle exercise stress testing, and serial assessments of postural tachycardia. They rested overnight, then had their second identical stress test, MRI and postexercise lumbar puncture. Subjects cycled at 70% of predicted heart rate (pHR = 220–Age) for 25 min then increased to 85% pHR. Lumbar puncture was performed 1 to 5 h after exercise.

Questionnaires

Subjects completed a series of questionnaires (SOM Table S2). Disability and impairment were assessed based on quality of life and the Medical Outcomes Survey Short Form 36 (SF-36)^{79,80}. Responses were converted from nominal and anchored ordinal scores to scales from 0 (severely impaired) to 100 (no impairment)⁸¹. The average of Vitality, Role Physical and Social Functioning (SF36 V, RP, SF) was calculated as it consistently gave the lowest scores and was superior to individual domains for differentiating CFS and Gulf War Illness (GWI) from SC.

All subjects completed the CFS Symptom Severity Questionnaire (CFSQ)²⁸. The 1994 Fukuda CFS criteria¹ were operationalized by scoring fatigue and 8 ancillary symptoms from the previous 6 months on an anchored ordinal scale with grades of none = 0, trivial = 1, mild = 2, moderate = 3 and severe = 4. Unlike the original Fukuda criteria, we required moderate or severe symptom severities for fatigue and at least four of the eight criteria for to be considered for ME/CFS diagnosis here.

Fatigue was corroborated using independent scales. The Revised Clinical Interview Schedule (CIS-R)⁸² consists of 6 topics used for interviews and was adapted as 6 nominal items to gauge overall fatigue and tiredness (range 0 to 6). The Chalder Fatigue questionnaire was assessed as the total score summed for 11 items (range 0

to 33)⁸³. The Multidimensional Fatigue Inventory (MFI) addressed five domains with ranges of 0 to 20 and sum (0 to 100)⁸⁴.

The McGill pain questionnaire⁸⁵ scored 11 “Sensory” pain descriptors and four “Affective” words (tiring, sickening, fearful, punishing). Severity was graded on an anchored ordinal scale: None = 0, Mild = 1, Moderate = 2, and Severe = 3, and summed for “Sensory” (range 0 to 33), “Affective” (range 0 to 12) and “Total” (range 0 to 45) scores.

Interoceptive symptoms were assessed from several questionnaires. Chronic Multisymptom Severity Inventory (CMSI) assessed interoceptive symptoms using the 0 to 4 point anchored ordinal scale⁸⁶. Domain scores were determined for Rheumatic which included pain and fatigue symptoms (range 0 to 44), Dyspnea (range 0 to 20), Cardiac (range 0 to 16), Headache (migraine and tension scored 0 to 4 each), Ear Sinus (range 0 to 20), Neuro (range 0 to 16), Irritable Bowel Syndrome based on Rome I criteria (range 0 to 32) [88], Bladder (range 0 to 16) and the sum of all items (range 0 to 172). An interoceptive CMSI score (CMSI no pain) was calculated by subtracting the Rheum domain from the sum of the CMSI (range 0 to 128). Migraines were assessed by International Headache Society criteria⁸⁷. Upper and lower airway symptoms were assessed using the Rhinitis Score⁸⁶ and Irritant Rhinitis Score⁸⁸. Systemic irritant symptoms were assessed using the Chemical Exposures questionnaire domain scores⁸⁹. The Composite Autonomic Symptom Score (COMPASS-31) graded symptoms conveyed by cranial nerve, autonomic and general afferent system pathways that are commonly associated with autonomic dysfunction⁹⁰.

Psychiatric disorders were screened by PRIMEMD questionnaire for Major Depressive Syndrome, Other Depressive Syndrome, Panic Syndrome and difficulty in completing the questionnaire⁹¹. Details of anxiety were examined with Generalized Anxiety Disorder 7 questionnaire (GAD7)⁹², Mood and Anxiety Questionnaire (MASQ)⁹³ and The Irritability Questionnaire⁹⁴. Major depression⁹⁵ and Somatic, Anhedonia and Depressed domains were probed with the Center for Epidemiology – Depression questionnaire^{96,97}. Scores ≥ 16 out of 60 have been used to infer risk of major depression but this is biased by somatic complaints such as fatigue that are common in major depressive disorder in the greater general population⁹⁸ but are also inherent to CFS criteria and diagnosis.

Psychological aspects of pain were examined with the Pain Beliefs and Perceptions questionnaire⁹⁹, Beliefs in Pain Control Questionnaire (BPCQ)¹⁰⁰, Pain Catastrophizing Scale¹⁰¹, and “Your Experiences with Pain” (Chronic Pain Stressor Scale, CPSS)¹⁰². Posttraumatic stress disorder (PTSD) was assessed by PTSD Check List Civilian (PCL-C)¹⁰³.

Dolorimetry

Central sensitization was assessed by dolorimetry. Dolorimetry was performed with a strain gauge (DPP gauge; Chatillion Products, Ametek Inc, Largo, FL) fitted with a 1 cm² rubber stopper with pressure applied at a rate of 0.5 to 1 kg/s against the 18 traditional tender points^{104–106}. The outcome point was the pressure that caused the subject to state that she was experiencing pain. A key aspect was to ensure that the patient felt in control of the process and had trust that the operator would stop pressing as soon as she indicated pain had developed. The mean of the 18 measurements was the dolorimetry pressure threshold. The coefficient of variability for dolorimetry was 9.3% for 57 women and 12.5% for 58 men who had serial measurements on 3 days by different staff members¹⁰⁶. The Pearson correlation coefficient between thumb pressure tender point counts and dolorimetry pressure thresholds was -0.862 (explained variance = 0.742).

Lumbar puncture

Identical methods were used in all subjects. Cerebrospinal fluid (20 ml) was drawn from the L1-L2 interspace using Gertie-Marx needles^{107,108} in the prone position under fluoroscopic guidance by interventional radiologists. Specimens were immediately placed on ice, centrifuged at 4 °C, specimens sent for routine laboratory studies, and aliquoted and stored at -80 °C within 1 h. After lumbar puncture subjects were informed strictly to rest in comfortable positions and to avoid straining actions and Valsalva maneuvers such as lifting luggage for 24 h before discharge.

Metabolomics

Targeted mass spectrometry approaches developed at Georgetown University^{42,109–112} were used to assess 179 metabolites and 220 lipids in cerebrospinal fluid in the nonexercise and postexercise cohorts of ME/CFS and SC subjects.

All LC-MS grade solvents including acetonitrile and water were purchased from Fisher Optima grade, Fisher Scientific. High purity formic acid (99%) was purchased from Thermo-Scientific. Debrisoquine and 4-nitrobenzoic acid were purchased from Sigma- Aldrich. EquiSPASH[®] LIPIDOMIX[®] quantitative mass spec internal standard and 15:0–18:1-d7-PA, C15 Ceramide-d7 (d18:1-d7/15:0) and 18:1 Chol (D7) ester were purchased from Avanti polar lipids. Internal standard for free fatty acid (FFA), dihydroceramides (DCER), hexosylceramides (HCER), lactosylceramides (LCER) were purchased from Sciex (Framingham, MA, USA) as Lipidzyzer platform kit.

The targeted metabolomics method quantitated 270 endogenous metabolites using QTRAP[®] 5500 LC-MS/MS System (Sciex). Cerebrospinal fluid samples were thawed then 150 μ L immediately mixed with 150 μ L of methanol/water 50/50, thoroughly mixed on a vortex mixer, incubated in an ultrasonic bath for 5 min and incubated on ice for 20 min. 150 μ L of ACN was added followed by incubation at -20°C for 20 min. After vortexing, samples were centrifuged at 13,000 RPM for 20 min at 4 °C. The supernatant was evaporated to dryness under nitrogen at 30 °C using a vacuum pump. The residue was reconstituted to 150 μ L by adding 150 μ L of 25 MeOH : 25 water : 50 acetonitrile containing 250 ng/mL of debrisoquine (DBQ) as internal standard for positive mode and 250 ng/mL of 4-nitrobenzoic acid as internal standard for negative mode. The tubes were

vortexed and incubated in the ultrasonic bath for 10 min then centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatants were transferred to MS vials for LC-MS analysis.

The quality control sample was made with 25 µL National Institute of Standards and Technology (NIST) standard plasma sample dissolved in 100 µL of extraction buffer (methanol/water 50/50) containing 250 ng/mL of debrisoquine (DBQ) as internal standard for positive mode and 250 ng/mL of 4-nitrobenzoic acid as internal standard for negative mode. The sample was vortexed for 30 s and incubated on ice for 20 min followed by addition of 100 µL of acetonitrile and incubation at -20 °C for 20 min. Samples were centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was transferred to MS vial for LC-MS analysis.

Five microliter of the prepared sample was injected onto a Kinetex 2.6 µm 100 Å 100 × 2.1 mm (Phenomenex, Torrance, CA, USA) using SIL-30 AC auto sampler (Shimadzu, Columbia, MD, USA) connected with a high flow LC-30AD solvent delivery unit (Shimadzu) and CBM-20 A communication bus module (Shimadzu) online with QTRAP 5500 (Sciex) operating in positive and negative ion mode. A binary solvent comprising of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was used. The extracted metabolites were resolved at 0.2 mL/min flow rate. The LC gradient conditions were as follows: Initial – 100% A, 0% B for 2.1 min; 14 min – 5% A, 95% B until 15 min; 15.1 min – 100% A, 0% B until 20 min. The auto sampler and oven were kept at 15 °C and 30 °C, respectively. Source and gas setting for the method were as follow: curtain gas = 40, CAD gas = 9, ion spray voltage = 1700 V in positive mode and ion spray voltage = 1600 V in negative mode, temperature = 350 °C, ion source gas 1 = 30 and ion source gas 2 = 50. The data were normalized to internal standard area and processed using Sciex OS software. To ensure high quality and reproducibility of LC-MS data, the column was conditioned using the quality control samples initially and after every 10 sample injections to monitor shifts in signal intensities and retention time as measures of reproducibility and data quality. The NIST plasma sample were run after every 20 samples to check the instrumental variance. Blank solvent was run after every 10 samples and before and after pooled quality control samples to minimize carry-over effects.

The targeted lipidomics method was designed to measure 21 classes of lipid molecules including free fatty acids (FFA), diacylglycerols (DAG), triacylglycerols (TAG), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidic acid (LPA), ceramides (CE), dihydroceramides (DCER), hexosylceramide (HCER), lactosylceramide (LCER), sphingomyelins (SM), acylcarnitines and cholesterol esters (CE) using QTRAP[®] 5500 LC-MS/MS System (Sciex).

Cerebrospinal fluid was thawed at room temperature and immediately processed by mixing 150 µL cerebrospinal fluid with 300 µL of isopropanol, vortexing serial incubations of 5 min in an ultrasonic bath, 20 min on ice, and incubation at -20 °C for 20 min. After vortexing, the samples were centrifuged at 13,000 RPM for 20 min at 4 °C. The supernatant was evaporated to dryness under nitrogen at 30 °C using a vacuum pump. Then, 150 µL isopropanol with internal standards was added to the residue to reconstitute the initial volume of CSF. The tubes were vortexed and incubated in the ultrasonic bath for 10 min. Samples were centrifuged at 13,000 rpm for 2 h at 4 °C. The supernatants were transferred to MS vials for LC-MS analysis.

NIST plasma sample (25 µL) was mixed with 125 µL of chilled isopropanol containing internal standards for lipid classes. The sample was vortexed for 30 s and incubated on ice for 30 min followed by incubation at -20 °C for 2 h. Samples were centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatants were transferred to MS vials for LC-MS analysis.

Five µL of each sample was injected onto a Xbridge amide 3.5 µm, 4.6 × 100 mm column (Waters, Milford, MA) using SIL-30 AC auto sampler (Shimadzu) connected with a high flow LC-30AD solvent delivery unit (Shimadzu) and CBM-20 A communication bus module (Shimadzu) online with QTRAP 5500 (Sciex) operating in positive and negative ion modes. A binary solvent comprising of acetonitrile/water 95/5 with 10 mM ammonium acetate as solvent A and acetonitrile/water 50/50 with 10 mM ammonium acetate as solvent B was used for the resolution. Lipids were resolved at 0.7 mL/min flow rate, initial gradient conditions started with 100% of solvent A, shifting towards 99.9% of solvent A over a time period of 3 min, 94% of solvent A over a time period of 3 min and 25% of solvent A over a period of 4 min. Finally, washing with 100% of B for 6 min and equilibrating to initial conditions (100% of solvent A) over a time period of 6 min using auto sampler temperature 15 °C and oven temperature 35 °C. Source and gas setting were as follow: curtain gas = 30, CAD gas = medium, ion spray voltage = 5.5 kV in positive mode and -4.5 kV in negative mode, temperature = 550 °C, nebulizing gas = 50 and heater gas = 60. The data were normalized to respective internal standard area for each class of lipid and processed using MultiQuant 3.0.3 (Sciex). The quality and reproducibility of LC-MS data was ensured using a number of measures. The column was conditioned using the pooled QC samples initially and after every 10 sample injections. The NIST plasma sample was run after every 20 samples to check the instrumental variance. Solvent blanks were run after every 10 samples and before and after pooled quality control samples to minimize carry-over effects.

The mass spectrometry abundances without log transformation or standardization were provided in SOM Table S2.

Statistical and bioinformatic analysis

Batch effects were examined in MetaboAnalyst 6.0^{113,114} and the reconciled nonexercise and postexercise data sets were used. Zeros were found in about 20 cells and one half the minimum value for each analyte was imputed. Data were log 10 transformed and autocorrected by subtracting the mean and dividing by the standard deviation. As a first approximation, ME/CFS and SC data were compared by Student's t-test with effect sizes of significant differences estimated by Hedge's *g*. Data were then assessed in SPSS version 29¹¹⁵ by multivariate general linear modeling with metabolites and lipids as dependent variables, disease (ME/CFS vs. SC), gender and exercise status (nonexercise vs. postexercise) as independent fixed factors with age and BMI^{26,116–118} as covariates and

correction by the Sidak method ($p < 0.05$) to control for multiple comparisons. Estimated marginal means were evaluated for each factor and their triple product (disease \times gender \times exercise). Significant differences between ME/CFS vs. SC for the main effect of disease and cross-products and Partial Eta Squared effect sizes were tabulated^{119–121}. Log transformed normalized and standardized analyte levels and questionnaire items were correlated using the nonparametric Spearman method.

Relationships were examined by pathway analysis and enrichment in MetaboAnalyst 6.0. Many of the secondary and less well studied metabolites were not linked to KEGG pathways and so their functions were detailed from The Human Metabolome Database MetaboCards^{122,123}. Previous studies in ME/CFS have identified alterations in metabolic modules for glycolysis, Tricarboxylic Acid Cycle (TCA), anaplerotic and other amino acid interactions with the TCA cycle, purine and pyrimidine metabolism and dysfunctional peroxisome and mitochondrial activities. In addition, our multivariate findings identified serine, 5-methyltetrahydrofolate (5MTHF) and other derivatives of the serine – glycine – one carbon pathway. The serine pathway provided a link to rationalize phospholipid synthesis in the setting of ME/CFS. KEGG pathways were used to integrate these metabolic modules into an interconnected general framework (Fig. 1). Metabolic modules were color coded. Metabolites that were significantly different were indicated in bold \pm italics and highlighted for ME/CFS > SC (yellow) and SC > ME/CFS (pink). Metabolites derived from the gut microbiome, diet, exposome or with poorly defined metabolism in humans were highlighted.

Supplementary online material (SOM) Table S1 contains the outcomes of the multivariate general linear model. SOM Table S2 contains the targeted mass spectrometry metabolomics and lipidomics abundances and questionnaire results. SOM Table S3 shows effect sizes for significant differences related to disease, gender and exercise in the multivariate general linear model. SOM Table S4 is a larger version of Fig. 5 Spearman correlation matrix for significant metabolites, lipids and questionnaire domain scores. Follow-up data analysis including Bayesian statistics are reported separately¹²⁴.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files. The results of the multivariate statistical analysis are in SOM Table S1. The raw mass spectrometry abundances of metabolomics and lipidomics with questionnaire data are in SOM Table S2. SOM Table S3 contained effect sizes for the univariate analysis of disease, exercise and gender status from the multivariate analysis. SOM Table S4 is a larger version of Fig. 5 Spearman correlation matrix for analytes and questionnaire items.

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References

1. Fukuda, K. et al. The chronic fatigue syndrome: A comprehensive approach to its definition and study. *Ann. Intern. Med.* **121**(12), 953–959 (1994).
2. Carruthers, B. M. Definitions and aetiology of myalgic encephalomyelitis: How the Canadian consensus clinical definition of myalgic encephalomyelitis works. *J. Clin. Pathol.* **60**(2), 117–119 (2007).
3. Carruthers, B. M. et al. Myalgic encephalomyelitis: International consensus criteria. *J. Intern. Med.* **270**(4), 327–338 (2011).
4. Beyond myalgic encephalomyelitis/chronic fatigue syndrome redefining an illness [Internet]. 2015 [cited 2022 Nov 16]. Available from: <https://nap.nationalacademies.org/read/19012/chapter/1>
5. Jason, L. A. et al. A community-based study of chronic fatigue syndrome. *Arch. Intern. Med.* **159**(18), 2129–2137 (1999).
6. Reyes, M. et al. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. *Arch. Intern. Med.* **163**(13), 1530–1536 (2003).
7. Lin, J. M. S. et al. The economic impact of chronic fatigue syndrome in Georgia: Direct and indirect costs. *Cost Eff. Resour. Alloc.* **9**(1), 1 (2011).
8. Bae, J. & Lin, J. M. S. Healthcare utilization in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS): Analysis of US ambulatory healthcare data, 2000–2009. *Front. Pediatr.* **7**, 185 (2019).
9. Vahratian, A., Lin, J. M. S., Bertolli, J. & Unger, E. R. Myalgic encephalomyelitis/chronic fatigue syndrome in adults: United States, 2021–2022. *NCHS Data Brief.* **488**, 1–8 (2023).
10. Ghali, A. et al. Factors influencing the prognosis of patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Diagnostics (Basel)* **12**(10), 2540 (2022).
11. Volberding, P. A. et al. (eds) *Long-term health effects of COVID-19* (National Academies Press, 2024).
12. Stussman, B. et al. Mixed methods system for the assessment of post-exertional malaise in myalgic encephalomyelitis/chronic fatigue syndrome: an exploratory study. *BMJ Neurol. Open* **6**(1), e000529 (2024).
13. Jason, L. A., Evans, M., So, S., Scott, J. & Brown, A. Problems in defining post-exertional malaise. *J. Prev. Interv. Commun.* **43**(1), 20–31 (2015).
14. Germain, A., Barupal, D. K., Levine, S. M. & Hanson, M. R. Comprehensive circulatory metabolomics in ME/CFS reveals disrupted metabolism of acyl lipids and steroids. *Metabolites* **10**(1), 34 (2020).
15. Hoel, F., Hoel, A., Pettersen, I. K., Rekeland, I. G., Risa, K. & Alme, K., et al. A map of metabolic phenotypes in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *JCI Insight.* **6**(16), (2021).
16. Che, X., Brydges, C. R., Yu, Y., Price, A., Joshi, S. & Roy, A., et al. Evidence for peroxisomal dysfunction and dysregulation of the CDP-choline pathway in myalgic encephalomyelitis/chronic fatigue syndrome. *medRxiv*, (2022).
17. Naviaux, R. K. et al. Metabolic features of chronic fatigue syndrome. *Proc. Natl. Acad. Sci. USA* **113**(37), E5472–E5480 (2016).
18. Glass, K. A., Germain, A., Huang, Y. V. & Hanson, M. R. Urine metabolomics exposes anomalous recovery after maximal exertion in female ME/CFS patients. *Int. J. Mol. Sci.* **24**(4), 3685 (2023).
19. <https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST000910&StudyType=MS&ResultType=1>. 2018.
20. Walitt, B. et al. Deep phenotyping of post-infectious myalgic encephalomyelitis/chronic fatigue syndrome. *Nat. Commun.* **15**(1), 907 (2024).
21. Li, K. et al. Cerebrospinal fluid and plasma metabolomics of acute endurance exercise. *FASEB J.* **36**(7), e22408 (2022).
22. Garner, R. S., Rayhan, R. U. & Baraniuk, J. N. Verification of exercise-induced transient postural tachycardia phenotype in Gulf War Illness. *Am J Transl Res.* **10**(10), 3254–3264 (2018).

23. Washington, S. D. et al. Exercise alters brain activation in Gulf War illness and myalgic encephalomyelitis/chronic fatigue syndrome. *Brain Commun.* **2**(2), fcaa070 (2020).
24. Baraniuk, J. N. & Shivapurkar, N. Exercise - induced changes in cerebrospinal fluid miRNAs in Gulf War Illness, chronic fatigue syndrome and sedentary control subjects. *Sci. Rep.* **7**(1), 15338 (2017).
25. Rayhan, R. U. & Baraniuk, J. N. Submaximal exercise provokes increased activation of the anterior default mode network during the resting state as a biomarker of postexertional malaise in myalgic encephalomyelitis/chronic fatigue syndrome. *Front. Neurosci.* **15**, 748426 (2021).
26. Costanzo, M. et al. Sex differences in the human metabolome. *Biol. Sex Differ.* **13**(1), 30 (2022).
27. Fluge, Ø. et al. Metabolic profiling indicates impaired pyruvate dehydrogenase function in myalgic encephalopathy/chronic fatigue syndrome. *JCI Insight* **1**(21), e89376 (2016).
28. Baraniuk, J. N. et al. A chronic fatigue syndrome (CFS) severity score based on case designation criteria. *Am. J. Transl. Res.* **5**(1), 53–68 (2013).
29. Wolfe, F. et al. The American college of rheumatology 1990 criteria for the classification of fibromyalgia. *Arthritis Rheum.* **33**(2), 160–172 (1990).
30. Wolfe, F. et al. The American College of Rheumatology preliminary diagnostic criteria for fibromyalgia and measurement of symptom severity. *Arthritis Care Res. (Hoboken)* **62**(5), 600–610 (2010).
31. Meiser, J. et al. Serine one-carbon catabolism with formate overflow. *Sci. Adv.* **2**(10), e1601273 (2016).
32. Zhao, E., Hou, J. & Cui, H. Serine-glycine-one-carbon metabolism: Vulnerabilities in MYCN-amplified neuroblastoma. *Oncogenesis* **9**(2), 14 (2020).
33. Sun, W. et al. Targeting serine-glycine-one-carbon metabolism as a vulnerability in cancers. *Biomark Res.* **11**(1), 48 (2023).
34. Vance, J. E. Phospholipid synthesis and transport in mammalian cells. *Traffic* **16**(1), 1–18 (2015).
35. Reus, L. M., Boltz, T., Francia, M., Bot, M., Ramesh, N. & Koromina, M., et al. Quantitative trait loci mapping of circulating metabolites in cerebrospinal fluid to uncover biological mechanisms involved in brain-related phenotypes. *bioRxiv*, (2023).
36. Ciregia, F. et al. Bottom-up proteomics suggests an association between differential expression of mitochondrial proteins and chronic fatigue syndrome. *Transl. Psychiatry* **6**(9), e904 (2016).
37. Cluntun, A. A., Lulkey, M. J., Cerione, R. A. & Locasale, J. W. Glutamine metabolism in cancer: Understanding the heterogeneity. *Trends Cancer* **3**(3), 169–180 (2017).
38. Garner, R. & Baraniuk, J. N. Orthostatic intolerance in chronic fatigue syndrome. *J. Transl. Med.* **17**(1), 185 (2019).
39. Germain, A., Giloteaux, L., Moore, G. E., Levine, S. M., Chia, J. K. & Keller, B. A., et al. Plasma metabolomics reveals disrupted response and recovery following maximal exercise in myalgic encephalomyelitis/chronic fatigue syndrome. *JCI Insight.* **7**(9), (2022).
40. Giloteaux, L. et al. Dysregulation of extracellular vesicle protein cargo in female myalgic encephalomyelitis/chronic fatigue syndrome cases and sedentary controls in response to maximal exercise. *J. Extracell. Vesicles* **13**(1), e12403 (2024).
41. Panyard, D. J. et al. Cerebrospinal fluid metabolomics identifies 19 brain-related phenotype associations. *Commun. Biol.* **4**(1), 63 (2021).
42. <https://biocrates.com/absoluteidq-p180-kit/>.
43. Carlsson, H. et al. Metabolomics of cerebrospinal fluid from healthy subjects reveal metabolites associated with ageing. *Metabolites* **11**(2), 126 (2021).
44. Hwangbo, N. et al. A metabolomic aging clock using human cerebrospinal fluid. *J. Gerontol. A Biol. Sci. Med. Sci.* **77**(4), 744–754 (2022).
45. Liu, F. C. et al. Exploring the aging process of cognitively healthy adults by analyzing cerebrospinal fluid metabolomics using liquid chromatography-tandem mass spectrometry. *BMC Geriatr.* **23**(1), 217 (2023).
46. Peters, K. et al. Metabolic drift in the aging nervous system is reflected in human cerebrospinal fluid. *Sci. Rep.* **11**(1), 18822 (2021).
47. Reavis, Z. W. et al. Sex and race differences of cerebrospinal fluid metabolites in healthy individuals. *Metabolomics* **17**(2), 13 (2021).
48. Borkowski, K. et al. Integration of plasma and CSF metabolomics with CSF proteomic reveals novel associations between lipid mediators and central nervous system vascular and energy metabolism. *Sci. Rep.* **13**(1), 13752 (2023).
49. DiSabato, D. J., Quan, N. & Godbout, J. P. Neuroinflammation: The devil is in the details. *J. Neurochem.* **139**(Suppl 2), 136–153 (2016).
50. Chen, S. et al. Cerebrospinal fluid metabolomic and proteomic characterization of neurologic post-acute sequelae of SARS-CoV-2 infection. *Brain Behav. Immun.* **115**, 209–222 (2024).
51. Neu, F. et al. Targeted metabolomics identifies accurate CSF metabolite biomarkers for the differentiation between COVID-19 with neurological involvement and CNS infections with neurotropic viral pathogens. *J. Transl. Med.* **22**(1), 620 (2024).
52. Plaatjie, O. N., van Furth, A. M. T., van der Kuip, M. & Mason, S. LC-MS metabolomics and lipidomics in cerebrospinal fluid from viral and bacterial CNS infections: A review. *Front. Neurol.* **15**, 1403312 (2024).
53. French, C. D. et al. NMR metabolomics of cerebrospinal fluid differentiates inflammatory diseases of the central nervous system. *PLoS Negl. Trop. Dis.* **12**(12), e0007045 (2018).
54. Ratuszny, D. et al. Identification of cerebrospinal fluid metabolites as biomarkers for enterovirus meningitis. *Int. J. Mol. Sci.* **20**(2), 337 (2019).
55. Cassol, E., Misra, V., Dutta, A., Morgello, S. & Gabuzda, D. Cerebrospinal fluid metabolomics reveals altered waste clearance and accelerated aging in HIV patients with neurocognitive impairment. *AIDS* **28**(11), 1579–1591 (2014).
56. Yan, J., Kuzhiumparambil, U., Bandodkar, S., Dale, R. C. & Fu, S. Cerebrospinal fluid metabolomics: Detection of neuroinflammation in human central nervous system disease. *Clin. Transl. Immunol.* **10**(8), e1318 (2021).
57. Al-Mekhlafi, A. et al. Elevated phospholipids and acylcarnitines C4 and C5 in cerebrospinal fluid distinguish viral CNS infections from autoimmune neuroinflammation. *J. Transl. Med.* **21**(1), 776 (2023).
58. Hanin, A. et al. Metabolomic changes in adults with status epilepticus: A human case-control study. *Epilepsia* **65**(4), 929–943 (2024).
59. Xu, L. et al. Cerebrospinal fluid metabolite alterations in patients with different etiologies, diagnoses, and prognoses of disorders of consciousness. *Brain Behav.* **13**(8), e3070 (2023).
60. Bäckryd, E., Thordeman, K., Gerdle, B. & Ghafouri, B. Cerebrospinal fluid metabolomics identified ongoing analgesic medication in neuropathic pain patients. *Biomedicine* **11**(9), 2525 (2023).
61. Gasparini, C. F., Smith, R. A. & Griffiths, L. R. Genetic and biochemical changes of the serotonergic system in migraine pathobiology. *J. Headache Pain* **18**(1), 20 (2017).
62. Byeon, S. K. et al. Cerebrospinal fluid lipidomics for biomarkers of Alzheimer's disease. *Mol. Omics* **17**(3), 454–463 (2021).
63. Andersen, A. D., Binzer, M., Stenager, E. & Gramsbergen, J. B. Cerebrospinal fluid biomarkers for Parkinson's disease - A systematic review. *Acta Neurol. Scand* **135**(1), 34–56 (2017).
64. Otto, C. et al. Comprehensive analysis of the cerebrospinal fluid and serum metabolome in neurological diseases. *J. Neuroinflammation* **21**(1), 234 (2024).
65. Herman, S., Åkerfeldt, T., Spjuth, O., Burman, J. & Kulth, K. Biochemical differences in cerebrospinal fluid between secondary progressive and relapsing-remitting multiple sclerosis. *Cells* **8**(2), 84 (2019).
66. Xie, K., Sun, Y., Li, X., Yang, S., Wang, M. & Zhang, Y., et al. Biomarkers and pathways in autism spectrum disorder: An individual meta-analysis based on proteomic and metabolomic data. *Eur. Arch. Psychiatry Clin. Neurosci.*, (2024).

67. Ogawa, S. et al. Reduced cerebrospinal fluid ethanolamine concentration in major depressive disorder. *Sci. Rep.* **15**(5), 7796 (2015).
68. Kaddurah-Daouk, R. et al. Cerebrospinal fluid metabolome in mood disorders-remission state has a unique metabolic profile. *Sci. Rep.* **2**, 667 (2012).
69. Pan, L. A. et al. Metabolomic disorders: Confirmed presence of potentially treatable abnormalities in patients with treatment refractory depression and suicidal behavior. *Psychol. Med.* **53**(13), 6046–6054 (2023).
70. Berner, J. & Acharjee, A. Cerebrospinal fluid metabolomes of treatment-resistant depression subtypes and ketamine response: A pilot study. *Discover Mental Health* **4**(1), 12 (2024).
71. Shang, P. et al. Identification of cerebrospinal fluid and serum metabolomic biomarkers in first episode psychosis patients. *Transl. Psychiatry* **12**(1), 229 (2022).
72. Lin, H. T. et al. 1H nuclear magnetic resonance (NMR)-based cerebrospinal fluid and plasma metabolomic analysis in type 2 diabetic patients and risk prediction for diabetic microangiopathy. *J. Clin. Med.* **8**(6), 874 (2019).
73. Weiss, N. et al. Cerebrospinal fluid metabolomics highlights dysregulation of energy metabolism in overt hepatic encephalopathy. *J. Hepatol.* **65**(6), 1120–1130 (2016).
74. Dakterzada, F. et al. Cerebrospinal fluid lipidomic fingerprint of obstructive sleep apnoea in Alzheimer's disease. *Alzheimers Res. Ther.* **15**(1), 134 (2023).
75. Batllori, M. et al. Effect of blood contamination of cerebrospinal fluid on amino acids, biogenic amines, pterins and vitamins. *Fluids Barriers CNS* **16**(1), 34 (2019).
76. Reeves, W. C. et al. Identification of ambiguities in the 1994 chronic fatigue syndrome research case definition and recommendations for resolution. *BMC Health Serv. Res.* **3**(1), 25 (2003).
77. Jones, J. F. et al. An evaluation of exclusionary medical/psychiatric conditions in the definition of chronic fatigue syndrome. *BMC Med.* **7**(1), 57 (2009).
78. Nater, U. M. et al. Psychiatric comorbidity in persons with chronic fatigue syndrome identified from the Georgia population. *Psychosom. Med.* **71**(5), 557–565 (2009).
79. Ware, J. E. & Sherbourne, C. D. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. *Med. Care* **30**(6), 473–483 (1992).
80. McHorney, C. A., Ware, J. E., Lu, J. F. & Sherbourne, C. D. The MOS 36-item Short-Form Health Survey (SF-36): III. Tests of data quality, scaling assumptions, and reliability across diverse patient groups. *Med. Care* **32**(1), 40–66 (1994).
81. Hays, R. D., Sherbourne, C. D. & Mazel, R. M. The RAND 36-Item Health Survey 1.0. *Health Econ.* **2**(3), 217–227 (1993).
82. Lewis, G., Pelosi, A. J., Araya, R. & Dunn, G. Measuring psychiatric disorder in the community: A standardized assessment for use by lay interviewers. *Psychol. Med.* **22**(2), 465–486 (1992).
83. Chalder, T. et al. Development of a fatigue scale. *J. Psychosom. Res.* **37**(2), 147–153 (1993).
84. Smets, E. M., Garssen, B., Bonke, B. & De Haes, J. C. The multidimensional fatigue inventory (MFI) psychometric qualities of an instrument to assess fatigue. *J. Psychosom. Res.* **39**(3), 315–325 (1995).
85. Melzack, R. The short-form McGill Pain Questionnaire. *Pain* **30**(2), 191–197 (1987).
86. Baraniuk, J. N., Clauw, D. J. & Gaumond, E. Rhinitis symptoms in chronic fatigue syndrome. *Ann. Allergy Asthma Immunol.* **81**(4), 359–365 (1998).
87. Headache Classification Committee of the International Headache Society (IHS). The International Classification of Headache Disorders, 3rd edition (beta version). *Cephalalgia*, **33**(9):629–808, (2013).
88. Baraniuk, J. N., Naranch, K., Maibach, H. & Clauw, D. J. Irritant rhinitis in allergic, nonallergic, control and chronic fatigue syndrome populations. *J. Chronic Fatigue Syndr.* **7**(2), 3–31 (2000).
89. Miller, C. S. & Prihoda, T. J. The Environmental Exposure and Sensitivity Inventory (EESI): A standardized approach for measuring chemical intolerances for research and clinical applications. *Toxicol. Ind. Health* **15**(3–4), 370–385 (1999).
90. Sletten, D. M., Suarez, G. A., Low, P. A., Mandrekar, J. & Singer, W. COMPASS 31: A refined and abbreviated composite autonomic symptom score. *Mayo Clin. Proc.* **87**(12), 1196–1201 (2012).
91. Spitzer, R. L., Kroenke, K. & Williams, J. B. Validation and utility of a self-report version of PRIME-MD: The PHQ primary care study primary care evaluation of mental disorders. Patient Health Questionnaire. *JAMA* **282**(18), 1737–1744 (1999).
92. Kroenke, K., Spitzer, R. L., Williams, J. B. W., Monahan, P. O. & Löwe, B. Anxiety disorders in primary care: Prevalence, impairment, comorbidity, and detection. *Ann. Intern. Med.* **146**(5), 317–325 (2007).
93. Wardenaar, K. J. et al. Development and validation of a 30-item short adaptation of the mood and anxiety symptoms questionnaire (MASQ). *Psychiatr. Res.* **179**(1), 101–106 (2010).
94. Craig, K. J., Hietanen, H., Markova, I. S. & Berrios, G. E. The irritability questionnaire: A new scale for the measurement of irritability. *Psychiatr. Res.* **159**(3), 367–375 (2008).
95. Martin, J. et al. Expert and self-assessment of lifetime symptoms and diagnosis of major depressive disorder in large-scale genetic studies in the general population: Comparison of a clinical interview and a self-administered checklist. *Psychiatr. Genet.* **27**(5), 187–196 (2017).
96. Radloff, L. S. The CES-D scale. *Appl. Psychol. Meas* **1**(3), 385–401 (1977).
97. Geisser, M. E., Roth, R. S. & Robinson, M. E. Assessing depression among persons with chronic pain using the Center for epidemiological studies-depression scale and the beck depression inventory: A comparative analysis. *Clin. J. Pain* **13**(2), 163–170 (1997).
98. Fuhrer, R. & Wessely, S. The epidemiology of fatigue and depression: A French primary-care study. *Psychol. Med.* **25**(5), 895–905 (1995).
99. Williams, D. A., Robinson, M. E. & Geisser, M. E. Pain beliefs: Assessment and utility. *Pain* **59**(1), 71–78 (1994).
100. Brown, C. A. The beliefs of people with chronic pain in relation to “important” treatment components. *Eur. J. Pain* **8**(4), 325–333 (2004).
101. Sullivan, M. J. L., Bishop, S. R. & Pivik, J. The pain catastrophizing scale: Development and validation. *Psychol. Assess.* **7**(4), 524–532 (1995).
102. Anderson, K. O., Dowds, B. N., Pelletz, R. E., Edwards, T. W. & Peeters-Asdourian, C. Development and initial validation of a scale to measure self-efficacy beliefs in patients with chronic pain. *Pain* **63**(1), 77–83 (1995).
103. Conybeare, D., Behar, E., Solomon, A., Newman, M. G. & Borkovec, T. D. The PTSD Checklist-Civilian Version: Reliability, validity, and factor structure in a nonclinical sample. *J. Clin. Psychol.* **68**(6), 699–713 (2012).
104. Bennett, R. M. Fibrositis: Misnomer for a common rheumatic disorder. *West J. Med.* **134**(5), 405–413 (1981).
105. Naranch, K. et al. A tender sinus does not always mean rhinosinusitis. *Otolaryngol. Head Neck Surg.* **127**(5), 387–397 (2002).
106. Surian, A. A. & Baraniuk, J. N. Systemic hyperalgesia in females with Gulf War illness, chronic fatigue syndrome and fibromyalgia. *Sci. Rep.* **10**(1), 5751 (2020).
107. Prager, J. M. et al. Spinal headaches after myelograms: Comparison of needle types. *AJR Am. J. Roentgenol.* **167**(5), 1289–1292 (1996).
108. Riley, E. T., Hamilton, C. L., Ratner, E. F. & Cohen, S. E. A comparison of the 24-gauge Sprotte and Gertie Marx spinal needles for combined spinal-epidural analgesia during labor. *Anesthesiology* **97**(3), 574–577 (2002).
109. Mandal, R. et al. Multi-platform characterization of the human cerebrospinal fluid metabolome: A comprehensive and quantitative update. *Genome Med.* **4**(4), 38 (2012).

110. Koal, T., Klavins, K., Seppi, D., Kemmler, G. & Humpel, C. Sphingomyelin SM(d18:1/18:0) is significantly enhanced in cerebrospinal fluid samples dichotomized by pathological amyloid- β 42, tau, and phospho-tau-181 levels. *J. Alzheimers Dis.* **44**(4), 1193–1201 (2015).
111. Cheema, A. K. et al. A metabolomic and lipidomic serum signature from nonhuman primates administered with a promising radiation countermeasure, gamma-tocotrienol. *Int. J. Mol. Sci.* **19**(1), 79 (2017).
112. Sheikh, K. D., Khanna, S., Byers, S. W., Fornace, A. & Cheema, A. K. Small molecule metabolite extraction strategy for improving LC/MS detection of cancer cell metabolome. *J. Biomol. Tech.* **22**(1), 1–4 (2011).
113. MetaboAnalyst 6.0 <https://dev.metaboanalyst.ca/home.xhtml>.
114. Pang, Z. et al. MetaboAnalyst 6.0: Towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Res.* **52**(W1), W398–406 (2024).
115. SPSS - <https://www.ibm.com/products/spss-statistics>.
116. Boca, S. M., Sinha, R., Cross, A. J., Moore, S. C. & Sampson, J. N. Testing multiple biological mediators simultaneously. *Bioinformatics* **30**(2), 214–220 (2014).
117. Boca, S. M. et al. Discovery of metabolic biomarkers for duchenne muscular dystrophy within a natural history study. *PLoS One* **11**(4), e0153461 (2016).
118. Boca, S. M. & Leek, J. T. A direct approach to estimating false discovery rates conditional on covariates. *PeerJ.* **6**, e6035 (2018).
119. Suurmond, R., van Rhee, H. & Hak, T. Introduction, comparison, and validation of meta-essentials: A free and simple tool for meta-analysis. *Res. Synth. Methods* **8**(4), 537–553 (2017).
120. Meta-Essentials [Internet]. Available from: <http://systematicreviewtools.com/tool.php?ref=Meta-Essentials>
121. Van Rhee, H. J., Suurmond, R. & Hak, T. User manual for Meta-Essentials: Workbooks for meta-analysis (version 1.4), (2015).
122. Metabocards - <https://hmdb.ca/metabolites/HMDB0000479>.
123. Wishart, D. S. et al. HMDB 50: The human metabolome database for 2022. *Nucleic Acids Res.* **50**(D1), D622–D631 (2022).
124. Baraniuk, J. N. Exertional exhaustion (postexertional malaise, PEM) evaluated by the effects of exercise on cerebrospinal fluid metabolomics, lipidomics and serine pathway in myalgic encephalomyelitis/chronic fatigue syndrome. *International Journal of Molecular Sciences* (ISSN 1422-0067, IF 4.9) Manuscript ID: ijms-3366404. Special Issue: Molecular pathologies and treatment for myalgic encephalomyelitis/chronic fatigue syndrome. Special Issue website: https://www.mdpi.com/journal/ijms/special_issues/P7GE9B0370.

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Author contributions

JNB was responsible for conception of the study, funding, analysis and interpretation of the metabolomics and lipidomics data, writing the manuscript and final approval.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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