Hematologic and Urinary Excretion Anomalies in Patients with Chronic Fatigue Syndrome

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Patients with chronic fatigue syndrome (CFS) have a broad and variable spectrum of signs and symptoms with variable onsets. This report outlines the results of a single-blind, cross-sectional research project that extensively investigated a large cohort of 100 CFS patients and 82 nonfatigued control subjects with the aim of performing a case-control evaluation of alterations in standard blood parameters and urinary amino and organic acid excretion profiles. Blood biochemistry and full blood counts were unremarkable and fell within normal laboratory ranges. However, the case-control comparison of the blood cell data revealed that CFS patients had a significant decrease in red cell distribution width and increases in mean platelet volume, neutrophil counts, and the neutrophil-lymphocyte ratio. Evaluation of the urine excretion parameters also revealed a number of anomalies. The overnight urine output and rate of amino acid excretion were both reduced in the CFS group ($P < 0.01$). Significant decreases in the urinary excretion of asparagine ($P < 0.0001$), phenylalanine ($P < 0.003$), the branch chain amino acids ($P < 0.005$), and succinic acid ($P < 0.0001$), as well as increases in 3-methylhistidine ($P < 0.05$) and tyrosine ($P < 0.05$) were observed. It was concluded that the urinary excretion and blood parameters data supported the hypothesis that alterations in physiologic homeostasis exist in CFS patients. Exp Biol Med 232:1041–1049, 2007

Key words: chronic fatigue syndrome; CFS; CFIDS; myalgic encephalomyelitis; ME; urine analyses; hematology; homeostasis

Introduction

Chronic fatigue syndrome (CFS) is a clinically defined illness that is characterized by unexplained, persistent, excessive and debilitating fatigue that presents in conjunction with a complex and variable constellation of other multisystemic symptoms (1, 2). Debilitating neuropsychologic, endocrinologic, immunologic and gastrointestinal symptoms are frequently associated with the disorder. Known also as myalgic encephalomyelitis (ME) and chronic fatigue and immune dysfunction syndrome (CFIDS), prevalence estimates suggest that the condition affects between 0.002% and 2.6% of the general population (3–8), including individuals of both sexes, all ages, and from many regions of the world (9).

Research hypotheses seeking to explain CFS have been diverse. However, despite substantial research efforts, details of the etiologic and pathophysiologic processes that contribute to CFS have not been forthcoming. Although physiologic anomalies have been noted in many studies to date, no single etiological agent or consistent cellular or biochemical alteration has been associated with the disorder. A major feature of CFS is the extremely diverse symptom presentation and the presence of variable types of onsets (10–12). This inherent heterogeneity has frustrated efforts to identify an etiological agent or agents and has contributed to

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the plethora of conflicting reports. The use of small patient groups has therefore been a primary limitation to the interpretation and extrapolation of CFS study findings in the past. The future investigation of patients with CFS should clearly involve larger patient numbers to address the problem of symptom diversity and minimize the potential confounding or bias introduced into data derived from various heterogenous cohorts.

Several studies have suggested that disturbances in amino acid homeostasis occur in CFS (13–18) and other clinically overlapping syndromes (19–22). In a recent study, CFS subjects were shown to have significant reductions in the urinary excretion of β-alanine, hydroxyproline, histidine, methionine, cystine, and phenylalanine compared with nonfatigued controls (18). Importantly, this report also showed that the excretion profiles of CFS patients were different from patients with depression and rheumatoid arthritis, suggesting that the urinary excretion in CFS patients reflects different metabolic perturbations compared with other patient groups. Significant reductions in plasma taurine, histidine, α-aminobutyric acid, and tyrosine were also noted for the CFS group. It was concluded that the results were consistent with inflammatory disease and warranted further investigation. However, the patient size used in this research project was small (30 subjects), and therefore caution should be applied in attempting to generalize these findings to all CFS patients.

The aim of this investigation was to assess urine metabolite excretion and blood cell parameters from 100 CFS patients who have been compared with 82 control subjects to test the hypothesis that CFS patients have a molecular and cellular basis to the disorder. This study forms part of a larger multidisciplinary investigation of subject parameters.

Materials and Methods

This research project was approved by the Medical Research Ethics Committee and Community Health Services for Royal North Shore Hospital (RNSH) and the University of Newcastle Human Ethical Review Committee, and informed consent was obtained from all subjects prior to their participation in the study.

Individuals with CFS and healthy age- and sex-matched control subjects were recruited by staff at RNSH over an 18-month period. The CFS group was recruited from sequential medical practitioner referrals to the RNSH CFS clinical group. The age- and sex-matched control subjects were recruited *via* friends or relatives of the CFS patients or *via* project advertisement at RNSH, the University of Sydney, and the University of Technology, Sydney. The recruited CFS patients had previously been diagnosed as having CFS by a clinician, and their diagnosis was confirmed by RNSH clinicians using the Oxford CFS case definition (23). Control subjects were asymptomatic and were excluded if they reported fatigue that influenced their lives, had any diagnosable illness or psychiatric disorder, or had evidence of a current or past history of any neurologic, endocrine, cardiovascular, hepatic, renal, haematologic, or psychiatric disease. Individuals reporting a history of drug abuse were not included in either study group. Patients unable or unwilling to comply with project requirements, unable to come into RNSH for clinical evaluation and sample collection or from outside the Sydney Metropolitan region were not enrolled in the study.

All subjects were clinically evaluated at the RNSH. On the night prior to clinical assessment, subjects were requested to fast from food and drink from 10:00 PM for collection of fasted morning urine and blood samples. Subjects were allowed to drink a small volume of water (less than 200 ml) throughout the night if necessary. For urine samples, subjects were asked to completely empty their bladder prior to sleeping (between 10:00 PM and 12:00 PM, this urine was not collected) and to collect all of the first-morning urine into a standard, sterile, 1-liter plastic bottle. Subjects recorded the time of both voids to provide an estimate of the excretion period and the volume of urine collected. Fasted whole-blood samples (10 ml) were collected from study subjects at RNSH by venipuncture into glass lithium-heparin vacutainers and were kept on ice. The blood samples were then used for the determination of blood cell parameters and biochemistry using standardized methodology.

Full blood counts were performed on blood samples collected from all study subjects. In addition, a comprehensive biochemical panel was performed on blood collected from CFS subjects as part of their clinical investigation and diagnosis. The panel included tests of kidney (urea, creatinine), liver (alanine amino transferase, aspartate amino transferase, gamma glutaryl transferase, bilirubin), thyroid (thyroxine, thyroid-stimulating hormone), and pancreatic (fasting plasma glucose) function, iron status (iron, iron binding capacity), and blood levels of electrolytes (sodium, potassium, bicarbonate, chloride, calcium, phosphate), proteins (albumin, total protein), folate, B12, and creatine kinase. Erythrocyte sedimentation rates and antinuclear antibody titers were also assessed.

The urine samples were coded, refrigerated at the collection point, and transported to the laboratory for processing within 24 hrs of collection. For each sample, a 10-ml aliquot of urine was centrifuged (1600 *g*; 5 mins; 4°C), and a 200-μl aliquot was transferred to a glass derivatization tube for freeze drying. The freeze-dried residue was then derivatized to form *N(O,S)*-heptafluorobutyryl-isobutyl (HFB-isobutyl) derivatives for analysis using a Hewlett Packard GC-MS (5971A mass-selective detector [MSD]; Santa Clara, CA) as previously described (14, 24). Quality control was achieved by running a standard mix of amino acids with each batch of analyses and by using internal standards and multirule Shewart plots to assess efficiency of sample processing and machine response (25).

Where possible, the chromatographic peaks were

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identified by HP-UX ChemStation computer searches of user-generated reference libraries (incorporating retention indices and mass spectra) and the WILEY database (John Wiley and Sons, Hoboken, NJ). Metabolite excretion rates were calculated by multiplying the concentration of metabolite per unit volume (milliliters) of urine by the volume of urine (milliliters) excreted per unit time (hours). Quantitative urine analysis usually involves the collection of 24-hr urine samples; however, a fasting morning urine sample was considered preferable in this study to minimize dietary influence on urine composition and standardize collection procedures.

All researchers were blinded to subject category and clinical and psychologic patient assessment. On completion of all laboratory analyses the data were decoded and grouped for statistical analyses. Data distributions were evaluated for compliance with parametric statistical requirements. All percentage data were arcsine transformed before analysis. Mean cell volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentrations did not require transformation. The remaining blood cell parameters and urine excretion rate data were log transformed to improve normality, linearity, and homoscedascity. Subject characteristics and blood and urine excretion parameters were assessed using unpaired independent Student's *t* tests, Mann-Whitney *U* tests and chi-square test of the independence of two variables according to the type and normality of the individual data sets. Multivariate group differences were assessed using multivariate standard and forward stepwise discriminant function analyses. These data were processed using Statistica (Versions 5.1 and 6.1; Statsoft, Tulsa, OK).

Results

A total of 114 CFS subjects enrolled in the study, and 14 subjects were excluded following clinical evaluation and review of medical history, with 73 females and 27 males forming the final CFS cohort. A total of 108 prospective control subjects were initially enrolled, and 26 subjects were excluded either because they were not prepared to comply with study requirements or because they did not meet the study inclusion criteria. The final number of healthy controls was 82, with 60 females and 22 males. There were no significant demographic differences between the two subject groups, with similar distributions of age and sex as shown in Table 1. A total of 82% and 90% of the Oxford-defined CFS subjects would also have qualified for a diagnosis of CFS according to the 1988 (1) and 1994 (2) CDC criteria, respectively, and no CFS subjects displayed fewer than 4 of the 11 original CDC minor criteria (1).

Review of the blood biochemistry panel assessed as part of the clinical workup of CFS patients found that the mean values were within the normal ranges. Some individuals did have values outside the normal ranges on one or more parameters; however, these deviations were not considered sufficiently extreme to warrant subject exclusion. Erythrocyte sedimentation rates (ESRs) were also determined for 80 of the 100 CFS subjects, with four female patients exceeding the normal range at values between 20 and 30 mm/hr. A total of 33 patients with CFS (33%) were positive for antinuclear antibodies (titer $\geq$ 1:40). However, all serologic results were below the level of 1:640 and, as such, were not considered high enough to necessitate subject exclusion.

Table 1. Demographic Characteristics of the Control and CFS groups[a]

Characteristic	Controls	CFS
No. of subjects	82	100
Mean age (±SD)	32.6 ± 14.0	29.1 ± 12.5
Age range	14–69 years	13–73 years
Percentage female	73.2	73.0

[a] Statistical methods. Student's *t*-test and chi-square probability, $\alpha = 0.05$. There were no significant differences between the two groups for any of the parameters.

The mean blood cell parameters were measured for both the CFS and control groups. All results were within the normal laboratory range, as shown in Table 2. However, multivariate evaluation using forward stepwise discriminant function analysis revealed significant differences in the patterns of blood cell parameters for CFS patients compared with controls (Wilks lambda = 0.90, canonical $R^2 = 0.10$, $F(6, 172) = 3.20$; $P < 0.006$). The primary factors differentiating the CFS patients from controls included an increased neutrophil cell count and a reduction in red cell distribution width (RDW). Case-control univariate analyses supported these findings and revealed that significant increases in mean platelet volume and in the neutrophil-lymphocyte ratio were also evident in patients with CFS (Table 2).

The urine volumes and estimated excretion rates for the CFS and control groups have been summarized in Table 3. The urine collection periods were very similar between CFS patients and control subjects, but the CFS cohort had significant reductions in urine excretion volumes compared with the control group. A reduction in the rate of urinary metabolite excretion was a major feature in the CFS group, reflecting significant reductions in excretion of essential branched-chain amino acids (BCAAs; $P < 0.005$) and nonessential amino acids ($P < 0.005$). There were no differences in excretion of the total organic acid components or the amino acid derivatives, which included β-alanine, ethanolamine, hippuric acid, and *S*-methylcysteine.

Multivariate investigations of both the excretion rate data and the percentage relative abundance data using standard discriminant function analysis were conducted to determine whether the amino acid metabolic profiles for the CFS patients could be differentiated from the control profiles. The results are summarized in Table 4. The analyses demonstrated that the urine excretion profile was significantly different in the CFS patients compared with the control subjects (excretion rate model, $P < 0.0004$;

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Table 2. Red and White Blood Cell Parameters for CFS ($n = 100$) and Control Subjects ($n = 81$)[a]

Variable	Control mean (SE)	CFS mean (SE)	*P*	Normal range
Red cell count (10^{12}/l)	4.62 (0.05)	4.61 (0.04)	NS	4.5–6.5
Hemoglobin (g/dl)				
Males	14.95 (0.20)	15.15 (0.10)	NS	14.0–18.0
Females	13.19 (0.10)	13.37 (0.10)	NS	12.0–16.0
Hematocrit (%)				
Males	43.66 (0.60)	44.18 (0.30)	NS	42.0–52.0
Females	38.66 (0.31)	39.05 (0.28)	NS	37.0–47.0
Mean corpuscular volume (fl)	86.70 (0.42)	87.74 (0.32)	NS	80.0–100.0
Mean cell hemoglobin (pg)	29.66 (0.17)	30.07 (0.13)	NS	27.0–32.0
Mean cell hemoglobin concentrate (g/dl)	34.17 (0.07)	34.25 (0.06)	NS	32.0–35.0
Red cell distribution width (%)	12.59 (0.09)	12.35 (0.09)	<0.03[b]	11.5–14.5
Platelet count (10^9/l)	256.8 (7.04)[c]	253.1 (6.07)	NS	150–450
Mean platelet volume (fl)	8.35 (0.11)	8.60 (0.10)	<0.02[b]	7.4–10.4
White cell count (10^9/l)	5.76 (0.15)	6.11 (0.14)	NS	4.0–11.0
Lymphocyte count (10^9/l)	1.94 (0.07)	1.95 (0.06)[d]	NS	1.5–4.0
Neutrophil count (10^9/l)	3.13 (0.09)	3.46 (0.11)[d]	<0.04	2.0–7.5
Eosinophil count (10^9/l)	0.20 (0.02)	0.19 (0.02)[d]	NS	0.04–0.4
Basophil count (10^9/l)	0.02 (0.00)	0.02 (0.00)[d]	NS	0.02–0.1
Monocyte count (10^9/l)	0.48 (0.02)	0.48 (0.01)[d]	NS	0.2–0.8
% lymphocytes	33.75 (0.69)	32.60 (0.81)[d]	NS	20–45
% neutrophils	54.09 (0.77)	56.17 (0.87)[d]	NS	40–75
% eosinophils	3.38 (0.30)	3.08 (0.21)[d]	NS	1–6
% basophils	0.38 (0.05)	0.33 (0.03)[d]	NS	1
% monocytes	8.35 (0.20)	7.86 (0.16)[d]	NS	2–10
Neutrophil-lymphocyte ratio	1.69 (0.05)	1.91 (0.08)[d]	<0.04	

[a] The normal range and units specific to each parameter are also presented. Statistical methods: Student's *t*-test, $\alpha = 0.05$. NS, not statistically significant.
[b] Mann-Whitney *U* test performed (probability estimate adjusted for ties) since transformation was unable to satisfactorily improve normality, linearity and homoscedascity of data.
[c] $n = 80$.
[d] $n = 99$.

percentage abundance model, $P < 0.008$). According to the standardized canonical coefficients, alterations in the excretion rate of seven urinary metabolites made significant and unique contributions to the discrimination between the CFS and healthy control groups ($P < 0.05$, data not shown). Specifically, increases in the urinary excretion of tyrosine, 3-methylhistidine and lysine, in addition to reductions in glutamic acid/glutamine, asparagine, serine and β-alanine ($P = 0.05$), were found to differentiate the CFS cohort from the control group. Review of the factor structure matrix revealed that the metabolites that correlated most strongly with the urine excretion rate discriminant function model were, in order of magnitude, asparagine (factor loading = 0.48); succinic acid (0.45); phenylalanine (0.35); proline (0.29); *S*-methylcysteine (0.29); alanine (0.28); the three BCAAs, valine (0.28), leucine (0.26) and isoleucine (0.26); 3-methylhistidine (–0.26); hippuric acid (0.26); and tyrosine (–0.25). These findings indicated that the group separation achieved by the primary discriminant function was correlated with changes in these metabolites with a positive and a negative factor loading indicating a reduction or an increase in the level of the respective variable in the CFS group. When the discriminant function generated from the analysis was used to classify patients *post hoc* into clinical categories based on the urine excretion profile, the urine excretion rate model was able to correctly classify 66% of healthy controls and 80% of the CFS patients into their respective groups on the basis of their urine excretion rate data. Similarly, for the percentage abundance model, 65% of controls and 73% of cases were correctly assigned to their groups on the basis of their urine metabolite percentage composition profiles.

Univariate analyses of the individual urine metabolite excretion rates (micromoles/hour) and percentage abundance data were performed to further assess the excretion of metabolites (Table 4). Reductions in urinary excretion rates of asparagine, alanine, proline, valine, leucine, isoleucine, phenylalanine, hippuric acid, succinic acid, and *S*-methylcysteine and increases in tyrosine and 3-methylhistidine were observed in the CFS patients compared with controls. These metabolites were identified in the multivariate analyses as primary differentiating factors, reaffirming their importance in distinguishing between the two groups.

Discussion

In the majority of studies, aberrations in blood biochemistry and blood cell parameters among CFS patients

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Table 3. Summary of Group Differences (CFS vs. Healthy Controls[a]) in Total Urine Volume, Estimated Urine Collection Periods, and Urine Metabolite Excretion Rates (μmol/hr)

	Controls, mean (SE)	CFS, mean (SE)	P^b
Urine collection periods and volumes			
Urine collection period (mins)	429.0 (14.0)	426.7 (12.6)	NS
Total urine volume (ml)	305.8 (14.7)	262.8 (13.1)	<0.02
Urine volume excreted per unit time (ml/hr)	45.4 (2.3)	38.2 (2.0)	<0.007
Urine metabolite excretion rate (μmol/hr)			
Total (measured) metabolites	1437.0 (98.9)	1136.8 (100.8)	<0.04
Total amino acids	423.6 (23.9)	334.5 (23.6)	<0.01
Essential amino acids[c]	28.0 (2.0)	27.2 (4.8)	NS
Branched chain[d]	9.8 (0.6)	7.6 (0.5)	<0.005
Nonessential amino acids[c]	394.5 (22.4)	306.2 (20.8)	<0.005
Transamination amino acids[e]	136.5 (7.7)	108.2 (6.8)	<0.0001
Total amino acid derivatives	282.1 (25.8)	231.2 (26.1)	NS
Total organic acids	693.1 (71.7)	535.9 (71.1)	NS

[a] Healthy control subjects, $n = 80$; CFS subjects, $n = 96$.
[b] Statistical method: Student's *t*-test on log-transformed urine metabolite data, $\alpha = 0.05$. NS, not statistically significant.
[c] Ornithine is not included in proteins and therefore is not classified as an essential or nonessential amino acid.
[d] Includes the amino acids valine, leucine, and isoleucine.
[e] Includes the amino acids alanine, aspartate, asparagine, glutamate/glutamine.

have been assessed through comparison with standard reference ranges (26). However, these ranges reflect blood characteristics for individuals with a wide diversity of genotypes and phenotypes, ages, and pre-history influences, and are extremely broad compared with the standard errors noted in most cohorts of healthy or CFS subjects. As a consequence, these comparisons have been restricted to the detection of gross changes in blood parameters that occur in association with highly specific pathology and are likely to miss more subtle perturbations. Consistent with this, comparisons of the standard blood parameters determined for the current CFS group against the reference ranges were generally unremarkable (Table 2). However, the case-control analyses of the standard blood cell data revealed that CFS subjects could be distinguished from healthy individuals on the basis of subtle differences in their blood cell profiles (Table 2).

The major blood cell parameter that differentiated CFS patients from the control group was an increase in the neutrophil count ($P < 0.05$). Increases in circulating neutrophils have been commonly observed in inflammation and in localized or generalized bacterial infections, especially with pyogenic bacteria, and are sometimes accompanied by fever due to release of leucocyte pyrogens (27). The augmented neutrophil count lead to an increased neutrophil-lymphocyte ratio, suggestive of a nonviral pathogen-like stimulus.

In addition, the mean RDW was decreased in CFS patients compared with healthy individuals ($P < 0.05$). RDW represents a sensitive measure of variation in red cell shape or anisocytosis (28). These changes may reflect alterations in red cell morphology, possibly as a result of oxidative damage as proposed by Richards *et al.* (29, 30) and are consistent with previous reports of altered erythrocyte morphology and rheology in patients with CFS (31–33). In a separate study, an increase in RDW was the principal blood cell parameter differentiating CFS patients from the healthy control population (34). These red blood cell findings collectively support the proposition that changes in the morphologic characteristics of erythrocyte populations may be evident among CFS patients (31–33, 35, 36). A reduction in RDW may reflect a dominance of one or two forms of erythrocytes. Both Simpson (31, 32) and Richards *et al.* (30) have noted a reduced proportion of normal cells and an increased percentage of nondiscocytic erythrocytes, particularly the stomatocytes, in subsets of CFS patients. Such changes may be contributing to CFS symptomatology. Nondiscocytes are poorly deformable, and an increased prevalence of these cells may impede capillary flow, oxygen delivery, and the removal of metabolic byproducts, such as carbon dioxide and lactic acid from body tissues (30). This may in turn lead to inefficient energy generation, fatigue, and impaired organ function.

The evaluations of urine excretion were specifically aimed at determining whether there was evidence for an altered metabolic homoeostasis in CFS subjects compared with healthy, nonfatigued controls. The results showed that the CFS patients had significantly reduced urine volumes for the overnight collections and significant reductions in the excretion of essential branch-chain amino acids and nonessential amino acids. Further analyses revealed that a range of amino acids and organic acids were anomalous between the CFS cohort and the control group. These findings were consistent with other studies that have reported anomalies in urinary and/or plasma amino and

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Table 4. Summary of Multivariate and Univariate Evaluations of Group Differences (CFS vs. Healthy Controls) in Individual Urinary Metabolite (a) Excretion Rates (μmol/hr) and (b) Percentage Abundance Composition

Multivariate analyses, standard discriminant function						
	(a) Excretion rate[a]			(b) Percentage abundance[b]		
Model statistics	Wilks' lambda = 0.69, Canonical R^2= 0.31 $F(27, 148) = 2.45$, $P < 0.0004$			Wilks' lambda = 0.75, Canonical R^2= 0.25 $F(27, 152) = 1.91$, $P < 0.008$		
Model *post hoc* classification	Controls 66.3%	CFS 80.2%	Overall 73.9%	Controls 65.4%	CFS 72.7%	Overall 69.4%

Univariate analyses						
	(a) Excretion rate[a]			(b) Percentage abundance[b]		
Metabolite	Control, mean (SE)	CFS, mean (SE)	P^c	Control, mean (SE)	CFS, mean (SE)	P^c
Glutamic acid[d]	31.3 (1.7)	28.0 (1.7)	NS	7.74 (0.31)	8.24 (0.36)	NS
Aspartic acid	6.5 (0.4)	6.1 (0.5)	NS	1.38 (0.07)	1.51 (0.07)	NS
Asparagine	83.5 (8.2)	47.4 (5.1)	<0.0001	1.77 (0.16)	1.18 (0.11)	<0.003
Alanine	98.7 (6.6)	74.1 (5.4)	<0.02	6.34 (0.39)	5.70 (0.31)	NS
Glycine	132.2 (10.4)	111.6 (10.8)	NS	18.90 (0.91)	18.56 (0.98)	NS
Serine	28.9 (1.6)	28.9 (4.0)	NS	7.27 (0.39)	7.78 (0.45)	NS
Threonine	10.6 (0.8)	9.0 (0.9)	NS	2.01 (0.11)	1.94 (0.10)	NS
Proline	12.7 (1.00)	9.2 (0.6)	<0.02	2.04 (0.14)	1.80 (0.11)	NS
Hydroxyproline	0.7 (0.1)	0.5 (0.05)	NS	0.48 (0.05)	0.48 (0.04)	NS
Lysine	5.4 (1.1)	9.0 (3.9)	NS	3.41 (0.48)	3.91 (0.80)	NS
Ornithine	1.1 (0.1)	1.1 (0.2)	NS	0.75 (0.06)	0.88 (0.06)	NS
Valine	3.7 (0.2)	2.9 (0.2)	<0.02	1.03 (0.06)	0.94 (0.05)	NS
Leucine	1.9 (0.1)	1.5 (0.1)	<0.03	0.40 (0.02)	0.37 (0.02)	NS
Isoleucine	4.1 (0.3)	3.2 (0.2)	<0.03	0.21 (0.01)	0.19 (0.01)	NS
Phenylalanine	2.3 (0.1)	1.6 (0.1)	<0.003	0.94 (0.05)	0.81 (0.05)	<0.03
Tyrosine	0.76 (0.11)	0.91 (0.10)	<0.04	0.50 (0.06)	0.82 (0.08)	<0.002
1-Methylhistidine	2.7 (0.7)	4.1 (0.9)	NS	1.82 (0.47)	3.53 (0.67)	<0.03
3-Methylhistidine	4.7 (0.6)	6.0 (0.8)	<0.03	1.81 (0.20)	2.61 (0.20)	<0.0009
β-Alanine	6.4 (0.7)	6.2 (0.6)	NS	0.91 (0.10)	0.97 (0.08)	NS
β-Aminoisobutyric acid	8.0 (1.2)	5.8 (0.9)	NS	1.32 (0.24)	1.07 (0.14)	NS
Ethanolamine	51.5 (4.0)	47.0 (3.8)	NS	2.74 (0.18)	3.29 (0.24)	NS
Hippuric acid	202.9 (23.9)	157.8 (24.9)	<0.03	20.27 (1.51)	16.73 (1.54)	<0.05
S-methylcysteine	5.3 (0.5)	3.75 (0.4)	<0.02	0.26 (0.02)	0.24 (0.02)	NS
Succinic acid	369.9 (28.2)	241.7 (27.6)	<0.0001	0.97 (0.09)	0.69 (0.05)	<0.004
Aconitic acid	27.3 (2.2)	22.8 (1.9)	NS	4.29 (0.27)	4.27 (0.24)	NS
Citric acid	291.0 (64.1)	267.3 (57.7)	NS	4.53 (0.73)	4.85 (0.68)	NS
Phenylacetic acid	4.8 (0.7)	4.1 (0.6)	NS	0.75 (0.08)	0.78 (0.09)	NS
Pyroglutamic acid	17.5 (1.4)	14.2 (1.4)	NS	2.51 (0.18)	2.51 (0.22)	NS

[a] Healthy control subjects, $n = 80$; CFS subjects, $n = 96$.
[b] Calculated from arbitrary area unit detector response values. Healthy control subjects, $n = 81$; CFS subjects, $n = 99$.
[c] Statistical methods: Student's *t*-test, $\alpha = 0.05$; NS = not statistically significant.
[d] Includes glutamine.

organic acids in subjects with CFS (13–15, 17, 18), and are suggestive of a disruption to normal metabolic homeostasis.

No individual urine or blood cell anomaly detected in this study was specific to CFS, and no single parameter changed consistently across the entire CFS group. Further comparison of the current research findings to those of other studies reporting changes in urine (13, 14, 18) and plasma (16–18) in clinically defined CFS also did not detect any specific anomaly consistently observed in all studies reviewed. These findings are compatible with CFS group heterogeneity and implicate the need to characterize CFS patients into more specific and homogeneous patient sets for clinical and pathologic investigation.

Although no single anomaly has been noted in all studies reviewed, the majority of studies, including the present investigation, have noted reductions in urine (13, 14, 18) and plasma (16–18) amino acid levels in patients with CFS compared with healthy controls. These findings collectively support the theory that patients with CFS display a generalized depletion of amino acids within the body's free amino acid pools that may be affecting nitrogen and energy metabolism, the availability of amino acids for tissue uptake and cellular metabolism, cell-to-cell communication and metabolic regulation. Two earlier studies have shown that protein synthesis was decreased in patients with CFS (37, 38). The depletion in amino acid precursors and a

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reduction in protein synthetic capacity could compromise the ability of cells and tissues to respond to regulatory inputs and thereby impair an organism's ability to deal with homeostatic disturbance. The reduction in the urinary output of asparagine in CFS patients noted in this study may be consistent with impaired protein synthesis, since asparagine is an important amino acid in protein structures, required for forming glycopeptides.

Depletion of intracellular ATP has been linked to CFS in a number of studies (39–41). Reductions in physiologic pools of transanimation and BCAAs, as suggested by the reduced urinary output data, could have significant impacts on energy metabolism. A decrease in the availability of amino acids for direct oxidation *via* the TCA cycle or for gluconeogenesis may impede the generation of cellular energy and promote fatigue. In addition, BCAAs are important sources of fuel for muscle tissue under nutritional and metabolic stress, where muscle oxidation of the preferred substrates (glucose and fatty acids) is diminished or impaired (42). Furthermore, studies with rats have demonstrated a 4-fold greater capacity of brain tissue to oxidize BCAA than was evident in muscle or liver tissue (43). A reduction in BCAA availability and cerebral uptake may therefore affect fuel utilization by decreasing the amount of these important oxidative substrates within the central nervous system. Consistent with this, glucose hypometabolism within the brain, as measured using positron emission tomogrpahy (PET) and 18F-fluorodeoxy-glucose, has been reported in ME/CFS patients compared with normal and depressed controls (44).

The potential influence of depleted BCAA levels on brain function warrants further consideration. Experimental evidence indicates that the brain uptake of BCAA exceeds that of all other amino acids (45, 46) and that these amino acids act as principal nitrogen donors for glutamate/glutamine synthesis within the brain (47–49). It has been estimated that one third and up to one half of the α-NH_2 groups of glutamate/glutamine in astrocytes are derived *via* transamination reactions involving leucine (47, 50–52). A series of vital transamination reactions occur within the CNS to facilitate nerve transmission and maintain nitrogen homeostasis (52). More specifically, evidence suggests that BCAAs such as leucine act as nitrogen donors in a series of cycles that operate to lower synaptic glutamic acid levels to maximize signal-to-noise ratios upon depolarisation (52). Deficits in BCAA availability may therefore have substantial implications on brain function through their effects on brain glutamate and glutamine homeostasis and indirectly through alteration of the availability of glutamate for oxidation and energy production. The glutamatergic neurons have been shown to be involved in many aspects of normal brain functions, including memory, cognition, learning, spatial recognition, and the maintenance of consciousness (53), many of which were impaired in CFS.

A reduction in peripheral leucine and other BCAAs may also impair the ability of the brain to dispose of waste nitrogen and thus lead to increased levels of toxic ammonia within the cerebral tissues. The passage of large neutral amino acids from the blood to the brain is connected to the countertransport of glutamine (54, 55). Given that the brain lacks the ability to synthesize urea, it has been proposed that this countertransport may function as a mechanism for the removal of excess nitrogen from the brain (52). A number of metabolic pathways in the brain produce ammonia. Decrements in the synthesis of glutamate would result in a reduction in the availability of glutamate for conversion to glutamine, which may in turn impede the removal of excess ammonia groups. This may have serious implications on neuronal function, given that brain tissue is highly sensitive to alterations in ammonia levels. Although it is premature to assign a definitive etiologic or pathobiologic role to these changes in relation to CFS, amino acid anomalies have been implicated in many well-recognized clinical conditions and are likely to have a significant impact on many aspects of physiology.

The CFS patients in the current study demonstrated an increased excretion of the catabolic markers tyrosine and 3-methylhistidine, findings that are consistent with an elevation in proteolysis. Elevated urinary outputs of tyrosine have previously been reported in a separate CFS patient cohort (14). Tyrosine excretion is a marker of degradation of short-term cytoplasmic proteins (nonmyofibrillar proteolysis) in the muscle, whereas 3-methylhistidine is a marker of the degradation of the contractile proteins actin and myosin (myofibrillar proteolysis; Ref. 56). A sustained proteolytic response may be consistent with reductions in cellular RNA (57–59) and protein synthesis (37, 38) within muscle tissue, as well as the observed upregulation of the activity of the 2–5A synthetase/Rnase L (60–65) and protein kinase RNA pathways (66) previously reported in CFS. Increases in the serum or plasma concentrations of cytokines thought to augment protein degradation, IL-1α (67) and TNF-α (68), have also been reported, although these findings have not been universally supported (12, 69–72). BCAAs have anabolic and anti-proteolytic effects on muscle protein metabolism (42), and their depletion has been associated with an augmentation in muscle protein proteolysis and a decline in muscle protein levels (42).

Conclusions. The results of this study revealed that patients with CFS/ME had anomalies in blood parameters, urine excretion volume, and urinary excretion of amino acids compared with age- and sex-matched nonfatigued controls. Reductions in overnight urinary output and a generalized depletion in the rate of amino acid excretion, in particular, depletions in the excretion of branched chain amino acids, were the most prominent alterations observed. These findings indicated significant disturbance to amino acid and nitrogen metabolism and homeostasis. Further investigation into the mechanisms underlying these changes and their etiologic and clinical significance is warranted.

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