AWARD NUMBER: W81XWH-15-1-0163

TITLE: An Integrated Genomics and Cell Biology Approach to Correlate Novel GWI Indicators of Infections and Neuroinflammatory Mechanisms with Targeted Drug Therapy

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REPORT DATE: MARCH 2019

TYPE OF REPORT: Final report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
MARCH 2019	Final report	1 Jul 2015 - 31 Dec 2018

4. TITLE AND SUBTITLE

An Integrated Genomics and Cell Biology Approach to Correlate Novel GWI Indicators of Infections and Neuroinflammatory Mechanisms with Targeted Drug Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-15-1-0163

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Nova Southeastern University
3301 College Avenue, Fort Lauderdale, FL
33314-7796

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Gulf War Illness (GWI) is a complex condition that is associated with variety of symptoms including fatigue, headache, memory problems, muscle and joint pain, gastrointestinal issues, neurological problems, hormonal imbalance and immune dysfunction. The condition appears to affect both men and women who were deployed to the GW, with up to one-third of these affected veterans remaining ill today. Currently, little is known about what causes the onset and progression of the disease. Therefore, diagnosis is made based on a process of elimination and treatment involves symptom management, rather than targeting the underlying mechanism of the disease. While little is known about the inner workings of GWI, recent data has suggested that the condition is likely caused by a combination of factors including an individual's genomic make-up and environmental exposures, such as toxic chemicals and/or illnesses. Further exacerbation or chronic stress exposure during and after war-time may also contribute to aggravation and persistence of GWI. The goal of our research efforts is to develop a better understanding of genomic characteristics as well as alterations in cellular mechanisms that contribute to the development of the disease. Identification of multiple disease targets will lead to designed therapeutic intervention. Specifically, our more detailed understanding of the dysfunction involved in GWI would greatly speed up the identification of promising biomarkers to help improve diagnosis and treatment of GWI.

There are two components to our research efforts: 1. genomic evaluation and 2. uncovering cellular mechanisms. In an effort to understand what is occurring on a genomic level, we aim to understand the changes that occur in RNA as well as in DNA activation/deactivation in individuals with GWI as compared to their healthy

counterparts exposed to similar war-time stressors. This will provide us with a better understanding of how changes in an individual's genomic make-up cause them to develop the illness versus those who remain healthy today.

While it is important to gain an understanding into what specific genes change their expression in the onset and progression of GWI, it is equally important to understand why these genes change their expression. Therefore, on a cellular level, we want to understand how the presence of prior and current illnesses can lead to the onset and progression of GWI as well as potentially alter specific mechanisms that are vital for cell survival, function and immune defense. We will test two known FDA-approved drugs for impacts on GWI intracellular mechanisms for possible reassignment. We will directly evaluate GWI-specific genomic differences and correlate these differences with alterations on fundamental cellular mechanisms. Our studies will contribute to the elucidation of differential mechanistic pathways that occur in GWI and their correlation to genomic changes, cellular activity, immunity and viral activity.

One of the most compelling aspects of this proposed research study is our ability to work closely and share our data with the previously DoD funded GWI consortium (GW120045, Dr. M. Morris, PI, Drs. N. Klimas and G. Broderick, Co-PIs). We also do not need to recruit new patients, as we will be able to access samples from the consortium. The consortium developed a translational model of GWI, integrating both clinical and basic research using a systems biology approach. This computational biology approach will aid our research team to identify signaling mechanisms relevant to GWI and outline the most promising biomarkers tied to these signaling pathways for selection and testing of therapeutic interventions to not only improve symptomatology but also reset normal well-coordinated signaling interactions. The consortium is designed for rapid identification of molecular targets and prediction of effective therapeutic interventions, which will help us to rapidly predict and assess potential therapeutic targets as well as add value to their research efforts.

The combination of genomics, cell biology and computational biology approaches will provide us with an improved understanding of the underlying mechanisms of GWI and potentially lead to beneficial targets for improved diagnosis. In addition, over the study period, a more detailed understanding of the dysfunctions involved in GWI would help to identify many candidate FDA-approved drugs that can be tested for impacts on GWI mechanisms and therefore be reassigned for more specific treatments that will address and possible revert the causes of disease.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	Unclassified	23	**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified			

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1. INTRODUCTION:

There are no specific biomarkers for diagnosis or management of GWI and treatment has been palliative, symptom-driven and physician-directed. The illness encompasses important pathophysiological mechanisms of relapse that reflect an overlap of stress-potentiated neuro-inflammatory dysfunction, possibly involving reactivation of latent viral infections, dysregulation in oxidative and nitrosative (O&NS) pathways and a lowered antioxidant status. The goal of our proposal is to find interconnecting links for effective drug treatment. Based on our preliminary results that indicate increased viral activity, here we focus on key cellular pathways related to immune system and metabolic functions as well as on control of gene expression in order to provide insight into regulatory mechanisms of GWI. We have found that GWI patients had elevated levels of miRNAs from viruses such as EBV, indicative of higher baseline viral activity and/or flaws in host immune response (unpublished). At the level of the genome, there are individual differences that govern predisposition and vulnerability to toxic chemicals/ infections. Therefore, not everyone exposed to the same toxic agent/pathogen will become ill. Responses to dysregulating agents depend on many levels of gene expression and cellular innate immunity, including the level of nucleo-cytoplasmic trafficking. Abnormal nuclear transport is associated with innate immune system deficiency and viral pathogenesis. Regulation of receptor-cargo complex formation by phosphorylation constitutes a key step for determining nuclear entry or exit of certain transcription factors. Intricately connected to this process is the JAK family of kinases. Therefore, unregulated NPC trafficking and dysfunctions in metabolic pathways may elucidate novel mechanisms of GWI disease progression.

2. KEYWORDS:

PBMCs -peripheral blood mononuclear cells;
RNAseq – technique for sequencing transcriptome;
CNV – copy number variation;
DEX –dexamethasone;
PMA- propidium monoazide;
STAT1 – Signal transducer and activator of transcription 1;
STAT3 - Signal transducer and activator of transcription 3;
Nup98 – Nucleoporin of ~ 98 KDa ;
Nup53 – Nucleoporin of ~ 53 KDa;
Nup153 – Nucleoporin of ~ 153 KDa;
Rae1 - Nucleoporin RNA export factor;
EBV – Epstein Barr Virus;
Mitochondrial function;
NPC – Nuclear pore complex;
Rta – EBV transcription activator protein;
Zta – EBV Zebra Immediate-Early Protein;
CD27 - lymphocyte costimulatory molecule that regulates T-cell, natural killer (NK) cell, B-cell, and plasma cell function, survival, and differentiation;
CD3 – receptor on T cells;
CD19 – receptors on B cells;
OKT3 – monoclonal antibody for stimulation of T cell growth;
IL-2 – Interleukin 2;
Sec13 – nucleoporin involved in vesicular transport and innate immune response.

3. ACCOMPLISHMENTS for 2017 - 2018:

- **Major goals for the project:**

Specific Aim 1: Use genomic approach to identify changes in gene expression that are characteristic of GWI and that differ from HC.

Major Task 1: Identify metabolic pathways affected at GWI and possible regulatory RNAs.
Subtask 1: (months 1 – 4): 100% completed (2016).
Subtask 2: (months 5 - 12):75% completed (2016)
Analysis of RNA-seq data for expression of transcripts (120 RNA samples).

Major Task 2: Investigate possible mechanisms of transcriptional regulation in GWI.
Subtask 1 (2016) (months 1 – 4): 90% completed (2018)

(2018)

- **What was accomplished:**

1) Major activities:

Raw RNAseq data have been pre-processed. Currently RNAseq data are being analyzed. All alignment of RNAseq data to the genome are finished (120 samples). We have used four different alignment programs (GSNAP, STAR, HISAT2 and MAPSPLICE) in order to find the best aligning rate.

To determine differential expression on gene level we quantified aligned reads using HTSeq program, and HTSeq output had been transferred into Biocunductor/R package edgeR. To build linear model we used the limma package that is incorporated into edgeR. Almost all contrasts (comparisons) have been run.

As it was determined, edgeR is not enough strict in evaluating False Discovery Rate. On the level of genes all samples have been re-analyzed using DESeq2. All contrasts (comparisons) have been run, and we are preparing the gene list for validation by Nanostring.

We evaluated the analysis of the differential splicing using the StringTie and Ballgown suite, and results were not satisfactory. Using StringTie and Ballgown suite we could not find any changes in the splicing. This software had other problems in identifying alternative splicing.

After evaluating various methods, we used DEXSeq to determine differential exon usage and differential exon expression. All comparisons between groups have been run for all aligners, and currently we determine the results that are common for at least two aligning programs.

2) Specific Objectives:

Compare differences in transcriptome expression between HCs and GWIs in order to identify specific targets for intervention and reestablishment of homeostasis.

3) Significant results or key outcomes:

At the baseline (T0) out of 72 differentially expressed genes (criteria: False Discovery Rate (FDR) < 10%, Fold Change (FC) more than 1.3 to either direction) 57 genes are protein-coding. 15 differentially expressed genes code for various regulatory RNAs representing antisense, long non-coding (lncRNA), pseudogenes, small nuclear (snRNA) and small nucleolar (snoRNA) RNAs.

We observed dysregulation of expression of some immune genes (CCR2, CCR9, CX3CR1, IL15, TLR8), genes encoding mitochondrial proteins (NDUFAF1, POLRMT1)

Significant under-expression of several snRNAs and snoRNAs reveal impaired splicing and RNA processing. Under-expression of signaling genes (NAB2, YES1, GPBAR1, etc.) indicates disturbance in immune and other signaling processes.

Currently we are creating list of differentially expressed genes and exons for validation by NanoString and subsequent system biology analysis.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We plan to validate results of RNAseq using custom Nanostring panel. This should be done by the end of 2018.

(2018)

Subtask 2 (months 5 - 10): 100% completed.

Evaluate CNV genome-wide using Agilent SurePrint G3 Human Genome CGH+SNP microarrays (40 microarrays).

Subtask 3 (months 11 – 13): 70% completed.

Analyze results of Agilent SurePrint G3 Human Genome CGH+SNP microarrays, identify differences in CNV between GWI patients and HC.

- **What was accomplished:**

1) Major activities:

We had to overcome some technical problems with the data analysis. Currently analysis of data is finished and validated using custom Nanostring panel.

2) Specific objectives:

Analysis of CNV differences between GWI and HC is one avenue towards elucidation of whether specific genes are differentially expressed based on genetic characteristics (predisposition).

3) Significant results or key outcomes:

We did not find any clear-cut amplifications or deletions specific for GWI patients. The custom Nanostring panel confirmed amplification in the region chr15:77332162-77332251 in DNA of 17 GWI patients out of 19. Amplification of this region was found in DNA of only 11 healthy controls out od 20. This intergenic region is located between two enhancers, which can regulate transcription of genes in B cells.

Major Task 3: Identify changes in methylation patterns of genomic DNA at GWI.

Subtask 1 (months 5 – 10): 100% completed.

Use the Illumina Infinium Human Methylation450 BeadChip arrays (120 DNA samples from 20 GWI patients and 20 healthy controls).

Subtask 2 (months 11 – 15): 100% completed.

Initial task was to analyze results of the Illumina Infinium Human Methylation450 BeadChip arrays, identify differences in the methylation patterns between GWI patients and HC. We analyzed results of DNA methylation assays that have been run on Illumina MethylationEPIC microarrays.

Subtask 3 (months 16-24): 100% completed.

Validate results of Illumina methylation arrays using pyrosequencing.

- **What was accomplished:**

1) Major activities:

We used new Illumina MethylationEPIC microarrays. These arrays have much better coverage (850,000 CpG sites) as compared to the Illumina Infinium Methylation450 BeadChip arrays (450,000 CpG sites).

Initially we analyzed all 120 assays. There were no differences between time points, so we restricted the final analysis and validation to the baseline (T0). Results have been validated using pyrosequencing.

2) Specific objectives:

To determine whether GWI methylation patterns significantly differ from HC. Differences in such patterns might be correlated to differential expression of symptoms-causing pathways and therefore correlate to epigenetic differences among the 2 groups.

3) Significant results or key outcomes:

The results of DNA methylation studies have been validated by pyrosequencing.

10,768 CpG sites were differentially methylated in genomic DNA of PBMC of GWI patients (FDR < 5%, mean difference in methylation > 0.05). Most of these sites are located in promoter areas.

We found that 47.6% of the differentially methylated promoters belong to protein-coding genes, and 52.4% belong to non-coding long and short RNAs (including intergenic lncRNAs, antisense RNAs, microRNAs, snRNAs, snoRNAs, etc.). Most of differentially methylated promoters are hyper-methylated, indicating possible repression of expression of corresponding genes.

CpG islands in promoters of immunoglobulins (IGKV1-6, IGHD1-20, IGDCC3, etc.) are hyper-methylated in PBMC of GWI patients.

Promoters of some other genes responsible for immune system (CYP2C9, IFNA6, etc.) were found to be hypo-methylated, but at the same time promoters of several other immune genes, such as were hyper-methylated, showing that the immune system is dis-regulated in GWI.

CpG islands in promoters of signaling (TXK, FGR, ZNF304, TYROBP, etc.), and metabolic process genes (DAO, UPB1, etc.) are preferentially hyper-methylated and so these genes are probably "switched off".

In addition to the proposed experiments we have run ELISA-like assays to assess the global methylation status of PBMC. We did not find significant differences in the global genomic DNA methylation levels between GWI patients and controls.

Our results have been accepted for publication:

Trivedi MS, Abreu MM, Sarria L, Rose N, Ahmed N, Beljanski V, Fletcher MA, Klimas NG, Nathanson L. Alterations in DNA Methylation Status Associated with Gulf War Illness. DNA Cell Biol. 2019 Mar 28. doi: 10.1089/dna.2018.4469. [Epub ahead of print]. PMID: 30920300.

Specific Aim II. **Cell Biology approach for characterization of latent viral/cytopathic reactivation and immune inflammatory response in GWI.**

Major Task 5: To determine whether a stressful trigger causes viral cytopathic effects on NPC functions of PBMCs.

Subtask 1 (months 1 – 6): 30% completed

NEED EXTENSION FOR COMPLETION (months 36 – 40)

Description:

In vitro culture of PBMCs in duplicates at baseline, t0 (20 GWI patients, 20 healthy controls) will be cultured after cell count determination using Moxi Z automated cell counter. Cell viability is determined by trypan blue exclusion assay. Cultured cells will be challenged in vitro with DEX (stress group: 20 GWI patients, 20 healthy controls in duplicates, total of 80 DEX-challenged samples). Control samples not treated with DEX (total of 80 non-challenged samples) will be assayed side by side with stress group.

Determination of mRNA cellular distribution: discrepancies between GWI and HC between in-vitro stressed and non-stressed (control) cells will follow by in-situ hybridization of total mRNA (oligo-d(T) probe) and confocal microscopy.

- **What was accomplished:**

1) Major activities:

For determination of mRNA cellular distribution: I have simplified and standardized the method for speed and ease of detection by using a new fluorophore from Thermo Fisher (Molecular Probes) SYTO™ RNASelect™ Green Fluorescent cell Stain. Now, instead of treating, fixing, permeabilizing, stabilizing RNA, etc. (oligo-d(T) probe procedure takes 4 days), the new SYTO detection probe is cell-permeable, non-toxic and is added straight to live cells in culture 30 minutes prior to fixation with 4% formaldehyde (or 100% cold methanol, depending on other staining requirements). Exemplary images for RNA detection and quantification are depicted in **Figure 1**.

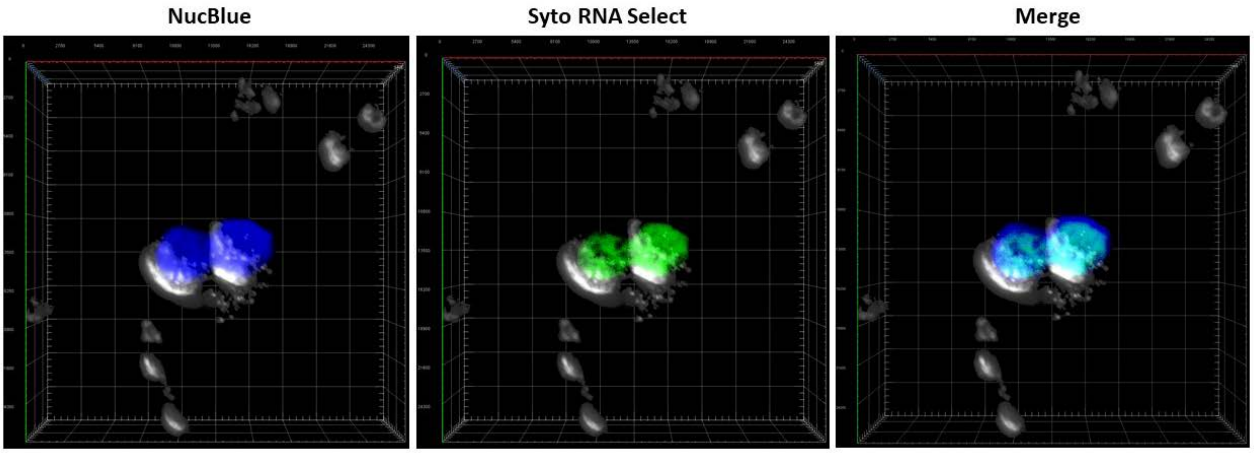


Figure 1. 3D representation of PBMC treated with NucBlue™ (blue) for localization of nuclei and SYTO™ RNASelect™ (green) for localization and quantitation of RNA. Right panel shows merged image with DIC contrast to emphasize cellular shape and extracellular vesicles.

I have reached out to collaborators at Myofrastand Inc, NY, in order to developed image analysis algorithms capable of analyzing segmentation of intracellular compartments as well as extracellular vesicles using the obtained confocal microscopy images. The staining method to identify DNA was NucBlue, Hoechst 33342 (Thermo Fisher). Active mitochondria were labeled with MitoTracker™ Orange CM-H2TMRos (Invitrogen™). Total RNA was labeled using two distinct methods – depending on compatible combination with availability of other fluorophores and/or antibodies. We have used either the method described above (oligo-d(T) probe for detection

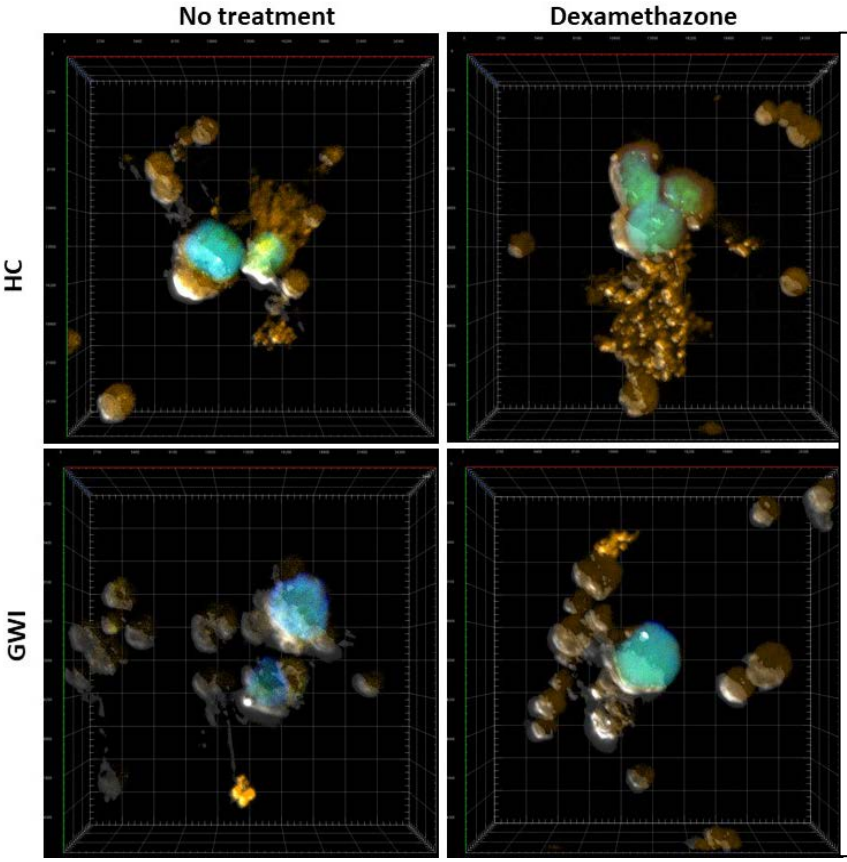


Figure 2. Dexamethasone treatment increases total active mitochondria and extracellular vesicles with mitochondria in GWI. 3D deconvolution images representing HC without treatment (upper left panel) and after 4 h DEX treatment (upper right panel). Green represents RNA. Blue indicates nucleus, orange shows active mitochondria and gray shadows were obtained with DIC contrast. Notice the overall increase in mitochondrial staining (orange color) and presence of vesicles after DEX treatment. GWI without treatment (lower left panel) and after 4 h DEX treatment (lower right panel). similar for both HC and GWI.

of mRNA), or SYTO™ RNASelect™ Green Fluorescent cell Stain (Invitrogen™). The SYTO RNASelect is a cell-permeant fluorophore that selectively stains total RNA.

Sets of GWI and HC PBMCs were prepared side-by-side for minimization of intra-experimental variables. In attempts to better analyze cellular behavior/response, I have tried to sort PBMCs according to cell type (using antibodies against CD3+ for T cells, against CD19+ for B cells and the remainder would be designated as natural killer cells) by using 2 different isolation methods: magnetic beads from StemCell Technologies and via flow citommetry/cell sorting via BD AriaFusion. Unfortunately, poor cell viability did not allow for successful sorting with either method. I have then proceeded without sorting by treating the designated cells with DEX as previously described. After fixation, each set of GWI and HC was manually scanned in order to identify cells that were morphologically non-apoptotic and by presence of nuclei (blue staining). At least 50 images were obtained per slide, and of those, at least 10 sets of Z-stacks (at optimal distance for 63X lens) were obtained. Each Z-stack dimension was determined according to cell size (beginning and ending of cell boarder as determined by blurring), rather than a "one-size-fits-all" fixed measurement approach. We have applied 3D deconvolution in order to obtain reconstituted images (**Figure 2**).

Image Analysis

I. Overview of initial process and normalization: Each 3D microscope image is a set of 2D images known as slices. The dimensions of the 2D images and distance between slices is obtained from the metadata information of the Zeiss.czi format microscopy images. All images are initially size normalized and are converted to .tiff format using ImageJ.

II. Cell detection and segmentation: Before cell detection and segmentation, our image processing pipeline performs object detection using otsu thresholding. Object detection is performed on the DIC image and all stained images. DIC image can capture any object whether it contains DNA, Mitochondria, RNA, etc. DNA stains are used to identify nuclei of cells. So, DIC objects that encompass the DNA objects are considered as entire cells and quantification of respective staining enclosed in these parameters are considered as intracellular. All the remaining DIC objects are considered as extracellular vesicles. Detailed description of the object detection pipeline used in DIC and stained images is given in the following subsections.

III. DIC image processing: Processing DIC images is challenging when compared to stained images because DIC images are typically noisy and blurry. Objects present in one slice cause blur in adjacent slices as shown in **Figure 3**. For this reason, we developed a selective algorithm that only takes into account image regions that are sharp. Those are subsequently stored for analysis in each slice. Initially, random noise in the images is reduced by smoothing the original input image using a small gaussian filter. We use the 'imfilter' matlab command for this purpose. Then, the sharp regions in the image are detected by taking the gradient of the image. This gradient image then gets a threshold for detection of object boundaries. Otsu thresholding, a type of auto-thresholding algorithm is used for this purpose. These object boundaries must be continuous across slices, for which we developed a slice smoothing method. The entire pipeline is shown in **Figure 4**. Intermediate images considered as "blurry" during DIC image processing are represented in **Figure 5**.

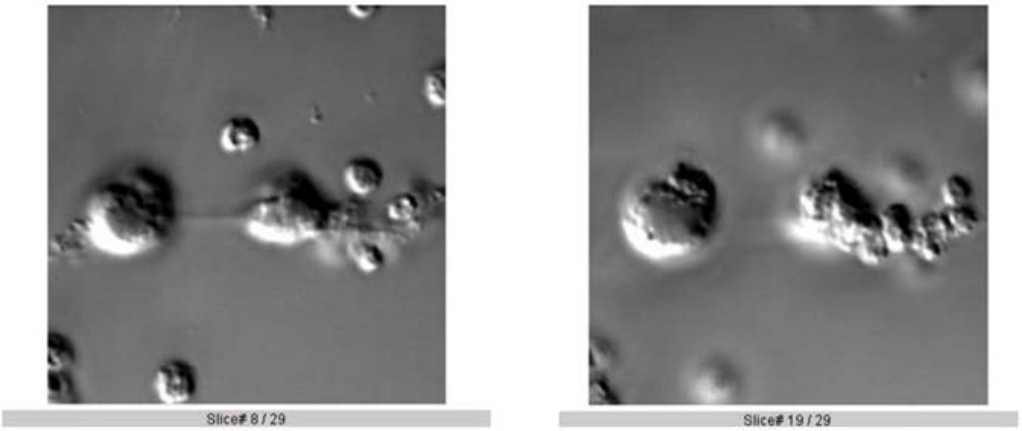


Figure 3. All identified objects in the left image are considered as real objects in that particular slice (8th) and are therefore sharp. These same objects when imaged from a different depth (Z-slice) are seen as blur (19^{th} of 29 slices, right panel). The larger objects are usually identified as cells based on nuclear staining (not shown) and smaller objects are considered as vesicles. Notice tubular-like structures connecting 2 cells and presence of very small, but well-defined vesicles on the upper-right quadrant (left panel).

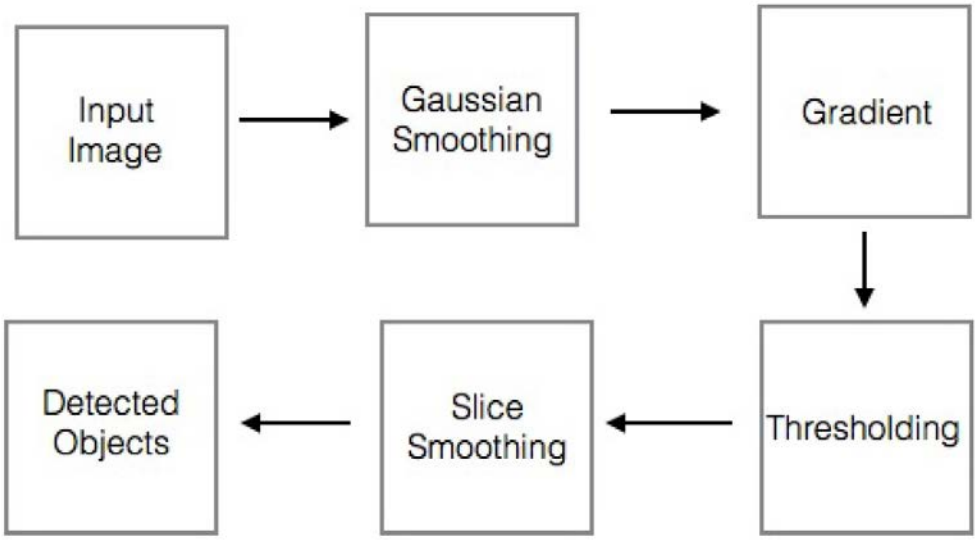


Figure 4. Image processing pipeline for DIC images.

IV. Processing Stained Images: The pipeline for stained images is almost the same as the one developed for DIC, except that a gradient calculation at the beginning is not necessary because blurring is not an issue. Smoothing and auto thresholding are sufficient for object detection. DIC images cannot directly receive threshold because of the half-moon patterns present at the objects locations. This sudden change in gradient causes auto thresholding algorithms to select only the bright parts as objects and neglect the dark (textured) on the surfaces. The image processing pipeline for stained images is as shown in **Figure 6**. Since stained images are not blurry like DIC, 3D gaussian smoothing can be initially performed to remove noise and causing minimal blur. An example of objects detected from stained images is shown in **Figure 7**.

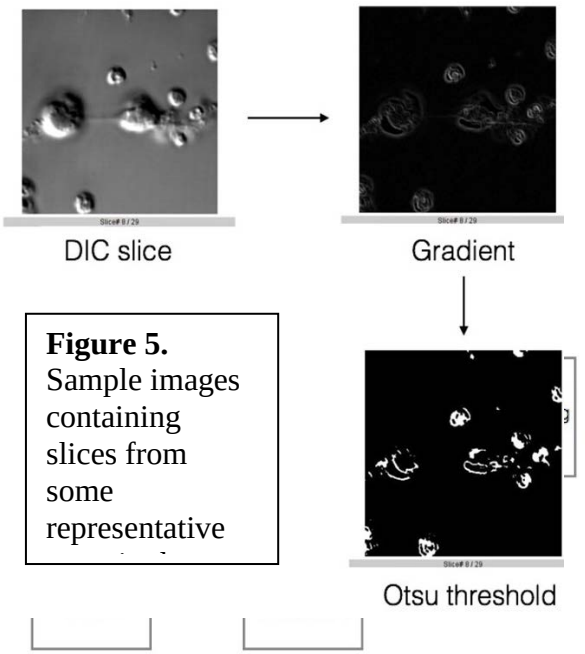


Figure 5. Sample images containing slices from some representative

Figure 6. Image processing pipeline for fluorophore- stained objects.

V. Intra and Extra- cellular objects: After detecting objects in DIC and stained fields of view, images are combined and all objects are taken into consideration. Objects are represented using binary method, i.e, a value of 1 indicates presence of object and value of zero represents absence. DIC object image contains all possible objects analyzed, including those containing Mitochondria, DAPI, RNA, Zta, Rta, etc. But, since object detection in DIC is relatively poor due to noise and blurs, we take advantage of objects obtained from the combined stained images as well. Blue stains show the presence of DNA and therefore the objects in which it is embedded are used to identify cells. All the remaining objects that did not contain blue are considered as vesicles. This can be observed by looking at the microscopy images. A max projection of all slices in DIC and stained images is shown in **Figure 8**A. Quantitation of extracellular vesicles with

mitochondria is represented in **Figure 8B**, Notice that upon DEX treatment, the number of vesicles with mitochondria decreases for HC, but increases for GWI.

VI. Extracting Quantitative information: Quantitative image data can be used to measure differences across image classes. In our case we extract the following information from each 3D microscope image:

1) Intracellular Mitochondria volume

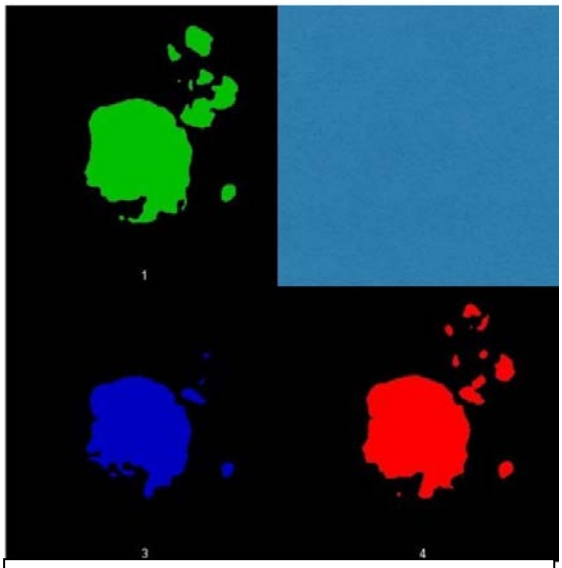
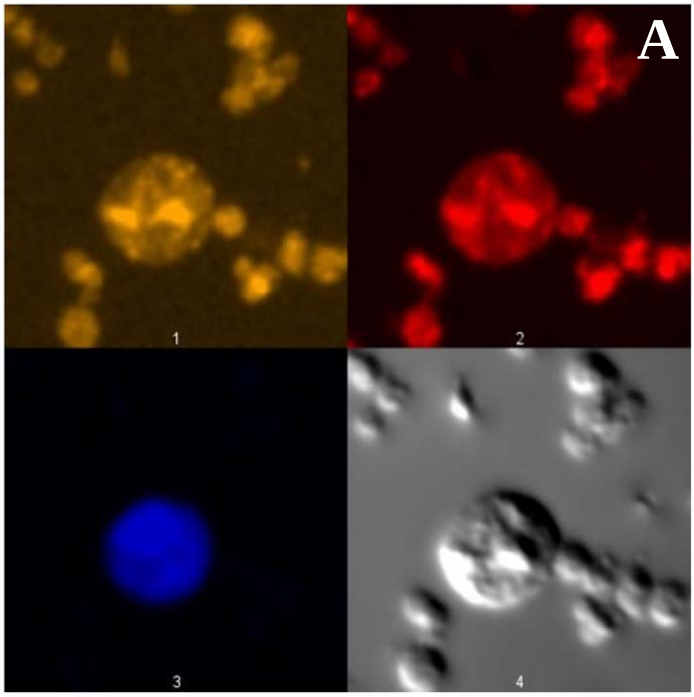


Figure 7. Binary images representing objects that contain RNA (green), DNA (blue) or Mitochondria (Red).

2) Extracellular Mitochondria volume
3) Total Mitochondria volume
4) Intracellular RNA volume
5) Extracellular RNA volume
6) Total RNA volume
7) Number of vesicles containing Mitochondria
8) Number of vesicles containing RNA

Note that all extracellular objects are classified as vesicles.

In order to perform unbiased quantitative analysis of the data obtained from cell painting, I teamed-up with Dr. Morris' postdoc, Dr. Jacqueline Machi. Dr. Machi has graphed the results and performed statistics using ANOVA (GraphPad Prizm).



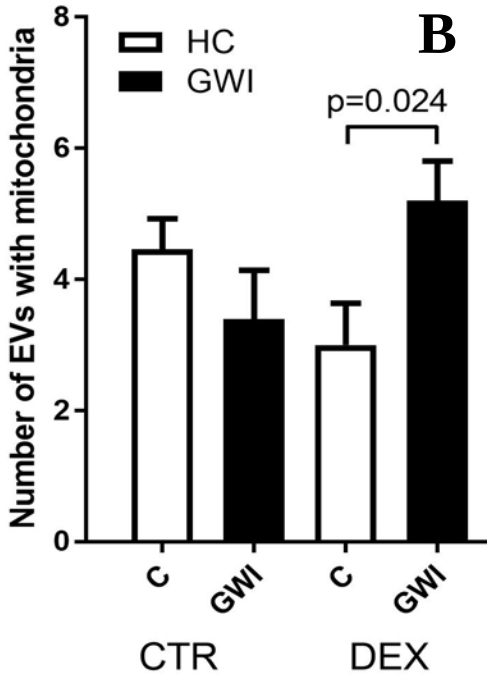


Figure 8. **(A)** Maximized projection of Mitochondria (1), RNA (2), nucleus (3), DIC (4). Representative slice of a 3D Z-stack acquisition. Notice the presence of extracellular objects that do not contain DNA. **(B)** Quantitation of extracellular vesicles that contain mitochondria in HC (white bars) and GWI (Black bars). Notice that number of EVs with mitochondria for HC decreases, while for GWI increases. These results are preliminary due to low n number analyzed at this point (2 HC and 2 GWI).

HCs show increased levels of intracellular mitochondria upon DEX treatment, while GWI does not show significant increase (**Fig. 9A**). Extracellular levels of mitochondria (in vesicles) slightly increased with DEX treatments for both HC and GWI (**Fig. 9B**). Notice that total levels of active mi[illegible]

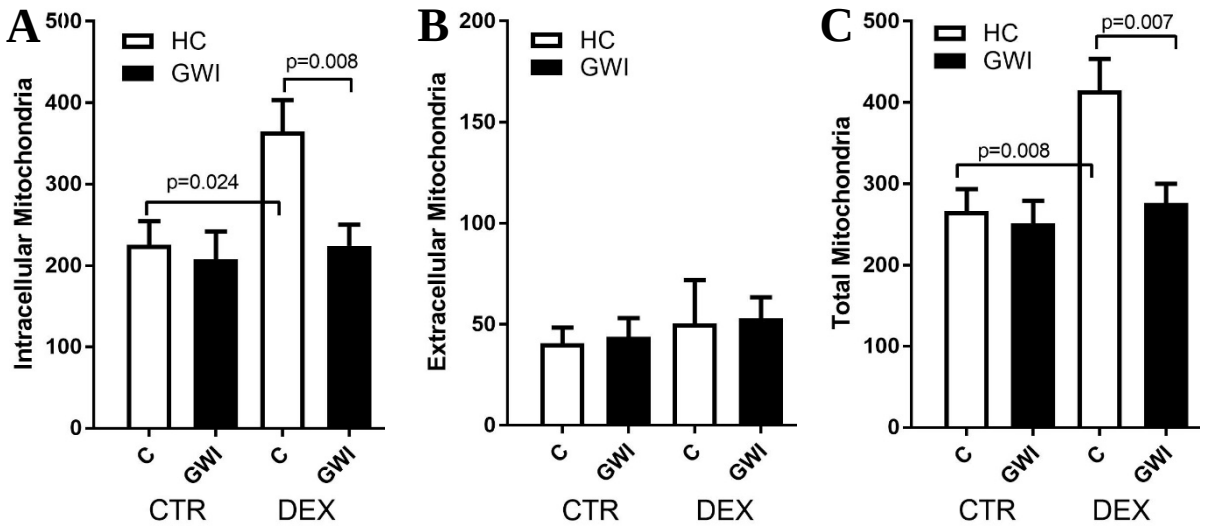


Figure 9. **Quantitation of mitochondria in PBMCs upon DEX treatment**. **(A)** Levels of intracellular mitochondria in HC increase upon DEX treatment, while remaining basically unchanged in GWI. **(B)** Levels of extracellular mitochondria that are supposedly in EVs slightly increase for both HC and GWI. **(C)** Total levels of active mitochondria significantly increase for HC, but are nearly unchanged for GWI.

[illegible]s of HCs and obtained numerous images (approximately 20 high-definition images for each condition, total of ~600 images for RNA distribution for each sample for a total of 2,400 images analyzed). Paired samples were handled simultaneously in order to avoid variables related to bleaching. Images were obtained with consistency of confocal parameters in order to allow quantification.

Coding for analysis of RNA quantity and distribution was described above. Initial analysis of 2 patients and 2 controls indicate that There is an overall increase in RNA content (both intracellular and cytoplasmic) when HC PBMCs are treated with DEX. GWI RNA levels did not differ among the 2 samples analyzed. We have collected more images of 4 additional GWI and 4 HC samples and set out for count/analysis.

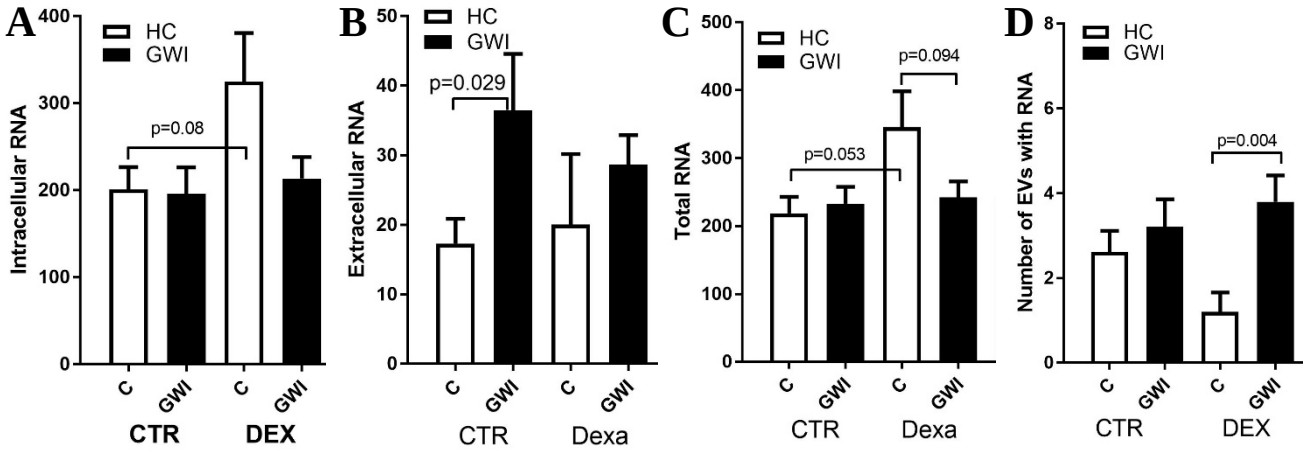


Figure 10. **Quantitation of RNA in PBMCs upon DEX treatment**. **(A)** Levels of intracellular RNA in HC increase upon DEX treatment, while remaining basically unchanged in GWI, similarly to levels of mitochondria. **(B)** Levels of extracellular RNA that are supposedly in EVs slightly increase for HC, but slightly decreased for GWI. Initial levels of extracellular RNA are much higher for GWI when compared to HC. **(C)** Total levels of RNA significantly increase for HC, but are nearly unchanged for GWI. **(D)** Number of EVs with RNA decrease for HC, but increase for GWI.

2) Specific objectives:
To explore discrepancies among PBMC total RNA levels and mitochondrial levels between HC and GWI before and after Dexamethasone (in vitro stress) treatment.

3) Significant results or key outcomes:
Statistical analysis of values obtained with MATLAB image analysis showed RNA distribution for 2 patients and 2 controls (above). The distribution was interpreted as intracellular, extracellular, or in EVs and total RNA. Although the n-number of this initial analysis is low, we have standardized the method for analysis of the remaining patient/control sets.

- **What do you plan to do during the next reporting period to accomplish the goals?**

We plan to use the extension period to finish processing the PBMCs from the remaining GWI/HC sets.

Subtask 2 (months 12 – 18): **30% completed**.
NEED EXTENSION FOR COMPLETION (months 41 – 46)

Stress-induced signal transduction and nucleocytoplasmic protein import patterns.
Determination of whether NPC components are differentially distributed in the cells.
Milestone: Determination of whether GWI cells present discrepancies or blockages on mRNA export and/or protein import/signal transduction.

- **What was accomplished:**

1) Major activities:

Standardization of immunostaining conditions for Nup98, Nup53/35, Nup153, Nup62. Developed pipeline for image analysis and quantitative measurements for each of the following objectives:

2) Specific objectives:

Preparation of slides for immunostaining using the following standardized combinations:

A) Nup62-800 (yellow) Rta (red) and Zta (green)

1) Expression levels of Nup62, Rta and Zta;
2) Possible co-localization of Rta and/or Zta with Nup62
3) Presence in extracellular vesicles
4) Nuclear vs cytoplasmic distribution
5) Relative number of EVs for each condition
6) Nuclear morphology
7) Vesiculation of cell membrane with presence of EBV proteins

B) Mitochondria (orange), Zta (green) and **Nup53/35** (red)

1) Expression levels and localization of mitochondria
2) Expression levels and localization of Zta (nuclear vs cytoplasmic)
3) Expression levels and localization of Nup35 (nuclear vs cytoplasmic)
4) Presence of mitochondria, Zta and/or Nup35 in EVs
5) Number of EVs for each condition
6) Nuclear morphology
7) Vesiculation of cell membrane with presence of Nup35, Zta and/or mitochondria

C) Mitochondria (orange), **Nup98** (green) and **Nup153** (red)

1) Expression levels and localization of mitochondria and Nup153
2) Expression levels and localization of Nup98 (nuclear vs cytoplasmic)

3) Significant results or key outcomes:

Confocal images were obtained for 20% of PBMC samples and are currently being analyzed as described above**.**

- **What do you plan to do during the next reporting period to accomplish the goals?**

We plan finish processing the PBMCs from the remaining GWI/HC sets according to the established protocol.

<u>Subtask 4 (months 18 - 30)</u>: **<u>100% completed</u>**.

- **What was accomplished:**

Determination of Active mitochondrial levels and utilization of active B12.

Vitamin B12, also called cobalamin, has important roles in function of brain and nervous system. It is involved in the metabolism of every cell in the human body. It aids in the formation of red blood cells, affects DNA synthesis, fatty acid and amino acid metabolism. Only bacteria and archaea have the enzymes needed to produce B12 and this process occurs in the human intestines. There are several forms of B12, but only the active form, holotranscobalamin (HoloTC), can interact with receptors at the surface of cells and be transported to the inside of cells in order to be available for the above-described functions. Although there are both rapidly and slowly equilibrating compartments in the body, cobalamin is distributed to those tissues in which the

endogenous pools appear to turn over rapidly [1]. Thus, changes in serum concentrations of active B12 will reflect either replacement of the rapidly turning over compartments or sequestration of the active form of the vitamin by bacterial microcompartments that most likely have escaped from the intestines via leaky gut mechanism. The latter is a novel hypothesis proposed here based on diligent literature investigation that is relevant to the present work. If changes in serum concentrations of active B12 are attributed to fact cellular absorption, than an overall change in the total levels of B12 would follow the same trend.

1) Major activities:

(A) Standardization of ELISAs and completion of serum samples testing. Serum samples were obtained from 20 GWI patients and 20 HCs according to the cohort described in methods. Briefly, the cohort uses serum from patients that had been subjected to exercise stress test. Blood had been collected at time zero plus 3 minutes (t0+3) and time 4 hours (t2) after beginning of stress test. Holotranscobalamine (active B12) ELISA kits from IBL were used according to manufacturer's instructions in order to measure the levels of a B12 form that can be readily uptaken by cells.

B) To explore discrepancies among active mitochondrial levels before and after DEX treatment and compare responses between GWI and HC. Accomplishments related to mitochondrial distribution are mentioned in the previous section.

2) Specific Objectives:
A) To determine the levels of both active and total B12 forms from patients and HC serum.
B) To measure relative mitochondrial levels based on quantitative confocal microscopy.

3) Significant results or key outcomes:

Active B12 and general B12 measurements were obtained from serum samples and analyzed by ANOVA (GraphPad Prizm). Results are shown in **Figure 11**.

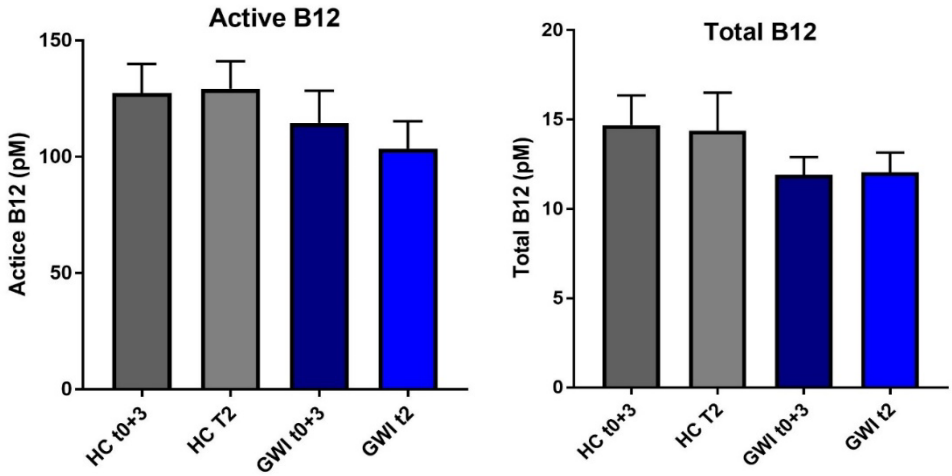


Figure 11. GWI active B12 levels decline after 4 h of exercise challenge, while total levels of B12 are increased. Left panel shown a plot of active B12 (pM) for HC at (t0+3min), HC at t2 (4 h after exercise challenge), GWI at (t0+3min) and GWI at t2. Notice that for HC, active levels of B12 are slightly increased, although not statistically significant, however, for GWI, active B12 levels are decreased after exercise challenge (left panel, blue columns). Total levels of B12 for HC are slightly decreased by the same proportion that active B12 is increased in the left panel. For GWI,

total B12 levels are slightly increased, showing a tendency for inverted behavior when compared to HC.

Major Task 7: Determination of whether NPC components are enhancing viral replication and/or budding on GWI.

Subtask 1 (months 24 - 30): 30% completed.
Multiple labeling Immunostaining of NE/NPC and viral components followed by microscopy and colocalization.

Subtask 2 (months 24 - 30): 30% completed.
Determination of whether NPC components are differentially distributed in the cells and associated with viral factors that promote replication and budding.

Major Task 9: Targeted Drug Testing

Subtask 1 (months 30 - 36): 30% completed.
NEED EXTENSION FOR COMPLETION (months 36 – 42)
Based on Specific Aim II data, determination of candidate drugs for testing.

- **What was accomplished:**

Choice of drugs for testing.

Based on the premise that intestinal dysbiosis alter immune system function and that the mechanisms that allow such phenotype might be deeply rooted in inter-kingdom communication between the microbiome and our mammalian cells, I've decided to test 2 drugs that have wide range of action, encompassing intestinal cells, immune cells, CNS, and mainly every organ system, the endocannabinoids:

i) **Anandamide** – Enzo Life Sciences cat# 89158-356
ii) **Cannabinoid receptor ligand (CRL)** – endogenous ligand for CB1 present in high levels in CNS. Enzo - VWR cat#89158-398

Among signaling molecules, endocannabinoids are among the oldest and most conserved [2]. The endocannabinoid system (ECS) has homoeostatic actions throughout the body and constitute a type of "master signaling" mechanism that modulate different receptor types, mainly CB1 in the CNS and CB2 in the periphery [3, 4]. ECS influence all organ systems, including the immune system, central nervous system, endocrine network, reproductive system and gastrointestinal functions [5-7]. **CB1** is not only found at the surface of cells, but also **in neuronal *mitochondrial (mt) membranes*** and modulate cellular respiration, energy production, ATP levels and cell viability [8], **placing it as central in mechanistic pathway for neurodegenerative diseases, and therefore a potential target for drug therapy.** However, research on naturally occurring modulators of CB receptors (i.e. molecules found in cannabis plants) is often enigmatically contradictory. Cannabinoids receptors are atypical messengers, which transfer the information from post terminal neuron to presynaptic neuron in a retrograde manner**. Two key components of cannabis, cannabidiol (CBD) and tetrahydrocannabinol (THC), can affect mitochondria directly and indirectly**. Interestingly, evidence suggests that **gut microbiome diversity dictates how neuronal mitochondria will respond to cannabinoid stimulus** [9]. Furthermore, a recent study suggests that ECS interferes with microbiome QS signaling, suppresses biofilm formation

and inhibits swimming motility of bacteria via down-regulation of QS cascade genes [10]. **We hypothesize** that endocannabinoids can ameliorate GWI stress-induced relapse symptoms associated with dysbiosis, gastro-intestinal epithelial gap junction integrity, vascular cell/systemic inflammation and cognition/memory deficits.

For the purpose of integrating the chosen drugs to the scope of this work, we will add add either anandimide or CRL to PBMCs in the presence of DEX. We will monitor active mitochondria levels and inflammatory cytokines levels.

- **Other achievements**:

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- **What opportunities for training and professional development has the project provided?**

Dr. Waziry is now actively involved in MATLAB image analysis via collaboration with Dr. Monica Pessanha, Myofrastand Inc., Dr. Arvind Rao at MD Anderson Image Analysis Center. They were very helpful with analysis design, coding and imaging for successful interpretation of metadata information obtained via confocal microscopy.

- **How were the results disseminated to communities of interest?**

We have participated in the 2018 Gulf War Illness Common Data Elements Meeting that was held at Nova Southeastern University in the month of March. During the brainstorming sessions, Dr. Waziry has contributed new ideas regarding:

A) **Inter-kingdom communication between the microbiome and our human cells**. This communication supposedly happens via stress hormones and metabolic biproducts of bacteria, which might be acting like hormones in the periphery as well as in the CNS. Based on the dysbiosis present in GWI patients, a shift of stress signals might enhance the activity of opportunistic pathogenic bacteria, therefore increasing propensity to leaky gut and partial translocation of bacterial microcompartments. Once these microcompartments enter the bloodstream, they might act like sponges that absorb available B vitamins out of the serum. Our bodies depend on bacteria for production of certain cofactors, especially vitamin B12. Bacterial microcompartments hold enzymes that interact with substrates as well as products of enzymatic pathways. These microcompartments, once in the serum, have no available substrate, therefore they might shift to the reverse path - or at least trap the "would-be-product" in a locked form making it unavailable for absorption by cells. As I have mentioned in the introduction of this grant, GWI patients have symptoms that resemble vitamin B12 deficiency, however, measurements of total B12 levels appear to be normal. Nevertheless, patients feel better after taking B12 injections. A mechanism that takes into account B12 sequestration via bacterial microcompartment trapping could at least explain these symptoms aspect.

B) Based on confocal observations of **lower mitochondrial levels in PBMCs of GWI** patients, Dr. Waziry suggested **that children of women who suffer from GWI should be tested for mitochondrial function**. This suggestion was based on the fact that mitochondria are inherited via the mother. On similar grounds of maternal inheritance and based on bacterial dysbiosis mentioned in item (A), I suggest here that **the microbiome of these children be tested as well**.

C) Following on inter-kingdom communication, dysbiosis, opportunistic pathogens and **high levels of oxidative stress** presented by GWI patients, Dr. Waziry suspects that a particular family of bacterial enzymes, **methionine gamma lyases (MGLs),** is getting absorbed by

patrolling immune cells. These enzymes break down methionine and potentially interferes with **SAM/glutathione pathway** as well as protein synthesis in general. Such interference can shift oxidative stress levels, therefore this mechanism might be central for elucidation of symptoms' causes.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Performing experiments has been particularly challenging due to the absence of a competent postdoc or technician. I have taken on the responsibility of conducting the molecular biology experiments. I am currently awaiting approval of an extension for the present studies. During the extension time I'll devote 100% of my efforts towards completion of the proposed studies.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project**?
  Completion of the experiments will elucidate major mechanistic aspects of the illness..

- **What was the impact on other disciplines?**
  GWI as well as ME/CFS might be the first illnesses to identify direct links between microbiome dysbiosis and human health. Elucidation of such mechanisms will revolutionize the fields of microbiology, immunology and mental health.

- **What was the impact on technology transfer**?
  Nothing to report.

- **What was the impact on society beyond science and technology?**
  Knowledge of the mechanisms and causes of the disease – which might be linked to pesticides that potentially alter membrane metabolism – will bring awareness and encourage modifications to common agricultural and food industry practices as well as usage of fossil fuels.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**.
  Nothing to report.

- **Actual or anticipated problems or delays and actions or plans to resolve them**.

The responsibility of completing Specific Aim II experiments still falls upon myself. I have devoted a substantial amount of time beyond my assigned effort during the past year in order to accomplish what is reported here. Nevertheless, I was informed in April, 2018 that my contract with NSU will not be renewed due to lack of funds. Depending upon approval of the extension for the current grant, I will be able to dedicate more time and effort towards completion of experiments, obtain better quality of data and be able to publish the findings.

- **Changes that had a significant impact on expenditures**.

As mentioned above, I was recently informed by NSU that there are no further funds for my employment. I have been working with the department in order to re-budget the remainder of the present DoD grant in order to cover grant-related expenses and my salary, so that I can remain employed by NSU in order to complete the studies proposed. Since last July, the department no longer supports my efforts to apply for new grants.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**.

Not applicable.

6. PRODUCTS:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project**?

Name:	Paula Waziry, M.A, Ph.D.
Project Role:	P.I.
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	9
Contribution to Project:	Dr. Waziry has performed allT/C of PBMCs, in vitro stress treatment, in-sity hybridization, Immunostaining, active mitochondrial staining, confocal microscopy, project development and troubleshooting, image analysis.
Funding Support:	DoD – present grant.

Name:	Lubov Nathanson, Ph.D.
Project Role:	Co-I.
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Dr. Nathanson has performed isolation of all RNA and DNA samples, quality control analysis and preparation of samples for RNA-seq, RNA-seq, CNV and methylation data analysis and validation.
Funding Support:	NSU KPCOM

Name:	Nancy Klimas, M.D.
Project Role:	Co-I.
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	1
Contribution to Project:	Dr. Klimas has overseen the project and contributed with direction to be taken in regards to cellular energy/mitochondrial function analysis.
Funding Support:	NSU KPCOM, VA, Miami

Name:	Mariana Morris, Ph.D.
Project Role:	Co-I.
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Dr. Morris has overseen the project and contributed with experimental design.
Funding Support:	NSU KPCOM

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  Yes. Dr. Waziry is no longer supported by other funding means or by NSU KPCOM.

- **What other organizations were involved as partners?**
  I have reached out to a colleague, Arvind Rao, at the Department of Bioinformatics and Computational Biology at The University of Texas MD Anderson Cancer Center, in order to receive guidance towards the development of the appropriated algorithms for qualitative/quantitative image analysis of metadata confocal microscopy. Dr. Rao assigned his postdoc, Dr. Srikanth Kuthuru, for coding and development of algorithms. The analysis method was specifically designed and developed for the present work and will lead to integration of image informatics of datasets to drug testing and eventually to clinical outcome.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

9. APPENDIX:

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