



# REPORT DOCUMENTATION PAGE

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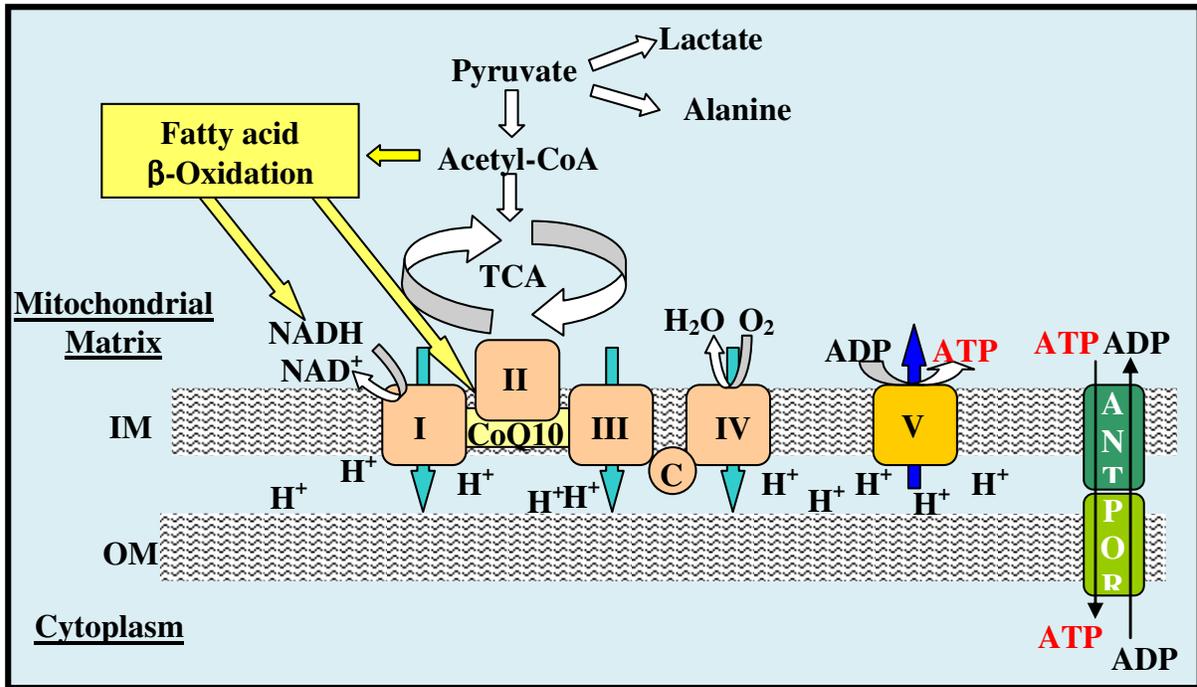
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## **Introduction:**

Gulf War syndrome (GWS) is associated with increased incidences of amyotrophic lateral sclerosis, pain syndromes, muscle complaints that include fatigue and myalgias, as well as other neurological symptoms. Approximately 100,000 individuals have medical complaints consistent with GWS. Clinical manifestations are similar to those identified in Chronic Fatigue Syndrome (CFS). Mitochondrial defects are identified pathologically, metabolically, and genetically in some patients with CFS. GWS has significant evidence for mitochondrial dysfunction with abnormalities in exercise physiology, abnormalities in mitochondrial morphology, biochemical defects in mitochondrial function, abnormalities in free radical generation affecting mitochondrial integrity, gene expression in genes affecting mitochondrial function, and mtDNA mutations (inherited, somatic, and sporadic during embryogenesis). Gene expression abnormalities in CFS show abnormalities in genes that are related to mitochondrial function. Hence, investigation of mitochondrial dysfunction in GWS is a priority.

Mitochondria are cytoplasmic structures with an inner and outer membrane separated by an intermembrane space. OXPHOS uses about 95% of the oxygen delivered to tissues, producing most of the ATP required by cells. Expression of genes involved in the OXPHOS pathway and the assembly of the five OXPHOS enzyme complexes Complex I (CI), Complex II (CII), Complex III (CIII, CIV and CV) within the inner mitochondrial membrane is a highly ordered and coordinated process directed by 37 genes in the mtDNA and as many as 1,500 genes in the nDNA. [1, 2] Optimal OXPHOS function requires aggregation of individual OXPHOS enzymes into supercomplexes. [3-7] Supercomplexes allow efficient formation of an electrochemical (proton) gradient created by CI, CIII, and CIV that is then used by CV to synthesize ATP (For review see [8]). Mitochondrial dysfunction causes over 50 pediatric and adult diseases. Patients with mitochondrial diseases can exhibit fatigue and myalgias as their only symptoms. Genetic defects producing mitochondrial dysfunction include: (1) inherited mutations in nDNA or mtDNA genes. (2) Sporadic mutations occurring during embryogenesis that are systemic or confined to specific tissues such as skeletal muscle. (3) Somatic mutations occurring through life due to aging, free radical damage, and exposure to environmental toxins or certain medications. Defects in OXPHOS have a broad array of cellular consequences including abnormal cellular calcium ( $Ca^{2+}$ ) regulation, impaired ATP generation, enhanced apoptosis, and increased free radical production. [9-12] In fibroblast cell lines harboring pathogenic mutations in CI genes, CI dysfunction causes depolarization of the mitochondrial membrane potential, resulting in a decreased supply of mitochondrial ATP to the  $Ca^{2+}$ -ATPases that control intracellular  $Ca^{2+}$  stores.  $Ca^{2+}$  content of these stores is then reduced, particularly in the endoplasmic reticulum. [13] Defects in any of these functions can lead to disease.

Interestingly, our experience to date with physicians in the Veteran's Administration Medical Centers consistently demonstrate negative attitudes or ambivalence toward veterans who have a diagnosis of Gulf War Syndrome as well as research in this area. There is a great deal of work needed to educate physicians in the VAMC about evaluation of patients with symptoms such as those associated with GWS. For example, none of the GWS surveyed to date have had exercise testing to quantitate and assess the physiologic basis of their fatigue.



**Figure 1. Overview of relationship between OXPHOS and beta-oxidation of fatty acids within the mitochondria. OXPHOS enzymes (CI-V) are located in the inner mitochondrial membrane. Beta-oxidation occurs in the matrix. Protons (H<sup>+</sup>) are actively pumped into the space between the inner membrane (IM) and outer membrane (OM). The electrochemical gradient is used to generate ATP by CV (ATP synthase).**

**Abbreviations: ADP: adenosine diphosphate; ANT: adenine nucleotide translocase; ATP: adenosine triphosphate; C (Cytochrome C), CoQ10: coenzyme Q10; FADH<sub>2</sub>: flavin adenine dinucleotide, reduced; O<sub>2</sub>: oxygen; NADH: Nicotinamide Adenine Dinucleotide, reduced; NAD: Nicotinamide Adenine Dinucleotide, oxidized; POR: porin; TCA: Tricarboxylic acid cycle (Kreb cycle).**

**Body:**

YEAR 1 of research (10/13/2009-7/14/2010) (9 months): Human Protection Approval obtained 10/13/2009. During this time period significant efforts were expended on (a) establishing funding distribution, (b) establishing recruitment mechanisms (physician letter, internet, Veteran's Administration Hospital, funding of reimbursement of travel to our facility). In addition, significant progress was made in developing laboratory control ranges needed for interpretation of data on GWS.

YEAR 2 of research (07/14/2010-07/14/2011) (12 months): Significant progress has been made for patient recruitment and analysis of controls and data from patients with chronic fatigue/fibromyalgia syndromes. Analysis of GWS samples is proceeding and is yielding important data for understanding this disorder. The data is summarized below.

***Brief Summary of SOW Tasks:***

**Task 1:** (Specific Aim 1) (Note: HRP Approval Granted 10/13/2009)

Fifty veterans with GWS who have fatigue and myalgias will be identified through the Atlanta Veterans Administration Medical Center and via website postings. Clinical records are requested and reviewed by the P. I. to confirm diagnosis of GWS. If the records are consistent with inclusion criteria, an appointment is made for clinical examination by the P.I., blood draw and skin biopsy. Modified criteria for chronic fatigue syndrome and fibromyalgia will be used as guides for patient inclusion criteria, thus allowing comparison of the GWS patient data with CFS/fibromyalgia patient data.

**Progress:**

1. 16 individuals have signed consent forms for the study and are enrolled.
2. 19 individuals are being evaluated for participation (records reviewed, consent process instituted, etc)

**Task 2:** (Specific Aim 2)

Characterize mitochondrial cellular energetics in GWS patients relative to age and gender matched controls using the following approaches: (1) high resolution respirometry of intact cells [EBV transformed lymphocytes, cultured fibroblasts], (2) quantitative analysis of individual mitochondrial proteins (denatured, Western blot), (3) analysis of intact OXPHOS enzyme complexes and supercomplexes (non-denatured, Blue Native and Clear Native gels), (4) in gel enzyme activity assessment of intact OXPHOS enzyme complexes and supercomplexes (Clear Native gel, in-gel activity measurements), (5) mtDNA copy number quantitation to assess for defects in regulating mtDNA replication, and (6) cellular coenzyme Q10 quantitation (endogenous synthesis is impaired in certain types of mitochondrial dysfunction). GWS data is compared to individuals analyzed in our laboratory who have a diagnosis of chronic fatigue syndrome/fibromyalgia.

**Progress:**

Considerable laboratory progress has been made.

1. We have established controls for laboratory analyses except for the EBV transformed cell lines. This work is underway.
2. We are completing analysis of the chronic fatigue syndrome/fibromyalgia patient population for publication and for comparison with the GWS patients.
3. We are proceeding with analyses on GWS cell lines enrolled in the study.

***Chronic fatigue syndrome/Fibromyalgia patient data (manuscript that will reference this grant is being prepared).***

Data from 59 patients (age range 15-64 years,  $\bar{x} \pm SD = 39 \pm 14.8$  years; 51 female (86.4%), 8 male (13.6%)) who met CDCP criteria for CFS and who met criteria for diagnosis of mitochondrial disease are summarized below. The female: male ratio in this group of patients is 6.4:1, which is similar to the ratio observed in Georgia CFS patients of 4.4:1. [14] Biochemical and genetic diagnosis of mitochondrial disease used the Nijmegen criteria that we modified for

adult mitochondrial disease diagnosis. [15] (Tables 1 and 2) Patients who scored in one of the follow diagnostic categories were classified as having CFS plus mitochondrial disease: (1) Probable, (2) Highly Probable, or (3) Definite. In this classification system, “Definite” is reserved for patients harboring a pathogenic mutation in a nDNA or mtDNA OXPHOS gene. Table 3 gives the distribution of Cardiopulmonary Exercise Testing (CPET), metabolic, and biochemical defects identified in these CFS plus mitochondrial disease patients. These approaches look at cellular energetics by various approaches and are highly correlative in these patients.

**A. CPET Testing in chronic fatigue/fibromyalgia patients:** CPET is essential in the assessment of patients complaining of exercise limitations due to fatigue. An abnormal maximum oxygen uptake (VO<sub>2</sub> max) and anaerobic threshold (AT) significantly increases the likelihood that the patient has mitochondrial dysfunction since these parameters reflect cellular oxidative phosphorylation capacity. [16] These parameters are highly reproducible [16] with approximately 5% variation between testing in the MET-TEST laboratory. CPET is abnormal in a high percentage of patients tested at 76.5% (39/51). All CPET testing used an electronically braked cycle ergometer and was performed by MET-TEST, Inc. ([www.mettest.net](http://www.mettest.net)). [17]

**B. Metabolic Testing in chronic fatigue/fibromyalgia patients:** Metabolic defects are common in this group of patients with mitochondrial disease with abnormal blood lactate, pyruvate, and alanine in 44.1% (26/59), 24.6% (26/59), and 10.2% (6/59), respectively. Samples were taken at rest with care not to produce false elevations during blood draw due to tourniquet use or due to sample processing. Lactate, pyruvate, and alanine elevations in blood are metabolic markers of OXPHOS dysfunction as outlined in Figure 1. With impairment of OXPHOS, pyruvate accumulates and is reduced by lactate dehydrogenase to lactate. When impairment of OXPHOS is more severe, pyruvate is transaminated by alanine transaminase to form alanine.

Additional abnormalities included a small fiber neuropathy in 35% (7/20) and cerebral folate defects. Cerebral folate deficiency (CFD) is characterized by decreased CSF 5-methyltetrahydrofolate (5-MTHF) below 50 nmol/Liter in the context of normal systemic folate metabolism. [18] CFD can occur in up to 40% of all patients with mitochondrial disease. [19] Due to the high prevalence of cerebral folate defects in mitochondrial disease, all study participants will be treated with folic acid 8 weeks prior to entry into the study. Small fiber neuropathies are associated with chronic pain syndromes and appear frequently in mitochondrial diseases associated with pain. [20, 21]

**C. Biochemical Testing:** The etiology of the defects identified by testing described in sections A and B above were confirmed with biochemical analysis that included assessment of OXPHOS function in live tissue (muscle and fibroblasts), OXPHOS enzymology of Complexes I-IV, muscle Coenzyme Q10 levels, analysis of selected OXPHOS subunits from Complexes I-V by Western blot, and by assessment of assembly of individual OXPHOS enzymes as well as supercomplexes. These approaches provide valuable information for investigating the mechanism of OXPHOS dysfunction. Optimal OXPHOS function requires aggregation of individual OXPHOS enzymes into supercomplexes which allows efficient and rapid transport of electrons. [3-7] Supercomplexes allow efficient formation of an electrochemical (proton) gradient created by Complexes I, III, and IV that is then used by CV to synthesize ATP. Failure

to form supercomplexes as well as impaired assembly of individual enzymes (Complexes I-V) is a part of the pathogenesis of many different classes of mitochondrial disease [22-32]. Live muscle that was assessed at the time of muscle biopsy and fibroblast cell lines showed significant defects in OXPHOS function that included abnormal electron transfer between enzyme complexes, reduced ability to generate ATP, and reduced ability to maintain an adequate proton gradient to drive ATP synthesis by CV (ATP synthase). These abnormalities were identified in 45.4% (25/55) of the live muscle samples and in 65% (26/40) of the fibroblasts. The OXPHOS enzymology of Complexes I-IV was abnormal in 94.9% (56/59) of the muscle samples. Impaired supercomplex formation and individual enzyme assembly is one of the main variables influencing the pattern of OXPHOS enzyme dysfunction.

Coenzyme Q10, an endogenously synthesized cofactor for electron transfer, was abnormal in 6.8% (4/59). This abnormality in CoQ10 is not related to dietary intake. These levels are likely related to defects associated with CoQ10 synthesis [33] and significantly impair electron transfer as well as increase mitochondrial degradation by mitophagy. [34] The OXPHOS defects in these patients produced significant impairments in the assembly of supercomplexes in muscle in 57.1% (28/49) and in the assembly and stability of individual OXPHOS enzyme complexes in 40.8% (24/59).

**D. Genetic Testing:** Careful assessment of the mtDNA revealed important categories of defects. MtDNA deletions were increased in 8.8% (5/59) of patients suggesting that genes controlling mtDNA replication and stability (Chromosome 10 open reading frame (C10ORF2), Deoxyguanosine kinase (DGUOK), Mitochondrial inner membrane protein (MPV17), Optic atrophy 1 (autosomal dominant) (OPA1), Polymerase (DNA directed), gamma (POLG1), Polymerase (DNA directed), gamma 2, accessory subunit (POLG2), Ribonucleotide reductase M2 B (TP53 inducible) (RRM2B), Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 (SLC25A4), Succinate-CoA ligase, ADP-forming, beta subunit (SUCLA2), Thymidine kinase 2, mitochondrial (TK2), Thymidine phosphorylase (TYMP) may harbor mutations or that free radical damage is increasing somatic mtDNA mutations. [35, 36] Interestingly, muscle mtDNA copy number was normal in all patients tested to date (59/59). The mtDNA was sequenced in 35 of the patients and revealed two classes of mutations: (a) 34.3% (12/35) of patients with mtDNA mutations requiring more investigation to determine their role in the patient's OXPHOS dysfunction and (b) 14.3% (5/35) of patients with pathogenic mtDNA mutations.

<b>Table 1. BIOCHEMICAL CRITERIA FOR MITOCHONDRIAL DISEASE OR MITOCHONDRIAL DYSFUNCTION</b>	
1.	Abnormal high resolution respirometry in muscle or fibroblasts (measurements <5% reference level) (Live (fresh tissue) assessment of CV function, coupling and protein leak across the mitochondrial membrane)
2.	Abnormal OXPHOS subunit immunohistochemistry or immunofluorescence in skeletal muscle tissue sections (Qualitative assessment of OXPHOS enzyme assembly within tissue sections. Defects in OXPHOS enzyme assembly are readily recognized by this testing.)
3.	Abnormal OXPHOS enzymology (single or multiple enzyme defects) (activity measurements <5% reference level) (Testing must be performed on mitochondria isolated from fresh (not frozen) tissue to minimize risk of artifacts caused by freezing skeletal muscle prior to mitochondrial isolation).

4.	Abnormal quantitative Western Blot of Selected OXPHOS subunits from Complexes I-V (levels <5% reference level for subunit) (Western blot can detect defects that are not evident by other techniques. [37])
5.	Abnormal muscle CoQ10 level (<50% of control mean) (Allows assessment for primary defects in Coenzyme Q10 synthesis. [38])
6.	Supercomplex evaluation: Optimal OXPHOS function requires aggregation of individual OXPHOS enzymes into supercomplexes, which allow efficient and rapid transport of electrons. [3-7] Supercomplexes allow efficient formation of an electrochemical (proton) gradient created by Complexes I, III, and IV that is then used by CV to synthesize ATP. Supercomplex formation is impaired in a variety of OXPHOS diseases. [22, 23, 26, 31, 32, 39-48]
a.	Abnormal supercomplex formation (Score 0.5 if present only in Blue Native OR OXPHOS Clear Native Immunoblot. Score 1 if present in BOTH tests.)
b.	Abnormal monomeric OXPHOS enzyme complex formation (Score 0.5 if present only in Blue Native OR OXPHOS Clear Native Immunoblot. Score 1 if present in BOTH tests.)
7.	Abnormal in-gel OXPHOS enzyme activity. (Qualitative in-gel assessment of OXPHOS enzyme activity in intact enzymes. This is particularly important in assessment of the ATPase activity of CV and CV assembly. [31, 39-42])

#### **Scoring for evaluation of Biochemical Criteria**

**Unlikely: Criteria - 1-7 are normal**

**Possible: Criteria – a single test is abnormal and the rest are normal or equivocal**

**Probable: Criteria- Two tests are abnormal**

**Highly Probable: >2 abnormal tests**

**Table 2. GENETIC CRITERIA FOR MITOCHONDRIAL DISEASE DIAGNOSIS**

1.	mtDNA depletion (decreased mtDNA copy number)
2.	Identification of confirmed pathogenic mtDNA or nDNA mutation
3.	Identification of provisional pathogenic mtDNA or nDNA mutation (i.e. mutation requires additional data supporting pathogenicity)
4.	No mutation identified

#### **Scoring for evaluation of Genetic Criteria**

**Definite: Criterion 1 or 2 is abnormal, Probable: Criteria for 3 are abnormal,**

**Indeterminate: Criterion 4 is present**

*Note: The failure to find a mutation does not exclude mitochondrial disease due to the large number of genes associated with mitochondrial disease and the large number of undiscovered genetic associations.*

**Table 3 Mitochondrial Dysfunction and CFS**

<b>Physiological Testing Summary</b>					
pulmonary Exercise Testing	Abnormal Testing	76.5% (39/51)	Metabolic Testing (Blood)	Abnormal Lactate (> 12 mg/dl)	44.1% (26 /59)
	Abnormal VO2 max			Abnormal	24.6%

	(<85% predicted VO2 max)	76.5% (39/51)		pyruvate (> 0.90 mg/dl)	(14/57)
	Abnormal Anaerobic Threshold (≤50% predicted VO2 max)	78.4% (40/51)		Abnormal Alanine (>563 μmol/L)	10.2% (6/59)
CSF 5- methyltetrahydrofolate	Abnormal (<50)	55.6% (5/9)	Small Fiber Neuropathy	Reduced small fiber nerve counts	35% (7/20)

### Biochemical Testing Summary

High Resolution respirometry (live cells)	Abnormal in muscle	45.4% (25/55)	OXPHOS Enzymology (Complexes I-IV) (muscle)	Abnormal Enzymology  94.9% (56/59)	CI: 16.9% (10/59) CIII: 1.7% (1/59) CI, III: 66.1% (39/59) CI, III, IV: 10.2% (6/59)
	Abnormal in fibroblasts	65% (26/40)			
Muscle CoQ10 level	Abnormal	6.8% (4/59)	OXPHOS Western blot (Selected subunits from Complexes I-V)	Abnormal	40.8% (24/59)
OXPHOS Supercomplex formation	Abnormally reduced formation	57.1% (28/49)	CV (ATPase) activity (Clear Native Gel)	Abnormal in- gel enzyme activity	15.1% (8/53)

### Mitochondrial DNA (MtDNA) Testing Summary

MtDNA Deletion mutations	Mild increase of mtDNA deletions (muscle)	8.8% (5/57)	MtDNA copy number	Muscle	Copy NORMAL normal in 100% (59/59)
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### MTDNA sequencing Data from Chronic Fatigue/Fibromyalgia Patients

<b>Equivocal mutations (significance requires additional investigation to delineate significance) 34.3% (12/35)</b>	<b>Abnormal mutations 14.3% (5/35)</b>
<p>1. 16S ribosomal RNA: 2887 T&gt;C; 0.07% of controls (2/2704); moderate evolutionary conservation; homoplasmic; n=1</p> <p>2. 16S ribosomal RNA: 2010T&gt;C; 0.04% in controls (1/2704); highly conserved.</p> <p>3. ATP synthase F0 subunit 6 (ATP6) gene (CV): 9095 T&gt;C Leucine 190 Proline; 0.11% of controls (3/2704); poor evolutionary conservation; homoplasmic n=2 (Two maternally related patients with CFS)</p>	<p>1. 12S ribosomal RNA gene: 1555A&gt;G [49]</p> <p>2. ND5 Gene (CI): 13057A&gt;G Threonine 241 Alanine; 0.04% in controls (1/2704); High evolutionary conservation; heteroplasmic; n=1</p> <p>3. Two variants in one patient: (a) tRNA-Leucine (UUR) gene: 3264 T&gt;C; 0% in controls (0/2704); high evolutionary conservation; homoplasmic. [50] (b) ND4</p>

<p>4. ND1 gene (CI): 3337 G&gt;A Valine 11 Methionine; 0.18% in controls (5/2704); poorly conserved; homoplasmic. n=1</p> <p>5. ND4L gene (CI): 10704 G&gt;A Valine 79 Isoleucine; 0% in controls (0/2704); Moderate evolutionary conservation, homoplasmic. n=1</p> <p>6. Two variants in one patient: (a) ND6 gene (CI): 14319 T&gt;C, Asparagine 119 Aspartate; 0.04% of controls (1/2704); highly conserved; homoplasmic (b) Cytochrome b (CIII): 15380 A&gt;G, Threonine212 Alanine; 0.07% in controls (2/2704); highly conserved. n=1</p> <p>7. Two variants in one patient: (a) COX3 gene (CIV) 9967T&gt;C, Valine 254 Alanine; 0% in controls (0/2704); highly conserved. (b) ATP6 gene (CV): 8725A&gt;G, Threonine 67 Alanine; 0.04% in control (1/2704); homoplasmic. N=1</p> <p>8. Cytochrome b (CIII): 14871 T&gt;C, Isoleucine 42 Threonine; 0.04% in controls (1/2704); moderately conserved; homoplasmic. N=1</p> <p>9. Cytochrome b (CIII): 15047 G&gt;A Glycine 101 Serine; 0.22% in controls (6/2704); highly conserved, homoplasmic. n=1</p> <p>10. Cytochrome b (CIII): 15011A&gt;G Methionine 89 Valine; 0% in controls (0/2704), poor conservation. N=1</p> <p>11. tRNA-Glutamine gene: 4363 T&gt;C; 0.04% in controls (1/2704); homoplasmic; n=1</p>	<p>gene (CI): 11253 T&gt;C Isoleucine 165 Threonine; 0.37% in controls (10/2704); poorly conserved, homoplasmic. Described in Leber optic atrophy and Parkinson disease. [51, 52]</p> <p>4. ND4 gene (CI): 11253 T&gt;C Isoleucine &gt; Threonine; 0.37% in controls (10/2704); poorly conserved, homoplasmic. Described in Leber optic atrophy and Parkinson disease. [51, 52]</p> <p>5. tRNA Serine (UCN) gene: 7468 C&gt;T, 0% in controls (0/2704); highly conserved. n=1</p>
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**Abbreviations:** VO2 max = maximum oxygen uptake during cycle ergometry exercise.

**GWS data:** Individuals with GWS show similar defects in mitochondrial function as do individuals with chronic fatigue syndrome/fibromyalgia. Data is summarized below (Table 4).

<b>Testing Summary: Table 4</b>	
<b>High Resolution Respiriometry: Assesses OXPHOS function in live cells</b>	
Fibroblasts	<b>ABNORMAL:</b> 2 of 3 patients tested show abnormal oxidative phosphorylation by high resolution respirometry. Both patients showed similar findings with abnormalities in the uncoupling ratio, the net

	routine flux control ratio, and the phosphorylation respiratory control ratio. Abnormal Uncoupling ratio and Net Routine Flux Control Ratio indicates a diminished capacity of the cells to respond to increased ATP demands (i.e. decreased reserve capacity). The abnormal phosphorylation control ratio indicates that a higher proportion of the maximum respiratory capacity is activated to drive ATP synthesis.
<b>OXPHOS Protein Chemistry</b>	
Clear Native Enzymology (Fibroblasts)	<b>ABNORMAL</b> To date all 4 patients tested show abnormal ATPase activity of Complex V. The entire OXPHOS enzyme is migrated into the gel and tested for ATPase activity. Appropriate controls are run with each gel.
Clear Native Immunoblot (Fibroblasts)	Normal analysis of Complexes I, III, IV and V for assembly and supercomplex formation in 2 patients tested to date.
<b>Tissue CoQ10 Levels (Reductions cause mitochondrial defects. CoQ10 is endogenously synthesized and is not affected by diet).</b>	
Leukocyte CoQ10 level	Unremarkable in six tested to date
Fibroblast CoQ10 level	<b>ABNORMAL</b> (<5% reference level) in both patients tested to date (GWS01, GWS02).
<b>Mitochondrial DNA (MtDNA) Testing Summary</b>	
<b>MtDNA Copy Number Assessment (mtDNA depletion testing)</b>	
Leukocytes	Copy NORMAL normal in patients tested. (5/5 tested. Testing is underway on 2 additional patients.)
Fibroblasts	<b>ABNORMAL</b> in 2 of 4 patients tested to date. One patient showed proliferation of mtDNA copy number which can be a compensatory reaction to an impairment of oxidative phosphorylation. A second patient (GWS2) had a DECREASE in mtDNA copy number consistent with depletion of the mtDNA in this tissue. Depletions in mtDNA cause defects in oxidative phosphorylation.

**Task 3:** (Specific Aim 3)

Assess the mitochondrial DNA (mtDNA) from each patient with GWS for mtDNA mutations by whole genome sequencing of leukocyte and skin cell mtDNA. Based on the findings from Specific Aim II, selected nuclear coded OXPHOS genes will be sequenced to assess for mutations that increase susceptibility to GWS.

**Mutations in the mtDNA identified in GWS patients to date**

<b>MtDNA Mutations in GWS: Table 5</b>	
<b>Patient</b>	<b>Mutation summary</b>
GWS01	tRNA Cysteine, 5814 T>C The frequency of this variant in multi-ethnic control populations is very low at 0.37% (10/2704). This mutation has been associated with mitochondrial encephalopathy in the following publications (Manfredi,

	G.,et al (1996). Human Mutation 7 (2): 158-163; Neuromuscul Disord 1997;7(3):156-9).
GWS01	ND5 (Complex I gene) 13924 C>T (Pro530Ser) This mutation has not been reported in control populations (0/2704). This amino acid position is strictly evolutionarily conserved which is an important criteria for pathogenicity. PolyPhen-2 analysis shows that this mutation is likely damaging to the ND5 polypeptide.
GWS02	ND4L (Complex I gene) 10704 G>A Valine (GTC) 79 Isoleucine (ATC). The frequency of this variant in multi-ethnic control populations is 0% (0/2704). This amino acid is moderately evolutionarily conserved.
GWS04	ND1 (Complex I gene) 3865 A>G (Ile187Val). The frequency of this variant in multi-ethnic control populations is 0% (0/2704). This amino acid is moderately evolutionarily conserved which is an important criteria for pathogenicity.
GWS05	COX3 (Complex IV) 9525 G>A (Ala107Thr) This mutation is not evolutionarily conserved. However, PolyPhen-2 analysis demonstrates that it may be damaging to the polypeptide.
GWS05	COX3 (Complex IV) 9804 G>A (Ala200Thr) This variant was originally identified in patients with Leber hereditary optic neuropathy (LHON) (at a higher frequency than controls; Biochemical and Biophysical Research Communications. 196 (2): 810-815; 1993). The role of this mutation in producing a disease is controversial (J Med Genet 2002;39:162–169; Hum Mutat. 2009 Jun;30(6):891-8).

**Progress:**

1. We have changed our laboratory approaches from capillary sequencing (Sanger sequencing) to Next Generation sequencing.
2. GWS samples are banked so that all can be run more efficiently by Next Generation Sequencing approaches.

**Key Research Accomplishments:**

1. The clinical features of the GWS patients are indistinguishable from CF/F syndromes.
2. OXPHOS Defects are an important cause of symptoms in patients with chronic fatigue/fibromyalgia (CF/F) syndromes.
3. CF/F patients (35%) have small fiber neuropathies. Small fiber neuropathies are important causes for sensory abnormalities (including pain) experienced by these patients. Although this study does not address this abnormality in GWS, the clinical features of the patients are indistinguishable from CF/F syndromes. Patients with GWS are likely to have a high incidence of small fiber neuropathy as well.
4. CF/F patients (55.6%) have cerebral folate defects. This is an important cause for cognitive symptoms that can respond to treatment with leucovorin (folinic acid). Although this study does not address this abnormality in GWS, the clinical features of the patients are indistinguishable from CF/F syndromes. Patients with GWS are likely to have a high incidence of cerebral folate defects as well.

5. 6.8% of CF/F syndrome patients have tissue deficiencies in CoQ10. This abnormality is observed in GWS patients. This defect can be treated with high levels of coenzyme Q10 treatment and is often associated with clinical improvement.
6. 65% of CF/F syndrome patients have defects in high resolution respirometry of fibroblasts. These same defects are observed in the GWS patients.
7. To date, mtDNA depletion has been confirmed in fibroblasts from one of the GWS cases. This is a significant defect that is associated with significant mitochondrial dysfunction. This same patient had abnormal high resolution respirometry in fibroblasts as well as a decrease in fibroblast CoQ10.
8. 15.1% of the CF/F syndrome patients show defects in the ATPase activity of Complex V. To date, each GWS patient tested shows this defect. This type of defect may directly affect ATP production by oxidative phosphorylation.
9. We have identified gene mutations in the mtDNA in the GWS patients that are similar to those observed in CF/F syndrome patients. In addition these mutations may account for or contribute to the clinical manifestations observed in GWS. Assessment of the mtDNA in GWS appears to be important in understanding the biochemical defects. Given the categories of biochemical defects (decreased CoQ10 and mtDNA depletion), nuclear gene mutations are likely.

#### **Reportable Outcomes:**

1. We are in the progress of preparing a manuscript for clinical and laboratory data of patients with chronic fatigue syndrome/fibromyalgia which will reference this grant.
2. While more data is required on GWS patients, the results to date are consistent with the hypothesis in the grant proposal linking GWS with mitochondrial defects.

#### **Conclusion:**

The data summarized above demonstrates the importance of mitochondrial defects in GWS as well as CF/F syndrome patients. In addition this data outlines important similarities between the two population of patients. Both share common pathologic processes.

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## Appendices

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