# Assessment of the Energy Metabolism in Patients with Chronic Fatigue Syndrome by Serum Fluorescence Emission

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**Abstract**

Chronic fatigue syndrome (CFS) is a debilitating fatigue illness that has unknown etiology and lacks an objective diagnostic marker. To examine the metabolic component of CFS, we determined serum NAD(P)H levels in 44 CFS patients and 30 healthy control subjects by measuring serum fluorescence emission at 450 nm. NAD(P)H concentrations in serum of CFS averaged $8.0 \pm 1.4$ (SD) uM while those in healthy controls averaged $10.8 \pm 0.8$ (SD) uM, a statistically significant difference. We conducted a sensitivity and specificity analysis. Using a cut-off concentration of 9.5 uM, we attained a sensitivity of 0.73 and a specificity of 1.0. Receiver operator characteristics (ROC) analysis yielded an area under the curve of 0.9. Serum NAD(P)H was compared to several endocrine and metabolic lab parameters. It was directly correlated with serum coenzyme Q10 levels and inversely correlated with urine hydroxyhemopyrrolin-2-one (HPL) levels. Based on these findings, we propose using serum NAD(P)H, measured as an intrinsic serum fluorescence emission, to monitor metabolism and fatigue status in subjects with CFS. Following patient NAD(P)H levels over time may aid in selecting therapeutic strategies and monitoring treatment outcome.

**Introduction**

Chronic fatigue syndrome (myalgic encephalomyelitis, myalgic encephalopathy, CFS, CFS/ME) is characterized by long-lasting disabling fatigue. It includes non-specific symptoms such as weakness, malaise, subjective fever, sore throat, lymph node pain, and decreased memory [1 – 4]. There are no conclusive diagnostic tests for CFS. It is diagnosed when debilitating fatigue symptoms of the sort listed above occur in the absence of psychiatric disorders, bipolar disorder, substance abuse, morbid obesity or any untreated or unresolved disease [5, 6].

Many potential causes for CFS/ME have been investigated, ranging from neurological and endocrine dysfunctions to immunological disorders and infections [5]. Possible neurological issues include disruptions in hypothalamic-pituitary-adrenal response and neurotransmitters, both of which have been associated with CFS [7 – 9]. Many, but not all, CFS patients have prior exposure to the Epstein-Barr virus [10 – 18]. Other evidence of an immunological component include increased numbers of activated T-cells, increased cytokine circulation, and T-helper cells, and altered natural killer cell activity in subjects with CFS [19 – 33]. A number of recent studies suggest that oxidative stress and alterations in mitochondrial function may contribute to CFS [34]. For example, one study linked CFS to reduced carnitine levels [35], an indication of compromised mitochondrial function, but this linkage was not observed elsewhere [36].

At present, the underlying mechanism for CFS is not known.

Since the cause of CFS is not known, finding reliable biomarkers for diagnosing it has proven difficult. Markers under study include altered gene expression profiles in leukocytes [37 – 41]. In particular, twelve genes have been identified for which mRNA levels change significantly with CFS compared to age and sex matched controls [42]. The attempt to monitor alterations in metabolism and homeostasis in CFS patients was made in study [43], by measuring urine metabolite levels via gas chromatography and mass spectroscopy. CFS patients showed increases in aminohydroxy-n-methylyrrolidine (CFS symptom marker 1), tyrosine, β-alanine, aconitic acid, and succinic acid along with reductions in alanine, glutamic acid, and an unidentified urine metabolite denoted CFS symptom marker 2. Another group has attempted to use the near-infrared spectra of serum to distinguish between CFS patients and healthy subjects [44].

The purpose of our study was to examine the use of serum NAD(P)H levels as a metabolic marker of fatigue state. We have developed a method of determining NAD(P)H levels based on fluorescence emission of serum at 450 nm [45]. Previously we have shown that cancer patients

have reduced serum NAD(P)H concentrations correlating with reported levels of fatigue [45]. The present manuscript details our data on serum NAD(P)H and chronic fatigue, along with correlations between serum NADH and other metabolic and endocrine markers.

**Material and Method**

*Participants*

The present study, conducted at the Riordan Clinic, consisted of forty-four CFS patients and thirty healthy volunteers. All subjects signed an informed consent approved by the Riordan Clinic Institutional Review Board. CFS patients were diagnosed according to the Centers for Disease Control criteria (released in 1994) with a spectrum of symptoms that existed for at least six months: new unexplained, persistent, or relapsing chronic fatigue not resolved by bed rest and severe enough to significantly reduce previous daily activity by 50%, along with headache, muscle pain, pain in multiple joints, and un-refreshing sleep. Other clinical conditions that may produce similar symptoms were excluded by thorough evaluation, based on the appropriate laboratory findings.

*Serum Fluorescence Emission*

Venous blood samples were obtained from fasting participants who were also asked not to take supplements and medicine for 24 hours before the blood drawing, a precaution necessary because fluorescence emission from vitamins and drugs can interfere with the serum fluorescence emission assay. Serum was separated from the blood by centrifugation at 3500 rpm for 15 minutes. All specimens were diluted by PBS (Phosphate Buffered Saline) using a ratio of 1:20 to measure the emission in the range of absorption less than 0.1 at the excitation wavelength. Fluorescence spectra were run using a SPEX spectrofluorometer (sensitivity 4000:1, double-grating spectrophotometers, excitation scans 315 - 340 nm and emission scans 330 - 600 nm).

Excitation scan wavelengths were chosen to exclude protein, which produces an intense emission peek at excitation wavelengths around 280 nm). The background curves for the solvent (PBS) were measured and subtracted from the fluorescence spectra of serum to remove background effects. Standard curves of emission intensity (counts per second) versus NADH concentration (nM) were made to allow conversion of the former to the latter.

*Other Biochemical Assays*

The HPL (hydroxyhemopyrrolin-2-one) assay is based on the extraction of pyrroles from urine with chloroform followed by reaction with Ehrlich's acid aldehyde reagent (0.5 g of p-dimethylaminobenzaldehyde, 2.5 ml sulfuric acid in 50 ml of methanol). This preparation yields a chromophore with an absorption maximum of 540 nm. For this test, random urine samples were collected and 200-500 mg of ascorbic acid was added as a preservative (2.0 ml of urine for analysis).

Serum coenzyme Q10 levels were determined as follows. After collection of human blood in EDTA containing tubes, plasma was separated from whole blood by centrifugation. Coenzyme Q10 was collected using a lipid extraction procedure and subjected to analysis without further purification. Human plasma Q10 was analyzed on the HPLC system (Hewlett Packard). The extract was separated on a reversed-phase C-18 column by methanol (66%) and hexane (34%) as a mobile phase, flow rate 1 ml/min and injection volume 50ul. Coenzyme Q10 was detected at 275 nm absorbance maximum by a UV detector. All other laboratory tests (hemoglobin, Free T3, EBV, Candida Ab) were performed by Riordan Clinic laboratory using standard clinical techniques.

*Statistics*

The nonparametric Mann-Whitney test was used to determine the magnitudes of between-group differences (Systat 13, Systat software, Inc). Values of $p<0.05$ were considered statistically

significant.    Sensitivity and Specificity at various NADH cut-offs were computed using the equations below.

Sensitivity = {True Positives} ÷ {True Positives + False Negatives}

Specificity = {True Negatives} ÷ {True Negatives + False Positives}

The diagnostic accuracy of coenzymes NAD(P)H levels was assessed in terms of true positive (sensitivity) versus true negative (1-specificity) using a nonparametric receiver operating characteristics (ROC) analyses [46 - 49].    Curve fitting was performed using Kaleidagraph (Synergy Software).

**Results**

*Characterization of Serum Fluorescence Emission*

Irradiation of the serum at wavelengths from 300 nm to 340 nm gave rise to emission in the 350 nm to 600 nm region, coinciding with the respective absorption and emission of NAD(P)H, along with other contributing molecules. For estimating the effect of different fluorescence components (proteins and coenzymes) on the native serum fluorescence, emission of the different fractions of fluorescent serum biomolecules, such as NAD(P)H, 3-hydroxyanthranilic acid, 4-pyridoxic acid, pyridoxal-5-phosphate, L-tryptophan, kynurinine, nicotinamide, and nicotinic acid (all ordered from Sigma), were compared.  More detailed description of the different fluorescent fractions of serum, and the procedure of calculations of contribution from different fractions of serum at the concentrations they would have in serum in total serum emission was presented in our study [45].

Figure 1 shows fluorescence emission data for serum, along with the emission of L-tryptophan,  and NAD(P)H as a function of emission wavelength.   At this range of
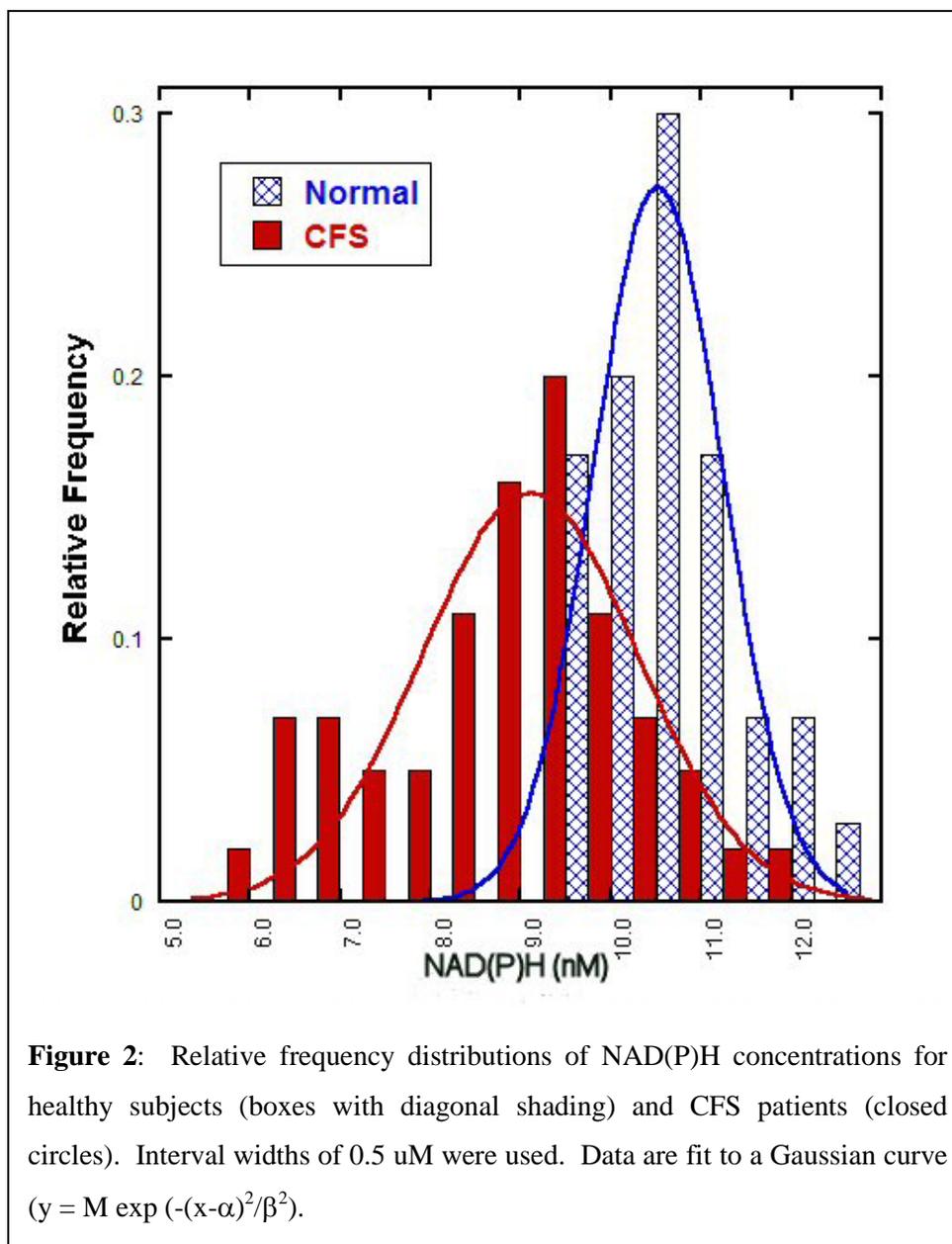
emission wavelengths, the serum signal can be totally accounted for by adding the L-tryptophan and NAD(P)H signals. To isolate NAD(P)H to the largest extent possible, we took future readings at the emission wavelength of 450 nm.
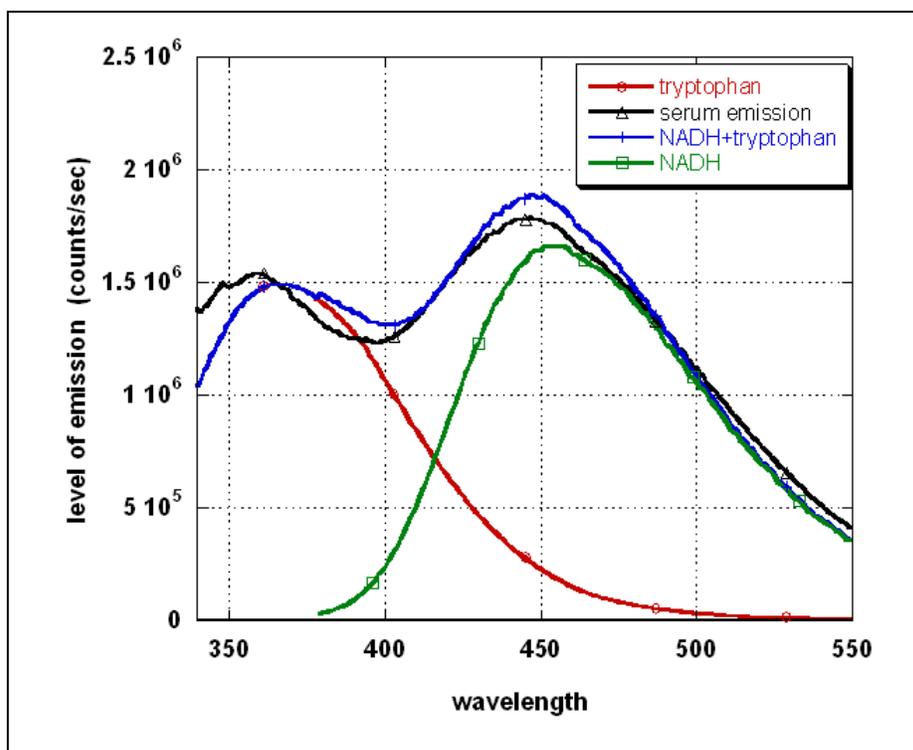


*NAD(P)H Levels using Fluorescence Emission.*

Serum NAD(P)H concentrations were determined for 44 subjects with CFS and 30 healthy controls. NAD(P)H concentrations in the serum of CFS subjects averaged $8.0 \pm 1.4$ (SD) uM while those in healthy controls averaged $10.8 \pm 0.8$ (SD) uM, a statistically significant difference ($p < 0.001$). Frequency distributions for NAD(P)H concentration in CFS patients and healthy controls are shown in Figure 2. While there is some overlap between the two groups in the 9 uM

– 11 nM range, most of the control subjects had NAD(P)H levels above 10 uM while most CFS subjects had NADH levels below 10 uM.



**Figure 2**:  Relative frequency distributions of NAD(P)H concentrations for healthy subjects (boxes with diagonal shading) and CFS patients (closed circles).  Interval widths of 0.5 uM were used.  Data are fit to a Gaussian curve ($y = M \exp (-(x-\alpha)^2/\beta^2)$.

The sensitivity and selectivity of NAD(P)H in matching CFS patients to control subjects is shown in Figure 3.  At a NAD(P)H cut-off of 9.5 uM, for example, the sensitivity is 0.73 and the specificity is 1.0.  A receiver operator characteristic (ROC) analysis (inset in Figure 3) yields an

area under the curve of 0.9. An AUC of 1.0 would represent perfect discrimination (between

CFS and controls), while an AUC of 0.5 would represent no discrimination.
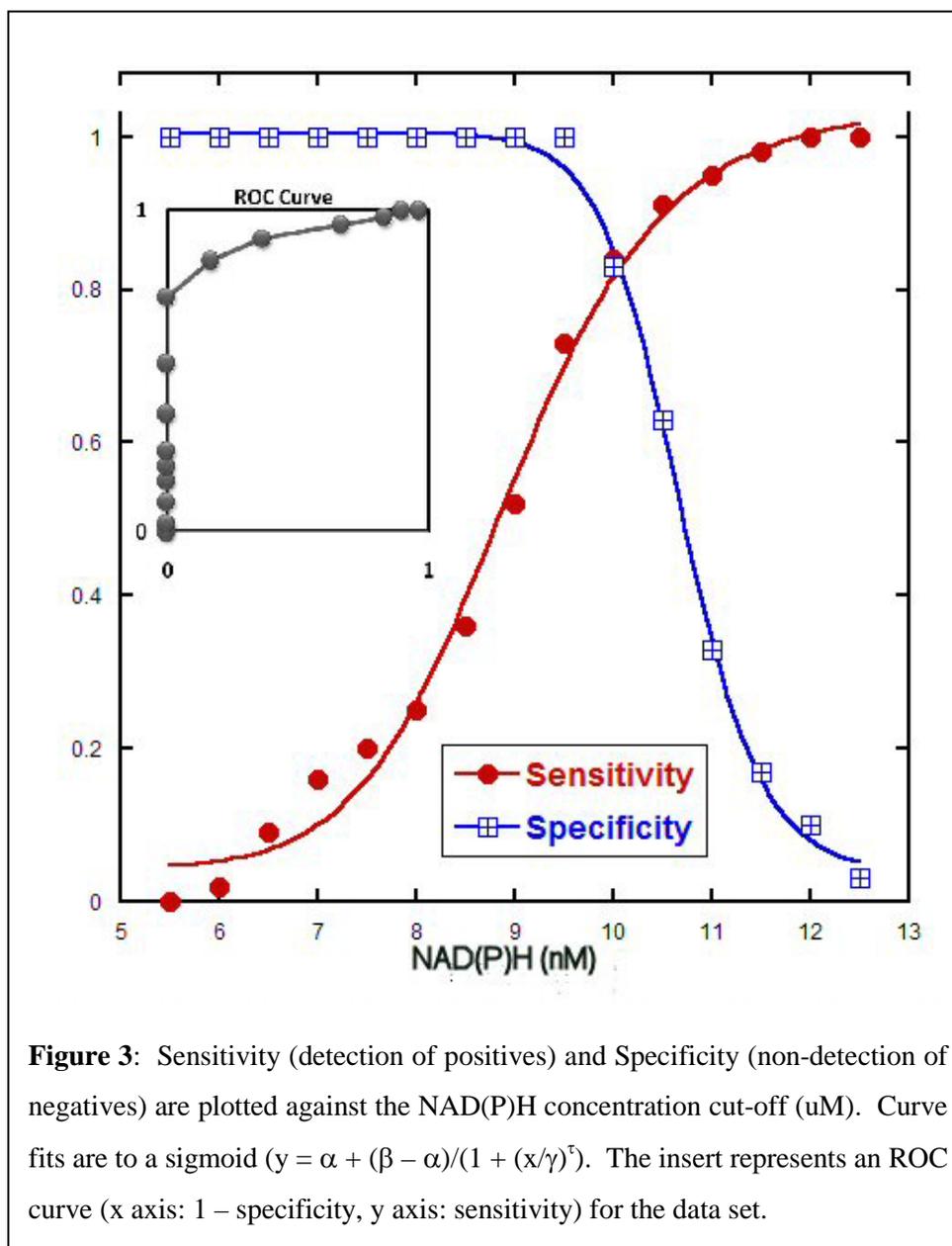


**Figure 3**: Sensitivity (detection of positives) and Specificity (non-detection of negatives) are plotted against the NAD(P)H concentration cut-off (uM). Curve fits are to a sigmoid ($y = \alpha + (\beta - \alpha)/(1 + (x/\gamma)^{\tau})$). The insert represents an ROC curve (x axis: 1 – specificity, y axis: sensitivity) for the data set.

We also compared NAD(P)H concentrations to other endocrine and metabolic parameters. Table

1 shows how averages for patients with CFS compare to normal ranges for each parameter.

Epstein Barr and Candida antibodies were, on average, well beyond the normal range in CFS
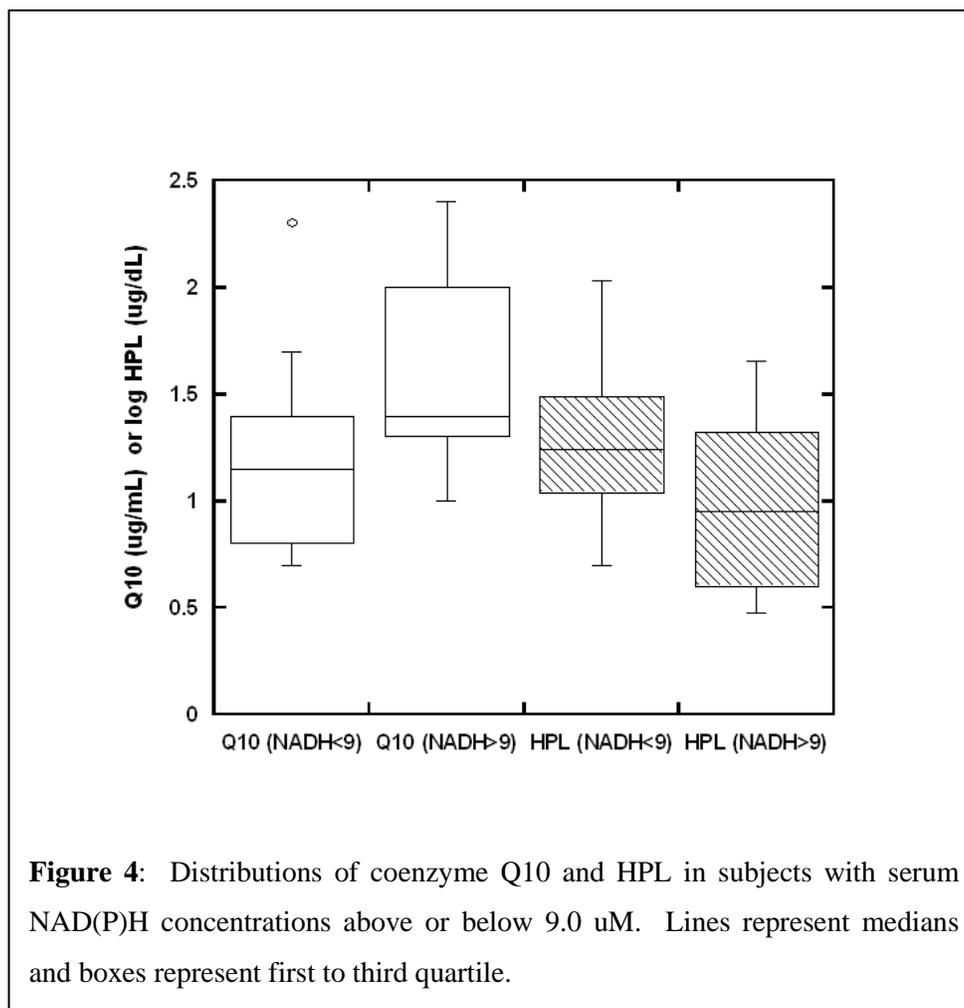
patients, consistent with the idea of an infection component to CFS. In contrast, free T3 levels were usually normal for CFS subjects in this study.

| Parameter | CFS | Lo-Normal | Hi-Normal |
|---|---|---|---|
| NAD(P)H (uM) | 8.8 ± 1.4 | 9.2 | 12.3 |
| Free T3 (pg/mL) | 3.0 ± 0.8 | 1.4 | 3.4 |
| Q10 (µg/mL) | 1.4 ± 0.6 | 1.3 | 2.1 |
| EBV early IgG (AU) | 50 ± 54 | 0 | 20 |
| Candida IgG (EU) | 33 ± 18 | 0 | 12.5 |
| Candida IgM (EU) | 22 ± 11 | 0 | 12.5 |
| Candida IgA (EU) | 28 ± 18 | 0 | 12.5 |
| Hemoglobin | 13.8 ± 1.2 | 12 | 16 |
| HPL ((µg/dL) | 20 ± 24 | 0 | 10 |

**Table 1**: Laboratory test parameters and NAD(P)H concentrations for patients with CFS (Mean ± SD given) compared with normal ranges. In the case of NADH, the normal range is ± 2SD from the control group mean. HPL represents hydroxyhemopyrrolin-2-one and EBV is Epstein-Barr virus.

A factor analysis, based on the correlation matrix between all variables given in Table 1, demonstrated that the best correlations were between the emission of NAD(P)H, serum coenzyme Q10 (r = 0.5, p<0.05) and urine HPL (r = 0.47, p<0.03). Distributions of coenzyme Q10 and HPL for high and low levels of NAD(P)H in serum are shown in Figure 4. As coenzyme Q10 is the component of a complex series of reactions that occur within mitochondria, the function of Q10 ultimately is linked to the generation of energy within the cells. The correlation between the

lower level of coenzyme Q10 and lower NAD(P)H signals for patients with CFS suggests lower



**Figure 4**: Distributions of coenzyme Q10 and HPL in subjects with serum NAD(P)H concentrations above or below 9.0 uM. Lines represent medians and boxes represent first to third quartile.

bioenergetics for these subjects. An inverse correlation was found between the level of serum emission and the level of HPL in urine.

**Discussion**

The measurement of fluorescence of reduced nicotinamide adenine dinucleotide in serum provides a non-invasive assay to estimate metabolism and fatigue levels in CFS patients. NAD(P)H concentrations were significantly lower in CFS patients compared to healthy controls; moreover, ROC analysis demonstrated the sensitivity and specificity of using NAD(P)H levels to

distinguish between CFS and control groups. NAD(P)H concentrations determined from serum fluorescence did not correlate with typical CBC parameters (RBC counts, WBC counts, hemoglobin, hematocrit, etc.), but do correlate with coenzyme Q10 (positive correlation) and urine HPL (negative correlation). These correlations are instructive, as all three variables can be thought of as metabolic markers. Coenzyme Q10 is essential for ATP production, and it exists in mitochondria in quantities ten-fold greater than those of other redox components. It is likely that decreases in serum NAD(P)H and Co-Q10 both indicate lower bioenergetics in CFS patient cells. HPL, on the other hand, is often elevated in patients with mental illness [50 – 52]. It is from subclass of monopyrroles, well known for bio-toxicity, and elevated HPL excretion classically associated with emotional stress [52]. Urine HPL may result from aberrations in porphyrin metabolism (as in conditions such as iron deficiency, unstable hemoglobin, RBC hemolysis). Since heme is tightly coupled to neuronal metabolic activity, heme depression may decrease of neuronal activity.

Our data supported by the measurements of metabolites in urine of CFS [43]. According to this study, the analysis of the metabolites excreted in urine of CFS patents demonstrated that the best molecule for separation of this state was aminohydro-N-methylpyrrolidine (referred as CFS urinary marker 1). The structure of this metabolite has similarity with HPL metabolite in urine that was measured in our study for CFS patients. Authors of the study [43], based on comparison of the CFS urinary marker 1 with neuroactive drug 3-amino-1-hydroxypyrrolidin-2-one (HA-966), which alter neurotransmitter activity and hypothalamic–pituitary-adrenal axis [53, 54], concluded that compounds with this structure may change the neuron metabolism or receptor function and may alter excitatory/inhibitory neurotransmission.

 As a result of our study, we proposed that fatigue level and metabolic slowdown in CFS patients can be evaluated and monitored by serum NAD(P)H concentration measurements. Following patient NAD(P)H levels over time may aid in selecting therapeutic strategies and monitoring treatment outcome. Future studies with more subjects, and with subjects being followed over time, are recommended.

 In addition, our previous studies demonstrated that the same procedure can be used for the evaluation of the level of fatigue in other pathological conditions, for example in cancer [45]. For cancer patients fatigue is one of the debilitating side effects of cancer and its treatment. We found that for cancer patients who complain on fatigue and exhaustion, the level of NAD(P)H in serum was lower than the normal range, but etiology was different. In these cancer patients, reduced NAD(P)H correlated with reduced RBC counts and hemoglobin, suggesting anemia as a cause.

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