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14. ABSTRACT Approximately 100,000 individuals have medical complaints consistent with Gulf War syndrome (GWS). Clinical manifestations of GWS are similar to those identified in Chronic Fatigue Syndrome (CFS) and mitochondrial defects are identified pathologically, metabolically, and genetically in some patients with CFS. In order to better understand and manage patients with GWS, the investigation of mitochondrial dysfunction in this syndrome is an important undertaking. During Year 3 of this grant, we have completed the validation of all laboratory testing that will be utilized and have had fifteen new subjects participate in the study. We have also been granted a no-cost extension to continue our study through July 2013. This extension will enable us to gather sufficient data to draw meaningful conclusions. The preliminary data support the hypothesis that important similarities exist between CFS and GWS patients.					
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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 of GWS are similar to those identified in Chronic Fatigue Syndrome (CFS) and 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. Oxidative phosphorylation (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 nuclear DNA (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 patients surveyed to date have had exercise testing to quantitate and assess the physiologic basis of their fatigue.

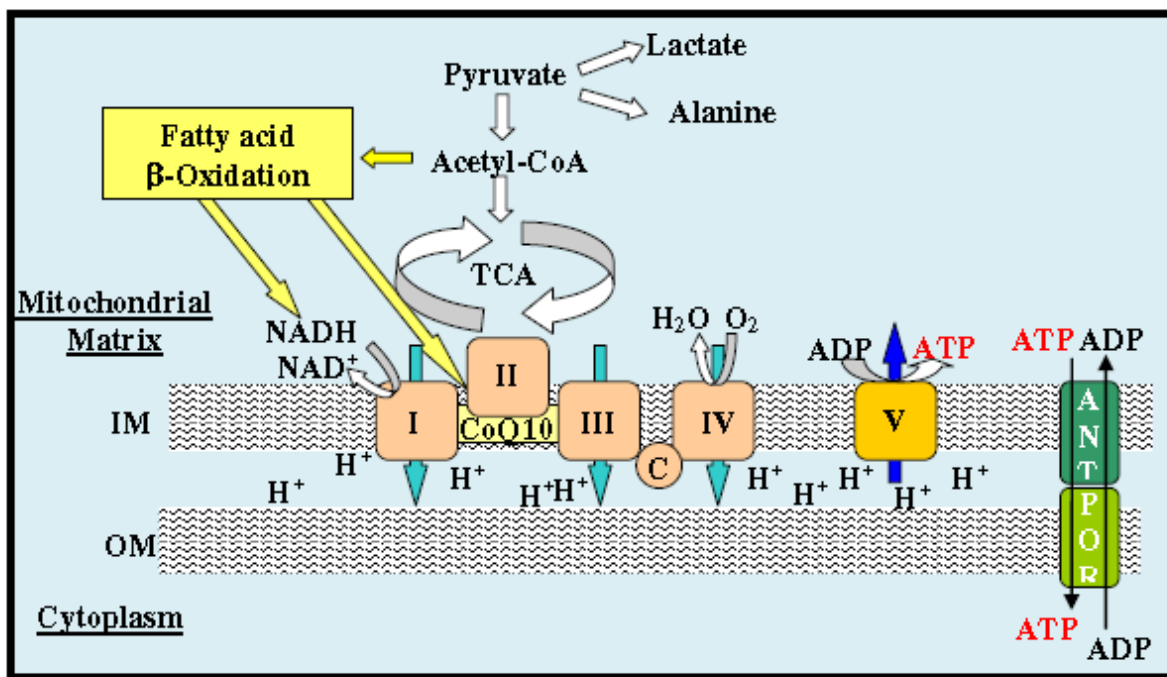


Figure 1. Overview of relationship between OXPHOS and beta-oxidation of fatty acids within the mitochondria. OXPHOS enzymes (CI-V) are located in the inner mitochondrial membrane. Beta-oxidation occurs in the matrix. Protons (H^+) are actively pumped into the space between the inner membrane (IM) and 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; FADH₂: flavin adenine dinucleotide, reduced; O₂: 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 was made for patient recruitment and analysis of controls and data from patients with chronic fatigue/fibromyalgia syndromes. Analysis of GWS samples was initiated and began yielding important data for understanding this disorder.

YEAR 3 of research (07/14/2011-07/14/2012) (12 months): All laboratory testing that will be performed in the completion of this grant has been validated. Additionally, we have made significant progress in enrolling subjects. Fifteen participants have completed the study during the past year and fifteen more are enrolled in the study and have been or are being scheduled for participation. Due to the time constraints that exist with receiving and reviewing medical records for these subjects and with scheduling them to be seen in clinic, we asked for, and received, a no-cost extension for this grant. This extension extends the grant period through July 14, 2013 and will enable us to collect sufficient data to draw meaningful conclusions from our study.

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 are being identified through various approaches that include notification of Veterans Administration Medical Centers (VAMC) and 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 are used as guides for patient inclusion criteria, thus allowing comparison of the

GWS patient data with CFS/fibromyalgia patient data. Based on published percentages of study groups showing evidence for mitochondrial defects, we predict that approximately 60-70% of GWS patients will harbor mitochondrial defects.

Progress during Year 3:

Recruitment is in the final stages in order to ensure that subject participation and sample analysis can be completed before the extended grant end date.

a. Website Registration

- i. The study is registered at ClinicalTrials.gov (identifier: NCT01264471; Registration date 12/20/2010)

- ii. The study is registered at the following sites:

<http://www.gulfweb.org/>

<http://www.ngwrc.org/>

<http://www.facebook.com/#!/groups/Gulfwarvet/>

<http://www.facebook.com/#!/groups/322000610955/>

http://www.immed.org/illness/gulfwar_illness_research.html

<http://cdmrp.army.mil/gwirp/default.shtml>

<http://www.desert-storm.com/soldiers/gwsmmap.html>

<https://www.facebook.com/#!/groups/90924811301/>

<https://www.facebook.com/#!/groups/149325998478946/>

- b. We obtained IRB approval to use an outside funding source to supply funds for travel to Atlanta for study participation. (Travel expenses are NOT charged to the DOD grant).

c. Recruitment of subjects:

- i. 22 individuals have completed the study (**15 during Year 3**)
- ii. 15 additional individuals have signed consent forms and are enrolled. These individuals have already been or are being scheduled for clinic visits.
- iii. 29 individuals have are being evaluated for participation. These 29 individuals have symptoms compatible with enrollment criteria and their health records have been or are being obtained for eligibility assessment.

- iv. Ten individuals have been disqualified since the last update due to exclusionary factors in their records.

Task 2: (Specific Aim 2)

To 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).

Progress during Year 3:

1. All analyses are established in our laboratory on muscle. These techniques have now been adapted for fibroblasts and EBV transformed lymphocytes for use with this grant. The status of each area of testing is outlined below.
 - a. High Resolution Respirometry
 - i. Fibroblast High Resolution Respirometry: 5%-95% reference intervals are validated for the parameters required for assessment of mitochondrial function. Reference ranges are established for the following parameters: uncoupling ratio, net routine flux control ratio, respiratory control ratio, leak flux control ratio, phosphorylation respiratory control ratio. Sample testing is currently underway and data analysis has begun. Of the participants that have been analyzed to date, 46% (6/13) have abnormal fibroblast respirometry results. This is nearly identical to the numbers that we see in patients with chronic fatigue

syndrome/fibromyalgia (47% = 35/74). As we analyze the data from the other testing that is being performed, we should be able to elucidate the mechanisms leading to these abnormalities.

- ii. High Resolution Respirometry on EBV transformed cell lines: 5%-95% reference intervals are validated for the parameters required for assessment of mitochondrial function. Reference ranges are established for the following parameters: uncoupling ratio, net routine flux control ratio, respiratory control ratio, leak flux control ratio, phosphorylation respiratory control ratio. These are shown in Table 1 below. Sample testing is currently underway and data analysis has begun.

Table 1: High resolution respirometry normal reference intervals for EBV transformed lymphocytes.

	5%	25%	Mean	75%	95%
Uncoupling Ratio	1.319	1.796	2.046	2.296	2.773
Net Routine Flux Control Ratio	0.316	0.350	0.373	0.397	0.430
Respiratory Control Ratio	7.10	9.28	10.79	12.30	14.47
Leak Flux Control Ratio	0.064	0.083	0.097	0.110	0.129
Phosphorylation Respiratory Control Ratio	0.218	0.253	0.277	0.301	0.335

- b. Western Blot (denatured oxidative phosphorylation subunit) Quantitative analysis of individual mitochondrial proteins. The technique has been established and validated for muscle, fibroblasts, and EBV transformed lymphocytes. We have developed more accurate approaches based on modification of the mitochondrial isolation procedure (immunocapture of mitochondria). After considerable adjustment of technique, we have very comparable results between a muscle mitochondria and fibroblast mitochondria. A comparison is shown in Figure 1. Sample testing is currently underway and data analysis is in progress.

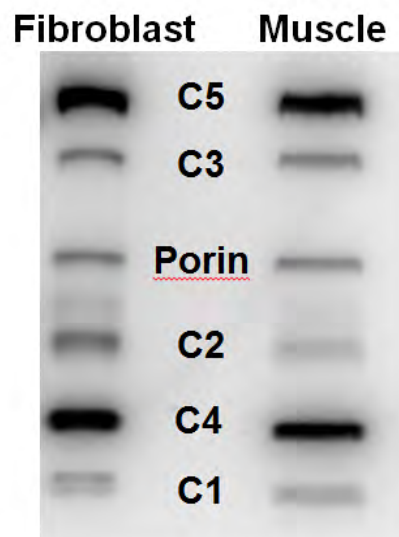


Figure 1. Representative Western Blot of selected mtDNA or nDNA coded subunits from Complexes I-V. Subunits tested: C1=ND6 subunit, Complex I, mtDNA coded; C2=30kDa subunit of Complex II, nuclear DNA coded; C3=core 2 subunit of Complex III, nuclear DNA coded; C4=subunit II of Complex IV, mtDNA coded; C5= F1 Alpha subunit of Complex V; nuclear DNA coded. The outer mitochondrial membrane porin is included for normalization of mitochondrial proteins.

- c. Blue Native and Clear Native Analyses (NON-denatured analysis of supercomplex formation and monomeric oxidative phosphorylation enzyme assembly). These approaches assess supercomplex formation and monomeric oxidative phosphorylation enzyme assembly. The process is validated for skeletal muscle and EBV transformed lymphocytes. GWS sample testing is currently underway and data analysis is in progress.

Despite initial data in fibroblasts indicating low mitochondrial protein concentration, we have begun the use of a glycolysis inhibition media during cell culture in order to upregulate these proteins and enhance visualization of the supercomplex formation and monomeric oxidative phosphorylation enzyme assembly on gels. Preliminary data with cells grown in these conditions looks promising.

- d. Clear Native Oxidative Phosphorylation Enzyme activity: This technique assesses activity of individual oxidative phosphorylation enzymes. Intact oxidative phosphorylation enzymes are isolated by gel electrophoresis and the activity assessed (as isolated enzymes). Sample testing is currently underway and data analysis is in progress. Some preliminary data is shown in Figure 2.

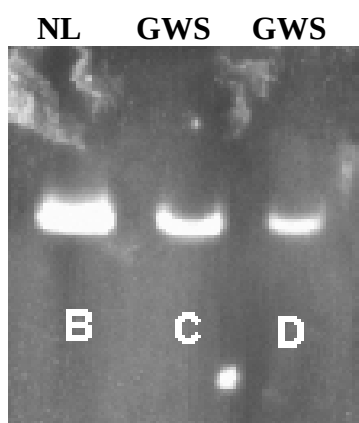


Figure 2. Clear Native oxidative phosphorylation enzyme activity analysis in fibroblasts. One control sample (B) and two GWS samples (C and D) are shown. Complex V ATPase activity appears compromised in the two GWS samples. NL = normal control

- e. mtDNA Copy Number Analysis: This technique is well validated for muscle and we have now established reference ranges in uncultured skin cells, fibroblasts, and EBV transformed cell lines. Sample testing is currently underway and data analysis is ongoing. Preliminary data suggest that a number of GWS patients may have depleted mtDNA copy numbers in their fibroblasts (<5% level). As we carefully evaluate the data from all of our testing, we will be able to better determine the significance of this finding.

- f. Cellular Coenzyme Q10 quantitation in fibroblasts and EBV transformed lymphocytes: Reference ranges have been established for these assays. Sample testing is currently underway and data analysis is being performed. The variability of CoQ10 levels in uncultured skin cells has made reference ranges difficult to interpret and these samples are not felt to be helpful in the assessment of GWS. The measurement of CoQ10 levels in CULTURED skin cells is proceeding.

Task 3: (Specific Aim 3)

To assess the mitochondrial DNA (mtDNA) from each patient with GWS for mtDNA mutations by whole genome sequencing of leukocyte and fibroblast 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.

Progress during Year 3:

1. Sequencing of fibroblast and leukocyte mtDNA from participants is underway and data analysis is in progress. As is commonly seen in patients with chronic fatigue syndrome/fibromyalgia, many rare or novel variants that have characteristics of pathogenic mutations have been detected in several of the GWS participants. These variants are summarized in Table 2. Interestingly, a few of the variants have been detected in only one tissue type (i.e., detected in fibroblasts and not in leukocytes or visa-versa) and several appear to be heteroplasmic from the sequencing data. Both of these properties can be characteristic of pathogenic mutations. The apparent heteroplasmy is currently being investigated by a restriction fragment length polymorphism (RFLP) approach.
2. We have augmented our sequencing approaches with Next Generation sequencing (NGS) and we have developed a NGS approach to assess key genes of cellular energetics.
3. Samples have been banked and selected genes based on the findings from Aim II will be assessed by NGS.

Table 2. mtDNA variants of possible significance detected in GWS participants.

Participant	Variant	Gene	Frequency (#/2704)	Homology	Additional Comments
GWS01	5814 T>C	tRNA Cysteine	10 (0.37%)	moderately conserved	This variant 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	7804 A>G, p.Leu73Leu	COX2	1 (0.04%)	N/A	This variant appears to be heteroplasmic in both leukocytes and fibroblasts. Mutations that do not alter an amino acid may be difficult to assess since they may produce abnormalities in structures NOT assessed by conventional analysis paradigms (e.g. mRNA expression and processing) (Science 2006:314 (5807):1930-1933).
GWS01	13924 C>T, p.Pro530Ser	ND5	4 (0.15%)	highly conserved	PolyPhen-2 predicts this variant to probably be damaging to the ND5 polypeptide structure and/or function.
GWS02	10704 G>A, p.Val79Ile	ND4L	0 (0%)	highly conserved	none
GWS04	3865 A>G, p.Ile187Val	ND1	1 (0.04%)	highly conserved	none
GWS04	2623 A>G	16s rRNA	0 (0%)	highly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending.
GWS04	11819 C>T, p.Leu354Leu	ND4	0 (0%)	N/A	This variant appears to be heteroplasmic in leukocytes ONLY. It was not detected in the fibroblast sample. Mutations that do not alter an amino acid may be difficult to assess since they may produce abnormalities in structures NOT assessed by conventional analysis paradigms (e.g. mRNA expression and processing) (Science 2006:314 (5807):1930-1933).
GWS05	9525 G>A, p.Ala107Thr	COX3	1 (0.04%)	poorly conserved	Both SIFT and PolyPhen-2 predict this variant to possibly be damaging to the COX3 polypeptide structure and/or function.
GWS05	9804 G>A, p.Ala200Thr	COX3	8 (0.30%)	highly conserved	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).
GWS06	2220 A>G	16s rRNA	3 (0.11%)	poorly conserved	none
GWS07	13468 C>A, p.Leu378Met	ND5	0 (0%)	highly conserved	Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND5 polypeptide structure and/or function.

Table 2. mtDNA variants of possible significance detected in GWS participants (continued)

Participant	Variant	Gene	Frequency (#/2704)	Homology	Additional Comments
GWS09	2636 G>A	16s rRNA	0 (0%)	strictly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending.
GWS09	15612 G>A, p.Ser172Leu	CYTB	0 (0%)	poorly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending.
GWS15	2557 C>T	16s rRNA	0 (0%)	poorly conserved	none
GWS15	14831 G>A, p.Ala29Thr	CYTB	5 (0.18%)	poorly conserved	This variant has been reported in association with LHON (Biochemical and Biophysical Research Communications (2002) 295 (2): 342-347), however, more recent data supports its status as a neutral polymorphism (American Journal of Human Genetics (2006) 78 (3): 487-497; Molecular Vision (2007) 13: 1516-1528; Human Mutation (Online) (2009) 30 (6): 891-898).
GWS16	914 A>G (appears heteroplasmic)	12s rRNA	0 (0%)	highly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending.
GWS16	7962 T>C, p.Leu126Ser	COX2	0 (0%)	poorly conserved	This variant is predicted by PolyPhen-2 to be damaging to the COX2 polypeptide structure and/or function.
GWS16	12788 C>T, p.Ser151Phe	ND5	0 (0%)	strictly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending. Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND5 polypeptide structure and/or function.
GWS16	12789 C>T, p.Ser151Ser (appears heteroplasmic)	ND5	0 (0%)	N/A	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in the leukocyte sample. Mutations that do not alter an amino acid may be difficult to assess since they may produce abnormalities in structures NOT assessed by conventional analysis paradigms (e.g. mRNA expression and processing) (Science 2006;314 (5807):1930-1933).
GWS16	14607 G>A, p.Pro23Ser (appears heteroplasmic)	ND6	0 (0%)	strictly conserved	This variant appears to be heteroplasmic in fibroblasts ONLY. It was not detected in leukocytes. RFLP analysis is pending. Both SIFT and PolyPhen-2 predict this variant to be damaging to the ND6 polypeptide structure and/or function.

Key Research Accomplishments:

1. While preliminary data analysis is ongoing, most analysis for the Gulf War Syndrome patients will take place during the last 3-4 months of the study when we have sufficient data to draw meaningful conclusions. Comparison of the Gulf War Syndrome data with appropriate normal controls and disease groups is essential for interpretation of the data. We are analyzing data from patients with known pathogenic mutations affecting oxidative phosphorylation as well as patients with chronic fatigue/fibromyalgia diagnoses. Comparison of data from these groups with the Gulf War Syndrome patients is important.
 - a. We have made considerable progress in characterizing the fatigue-myalgia population that is being used for comparison with GWS. We have performed detailed characterization of approximately 110 individuals with fatigue-myalgia. The characterization is clinical, metabolic, biochemical, and genetic.
2. Since our last update, it is still true that, to date, the data suggest that patients with GWS have a similar cellular energetics profile as those patients with chronic fatigue/fibromyalgia who harbor oxidative phosphorylation defects. As we continue to analyze the data, there appears to be no difference in the fatigue-myalgia patients and the GWS patients.
3. This observation is very important since it implies that these patients with GWS are at increased risk for developing cerebral folate defects as well as small fiber neuropathies. Cerebral folate defects are treatable metabolic defects.

Progress during Year 3:

An essential aspect of the Gulf War Syndrome patient analysis is comparison of data with patients who harbor known mitochondrial mutations (nuclear DNA or mtDNA) as well as patients who have chronic fatigue or fibromyalgia. We are continuing to work on the three manuscripts that will reference this grant mentioned in the last quarterly update. Of significance, more recent data has led to expansion of the second manuscript to include mutations other than Complex V (ATP synthase). The topics of each manuscript are summarized below. As evaluation of these data sets are completed, the results will be included in the DOD reports.

1. The first manuscript focuses on the characteristics of patients who harbor mutations in SURF1 (an assembly factor for Complex IV). This paper is directly relevant to this grant

because it further defines how the types of changes that are observed in patients with OXPHOS defects are caused by various mechanisms.

2. The second manuscript is a detailed assessment of the supercomplexes in patients who harbor mutations in both nuclear and mitochondrial DNA. This paper is directly relevant to this grant because it further defines how the types of changes that are observed in patients with OXPHOS defects by various mechanisms affect these techniques. Both of these papers are essential to assessing the complex data sets anticipated in the Gulf War Syndrome patients.
3. The third manuscript describes over 100 patients diagnosed with chronic fatigue and myalgia by the techniques used in this grant. This group is essential to characterize and compare with the Gulf War Syndrome patients as discussed above.

Reportable Outcomes:

1. We are in the process 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 are required on GWS patients, the results to date are consistent with the hypothesis in the grant proposal linking GWS with mitochondrial defects.

Conclusion:

During Year 3, we have completed the laboratory validation of all assays for all tissue types that will be utilized in this grant. Additionally, we have had fifteen new participants (for a total of 22). These numbers are approaching levels where we will be able to draw meaningful conclusions from our data. The no-cost extension that we received will allow us to evaluate a sufficient number of subjects. The preliminary data summarized above demonstrates the importance of mitochondrial defects in GWS patients. These data also outline important similarities between chronic fatigue/fibromyalgia and GWS patients and support the hypothesis that both share common pathologic processes.

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Appendices

None.