Title: Dysregulation of the Kennedy Pathway and Tricarboxylic Acid Cycle in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

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Abstract

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic and debilitating disease that is characterized by unexplained physical fatigue unrelieved by rest. Symptoms also include cognitive and sensory dysfunction, sleeping disturbances, orthostatic intolerance and gastrointestinal problems. The pathogenesis is not fully understood. Using regression, Bayesian and enrichment analyses, we conducted targeted and untargeted metabolomic analysis of 888 metabolic analytes in plasma samples of 106 ME/CFS cases and 91 frequency-matched healthy controls. In ME/CFS cases, the regression, Bayesian and enrichment analyses all revealed abnormal levels of several membrane lipids indicating dysregulation of the Kennedy pathway: decreased plasma levels of plasmalogens, phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, and phospholipid ethers. Enrichment analyses revealed decreased levels of cholines, ceramides and carnitines, and increased levels of long chain triglycerides, dicarboxylic acids, hydroxy-eicosapentaenoic acid, and the tricarboxylic acid cycle intermediates alpha-ketoglutarate and succinate. Using machine learning algorithms with selected metabolites as predictors, we were able to differentiate female ME/CFS cases from female controls (highest AUC=0.794) and ME/CFS cases without self-reported irritable bowel syndrome (sr-IBS) from controls without sr-IBS (highest AUC=0.873). Our findings are consistent with earlier ME/CFS work indicating compromised energy metabolism and redox imbalance, and highlight specific abnormalities that may provide insights into the pathogenesis of ME/CFS.

Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a disease of unknown cause that is defined by impairment from fatigue lasting longer than six months, unrefreshing sleep, post-exertional malaise, and either cognitive dysfunction or orthostatic intolerance[1]. People with ME/CFS often report additional symptoms, such as gastrointestinal disturbances, influenza-like symptoms, and chronic pain[2]. It is estimated that ME/CFS affects between 0.4% to 2.5% of the global population, and 1.5 to 2.5 million people in the United States alone[1, 3]. There are no approved diagnostic tests for ME/CFS; medical providers must assess medical history, conduct a physical examination and exclude other disorders for diagnosis[4, 5].

Prior metabolomic studies of patients with ME/CFS have provided insights into the potential pathogenesis and course of the disease, demonstrating disturbances in energy, lipid, amino acid, and redox metabolism[6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. Studies continue in the ongoing pursuit of identifying a metabolomic signature as biomarker for ME/CFS. However, there is a lack of consistency in the metabolites that are altered in ME/CFS patients across these studies. This may be the result of the heterogeneity of this disease.

Metabolic dimensions of ME/CFS may be related to sex; women are disproportionately affected by ME/CFS[1, 17]. Naviaux et al. (2016) found differences in metabolic pathway disturbances and altered metabolite levels when stratifying ME/CFS cases by sex[15]. Other have also reported sex-specific differences in plasma biomarkers[14, 18, 19].

Comorbid gastrointestinal (GI) symptoms constitute a potential subtype in ME/CFS [11, 12, 14, 15, 19, 20, 21, 22, 23]. Among those with ME/CFS, the presence or absence of self-reported physician diagnosed irritable bowel syndrome (sr-IBS), in particular, has highlighted differences in the plasma proteome relating to immune dysregulation and altered levels of metabolites within the metabolome[14, 19]. In a fecal metagenomics study, Nagy-Szakal et al. (2017) identified eleven bacterial species delineating differences between

ME/CFS patients with and without sr-IBS and found relations between bacterial taxa and symptoms relating to fatigue and pain[23].

In this study, we report targeted and untargeted analyses of 888 metabolic analytes comprising of primary metabolites, biogenic amines, complex lipids, and oxylipins in plasma of ME/CFS cases and controls. We identified altered metabolomic profiles between ME/CFS patients, controls, and subgroups within ME/CFS patients based on sex and sr-IBS.

Materials and Methods

Study population.

Our starting population comprised 177 ME/CFS cases and 177 controls in ME/CFS clinics in Incline Village, NV; Miami FL; New York, NY; Salt Lake City, UT; and Palo Alto, CA. All ME/CFS cases met the 1994 CDC Fukuda[24] and Canadian consensus criteria for ME/CFS[25]. All ME/CFS cases completed standardized screening and assessment instruments including medical history and symptom rating scales as well as a physical examination. Controls were matched to cases on age, sex, race/ethnicity, geographic/clinical site, and date of sampling (±30 days). Based on screening criteria, we excluded 5 ME/CFS cases that met any exclusion criteria from the 1994 CDC Fukuda and/or Canadian consensus criteria for ME/CFS such as having chronic infections, rheumatic and chronic inflammatory diseases, neurological disorders, psychiatric conditions, or were taking any immunomodulatory medication. Controls underwent the same screening process as ME/CFS subjects and were excluded if they reported ME/CFS or other conditions deemed by the recruiting physician to be inconsistent with a healthy control population. Controls were also excluded if they had a history of substance abuse, psychiatric illness, antibiotics in the prior three months, immunomodulatory medications in the prior year, and clinically significant findings on physical exam or screening laboratory tests. One control was excluded after prescreening based on these criteria. Additionally, 21 participants were excluded prior to baseline due to withdrawal from the study (n=18), loss to follow-up (n=2), and enrollment capacity (n=1). The baseline questionnaire was completed by with 327 participants. During the study, an additional 63 participants were excluded for study protocol deviations (n=25), loss to follow-up (n=25), and withdrawal from the study (n=13), resulting in a total of 264 participants.

For the analysis reported here, a sub-cohort was established based on complete survey and biospecimen data (blood, saliva and stool) at the first and last time points of the study and key demographic characteristics were frequency-matched to ensure that the nested cohort was similar to the full cohort. This sub-cohort consisted of 106 ME/CFS cases and 91 controls; the derivation of the sub-cohort is summarized in **Figure 1**. All participants provided informed written consent in accordance with protocols approved by the Institutional Review Board at Columbia University Irving Medical Center.

Plasma collection.

Blood samples were collected into BD VacutainerTM Cell Preparation Tubes (CPT) with ethylenediaminetetraacetic acid (EDTA) anticoagulant between January, 2016 and June, 2016, and centrifuged to pellet red blood cells. The plasma was shipped to Columbia University at 4°C. After aliquoting, samples were stored at −80°C until thawed for metabolomics analyses. All the samples were analyzed within two years of collection.

Clinical assessment.

Clinical symptoms and baseline health status were assessed on the day of physical examination and biological sample collection from both case and control subjects using the following instruments: the Short Form 36 Health Survey (SF-36), the Multidimensional Fatigue Inventory (MFI), DePaul Symptom Questionnaire (DSQ)[26], and Pittsburgh Sleep Quality Index (PSQI)[27]. The SF-36 includes the following subject-reported evaluations about current health status: physical and social functioning, physical and emotional limitations, vitality, pain, and general and mental health[28]. The MFI comprises of a 20-item self-reported questionnaire focused on general, physical and mental fatigue, reduced activity, and reduced motivation[29]. Cognitive function was tested based on the DSQ questionnaire data and was scored using a standard cognitive disturbance definition as well as a modified definition based on a subset of questionnaire variables. Sleeping disturbances linked to ME/CFS were tested and scored based on DSQ and PSQI questionnaire items. Each instrument was transformed into a 0–100 scale to facilitate combination and comparison wherein a score of 100 is equivalent to maximum disability or severity and a score of zero is equivalent to no disability or disturbance.

A diagnosis of sr-IBS was based on answers in the medical history form. Subjects were asked if they had received a previous IBS diagnosis by a physician and the date of that diagnosis. Of the 106 subjects with ME/CFS, 35 (33.0%) had sr-IBS. Of the 91 control subjects, 3 (3.3%) had sr-IBS.

Metabolomics analysis.

Samples were stored at −80°C before analysis. Untargeted metabolomics data were acquired using three chromatography/mass spectrometry-based assays (MS): (1) Primary metabolites such as mono- and disaccharides, hydroxyl- and amino acids were measured by gas chromatography/time-of-flight mass spectrometry (GC-TOF MS[30]) including data alignment and compound annotation using the BinBase database algorithm[31]. (2) Biogenic amines including microbial compounds such as trimethylamine N-oxide (TMAO), methylated and acetylated amino acids and short di- and tripeptides were measured by hydrophilic interaction liquid chromatography/quadrupole time-of-flight mass spectrometry (HILIC-QTOF MS). (3) Complex lipids including phosphoglycerolipids, triacylglycerides, sphingolipids, and free fatty acids were analyzed by liquid chromatography (LC)/quadrupole time-of-flight mass spectrometry (CSH-QTOF MS[32]). Targeted bioactive oxylipin assay included thromboxanes, prostaglandins, and hdyroxy-, keto- and epoxy-lipins. All LC-MS/MS data included diverse sets of internal standards. LC-MS data were processed by MS-DIAL vs. 4.0 software[33], and the compounds were annotated based on accurate mass, retention time and MS/MS fragment matching using LipidBlast[34] and Massbank of North America libraries[35]. MS-FLO was used to remove erroneous peaks and reduce the false discovery rate in LC datasets[36]. A total of 821 known metabolites were annotated. Some complex lipids were annotated in both positive (ESI+) and negative (ESI-) ion modes, resulting in a total of 888 metabolic analytes that were included in our analysis. Data were normalized by SERRF[37]. Residual technical errors were assessed by coefficients of variation (CV) for known metabolites.

Statistical analyses.

For each metabolic analyte, zero values reflecting a measurement below the detection limit, were replaced with 50% of its smallest available value. In each of the four metabolomics panels, outliers were identified through principal component analysis (PCA). In primary metabolites (PM), 6 outliers (4 cases and 2 controls) were identified and removed; in complex lipids (CL), there were 5 outliers (3 cases and 2

controls); in oxylipins (OL), there was 1 outlier (1 case); in biogenic amines (BA), 4 outliers (3 cases and 1 control) were eliminated.

To compare the levels of each metabolite between ME/CFS cases and controls, we employed a variety of regression models with the metabolite level as the dependent variable and the binary case/control status as the independent variable, adjusting for all the matching variables (age, sex, race/ethnicity, geographic/clinical site, and season of sampling), body mass index (BMI) and sr-IBS. We considered two options for the dependent variable: 1) original metabolite levels, and 2) natural log-transformed metabolite levels. Before log-transformation, if necessary, all data points in metabolic analytes were multiplied by a minimal factor to keep the feature on a positive domain. Four regression models were considered: Gaussian regression with identity link, Gaussian regression with log link, lognormal regression and Gamma regression with log link. The Bayesian information criterion (BIC) was used to select the best fitting transformation/regression combination. We then calculated the estimated coefficient for the case/control status, together with its 95% confidence interval (95% CI) and p-value. Multiple comparisons over all metabolites were corrected using the Benjamini-Hochberg procedure[38] controlling the false discovery rate (FDR) at the 0.15 level. Additionally, chemical enrichment analyses were performed using ChemRICH[39] to determine chemical classes that were significantly altered between groups. ChemRICH does not rely upon background databases for statistical calculations and provides enrichment analysis based upon chemical structure, as opposed to defined pathways that can be inherently flawed[39].

For each metabolite, we also conducted Bayesian analysis with the best fitting transformation/regression combination using R packages "rstanarm"[40] and "bayestestR"[41]. Default (weakly informative) prior distributions from rstanarm were applied adjusting the scales of the priors internally. We then calculated the Bayes factors (BFs) and 95% highest density credible intervals (HDIs). The BF of a single parameter indicates the degree by which the mass of the posterior distribution has shifted further away from or closer to the null value (zero), relative to the prior distribution[42]. Hence, the BF measures the strength of evidence in favor of the alternative hypothesis ($\beta \neq 0$) over the null hypothesis ($\beta = 0$). The 95% credible interval in the Bayesian framework is the range, within which the effect has 95% probability of falling, given the observed data. It has a different interpretation from the 95% confidence interval in the frequentist framework which instead signifies that with a large number of repeated samples, 95% of such calculated confidence intervals would include the true value of the parameter. We considered a metabolite significantly associated with ME/CFS if it satisfied the following criteria: 1) FDR adjusted p-value < 0.15, 2) BF > 3, and 3) 95% HDIs not covering 0. Jeffreys (1961)[43] suggested that the strength of evidence for the alternative hypothesis compared to the null hypothesis is regarded as noteworthy if BFs are above 3.

Naviaux et al. (2016)[15] showed that potential diagnostic metabolites for ME/CFS in targeted metabolomics are different between male and female subjects. Accordingly, we conducted sex-stratified analyses in addition to analyses with the whole cohort. In our previous work with a different cohort, sr-IBS comorbidity was identified as the strongest driving factor in the separation of topological networks based on fecal microbiome and plasma metabolic pathways[14, 23]. We subsequently found different patterns in the relationships between plasma proteomic profiling and ME/CFS when comparing ME/CFS with or without sr-IBS to healthy controls[19]. Given this precedent, we tested the hypothesis that sr-IBS subgroups in ME/CFS patients have altered metabolic profiles in a stratified analysis. As there were only 3 control subjects with sr-IBS, we focused on the comparison of ME/CFS subjects without sr-IBS versus controls without sr-IBS.

To explore the utility of the metabolomics assay as a biomarker tool for ME/CFS, we employed four machine learning algorithms: least absolute shrinkage and selection operator (Lasso)[44], adaptive Lasso (AdaLasso)[45], Random Forests (RF)[46] and XGBoost[47]. AdaLasso is different from Lasso in that AdaLasso has the oracle property that leads to consistent variable selection whereas Lasso is only consistent for variable selection under certain conditions on the shrinkage parameters and correlations[48]. However, neither outperforms the other consistently in predictions. For each of the algorithm, three sets of predictors were considered: 1) all metabolites, 2) metabolites with BF>1, and 3) metabolites with BF>3. The predictive models were first trained in the 80% randomly-selected training set using 10-fold cross-validation; the remaining 20% of the study population was used as the independent test set to validate model performance. We also applied the Bayesian Model Averaging (BMA) method[49] that combines the predictions of multiple models using weighted averages in which the weights are Bayesian posterior probabilities that the given model is the true model, conditional on the training data. The predictive performance of the 5 models (Lasso, AdaLasso, RF, XGBoost and Model Average) using the three sets of predictors in the test set was evaluated using Area under the Receiver Operating Characteristic curve (AUROC) values and Receiver Operating Characteristic (ROC) curves.

Data analyses were performed using MATLAB Statistics Toolbox R2013a (MathWorks, Inc., Natick, MA) and R version 3.6.3 (RStudio, Inc., Boston, MA). All p-values were 2-tailed.

Results

Study population characteristics.

The study included plasma samples from 106 ME/CFS cases and 91 healthy controls recruited from five sites across the United States. Demographic and clinical characteristics of the study population are shown in **Table 1**. ME/CFS cases and controls were similar for all the frequency matching variables except season of collection (*Chi-squared p* = 0.004). We adjusted for all the matching variables, BMI and sr-IBS in our statistical analyses to account for confounding. All scales in SF-36 and MFI were significantly different between the two cohorts (*Wilcoxon rank-sum p* < 0.001). The study population is similar to the prescreened cohort that consisted of 177 ME/CFS cases and 177 controls in sex (*Chi-squared p*=0.60), race (*Chi-squared p*=0.66) and age (*Wilcoxon rank-sum p*=0.65).

Metabolomic dataset.

Targeted and untargeted mass spectrometry platforms yielded data for 888 metabolic analytes comprising 100 primary metabolites (PM), 237 biogenic amines (BA), 480 complex lipids (CL), and 71 bioactive oxylipins (OL). **Supplementary Table S1** shows the sample mean and the standard deviation (SD) of levels of each metabolite within all ME/CFS cases, all controls, female ME/CFS cases, female controls, male ME/CFS cases, male controls, ME/CFS cases without sr-IBS and controls without sr-IBS.

ME/CFS is associated with altered metabolomic profile.

In PM, BA and CL panels, lognormal regression models with log-transformed metabolite levels as dependent variables had the lowest BIC values and best fit the data; the estimated coefficients can be interpreted as the differences in the mean values of log-log transformation of metabolite levels between cases and controls. In OL panel, a mixture of lognormal and log-link Gamma regression models with original metabolite levels as dependent variables best fit the data. For lognormal regression models, the

estimated coefficients are interpreted as the mean differences of log transformation of metabolite levels between two groups. For log-link Gamma regression models, the estimated coefficients are interpreted as the log of fold change between two groups.

We did not identify any metabolite as significantly associated with ME/CFS in the PM panel. In the BA panel, levels of acetaminophen were increased in ME/CFS cases compared to controls. In the CL panel, we found decreased levels of plasmalogens, unsaturated phospholipid ethers, unsaturated phosphatidylcholines (PC), an unsaturated sphingomyelin (SM), and an unsaturated lysophosphatidylcholines (LPC) in ME/CFS cases compared to controls. In the OL panel, decreased levels of Resolvin D1 were observed in ME/CFS cases compared to controls. **Table 2** shows the estimated coefficients in the regression models of these metabolites, their associated 95% CIs, p-values, FDR adjusted p-values and BFs. Because we used weakly informative priors in Bayesian analysis, the 95% HDIs were extremely similar to the 95% CIs. We report estimations of HDIs in **Supplementary Table S2** where estimations for all metabolites are shown.

Set enrichment analysis of the results from the regression models (**Figure 2A**) revealed that ME/CFS subjects had reduced levels of plasmalogens, sphingomyelins, unsaturated phospholipid ethers, unsaturated ceramides, carnitines, saturated lysophospholipids, unsaturated lysophosphoethanolamines, unsaturated lysophosphatidylcholines, saturated triglycerides and prostaglandins. The majority of unsaturated phosphatidylcholines were also down-regulated in ME/CFS cases. Increased levels of hydroxy-eicosapentaenoic acid (HEPE), dicarboxylic acids, and the majority of unsaturated long chain triglycerides were found in ME/CFS cases compared to controls. There were mixed directional alterations in the food exposome and epoxy fatty acids (EpODE). Complete data from ChemRICH enrichment analysis are provided in **Supplementary Table S3**. Data from compound-level enrichment analysis for the significantly altered metabolic clusters are illustrated in **Supplementary Table S4**. Levels of choline in food exposome were reduced in ME/CFS (estimated coefficient β=-0.009, p=0.004); levels of succinic acid (β=0.022, p=0.007) and alpha-ketoglutarate (β=0.016, p=0.048) in dicarboxylic acids were elevated in ME/CFS.

Altered metabolomic profiles in female and male ME/CFS patients.

Naviaux et al. (2016)[15] discovered that women with ME/CFS, but not men, had disturbed fatty acid and endocannabinoid metabolism. Accordingly, we repeated separately the analyses in female and male cohorts in our study population.

In female subjects, regression and Bayesian analyses (**Table 2**) revealed that levels of phosphatidylcholines (PC), phosphatidylethanolamines (PE) and sphingomyelins (SM) in the CL panel were decreased in ME/CFS patients compared to controls. In the BA panel, levels of two drug metabolites, alprazolam and acyclovir, were up-regulated in ME/CFS patients. We did not find the elevated levels of acetaminophen in female subjects that were observed in the entire ME/CFS (male and female population), presumably due to loss of power. Enrichment analysis in female subjects (**Figure 2B**) identified dysregulations in the same metabolic clusters as in the overall population. Complete data from enrichment analysis in female subjects are shown in **Supplementary Table S6**. In contrast, we did not find any metabolites significantly associated with risk of ME/CFS in male subjects. This may be due to limited sample size. **Supplementary Table S5** shows the regression and Bayesian estimations for all metabolites in male and female cohorts.

Altered metabolomics profile in ME/CFS patients without sr-IBS.

Due to the limited sample size of subjects with sr-IBS (35 ME/CFS cases and 3 controls), we only compared levels of metabolites between ME/CFS cases without sr-IBS and controls without sr-IBS. Levels of phosphatidylcholines (PC) and phosphatidylethanolmines (PE) were decreased in ME/CFS patients in this subgroup (**Table 2**). In the ChemRICH enrichment analysis, the dysregulations in metabolite clusters found to be dysregulated in the subgroup without sr-IBS (**Figure 2C**) were all identified in the overall population (**Figure 2A).** Complete data pertaining to the regression, Bayesian and enrichment analyses are shown in **Supplementary Tables S7** and **S8**.

Assessment of the metabolomics assay as a potential diagnostic tool for ME/CFS.

We considered three sets of metabolites as predictors to distinguish ME/CFS cases from controls, including all metabolites, metabolites with BF>1 and metabolites with BF>3. Each set of predictors was fitted in five different machine learning classifiers: Lasso, adaptive Lasso (AdaLasso), Random Forests (RF), XGBoost and Model Average. The classifiers were first trained in the 80% randomly-selected training set and then validated in the remaining 20% test set. **Figure 3A**, **Figure 3B**, and **Figure 3C** show the ROC curves and the AUROC values differentiating all ME/CFS cases from all controls, female ME/CFS from female controls, and ME/CFS without sr-IBS from controls without sr-IBS, respectively, in the test set. Although classifiers did not differentiate all ME/CFS from all controls, Lasso with BF>1 metabolites as predictors distinguished female ME/CFS patients from female controls with an AUROC value of 0.794 (95% CI: 0.612-0.976) and Lasso with BF>3 metabolites distinguished ME/CFS without sr-IBS from controls without sr-IBS with an AUROC value of 0.873 (95% CI: 0.747-0.999). The AUROC values and their associated 95% CIs of all the classifiers are shown in **Supplementary Table S9**.

Metabolites significantly associated with ME/CFS or ME/CFS subgroups do not strongly correlate with duration of illness or symptom severity scores.

We investigated whether the plasma levels of metabolites, defined by their associations with ME/CFS and ME/CFS subgroups (**Table 2**), correlated with duration of illness in years, Short Form 36 Health Survey (SF-36) scales and Multidimensional Fatigue Inventory (MFI) scales using Spearman's correlation tests. None of the correlation coefficients exceeded the absolute magnitude of 0.5 (data not shown).

Discussion

Since the first reports of large-scale metabolomic studies in people with ME/CFS were published in 2016 by Naviaux[15], Yamano[16], and Fluge[9], several research teams, including our own[14] have reported metabolomic analyses of plasma. The majority describe abnormalities in energy metabolism, with most reporting decreased levels of phospholipids and suggesting abnormalities in mitochondrial activity that could contribute to fatigue and cognitive dysfunction. Here we report confirmation of decreased levels of phospholipids, including phosphatidylcholine and sphingolipids, and provide evidence for dysregulation of the Kennedy pathway and the tricarboxylic acid cycle. Our findings provide potential insights into the pathobiology of clinical features of ME/CFS by providing a mechanistic framework for understanding compromised energy production, loss of integrity of cellular and mitochondrial membranes, inflammation, impaired cognition, dysregulated autonomic function, impediments to repair of tissue injury, and redox imbalance.

The Kennedy pathway is responsible for the biosynthesis of phosphatidylcholines (PC) and phosphatidylethanolamines (PE), the two most abundant phospholipids in mammalian cells[50]. PC, the most abundant phospholipid in the mitochondrial membranes[51, 52], is sourced from endoplasmic reticulum. PE is synthesized in mitochondria by the decarboxylation of phosphatidylserine by phosphatidylserine decarboxylase 1 (Psd1) at the inner mitochondrial membrane[53]. PC and PE are essential to the formation of intermediate structures in membrane fusion and fission events, for stabilizing membrane proteins into their optimal conformations, and for actin-filament disassembly in the end stage of cytokinesis[54, 55, 56]. In people with ME/CFS, we found decreased levels of PC and PE and their downstream products: ceramides, sphingomyelins, lysophosphatidycholines, phospholipid ethers, prostaglandin D2 (PGD2) and prostaglandin F2α (PGF2α).

One critical functional implication of reduced levels of PC and PE is impaired oxidative phosphorylation. PC depletion specifically affects the function of inner membrane protein translocases of mitochondria, including the TIM23 complex[57]. PE synthesis is critical for cytochrome bc1 complex III function in the mitochondrial inner membrane[53]. Preprotein binding to the TIM/TOM complex, which translocates proteins produced from nuclear DNA through the mitochondrial membrane for use in oxidative phosphorylation, is disturbed in PE-deficient mitochondria[58, 59]. Cytochrome c oxidase activity in the respiratory chain complex is also decreased with PE-deficiency[60, 61]. Reduced import of PE into the mitochondria results in the formation of respiration deficient cells[55], and in mitochondrial dysfunction. Finally, reduced levels of lysophosphatidycholines and phospholipid ethers, as well as of PC and PE, can impede mitochondrial respiration[55]. Reduced synthesis of PGF2α and PGD2 in phospholipase A2γ-deficient mice induces mitochondrial dysfunction as well as oxidative stress that can contribute to further mitochondrial damage[62].

Because PE and PC, and downstream metabolites in the Kennedy pathway, are important components of the lipid bilayer, the reduction in their levels has implications for signaling. Alteration in the levels or conformation of membrane components can adversely affect the functionality of proteins embedded in the membranes such as G protein coupled receptors (GPCRs). Phospholipids can act as direct allosteric modulators of GPCR activity through the lipid head group that affect ligand binding (agonist and antagonist) and receptor activation. For example, PE favors antagonist binding and stabilizes the inactive state of the receptor, whereas phosphatidylglycerols favor agonist binding and activation[63].

Both PE and PC are precursors to many biologically active molecules that can act as second messengers. Prominent among them are Di-acyl glycerol (DAG), fatty acids, phosphatidic acid, lysophosphatidic acid, N-arachidonylethanolamine, N-palmitoylethanolamine, N-steroylethanolamine and arachidonic acid[64, 65, 66]. Ceramides, are not only structural components of membranes, but can also act as second messengers in modulating a range of cellular signaling pathways[67].

Metabolomic analyses also revealed reductions in levels of plasmalogens and of resolvin D1. Plasmalogens are compounds with antioxidant functions that are synthesized by peroxisomes. Resolvin D1, a derivative of docosahexanoic acid (DHA), may contribute to resolution of inflammation by targeting dead cells for clearance by macrophages.[68] As previously reported, [14, 69] we found a significant reduction in levels of carnitine. Carnitine regulates the cellular to mitochondrial ratio of free CoA to Acyl-CoA, removes the unwanted acyl groups and plays a key role in the transport of long-chain fatty acids from cytoplasm to the mitochondrial matrix for oxidation[70]. Depletion of carnitine reduces the generation of ATP from fatty acids, and may adversely impact the integrity of cell and mitochondrial membranes, and responses to inflammation and oxidative stress[70]. Finally, reduced levels of carnitine threaten the integrity of cell and mitochondrial membranes, increase oxidative stress, and reduce the

ability to counter inflammation[71]. We also observed increased levels of long-chain triglycerides in ME/CFS. Depletion of carnitine leads to the accumulation of long-chain triglycerides that become targets for lipid peroxidation by mitochondria[72]. The accumulation of toxic lipid peroxidation products can lead to mitochondrial membrane damage[18].

The tricarboxylic acid (TCA) cycle is a conserved pathway in aerobic organisms through which the acetyl-CoA from carbohydrates, fats and proteins is converted into ATP. We observed elevated levels of two TCA cycle intermediates, the dicarboxylic acids α-ketoglutarate (α-KG) and succinate in ME/CFS. Increased levels of α-KG have been reported previously in ME/CFS patients[11], although we are not aware of previous reports of elevated levels of succinate. Abnormal levels of TCA cycle intermediates suggest inefficiencies in ATP production that may contribute to the fatigue and post-exertional malaise reported in ME/CFS. Increases in α-KG levels have been reported to induce severe metabolic impairment of pyruvate oxidation in the tricarboxylic acid cycle, leading to cell death[73]. Succinate accumulation has been reported to induce HIF-1α stabilization as well as the transcriptional activation of the pro-inflammatory cytokine IL-1β[73]. Elevated succinate levels contribute to increased oxidative stress and neuronal degeneration in rat models[74]. Oxidative stress, in turn, augments nitrosative stress[75]. Nitrosative stress, which has been documented in people with ME/CFS[76], can lead to the increased production of peroxynitrite and downregulate the function of both alpha-ketoglutarate dehydrogenase and succinate dehydrogenase[75, 76, 77]. Infection is a common cause of nitrosative stress, and many ME/CFS patients report symptoms consistent with system infection prior to diagnosis.

Choline depletion was another potentially important finding in our study. Choline is an essential nutrient; 95% of it is utilized in the synthesis of PC via the Kennedy pathway[50]. The remaining 5% exists as either free choline or is used in the synthesis of phosphocholine, glycerophosphocholine, CDP-choline, acetylcholine, and other choline-containing phospholipids like sphingomyelin, plasmalogens and lysophosphatidylcholine. Each of these compounds contributes to maintenance of the structure and signaling functionality of the plasma membrane[50, 66]. IgG autoantibodies that specifically target GPCRs have been reported, even in healthy individuals, but are more commonly found in ME/CFS[78, 79], particularly to autonomic nervous system targets including the M3 Acetylcholine receptor (M3AChR) and β2 Adrenergic receptor (β2AdR). Agonists for each of these receptors have choline precursors, acetylcholine (AC) and epinephrine (adrenaline), respectively. Choline also plays a role in the production of epinephrine, by donating the methyl group. Thus, choline deficiency could potentially lead to the autonomic dysfunction that is found in many people with ME/CFS, with reduced tissue blood flow and oxygen supply, leading to hypoxia, ischemia and fatigue[80].

Impairments in cognition have been reported in ME/CFS[81, 82]. Our metabolomic data revealed reductions in levels of sphingomyelin, ceramides, and plasmalogens that may contribute to central nervous system dysfunction. Reduced levels of sphingomyelins have been reported in neurological disorders such as Alzheimer's disease (AD)[83, 84], Parkinson's disease (PD)[85] and multiple sclerosis[86]. In these examples of neurodegeneration, levels of sphingomyelin were reduced, but ceramide was increased[87]. In contrast, our data show reduced levels of both sphingomyelin and ceramide. Reduced levels of ceramide are associated with decreased cell survival, aberrant Purkinje cell dendritic differentiation[88], and neurons having shorter axon plexus and fewer axonal branches[89]. Reductions in mitochondrial ceramide levels have also been shown to result in neuronal degeneration and reduced mitochondrial respiratory function as manifest with decreased mitochondrial basal and maximal oxygen consumption rates and decreases in spare respiratory capacity[89]. Reduced plasmalogen levels have been observed AD and PD[90, 91, 92]. Plasmalogen deficiencies may increase vulnerability of neural membranes to oxidative stress,

destabilize membranes, and impair muscarinic cholinergic signaling and abnormal amyloid precursor processing[91, 93, 94, 95].

Conclusion

Our findings indicate a series of interconnected metabolic alterations in people with ME/CFS, that are consistent with two central abnormalities that may contribute to the pathogenesis of ME/CFS: disturbances in the Kennedy Pathway leading to reductions in levels of PC and PE and structural and functional disturbances of cellular and mitochondrial membranes, and reductions in levels of α-ketoglutarate and succinate that are consistent with an impairment in the TCA cycle.

Acknowledgements

This study was funded by National Institutes of Health U54 AI138370 (Center for Solutions for ME/CFS). We are grateful to Kelly Magnus of the Center for Infection and Immunity at Columbia University for assistance with manuscript preparation, to Kelly Paglia of the West Coast Metabolomics Center at University of California, Davis for her support and coordination, to the Chronic Fatigue Initiative of the Hutchins Family Foundation and the ME/CFS patients who provided the samples and inspiration that enabled our work. We dedicate this paper to the memory of Bohyun Lee and her contributions to research in ME/CFS.

Ethics Statement

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Author contributions: X.C., B.L., D.K.B., O.F. and W.I.L. developed the experimental design; S.L., D.L.P., S.D.V., L.B. and J.G.M. contributed to case and control recruitment and characterization; X.C., C.R.B., Y.Y., B.L., A.C. and D.M.P. contributed to biostatistical analysis. X.C., C.R.B., B.L., D.K.B. and A.C. contributed to bioinformatics analysis; X.C., A.R.P., S.J., A.R., A.L.K. and W.I.L contributed to the data interpretation and functional analysis; X.C., C.R.B., A.R.P., Y.Y., S.J., A.R., B.L., D.K.B., A.C., D.M.P., S.L., D.L.P., S.D.V., L.B., M.H., J.G.M., A.L.K., O.F. and W.I.L. contributed to the writing and the review of manuscript before submission for publication. All authors read and approved the final manuscript.

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Figure 1. Pipeline for sample selection.

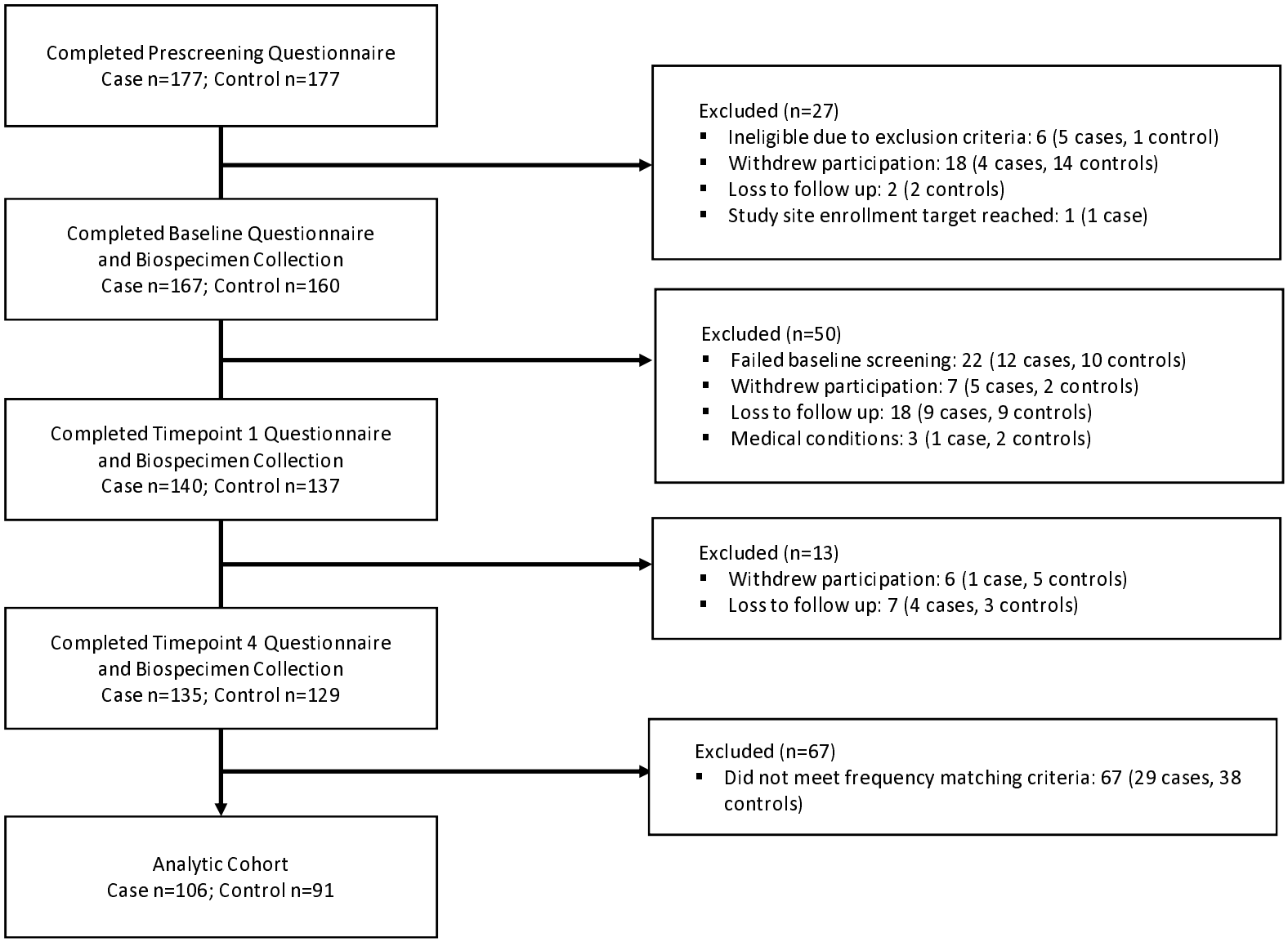
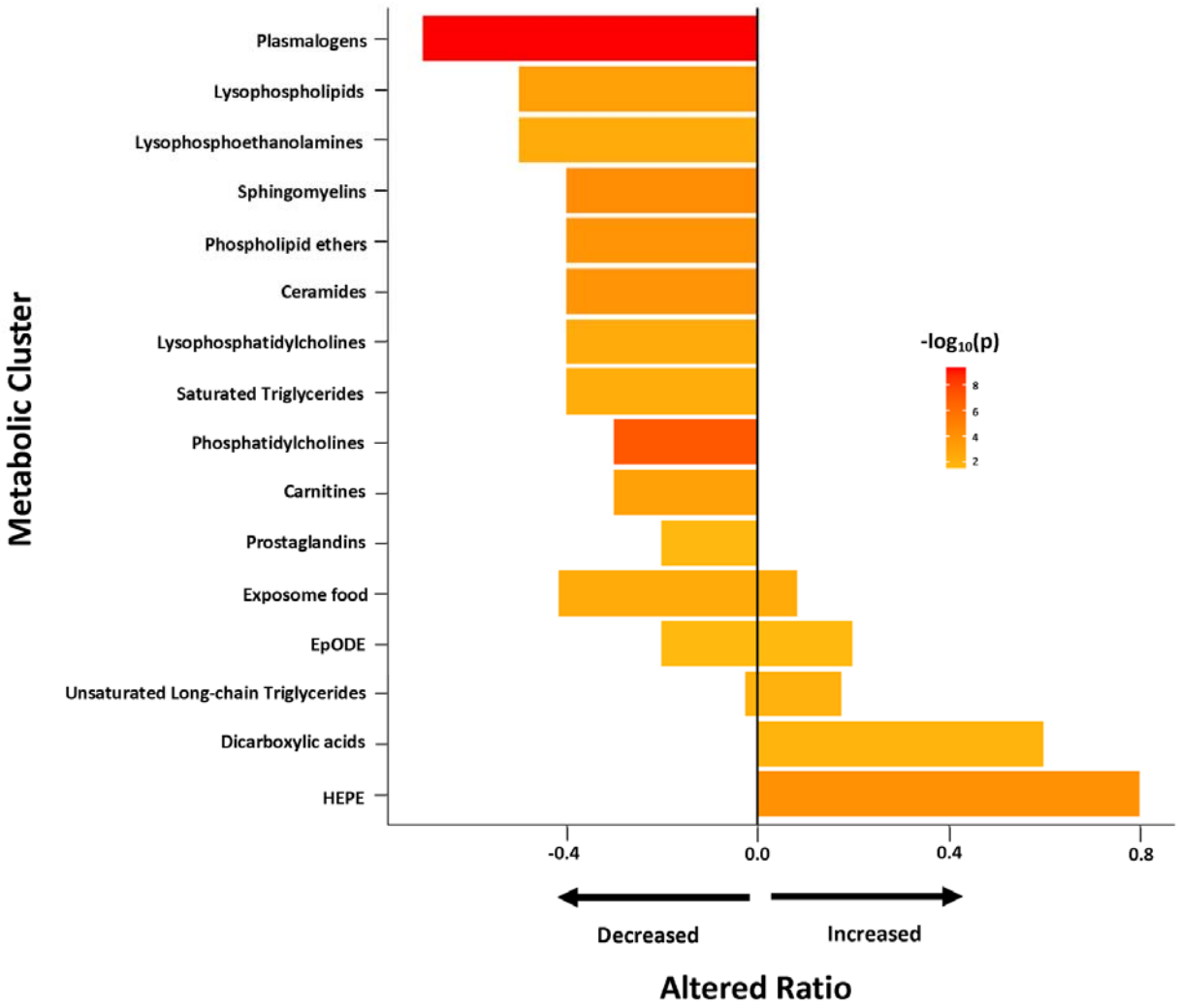


Figure 2. Chemical enrichment analyses using ChemRICH

a



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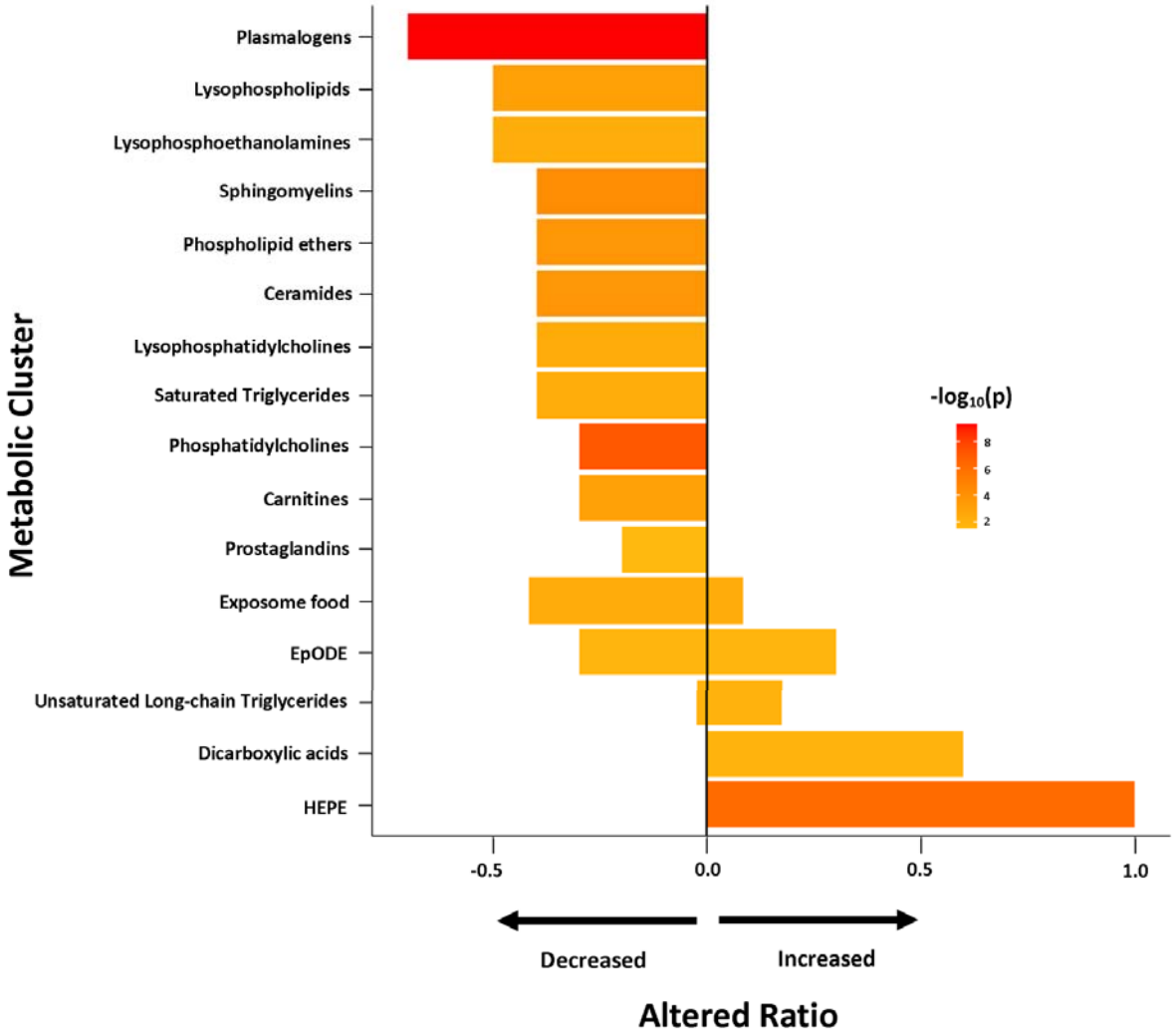

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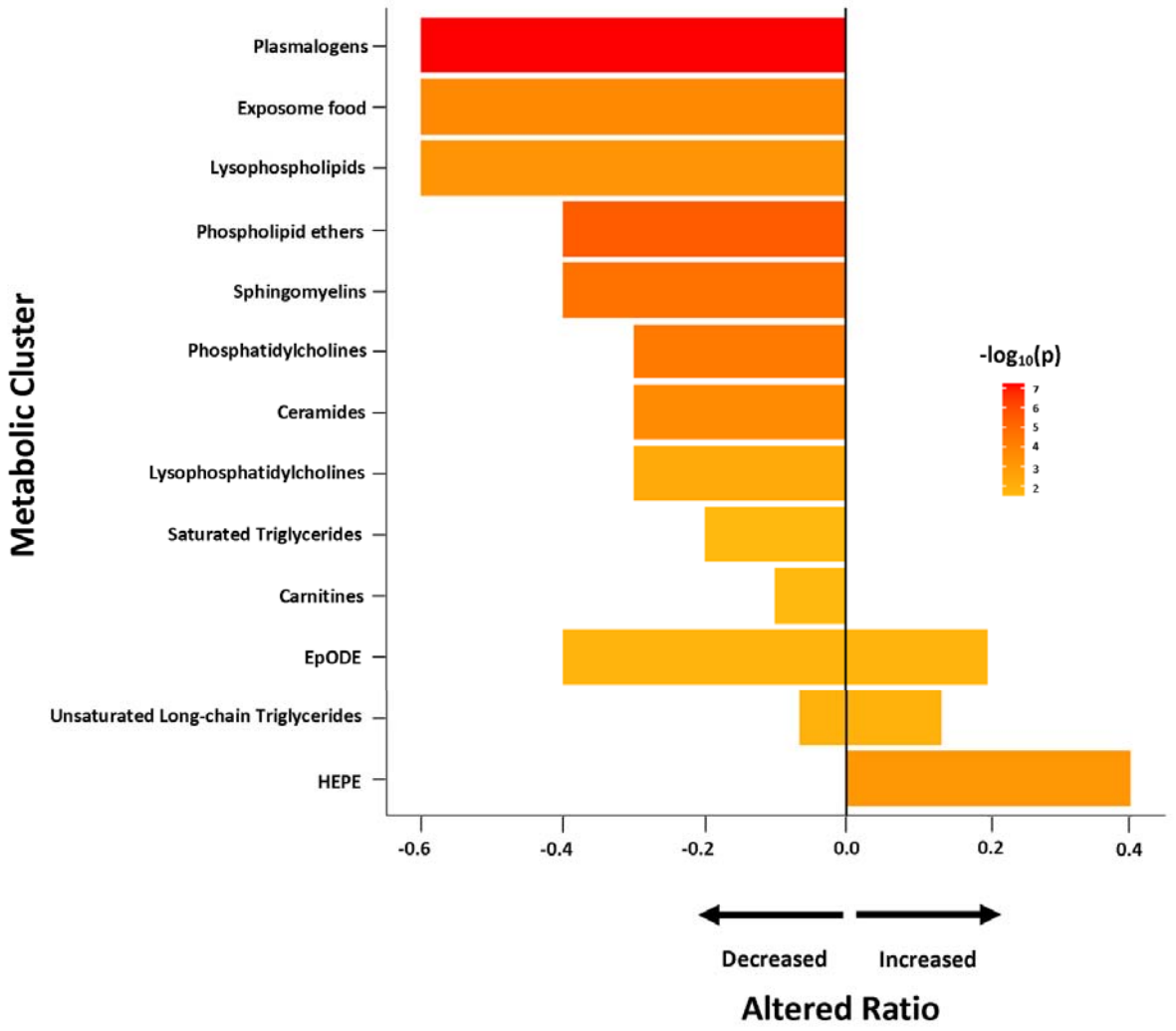
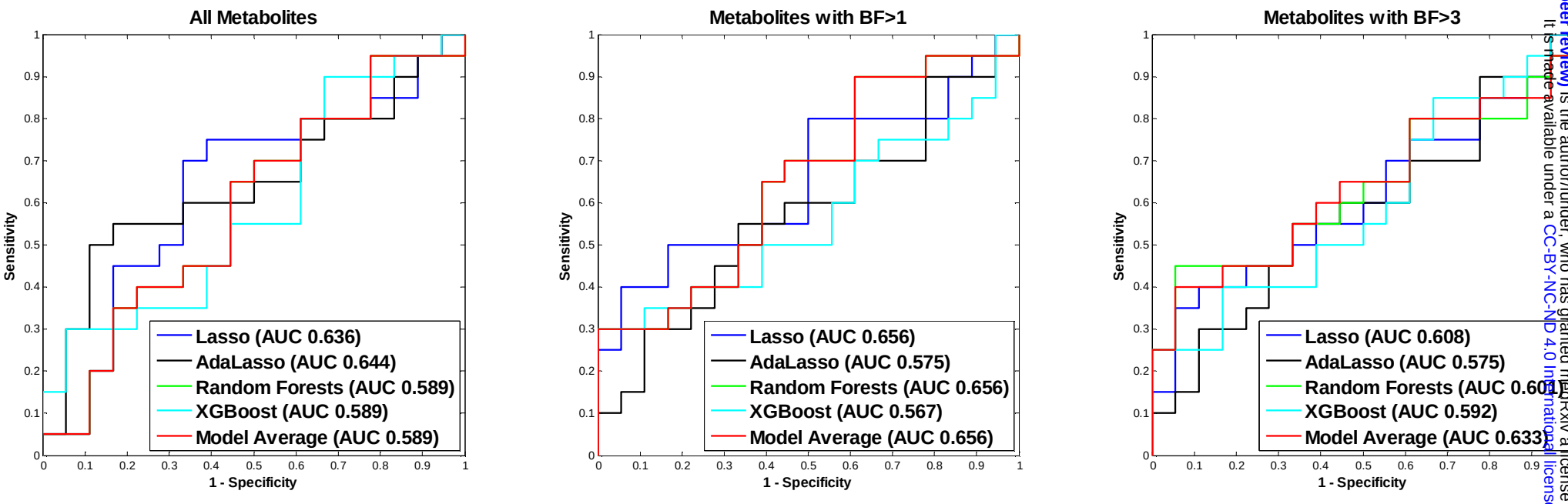


a All ME/CFS v. controls. **b** Female ME/CFS v. female controls. **c** ME/CFS without sr-IBS v. controls without sr-IBS. The length of the bar represents altered ratio for each metabolic cluster. A bar restricted to the left of the centered vertical line indicates a metabolic cluster that is lower in ME/CFS patients. A bar restricted to the right of the centered vertical line indicates a metabolic cluster that is higher in ME/CFS patients. A bar that crosses the vertical line indicates a metabolic cluster that is dysregulated in mixed directions. The color represents significance. EpODE: epoxy octadecadienoic acid. HEPE: hydroxy eicosapentaenoic acid. ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome. sr-IBS: self-reported physician diagnosed irritable bowl syndrome.

Figure 3. ME/CFS predictive modeling.

a



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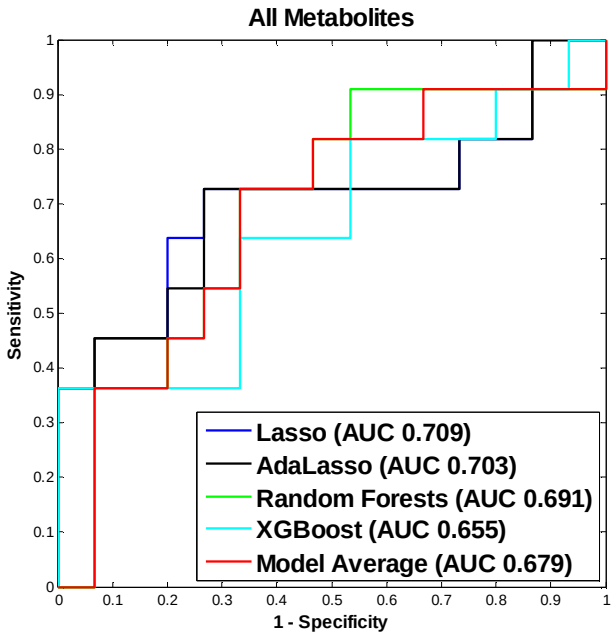

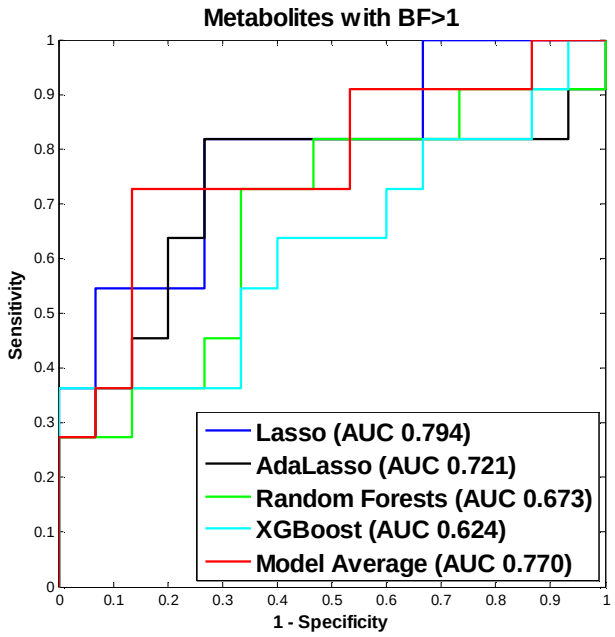

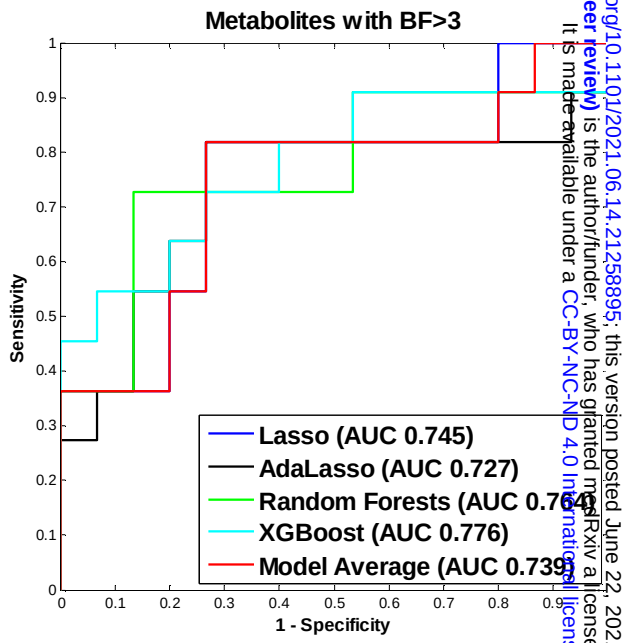

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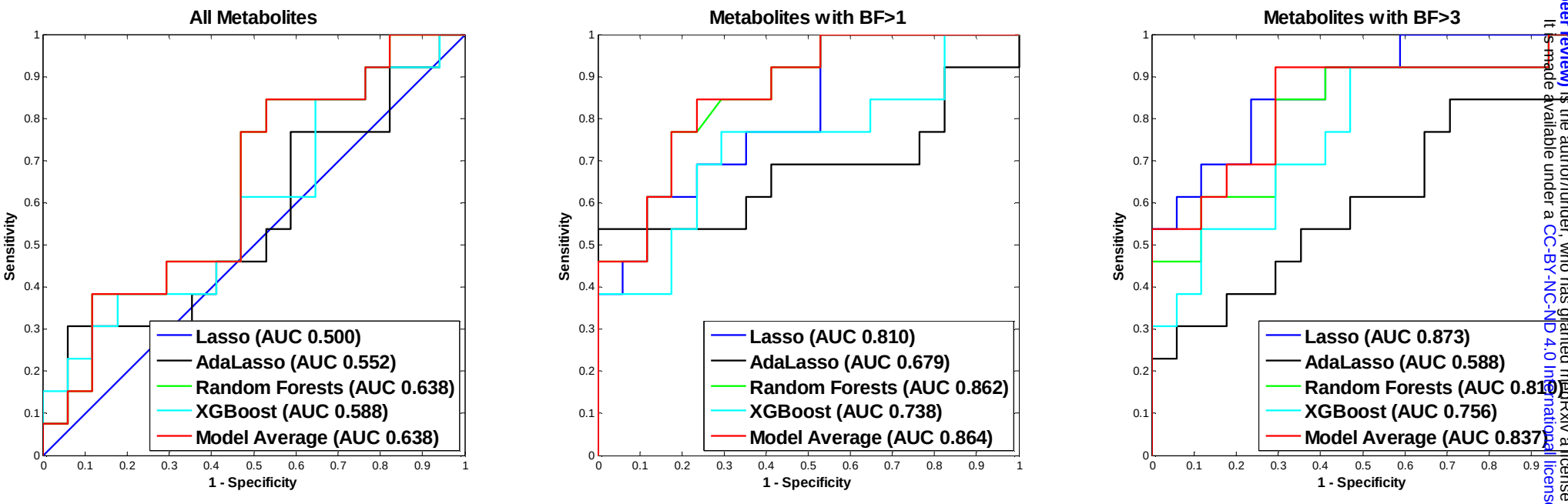
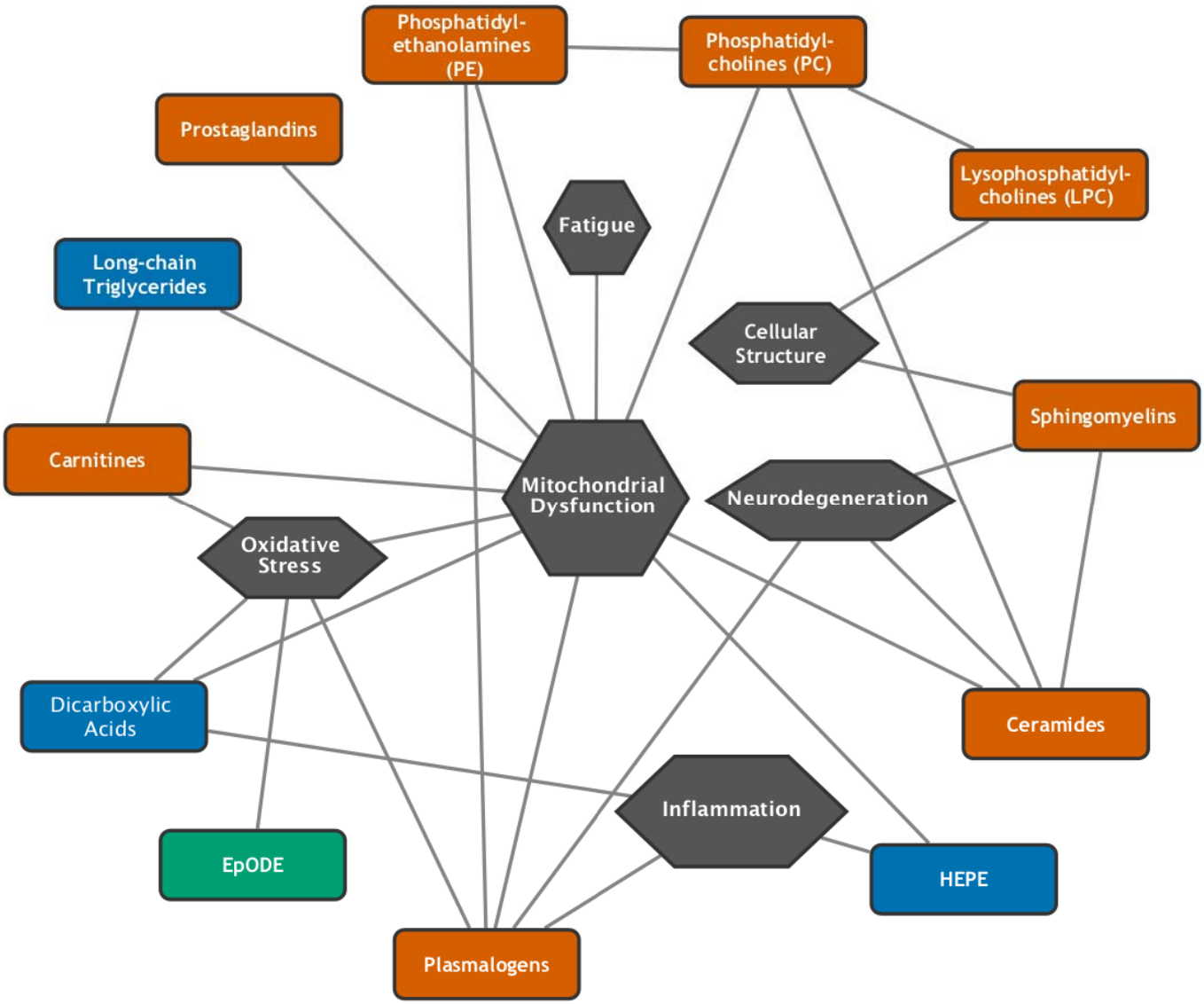


a Overall population. b When only ONS participants to differentiate ME/CFS cases from healthy controls, we employed five machine learning algorithms, adaptive lasso (AdaLasso), Random Forests (RF), XGBoost, and Bayesian Model Averaging (Model average). For each algorithm, three sets of predictors were considered: 1) all metabolites, 2) metabolites with BF factor >1, and 3) metabolites with BF factor >3. The predictive models were first trained in the 80% randomly selected training set using 10-fold cross-validation, and the remaining 20% of the study population was used as the independent test set to validate model performance. ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome. SR: self-reported physician diagnosed irritable bowel syndrome. BF: Bayes factor. AUC: area under the receiver operating characteristic curve.

Figure 4. Functional interaction network of altered metabolic clusters in ME/CFS



Metabolite levels that are decreased (orange), increased (blue), or mixed in direction (green) in the enrichment assay have been associated with oxidative stress, mitochondrial dysfunction, and neurodegeneration.

Table 1. Subject characteristics.

Subject Characteristics		ME/CFS (n=106)	Controls (n=91)	p-value[5]
Sex	Female	75	69	0.42
	Male	31	22	
Age	Mean ± SD	47.8 ± 13.7	47.0 ± 14.1	0.78
	Median (Range)	51.0 (21.6-70.0)	50.6 (21.2-68.2)	
Race and Ethnicity	White and not Hispanic	93	85	0.40
	Hispanic	6	3	
	Not White and not Hispanic	7	3	
Site of Collection	Incline Village, NV	23	17	0.30
	Miami, FL	15	5	
	New York, NY	17	19	
	Salt Lake City, UT	32	32	
	Palo Alto, CA	19	19	
Season of Collection	Winter	33	14	0.004
	Spring	72	70	
	Summer	1	7	
sr-IBS Comorbidity[1]	Yes	35	3	<0.001
	No	71	88	
BMI	Mean ± SD	26.1 ± 5.2	25.2 ± 4.7	0.31
	Median (Range)	25.0 (18.1-41.2)	25.1 (16.9-38.7)	
Duration of ME/CFS[2]	Mean ± SD	15.0 ± 9.8	n/a	n/a
	Median (Range)	14.4 (1.2-44.2)	n/a	
	≥ 3 years	92	n/a	
	< 3 years	8	n/a	
SF-36 Scales[3] Mean Score ± SD	Emotional Limitations	62.3 ± 43.7	94.9 ± 18.5	<0.001
	Emotional Well-being	69.6 ± 18.1	84.5 ± 10.5	<0.001
	General Health	24.9 ± 16.6	85.0 ± 12.3	<0.001
	Pain	45.5 ± 26.9	89.9 ± 12.7	<0.001
	Physical Functioning	39.9 ± 22.9	94.8 ± 11.9	<0.001
	Physical Limitations	3.3 ± 12.9	94.2 ± 18.7	<0.001
	Social Functioning	30.1 ± 25.6	93.5 ± 13.4	<0.001
	Vitality	14.3 ± 18.1	75.5 ± 13.9	<0.001
MFI Scales[4] Mean Score ± SD	General Fatigue	83.3 ± 20.3	22.4 ± 19.5	<0.001
	Mental Fatigue	60.3 ± 23.6	19.6 ± 21.2	<0.001
	Physical Fatigue	80.7 ± 20.4	17.3 ± 17.7	<0.001
	Reduced Activity	74.9 ± 22.7	16.7 ± 19.8	<0.001
	Reduced Motivation	48.4 ± 26.3	20.7 ± 23.5	<0.001

[1]Prior physician diagnosed irritable bowel syndrome, self-reported on the questionnaire. [2]Only 90 responses were received for this item. [3]36-Item Short Form Health Survey; scored on 0-100 scale with 0 = poor health status and 100 = excellent health status. [4]Multidimensional Fatigue Inventory; scored on 0-100 scale with 0 = no fatigue and 100 = greater fatigue. [5]For categorial variable, p-values were derived from Chi-squared tests; for continuous variables, p-values were derived from Wilcoxon rank-sum tests. SD: standard deviation. ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome

Table 2. Metabolites significantly associated with ME/CFS or ME/CFS subgroups.

Metabolite	Enrichment Cluster	Regression Model	ME/CFS vs. Control				
			Estimated Coefficient	95% CI	p-value	FDR	BayesFactor
Biogenic Amines (BA)							
Acetominophen	drugs	Lognormal	**0.068**	**(0.028, 0.108)**	**0.001**	**0.103**	**3.035**
Alprazolam	drugs	Lognormal	0.063	(0.016, 0.109)	0.009	0.178	0.963
Acyclovir	drugs	Lognormal	0.112	(0.038, 0.185)	0.003	0.139	2.674
Complex Lipids (CL)							
PE (p-36:2)/PE (o-36:3) - ESI(+)	plasmalogens	Lognormal	**-0.028**	**(-0.043, -0.013)**	**0.000**	**0.074**	**20.935**
PE (p-34:2)/PE (o-34:3)	plasmalogens	Lognormal	**-0.037**	**(-0.060, -0.014)**	**0.002**	**0.126**	**5.662**
SM (d40:3)	sphingomyelins	Lognormal	-0.025	(-0.043, -0.007)	0.007	0.173	1.222
LPC (18:2) - ESI(-)	unsaturated lysophosphatidylcholines	Lognormal	**-0.019**	**(-0.032, -0.007)**	**0.003**	**0.139**	**4.102**
PC (36:2)	unsaturated phosphatidylcholines	Lognormal	**-0.007**	**(-0.011, -0.003)**	**0.000**	**0.074**	**11.241**
PC (36:4) A - ESI(+)	unsaturated phosphatidylcholines	Lognormal	**-0.018**	**(-0.028, -0.008)**	**0.000**	**0.074**	**8.134**
PC (36:4) A - ESI(-)	unsaturated phosphatidylcholines	Lognormal	**-0.019**	**(-0.031, -0.008)**	**0.001**	**0.103**	**4.032**
PC (32:2) - ESI(-)	unsaturated phosphatidylcholines	Lognormal	**-0.027**	**(-0.043, -0.010)**	**0.002**	**0.135**	**7.389**
PC 34:4e	unsaturated phosphatidylcholines	Lognormal	**-0.022**	**(-0.036, -0.008)**	**0.003**	**0.139**	**4.327**
PC (34:2) - ESI(+)	unsaturated phosphatidylcholines	Lognormal	-0.005	(-0.008, -0.002)	0.003	0.139	2.039
PC (p-34:2)/PC (o-34:3) - ESI(+)	unsaturated phospholipid ethers	Lognormal	**-0.018**	**(-0.027, -0.009)**	**0.000**	**0.062**	**44.620**
PC (p-34:1)/PC (o-34:2)	unsaturated phospholipid ethers	Lognormal	**-0.021**	**(-0.032, -0.010)**	**0.000**	**0.062**	**178.678**
PC (p-36:1)/PC (o-36:2)	unsaturated phospholipid ethers	Lognormal	**-0.055**	**(-0.086, -0.024)**	**0.001**	**0.074**	**11.555**
PC (p-34:2)/PC (o-34:3) - ESI(-)	unsaturated phospholipid ethers	Lognormal	**-0.020**	**(-0.032, -0.009)**	**0.001**	**0.074**	**12.281**
PC (p-36:4)/PC (o-36:5) - ESI(-)	unsaturated phospholipid ethers	Lognormal	**-0.021**	**(-0.034, -0.009)**	**0.001**	**0.103**	**7.046**
PC (p-34:1)/PC (o-34:2) A	unsaturated phospholipid ethers	Lognormal	**-0.027**	**(-0.044, -0.011)**	**0.002**	**0.125**	**5.655**
Oxylipins (OL)							
Resolvin D1	OH-FA_22_6_1	Gamma	**-0.528**	**(-0.846, -0.210)**	**0.002**	**0.134**	**6.635**

Metabolite	Enrichment Cluster	Regression Model	Female ME/CFS vs. Female Control				
			Estimated Coefficient	95% CI	p-value	FDR	BayesFactor
Biogenic Amines (BA)							
Acetominophen	drugs	Lognormal	0.064	(0.016, 0.113)	0.010	0.211	1.172
Alprazolam	drugs	Lognormal	**0.081**	**(0.030, 0.132)**	**0.002**	**0.121**	**3.486**
Acyclovir	drugs	Lognormal	**0.152**	**(0.057, 0.247)**	**0.002**	**0.121**	**3.179**
Complex Lipids (CL)							
PE (p-36:2)/PE (o-36:3) - ESI(+)	plasmalogens	Lognormal	**-0.033**	**(-0.049, -0.017)**	**0.000**	**0.048**	**24.602**
PE (p-34:2)/PE (o-34:3)	plasmalogens	Lognormal	**-0.042**	**(-0.066, -0.018)**	**0.001**	**0.064**	**6.155**
SM (d40:3)	sphingomyelins	Lognormal	**-0.035**	**(-0.055, -0.014)**	**0.001**	**0.064**	**6.392**
LPC (18:2) - ESI(-)	unsaturated lysophosphatidylcholines	Lognormal	-0.022	(-0.036, -0.007)	0.005	0.169	2.295
PC (36:2)	unsaturated phosphatidylcholines	Lognormal	**-0.009**	**(-0.013, -0.004)**	**0.000**	**0.054**	**14.972**
PC (36:4) A - ESI(+)	unsaturated phosphatidylcholines	Lognormal	**-0.022**	**(-0.033, -0.010)**	**0.000**	**0.054**	**8.061**
PC (36:4) A - ESI(-)	unsaturated phosphatidylcholines	Lognormal	**-0.025**	**(-0.038, -0.011)**	**0.000**	**0.054**	**11.432**
PC (32:2) - ESI(-)	unsaturated phosphatidylcholines	Lognormal	-0.023	(-0.041, -0.006)	0.011	0.218	0.909
PC 34:4e	unsaturated phosphatidylcholines	Lognormal	**-0.029**	**(-0.045, -0.014)**	**0.000**	**0.054**	**13.135**
PC (34:2) - ESI(+)	unsaturated phosphatidylcholines	Lognormal	**-0.006**	**(-0.010, -0.003)**	**0.000**	**0.054**	**6.913**
PC (p-34:2)/PC (o-34:3) - ESI(+)	unsaturated phospholipid ethers	Lognormal	**-0.022**	**(-0.032, -0.011)**	**0.000**	**0.048**	**36.107**
PC (p-34:1)/PC (o-34:2)	unsaturated phospholipid ethers	Lognormal	**-0.025**	**(-0.038, -0.012)**	**0.000**	**0.054**	**26.013**
PC (p-36:1)/PC (o-36:2)	unsaturated phospholipid ethers	Lognormal	**-0.067**	**(-0.106, -0.029)**	**0.001**	**0.064**	**5.458**
PC (p-34:2)/PC (o-34:3) - ESI(-)	unsaturated phospholipid ethers	Lognormal	**-0.025**	**(-0.038, -0.011)**	**0.001**	**0.054**	**7.542**
PC (p-36:4)/PC (o-36:5) - ESI(-)	unsaturated phospholipid ethers	Lognormal	**-0.025**	**(-0.040, -0.011)**	**0.001**	**0.064**	**7.044**
PC (p-34:1)/PC (o-34:2) A	unsaturated phospholipid ethers	Lognormal	**-0.035**	**(-0.055, -0.015)**	**0.001**	**0.064**	**6.285**
Oxylipins (OL)							
Resolvin D1	OH-FA_22_6_1	Gamma	-0.513	(-0.871, -0.156)	0.006	0.183	1.811

Metabolite	Enrichment Cluster	Regression Model	Male ME/CFS vs. Male Control				
			Estimated Coefficient	95% CI	p-value	FDR	BayesFactor
Biogenic Amines (BA)							
Acetominophen	drugs	Lognormal	0.064	(-0.020, 0.148)	0.143	0.864	0.200
Alprazolam	drugs	Lognormal	0.017	(-0.090, 0.123)	0.761	0.956	0.068
Acyclovir	drugs	Lognormal	0.013	(-0.093, 0.120)	0.806	0.956	0.066
Complex Lipids (CL)							
PE (p-36:2)/PE (o-36:3) - ESI(+)	plasmalogens	Lognormal	-0.020	(-0.059, 0.018)	0.310	0.864	0.117
PE (p-34:2)/PE (o-34:3)	plasmalogens	Lognormal	-0.032	(-0.090, 0.027)	0.291	0.864	0.119
SM (d40:3)	sphingomyelins	Lognormal	-0.002	(-0.040, 0.037)	0.933	0.992	0.060
LPC (18:2) - ESI(-)	unsaturated lysophosphatidylcholines	Lognormal	-0.022	(-0.043, -0.001)	0.048	0.864	0.426
PC (36:2)	unsaturated phosphatidylcholines	Lognormal	-0.004	(-0.011, 0.003)	0.271	0.864	0.106
PC (36:4) A - ESI(+)	unsaturated phosphatidylcholines	Lognormal	-0.015	(-0.034, 0.004)	0.128	0.864	0.174
PC (36:4) A - ESI(-)	unsaturated phosphatidylcholines	Lognormal	-0.014	(-0.035, 0.006)	0.181	0.864	0.151
PC (32:2) - ESI(-)	unsaturated phosphatidylcholines	Lognormal	-0.041	(-0.084, 0.001)	0.065	0.864	0.438
PC 34:4e	unsaturated phosphatidylcholines	Lognormal	-0.007	(-0.040, 0.025)	0.666	0.928	0.073
PC (34:2) - ESI(+)	unsaturated phosphatidylcholines	Lognormal	0.000	(-0.006, 0.006)	0.900	0.976	0.061
PC (p-34:2)/PC (o-34:3) - ESI(+)	unsaturated phospholipid ethers	Lognormal	-0.010	(-0.031, 0.011)	0.343	0.864	0.106
PC (p-34:1)/PC (o-34:2)	unsaturated phospholipid ethers	Lognormal	-0.009	(-0.028, 0.010)	0.365	0.873	0.091
PC (p-36:1)/PC (o-36:2)	unsaturated phospholipid ethers	Lognormal	-0.020	(-0.061, 0.021)	0.348	0.864	0.082
PC (p-34:2)/PC (o-34:3) - ESI(-)	unsaturated phospholipid ethers	Lognormal	-0.016	(-0.038, 0.005)	0.144	0.864	0.169
PC (p-36:4)/PC (o-36:5) - ESI(-)	unsaturated phospholipid ethers	Lognormal	-0.019	(-0.046, 0.008)	0.176	0.864	0.169
PC (p-34:1)/PC (o-34:2) A	unsaturated phospholipid ethers	Lognormal	-0.013	(-0.042, 0.015)	0.363	0.873	0.107
Oxylipins (OL)							
Resolvin D1	OH-FA_22_6_1	Gamma	-0.282	(-0.991, 0.426)	0.439	0.881	0.099

Metabolite	Enrichment Cluster	Regression Model	ME/CFS without sr-IBS vs. Control without sr-IBS				
			Estimated Coefficient	95% CI	p-value	FDR	BayesFactor
Biogenic Amines (BA)							
Acetominophen	drugs	Lognormal	0.066	(0.023, 0.109)	0.003	0.291	2.586
Alprazolam	drugs	Lognormal	0.068	(0.018, 0.119)	0.008	0.310	0.850
Acyclovir	drugs	Lognormal	0.104	(0.031, 0.177)	0.006	0.310	1.688
Complex Lipids (CL)							
PE (p-36:2)/PE (o-36:3) - ESI(+)	plasmalogens	Lognormal	**-0.029**	**(-0.045, -0.013)**	**0.000**	**0.081**	**6.915**
PE (p-34:2)/PE (o-34:3)	plasmalogens	Lognormal	-0.036	(-0.060, -0.012)	0.004	0.310	2.620
SM (d40:3)	sphingomyelins	Lognormal	-0.026	(-0.046, -0.006)	0.013	0.310	1.103
LPC (18:2) - ESI(-)	unsaturated lysophosphatidylcholines	Lognormal	-0.017	(-0.030, -0.003)	0.016	0.310	0.720
PC (36:2)	unsaturated phosphatidylcholines	Lognormal	**-0.008**	**(-0.012, -0.004)**	**0.000**	**0.076**	**8.626**
PC (36:4) A - ESI(+)	unsaturated phosphatidylcholines	Lognormal	-0.017	(-0.028, -0.007)	0.002	0.218	3.025
PC (36:4) A - ESI(-)	unsaturated phosphatidylcholines	Lognormal	-0.018	(-0.030, -0.006)	0.005	0.310	2.130
PC (32:2) - ESI(-)	unsaturated phosphatidylcholines	Lognormal	-0.024	(-0.040, -0.007)	0.006	0.310	1.525
PC 34:4e	unsaturated phosphatidylcholines	Lognormal	-0.023	(-0.037, -0.008)	0.003	0.248	3.559
PC (34:2) - ESI(+)	unsaturated phosphatidylcholines	Lognormal	-0.004	(-0.007, -0.001)	0.021	0.327	0.553
PC (p-34:2)/PC (o-34:3) - ESI(+)	unsaturated phospholipid ethers	Lognormal	**-0.019**	**(-0.028, -0.010)**	**0.000**	**0.076**	**16.961**
PC (p-34:1)/PC (o-34:2)	unsaturated phospholipid ethers	Lognormal	**-0.022**	**(-0.033, -0.010)**	**0.000**	**0.076**	**30.007**
PC (p-36:1)/PC (o-36:2)	unsaturated phospholipid ethers	Lognormal	-0.052	(-0.084, -0.020)	0.002	0.218	4.636
PC (p-34:2)/PC (o-34:3) - ESI(-)	unsaturated phospholipid ethers	Lognormal	-0.019	(-0.030, -0.007)	0.002	0.248	3.502
PC (p-36:4)/PC (o-36:5) - ESI(-)	unsaturated phospholipid ethers	Lognormal	-0.020	(-0.032, -0.008)	0.001	0.218	6.200
PC (p-34:1)/PC (o-34:2) A	unsaturated phospholipid ethers	Lognormal	-0.025	(-0.042, -0.008)	0.005	0.310	1.458
Oxylipins (OL)							
Resolvin D1	OH-FA_22_6_1	Gamma	-0.379	(-0.683, -0.073)	0.016	0.310	0.597

For ME/CFS vs. controls, regression models were adjusted for age, sex, race/ethnicity, geographic/clinical site, season of sampling, body mass index, sr-IBS. In the sex-stratified comparisons, regression models were not adjusted for sex. In comparisons within subjects without sr-IBS, regression models were not adjusted for sr-IBS. For lognormal regression, estimated coefficients are interpreted as the differences in the mean values of log-log transformation of metabolite levels between cases and controls. For Gamma regression, estimated coefficients are interpreted as the log of fold change between two groups. Estimations in **bold** are signficant in the corresponding comparisons. Criteria for significance: 1) FDR adjusted p-value from regression model < 0.15, 2) BayesFactor > 3, and 3) 95% highest density credible intervals not covering 0. The credible intervals were extremely similar to the confidence intervals and are shown in Supplementary Table S2, S5, and S7. No primary metabolites were found to be significantly associated with ME/CFS. ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome. sr-IBS: self-reported irritable bowel syndrome. CI: confidence interval. FDR: false discovery rate adjusted p-value.