Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness

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Long title: Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness

Short title: Low mitochondrial lipids in GWI

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Highlights

- Translational studies of mitochondrial lipid changes in GWI.
- Long-term memory deficits are present in a GWI mouse model
- Mitochondrial lipids are decreased in the brains of a GWI mouse model.
- Plasma mitochondrial lipids are decreased in a GWI mouse and in veterans with GWI.
- Targeting mitochondria may be useful for developing GWI therapies and biomarkers.

Abstract:

Gulf War Illness (GWI) affects 25% of veterans from the 1990-1991 Gulf War (GW) and is accompanied by damage to the brain regions involved in memory processing. After twenty-five years, the chronic pathobiology of GWI is still unexplained. To address this problem, we examined the long-term consequences of GW exposures in an established GWI mouse model to identify biological processes that are relevant to the chronic symptoms of GWI. Three-month old male C57BL6 mice were exposed for 10 days to GW agents (pyridostigmine bromide and permethrin). Barnes Maze testing conducted at 15- and 16-months post-exposure revealed learning and memory impairment. Immunohistochemical analyses showed astroglia and microglia activation in the hippocampi of exposed mice. Proteomic studies identified perturbation of mitochondria function and metabolomics data showed decreases in the Krebs cycle compounds, lactate, β-hydroxybutyrate and glycerol-3 phosphate in the brains of exposed mice. Lipidomics data showed decreases in fatty acids, acylcarnitines and phospholipids, including cardiolipins in the brains of exposed mice. Pilot biomarker studies showed that plasma from exposed mice and veterans with GWI had increases in odd-chain, and decreases in long-chain, acylcarnitines compared to their respective controls. Very long-chain acylcarnitines were decreased in veterans with GWI compared to controls. These studies suggest that mitochondrial lipid disturbances might be associated with GWI and that further investigation is required to determine its role in the pathophysiology of this illness. Targeting mitochondrial function may provide effective therapies for GWI, and that lipid abnormalities could serve as biomarkers of GWI.

Keywords: Gulf War Illness; pyridostigmine bromide; pesticides; mitochondria; cardiolipin; acylcarnitine

1. Introduction:

Nearly 250,000 veterans from the 1990-1991 Gulf War (GW) continue to suffer from Gulf War Illness (GWI), which is a chronic and debilitating condition with a central nervous system (CNS) component (White et al., 2016; Binns et al., 2008) that is characterized by reduced volume and hypometabolism in the hippocampi of veterans with GWI (White et al., 2016). A functional imaging study showed that deficits in working memory are associated with an abnormal activation of the prefrontal cortex of ill GW veterans (Hubbard et al., 2014). A diffusion tensor imaging study suggested that compared to controls, veterans with GWI have atrophy of the axonal tracts that link the cortical gray matter regions involved in fatigue, pain and cognition (Rayhan et al., 2013b). Current treatments for GWI are based on symptom management due to a lack of understanding of the complex pathophysiology of GWI. Many GW veterans have not been able to receive an appropriate diagnosis of their conditions due to the unavailability of objective biological markers that can quantify the underlying pathology. Hence, investigations into chronic pathophysiology of GWI is required for developing treatments and diagnostic biomarkers of this illness.

Reports compiled by the Research Advisory Committee (RAC) on Gulf War Veterans' Illnesses implicate exposure to GW agents, such as the acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB) and pesticides permethrin (PER), in the pathogenesis of GWI (White et al., 2016; Binns et al., 2008). Evidence for a causal role of GW agents in GWI comes from studies showing elevated rates and severity of illness among veterans who consumed a large quantity of PB tablets and also applied pesticides (White et al., 2016; Binns et al., 2008; Steele et al., 2012). A case-control study of 300 GW veterans showed that compared to controls, veterans with GWI had a higher prevalence of exposure to PB and pesticides (White et al., 2016; Steele et al., 2012). Given the CNS dysfunction in

GWI, identifying molecular changes in the brain necessitates the use of well-established animal models of GWI. We developed a GWI mouse model using combined exposure to PB and PER that exhibits anxiety and cognitive problems that are similar to the symptoms reported by veterans with GWI (White et al., 2016; Abdullah et al., 2011; Zakirova et al., 2015; Sullivan et al., 2003). Hence, this mouse model captures one of the key clinical features of GWI and is suitable for examining long-term changes that are relevant to the current clinical condition of veterans with GWI.

Histopathological analyses of this PB+PER mouse model revealed an increase in astroglia activation in exposed mice (Abdullah et al., 2011; Zakirova et al., 2015), which is also observed in several other rodent models of GWI (Abdullah et al., 2012; Abdullah et al., 2011; Zakirova et al., 2015; Parihar et al., 2013; Ojo et al., 2014). In the brain, astroglia play a fundamental role in providing support to neurons by maintaining the extracellular environment and delivering nutrients to neurons that are acquired from the peripheral circulation. For instance, astroglia take up glucose from the brain capillaries via their end-feet and deliver to neuronal axons (Baltan 2015). While glucose is the primary energy source which fuels the tricarboxylic acid (TCA) cycle, other nutrients, such as ketone bodies and lactate, are used for neuroenergetics when the glucose supply runs low or during high neuronal demand (Logica et al., 2016). In the brain, β-oxidation of free fatty acids (FFA) yields acetyl-CoA molecules for the TCA cycle to generate ATP (Guzmán, & Blázquez 2004). Importantly, astroglia are the primary cell type in the brain that can store glucose as glycogen which can be converted to lactate and transported to neurons via mono-carboxylate transporters (MCT) (Newington et al., 2013). In this regard, a recent imaging study showed impaired lactate utilization in the brains of veterans with GWI (Rayhan et al., 2013a), which could suggest potential disruption of the astroglia-neuron shuttle in relation to GWI.

The goal of this current work is to elucidate long-term biological disturbances associated with GW agent exposure. Several decades have now elapsed since veterans were exposed to GW agents, and, therefore, an interaction between exposure to these chemicals and aging is also a concern for

this vulnerable population (Institute of Medicine 2013). Given the complexity of biological response to GW agents, the heterogeneity of the clinical presentation of GWI and the chronicity of the illness (White et al., 2016), we used omic approaches (proteomics, lipidomics and metabolomics) to identify biological pathways associated with GW agent exposure. These analyses pointed to mitochondrial lipid and energetic disruptions in our mouse model. In a pilot study, we examined the translational value of mitochondria-specific lipids (acylcarnitines) in plasma and showed that these lipids have the potential to differentiate veterans with GWI from controls. Targeting mitochondrial lipid metabolism may be an avenue for developing treatments for the chronic and persistent CNS problems currently experienced by veterans with GWI. These lipids may also be useful for identifying blood biomarkers of GWI.

2. Materials and Methods:

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee and performed as previously described (Abdullah et al., 2011; Zakirova et al., 2015). Male C57BL6 mice (3-months of age, weight 25 g ± 0.7 SD) were co-administered 0.7 mg/kg of PB and 200 mg/kg of PER in a single intraperitoneal injection volume of 50 µl in dimethyl sulfoxide (DMSO) or the same volume of DMSO alone (control) daily for 10 consecutive days (n = 10 for controls, n = 9 for GW agent exposed mice). The doses of PB and PER are less than 1/5 and 1/2 of the known LD_{50} values in rodents, respectively (Chaney et al., 2002; Williamson et al., 1989). Given the paucity of information on doses and routes of PER exposure, we used 200 mg/kg of PER to mimic a high-level exposure that is similar to that used in mice by previous studies showing adverse behavioral or pathological outcomes (Abdullah et al., 2011; Zakirova et al., 2015; Dodd, & Klein 2009; Pittman et al., 2003). The cis/trans ratio of PER were based on the recommended by the World Health Organization (25% cis and 75% trans) (WHO 2003). The dose of 0.7 mg/kg of PB have been shown to elicit neurobehavioral deficits relevant to GWI symptom presentation (Zakirova et al., 2015).

Experimenters for neurobehavioral, neuropathological, lipidomics, metabolomics and proteomic analyses were blinded to the exposure assignment. For these studies, post-exposure time-points of 15- and 16-months were chosen at which point these mice were 18- and 19-months of age, respectively.

2.2. Human subjects

Plasma samples from 12 veterans with GWI and 8 healthy controls who were deployed to the GW were assessed in this pilot study. Plasma samples from GW veterans were provided by our collaborators at the Boston University and NOVA Southeastern University sites from an established biorepository of samples from GW veterans who agreed to share their blood samples from prior studies including the Boston Gulf War Illness Consortium (GWIC) and the Dynamic Modeling of GWI study from NOVA Southeastern University and the South Florida Veterans Affairs Foundation for Research and Education, Inc. The GWI biorepository is approved by the institutional review board (IRB) at the Boston University, the NOVA Southeastern University and the Miami Veterans Administration Medical Center (VAMC). Samples from the GWI biorepository were all collected from the Boston and Miami sites using the same written standard operating procedures for performing phlebotomy, plasma separation and aliquoting. All samples were stored at -80^{o}C and were not previously thawed and refrozen. Gulf War veteran participants were consented into their respective studies using the International Conference on Harmonization Good Clinical Practice guidelines. The Kansas GWI criteria (Steele 2000) were used to determine GWI case and control status. The Kansas GWI criteria require that 1990-1991 GW veterans endorse symptoms in at least 3 out of 6 symptom domains (fatigue/sleep problems, pain, cognitive, mood symptoms, gastrointestinal symptoms, respiratory symptoms and skin abnormalities). Controls veterans were also from the 1990-1991 GW who did not endorse 3 out of 6 symptom domains associated with the Kansas criteria. Subjects also completed demographics and health symptom questionnaires including the Pittsburgh Sleep Quality Index (PSQI), Visual Analog Scale (VAS) for pain, Multi-dimensional Fatigue Inventory (MFI-20)

questionnaire, Medical Outcome Study Short Form 36-veteran version (SF-36V), and Profile of Mood States (POMS). Study participants were excluded if they reported being diagnosed with another medical condition that could explain the above mentioned symptoms. According to the Kansas GWI case definition exclusions, veterans with a history of prior CNS or major psychiatric disorders that may affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson's Disease, Alzheimer's disease, schizophrenia) would be ineligible. For the purposes of current translational studies and due to the known interactions between GWI and gender (Abdullah et al., 2012; Smylie et al., 2013), only male subjects were evaluated since the mouse studies were only comprised of male mice. Both control GW veterans (n = 8) and veterans with GWI (n = 12) were matched for age (mean age in controls = 49.3 ± 2.6 SE and GWI = 46.1 ± 1.6 SE) and ethnic background (among controls, 5 subjects were white, 1 Hispanic and 2 African American and among GWI, 4 subjects were white, 2 Hispanic, 5 African American and 3 Asian).

2.3. Behavioral testing

The Barnes maze acquisition trials were conducted at 15-months post-exposure (daily over 4 days) for 3 min per trial as previously described (Sunyer et al., 2007). Briefly, four trials were administered per mouse with an inter-trial interval of 15 min. Each mouse was placed in the middle of the maze and the trial ended when the mouse entered the escape box or after 3 min. Bright flood lamps were used as motivators for mice to enter the escape box, where they stayed for 1 min. If a mouse did not reach the target hole within 3 min, the experimenter guided it to the escape box. A probe trial was conducted 24 hrs after the last training session. The escape box was removed for the probe trials. Each mouse was placed in the middle of the maze and allowed to explore for a fixed interval of 1.5 min. Another probe trial was administered at 16 months post-exposure to assess long-term memory. The Ethovision software was used to track mouse movement and total distance traveled by each mouse on the platform was used to calculate the path length. Animals were subsequently euthanized for the neuropathological and biochemical studies.

2.4. Neuropathological analyses

Animals were transcardially perfused with phosphate buffered saline (PBS) followed by an overnight fixation of the brains in 4% paraformaldehyde and embedded with paraffin. Sagittal sections (lateral 0.2-0.4mm) were prepared and rehydrated in an ethanol gradient before the staining procedures. Glial fibrillary acid protein (GFAP; 1:10,000, Dako, Carpinteria, CA, USA) and ionized calcium binding adaptor molecule 1 (Iba1) antibodies (1:5,000; Abcam, Cambridge, MA, USA) were used to stain astroglia and microglia, respectively. Primary antibodies were localized using Vectastain ABC kits (Vector Laboratories, Burlingame, CA, USA) as previously described (Abdullah et al., 2011; Mouzon et al., 2014). ImagePro plus software (Media Cybernetics, Rockville, MD, USA) was used to analyze the stained sagittal sections (4 randomly selected non-overlapping areas of 100 μm^2 of cerebral cortices and the dentate gyri). The stained areas were calculated and expressed as a percentage of the field of view.

2.5 Proteomics

Brain tissue was homogenized in PBS containing 1M sodium chloride (NaCl), and clarified by centrifugation. The supernatant was fractionated into soluble and membrane fractions via methanolic PBS precipitation. Protein from 100µg of each sample underwent reduction and alkylation at cysteine residues to avoid self aggregation prior to trypsinization. The digest was 4-plex labeled using Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) according to manufacturer's instructions (SCIEX, Framingham, MA, USA). The groups were paired and mixed according to experimental design and fractionated by reversed phase chromatography and analyzed by capillary liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) using a Easy Ultra Performance Liquid Chromatography (UPLC) interfaced with a Q-Exactive Orbitrap MS (Thermo). Higher-energy C-trap dissociation was used with the minimum m/z set to 100 to ensure the presence of the reporter ions in all MS^2 spectra for quantification. Following quantification, differentially expressed proteins were investigated using Ingenuity

Pathways Analysis (IPA, Ingenuity® Systems, Redwood City, CA, USA) (see supplementary methods for more details).

2.6. Metabolomics

Brain homogenate from whole left hemisphere (50µl) containing 8mg wet weight of tissue were prepared as previously described (Abdullah et al., 2012) and combined with 5µl of deuterium (d) labeled internal standard (IS) mix containing 200 µg/ml d_3-stearic acid and 5 µg/ml d_5-phenylalanine and 200µl of 80% methanol. The mixture was heated for 2 min at 80^{o}C and cooled for 5 min before centrifuging for 10 min at 20,000 x g. The supernatant was dried under vacuum and then aldehydes and ketones were derivatized to their methoximes by addition of 10 µl of methoxylamine hydrochloride in pyridine and heating at 30^{o}C for 90 min. Functional groups containing active hydrogen were converted to their trimethylsilyl derivatives by addition of 90 µl of N-Methyl-N-trimethylsilyltrifluoroacetamide containing 1% trimethylchlorosilane followed by incubation at 60^{o}C for 30 min. Derivatized samples were analyzed on Agilent 7200 GC-QTOF (Agilent, Santa Clara, CA, USA). One µl of sample was injected (split 1:10, injector temperature 250^{o}C and hydrogen as the carrier gas) onto a DB5-MS column (30 m X 0.25 mm ID, 0.25 µm film thickness). The column temperature was held at 60^{o}C for 1 min and then programmed to 325^{o}C at 10^{o}C/min with a 3.5 min final hold. Electron impact ionization (EI) was used at 70 eV with the source at 250^{o}C. Spectra were acquired from m/z 40 – 600 at the rate of 5 spectra/second, mass accuracy <2 ppm and mass resolution greater than 13,500 full width at half maximum (FWHM). Metabolites were identified by searching spectra from our dataset with those deposited in the Golm database (http://gmd.mpimp-golm.mpg.de/) and the Fiehn gas chromatography/mass spectrometry (GC/MS) metabolomics retention time locked library (Agilent Technologies, created 21 Jul 2013). Spectra were exported and normalized against d_5-phenyalanine for amino acids and d_3-stearic for all other metabolites) followed by statistical analyses using SPSS version 22 (IBM Corporation, Armonk, NY, USA).

2.7. Lipidomics

Brain homogenates for lipidomics were prepared as for metabolomics studies described above.

Cardiolipin analysis: Cardiolipin (CL) extraction was carried out using modified Bligh-Dyer method described elsewhere and in the supplemental section (Sparagna et al., 2005). The lower organic phase was collected and dried for liquid chromatography/mass spectrometry (LC/MS) analysis. Normal phase high-pressure liquid chromatography (HPLC) was carried out using a 1 mm ID x 100 mm column containing 3 µm Ultra silica particles (Restek, Bellefonte, PA, USA). A gradient was run from 50% solvent B (isopropanol: hexane 40:30 v/v) in solvent A (isopropanol:hexane:20mM ammonium acetate 40:30:7 v/v/v) to 95% B in 14 min with a 6 min hold at the final conditions. The flow rate was 50 µl/min with the column temperature at 40°C. Negative ion electrospray spectra were acquired at 100,000 resolution with a scan range from m/z 590 to 900 on a LTQ-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA, USA). All spectra were obtained with a 200 ms maximum ion injection time. The acquired spectra of doubly-charged ions corresponding to individual CL species were deconvoluted (converted to an uncharged spectra) and deisotoped using the Xtract utility in the Xcalibur software (Thermo Scientific, Waltham, MA USA) and quantified with reference to the internal standard. Determination of FA composition of individual CL species is based on reported literature (Monteiro-Cardoso et al., 2015).

Acylcarnitine analyses: Aclycarnitine was extracted from 50µl of brain homogenate or 50µl of plasma to which 5µl of labeled internal standard mix (Nsk-b-1, Cambridge isotope laboratories, MA, USA) was added with 500µl of 25% methanol in acetonitrile (ACN) followed by centrifugation at 20,000 x g. Dried extracts were resuspended in 5% aqueous methanol and subjected to LC/MS using a 2.1mm ID x 50mm Kinetex HILIC column containing 1.7 µm particles (Phenomenex, Torrance, CA, USA). A gradient was run from 95% solvent A (ACN:water:100mM ammonium formate,90:5:5 v/v) in 5% solvent B (ACN:water:100mM ammonium formate,50:45:5 v/v) to 70% B in 10 min with a 10 min hold at the final conditions. The flow rate was 250 µl/min with the column temperature at 40°C. Positive ion electrospray spectra was acquired in the FTMS mode at 30,000

resolution with a scan range from m/z 150 to 1000 on a LTQ-Orbitrap MS (Thermo Scientific). All spectra were obtained with a 100 ms maximum ion injection time and summing 2 microscans.

Phospholipid analyses: Lipids were extracted from mouse brain homogenates and plasma using the Folch method (Folch et al., 1957). Internal standards for each phospholipid (PL) class were added to control for technical variability and for quantification, see procedures elsewhere (Abdullah et al., 2014). Phospholipids were separated using normal phase LC/MS with a Thermo LTQ-XL mass spectrometer (Thermo-Fisher, MI, USA) as previously described (Abdullah et al., 2014). Mass spectra were processed using LipidomeDB online to identify and quantify PL molecular species (Zhou et al., 2011). Within each class, the molecular species that were identified were totaled to generate total phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylinositol (PI) values.

Total Fatty acids: Brain homogenate prepared as above was combined with 14:0 and 17:0 fatty acid (FA) as internal standards, extracted using the Folch method (Folch et al., 1957) and total FA were prepared followed by saponification of lipids and followed by preparation of methyl esters of these FA (see supplemental section for more details). The fatty acid methyl esters were dissolved in isooctane for GC/MS analysis using an Agilent 5975 Turbo GC/MS system (Agilent, Wilmington, DE USA). An Agilent HP-88 Fused Silica Capillary column 30 m x 250 μm x 0.20 μm film thickness was used with hydrogen as the carrier gas using a splittless injection. The GC oven temperature was held at 70°C for 3 min, increased at a rate of 100°C per min up to 140°C then programmed at a rate of 3°C per min up to 210°C. MS was performed with positive ion ammonia chemical ionization and spectra acquired from m/z 200 – 440.

2.8. Statistical analyses

If data were normally distributed (based on the histogram plots and skewness/kurtosis measures), a mixed linear model (MLM) regression was employed to examine the independent effects of exposure and time and any potential interactions between them on the neurobehavioral outcomes.

As applicable, skewed data were either normalized using log transformation for MLM or analyzed using generalized estimating equation (GEE) to test the statistical significance of skewed data and p-values were calculated using the Wald test. For neurobehavioral studies, the total path length to the target hole was considered an outcome factor where exposure, acquisition days and trials were considered fixed factors and each mouse was a random factor. Neuropathology data were analyzed using the MLM or GEE depending on the distribution of the dataset where exposure and replicates were fixed factors and mouse was a random factor. For lipidomics, principal component analysis (PCA) were performed to achieve the dimension reduction and minimize multi-colinearity followed by MLM regression as previously described (Abdullah et al., 2012; Graessler et al., 2009). Variables with eigenvalues of >1 were retained, PCA was used for extracting components, and varimax with Kaiser normalization was used for generating linear combinations to maximize variance loading in order to simplify and clarify the data structure. The Anderson–Rubin method was used for exporting uncorrelated scores in order to perform MLM regression analyses on each component and the outcome measures, while adjusting for fixed (exposure and replication) and random factors (mouse). Metabolomics data were analyzed using MLM with fixed factor being exposure and replication and random factor being mouse.

3.0. Results:

3.1. Neurobehavioral deficits accompany brain mitochondrial disturbances in GW-agent exposed mice.

We observed neurobehavioral deficits in the Barnes Maze test at 15- and 16-months post-exposure. During the acquisition trials conducted at 15-months post-exposure, mice were allowed to explore the arena for 3 min and escape into the visible box through one of the holes in the circular platform. Control mice learned to escape, which corresponded with a significantly shorter path length over training days 1 through 4. However, GW agent exposed mice had similar path length throughout the training period. During the probe trials, conducted on day 5 at 15-months post-exposure and

another 30 days later at 16-months post-exposure (without retraining), the visible escape box was removed and each mouse was allowed to explore the arena for 1.5 min. At both trials, exposed mice had longer path length than controls indicating poor spatial memory (Fig. 1A-B). Neuropathological analyses show small but significant astroglia and microglia activation within the dentate gyri of exposed animals at 16-months post-exposure (Fig. 1C-N). There were no differences between the two groups for astroglia and microglia activation within the cortices or other hippocampal regions. Differentially expressed proteins from the proteomic studies were uploaded into the IPA knowledgebase. Biological functions (biofunctions), such as lipid metabolism, energy production, cell cycle, molecular transport, inflammatory responses and immune cell trafficking were shown to be modulated at 16-months post-exposure to GW agents. Glycolysis I, sucrose degradation V and gluconeogenesis I were the three most modulated canonical pathways following GW agent exposure (Fig. 2). Additional canonical pathways affected by GW agents included, mitochondria dysfunction, pyruvate fermentation to lactate and creatine phosphate biosynthesis. Metabolomic data (Fig. 3) also supported mitochondria dysfunction by showing that lactate, β-hydroxybutyrate (β-HB) and the Krebs cycle metabolites, such as succinic acid, fumaric acid, malic acid, citric acid and isocitric acid were significantly lower in exposed mice compared to controls. Glycerol-3-phosphate and glyceric acid 3-phosphate were also lower in exposed mice ($p < 0.05$).

3.2. Long-term decreases in brain lipids in GW agent exposed mice.

Lipidomic analyses were performed to examine mitochondria specific lipids, CL and acylcarnitines. Relative to control mice, exposed mice had lower levels of CL, particularly long-chain unsaturated species (Fig. 4A). Long-chain acylcarnitine species (Fig. 4B) were also decreased in the brains of GW agent exposed mice compared to control mice ($p < 0.05$). Significant decreases in PE, PC, PI, and LPC were also observed in exposed mice compared to controls (Fig. 4C). Supplemental figure 1 shows the molecular species distribution for each PL class associated with GW agent exposure; a

number of these were essential polyunsaturated fatty acid (PUFA) containing molecular species. Total fatty acid (FA) analysis (Fig. 4D) showed decreases in omega-3 and omega-6 PUFA ($p < 0.05$), where most substantial decreases were observed in omega-3 docosahexaenoic acid (DHA, 22:6n3) and omega-6 arachidonic acid (AA, 20:4n6) and adrenic acid (22:4n6).

3.3. Plasma acylcarnitines alterations in GW-agent exposed mice and GWI veterans

Acylcarnitines are the most abundant mitochondrial lipids that are present in appreciable quantities in plasma. We examined whether these lipids were also altered in plasma samples from our mouse model and in male GW veterans with GWI (46.1 ± 0.92 SE years) compared to male control GW veterans (49.3 ± 1.43 SE years). Figure 5 shows the distribution of acylcarnitine species in plasma from control and exposed mice at 16-months post-exposure. While short-chain (< 6 carbons) acylcarnitine species did not differ between control and exposed mice, odd-chain species (3, 5 and 7) were increased, whereas medium- (6 to 12) and long-chain (14 to 20) acylcarnitine species were decreased in GW agent exposed compared to control mice ($p < 0.05$). In human plasma, we observed an increase in odd-chain ($p < 0.05$) and a trend for decrease in long-chain acylcarnitines in veterans with GWI compared to control veterans ($p = 0.08$). We also detected very long-chain acylcarnitine species (C24:0 to C26:0) and found these species to be also decreased in veterans with GWI compared to controls ($p < 0.05$). Figure 6 shows the distribution of acylcarnitine species in veterans with GWI and in healthy controls. There were no differences between GWI and control subjects for short-chain and medium-chain acylcarnitine species (data not shown).

4.0 Discussion:

To date, GWI remains difficult to diagnose and there are no effective treatments that target the underlying pathology. Given the prominent CNS component to this illness, the use of animal models is necessary for molecular characterization of the brain in order to identify biological targets for treating GWI. Owing to the complexity of biological responses to GW agents and a heterogeneous presentation of clinical symptoms in patients, it has been challenging to identify molecular targets for

therapeutic intervention. Our omic analyses presented herein point to long-term mitochondrial lipid and bioenergetic disturbances following subacute exposure to GW agents. The internal consistency of these findings is demonstrated in proteomic data showing disruption of pathways which regulate mitochondrial bioenergetics, metabolomics data showing changes in mitochondrial metabolites corresponding with these pathways and lipidomics data showing decreases in several classes of lipids that are specific to mitochondria in exposed mice. These GW-agent induced long-term mitochondria disturbances are accompanied with astroglia and microglia activation in the hippocampus and coincide with impaired learning and spatial memory. We also show that mitochondria specific lipids are altered in plasma from this GWI mouse model and in veterans with GWI. As such, these lipids, with further validation, could be useful as translational biomarkers of GWI. Collectively, these studies suggest that mitochondria lipid disturbances in GW agent exposed mice are also present in veterans with GWI and may be associated with long-term consequence of GW agent exposure that are relevant to current clinical status of GWI.

Cognitive deficits are among the most common complaints in veterans with GWI with affected veterans performing poorly on neuropsychological tests in tasks relating to executive functioning and memory compared to controls (Sullivan et al., 2003; Anger et al., 1999; Sillanpaa et al., 1997; Binder et al., 1999; Bunegin et al., 2001; Lange et al., 2001; Storzbach et al., 2000). Using a GWI mouse model, we demonstrate that cognitive deficits are present at 16-months post-exposure in mice exposed to GW agents at an early age. Despite being 18-months of age, the path length for control mice improved significantly over the training period while GW agent exposed mice of same age had similar performance throughout the training, suggesting impaired learning in exposed mice. Gulf War agent exposed mice exhibited persistent spatial memory deficits on probe trials conducted at 15- and 16-months post-exposure, having longer path length than control mice. Our studies are consistent with previous studies in showing that cognitive impairment is a persistent feature of GWI as observed

in rodent models of GWI (Abdullah et al., 2011; Zakirova et al., 2015; Abdullah et al., 2012; Parihar et al., 2013; Terry 2012).

Accompanying neuropathology in this mouse model included the presence of activated astroglia and microglia in the hippocampi of exposed mice relative to controls. Glial cells perform a variety of functions in the brain including immune surveillance and maintenance of energy homeostasis, dysregulation of which is thought to propagate age related cognitive dysfunction (Bilbo et al., 2012). Brain inflammation, as determined by increases in pro-inflammatory cytokines and microglia activation, is supported by studies in other rodent models of GWI (Parihar et al., 2013; O'Callaghan et al., 2015). This is consistent with clinical studies showing altered pro-inflammatory cytokines in veterans with GWI relative to controls (Skowera et al., 2004; Whistler et al., 2009; Broderick et al., 2013; Parkitny et al., 2015). Astroglia are key regulators of the nutrient supply to the brain, converting FA to β-HB, which serves as an energy source when the glucose supply is low or during high neuronal activity (Guzmán, & Blázquez 2004). Astroglia produce lactate through glycolysis, which is then transported to neurons via specialized MCT and serves as an alternative fuel for ATP production in neurons (Stobart, & Anderson 2013; Rayhan et al., 2013b). A small case-control functional magnetic resonance (fMRI) imaging study showed uncoupling of lactate utilization and glutamate to glutamine conversion during exercise in a subset of veterans with GWI, which could suggest deregulation of the astroglia-neuron lactate shuttle could potentially be associated with CNS bioenergetic disturbances in GWI (Rayhan et al., 2013a). In agreement with this, our proteomic studies identified that GW agent exposure was associated with bioenergetics, particularly pathways related to conversion of pyruvate to lactate and gluconeogenesis. These findings were confirmed by metabolomic studies showing a reduction of the Krebs cycle metabolites, lactate, pyruvate, β-HB and glycerol phosphates in the brains of GW agent exposed mice.

We observed that CL, a key mitochondrial lipid, is dramatically reduced in the brains of GW agent exposed mice. Cardiolipin represents 20% of total mitochondrial lipids (Horvath et al., 2015) and a

reduction in CL is a strong indicator of decreased activity of the electron transport chain (ETC) (Shen et al., 2015). Mitochondrial bioenergetics rely on adequate quantities of CL and a reduction of CL suggests impaired ATP production. Studies have shown that exposure to certain pesticides lowers CL in different brain regions (Astiz et al., 2009; Czerniczyniec et al., 2013). There is some evidence that mitochondrial disturbances might be present in veterans with GWI. A small matched case-control study of ^{31}Phosphorus Magnetic Resonance Spectroscopy showed that veterans with GWI had prolonged post-exercise recovery of muscle phosphocreatine following exercise compared to controls, suggesting defective energy metabolism in the mitochondria (Koslik et al., 2014). As mentioned above, a fMRI study showed impaired lactate utilization as an alternative energy supply in the brains of veterans with GWI (Rayhan et al., 2013a). Support for a possible association of mitochondria dysfunction in GWI also comes from animal studies. In rats, chronic exposure to an organophosphate (OP) pesticide dichlorvos was associated with increased mitochondrial reactive oxygen species production, decreased manganese superoxide dismutase activity and increased lipid peroxidation (Binukumar et al., 2010). Dynamic measurements of mitochondria following chlorpyrifos exposure showed impaired mitochondrial electron transport and decreases in mitochondrial length, number and axonal movement in rodents exposed to GW agents (Binukumar et al., 2010; Middlemore-Risher et al., 2011).

In addition to CL, mitochondria contain PC, PE, and PI where PC and PE together comprise 60% and PI comprise 10% of total mitochondrial PL (Mejia, & Hatch 2016). Acylglycerophosphate acyltransferase 4 (AGPAT4) is abundant in the brain and resides in the outer mitochondria membrane (Bradley et al., 2015). Although AGPAT4 catalyzes the formation of phosphatidic acid (PA), this reaction is a branching point for the Kennedy pathway, regulating conversion of PC from methylation of PE. As such, this enzyme has the potential to alter the composition of PL classes within the mitochondria. In fact, a recent *in vivo* study demonstrated that loss of this enzyme resulted in decrease in PC, PE and PI (Bradley et al., 2015). Since we also observe decreases in these PL

classes in the brains of GW agent exposed mice, interrogation of lipid metabolism by AGPAT4 may assist in understanding of the pathophysiology of GWI.

Acylcarnitines transport FA to the mitochondrial inner membrane for β-oxidation and maintain the balance between the free and esterified CoA (Corso et al., 2011). Acylcarnitines have been proposed as biomarkers of metabolic disorders (i.e. diabetes), disorders caused by environmental exposures and neurodegenerative diseases (De Palo et al., 1981; Mihalik et al., 2010; McGill et al., 2014). Therefore, we examined their levels in plasma from our GWI mouse model and veterans with GWI compared to their respective controls. Odd-chain acylcarnitines were elevated in GW agent exposed and in veterans with GWI relative to their respective controls. There were no differences in the brain concentrations of odd-chain acylcarnitines between control and exposed mice. As such, plasma changes in these acylcarnitines suggest that these effects are peripheral rather than CNS manifestation of this illness. An elevation of odd-chain acylcarnitines in plasma is indicative of methylmalonyl-CoA mutase deficiencies (Jones et al., 2010), a mitochondrial enzyme which is a part of the metabolic pathways that catabolize certain amino acids, odd-chain fatty acids and cholesterol to generate metabolites for the Krebs cycle (Hörster, & Hoffmann 2004). Deficiencies of this enzyme are shown to accompany mild brain edema in a few case studies (Lee et al., 2008). We only detected even carbon long-chain and very long-chain acylcarnitines in plasma. Long-chain acylcarnitines were generally decreased in both the brain and plasma of exposed mice and decreased in plasma from veterans with GWI. We were unable to detect very long-chain acylcarnitines in mice but these species were detectable in human plasma and were lower in veterans with GWI compared to controls. Together, decreases in long-chain and very long-chain acylcarnitines suggest acetyl-CoA shortage or impaired activity of enzymes that catalyze transfer of the acetyl group from CoA to carnitine (van Vlies et al., 2005). Hence, confirmatory analyses in larger cohorts of GW veterans are required to better understand the biological relevance of the

carnitine shuttle in the pathobiology of GWI and to determine if plasma acylcarnitine levels could potentially serve as biomarkers of GWI.

5.0 Conclusion:

Gulf War Illness is primarily characterized by symptoms of fatigue, joint pain and impaired cognition. Among the multisymptom presentation of GWI, memory impairment represents one of the most common and debilitating symptoms (White et al., 2016; Smith et al., 2013; Binns et al., 2008). We observed mitochondrial lipid disturbances in a well-validated GWI mouse model exhibiting glia activation which corresponded with a functional outcome of neurobehavioral deficits long after subacute exposure to GW agents indicating the chronic nature of the effects. In addition, we provide pilot evidence that mitochondrial lipid alterations are also present in veterans with GWI. Hence, this mouse model displays both cognitive impairment and mitochondrial lipid defects seen in veterans with GWI. Although considerable work remains to determine if mitochondria dysfunction has an important pathological role in GWI, these studies indicate further investigation of mitochondrial function could provide a better understanding of the pathobiology of GWI. These studies also suggest that targeting mitochondria lipid metabolism may lead to therapies for treating the symptoms of GWI.

Author's contributions:

LA, JE, HM, TN, JR, GC, MH, TE, ZZ, GA, CB, SB, BM NK, and KS contributed to data and sample collection and analysis. LA, MM, and FC provided overall guidance on the study design, conduct, implementation and interpretation of the results. All authors contributed to the preparation and finalization of the manuscript.

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Competing interests: None

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Figure 1: The neurobehavioral and neuropathological deficits in GW agent exposed mice at 16-months post-exposure. Barnes Maze: (A) Mean ± SEM in control (n = 10) and exposed mice (n = 9). The total path length to the target hole was shorter for controls vs. exposed mice and there was an overall interaction between exposure and trials for all of the training period during the acquisition trials ($p < 0.05$). *Post-hoc* analyses for each training day showed that the interactions between trials and exposure were significant for days 1, 2 and 4 only, $^*p < 0.05$. (B) For the probe trials at 15- and 16-months post-exposure, larger path length for exposed mice compared to controls is indicative of spatial memory deficits. Post-hoc analyses showed a significant net decrease in exposed mice relative to controls at 15- months ($^*p < 0.05$) and $p = 0.051$ for 16-months post-exposure. (C-H) Sagittal sections 0.4 mm lateral to midline in the layer III/IV of the cortex. Staining of astroglia with GFAP and (I-N) microglia with iba1. (C-E) There were no differences between the groups for GFAP within the cortex. (E-F) Compared to controls, exposed mice had 1.7 fold increase in GFAP in the DG, $^*p < 0.05$. (F) The arrow points to an activated astroglia with highly branched processes and hypertrophied cell body. The insert shows an example of activated astroglia. (I-J) There were no differences between the two groups for iba1 in the cortex. (K-L) Iba1 staining in DG was 1.2 fold higher in exposed vs. controls. The arrows point to activated microglia cell with hypertrophic cell body,$^*p < 0.05$. The insert shows an example of activated microglia.

Figure 2. Ingenuity Pathway Analysis (IPA) identified networks, biofunctions and canonical pathways modulated by GW agent exposure: Brain proteins that were differentially expressed at 16-months post-exposure to GW agents were interrogated using IPA. (A) Differentially expressed proteins were further interrogated using IPA, and a ‘‘Core Analysis’’ was performed, termed biofunctions. The blue bars represent biological functions that were significantly modulated by GW exposures. The red line designated by the term “threshold” indicates the statistical significance cut-off

of -log(p-value) 1.5 (based on right-sided Fishers exact test). Lipid metabolism, immune cell trafficking, energy production and molecular transport were affected by GW agent exposure. (B) Canonical pathways (gray bars) most significant to the dataset were determined based on the ratio (exhibited as solid black line with dots) of the number of proteins mapping to a particular pathway divided by total number of proteins significantly modulated by GW agent exposure. A right-sided Fishers exact test to calculate a p value (line named threshold indicating the statistical cut-off of -log (p-value) 1.5). Canonical pathways included glycolysis, gluconeogenesis, glycerol-3-phosphate shuttle and mitochondria dysfunction were associated with GW agent exposure.

Figure 3: Metabolomic analyses showing decreases in lipid metabolites, lactate, the Krebs cycle compounds and glycerol phosphate following GW agent exposure. Mean ± SEM (expressed as percentage of control, n = 4 per group). (A) Metabolites within the TCA cycle, (B) lactate, β-HB, (C) glycerol-3-phosphate, glycerol-2-phosphate and glyceric acid phosphate were all decreased in exposed relative to control mice. *$p < 0.05$, **$p < 0.01$.

Figure 4. Brain lipid profiles show significant decreases in cardiolipin, phospholipids, acylcarnitines and total fatty acids in GW agent exposed mice relative to controls. Mean ± SEM (expressed as percentage of control, n = 4 per group). (A) While most CL species were decreased in exposed mice compared to controls, those with highly unsaturated double-bonds were the lowest following GW exposure. (B) Several long chain acylcarnitine species were decreased in GW agent exposed mice. (C) Individual molecular species for each PL class identified by LC/MS were summed after lipidomeDB analyses to calculate total PL levels within each class. Relative to controls, PC and LPC were 8% lower, whereas total PE was 16% and total PI was 18% lower in exposed mice. (D) Major omega-3 fatty acids that are highly enriched in the brain, such as omega-3 DPA (22:5n3) and

DHA (22:6n3) and omega-6 AA (20:4n6) and adrenal acid (22:4n6) were decreased in GW agent exposed mice than controls.*$p < 0.05$, **$p < 0.01$.

Figure 5. Plasma acylcarnitine profiles in GW agent exposed mice relative to controls. Mean ± SEM (expressed as percentage of control, n = 4 per group). (A) odd-chain acylcarnitine species were increased while (B) medium, and (C) long-chain acylcarnitine species were decreased in exposed mice compared to controls. (D) Individual molecular species of acylcarnitine that were significantly increased after PCA followed by MLM analyses. Odd-chain C3:0 and C5:0 were significantly elevated in exposed animals but the remaining species were decreased in the GW agent exposed mice compared to control mice.*$p < 0.05$,**$p < 0.01$

Figure 6. Plasma acylcarnitine profiles in veterans with GWI compared to GW control veterans. Mean ± SEM (n = 8 for controls and n = 12 for GWI). (A) odd-chain acylcarnitine species were increased while (B) long-, and (C) very long-chain acylcarnitine species were decreased in veterans with GWI compared to controls. (D) Individual molecular species of acylcarnitine examined with PCA followed by MLM analyses. C18:0, C18:3 and C26:0 were marginally significant ($p = 0.06$, $p = 0.09$ and $p = 0.09$, respectively). The remaining long- and very-long chain species were significant at $p < 0.05$. Odd-chain C3:0 was not identified as part of the PCA associated with GWI but significantly elevated in veterans with GWI compared to controls ($p = 0.05$).

Figure 1A-B

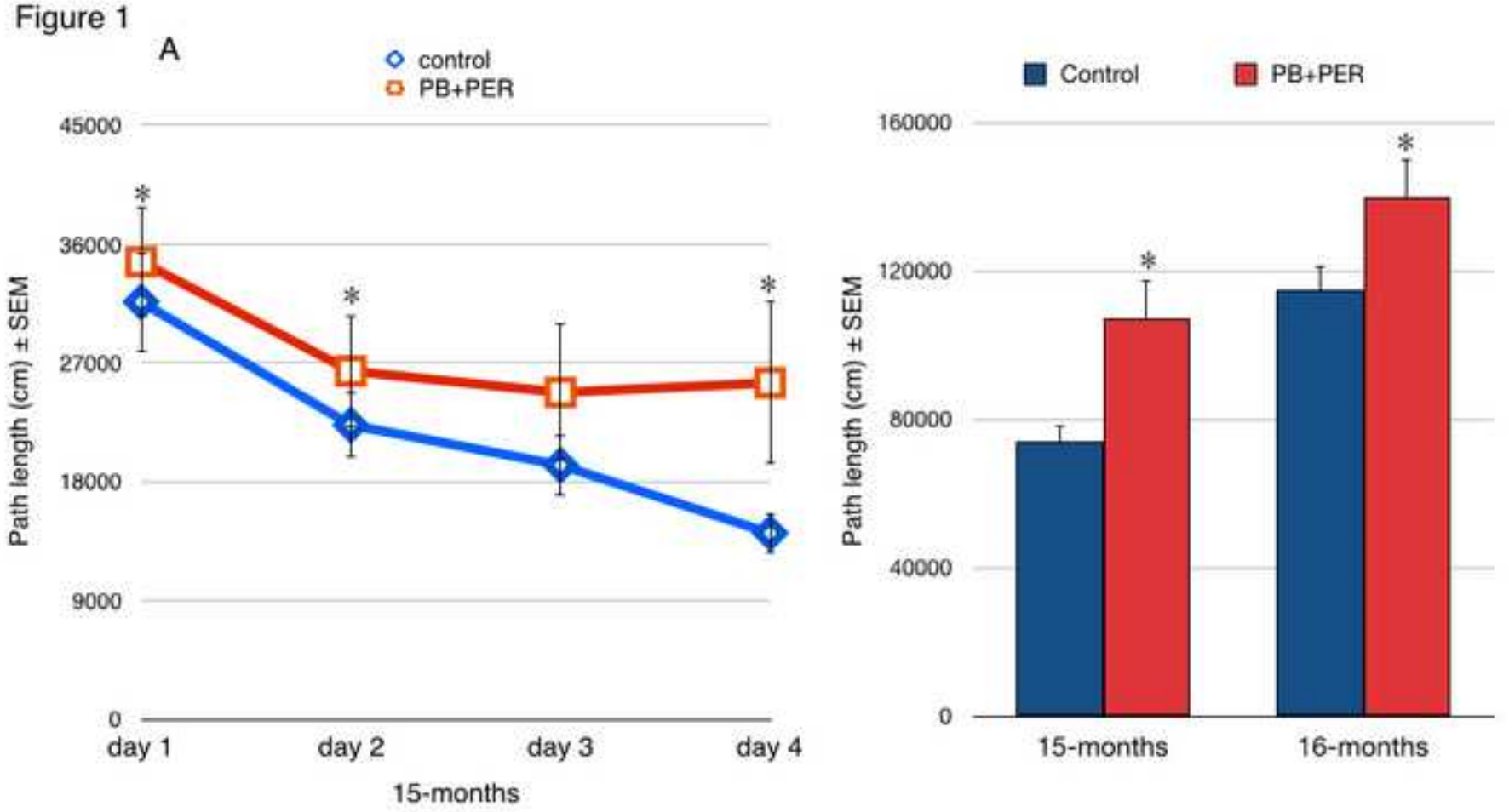
Figure 1
A
control
PB+PER
45000
36000
27000
18000
9000
0
Path length (cm) ± SEM
day 1
day 2
day 3
day 4
15-months
Control
PB+PER
160000
120000
80000
40000
0
Path length (cm) ± SEM
15-months
16-months

Figure 1C-N

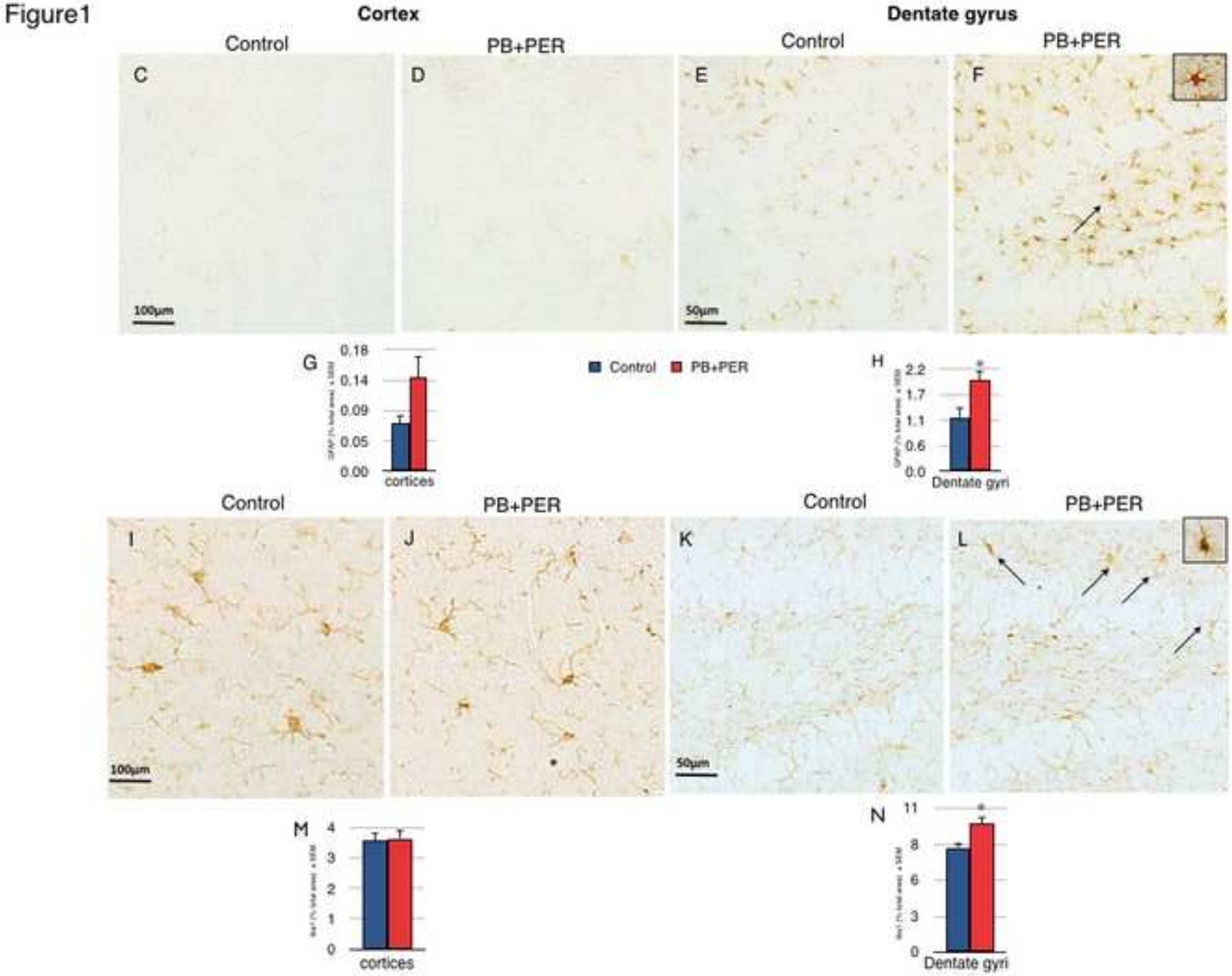
Figure1
Cortex
Dentate gyrus
Control
PB+PER
Control
PB+PER
C
D
E
F
100µm
50µm
G
0.18
0.14
0.09
0.05
0.00
cortices
Control
PB+PER
H
2.2
1.7
1.1
0.6
0.0
Dentate gyri
Control
PB+PER
Control
PB+PER
I
J
K
L
100µm
50µm
M
4
3
2
1
0
cortices
N
11
8
6
3
0
Dentate gyri

Figure 2

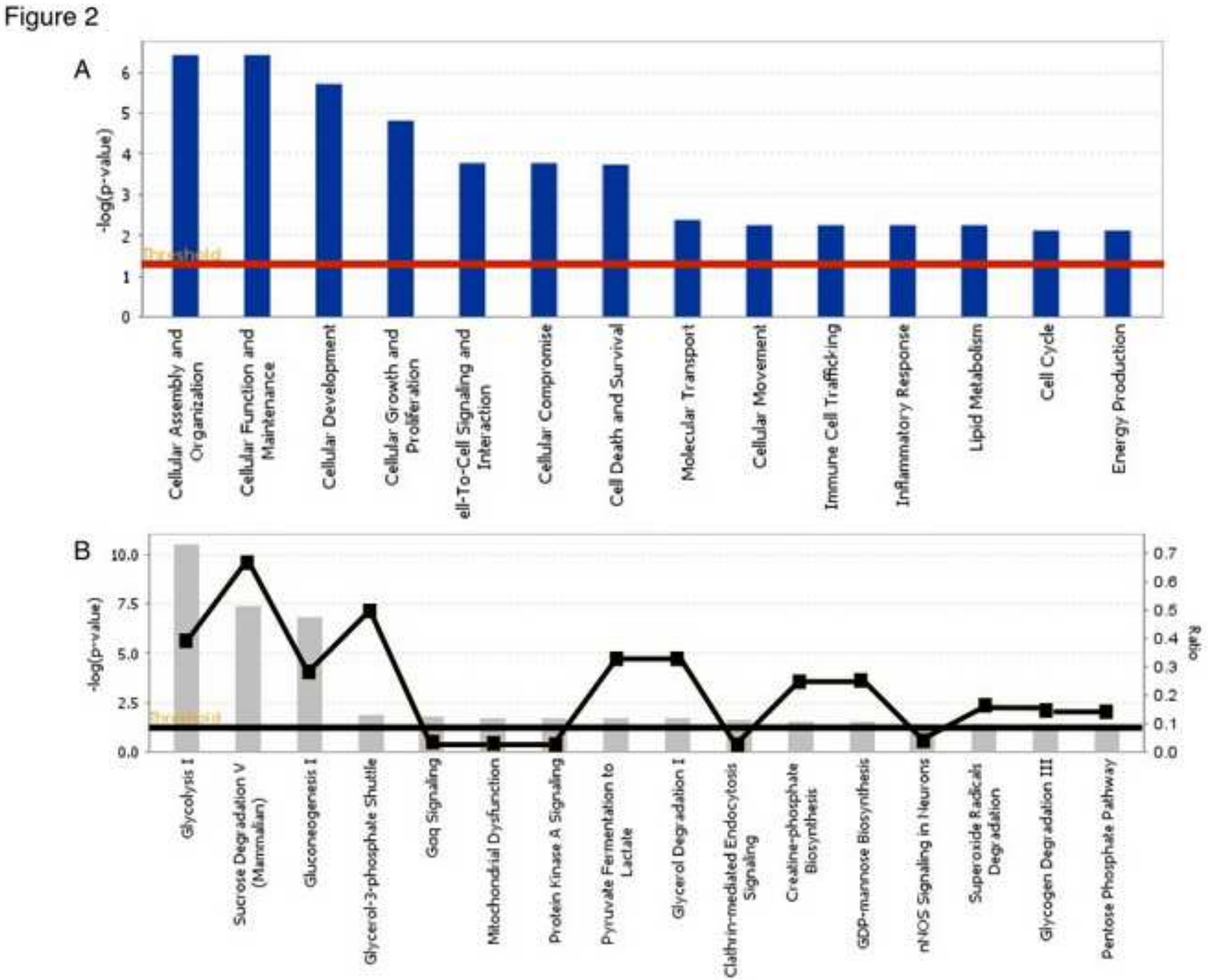
Figure 2
A
-log(p-value)
0
1
2
3
4
5
6
Threshold
Cellular Assembly and Organization
Cellular Function and Maintenance
Cellular Development
Cellular Growth and Proliferation
ell-To-Cell Signaling and Interaction
Cellular Compromise
Cell Death and Survival
Molecular Transport
Cellular Movement
Immune Cell Trafficking
Inflammatory Response
Lipid Metabolism
Cell Cycle
Energy Production
B
-log(p-value)
0.0
2.5
5.0
7.5
10.0
Ratio
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Glycolysis I
Sucrose Degradation V (Mammalian)
Gluconeogenesis I
Glycerol-3-phosphate Shuttle
Gαq Signaling
Mitochondrial Dysfunction
Protein Kinase A Signaling
Pyruvate Fermentation to Lactate
Glycerol Degradation I
Clathrin-mediated Endocytosis Signaling
Creatine-phosphate Biosynthesis
GDP-mannose Biosynthesis
nNOS Signaling in Neurons
Superoxide Radicals Degradation
Glycogen Degradation III
Pentose Phosphate Pathway

Figure 3

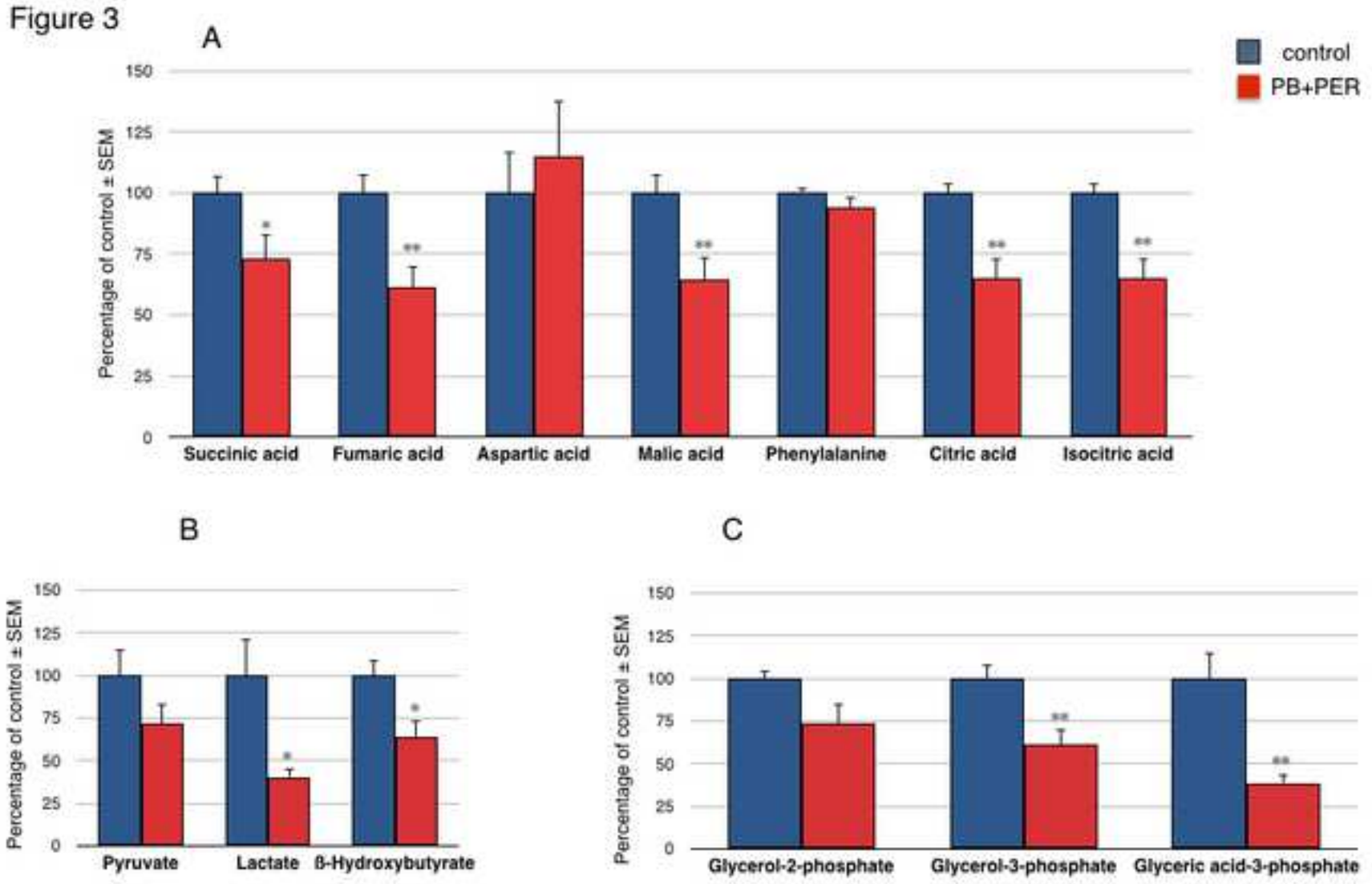
Figure 3
A
control
PB+PER
Percentage of control ± SEM
150
125
100
75
50
25
0
Succinic acid
Fumaric acid
Aspartic acid
Malic acid
Phenylalanine
Citric acid
Isocitric acid
B
Pyruvate
Lactate
ß-Hydroxybutyrate
C
Glycerol-2-phosphate
Glycerol-3-phosphate
Glyceric acid-3-phosphate

Figure 4

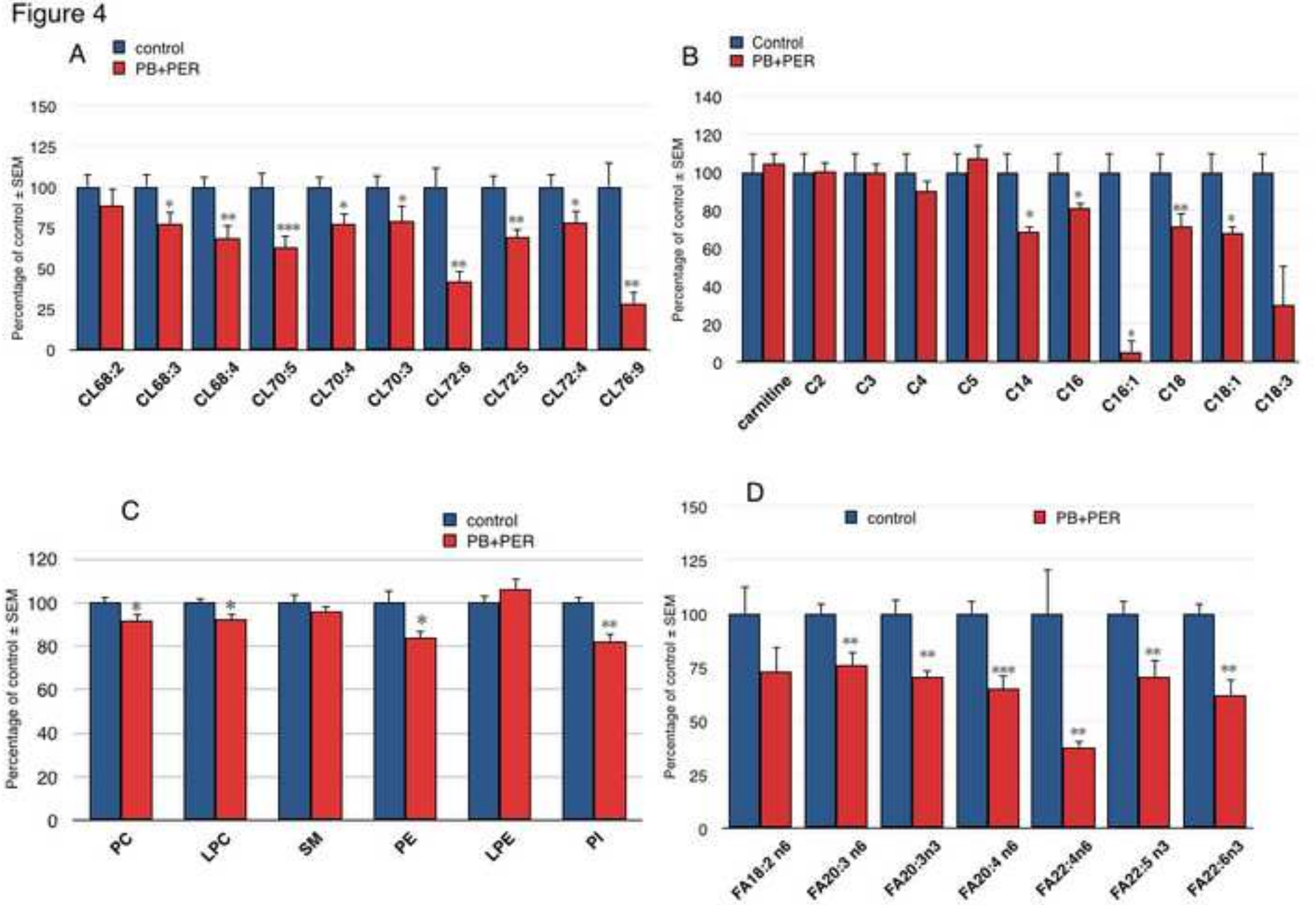
Figure 4
A
control
PB+PER
Percentage of control ± SEM
150
125
100
75
50
25
0
CL68:2
CL68:3
CL68:4
CL70:5
CL70:4
CL70:3
CL72:6
CL72:5
CL72:4
CL76:9
B
Control
PB+PER
Percentage of control ± SEM
140
120
100
80
60
40
20
0
carnitine
C2
C3
C4
C5
C14
C16
C16:1
C18
C18:1
C18:3
C
control
PB+PER
Percentage of control ± SEM
120
100
80
60
40
20
0
PC
LPC
SM
PE
LPE
PI
D
control
PB+PER
Percentage of control ± SEM
150
125
100
75
50
25
0
FA18:2 n6
FA20:3 n6
FA20:3n3
FA20:4 n6
FA22:4n6
FA22:5 n3
FA22:6n3

Figure 5

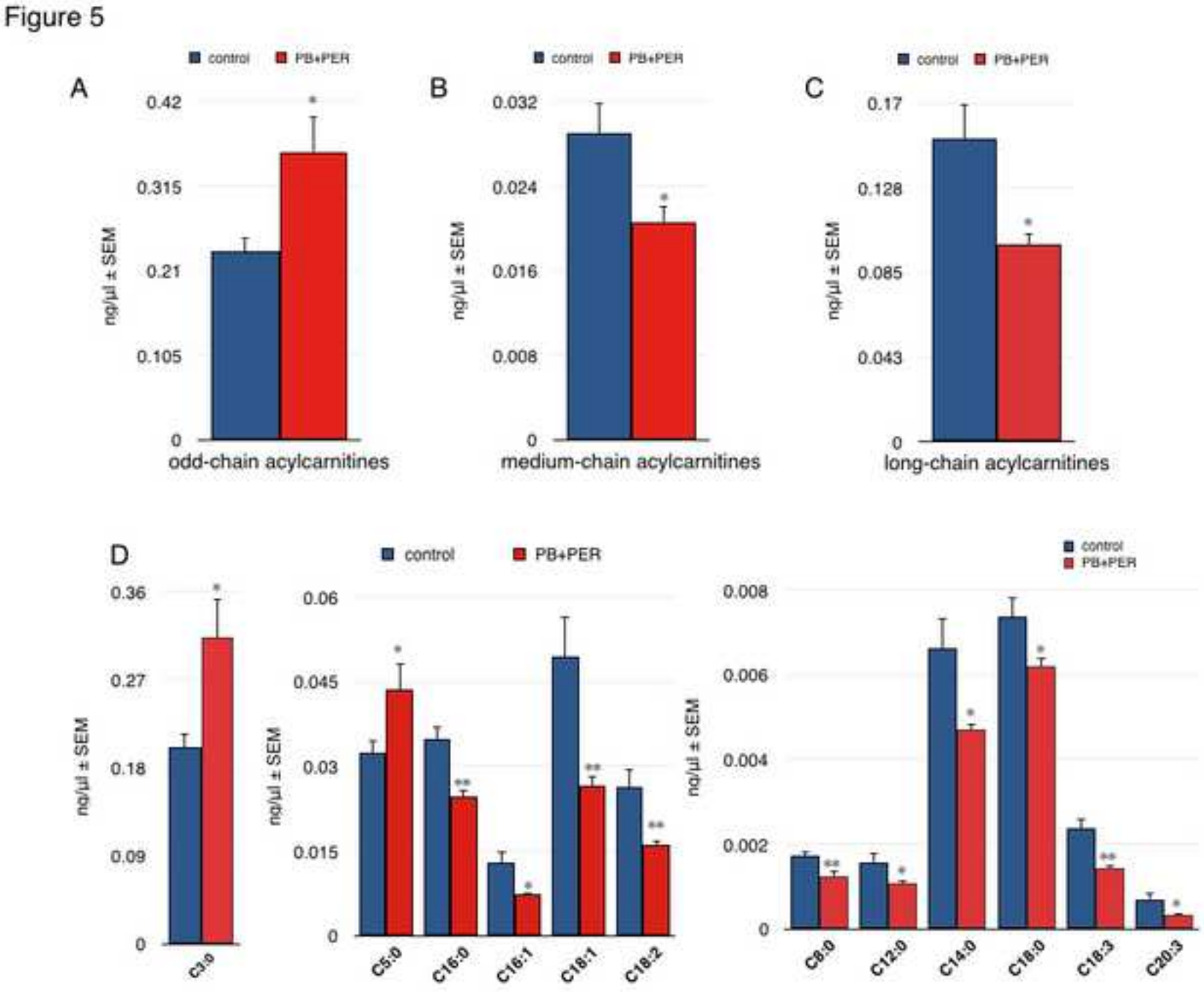
Figure 5
A
control
PB+PER
0.42
0.315
0.21
0.105
0
ng/µl ± SEM
odd-chain acylcarnitines
B
0.032
0.024
0.016
0.008
medium-chain acylcarnitines
C
0.17
0.128
0.085
0.043
long-chain acylcarnitines
D
0.36
0.27
0.18
0.09
C3:0
0.06
0.045
0.03
0.015
C5:0
C16:0
C16:1
C18:1
C18:2
0.008
0.006
0.004
0.002
C8:0
C12:0
C14:0
C18:0
C18:3
C20:3

Figure 6

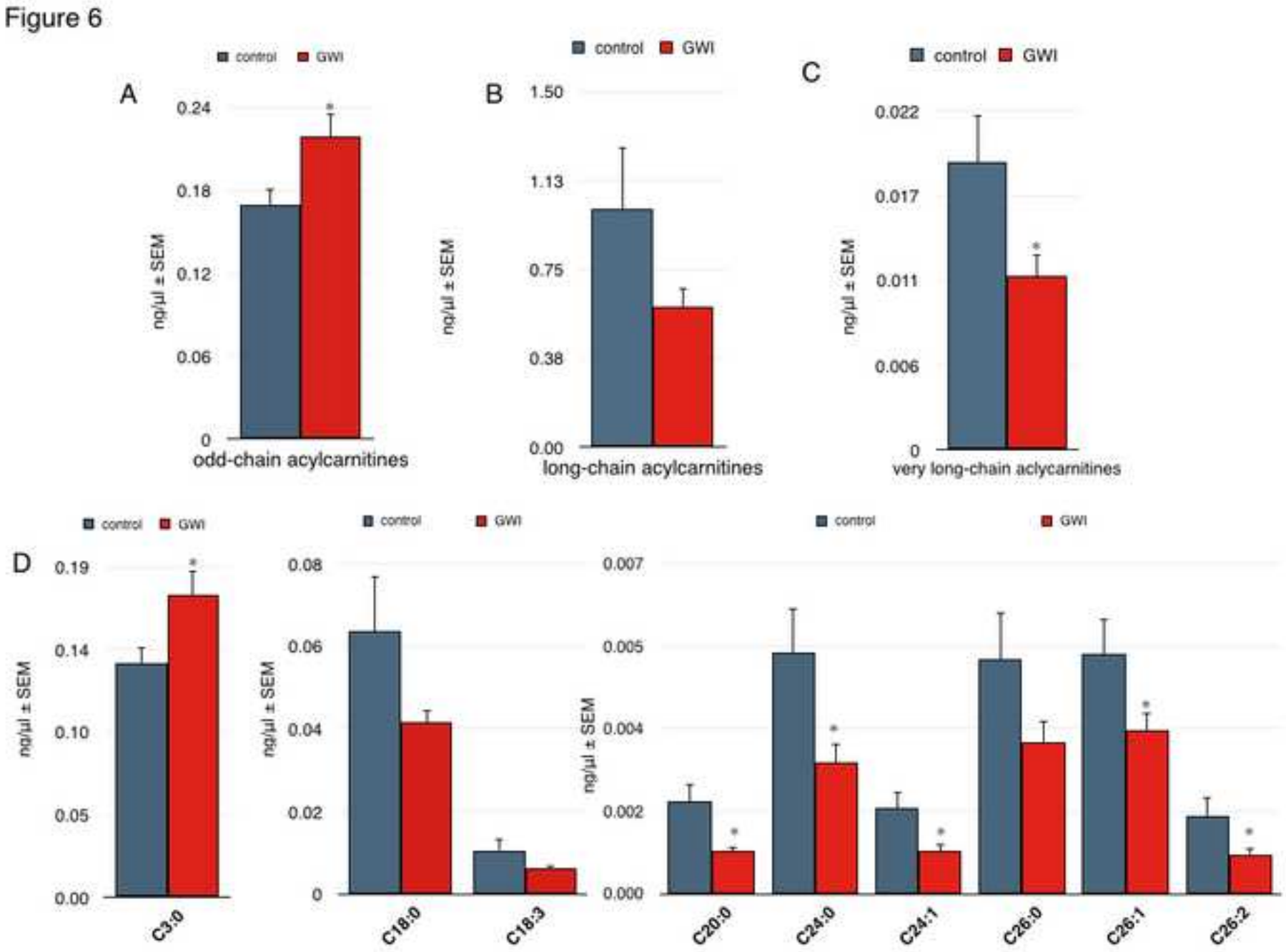
Figure 6
control
GWI
A
ng/µl ± SEM
0.24
0.18
0.12
0.06
0
odd-chain acylcarnitines
B
1.50
1.13
0.75
0.38
0.00
long-chain acylcarnitines
C
0.022
0.017
0.011
0.006
0
very long-chain aclycarnitines
D
0.19
0.14
0.10
0.05
0.00
C3:0
0.08
0.06
0.04
0.02
0
C18:0
C18:3
0.007
0.005
0.004
0.002
0.000
C20:0
C24:0
C24:1
C26:0
C26:1
C26:2