Correction

**MEDICAL SCIENCES**

The editor, R.W.D., wishes to note that he has consulted with R.K.N. with respect to R.W.D.’s son’s metabolism and with E.G. with respect to R.W.D.’s son’s care. These consultations played no role in the work or the editing of this paper. R.W.D.’s son was not a subject in this study. After this research study was completed, written, and edited, R.W.D., as chair of the Open Medicine Foundation (OMF) Scientific Advisory Board, asked R.K.N. to join the board and began collaborating with him and E.G. on follow-up studies related to this work. Thus, R.W.D. now has a professional relationship with R.K.N. and E.G. The OMF provided a grant to R.K.N. to support the follow-up studies. The OMF and R.W.D. played no role in the work described in this paper.

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Metabolic features of chronic fatigue syndrome

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Edited by Ronald W. Davis, Stanford University School of Medicine, Stanford, CA, and approved July 13, 2016 (received for review May 11, 2016)

More than 2 million people in the United States have myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). We performed targeted, broad-spectrum metabolomics to gain insights into the biology of CFS. We studied a total of 84 subjects using these methods. Forty-five subjects (n = 22 men and 23 women) met diagnostic criteria for ME/CFS by Institute of Medicine, Canadian, and Fukuda criteria. Thirty-nine subjects (n = 18 men and 21 women) were age- and sex-matched normal controls. Males with CFS were 53 (± 2.8) y old (mean ± SEM; range, 21–67 y). Females were 52 (± 2.5) y old (range, 20–67 y). The Karnofsky performance scores were 62 (± 3.2) for males and 54 (± 3.3) for females. We targeted 612 metabolites in plasma from 63 biochemical pathways by hydrophilic interaction liquid chromatography, electrospray ionization, and tandem mass spectrometry in a single-injection method. Patients with CFS showed abnormalities in 20 metabolic pathways. Eighty percent of the diagnostic metabolites were decreased, consistent with a hypometabolic syndrome. Pathway abnormalities included sphingolipid, phospholipid, purine, cholesterol, microbiome, pyrroline-5-carboxylate, riboflavin, branch chain amino acid, peroxisomal, and mitochondrial metabolism. Area under the receiver operator characteristic curve analysis showed diagnostic accuracies of 94% [95% confidence interval (CI), 84–100%] in males using eight metabolites and 96% (95% CI, 86–100%) in females using 13 metabolites. Our data show that despite the heterogeneity of factors leading to CFS, the cellular metabolic response in patients was homogeneous, statistically robust, and chemically similar to the evolutionarily conserved persistence response to environmental stress known as dauer.

Significance

Chronic fatigue syndrome is a multisystem disease that causes long-term pain and disability. It is difficult to diagnose because of its protean symptoms and the lack of a diagnostic laboratory test. We report that targeted, broad-spectrum metabolomics of plasma not only revealed a characteristic chemical signature but also revealed an unexpected underlying biology. Metabolomics showed that chronic fatigue syndrome is a highly concerted hypometabolic response to environmental stress that traces to mitochondria and was similar to the classically studied developmental state of dauer. This discovery opens a fresh path for the rational development of new therapeutics and identifies metabolomics as a powerful tool to identify the chemical differences that contribute to health and disease.


The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the NIH Metabolomics Data Repository and Coordinating Center (DRCC) (accession no. ST000450).

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both men and women and that targeted metabolomics can be used to uncover biological insights that may prove useful for both diagnosis and personalized treatment.

Results

Demographics. The 84 subjects in this study were recruited from 51 zip codes around the United States and Canada (SI Appendix, Fig. S1). Eighty subjects were from California. All CFS subjects met the 2015 diagnostic criteria published by the IOM (4), the Canadian working group (8), and Fukuda et al. (9). The IOM criteria are listed in Box 1. Although the IOM has suggested the use of the new name, “systemic exertion intolerance disease” (SEID), we will use the term “CFS” to refer to the same disease meeting the above criteria. The average age of men with CFS in this study was 53 (±2.8) (Table 1). The average age of the women with CFS was 52 (±2.5). The average age of onset was 30 (±2.6) y for the men and 33 (±2.3) y for the women. The average duration of illness was 21 (±3.0) y for men and 17 (±2.3) y for women. The Karnofsky quality of life performance score (10) for men was 62 (±3.2) and 54 (±3.3) for women (Table 1).

A Homogeneous Metabolic Response to Heterogeneous Triggers. Although the current study was not designed to examine the role of different triggering events, we collected some basic data. Possible triggering events fell broadly into five groups: biological (viral, bacterial, fungal/mold, and parasitic infections), chemical exposures, physical trauma, psychological trauma, and unknown. The specific biological and chemical exposures and the precise nature of the physical and psychological traumas were diverse, numbering more than a dozen in just this small sample. Several patients had multiple triggers that converged in the same year. Although biological triggers were most common, no single infectious agent or other stressor was statistically more prevalent, and comprehensive testing for biological exposures in the control group was beyond the scope of this study.

Despite the heterogeneity of triggers, the cellular response to these environmental stressors in patients who developed CFS was homogeneous and statistically robust. These data supported the notion that it is the unified cellular response, and not the specific trigger, that lies at the root of the metabolic features of CFS.

Metabolomics Revealed a Chemical Signature of CFS. Multivariate analysis was used to identify the pattern of chemical abnormalities in CFS compared with healthy controls. In the three-dimensional plot of the results (Fig. 1A and B), we found that both males and females with chronic fatigue had a chemical signature that was distinct from healthy controls. The relative pathway impact and statistical significance were visualized in Fig. 1C and D. Diagnostic and personalized metabolites are illustrated in Fig. 1E. The nine biochemical pathway disturbances that were common to both males and females with CFS were visualized in a Venn diagram (Fig. 1E). Eleven pathways were represented by metabolite disturbances that showed a degree of sex specificity. The biochemical pathways and metabolites that were altered in CFS were then ranked and tabulated (Tables 2 and 3 and SI Appendix, Figs. S2A and B and S3A and B) and visualized by Cytoscape pathway analysis (SI Appendix, Fig. S4 A and B). The dominant finding from the pathway analysis was that sphingolipid abnormalities constituted close to 50% of all of the metabolic disturbances associated with CFS in both males and females. Phospholipid abnormalities constituted 16% of the metabolic disturbances in males and 26% in females (Tables 2 and 3).

Table 1. Demographics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chronic fatigue</td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td>Mean (SEM) Range</td>
<td>Mean (SEM) Range</td>
</tr>
<tr>
<td>Subject number, n = 84</td>
<td>22 (2.8) 21–67</td>
<td>18</td>
</tr>
<tr>
<td>Age, y</td>
<td>53 (3.5) 23–69</td>
<td>ns</td>
</tr>
<tr>
<td>Age of onset, y</td>
<td>30 (2.6) 13–54</td>
<td>n/a</td>
</tr>
<tr>
<td>Duration of Illness, y</td>
<td>21 (3.0) 3–49</td>
<td>n/a</td>
</tr>
<tr>
<td>Karnofsky performance</td>
<td>62 (3.2) 30–90</td>
<td>4 × 10⁻¹³</td>
</tr>
<tr>
<td>score</td>
<td>100 (0) 100</td>
<td></td>
</tr>
<tr>
<td>Number of medications</td>
<td>4.1 (0.9) 0–16</td>
<td>0.0005</td>
</tr>
<tr>
<td>Years of education*</td>
<td>16 (0.8) 8–21</td>
<td>10–25</td>
</tr>
<tr>
<td>BMI</td>
<td>25.0 (0.7) 17–31</td>
<td>21–34</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White/non-Hispanic</td>
<td>22 (2.8) 17–31</td>
<td>ns</td>
</tr>
<tr>
<td>Hispanic</td>
<td>0 (2) 0</td>
<td>0</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (2) 0</td>
<td>0</td>
</tr>
<tr>
<td>Native American</td>
<td>0 (2) 0</td>
<td>0</td>
</tr>
<tr>
<td>African American</td>
<td>0 (2) 0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Years of education: High school = 12; AA = 14; bachelor’s = 16; master’s = 18; JD = 19; MD, DO, or ND = 20; PhD = 21; MD–PhD = 25.
Metabolites Correlated with the Clinical Severity of CFS. We next examined how each of the top 25 metabolite abnormalities was related to clinical functional status by Spearman correlation analysis. Each of these metabolites was found to have false discovery rates (FDRs) of less than 10% (SI Appendix, Table S1A and B). A list of the top 61 metabolites appears in SI Appendix, Table S1C and D. Twenty-one of the top 25 (84%) discriminating metabolites were low. These findings were consistent with the notion that CFS is a coordinated hypometabolic state.

Sphingolipids and Glycosphingolipids Were Decreased. The largest disturbances in the chemical signature of CFS were produced by widespread decrease in plasma sphingo- and glycosphingolipids (Fig. 1C and D and Tables 2 and 3). Thirty molecular species of sphingolipids were decreased in males, and 21 were decreased in females. Sphingolipid and glycosphingolipid abnormalities explained 55% of the metabolic impact in males and 44% in females (Tables 2 and 3). Measured glycosphingolipids included glucosyl- (GC), dihexosyl- (DHC), and trihexosyl- (THC) ceramides. In males, over 50% (16/30) of the sphingolipids that were decreased were ceramides, and 47% (14/30) were sphingomyelin species. In females, 86% (18/21) were ceramides and 14% (3/21) were sphingomyelins in females (SI Appendix, Table S1A–D). In general, females with chronic fatigue retained more sphingomyelin species in the normal range than males. The low sphingolipid profile in CFS appears to be an adaptive response that is opposite to the increased sphingolipids observed in metabolic syndrome (11) and the acute cell danger response (CDR) (7) and ultimately may represent a fundamental response to oppose the spread of persistent viral and intracellular bacterial infections.
Phospholipids Were Decreased. Several plasma phosphatidylcholine (PC) phospholipids were decreased in both males and females with CFS (Tables 2 and 3). In contrast, we found that a very specific molecular species of phospholipid, PC18:1/22:6, containing the essential omega 3 fatty acid docosahexaenoic acid (DHA, C22:6) and oleic acid (C18:1) was increased. This pattern is opposite to that seen in response to acute infection and the CDR (12) and metabolic syndrome (13).

Purines Were Decreased. Plasma uric acid was decreased in males with CFS (Table 2, Males). Uric acid is the end product of purine metabolism and an important antioxidant molecule (14, 15). Plasma adenosine was decreased in females (Table 3, Females). Adenosine is produced from ATP and ADP released from cell surface ectonucleotidases, and by S-adenosylhomocysteine hydrolase (SAHH), during acute infection, inflammation, or stress (16, 17). The decrease in plasma purines in CFS is consistent with decreased synthesis and/or turnover (flux) of ATP and GTP and decreased reserve capacity caused in part by a generalized decrease in the ability to restore high-energy phosphate stores after exertion.

Aromatic Amino Acid Metabolites from the Microbiome Were Decreased. Plasma 4-hydroxyphenyllactic acid (HPLA) was decreased in males with CFS (Table 2, Males). Plasma phenyllactic acid (PLA) was decreased in females (Table 3, Females). HPLA is a microbiome metabolite of tyrosine. PLA is a microbiome metabolite of phenylalanine. This pattern is also opposite of what is found during acute inflammation and infection (18).

Flavin Adenine Dinucleotide (FAD) Was Decreased. Plasma FAD was decreased in both males and females with CFS (Tables 2 and 3). FAD is synthesized from riboflavin (vitamin B2) and ATP. The gastrointestinal absorption, distribution, and transporter-mediated uptake of FAD are carefully regulated during health and disease (19). FAD is mobilized from tissues, increased in the plasma, and renal secretion is increased under conditions of stress or infection (20). FAD is an important cofactor for fatty acid oxidation and sterol synthesis and is required for activation and oxidation of vitamin B6 (pyridoxine); lipoid acid metabolism (E3 subunit) needed for pyruvate, alpha-ketoglutarate, and branched chain amino acid oxidation; vitamin A activation; 5-methyltetrahydrofolic acid synthesis; niacin and NAD synthesis; and glutathione reduction. Functional deficiency of riboflavin can be produced by dietary and environmental factors (21). Severe riboflavin deficiency can present with a plasma acyl-carnitine pattern similar to multiple acyl-CoA dehydrogenase deficiency (MADD), also known as glutaric aciduria type II (GAI) (22). GAI-like acyl-carnitine abnormalities did not appear in CFS patients.

Cholesterol and Bile Acid Synthesis Through the Lathosterol Pathway Were Decreased. Plasma lathosterol was decreased in both males and females with CFS (Tables 2 and 3). Total plasma cholesterol, desmosterol, cortisol, and aldosterone were normal in both males and females with CFS. Two pathways are used in mammalian cells to synthesize cholesterol. These are the Kandutsch–Russell (K–R) pathway through lathosterol and the Bloch pathway through desmosterol (23). The K–R pathway is preferred for cholesterol synthesis in the brain, heart, skeletal muscle, and skin, making up as much as 80% of cholesterol synthesis in these tissues under baseline conditions (23). The Bloch pathway is normally used preferentially in certain metabolic stress-response tissues like the gonads, spleen, adrenal glands, kidney, and adipose tissue. Under baseline conditions of health, the liver uses a nearly equal blend of Bloch and K–R pathways. Our data are consistent with increased flux through the desmosterol pathway to maintain normal cellular levels of cholesterol. The desmosterol pathway corresponds to the stress-inducible arm of de novo cholesterol and sterol synthesis.

Plasma chenodeoxycholic acid (CDCA) was decreased in females (Table 3, Females). CDCA is a primary bile acid made from cholesterol. Decreased cholesterol flux can result in decreased substrate for bile acid synthesis needed for normal fat digestion and microbiome signaling (24). The absence of adequate bile acid delivery can lead to a loss in intestinal mucosal integrity and leaky gut via a cascade of events stemming in part from disrupted farnesoid X receptor signaling (25).

Pyrrrole-5-Carboxylate and Arginine Were Increased. Pyrrole-5-carboxylic acid (PSC) was increased in both males and females with CFS (Tables 2 and 3). PSC production is a well-studied response to stress in plants (26) and mammals (27, 28). PSC can be produced by the stress-induced oxidation of proline and hydroxyproline from collagen turnover via the enzyme proline oxidase or from glutamate oxidation via P5CS (PSCS). PSC is converted to glutamate semialdehyde (GSA) non-enzymatically, then to ornithine under stress conditions. This reaction is catalyzed by what is often considered the reverse reaction of the mitochondrial enzyme, ornithine amino transferase (OAT). Hydroxyproline was increased in females with CFS.
chronic fatigue (Table 3, Females). Hydroxyproline is converted to proline, then to PSC and GSA, which is then used as the precursor for arginine (Arg) synthesis from ornithine in the epithelial of the small intestine under conditions of decreased calorie or protein intake (28). Another metabolic fate of hydroxyproline is glyoxylate, which can be transaminated in mitochondria to produce glycine and metabolized in peroxisomes to oxalate and peroxide for cell defense and innate and antiviral immunity (29).

Plasma Arg levels were also increased in chronic fatigue males and females. Arg is both a source of urea by arginase in the urea cycle, but more importantly, it is an activator of N-acetylglutamate (NAG) synthesis. NAG is the obligate activator of carbamoyl phosphate synthetase I (CPS-I). CPS-I is required for the introduction of ammonia into the urea cycle via the synthesis of citrulline from ornithine and carbamoylphosphate by ornithine transcarbamoylase (OTC). Citrulline, ornithine, proline, glutamine, and glutamate levels were all normal. Under stress conditions, proline from collagen breakdown is shunted to Arg synthesis to proline, then to P5C and GSA, which is then used as the precursor for arginine (Arg) synthesis from ornithine in the epithelial of the small intestine under conditions of decreased calorie or protein intake (28).

Diagnostic vs. Personalized Metabolic Disturbances. We classified all of the metabolite abnormalities in each patient as either being one of the abnormalities that defined CFS patients as a group (Fig. 1F, Tables 2 and 3, and SI Appendix, Table S1 A–D) or as abnormalities that differed from controls but did not contribute to the CFS diagnosis. CFS patients had an average of 10 (±1.0) metabolite abnormalities that contributed to the CFS diagnosis and 30 (±2.0) metabolites that were abnormal but noncontributory for purposes of CFS diagnosis (Fig. 1F and SI Appendix, Fig. S5). This means that 75% of the chemical abnormalities identified by metabolomic analysis were personalized, and 25% provided diagnostic group information. Our clinical experience suggests that symptom improvements can be achieved more reliably by addressing the personalized abnormalities rather than by assuming a chemical abnormality without actual measurement.

Assessment of Metabolomics as a Diagnostic Test in CFS. After identifying over 60 metabolites that differed between CFS and controls in both males and females (SI Appendix, Table S1 A–D), we set out to find smaller sets of analytes that could be used for diagnosis. Samples of 5–15 of the top 60 metabolites were manually selected to broadly interrogate several of the discriminating biochemical pathways (Tables 2 and 3) in males and females. The performance of each classifier set of metabolites was then tested by area under the receiver operator characteristic (AUROC) curve analysis. We found that the exact specification of metabolites in the classifier was flexible. Using both forward selection and backward elimination methods (34), we found that once a set of 5–15 analytes was found, the addition or removal of one or a few analytes had little effect on the overall quality of the classifier. In males, we found a set of 8 analytes performed well (Fig. 2A). In females, we found a set of 13 analytes performed well (Fig. 2B). We found that even single-analyte classification methods performed surprisingly well in this small sample of 84 subjects (Table 4). However, single biomarkers are biologically implausible as a diagnostic test for complex diseases like CFS and are likely to perform poorly in larger populations. By using classifiers constructed from 5 to 15 metabolites, natural biological variation is more readily accommodated and diagnostic accuracy is more robust. We also performed a principal components analysis (PCA)

### Table 3. Biochemical pathway abnormalities in CFS, females

<table>
<thead>
<tr>
<th>No.</th>
<th>Pathway name</th>
<th>Measured metabolites in the pathway, N</th>
<th>Expected pathway proportion, P = N/421</th>
<th>Expected hits in sample of 61, P *, 61</th>
<th>Observed hits in the top 61 metabolites</th>
<th>Fold enrichment, obs/exp</th>
<th>Impact, sum VIP score</th>
<th>Fraction of impact explained, % of 117.3</th>
<th>Increased</th>
<th>Decreased</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sphingolipids</td>
<td>71</td>
<td>0.169</td>
<td>10.29</td>
<td>21</td>
<td>2.0</td>
<td>41.1</td>
<td>35%</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Phospholipids</td>
<td>77</td>
<td>0.183</td>
<td>11.16</td>
<td>17</td>
<td>1.5</td>
<td>31.0</td>
<td>26%</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Glycosphingolipids</td>
<td>12</td>
<td>0.029</td>
<td>1.74</td>
<td>5</td>
<td>2.9</td>
<td>11.1</td>
<td>9%</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Purines</td>
<td>20</td>
<td>0.048</td>
<td>2.90</td>
<td>3</td>
<td>1.0</td>
<td>6.0</td>
<td>5%</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Microbiome metabolism</td>
<td>21</td>
<td>0.050</td>
<td>3.04</td>
<td>3</td>
<td>1.0</td>
<td>5.3</td>
<td>5%</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Fatty acid oxidation and synthesis</td>
<td>36</td>
<td>0.086</td>
<td>5.22</td>
<td>2</td>
<td>0.4</td>
<td>4.0</td>
<td>3%</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>PSC, Arg, Ornithine, Pro steroids</td>
<td>6</td>
<td>0.014</td>
<td>0.87</td>
<td>2</td>
<td>2.3</td>
<td>3.6</td>
<td>3%</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Cholesterol, nongonadal</td>
<td>16</td>
<td>0.038</td>
<td>2.32</td>
<td>1</td>
<td>0.4</td>
<td>2.5</td>
<td>2%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>Collagen/hydroxyproline metabolism</td>
<td>2</td>
<td>0.005</td>
<td>0.29</td>
<td>1</td>
<td>3.5</td>
<td>2.4</td>
<td>2%</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Vitamin B2 (riboflavin)</td>
<td>2</td>
<td>0.005</td>
<td>0.29</td>
<td>1</td>
<td>3.5</td>
<td>2.1</td>
<td>2%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>Bile salt metabolism</td>
<td>7</td>
<td>0.017</td>
<td>1.01</td>
<td>1</td>
<td>1.0</td>
<td>1.9</td>
<td>2%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>Endocannabinoids</td>
<td>2</td>
<td>0.005</td>
<td>0.29</td>
<td>1</td>
<td>3.5</td>
<td>1.7</td>
<td>1%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Branch chain amino acids</td>
<td>10</td>
<td>0.024</td>
<td>1.45</td>
<td>1</td>
<td>0.7</td>
<td>1.6</td>
<td>1%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>Vitamin B12 (cobalamin) metabolism</td>
<td>2</td>
<td>0.005</td>
<td>0.29</td>
<td>1</td>
<td>3.5</td>
<td>1.6</td>
<td>1%</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>Amino-sugar, galactose, and nonglucose</td>
<td>4</td>
<td>0.010</td>
<td>0.58</td>
<td>1</td>
<td>1.7</td>
<td>1.5</td>
<td>1%</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Subtotal: 13 48 61
to identify orthogonal components of the metabolomic signature (SI Appendix, Table S4 A and B and Fig. S7 A and B). However, we have found PCA to be less robust than partial least squares discriminant analysis (PLS-DA) and random forest (RF) analysis in identifying diagnostically useful metabolites in independent clinical settings.

Metabolic Similarities Between CFS and Dauer. Many of the pathways and metabolites that were abnormal in CFS are also known to be features of dauer, a well-studied, long-lived survival and persistence state triggered by environmental stress (35, 36) (Table 5). Interestingly, we found that the direction of CFS abnormalities was opposite to metabolic syndrome (37) and opposite to the metabolic response to infection, inflammation, or environmental stress that has been called the CDR (7). For example, cholesterol, phospholipids, sphingolipid, and purine metabolism are all decreased in CFS and dauer but are increased in metabolic syndrome and the stereotyped CDR (Table 5). These facts suggest that CFS is an evolutionarily conserved, genetically regulated, hypometabolic state similar to dauer that permits survival and persistence under conditions of environmental stress but at the cost of severely curtailed function and quality of life.

Discussion

The purpose of this study was to test the utility of targeted metabolomics in the diagnosis of CFS. We found that patients meeting the criteria for CFS recommended by the IOM (4), Canadian working group (8), and Fukuda et al. (9) had objective chemical abnormalities that clearly distinguished them from controls. In addition, pathway analysis revealed that all nine of the pathways disturbed in both men and women with CFS were related to the CDR (7). However, in contrast to an acute CDR, in which plasma sphingolipids and phospholipids are increased, these pathways were decreased, suggesting a postexposure adaptation or mitoellular hormesis (38, 39) in response to pathologically persistent or recurrent cell damage signaling (6, 7).

Hypometabolism, Dauer, and CFS. Our results show that the metabolic features of CFS are consistent with a hypometabolic state. Sphingolipids, glycosphingolipids, phospholipids, purines, micro-bione aromatic amino acid and branch chain amino acid metabolites, FAD, and lathosterol were decreased. The decreases in these metabolites correlated with disease severity as measured by Karnofsky scores (SI Appendix, Table S1 A–D). Much research has been done on the hypometabolic phenotype in other biologic systems, including dauer (35), diapause (40), hibernation (41), estivation (42), torpor (43), ischemic preconditioning (44), ER stress (45), the unfolded protein response (46), autophagy (47, 48), and caloric restriction (49). Dauer, which means persistence or long-lived in German, is an example of one well-studied system. The developmental stage of dauer is a hypometabolic state capable of living efficiently by altering a number of basic mitochondrial functions, fuel preferences, behavior, and physical features. Dauer is comprised of an evolutionarily conserved and synergetic suite of metabolic and structural changes that are triggered by exposure to adverse environmental conditions. Entry into dauer confers a survival advantage in harsh conditions (35). When the dauer response is blocked by certain mutations (dauer defective), animals are short-lived when exposed to environmental stress. These mutations show that the latent ability to enter into a hypometabolic state during times of environmental threat is adaptive, even though it comes at the cost of decreasing the optimal functional capacity. Similar to dauer, CFS appears to represent a hypometabolic survival state that is triggered by environmental stress. The metabolic features of CFS and dauer correspond to the same pathways that characterize the acute CDR and metabolic syndrome (50) but are regulated in the opposite direction. For example, cholesterol, phospholipids, and uric acid are often elevated in the acute CDR and metabolic syndrome, but these metabolites were decreased in CFS patients. A prediction based on these findings is that patients with CFS would be more resistant to the constellation of hypertension, dyslipidemia, central obesity, and insulin resistance that increase all-cause mortality associated with metabolic syndrome (37), but at the cost of significant long-term disability, pain, and suffering.

The Importance of Mitochondria, Redox, and NADPH Metabolism in Chronic Fatigue. All of the metabolic abnormalities that we identified in CFS were either directly regulated by redox or the availability of NADPH. About 60% of NADPH is produced by the pentose phosphate pathway under baseline conditions. The other 40% is produced by the combined flux through five NADP+ dependent enzymes: (i) malic enzyme (ME), (ii) isocitrate dehydrogenase (IDH), (iii) glutamate dehydrogenase (GDH), (iv) nicotinamide nucleotide transhydrogenase (NNT), and (v) methylene tetrahydrofolate dehydrogenase 2-like protein (MTHFD2L). Each of these enzymes has at least one mitochondrial isoform and is known to be up-regulated under conditions of environmental or developmental stress. It has recently been shown that mitochondrial MTHFD2L is responsible for producing 20–40% of cellular NADPH by the oxidation of methylene tetrahydrofolate acid to 10-formyl tetrahydrofolate (51). These data show that folates are important not only in methylation reactions but also in regulating intracellular redox and NADPH levels (SI Appendix, Fig. S6). A number of single nucleotide polymorphisms (SNPs) have been identified in the MTHFD2L gene that correlate with the CDR and interleukin 1p (IL1β) production triggered by smallpox vaccination (52). Mitochondrial pools of NADPH are in continuous communication with NADH levels through the enzyme NNT. Therefore, NADPH acts as a global barometer of cellular fuel status by interrogating both mitochondrial electron (NADH) consumption and the availability of cytoplasmic reducing.

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equivalents as NADPH. When mitochondrial electron transport decreases for any reason, fewer molecules of oxygen are converted to water (H₂O) by cytochrome c oxidase. If capillary delivery of oxygen to the cell is unmatched, the concentration of dissolved oxygen rises in the cell like water in a bowl in response to instantaneous decreases in mitochondrial oxygen consumption. This activates scores of enzymes that are kinetically regulated by the availability of dissolved oxygen and can act as oxygen sensors. Some of these include NADPH oxidases like Nox4 (33) that make hydrogen peroxide (H₂O₂) from the excess diatomic oxygen (O₂) to initiate the oxidative shielding response (6). When reduced (NADPH) and total (NADPH plus NADP⁺) pools are low, sterol, fatty acid, protein, and nucleotide synthesis fall to baseline survival levels. When NADPH levels are higher, metabolism is shifted from persistence to normal cell function and growth, anabolic pathways are stimulated, biomass is created, and carbons and electrons are stored as biopolymers for cell growth and repair in the form of lipids, protein, glycogen, glycans, and nucleic acids.

It is important to emphasize that NADPH is neither the problem nor the solution by itself. It is a messenger and cofactor. NADPH cannot work without the availability of hundreds of carbon skeletons of intermediary metabolism needed to carry out the message—the signal that fuel stores are either replete or insufficient. If capillary delivery of the signal that fuel stores are either replete or insufficient, the message will help clinicians monitor individualized responses to treatments for CFS are likely to be achieved by careful attention to nutrition, metabolism, triggers, stressors, and physical activity as an integrated system, combined with a systems biological understanding of the triggers of the CDR (7) and dauer entry and exit (35).

Conclusions

CFS has a chemical signature that can be identified using targeted plasma metabolomics. Receiver operator characteristic (ROC) curve analysis showed a diagnostic accuracy that exceeded 90%. The pattern and directionality of these changes showed that CFS is a conserved, hypometabolic response to environmental stress similar to dauer (35). Only about 25% of the metabolite disturbances found in each person were needed for the diagnosis of CFS. About 75% of the metabolite abnormalities were unique to the individual and useful in guiding personalized treatment. The study of larger cohorts from diverse geographical areas, and comparison with related medical disorders like depression and posttraumatic stress disorder, will be needed to validate the universality and specificity of these findings. The finding of an objective chemical signature in CFS helps to remove diagnostic uncertainty, will help clinicians monitor individualized responses to treatment, and will facilitate multicenter clinical trials.

Materials and Methods

Patients and Controls. This study was approved by the University of California, San Diego Institutional Review Board (IRB Project 140072) and conformed to the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects (54). Patients and controls were recruited prospectively over a 1-y period, June 2014–May 2015. Signed informed consent was obtained from all subjects. All CFS patients met the 2015 IOM (4), Canadian (8), and Fukuda (9) diagnostic criteria for CFS. Healthy controls were age- and sex-matched volunteers without CFS. The total

Table 4. Diagnostic accuracy of targeted plasma metabolomics in CFS

<table>
<thead>
<tr>
<th>Sex</th>
<th>Classifiers</th>
<th>AUROC*</th>
<th>95% CI</th>
<th>rdCV²</th>
<th>accuracy</th>
<th>Permutation⁠³</th>
<th>P value</th>
<th>2 × 2 sensitivity</th>
<th>2 × 2 specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>8-analyte example⁴</td>
<td>0.94</td>
<td>0.84–1.0</td>
<td>0.84</td>
<td>0.001</td>
<td>0.90</td>
<td>0.91</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-analyte example⁴</td>
<td>0.71</td>
<td>0.50–0.88</td>
<td>0.62</td>
<td>0.009</td>
<td>0.72</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>13-analyte example⁵</td>
<td>0.96</td>
<td>0.87–1.0</td>
<td>0.90</td>
<td>0.001</td>
<td>0.93</td>
<td>0.91</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-analyte example⁵</td>
<td>0.68</td>
<td>0.42–0.86</td>
<td>0.58</td>
<td>0.009</td>
<td>0.68</td>
<td>0.70</td>
<td>0.67</td>
<td></td>
</tr>
</tbody>
</table>

n = 18 control males and 22 CFS males, and n = 21 control females and 23 CFS females.

*AUROC, area under the receiver operator curve reflects the overall accuracy of diagnosis using these analytes.

²rdCV, repeated random subsample (2/3 in, 1/3 out) double cross-validation.

³Permutation P values represent the probability that the RF classification of cases and controls using the specified analytes could be obtained by chance.

⁴Values calculated by standard 2 × 2 contingency table analysis.

⁵8-analytes in males, phosphatidyl choline PC(16:0/16:0), glucosylceramide GC(18:1/16:0), 1-PSC, FAD, pyrogallic acid (also known as 5-oxoproline), HICA, L-serine, and lathosterol.

¹1-analyte: phosphatidyl choline PC(16:0/16:0).

²13-analytes in females: THC(18:1/24:0), phosphatidyl choline PC(16:0/16:0), hydroxyproline, ceramide(d18:1/22:2), lathosterol, adenosine, phosphatidylinositol PI(16:0/16:0), FAD, 2-octanoylcarnitine, phosphatidyl choline plasmalogens PC(22:6/18:0), phosphatidyl choline PC(18:1/22:6), 1-PSC, and CDCA.

Table 5. Metabolic similarities and contrasts between CFS and Dauer, cell danger and metabolic syndrome

<table>
<thead>
<tr>
<th>Plasma metabolites</th>
<th>CFS</th>
<th>Dauer</th>
<th>CDR (7)</th>
<th>Metabolic syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingolipids</td>
<td>Decreased (M + F)⁴</td>
<td>Decreased (62)</td>
<td>Increased (63)</td>
<td>Increased (64)</td>
</tr>
<tr>
<td>Glycosphingolipids</td>
<td>Decreased (M + F)</td>
<td>Decreased (62)</td>
<td>Increased (63)</td>
<td>Increased (65)</td>
</tr>
<tr>
<td>Phospholipids, most species</td>
<td>Decreased (M + F)</td>
<td>Decreased (66)</td>
<td>Increased (67)</td>
<td>Increased (68)</td>
</tr>
<tr>
<td>PC(18:1/22:6)—Oleoyl/DHA phospholipids</td>
<td>Increased (M + F)</td>
<td>No data</td>
<td>Decreased (67)</td>
<td>Decreased (13)</td>
</tr>
<tr>
<td>Cholesterol, sterol synthesis</td>
<td>Decreased (M + F)</td>
<td>Decreased (69)</td>
<td>Increased (70)</td>
<td>Increased (71)</td>
</tr>
<tr>
<td>Purines</td>
<td>Decreased (M + F)</td>
<td>Decreased (72)</td>
<td>Increased (73)</td>
<td>Increased (74)</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Decreased (M)</td>
<td>N/A¹</td>
<td>Increased (75)</td>
<td>Increased (76)</td>
</tr>
<tr>
<td>PSC/Arg</td>
<td>Increased (M + F)</td>
<td>No data</td>
<td>Decreased (77)</td>
<td>No data</td>
</tr>
<tr>
<td>FAD/Riboflavin</td>
<td>Decreased (M + F)</td>
<td>Decreased (72)</td>
<td>Increased (20)</td>
<td>No data</td>
</tr>
</tbody>
</table>

⁴F, females only; M, males only; M + F, males and females.

¹N/A, the end products of purine metabolism in worms are glyoxylate and ammonia, not uric acid.
number of subjects analyzed in this study was 84. This included 23 females and 22 males with CPS and 18 male and 21 female controls.

Metabolomics. Targeted, broad-spectrum, chemometric analysis of 612 metabolites from 63 biochemical pathways was performed as described (55) with minor modifications. Over 420 metabolites were detectable in all plasma samples. Regular quality control experiments showed metabolite AUC correlations over 0.98 and relative SDs of 9–12% (SI Appendix, Tables S2 and S3). See SI Appendix, SI Methods for details.

Data Analysis. Metabolomic data were log-transformed, scaled by control SDs, and analyzed by multivariate PLSDA, PCA, t test, univariate ANOVA with pairwise comparisons, and post hoc correction for multiple hypothesis testing using Fisher’s least significant difference method in MetaboAnalyst (56), or the FDR method of Benjamini and Hochberg (57). Metabolites with variable importance in projection (VIP) scores determined by PLSDA that were greater than 1.5 were considered significant. Metabolite correlations with Karnofsky performance scores were calculated by Pearson parametric and Spearman nonparametric methods implemented in STATA (Stata/SE12.1, StataCorp), Prism (Prism 6, GraphPad Software), or R. Significant metabolites were grouped into pathways and their VIP scores summed to determine the rank-ordered significance of each biochemical pathway. Sets of 5–15 metabolites were selected manually from the top 50 significant metabolites as candidate diagnostic classifiers using two multivariate methods: RFs (58) and linear support vector machine (SVM) implemented in MetaboAnalyst. The diagnostic performance of the selected classifiers was then visualized and quantified by AUROC curve analysis (34). Classifier robustness was estimated by repeated double cross-validation (rdCV) (59) and permutation testing 1,000 times in MetaboAnalyst. Confidence intervals for the ROC curves were calculated by bootstrap resampling. Sensitivity, specificity, accuracy, positive predictive value, negative predictive value, and number of misclassifications (60) were estimated by conventional 2 x 2 contingency table analysis. P values calculated by Fisher’s exact test in Prism.

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See the list of references above for a comprehensive bibliography on the topic of aging and its metabolic and cellular pathways. The references cover a wide range of studies, from molecular biology to clinical studies, highlighting the complex interactions between aging, metabolism, and disease.

For example, the reference 48 by Hood DA et al. (2015) discusses exercise and the regulation of mitochondrial turnover, which is a critical aspect of aging research. The reference 49 by Long YC et al. (2014) explores the biochemistry and cell biology of aging, providing insights into the mechanisms underlying aging processes.

The reference 50 by Alberti KG et al. (2013) addresses the metabolic syndrome and its components, offering a comprehensive overview of the dysregulation observed in this condition. The reference 51 by Fan J et al. (2014) presents a quantitative flux analysis revealing folate-dependent NADPH production, which is crucial for understanding the metabolism of aging cells.

The reference 52 by Kennedy RB et al. (2012) investigates genome-wide analysis of polymorphisms associated with mitochondrial turnover, providing insights into the genetic basis of aging.

The reference 53 by Nisimoto Y et al. (2014) describes the role of hydrogen peroxide-generating oxygen sensors in the regulation of cytokine responses in smallpox vaccine recipients.

The reference 54 by World Medical Association (2013) emphasizes the importance of the Helsinki Declaration in ethical medical research.

The reference 55 by Naviaux JC et al. (2015) discusses antipurinergic therapy and its correcting effects on autism-like features in the Fragile X (Fmr1 knockout) mouse model.

The reference 56 by Xia J et al. (2015) introduces MetaboAnalyst 3.0, a powerful tool for metabolomics data analysis.

The reference 57 by Benjamini Y et al. (1995) presents a method for controlling the false discovery rate in multiple testing, which is crucial for interpreting the results of metabolomics studies.

The reference 58 by Breiman L (2001) introduces Random Forests, a machine learning algorithm that is widely used in metabolomics research.

The reference 59 by Filzmoser P et al. (2009) describes a repeated double cross validation method for metabolomics studies.

The reference 60 by Szymanska E et al. (2012) introduces Double-check, a method for validating metabolomics models.

The reference 61 by Xi B et al. (2014) presents a statistical analysis and modeling of mass spectrometry-based metabolomics data.


The reference 63 by Schneider-Schaulies J et al. (2015) explores the role of sphingolipids in viral infection.

The reference 64 by Chaurasia B et al. (2015) examines the role of ceramides in lipotoxicity and metabolic disorders.

The reference 65 by Inokuchi J (2014) examines the role of GM3 in diabetes.

The reference 66 by Abusharkh SE et al. (2014) investigates the role of phospholipid headgroup composition and trehalose in the desiccation tolerance of Caenorhabditis elegans.

The reference 67 by Liu JL et al. (2013) describes the impact of mitochondrial oxidative stress on bile acid-like molecules in C. elegans, providing a new perspective on human metabolic diseases.

The reference 68 by Ng TW et al. (2014) explores the dose-dependent effects of rosuvastatin on plasma sphingolipidome and phospholipidome in the metabolic syndrome.

The reference 69 by Haneklaus M (2013) examines the role of uric acid as a modulator of glucose and lipid metabolism.

The reference 70 by Osgood K et al. (2013) discusses the serum uric acid levels in current and future components of the metabolic syndrome.

The reference 71 by Lamkanfi M et al. (2009) introduces inflammasomes as guardians of cytosolic sanctity.

The reference 72 by Lima WG et al. (2015) examines the role of uric acid as a modulator of glucose and lipid metabolism.

The reference 73 by Kao CC et al. (2009) explores the role of arginine, citrulline, and nitric oxide metabolism in sepsis.

These references, among others, provide a comprehensive understanding of the metabolic and cellular pathways involved in aging and aging-related diseases.