Exercise – induced changes in cerebrospinal fluid miRNAs in Gulf War Illness, Chronic Fatigue Syndrome and sedentary control subjects

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Gulf War Illness (GWI) and Chronic Fatigue Syndrome (CFS) have similar profiles of pain, fatigue, cognitive dysfunction and exertional exhaustion. Post-exertional malaise suggests exercise alters central nervous system functions. Lumbar punctures were performed in GWI, CFS and control subjects after (i) overnight rest (nonexercise) or (ii) submaximal bicycle exercise. Exercise induced postural tachycardia in one third of GWI subjects (Stress Test Activated Reversible Tachycardia, START). The remainder were Stress Test Originated Phantom Perception (STOPP) subjects. MicroRNAs (miRNA) in cerebrospinal fluid were amplified by quantitative PCR. Levels were equivalent between nonexercise GWI (n = 22), CFS (n = 43) and control (n = 22) groups. After exercise, START (n = 22) had significantly lower miR-22-3p than control (n = 15) and STOPP (n = 42), but higher miR-9-3p than STOPP. All post-exercise groups had significantly reduced miR-328 and miR-608 compared to nonexercise groups; these may be markers of exercise effects on the brain. Six miRNAs were significantly elevated and 12 diminished in post-exercise START, STOPP and control compared to nonexercise groups. CFS had 12 diminished miRNAs after exercise. Despite symptom overlap of CFS, GWI and other illnesses in their differential diagnosis, exercise-induced miRNA patterns in cerebrospinal fluid indicated distinct mechanisms for post-exertional malaise in CFS and START and STOPP phenotypes of GWI.

Chronic Fatigue Syndrome (CFS) and Gulf War Illness (GWI) are nociceptive, interoceptive, fatiguing illnesses that are currently defined by symptoms and exclusion of other conditions in their extensive differential diagnoses. CFS developed from medical traditions of neurasthenia and viral infection, and GWI from “signs, symptoms, and ill-defined conditions (SSID; International Classification of Diseases-9th Revision, Clinical Modification (ICD-9-CM) codes 780-799)”12. These legacies are being revised based on new discoveries about disease pathogenesis. With the revisions comes an increasing need for objective biomarkers to define and diagnose these diseases.

The 1994 Center for Disease Control (CDC) criteria for CFS are: (a) moderate or severe, persistent and sustained fatigue lasting more than 6 months and causing impairment of daily activities, plus (b) moderate or severe complaints of at least 4 of 8 ancillary criteria: short term memory or problems with concentration, sore throat, sore lymph nodes, myalgia, arthralgia, sleep disturbances, new onset headaches that include migraine, and post-exertional malaise (Fig. 1). Post-exertional malaise, also referred to as exertional exhaustion, is an unique characteristic of CFS that is shared by GWI subjects.

Twenty six years after the First Persian Gulf War, 25% to 32% of the nearly 697,000 U.S. veterans of that conflict continue to have cognitive and physical exhaustion that is made worse by effort (exertional exhaustion), systemic pain and hyperalgesia, migraines, gastrointestinal distress with severe diarrhea, and other medical problems (Table 1). Epidemiological risk factors include exposures to low dose sarin and cyclosarin from munitions such as the demolition at Khamisiyah, Iraq; pyridostigmine bromide taken for nerve agent prophylaxis; and...
Figure 1. Overlap of diagnostic criteria for CFS, GWI and major depressive disorder. Diagnostic protocols for CFS, GWI and depression selected different sets of primary and ancillary symptoms. CFS requires fatigue, then confirmation with ≥4 of 8 ancillary criteria. Active depression and other psychiatric diseases are exclusionary diagnoses for CFS. GWI requires 3 of 7 categories of symptoms. Depression requires depressed affect and anhedonia, then sufficient supporting complaints.

| Group | Non-exercise groups | Post-exercise groups |
|-------|---------------------|----------------------|
|       | sc0 | cfs0 | gw00 | SC | START | STOPP | CFS |
| N     | 22  | 43   | 22   | 15 | 22    | 42    | 16  |
| Age   | 42.0 ± 13.1 | 45.5 ± 10.6 | 49.2 ± 9.7 | 44.3 ± 11.5 | 45.3 ± 8.7 | 46.6 ± 8.2 | 47.0 ± 10.6 |
| Male* | 11 (48%) | 9 (22%) | 9 (41%) | 11 (85%) | 8 (80%) | 18 (82%) | 2 (29%) |

CFS Severity Questionnaire (CFSQ)

| Fatigue | 1.3 ± 1.4 | 3.7 ± 0.4 | 3.5 ± 0.5 | 5.3 ± 0.5 | 5.5 ± 0.5 | 3.9 ± 0.4 |
|---------|------------|-----------|-----------|-----------|-----------|-----------|
| memory, concentration | 1.0 ± 1.2 | 3.1 ± 0.7 | 2.8 ± 1.0 | 1.0 ± 1.2 | 3.1 ± 0.9 | 2.9 ± 0.8 | 2.7 ± 1.0 |
| sore throat | 0.3 ± 0.8 | 1.5 ± 1.0 | 1.5 ± 1.1 | 0.2 ± 0.6 | 2.1 ± 1.3 | 1.4 ± 1.3 | 1.7 ± 3.3 |
| sore lymph nodes | 0.2 ± 0.5 | 1.4 ± 1.3 | 1.8 ± 1.5 | 0.1 ± 0.3 | 1.9 ± 1.3 | 1.0 ± 1.1 | 1.3 ± 1.4 |
| muscle pain | 1.3 ± 1.4 | 3.1 ± 1.1 | 3.0 ± 1.3 | 0.9 ± 0.1 | 3.3 ± 0.5 | 3.1 ± 0.8 | 2.9 ± 1.2 |
| joint pain | 0.9 ± 1.1 | 2.6 ± 1.3 | 2.8 ± 1.2 | 1.1 ± 1.0 | 3.2 ± 1.3 | 5.2 ± 0.8 | 2.6 ± 1.1 |
| headache | 1.0 ± 1.4 | 2.5 ± 1.3 | 2.5 ± 1.2 | 1.0 ± 1.4 | 3.3 ± 1.1 | 2.4 ± 1.2 | 1.7 ± 1.7 |
| disrupted sleep | 1.4 ± 1.5 | 3.5 ± 0.7 | 3.6 ± 0.6 | 1.5 ± 1.2 | 3.6 ± 0.5 | 3.1 ± 0.0 | 3.3 ± 0.5 |
| exertional exhaustion | 1.3 ± 1.6 | 3.3 ± 1.0 | 3.4 ± 1.2 | 0.4 ± 0.8 | 3.7 ± 0.5 | 3.3 ± 0.8 | 3.4 ± 0.5 |
| CFSQ Sum8 | 7.4 ± 7.0 | 21.1 ± 4.6 | 21.4 ± 5.2 | 6.2 ± 5.6 | 24.2 ± 3.7 | 20.9 ± 5.1 | 19.6 ± 5.6 |

SF-36 Quality of Life

| Physical function | 83.0 ± 26.0 | 38.0 ± 20.5 | 44.0 ± 26.9 | 87.3 ± 22.9 | 43.3 ± 27.2 | 43.1 ± 23.2 | 36.4 ± 19.7 |
| Role physical | 59.8 ± 47.5 | 6.4 ± 19.5 | 8.8 ± 18.6 | 86.5 ± 30.0 | 0.0 ± 0.0 | 15.5 ± 39.0 | 0.0 ± 0.0 |
| Bodily pain | 73.0 ± 32.7 | 30.0 ± 21.0 | 38.0 ± 26.1 | 75.3 ± 21.5 | 20.1 ± 15.6 | 30.5 ± 18.4 | 39.0 ± 25.7 |
| General health | 67.5 ± 20.6 | 32.7 ± 19.0 | 31.8 ± 19.7 | 75.5 ± 13.0 | 17.4 ± 12.7 | 29.4 ± 19.8 | 24.3 ± 14.3 |
| Vitality | 54.6 ± 28.6 | 11.6 ± 11.7 | 20.5 ± 13.5 | 63.8 ± 12.4 | 12.2 ± 11.8 | 14.0 ± 13.6 | 8.6 ± 9.4 |
| Social function | 79.9 ± 29.6 | 24.6 ± 21.3 | 28.1 ± 21.8 | 84.6 ± 15.4 | 18.1 ± 16.7 | 30.4 ± 24.5 | 19.6 ± 12.2 |

Chronic Multisymptom Index (CMSI)

| Rheumatic | 7.0 ± 9.2 | 19.2 ± 6.9 | 21.3 ± 9.6 | 3.9 ± 4.2 | 23.1 ± 7.2 | 20.9 ± 5.1 | 20.3 ± 7.5 |
| Dyspnea | 2.0 ± 3.4 | 6.8 ± 6.3 | 8.2 ± 5.2 | 1.7 ± 2.0 | 12.0 ± 6.1 | 7.0 ± 4.5 | 9.1 ± 5.2 |
| Neurological | 2.0 ± 3.7 | 7.5 ± 3.8 | 8.3 ± 4.0 | 1.1 ± 1.3 | 9.2 ± 3.4 | 8.7 ± 2.5 | 7.4 ± 3.5 |
| CMSI Sum52 | 24.2 ± 28.9 | 58.4 ± 25.3 | 77.2 ± 34.1 | 12.6 ± 11.2 | 89.7 ± 26.3 | 72.2 ± 23.5 | 64.3 ± 30.4 |
| CESD | 9.2 ± 8.1 | 20.9 ± 9.0 | 27.7 ± 12.9 | 8.8 ± 7.6 | 33.2 ± 8.4 | 24.0 ± 10.8 | 17.9 ± 10.0 |
| %CESD ≥ 16 | 19.0% | 68.6% | 81.0% | 33.3% | 85.7% | 73.2% | 53.3% |
| GAD7 | 0.50 ± 0.02 | 0.17 ± 5.5 | 8.83 ± 6.77 | 4.17 ± 6.0 | 2.25 ± 4.77 | 7.81 ± 5.8 | 4.67 ± 5.3 |
| FM 1990* | 0/22 (5%) | 15/58 (39%) | 9/19 (47%) | 0/13 (0%) | 5/10 (50%) | 9/22 (41%) | 2/6 (33%) |
| IgG/albumin | 0.13 ± 0.04 | 0.11 ± 0.03 | 0.12 ± 0.03 | 0.12 ± 0.02 | 0.13 ± 0.04 | 0.12 ± 0.03 | 0.14 ± 0.03 |

Table 1. Demographics and symptom severities. Mean ± SD. *p < 0.002 by contingency tables between all groups. FDR < 0.010 after significant ANOVA: sc0 vs cfs0; sc0 vs gw00; SC vs START; SC vs STOPP; START vs CFS.
personal pesticides. Objective findings that distinguish GWI subjects from their unaffected deployed and non-deployed peers and civilians include cerebrospinal fluid proteomics, low activity butryrycholinesterase alleles in GWI cases who used pyridostigmine (odds ratio = 40.0)19, brain white matter dysfunction with increased axial diffusivity by diffusion tensor imaging and mitochondrial dysfunction22,24. A logical hypothesis is that Gulf War era exposures to cholinesterase inhibitors caused acute acetylcholine neurotoxicity in persons with genetically reduced levels of acetylcholinesterase activity21, followed by chronic progression of the initial lesion22,23,24.

We have reported that GWI veterans can be divided into two phenotypes based on responses to the physiological stressor of submaximal exercise testing25. One third of subjects developed new postural tachycardia after exercise and were positively selected as the START (Stress Test Activated Reversible Tachycardia) group. START subjects had: (i) exercise - induced postural tachycardia, (ii) increased blood oxygenation level dependent (BOLD) signal in the cerebellar vermis during a cognitive task before exercise, (iii) reduced BOLD signals during a working memory task after exercise, and (iv) reduced brainstem volumes suggesting atrophy. The remainder formed the Stress Test Originated Phantom Perception (STOPP) group because they had significantly greater BOLD activation of basal ganglia and anterior insula during cognitive testing than sedentary controls (SC) and START. That pattern was similar to phantom limb pain. The two phenotypes suggest there were two mechanisms of initial injury or on-going progression that will require different diagnostic and treatment approaches.

Cerebrospinal fluid was extensively assayed for micro-RNAs (miRNA), proteomics, metabolomics, and other analytes to interrogate the central neurotoxic pathologies proposed in GWI27 and CFS16,17. miRNAs are ~22 nucleotide long, single-stranded RNAs transcribed from genomic DNA28. They form the RNA-induced silencing complex (RISC) and bind to complementary sequences in the 3′ untranslated region of mRNAs to repress translation or promote mRNA degradation. miRNAs dynamically fine-tune the expression of most cellular proteins.

miRNAs are primary microRNAs (miRNA) that are processed from a longer transcript to a ~22 nucleotide long, single-stranded RNA. They are processed through the microprocessor complex (MEC) and bind to complementary sequences in the 3′ untranslated region of mRNAs to repress translation or promote mRNA degradation. miRNAs dynamically fine-tune the expression of most cellular proteins.

First, we hypothesized that CFS (cfs0), GWI (gwi0) and sedentary control (sc0) subjects at rest (nonexercise, lower case italics with 0) would have significant differences in cerebrospinal fluid biomarkers from each other. The nonexercise groups rested overnight and had no exercise before their lumbar punctures. Nonexercise miRNA patterns were predicted to be different from other conditions in the differential diagnosis such as depression and fibromyalgia. Second, differences would be magnified in post-exercise SC, CFS, and the exercise-defined START and STOPP phenotypes of GWI subjects when compared to each other (upper case italics to denote post-exercise). Third, differences between the post-exercise groups and their appropriate nonexercise comparison groups (SC vs. sc0, CFS vs. cfs0, START vs. gwi0, STOPP vs. gwi0) would model the effects of exercise on the central nervous system and the pathology of exertional exhaustion.

Methods
Clinical information. All subjects gave written informed consent. The protocol was approved by the Georgetown University Institutional Review Board and the Human Research Protection Office of the Department of Defense Congressionally Directed Medical Research Program. Lumbar puncture, quantitative PCR, and other investigations were performed in accordance with currently published standards, guidelines (MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments http://miqe.gene-quantification.info/) and World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects (http://www.wma.net/en/30publications/10policies/b3/). The investigations were not considered clinical trials using the World Health Organization (WHO) definition (http://www.who.int/ictrp/en/).

GWI and healthy veterans, non-military, sedentary control (SC) subjects completed questionnaires for case designation criteria of GWI (Fig. 2) and CFS (Fig. 3), common symptoms in CFS and GWI, quality of life41, Generalized Anxiety Disorder 742, and Center for Epidemiologic Studies Depression Scale43 scores. Fibromyalgia was assessed by pain plus tenderness (1990 criteria)44. Clinical and methodological details were published previously25. All subjects had submaximal bicycle exercise stress tests on 2 consecutive days with magnetic resonance imaging before and afterwards, followed by a lumbar puncture25. Subjects cycled at 70% of age predicted maximum heart rate for 25 min followed by stepwise increases in bicycle resistance to reach 85% predicted heart rate25. Exercise was required to induce postural tachycardia that defined the START phenotype. Cerebrospinal fluid total protein, albumin and IgG were measured and aliquots were frozen at −80 °C until thawed for miRNA extraction.

Quantitative PCR. miRNA analysis was completed in blinded fashion by N.S. without knowledge of subject diagnosis. Total RNA was isolated by mixing 0.5 ml cerebrospinal fluid 1:10 with QIAzoTM lysis reagent (Qiagen) and 0.1 ml CHCl3 before vortexing for 1 min. miRNA was extracted from the upper phase using miRNAeasy Mini Kits (Qiagen). miRNA levels were estimated by optical density of cDNA after reverse transcription with miScriptII RT kits (Qiagen). miRNA expression profiling used miScript PCR arrays for 380 miRNAs and miScript SYBRgreen PCR kits (Qiagen) on an ABI 7900 HT Real-Time PCR system (Applied Biosystems) and manufacturer’s protocol.

miRNA selection. The first level of constraint required miRNAs to be detectable with PCR cycle threshold (Ct) ≤ 35. miRNAs with Ct > 35 were designated as “undetectable” and 35 was imputed as their Ct. Second, in order to be considered a viable biomarker, miRNA had to detectable with Ct ≤ 35 in at least two thirds of subjects in a group.

miRNA normalization and ∆∆Ct. Four normalization strategies and ∆∆Ct computations were compared.
The N0 (no normalizer) analysis used the entire dataset with 35 imputed for all Ct > 35. The average ΔCt was calculated for each group, then ΔΔCt determined between each pair of groups. Significantly different miRNAs were detected by one-way ANOVA followed by Tukey's Honest Significant Difference (HSD; p < 0.05). Student's 2-tailed unpaired t-tests were computed for all miRNAs and pairs of groups, and False Discovery Rates (FDR) calculated to correct for multiple comparisons. FDR ≤ 0.10 was used as the next constraint to detect significant differences in ΔΔCt. The other normalizers used 2 (N2), 3 (N3) and 6 (N6) miRNAs. For each individual, ΔCt was calculated as the difference of the N2, N3 or N6 normalizer minus the Ct for each of the other miRNAs. Average ΔCt and ΔΔCt were calculated.

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**Figure 2.** Kansas Criteria for Gulf War Illness scoring form based on Steele. ©JNBaraniukMD_17g13. Used with permission of the copyright holder.
Outcomes were: (i) differences between non-exercise groups (sc0, cfs0 and gwi0), (ii) differences between groups after exercise (SC, START, STOPP, CFS), and (iii) exercise–induced differences between each post-exercise group and its appropriate non-exercise control group. ΔΔCt data were reported as mean ± SD to allow calculation of Cohen’s d (mean difference/SDpooled) and to predict future sample sizes42. Receiver operating characteristics were calculated for each significant ΔΔCt in SPSS 24.

Results

Demographics. CFS groups (cfs0, CFS) had more females, and GWI groups (gwi0, START, STOPP) more males (Table 2). Quality of life31, fatigue, cognitive, sleep, pain and interoceptive symptoms29,30 were significantly impaired in GWI and CFS groups compared to sedentary control subjects. Fibromyalgia (1990 criteria) was more prevalent in CFS and GWI than controls 34. The case designation criteria of GWI, CFS, generalized anxiety and depression share fatigue, sleep, cognition, and sympathetic nervous system symptoms 32. The Center for Epidemiology – Depression questionnaire found depression in 78.3% of GWI, 64.0% of CFS and 25.0% of control subjects33. Generalized Anxiety Disorder 7 scores were significantly higher for gwi0 and START compared to sc0 (FDR < 0.01) and SC (FDR < 0.01), respectively33. Cerebrospinal fluid total protein, albumin, IgG and their ratios were equivalent between groups36.

Normalizers. The raw data were placed in Supplementary Table S1. The statistical constraints reduced the number of miRNAs that were candidates for biomarkers and normalizers down to 88 (Supplementary Table S2). Supplementary Tableas the raw Ct data with 35 imputed for all Ct > 35 (Supplementary Table S3).

The N2 normalizer used miR-489 and miR-490-3p because they (i) were detected in all subjects with Ct ≤ 35, (ii) were abundant in cerebrospinal fluid, (iii) had small variances (25.2 ± 0.8 and 25.5 ± 0.9, respectively, mean ± SD) with narrow ranges for Ct (minimum 22.8 to maximum 27.8, and minimum 22.3 to maximum 28.1, respectively), and (iv) were not significantly different between groups (ANOVA > 0.05 and FDR > 0.10 for each pairing) (Fig. 4).

The N3 normalizer was the mean of miR-489, miR-490-3p and miR-127-3p (29.3 ± 1.2, mean ± SD, minimum 24.2, maximum 35). Two Ct values were 35; the averages for the 2 groups were imputed instead of 35 for this normalizer (26.6 ± 2.1, mean ± SD).

The N6 normalizer added miR-124-3p (30.6 ± 1.7, mean ± SD), miR-183-3p (31.8 ± 1.0, mean ± SD), and miR-433 (29.9 ± 1.5, mean ± SD). Two subjects each had 35 imputed for miR-127-3p, miR-183-3p and miR-433; the N6 normalizer included these values (28.7 ± 2.8, mean ± SD). Ct data for these 6 miRNAs were shown in Fig. 4.

All normalizer miRNAs had ΔΔCt < 1.0, ANOVA > 0.05, FDR > 0.10 and were detectable in at least 180 of the 182 subjects (Supplementary Table S2).

The N0 normalizer selected 31 miRNAs that met the significance criteria of (i) being detected with Ct ≤ 35 in more than two thirds of subjects in at least 1 of the 7 groups, (ii) HSD ≤ 0.05, and (iii) FDR ≤ 0.10. N2 identified 21, N3 had 24, and N6 found 23 significant miRNAs. The intersection of the 4 normalizers identified 18 miRNAs with at least 1 significant difference between groups (Fig. 5). One was added by the intersection of 3 normalizers.
There was excellent agreement for the magnitudes of ΔΔCt between the 4 normalizers (Supplementary Tables S4 and S5). In contrast, N0 selected 9 additional miRNAs that were not found with N2, N3 and N6 (Fig. 5). These were considered false positive results.

**Nonexercise groups.** None of the miRNAs were significantly different between nonexercise groups using our stringent criteria. miR-22-3p ΔCt values were higher in cfs0 than sc0, but the differences were not significant after ANOVA and Tukey tests.

**Post-exercise groups.** miR-22-3p and miR-9-3p were the only miRNAs to be significantly different between the post-exercise groups (Fig. 6). miR-22-3p was an anomaly by having wide ranges of Ct in all groups. miR-22-3p was virtually not detectable in START, and so levels in START were significantly diminished compared to SC and STOPP (Supplementary Table S6). The reduction in START, but relative increase in STOPP, supported the presence of 2 phenotypes of GWI. Specificities and sensitivities were 0.76 for START versus SC (Ct threshold of 29) and START versus STOPP (Ct threshold of 33) (Table 2).

miR-9-3p demonstrated a different trend. Only the START group had detectable levels (Ct ≤ 35) in more than two thirds of subjects. The difference between START and STOPP was small (Fig. 6, Supplementary Table S6) but significant (HSD < 0.05, FDR < 0.05). The low specificity and sensitivity of 65% at a threshold of Ct = 33 reflected the low levels of miR-9-3p in cerebrospinal fluid.

| miRNA     | Group 1 | Group 2 | Specificity | Threshold | Sensitivity | AUC  |
|-----------|---------|---------|-------------|-----------|-------------|------|
| miR-22-3p | SC      | sc0     | 0.80        | 32        | 0.80        | 0.81 |
| miR-204-5p| SC      | sc0     | 0.80        | 31        | 0.80        | 0.91 |
| miR-99b-5p| SC      | sc0     | 0.73        | 33        | 0.73        | 0.82 |
| miR-30d-5p| SC      | sc0     | 0.72        | 33        | 0.72        | 0.89 |
| miR-425-3p| SC      | sc0     | 0.7         | 33        | 0.7         | 0.79 |
| miR-328   | sc0     | SC      | 0.74        | 23        | 0.74        | 0.79 |
| miR-608   | sc0     | SC      | 0.78        | 28        | 0.78        | 0.80 |
| miR-99b-5p| START   | gwi0    | 0.76        | 33        | 0.76        | 0.84 |
| miR-425-3p| START   | gwi0    | 0.72        | 33        | 0.72        | 0.84 |
| miR-370   | START   | gwi0    | 0.72        | 31        | 0.72        | 0.84 |
| miR-328   | gwi0    | START   | 0.91        | 25        | 0.91        | 0.98 |
| let-7i-5p | gwi0    | START   | 0.78        | 33        | 0.78        | 0.80 |
| miR-200a-5p| gwi0  | START   | 0.83        | 33        | 0.83        | 0.88 |
| miR-608   | gwi0    | START   | 0.82        | 30        | 0.82        | 0.88 |
| miR-93-3p | gwi0    | START   | 0.77        | 34        | 0.77        | 0.84 |
| miR-204-5p| STOPP   | gwi0    | 0.62        | 31        | 0.62        | 0.72 |
| miR-99b-5p| STOPP   | gwi0    | 0.75        | 33        | 0.75        | 0.84 |
| miR-328   | gwi0    | STOPP   | 0.86        | 23        | 0.86        | 0.87 |
| let-7i-5p | gwi0    | STOPP   | 0.72        | 32        | 0.72        | 0.78 |
| miR-200a-5p| gwi0  | STOPP   | 0.71        | 31        | 0.71        | 0.73 |
| miR-608   | gwi0    | STOPP   | 0.79        | 28        | 0.79        | 0.77 |
| miR-93-3p | gwi0    | STOPP   | 0.75        | 32        | 0.75        | 0.76 |
| miR-328   | cfs0    | CFS     | 0.84        | 24        | 0.84        | 0.90 |
| miR-608   | cfs0    | CFS     | 0.76        | 29        | 0.76        | 0.84 |
| let-7i-5p | cfs0    | CFS     | 0.68        | 31        | 0.68        | 0.74 |
| miR-200a-5p| cfs0  | CFS     | 0.72        | 32        | 0.72        | 0.82 |
| miR-93-3p | cfs0    | CFS     | 0.77        | 33        | 0.77        | 0.83 |
| miR-126-5p| cfs0    | CFS     | 0.74        | 32        | 0.75        | 0.88 |
| miR-19b-3p| cfs0    | CFS     | 0.63        | 34        | 0.63        | 0.79 |
| miR-505-3p| cfs0    | CFS     | 0.65        | 34        | 0.65        | 0.82 |
| miR-92a-3p| cfs0    | CFS     | 0.81        | 29        | 0.81        | 0.86 |
| miR-186-3p| cfs0    | CFS     | 0.67        | 35        | 0.67        | 0.79 |
| miR-323b-5p| cfs0  | CFS     | 0.75        | 32        | 0.75        | 0.84 |
| miR-532-5p| cfs0    | CFS     | 0.70        | 34        | 0.70        | 0.73 |

Table 2. Receiver operating characteristics.

miRNAs elevated after exercise compared to nonexercise groups. Exercise elevated the levels of several miRNAs compared to appropriate nonexercise groups. SC had higher levels than sc0 for miR-22-3p (Fig. 6), miR-30d-5p, miR-204-5p, miR-425-3p, and miR-99b-5p (Fig. 7). Specificities and sensitivities for miR-204-5p and miR-22-3p were 0.80 at thresholds of 31 and 32, respectively.
miR-425-3p and miR-99b-5p when compared to gwi0. miR-370 was detected in almost all cerebrospinal fluid samples, but only START had a significant elevation compared to nonexercise (ΔΔCt = 1.7 ± 2.1 versus gwi0, mean ± SD). STOPP shared the exercise – induced elevation of miR-99-5p with SC and START. Specificities and sensitivities for miR-99b-5p were about 0.75 at Ct thresholds of 33 for SC, START and STOPP compared to their nonexercise controls.

Figure 4. Normalizer miRNAs. Data are shown as Ct for each miRNA (N0 normalizer). The N2 normalizer used (a) miR-489 and (b) miR-490-3p. N3 added (c) miR-127-3p. The N6 normalizer added (d) miR-433, (e) miR-124-3p, and (f) miR-183-3p. Each miRNA had ΔΔCt < 1.0 between groups, ANOVA > 0.05, FDR > 0.10, and were detectable in at least 180 of the 182 subjects. The blue line indicates Ct = 35. Nonexercise groups were control (sc0, grey circles), Chronic Fatigue Syndrome (cfs0, grey triangles), and Gulf War Illness (gwi0, grey diamonds). Post-exercise groups were control (SC, yellow circles), Chronic Fatigue Syndrome (CFS, blue triangles), and the Gulf War Illness START (green squares) and STOPP (red diamonds) phenotypes. Mean ± SD.

Figure 5. Intersection of miRNAs from each normalizer. The intersection of N0, N2, N3, and N6 identified 16 miRNAs that had at least 1 significant difference between groups (central yellow boxes). N0, N2 and N6 and N2, N3 and N6 added one each. Pairs of normalizers identified 6 miRNAs that were not considered significant. N0 was least selective as it identified an additional 12 miRNAs that were considered false positives. Therefore, the miRNAs selected by 3 or 4 normalizers were the set of significantly different miRNAs.

START had elevated levels of miR-425-3p and miR-99b-5p when compared to gwi0. miR-370 was detected in almost all cerebrospinal fluid samples, but only START had a significant elevation compared to nonexercise (ΔΔCt = 1.7 ± 2.1 versus gwi0, mean ± SD). STOPP shared the exercise – induced elevation of miR-99-5p with SC and START. Specificities and sensitivities for miR-99b-5p were about 0.75 at Ct thresholds of 33 for SC, START and STOPP compared to their nonexercise controls.

CFS did not have any elevations of miRNA levels compared to cfs0.

miRNAs diminished after exercise compared to nonexercise groups. miR-328 and miR-608 were significantly diminished by exercise in SC, CFS, START and STOPP compared to the nonexercise sc0, cfs0, and gwi0 groups (Fig. 8). These miRNAs were detectable in almost all cerebrospinal fluid specimens in this study.
Figure 6. miRNA differences between post-exercise groups. (a) miR-22-3p was not detectable in most of the START subjects (green squares above the blue line at Ct = 35). START had significantly less miR-22-3p than SC (yellow circles) and STOPP (red diamonds) as indicated by bars over top of the groups (HSD < 0.05, FDR < 0.10). In addition, SC had significantly more miR-22-3p than sc0 (grey circles). (b) miR-9-3p was detected in START, but was found in fewer than two thirds of subjects in the other groups. START had significantly more miRNA expression than STOPP (ΔΔCt = 1.6 ± 1.4, mean ± SD, HSD < 0.05, FDR < 0.10).

Figure 7. miRNAs that were significantly elevated in post-exercise compared to appropriate nonexercise control groups. Significant differences between groups were indicated by the bars at the top of the graphs for (a) miR-99b-5p, (b) miR-425-3p, (c) miR-30d-5p, (d) miR-204-5p, and (e) miR-370 (HSD ≤ 0.05, FDR ≤ 0.10, detected with Ct ≤ 35 in more than two thirds of one group per pair). The horizontal blue line indicated Ct = 35. Mean ± SD.
Specificities and sensitivities for miR-328 ranged from 0.74 at Ct = 23 for SC, 0.84 for CFS, 0.86 for STOPP and 0.91 for START (Ct thresholds of 23 to 25). Specificities and sensitivities for miR-608 ranged from 0.78 to 0.83 (thresholds = 28). Diminished miR-328 and miR-608 may be a consequence of exercise that affected all subjects regardless of their disease status.

miR-let-7i-5p, miR-200a-5p and miR-93-3p were significantly reduced in START, STOPP and CFS compared to their gwi0 and cfs0 nonexercise controls (Fig. 9). They were unchanged between SC and sc0 groups.

CFS was distinguished from the other groups by having significant reductions of miR-126-5p, miR-186-3p, miR-19b-3p, miR-92a-3p and miR-505-3p compared to the nonexercise cfs0 group (Fig. 10). Specificities and sensitivities were about 0.82 for miR-328, miR-608 and miR-92a-3p. The large number of exercise-induced reductions in miRNAs differentiated CFS from SC and the GWI phenotypes.

Gender. Cerebrospinal fluid miRNA levels for females and males in the nonexercise and the post-exercise groups were equivalent except for a significantly higher level of miR-9-3p in START than STOPP males ($\Delta\Delta C_{t} = 1.7 \pm 1.4$)\(^4\).

The only exercise-induced change in females was a reduction in miR-328 in the STOPP group compared to gwi0 (5.7 ± 0.8). Samples sizes for the post-exercise SC and START females (n = 3 each) were too small to infer meaningful differences.

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**Figure 8.** Decreased (a) miR-328 and (b) miR-608 levels in SC, CFS, START and STOPP groups after exercise.

**Figure 9.** miRNAs reduced by exercise. GWI phenotypes (START and STOPP) and CFS all had reductions in (a) miR-let-7i-5p, (b) miR-200a-5p and (c) miR-93-3p. Sedentary controls had no changes.
The post-exercise control males had significantly elevated miR-204-3p (4.4 ± 2.8), miR-30d-5p (3.4 ± 2.3) and miR-30a-5p (2.9 ± 2.0) compared to nonexercise males. miR-328 was reduced by exercise in STOPP (7.2 ± 5.4), STOPP (6.9 ± 4.8) and CFS (5.7 ± 3.7) males compared to nonexercise males. Control males had a similar magnitude change that was not significant by FDR (3.3 ± 3.2). STOPP males had significantly diminished miR-608 (4.6 ± 3.2) and miR-200a-5p (3.7 ± 2.1).

These differences were consistent with the overall group effects

**Discussion**

This is the first description of the effects of exercise on cerebrospinal fluid miRNA expression in healthy subjects. Exercise diminished miR-328 and miR-608 in all subjects suggesting a general effect on the brain (Fig. 8, Supplementary Table S5). Exercise caused distinct patterns of miRNA changes in CFS and the START and STOPP phenotypes of GWI indicating significant pathophysiological differences between conditions.

Unlike our starting hypothesis, there were no differences in miRNA levels between the nonexercise groups of control, CFS and GWI subjects. Therefore, baseline levels of cerebrospinal fluid miRNAs may not be useful for diagnosis of CFS or GWI.

The only significant differences between groups after exercise were diminished miR-22-3p in compared to SC and STOPP, and elevated miR-9-3p in START compared to STOPP (Fig. 6). These differences between START and STOPP support our 2 phenotypes of GWI.

The most striking findings were the changes between post-exercise groups and their appropriate nonexercise comparison groups. SC had 5 elevated miRNAs after exercise, compared to 3 for START, 1 for STOPP, and none in CFS (Fig. 7, Supplementary Table S4).

The reduction of miR-608 after exercise has implications for the cholinergic hypothesis of GWI pathophysiology because it targets acetylcholinesterase and interleukin-6 (IL6) mRNAs. miR-608 binds weakly to the single-nucleotide polymorphism rs17228616 allele in the 3′-untranslated region of acetylcholinesterase mRNA. Homozygotes for rs17228616 have reduced affinity for miR-608. This promotes mRNA stability and increases acetylcholinesterase protein translation. As a consequence, more miR-608 is available to bind to IL6 mRNA and reduces its translation. This allele also contributes to reduced cortisol and elevated blood pressure. Because rs17228616 promotes higher acetylcholinestase activity, it may be relatively protective against nerve agent and pyridostigmine bromide exposure.

miR-let-7i-5p, miR-93-3p and miR-200a-5p were significantly diminished after exercise in START, STOPP and CFS, but not SC (Fig. 9). This was consistent with a cardinal finding in CFS and GWI: function may appear normal when rested, but will deteriorate after a physiological stressor. miR-let-7i was reduced in plasma after exercise in athletes. miR-let-7i has decreased expression in the prefrontal cortex of FSL rats in a model of...
IL6 is a target of miR-let-7i, and, as predicted, this cytokine was significantly elevated in the brains of these rats. When FSL rats were given access to running wheels, their miR-let-7i expression was increased and IL6 reduced. Modulation of miR-let-7i and IL-6 may contribute to exercise-induced benefits in “inflammatory” depression. miR-let-7i also contributes to the regulation of acetycholine’s muscarinic and α4β2 nicotinic receptors and epigenetic regulation of acetylcholinesterase. These animal models may not be appropriate for CFS or GW1 because human subjects develop exertional exhaustion after exercise, and are unlikely to significantly increase spontaneous exercise levels when provided with a treadmill.

The CFS group had 12 miRNAs reduced after exercise. miR-186-3p was decreased in aging mice where it targets β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) mRNA. Diminished miR-186-3p allows increased BACE1 mRNA translation and cleavage of amyloid peptides that increase the risk for brain disease. miR-19b-3p was reduced in serum from Alzheimer’s patients, and targeted signal transduction and activator of transcription 3 (STAT3) mRNA in a murine model. miR-92a-3p was increased in glioblastoma and targeted BCL2L11 to reduce tumor apoptosis. Its reduction after exercise may promote apoptosis in CFS. miR-126-5p was highly expressed in endothelial cells where it targets vascular (VCAM), intercellular (ICAM) and activated leukocyte (ALCAM) cell adhesion molecule mRNAs and so reduces transendothelial migration. This is relevant for immune cell influx into the brain, and hypotheses of neuroinflammation in CFS pathogenesis.

Neurons may be the sources of miR-124-3p, miR-127-3p, miR-433, and miR-323b-5p (Figs 4 and 10). There was little overlap with the miRNAs synthesized in astrocytes, oligodendrocytes and microglia.

The choroid plexus epithelium may be a primary source of miRNAs in cerebrospinal fluid. Epithelial cells form a monolayer linked by tight junctions that creates the “shrink wrapped” cellular barrier around fenestrated capillaries. Interferon-γ and other mediators generated by exercise, inflammation, and other stressors act directly on choroid plexus to modulate barrier permeability, plasma protein transport, protein synthesis and secretion of nutrients into cerebrospinal fluid. miR-328, which was present in all subjects and reduced after exercise (Fig. 8), binds to the 3′-untranslated regions of CD44 and collagen type 1 1 α1 mRNAs to modulate extracellular barrier functions. Choroid plexus miRNAs are packaged into extracellular vesicles and released into cerebrospinal fluid. Downstream targets include subventricular neural stem cells, mature neurons, astrocytes, oligodendrocytes, microglia, meningeal and central immune cells. Blockade of extracellular vesicle secretion from choroid plexus cells decreased brain inflammation in a mouse model of lipopolysaccharide-induced inflammation. Choroid plexus miRNAs may be novel drug targets to modulate acute illness behaviours, fever, and chronic pain in systemic illnesses.

Choroid plexus is dysfunctional in Alzheimer’s disease. This provides the rationale to consider the role of the blood–cerebrospinal fluid barrier in the cognitive dysfunction of CFS and GW1. There are numerous reports of elevated and diminished miRNAs in cerebrospinal fluid in Alzheimer’s disease, but none matched the patterns of our groups. miR-let-7i-5p was elevated in Alzheimer’s, but levels were equivalent in nonexercise groups (Fig. 8).

Depression is in the differential diagnosis because of the shared ancillary diagnostic criteria (Fig. 1). Major depressive disorder is defined by affective dysfunction with sadness, flat affect and anhedonia as essential features, followed by secondary criteria including fatigue, cognitive, sleep, and somatic dysfunction. However, screening questionnaires for depression emphasize somatic symptoms. Complaints of fatigue, sleep, and cognitive dysfunction will inflate total questionnaire scores, and may lead to false positive inference of major depressive disorder even if anhedonia or affective complaints are absent. This is particularly problematic in CFS and GW1 where these features are diagnostic criteria (Fig. 1). As a result, Center for Epidemiology – Depression (CESD) scores were significantly elevated in GW1 (78.3%), CFS (64.0%) and control (25.0%) groups (Table 1).

Quantitative PCR of miRNAs offers a more objective solution. miR-16 in cerebrospinal fluid was significantly lower in major depressive disorder patients than control subjects. However, this was not confirmed in an independent group who had a different pattern of 11 significantly elevated and 5 reduced miRNAs. Our data did not confirm either of these findings because only 3 of the miRNAs were detected with Ct ≤ 35 in more than two thirds of our nonexercise group. miR-425-3p was significantly reduced in depression patients, and was detected in about half of all nonexercise subjects. It was increased after exercise in SC, START and STOPP but not CFS (Fig. 7). This lack of reproducibility highlights the need to independently verify miRNA findings, and supports our rationale for strict statistical criteria to define potential miRNA biomarkers.

The pain and tenderness of GW1 subjects (Table 1) indicated systemic hyperalgesia and suggested parallels with fibromyalgia. Nine miRNAs were virtually undetectable in 10 fibromyalgia women compared to 8 healthy control women. miR-99b-5p and miR-29a-3p were absent in fibromyalgia, but were detected in more than two thirds of our participants. miR-99b-5p was significantly increased after exercise in SC, START and STOPP (Fig. 7). The other 7 miRNAs were detected in less than half of our specimens. This suggested that GW1 and CFS were distinct from fibromyalgia.

Limitations to the diagnostic use of quantitative miRNA analysis in cerebrospinal fluid include the remarkable lack of consensus about miRNA levels in control subjects. This can be remedied by standardization of reagents and protocols, open source sharing of study outcomes, and meta-analysis of the raw data. The yield of extracted miRNA and detectability were improved with 0.5 ml instead of 0.2 ml of cerebrospinal fluid. QPCR with Ct cut-offs ≤ 35 cycles reduced amplification artifacts. The wide range of miR-22-3p Ct values (Fig. 6) may be due to commercial changes in reagents designed to improve miRNA detection. Highly abundant miRNAs that were detected with Ct ≤ 35 in all subjects were used as normalizers (Fig. 4) rather than the global average level, miR-423-5p, miR-124-3p or U6. Constraints included (i) significant ANOVA and Tukey HSD between groups, (ii) significant FDR to correct for multiple comparisons, and (iii) focusing on miRNAs that were detected in more than two thirds of subjects per group that may be viable biomarker candidates for use in the general population. Ages were comparable between groups (Table 1) and there were no differences in expression between males and females. Next generation sequencing is an excellent discovery tool but needs...
careful internal standardization to be as sensitive as QPCR for quantification. Adequate sample sizes were essential because our initial findings with about a dozen subjects per group showed differences between START and STOPP after exercise, but these differences eventually regressed to the mean as more subjects were analyzed. This is especially pertinent to smaller studies examining the differential diagnosis of CFS and GWI. 40–42 Limitations of the testing paradigm include the intensive nature of the exercise and magnetic resonance imaging characterization of GWI subjects to determine their phenotypes. Lumbar puncture was required to obtain the cerebrospinal fluid miRNA biomarkers, but this procedure is not a contraindication to making an objective diagnosis of GWI. On the contrary, magnetic resonance imaging with cerebrospinal fluid QPCR miRNA profiling may be complementary tools for diagnosis of CFS, GWI and their subtypes.

Conclusions
Cerebrospinal fluid miRNA levels were equivalent between SC, CFS and GWI subjects who had rested before exercise (nonexercise groups). miRNA levels were different from the ones that are altered in depression, fibromyalgia, and Alzheimer’s disease suggesting that these are all distinct diseases, or that the data from those smaller studies could not be replicated in this larger study. miRNA levels were equivalent between the post-exercise SC, CFS and GWI phenotypes of START and STOPP with the exception of miR-22-3p and miR-9-3p that significantly distinguished START from STOPP. This adds another layer of evidence to support neurotoxic pathology in GWI 47 and these 2 phenotypes of GWI veterans. Post-exercise levels were significantly elevated (n = 6) or diminished (n = 12) compared to the nonexercise comparison groups. miR-328 and miR-608 were elevated in SC, CFS, START and STOPP and may be a global marker of the exercise stressor on the choroid plexus and brain. CFS had 12 diminished and zero elevated miRNAs after exercise indicating its pathophysiology and responses to exercise were unique compared to GWI and control subjects. Despite the symptom overlap of CFS, GWI and other illnesses in the differential diagnosis (Fig. 1), the distinct exercise-induced miRNA patterns in cerebrospinal fluid imply separate mechanisms for post-exertional malaise in these diseases.

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Author Contributions
J.N.B. organized the studies. N.S. performed the QPCR in blinded fashion. J.N.B. and N.S. wrote the paper.

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