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Neurorestorative targets of dietary long-chain omega-3 fatty acids in neurological injury

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Abstract

Long-chain omega-3 polyunsaturated fatty acids (LC-O3PUFAs) exhibit therapeutic potential for the treatment and prevention of the neurological deficits associated with spinal cord injury (SCI). However, the mechanisms implicated in these protective responses remain unclear. The objective of the present functional metabolomics study was to identify and define the dominant metabolic pathways targeted by dietary LC-O3PUFAs. Sprague-Dawley rats were fed rodent purified chows containing menhaden fish oil-derived LC-O3PUFAs for 8 weeks before being subjected to sham or spinal cord contusion surgeries. We show, through untargeted metabolomics, that dietary LC-O3PUFAs regulate important biochemical signatures associated with amino acid metabolism and free radical scavenging in both the injured and sham-operated spinal cord. Of particular significance, the spinal cord metabolome of animals fed with LC-O3PUFAs exhibited reduced glucose levels (−48%) and polar uncharged/hydrophobic amino acids (<−20%) while showing significant increases in the levels of antioxidant/anti-inflammatory amino acids and peptides metabolites, including β-alanine (+24%), carnosine (+33%), homocarnosine (+27%), kynurenine (+88%), when compared to animals receiving control diets ($p < 0.05$). Further, we found that dietary LC-O3PUFAs impacted the levels of neurotransmitters and the mitochondrial metabolism, as evidenced by significant increases in the levels of N-acetylglutamate (+43%) and acetyl-CoA levels (+27%), respectively. Interestingly, this dietary intervention resulted in a global correction of the pro-oxidant metabolic profile that characterized the SCI-mediated sensorimotor dysfunction. In summary, the significant benefits of metabolic homeostasis and increased antioxidant defenses unlock important neurorestorative pathways of dietary LC-O3PUFAs against SCI.

Keywords

Metabolomics; DHA metabolome; Spinal cord injury; ROS; Neuroprotection; Pain

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Conflict of interest
The authors declare that they have no conflict of interest.

Introduction

Spinal cord injury (SCI) is a major debilitating neurological condition that affects several million people worldwide. The initial physical insult sustained in SCI triggers a longer secondary damage that leads to inflammation, demyelination, and apoptosis ultimately leading to dysfunction [1]. This secondary injury phase is characterized by metabolic alterations, glutamate-induced excitotoxicity, and oxidative stress [2–5]. The levels of these reactive oxygen (ROS) and nitrogen species (NOS) increase considerably when the metabolism is compromised, which can result in irreversible damage to cell membrane lipids, proteins and nucleic acids. ROS scavengers, including catalase, glutathione (GSH), and superoxide dismutase (SOD), are endogenous defense mechanisms that combat oxidative damage. Although there is no current cure for SCI, accumulating clinical and experimental evidence support interventions that target these restorative pathways and hold tremendous promise in ameliorating neurological dysfunction [6].

Long-chain omega-3 polyunsaturated fatty acids (LC-O3PUFAs) modulate multiple pathways that contribute to secondary damage following SCI [7–19]. We recently demonstrated that the administration of LC-O3PUFAs restores the cord lipid homeostasis, confers neuroprotection, prevents sensorimotor dysfunction and neuropathic pain, and facilitates locomotor recovery following acute and chronic SCI when administered before the injury [17–19]. However, there is very limited understanding of the pathways activated by dietary LC-O3PUFAs in the injured central nervous system.

Dietary fatty acids exert potent effects on cellular metabolism through tightly regulated mechanisms at the transcriptional, posttranscriptional, translational, or posttranslational levels. This study investigates the global non-lipid targets of nutritional LC-O3PUFAs, which offers the advantage of linking dietary LC-O3PUFA-gene interactions to distinctive metabolites and small molecules. This study represents the first unbiased attempt to identify the biologically meaningful metabolic networks that are influenced by dietary LC-O3PUFAs during the acute and chronic injury phases following SCI. In addition, we define the putative biochemical signatures associated with resiliency against SCI.

Materials and Methods

Animals

All animal studies were performed in compliance with the Loma Linda University School of Medicine regulations and institutional guidelines consistent with the NIH Guide for the Care and Use of Laboratory Animals. Female Sprague-Dawley rats were received from Charles River Laboratories (Portage, MI) and single housed in environmentally enriched cages on alternating 12 h light/dark cycles after being acclimated to the new environment for one week.

Experimental design and diets

Young adult rats (185–200 grams) were fed control or fish oil-enriched diets for 8 weeks were subjected to sham injury or spinal cord injury and subsequently allowed to recover for 1 or 8 weeks after trauma. Spinal cord tissue was collected for global metabolic profiling (n

= 7–10 samples per group). This study used two independent cohorts (cohort 1: at least 7 animals per diet group, allowed to survive until 1 weeks post-operation; cohort 2: at least 8 animals per diet group, allowed to survive 8 weeks post-operation). Behavioral data from rats in cohort 2 was previously reported [18,19]. Figure 1 summarizes the timeline outlining the experimental design. For each group, the average animal weight and daily food consumption is expressed in grams ± standard deviation.

Custom AIN-93G-based diets were prepared with modifications to the omega-3 fatty acid source as described previously [18,19]. Typical analysis of the AIN-93G formulation reveals 7.1% fat, containing cholesterol (0 ppm), linoleic acid (3.58%), linolenic acid (0.55%), arachidonic acid (0%), omega-3 fatty acids (0.55%), total saturated fatty acids (1.05%), total monosaturated fatty acids (1.54%), and polyunsaturated fatty acids (3.78%). In our study, the dietary omega-3 fatty acids were supplied as either soybean oil (control chow) or menhaden fish oil (DHA = 12.82-gm and EPA = 6.91-gm per 100 gm of fish oil). Because the fish oil-based diet contained 6.23 grams of fish oil per 100 grams of diet, we estimate that feeding a 270-gram rat with approximately 20 grams of diets (or 1.25 grams of fish oil per day) should result in a daily intake of approximately 60 mg of DHA and 32 mg of EPA per 100 grams of body weight. The total absolute amount of ingested LC-O3PUFAs may vary when additional sources of omega-3 in the AIN-93G diet are considered. Mass-spec analysis further revealed that the level of cholesterol in the menhaden fish oil was 0.582-g/100 g. Cholesterol was added to control diets to match this level. The diets were stored at 4 C and used fresh. The amount of food ingested was recorded daily during weekdays and averaged during weekends. Table 1 summarizes the composition of the diets.

Surgical and Post-Operative Procedures

Eight weeks after the dietary intervention, animals were deeply anesthetized with a mixture of ketamine/xylazine (80 mg/kg and 10 mg/kg, respectively). The New York University (NYU) Impactor was used to generate a contusive lesion to the thoracic 10 level of the spinal cord [20]. The spinal cord was subjected to weight drop impact using a 10-g rod released from a height of 12.5-mm. Sham animals received only a laminectomy surgery. The animals body temperature was maintained at 37°C during surgery. The muscle layers were then sutured and the skin layers closed. Postoperative care of SCI rats included manual bladder expression at least two times a day until the return of spontaneous urination. Cefazolin (Bristol Myers Squibb, New York, NY; 25 mg/kg, s.q.) and Buprenex® (buprenorphine; Reckett and Colman Pharmaceuticals, Inc. Richmond, VA; 0.05 mg/kg, s.c.) were also given to all rats for 5 and 3 consecutive days, respectively. Animals were allowed to survive for 1 or 8 weeks post-operation and the spinal cord tissue dissected for metabolomics analysis.

Metabolomic Profiling

Global metabolic profiling was performed as previously described [19,18]. Animals were deeply anesthetized and perfused with ice-cold PBS. The spinal cord tissue (75–100 mg) was dissected and put into liquid nitrogen and then stored at −80°C until use. The tissue samples were homogenized in water and the protein precipitated with methanol containing four standards to report on extraction efficiency. The resulting supernatant was split into

equal aliquots for analysis on the three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS^2) optimized for basic species, UHPLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). The metabolites were identified by comparing the ion features in the experimental samples and to a reference library of chemical standards that includes retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra. The biochemical features were curated by visual inspection for quality control using the software developed at Metabolon [21].

Metabolomics Analyses

The false discovery rate (FDR) was calculated as previously reported [18,22,23]. The q-value describes the false discovery rate and takes into account the multiple comparisons.

The partial least square-discriminant analysis (PLS-DA) was used to identify predictors between groups as previously described [19,18]. This regression method provides information that can predict the class membership (Y) via linear combination of original variables (X). We performed the separation distance permutation to assess the significance of class discriminations between groups. The variable importance in projection (VIP) measures the impact of each metabolite in the model. VIP is a weighted sum of squares of the PLS loadings and takes into account the amount of explained Y-variation in each dimension. Biochemicals with values above 1 are considered important contributors to the group discriminations.

The metabolomic functional analyses were generated through the use of Ingenuity Pathways Analysis (IPA) (www.ingenuity.com). The relative metabolite ratios obtained from metabolomics analyses were converted to fold change values by the IPA software. The data set was filtered to include metabolites that were associated with biological functions in the Ingenuity Pathways Knowledge Base. The IPA algorithm uses the *p*-value ($p < 0.05$) to determine the probability that each biological function assigned to that data set is due to random chance alone. This value is calculated by considering the number of metabolites in the dataset that participate in that function and the total number of features that are known to be associated with that function in the IPA knowledge database. The level of statistical significance was set at a *p*-value less than 0.05, suggesting non-random associations. We also used established analytical approaches to aid in the interpretation of our large data set [24]. For a biochemical reaction in which $(A + B)_{substrates}$ yield $(C + D)_{products}$, then low levels of A and/or B with concomitant increases in C and/or D suggest increased metabolism towards formation of products. The inverse scenario was interpreted as accumulation of substrates.

Statistical Analysis

Statistical analyses were performed using SPSS version 20.0 (IBM: SPSS, Armonk, New York), Prism 5 software v5d (GraphPad Software Inc., San Diego, CA), the “R” program (http://cran.r-project.org/), metaboanalyst [25,26], and IPA. ANOVA contrasts were used to identify features that differed significantly between groups. Associations were made with the Spearman’s rank correlation tests in order to explore relationships between the oxidative

profile and the functional phenotype. Data are presented as mean ± SD, unless otherwise specified. The differences were considered statistically significant at $p < 0.05$.

Results

Our previous reports showed that preventive administration of docosahexaenoic acid (DHA) or consumption of a diet rich in long-chain omega-3 polyunsaturated fatty acids (LC-O3PUFAs) confers potent prophylaxis against multiple SCI co-morbidities and improves functional recovery [17,19,18]. However, the mechanisms underlying these beneficial effects remain largely unknown. The goal of this study was to characterize the impact of LC-O3PUFA dietary supplementation on the spinal cord non-lipid metabolic responses during the acute and chronic phases of SCI recovery as well as in the sham-operated spinal cord. The study design is summarized in Figure 1.

The ability to measure and study dietary LC-O3PUFA's targets and derivatives has been facilitated by the availability of untargeted metabolomics. Because the neurometabolome is tightly regulated, this technique allows for detection of very subtle alterations in biochemical pathways [27]. The partial least square-discriminant analysis (PLS-DA) score plot was obtained using the variation scores of the first three principal components. In the generated regression models, these components explained more than 50% of the differences between groups at 1 and 8 weeks post-surgery (Figure 2A and Figure 3A). Each plot mark corresponds to a rat in the study and the variability in metabolite levels that were detected for that animal. Permutation analyses validated the class discrimination (observed test statistic $p < 0.05$ in both models). PLS analyses revealed that the diet enriched in LC-O3PUFAs had a significant impact in the levels of selective carbohydrates, amino acids, and small peptides with antioxidant capabilities. These small molecules showed the strongest influence to the observed metabolomics differences between groups. This was evidenced by variable importance in projection (VIP) values above 1 (Figure 2B and Figure 3B).

Here, LC-MS/MS data was analyzed using Ingenuity Pathway Analysis software, which as expected revealed that dietary LC-O3PUFAs preferentially target pathways associated with cellular homeostasis and neurological function (Figure 4). In particular, we found that the most significant regulated functions in animals fed diets with enriched in LC-O3PUFAs were related to the transport and metabolism of amino acids and neurotransmitter systems and Ca^{2+}-mediated cell signaling, supporting the role of LC-O3PUFAs as crucial regulators of the neuron-glia signaling network [28]. In addition, the dietary LC-O3PUFAs had a robust impact on the metabolism of peptides and amino acids implicated in the regulation of reactive oxygen and nitrogen species. Table 2 summarizes the most significant functions and the number of molecules that were altered by the LC-O3PUFA-rich diet in sham and injured animals.

Dietary LC-O3PUFAs Improve Cellular Bioenergetics and Antioxidant Metabolism in Sham Animals

To gain insight into the potential biochemical targets implicated in the neuroprophylactic responses, we investigated the neurometabolome of sham-operated animals. We found that the diet rich in LC-O3PUFAs significantly altered the metabolism of distinctive amino acids

and carbohydrates when compared to sham animals receiving control diets ($p < 0.05$). For instance, ANOVA contrasts revealed that the LC-O3PUFA-rich diet increased the spinal cord levels of carnosine (+33%), homocarnosine (+27%), and the major protective precursor, β-alanine (+24%). The levels of 4-guanidinobutanoate (+34%), which is a common byproduct of arginine metabolism and distant precursor of homocarnosine, were increased in the spinal cord of sham-operated rats fed with LC-O3PUFAs when compared to control animals. Recent evidence suggests that the biochemical pathways implicated in the metabolism of these neuroprotective peptides may protect the spinal cord from inflammation and tissue damage after SCI [29,30]. The diet slightly decreased the levels of the precursor/metabolite amino acid glutamine (−14%) while glutamate levels remained stable and led to significantly higher Glu/Gln ratios (data not shown), suggesting reduced glutamine synthesis or accelerated recycling/catabolism. We found that the dietary intervention regulated the tryptophan metabolism, as evidenced by increased levels of kynurenine (88%) in the spinal cord of animals fed LC-O3PUFA-rich diets when compared to controls. Kynurenines are important modulators of oxidative stress and neurodegeneration [31,32].

Our results demonstrate a significant reduction in key metabolites implicated in the pentose phosphate pathway, including glucose (−48%), glucose-6-phosphate (−24%) and sedoheptulose-7-phosphate (−46%). Further, the diet increased the levels of acetyl CoA (27%) when compared to the control diet fed animals. Together with the findings showing a reduction in the levels of polar uncharged and hydrophobic side chain amino acid derivatives, including threonine levels (−28% from control), phosphoserine (−33%), betaine (−23%), and 3-hydroxyisobutyrate (−31%), these results suggest an increased protein turnover and/or amino acid shunting to energetic pathways.

We found that the diet rich in LC-O3PUFA had significantly lower levels of ergothioneine (−30%) at 8 weeks post-operation, suggesting differences in the levels of this naturally occurring amino acid between the dietary oils. The major metabolomic changes observed in sham-operated rats consuming LC-O3PUFAs are summarized in Table 3. Both IPA and metaboanalyst bioinformatics software were employed to gain insights into the major metabolic targets of dietary LC-O3PUFAs in the sham-operated spinal cord (Figure 5).

Dietary LC-O3PUFAs Target Amino Acid Systems and Complex Carbohydrates at One-Week Post-SCI

We found that the diet rich in LC-O3PUFAs selectively targeted the metabolism of molecules implicated in oxidative protection and amino acid turnover at one week post-injury. In particular, the rats fed with LC-O3PUFAs showed increased levels of cystathionine (+43%), ornithine (+76%), urea (+36%), and hippurate (+219) when compared to injured animals fed control diets.

Another novel finding of this study is that dietary LC-O3PUFAs dramatically upregulated the levels of heme (+292%) in the spinal cord of injured rats at 1 week post-injury (wpi), suggesting increased levels of proteins containing protective heme groups.

Similar to the diet effects in sham-operated rats, we found that the dietary intervention resulted in selective modulation of carbohydrates and the glutamate neurotransmitter system.

For instance, we found that dietary LC-O3PUFAs increased the levels of the glucose-containing saccharides maltose (+62%) and maltoriose (+218%). The diet rich in LC-O3PUFAs increased the levels of N-acetylglutamate (+43%) when compared to injured animals fed control diets.

The dietary intervention slightly decreased the levels of methionine (−9%), arginine (−10%), hypoxanthine (−10%), and phosphopantetheine (−16%) when compared to controls at one-week post-SCI ($p < 0.05$). The metabolomic alterations at 1 wpi are summarized in Table 4.

Dietary LC-O3PUFAs Increase Antioxidant Defenses and Prevent GSH Depletion in the Chronically Injured Spinal Cord

Of particular significance, the animals receiving the LC-O3PUFA-rich diet showed increased glutathione turnover (GSH, +42% and GSSH, +34%) at 8 wpi, suggesting improved antioxidant defenses. The diet slightly increased the levels of γ-glutamylglutamine (+15). This finding provides further evidence of the impact of dietary LC-O3PUFAs in the modulation of the glutamatergic system.

Interestingly, the LC-O3PUFA-rich diet increased the levels of biomarkers associated with cell proliferation and epigenetic mechanism, including cytidine (+20%), uridine (+15%), and N-acetyllisine (+49%) when compared to control fed rats at 8 wpi. We also detected decreased levels of asparagine (−25%), 2-aminobutyrate (−46%), anserine (−50%), and ergothioneine (−48%) in rats consuming LC-O3PUFAs at 8 weeks post-SCI. The metabolic signatures of dietary LC-O3PUFAs at 8 wpi are summarized in Table 4. IPA-assisted metabolic maps were generated to facilitate the understanding of the complex pathways regulated by dietary LC-O3PUFAs (Figure 6). The complete non-lipid metabolomic profile detected in the spinal cord of the studied groups is represented in Table 5 and Table 6.

Neural Oxidative Status is Significantly Associated with Sensorimotor Function after SCI

To determine the relationship between the spinal cord oxidative status and sensorimotor functional recovery, we performed Spearman correlation analyses. We found that the oxidative status biomarker (GSH:GSSH ratio) was associated with motor (r value = 0.76, $p < 0.0001$; $n = 49$ pairs when combining both timepoints and when normalizing the data by removing highest and lowest scores from each group) and sensory function (r value = −0.54, $p < 0.01$; $n = 31$ pairs when using rats at 8 weeks post-operation). These findings support that oxidative stress plays an important role in determining functional outcomes after SCI and suggest its potential applicability to evaluate prognosis for neurological improvements in injured patients. The results underscore the importance of dietary LC-O3PUFAs in modulating the oxidative status.

Discussion

Rats fed a diet rich in long-chain omega-3 polyunsaturated fatty acids (LC-O3PUFAs) are more resilient in withstanding the secondary damage events and sensorimotor dysfunction associated with spinal cord injury (SCI) [17,19,18]. The present report defines the major underlying non-lipid targets of dietary LC-O3PUFAs through the unbiased interrogation of spinal cord tissue metabolite levels. We now report that dietary LC-O3PUFAs have a

profound impact in the neural bioenergetics and antioxidant metabolomic profile. This was evidenced by marked and selective changes in the levels of carbohydrates, amino acids and nucleic acids.

Injury to the spinal cord leads to a complex cascade of pathophysiological processes that result in neural dysfunction. Multiple acute processes have been proposed that contribute to inflammation, cell death and dysfunction, and include ischemia, edema, cell membrane derangements, neurotransmitter and ionic imbalances, compromised energy metabolism, and production of free radicals. Our central guiding hypothesis is that prophylaxis and very early therapeutic interventions may be required to reduce the physical and psychological burden of disease on individuals at risk or afflicted by SCI.

LC-O3PUFAs confer prophylaxis against SCI

Along these lines, there are several clinical and occupational scenarios that present a significant risk of being affected by SCI. These situations include but are not limited to open repair for ruptured abdominal aortic aneurysm, thoracic endovascular aortic repair (TEVAR), amyotrophic lateral sclerosis, atherosclerosis, cerebral palsy, spina bifida, vitamin B12 deficiency, multiple sclerosis, iatrogenic ischemia, syringomyelia, spondylolysis, disc herniations, radiation toxicity, and tumors, sports, and military conflicts [33–37].

LC-O3PUFAs target antioxidant systems, carbohydrate-amino acid bioenergetics and peptides in the sham-operated spinal cord

The neural metabolism slows considerably in the early phase of SCI, resulting in alterations to the energetic metabolism. Further, the production of reactive oxygen or nitrogen species that accompany the pathophysiology of SCI can lead to deleterious lipid and protein alterations. This increased oxidative and nitrative stress requires metabolic pathways to be redesigned to satisfy large demands for antioxidants. This study demonstrates that dietary LC-O3PUFAs can target the metabolism of carnosine and homocarnosine. These dipeptides are derived from histidine and GABA and represent major endogenous antioxidant/anti-inflammatory chemicals in the spinal cord. For instance, administration of carnosine to SCI animals decreases immune cell infiltration, inducible nitric oxide synthase expression, pro-inflammatory cytokine expression, and apoptosis while improving motor function [30]. Further, carnosine attenuates nociceptive responses in inflammatory pain models [38]. We propose that, these dipeptides could be partly responsible for the locomotor recovery and marked antinociceptive behaviors that are exhibited by animals consuming these long-chain fatty acids.

Glutamine is an important precursor of releasable glutamate and also one of the most effective substrates and modulators of glucose metabolism. The metabolism of glutamine is tightly regulated as it plays major roles in neurotransmission, immunomodulation, and glutathione synthesis [39,40] and in SCI [41]. Further, recent studies suggest that metabolic ratios evaluating chemical changes in glutamine-glutamate cycling may have prognostic value in neurotrauma and could help explain the state of gliosis and hyperexcitability often observed in SCI [42,43,4,44]. This study reveals that dietary LC-O3PUFAs target the

glutamine-glutamate cycling even in sham animals. Because the LC-O3PUFA-rich diet did not alter the levels of glutamate in the spinal cord, this effect could reflect a reduced glutamine synthesis or accelerated glutamine recycling/catabolism. These findings imply that dietary LC-O3PUFAs modulate the expression of glutamine synthetase, which has been shown to protect from glutamate-induced excitotoxicity in SCI [45]. The metabolism of tryptophan has also been implicated in the pathophysiology of SCI [46,47]. Here, we provide evidence suggesting that the metabolism of tryptophan is geared towards the generation of kynurenines in the rats receiving LC-O3PUFA-rich diets. These metabolites are important regulators of mitochondrial homeostasis and oxidative stress, inflammation, and glutamate excitotoxicity [31].

The data shows that the levels of metabolic substrates and intermediates of glycolysis such as glucose, glucose-6-P, fructose, and sedoheptulose-7-P are significantly reduced by the LC-O3PUFA diet in the sham-operated animals. These observations together with our previous findings demonstrating that LC-O3PUFAs increase the levels of palmitoleate (a marker of *de novo* lipogenesis) suggest that these fatty acids reprogram the neural cell glucose bioenergetics [19]. LC-O3PUFAs may stimulate energy production largely by oxidative phosphorylation via the tricarboxylic acid (TCA) cycle and pentose phosphate pathway (PPP) rather than glycolysis. This aneplerotic flux could have the advantage of diverting glycolytic intermediates into various biosynthetic pathways, which are essential for the synthesis of necessary macromolecules (i.e. amino acids, nucleosides and lipids required for assembling new cells). This idea is supported by recent findings showing that dietary LC-O3PUFAs reduce glycolysis by decreasing the expression of glycolytic enzymes [48]. The reduced levels of selective amino acids with concomitant increases in the levels of urea and ornithine further support the strong influence of dietary PUFAs in modulating neural bioenergetics. Interestingly, the carbon skeleton of most of the amino acids that were reduced by the LC-O3PUFA diet can also feed into the TCA cycle and converted to pyruvate, acetyl CoA, acetoacetate and succinyl CoA, suggesting a selective shunting of amino acids into the cycle. This observation indicates that the diet rich in LC-O3PUFAs may increase the protein and amino acid turnover and/or storage as fat and glycogen. Again, this could result in a more efficient system to handle excess amino nitrogen, which could be beneficial in situations in which neurons are starving or exposed to toxic levels of neurotransmitters such as during SCI.

Together, these observations lead us to conclude that the ability to regulate the neural cell bioenergetics and to increase and sustain stable levels of endogenous antioxidants may represent important mechanisms by which dietary LC-O3PUFAs confer prophylaxis against SCI. Through building a reservoir of protective molecules and by improving the cellular energetics and antioxidant availability, neurons and glia are likely to be more resistant against calcium overload, glutamate toxicity, cell death, and dysfunction after SCI.

Administration of LC-O3PUFAs in the diet improves the spinal cord redox potential in the injured spinal cord

Chronic oxidative damage after SCI results in neuroinflammation and maladaptive plasticity leading to sensorimotor abnormalities [49]. The SH-containing tripeptide glutathione is

effective in the protection of SH-carrying proteins against oxygen radicals and may thereby be especially important for neuroprotection [50,51]. The glutathione metabolism is rapidly activated following SCI [52] and its experimental depletion in SCI leads to appreciable worsening of neutrophil infiltration, lipid peroxidation, apoptosis, and loss of hind leg movement [53]. Thus, the intracellular glutathione pool could be important for limiting oxidative stress–induced neuronal injury. Our data suggest that glutathione is a molecular target of dietary LC-O3PUFAs and could represent a key mechanism conferring protection in SCI rats and is inline with previous findings in rat hippocampal cultures [54].

Another finding that reflects an enhanced antioxidant system in rats fed with LC-O3PUFAs are the increased levels of sulfur amino acids. The increase in cystathionine and decrease of methionine observed in rats fed diets rich in LC-O3PUFAs suggests an increase in transulfuration pathway. This observation is supported by a study indicating that LC-O3PUFAs are capable of increasing the expression of cystathionine-γ-lyase (CSL), which catalyzes the splitting of cystathionine into cysteine and α-ketobutyrate [55].

A novel finding of the study is the effect of the diet in increasing the levels of heme in the spinal cord, suggesting that dietary LC-O3PUFAs regulate the metabolism of heme-containing proteins such as hemoproteins. Hemoproteins contain a globin fold, the structural hallmark of the tissue hemoglobins [56,57], which enables the binding of oxygen, nitric oxide, or free radicals. The globin fold also enables the tissue hemoglobin to serve as either a facilitator of oxygen transport within a cell, a scavenger of nitric oxide, and/or an enzyme with peroxidase activity. Hemoproteins such as cytoglobin (CYGB) neuroglobin (Ngb) have been implicated in mediating neurorestorative responses in models of hypoxia-ischemia and spinal cord trauma [58–60]. Although LC-O3PUFAs may also prevent the degradation of these key proteins, the effects of dietary LC-O3PUFAs on this important family needs to be clarified and further characterized.

Dietary LC-O3PUFAs increase the levels of glucose-containing oligosaccharides during the acute injury phase

Of particular significance we found that the diet rich in LC-O3PUFAs elevated the levels of oligosaccharides containing only glucose (i.e., maltose and maltotriose). Two potential mechanisms may be involved in this process: (1) the LC-O3PUFA diet facilitates the carbohydrate accumulation through glycogenesis or (2) the LC-O3PUFA diet may prevent the depletion of glycogen, a hallmark of ischemic damage. These mechanisms can facilitate energy production and offer metabolic alternatives to the damaged cord. For instance, LC-O3PUFAs could be important regulators of brain energy metabolism by affecting glucose utilization, the expression of the glucose transporter-1 (GLUT1), and the metabolism of glycans and glycoproteins, supporting the results presented herein [61,62].

LC-O3PUFAs increase the levels of pyrimidines and epigenetic biomarkers

Studies have shown that brain phosphatide synthesis requires three circulating compounds: DHA, uridine, and choline [63]. Interestingly, oral administration of these precursors increases the levels of phosphatides, synaptic proteins and number of dendritic spines in the brain [63]. The increased levels of these precursors in the animals receiving LC-O3PUFAs

in the diet support our previous electrophysiological findings showing increased spinal plasticity and repair responses in SCI animals pretreated with DHA injections [17].

This study shows that dietary intake of LC-O3PUFAs increased the levels of acetyllisine. It is thus reasonable to propose that this increased acetylation levels of lysine could reflect potential epigenetic mechanisms mediated by the diet. In fact, DHA has been reported to increase the acetylation of H3 and Bcl-2 levels, promoting gene expression and suggesting a mechanism for the neuroprotective roles of DHA [64]. Our own studies provide further support to this genetic regulation by DHA [65].

In summary, dietary-essential LC-O3PUFAs accrete in the spinal cord cell membranes and alter multiple crucial metabolic pathways implicated in cell bioenergetics, signal transduction, gene expression, synaptic plasticity, and calcium regulation. Although DHA and EPA structure is an excellent target for lipid peroxidation and may function as free-radical scavenger, we demonstrate that LC-O3PUFA accretion in advance of injury may promote neural resilience against SCI by regulating the neuron-glia metabolic network. Accumulated LC-O3PUFAs can be released from cell membranes at the onset of injury and trigger the docosanoid pathway and DHA-derived messengers, including docosatrienes, resolvins, and neuroprotectins, such as neuroprotectin D1 [66–70].

Conclusions

Small-molecule profiling defined the underlying biologic state and distinctive non-lipid metabolic signatures associated with dietary LC-O3PUFA. We demonstrated that the consumption of a diet rich in LC-O3PUFAs has a wide-ranging impact on the spinal cord metabolism. These changes may explain some of the observed differences in the phenotype of animals fed LC-O3PUFA-rich diets after SCI. Global neurometabolomic analyses uncovered the LC-O3PUFA metabolome as quite diverse and defined various metabolic pathways, including amino acid neurotransmitter systems and antioxidant defenses as its principal non-lipid targets *in vivo*. These changes demonstrate that LC-O3PUFAs are able to influence signaling networks beyond those associated with lipid metabolism. The rapid treatment of SCI animals with DHA and EPA has been shown to ameliorate several of the secondary biological responses that accompany the physical trauma. We have shown that these protective effects can be expanded to prophylactic interventions, which may be a preventive measure to provide critical protection to individuals with known risk for SCI, including surgical patients, contact sports athletes, soldiers, and first responders. Although dietary LC-O3PUFAs may lack target specificity when compared to other therapeutics, diet may be more effective with respect to costs and safety concerns and deserve serious consideration in clinical applications. Through defining the protective mechanism of dietary LC-O3PUFA, we hope that this study serves to inspire future studies on the identification and validation of novel prophylactic and therapeutic targets for nervous system lesions.

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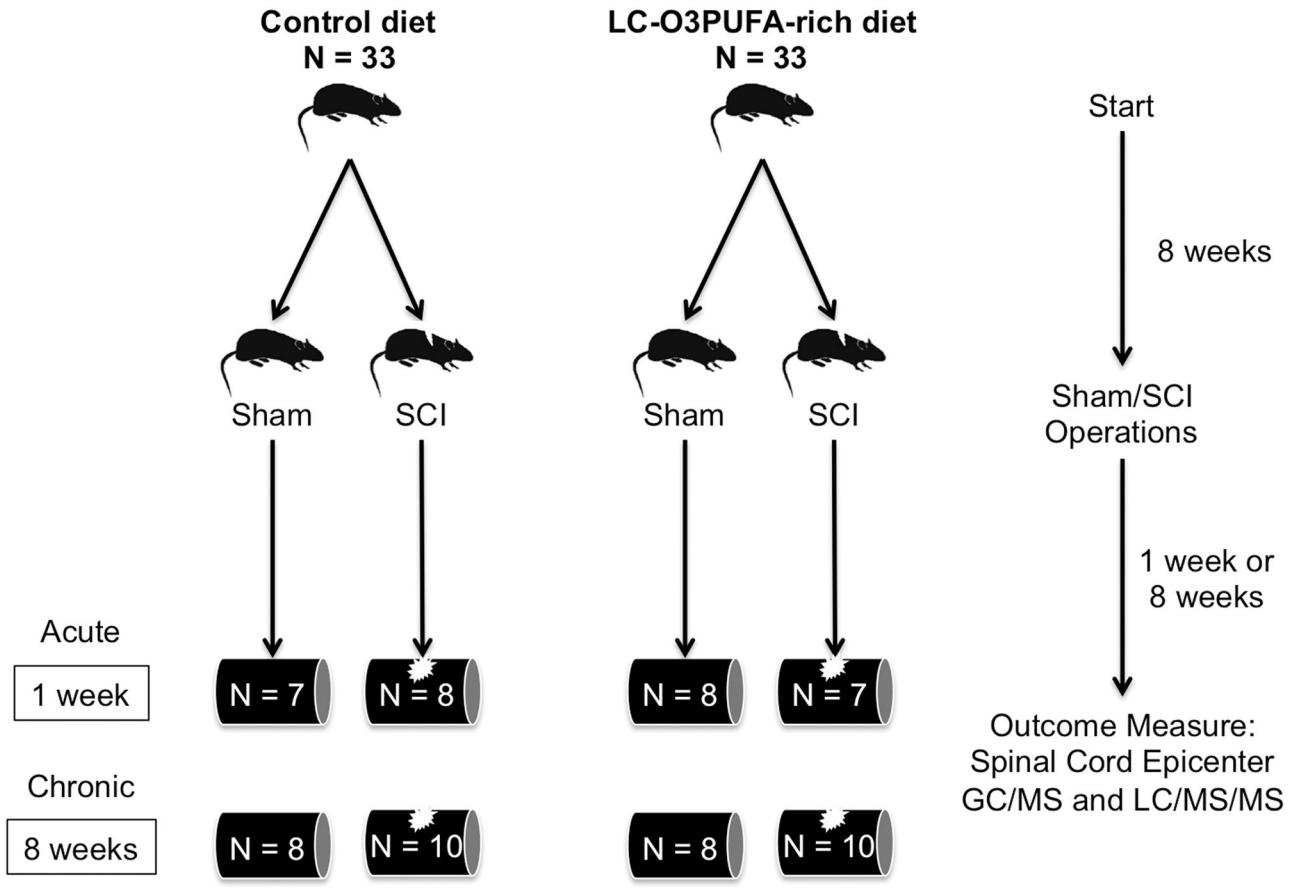

	Control:Sham	Control:SCI	LC-O3PUFA:Sham	LC-O3PUFA:SCI	
Weights	267.2 ± 28.22	259.3 ± 21.82	281.7 ± 41.12	276.7 ± 32.34	(grams ± SD)
Diet / day	19.41 ± 4.08	18.73 ± 4.45	20.28 ± 4.84	18.91 ± 5.26	(grams ± SD)

Figure 1. Timeline outlining experimental design and animal groups

Rats were fed control or LC-O3PUFA-enriched chows for 8 weeks before being subjected to sham or SCI operations. Rats were removed for terminal global metabolomics analyses during acute and chronic injury stages. Values for weights and food consumption are average grams (g) ± S.D; *n* = at least 16 rats per group.

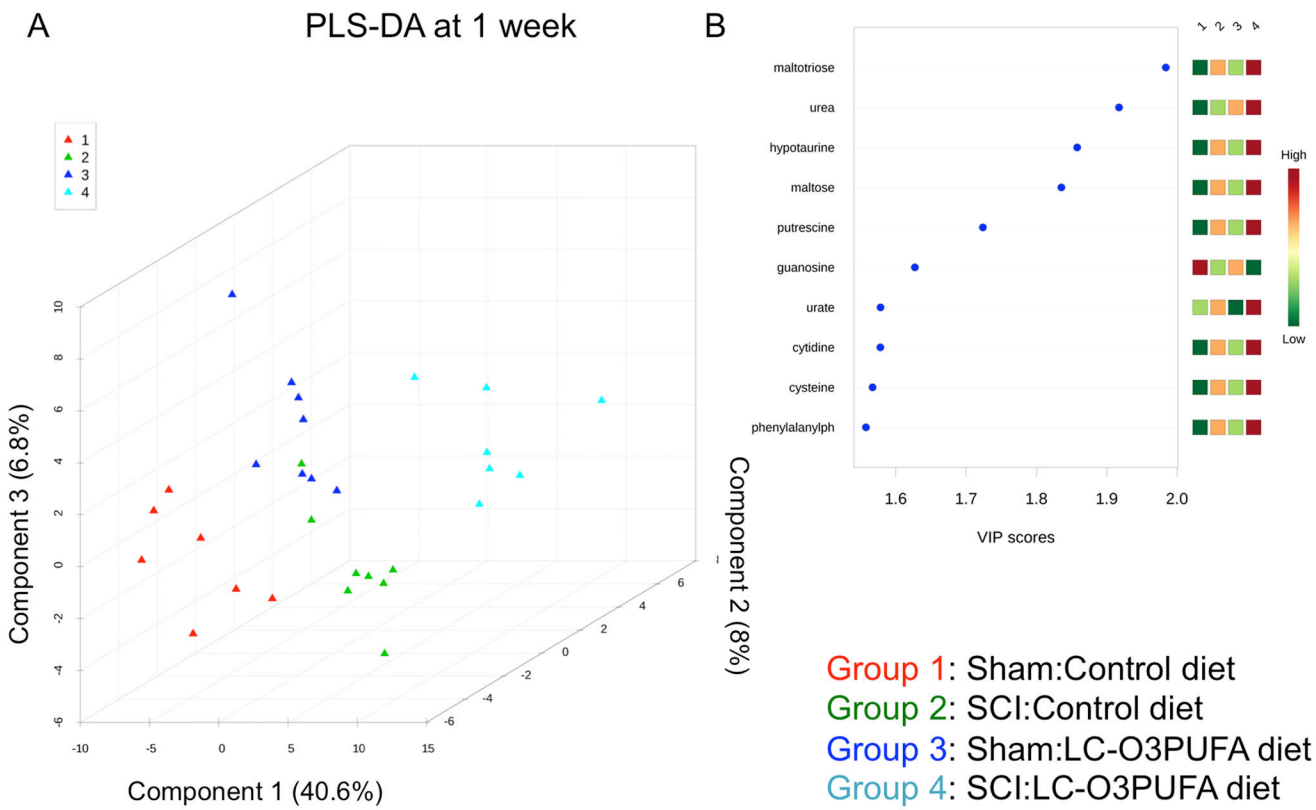

Figure 2. Dietary LC-O3PUFAs significantly modulate the non-lipid spinal cord metabolome during acute injury stages

(A) Partial least square discriminant analysis (PLS-DA) distinguished subgroups based on operation and dietary intake at 1 week post-operation. This model was constructed using scaled intensity peaks of the global non-lipid detected features. Permutation provided statistically significant separations between subgroups ($p < 0.05$; not shown). (B) The variable influence on projection (VIP) reflects the importance of amino acids and antioxidant peptides in the generated PLS model.

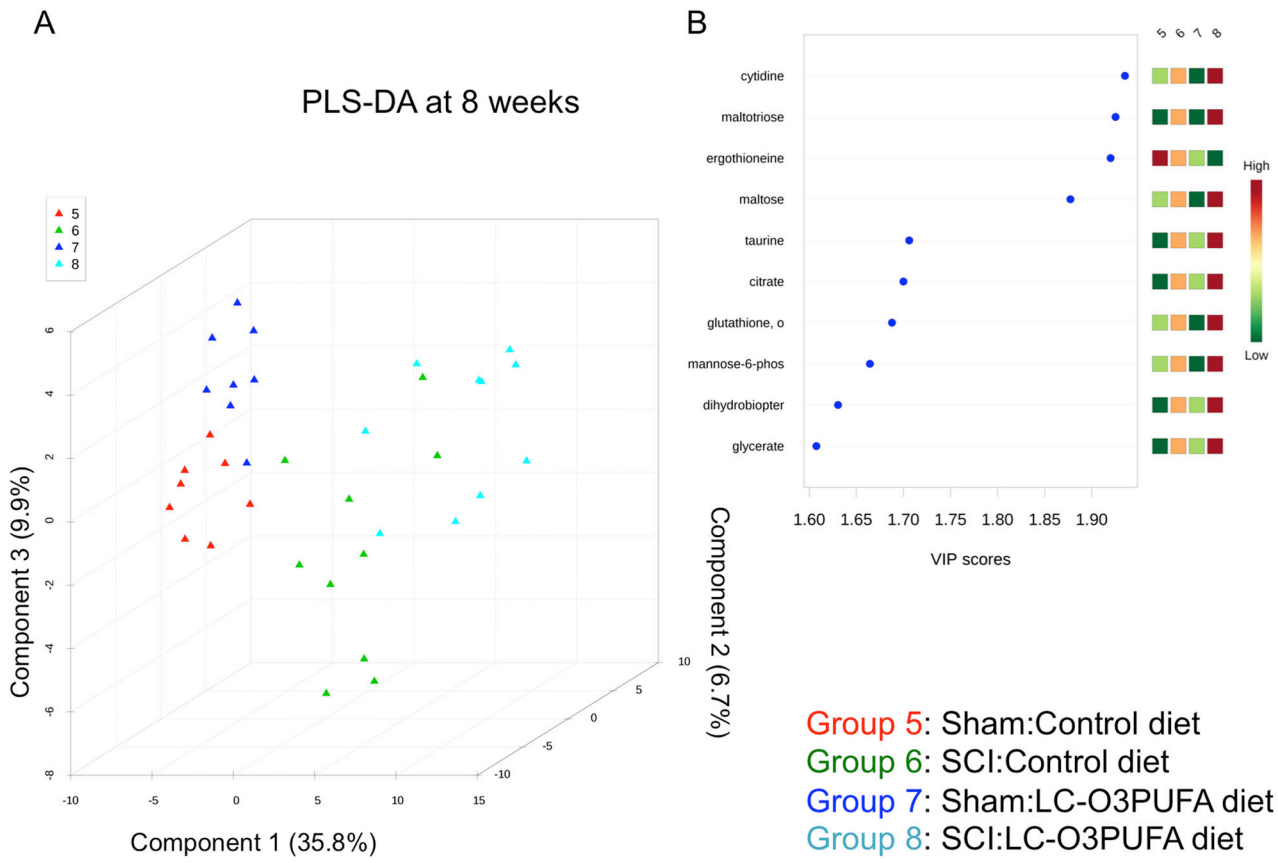

Figure 3. Dietary LC-O3PUFAs significantly modulate the non-lipid spinal cord metabolome during chronic injury stages

(A) Partial least square discriminant analysis (PLS-DA) discriminated between groups based on dietary intake at 8 weeks post-operation. The model was generated using scaled intensity peaks of the global non-lipid detected features. Permutation provided statistically significant separations between subgroups ($p < 0.05$; data not shown). (B) The variable influence on projection (VIP) indicates the importance of carbohydrates, amino acids, and antioxidant peptides at 8 week-post operations.

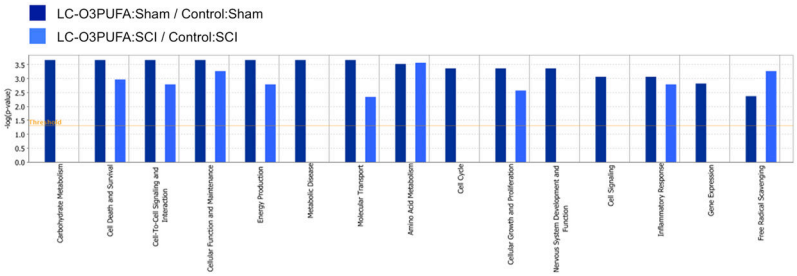

Figure 4. Results of LC-MS/MS data analysis using Ingenuity Pathways Analysis (IPA) software

The data set was filtered for non-lipid small-molecules that met the 1.5 fold cut-off criteria. These metabolites were associated with biological functions in the IPA Knowledge Base. The *p*-value was calculated using right-tailed Fischer's exact test and represents the probability that each biological function assigned to that data set is due to random chance alone. P-value < 0.05 were considered statistically significant.

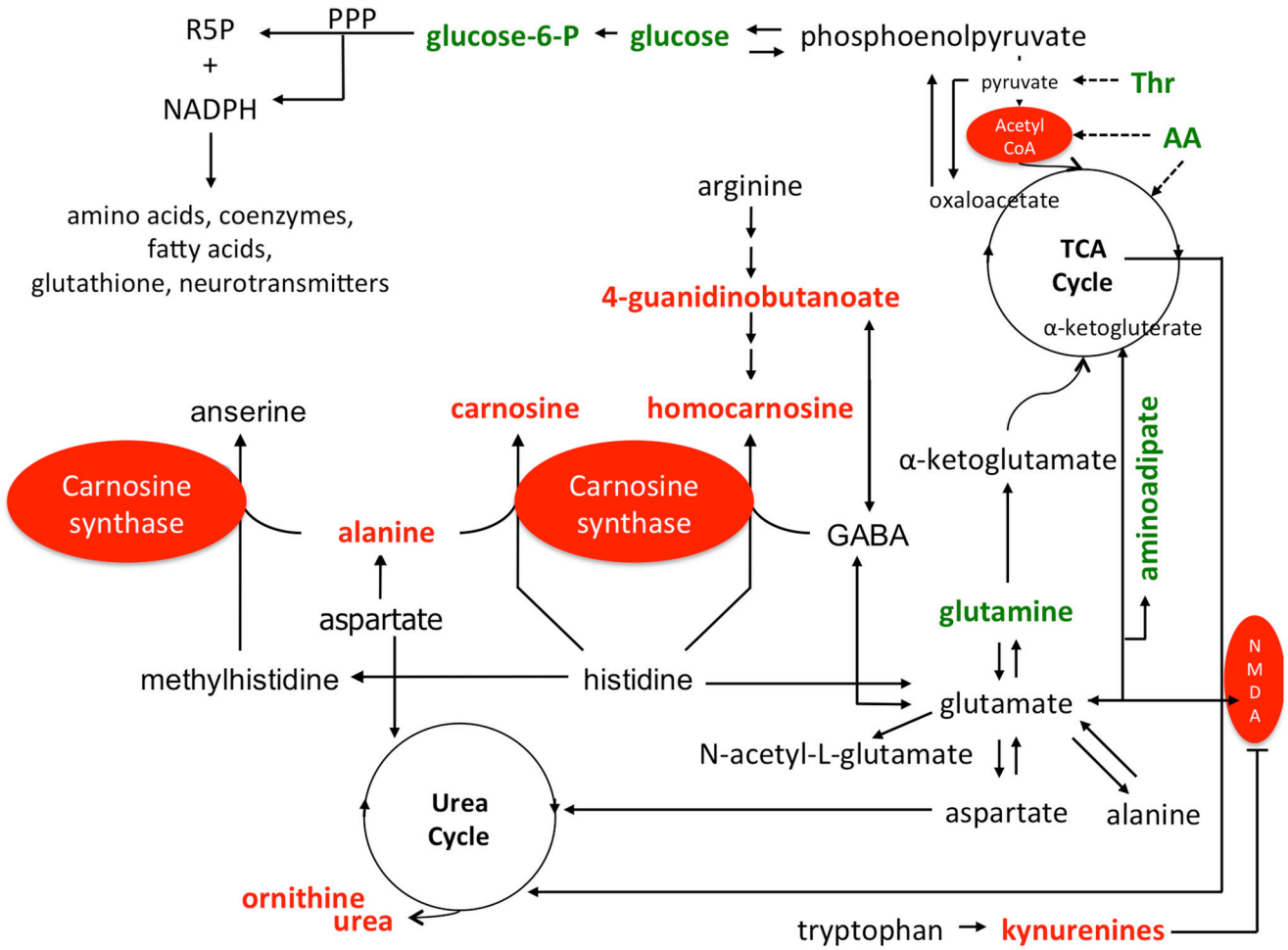

Figure 5. Metabolic pathways targeted by dietary LC-O3PUFA in the sham rat spinal cord

Dietary LC-O3PUFAs target the metabolism of carnosine and homocarnosine, as evidenced by increased levels of alanine, carnosine, homocarnosine, and 4-guanidinobutanoate in the spinal cord of animals fed with LC-O3PUFAs. The diet rich in LC-O3PUFAs also altered the glutamine-glutamate cycling. A distinctive group of amino acid systems were affected by the diet, including threonine and tryptophan. In particular, animals fed with LC-O3PUFAs showed dramatic increases in the levels of kynurenines, which can regulate mitochondrial homeostasis and oxidative stress, inflammation, and glutamate excitotoxicity through NMDA receptor inhibition. Notably, the diet increased the levels of ornithine and urea, while decreasing glucose and glucose-6-P levels, showing selective alterations in the spinal cord cell bioenergetics. This support that LC-O3PUFAs fuel energy production largely by oxidative phosphorylation via the tricarboxylic acid (TCA) cycle and pentose phosphate pathway (PPP) rather than glycolysis, which are essential pathways for the synthesis of necessary macromolecules (i.e. amino acids, neurotransmitters, glutathione, nucleosides and lipids required for assembling new cells). These pathways may represent important mechanisms by which dietary LC-O3PUFAs confer prophylaxis against neurodegeneration and dysfunction in SCI. This reservoir of protective molecules and antioxidant bioavailability is expected to make neurons and glia more resistant against

calcium overload, glutamate toxicity, and cell death following SCI. Metabolites in red increased with the dietary intervention. Features in green decreased with the LC-O3PUFA diet when compared to controls. Putative enzymatic/receptor targets are highlighted in red ovals. Abbreviations: AA, amino acid (polar); GABA, gamma-aminobutyrate; NADPH, nicotinamide adenine dinucleotide phosphate; NMDA, N-methyl-D-aspartate; PPP, pentose phosphate pathway; R5P, ribose 5-phosphate; Thr, threonine.

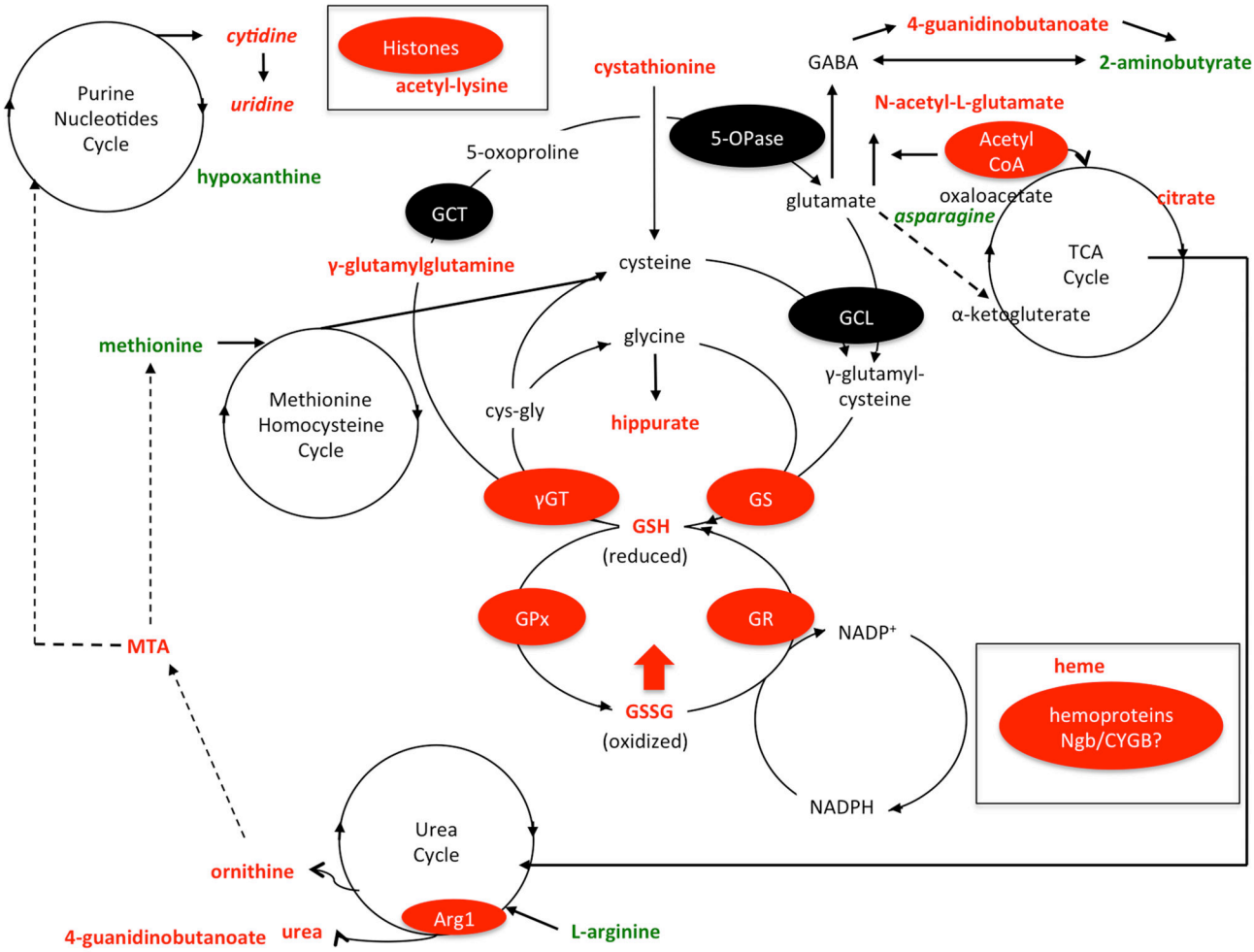

Figure 6. Metabolic pathways targeted by dietary LC-O3PUFA in the injured spinal cord

Although we did not characterize the specific sources of ROS in the present study, our metabolomics dataset supports the role of mitochondrial dysfunction as a major source during both acute and chronic injury stages. Interestingly, we identified glutathione (GSH) metabolism as a molecular target of dietary LC-O3PUFAs. For instance, the animals receiving the dietary intervention showed increased spinal cord levels of γ-glutamylglutamine, cystathione, hippurate, GSH, and GSSH, suggesting increased production and/or reduced depletion of antioxidant pools after SCI. Notably, the levels of heme were increased in the spinal cord of rats exposed to the LC-O3PUFA-rich diet, proposing a novel protective mechanism for LC-O3PUFAs. Similar to the findings observed in sham rats, LC-O3PUFAs altered the TCA and Urea cycle. The increased levels of purine nucleotides and acetyl-lysine suggests a mechanism for which chronic dietary supplementation with LC-O3PUFAs modulates plasticity, growth, and gene expression. Metabolites in red increased with the dietary intervention. Features in green decreased with the LC-O3PUFA diet when compared to controls. Putative enzymatic and protein targets are highlighted in red ovals. Abbreviations: 5-OPase, 5-oxoprolinase; Arg1, arginase; CYGB, cytoglobin; cys-gly, cysteine-glycine; GSH, glutathione, reduced; GSSH, glutathione disulfide, oxidized; GCL, γ-glutamylcysteine ligase; GS, glutathione synthase; GPx,

glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; γGT, γ-glutamyltransferase; GCT, γ-glutamylcylotransferase; MTA, 5′-methylthioadenosine; Ngb, neuroglobin; TCA, tricarboxylic acid.

Table 1

Detailed compositional analysis of AIN-93G-based diets

The level of dietary fat was approximately 6% of dry weight supplied as either soybean oil (control chow) or menhaden fish oil (LC-O3PUFA-enriched chow). Gas chromatography coupled with mass spectrometry demonstrated that the level of DHA and eicosapentaenoic acid (EPA) in the menhaden fish oil was 12.82-g and 6.91-g, respectively, per 100g of diet. The level of cholesterol was 0.582-g/100g. This amount was added to control diets to make the levels consistent with that of the fish oil diet.

Ingredient	AIN-93G Control diet (%)	AIN-93G Fish oil-enriched diet (%)
Casein	20	20
L-Cystine	0.3	0.3
Corn starch	39.7	39.7
Maltodextrin	13.2	13.2
Sucrose	10	10
Fiber	5	5
Vitamin mix	1	1
Mineral mix	3.5	3.5
Choline bitartrate	0.25	0.25
tBHQ	0.0014	0.0014
Soybean oil	7	0.77
Fish oil (DHA + EPA + cholesterol)	0	**6.23**
Cholesterol (added to match fish oil levels)	0.0121	0
Omega-3 Fatty Acids in Oils		
DHA	<1 g/100 g soybean oil	**12.82 g/100 g fish oil**
EPA	<1 g/100 g soybean oil	**6.91 g/100 g fish oil**
omega-6:omega-3	7.5:1	**1:3.7**

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Table 2

Major pathways and functions associated with dietary consumption of LC-O3PUFAs in rats

Diet Effect in Sham Rats

Category	Diseases or Functions Annotation	p-Value	# Molecules
Cellular Function and Maintenance	cellular homeostasis	1.94E-04	17
Molecular Transport	transport of molecule	8.00E-04	16
Small Molecule Biochemistry	synthesis of nitric oxide	1.19E-09	15
Cell Signaling	synthesis of nitric oxide	1.19E-09	15
Free Radical Scavenging	metabolism of reactive oxygen species	1.68E-06	15
Amino Acid Metabolism	transport of amino acids	2.84E-11	12
Molecular Transport	transport of amino acids	2.84E-11	12
Small Molecule Biochemistry	transport of amino acids	2.84E-11	12
Molecular Transport	quantity of Ca2+	5.72E-06	12
Cell Signaling	quantity of Ca2+	5.72E-06	12

Diet Effect in SCI Rats

Category	Diseases or Functions Annotation	p-Value	# Molecules
Cellular Function and Maintenance	cellular homeostasis	2.93E-05	14
Free Radical Scavenging	synthesis of reactive oxygen species	9.76E-07	12
Protein Synthesis	metabolism of protein	1.02E-06	12
Molecular Transport	transport of molecule	6.82E-04	12
Small Molecule Biochemistry	synthesis of nitric oxide	3.62E-08	11
Cell Signaling	synthesis of nitric oxide	3.62E-08	11
Cell-To-Cell Signaling and Interaction	activation of blood cells	4.15E-06	11
Cell Death and Survival	cell survival	1.64E-03	11
Cellular Growth and Proliferation	growth of bacteria	7.29E-11	10
Cell Signaling	quantity of Ca2+	2.45E-06	10

Table 3

Significant metabolites targeted by dietary LC-O3PUFAs in sham rats

Fold changes when compared to sham animals receiving control diets. The additional columns depict the individual effects of diet and SCI in the modulating the levels of each feature. Up arrow, upregulation; down arrow, downregulation; =, not significant change.

One Week Post-Sham Operation (LC-O3PUFA Diet/Control Diet)

Metabolite	Fold-chage	Diet Effect	SCI Effect
threonine	0.72	↑	=
beta-alanine	1.24	↓	=
glutamine	0.86	↑	=
2-aminoadipate	0.61	↑	=
kynurenine	1.88	↓	=
urea	1.44	↓	=
carnosine	1.33	↓	↑
homocarnosine	1.27	↓	↑

Eight Weeks Post-Sham Operation (LC-O3PUFA Diet/Control Diet)

Metabolite	Fold-chage	Diet Effect	SCI Effect
amino acids with polar uncharged and hydrophobic side chains: serine, threonine and valine, leucine, isoleucine metabolism	<0.80	↑	=
4-guanidinobutanoate	1.34	↓	=
fructose	0.71	↑	=
glucose-6P	0.76	↑	↓
glucose	0.52	↑	↓
sedoheptulose-7-phosphate	0.54	↑	↓
N1-methyladenosine	0.74	↑	=
adenosine 2′-monophosphate (2′-AMP)	0.79	↑	↓
acetyl CoA	1.27	↓	↑
ergothioneine	0.70	↑	=

Table 4

Important small-molecule targets of LC-O3PUFAs in spinal cord injured rats

Fold changes when compared to injured rats receiving control diets. The additional columns illustrate the individual effects of diet and SCI in the regulating the levels of each biochemical. Up arrow, upregulation; down arrow, downregulation; =, not significant change.

One Week Post-SCI (LC-O3PUFA Diet/Control Diet)

Metabolite	Fold-chage	Diet Effect	SCI Effect
N-acetylglutamate	1.43	↓	↑
cystathionine	1.43	↓	=
Methionine and acetylmethionine	0.91, 0.88	↓	↓
arginine	0.90	↑	↓
ornithine	1.76	↓	↓
urea	1.36	↓	=
maltose and maltotriose	1.62, 2.18	↓	↓
hypoxanthine	0.90	↑	=
heme	2.92	↓	=
phosphopantetheine	0.84	↑	↑
hippurate	2.19	↓	=

Eight Weeks Post-SCI (LC-O3PUFA Diet/Control Diet)

Metabolite	Fold-chage	Diet Effect	SCI Effect
asparagine	0.75	↑	=
N2-acetyllysine	1.49	↓	=
2-aminobutyrate	0.54	↑	=
5-methylthioadenosine (MTA)	1.19	↓	=
4-guanidinobutanoate	1.27	↓	=
glutathione, reduced (GSH)	1.42	↓	=
glutathione, oxidized (GSSG)	1.34	↓	↓
anserine	0.50	↑	=
gamma-glutamylglutamine	1.15	↓	↑

Eight Weeks Post-SCI (LC-O3PUFA Diet/Control Diet)

Metabolite	Fold-chage	Diet Effect	SCI Effect
maltose	1.71	↓	↓
maltriose	1.17	↓	=
citrate	1.30	↓	↓
cytidine	1.20	↓	↓
uridine	1.15	↓	=
methylphosphate	1.26	↓	=
ergothioneine	0.52	↑	=

Table 5

Complete non-lipid metabolomic profile at one-week post-operation

Data represents averages of scaled metabolite amount ± standard deviation. For each detected metabolite, the raw area counts were re-scaled to set the median metabolite relative amount equal to 1. The Human Metabolome Database (HMDB) identifier has been provided. The green color in the heat map indicates averaged median values equal or lower than 1.5-fold from the median metabolite amount of 1, whereas red indicates averaged median values equal or higher than 1.5-fold from the median metabolite amount.

	Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
Amino Acids	2-aminoadipate	HMDB00510	Lysine Metabolism	1.18	0.28	1.07	0.25	1.07	0.25	0.65	0.27
	2-aminobutyrate	HMDB00650	Butanoate metabolism	1.66	0.90	0.79	0.29	0.79	0.29	0.69	0.35
	2-methylbutyroylcarnitine (C5)	HMDB00378	Valine, leucine and isoleucine metabolism	1.14	0.36	0.38	0.03	0.38	0.03	0.38	0.04
	3-(4-hydroxyphenyl)lactate (HPLA)	HMDB00755	Phenylalanine & tyrosine metabolism	1.41	0.36	0.74	0.21	0.74	0.21	0.75	0.08
	3-hydroxyisobutyrate	HMDB00336	Valine, leucine and isoleucine metabolism	1.04	0.26	0.84	0.36	0.84	0.36	0.79	0.29
	3-phosphoserine	HMDB00272	Glycine, serine and threonine metabolism	0.94	0.37	1.01	0.33	1.01	0.33	1.01	0.28
	4-guanidinobutanoate	HMDB03464	Guanidino and acetamido metabolism	1.22	0.18	0.80	0.26	0.80	0.26	0.82	0.20
	5-aminovalerate	HMDB03355	Urea cycle; arginine-, proline-, metabolism	1.12	0.12	0.80	0.15	0.80	0.15	0.79	0.16
	5-methylthioadenosine (MTA)	HMDB01173	Polyamine metabolism	0.88	0.16	1.08	0.17	1.08	0.17	1.11	0.14
	5-oxoproline	HMDB00267	Glutathione metabolism	0.93	0.07	1.08	0.09	1.08	0.09	1.09	0.13
	alanine	HMDB00161	Alanine and aspartate metabolism	1.17	0.16	0.78	0.10	0.78	0.10	0.82	0.10
	arginine	HMDB00517	Urea cycle; arginine-, proline-, metabolism	1.08	0.10	0.97	0.08	0.97	0.08	0.96	0.07
	asparagine	HMDB00168	Alanine and aspartate metabolism	1.34	0.21	0.77	0.19	0.77	0.19	0.77	0.12
	aspartate	HMDB00191	Alanine and aspartate metabolism	0.81	0.06	1.26	0.17	1.26	0.17	1.39	0.13
	beta-alanine	HMDB00056	Alanine and aspartate metabolism	0.92	0.10	0.90	0.23	0.90	0.23	1.12	0.24
	betaine	HMDB0043	Glycine, serine and threonine metebolism	1.42	0.11	0.65	0.15	0.65	0.15	0.70	0.11
	C-glycosyltryptophan*		Tryptophan metabolism	1.41	0.59	0.93	0.23	0.93	0.23	0.94	0.18
	citrulline	HMDB00904	Urea cycle; arginine-, proline-, metabolism	1.64	0.35	0.61	0.15	0.61	0.15	0.53	0.10
	creatine	HMDB00064	Creatine metabolism	0.88	0.12	1.05	0.17	1.05	0.17	1.06	0.21
	creatinine	HMDB00562	Creatine metabolism	0.95	0.18	1.10	0.22	1.10	0.22	1.14	0.19
	cystathionine	HMDB00099	Cysteine, methionine, SAM, taurine metabolism	0.94	0.26	1.06	0.24	1.06	0.24	1.01	0.17
	cysteine	HMDB00574	Cysteine, methionine, SAM, taurine metabolism	1.04	0.15	0.90	0.15	0.90	0.15	0.95	0.09
	cysteine-glutathione disulfide	HMDB00656	Glutathione metabolism	1.49	0.43	0.81	0.12	0.81	0.12	0.79	0.23
	cystine	HMDB00192	Cysteine, methionine, SAM, taurine metabolism	0.99	0.35	0.99	0.32	0.99	0.32	0.72	0.15
	gamma-aminobutyrate (GABA)	HMDB00112	Glutamate metabolism	0.72	0.09	1.29	0.15	1.29	0.15	1.40	0.23
	glutamate	HMDB03339	Glutamate metabolism	0.86	0.07	1.10	0.07	1.10	0.07	1.14	0.11

Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
			Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
glutamine	HMDB00641	Glutamate metabolism	0.96	0.11	1.05	0.11	1.05	0.11	0.90	0.14
glutathione, oxidized (GSSG)	HMDB03337	Glutathione metabolism	1.80	1.87	0.95	0.27	0.95	0.27	0.97	0.35
glutathione, reduced (GSH)	HMDB00125	Glutathione metabolism	1.06	0.34	1.07	0.26	1.07	0.26	1.15	0.23
glycine	HMDB00123	Glycine, serine and threonine metabolism	0.95	0.12	1.08	0.14	1.08	0.14	1.15	0.13
histidine	HMDB00177	Histidine metabolism	1.45	0.26	0.72	0.08	0.72	0.08	0.75	0.09
hydroxyisovaleroylcarnitine (C5)		Valine, leucine and isoleucine metabolism	1.68	0.32	0.67	0.14	0.67	0.14	0.67	0.16
hypotaurine	HMDB00965	Cysteine, methionine, SAM, taurine metabolism	1.74	0.28	0.54	0.14	0.54	0.14	0.62	0.11
isoleucine	HMDB00172	Valine, leucine and isoleucine metabolism	1.43	0.15	0.82	0.07	0.82	0.07	0.82	0.07
isovalerylcarnitine (C5)	HMDB00688	Valine, leucine and isoleucine metabolism	1.41	0.55	0.23	0.11	0.23	0.11	0.19	0.10
kynurenine	HMDB00684	Tryptophan metabolism	2.15	3.86	2.58	5.01	2.58	5.01	4.84	4.10
leucine	HMDB00687	Valine, leucine and isoleucine metabolism	1.43	0.10	0.79	0.05	0.79	0.05	0.80	0.07
lysine	HMDB00182	Lysine metabolism	1.22	0.31	0.89	0.11	0.89	0.11	0.93	0.05
methionine	HMDB00696	Cysteine, methionine, SAM, taurine metabolism	1.28	0.11	0.86	0.06	0.86	0.06	0.88	0.06
N-acetyl-aspartyl-glutamate (NAAG)	HMDB01067	Glutamate metabolism	0.71	0.05	1.18	0.06	1.18	0.06	1.14	0.05
N-acetylalanine	HMDB00766	Alanine and aspartate metabolism	1.25	0.15	0.88	0.10	0.88	0.10	0.95	0.05
N-acetylaspartate (NAA)	HMDB00812	Alanine and aspartate metabolism	0.49	0.16	1.46	0.16	1.46	0.16	1.31	0.23
N-acetylglutamate	HMDB01138	Glutamate metabolism	0.61	0.20	1.23	0.35	1.23	0.35	1.34	0.28
N-acetylmethionine	HMDB11745	Cysteine, methionine, SAM, taurine metabolism	1.58	0.16	0.62	0.04	0.62	0.04	0.67	0.09
N-acetylthreonine		Glycine, serine and threonine metabolism	1.27	0.29	0.78	0.10	0.78	0.10	0.77	0.11
N2-acetyllysine	HMDB00446	Lysine metabolism	1.04	0.28	1.18	0.22	1.18	0.22	1.08	0.24
N6-acetyllysine	HMDB00206	Lysine metabolism	1.12	0.18	0.91	0.24	0.91	0.24	0.93	0.13
ornithine	HMDB03374	Urea cycle; arginine-, proline-, metabolism	1.82	0.65	0.84	0.11	0.84	0.11	0.85	0.08
phenylalanine	HMDB00159	Phenylalanine & tyrosine metabolism	1.44	0.15	0.83	0.09	0.83	0.09	0.83	0.08
pipecolate	HMDB00070	Lysine metabolism	0.99	0.29	0.72	0.36	0.72	0.36	0.66	0.28
proline	HMDB00162	Urea cycle; arginine-, proline-, metabolism	1.27	0.12	0.88	0.08	0.88	0.08	0.83	0.06
putrescine	HMDB01414	Polyamine metabolism	1.42	0.24	0.42	0.05	0.42	0.05	0.52	0.17
S-adenosylhomocysteine (SAH)	HMDB00939	Cysteine, methionine, SAM, taurine metabolism	0.84	0.12	1.14	0.12	1.14	0.12	1.10	0.10
sarcosine (N-Methylglycine)	HMDB00271	Glycine, serine and threonine metabolism	0.97	0.13	1.06	0.15	1.06	0.15	1.01	0.23
serine	HMDB00187	Glycine, serine and threonine metabolism	1.21	0.19	0.77	0.13	0.77	0.13	0.80	0.10
spermidine	HMDB01257	Polyamine metabolism	1.18	0.74	0.95	0.11	0.95	0.11	1.14	0.30
taurine	HMDB00251	Cysteine, methionine, SAM, taurine metabolism	1.70	0.51	0.72	0.22	0.72	0.22	0.78	0.18
threonine	HMDB00167	Glycine, serine and threonine metabolism	1.11	0.13	0.98	0.21	0.98	0.21	0.71	0.16
hydroxyproline	HMDB00725	Urea cycle; arginine-, proline-, metabolism	1.48	0.67	0.64	0.23	0.64	0.23	0.48	0.15

	Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	tryptophan	HMDB00929	Tryptophan metabolism	1.23	0.10	0.82	0.11	0.82	0.11	0.83	0.13
	tyrosine	HMDB00158	Phenylalanine & tyrosine metabolism	1.37	0.13	0.75	0.08	0.75	0.08	0.77	0.07
	urea	HMDB00294	Urea cycle; arginine-, proline-, metabolism	0.85	0.28	0.75	0.21	0.75	0.21	1.07	0.26
	valine	HMDB00883	Valine, leucine and isoleucine metabolism	1.42	0.11	0.84	0.06	0.84	0.06	0.85	0.06
Carbohydrates	dihydroxyacetone	HMDB01882	Glycolysis, gluconeogenesis, pyruvate metabolism	1.18	0.41	0.84	0.51	0.84	0.51	1.22	0.59
	1, 5-anhydroglucitol (1, 5-AG)	HMDB02712	Glycolysis, gluconeogenesis, pyruvate metabolism	1.24	0.23	0.74	0.22	0.74	0.22	0.81	0.29
	3-phosphoglycerate	HMDB00807	Glycolysis, gluconeogenesis, pyruvate metabolism	0.99	0.20	1.03	0.14	1.03	0.14	0.93	0.16
	arabinose	HMDB00646	Nucleotide sugars, pentose metabolism	0.98	0.15	0.98	0.14	0.98	0.14	1.08	0.18
	arabitol	HMDB01851	Nucleotide sugars, pentose metabolism	1.05	0.52	1.21	0.36	1.21	0.36	0.99	0.29
	dihydroxyacetone phosphate (DHAP)	HMDB01473	Glycolysis, gluconeogenesis, pyruvate metabolism	0.92	0.22	0.94	0.19	0.94	0.19	1.00	0.22
	erythronate*	HMDB00613	Aminosugars metabolism	1.26	0.29	1.02	0.14	1.02	0.14	0.80	0.11
	fructose	HMDB00660	Fructose, mannose, galactose, starch, and sucrose metabolism	1.24	0.40	0.79	0.13	0.79	0.13	0.91	0.34
	fructose 6-phosphate	HMDB00124	Glycolysis, gluconeogenesis, pyruvate metabolism	1.62	0.47	0.80	0.10	0.80	0.10	0.82	0.26
	glucose	HMDB00122	Glycolysis, gluconeogenesis, pyruvate metabolism	2.26	1.26	0.22	0.06	0.22	0.06	0.38	0.36
	glucose 6-phosphate	HMDB01401	Glycolysis, gluconeogenesis, pyruvate metabolism	1.54	0.47	0.68	0.10	0.68	0.10	0.72	0.24
	glycerate	HMDB00139	Glycolysis, gluconeogenesis, pyruvate metabolism	1.28	0.31	0.95	0.32	0.95	0.32	0.93	0.20
	Isobar: hexose diphosphates		Glycolysis, gluconeogenesis, pyruvate metabolism	1.00	0.39	0.86	0.26	0.86	0.26	1.00	0.32
	Isobar: ribulose 5-phosphate, xylulose 5-phosphate		Nucleotide sugars, pentose metabolism	1.05	0.24	0.94	0.09	0.94	0.09	0.96	0.18
	lactate	HMDB00190	Glycolysis, gluconeogenesis, pyruvate metabolism	1.01	0.12	0.99	0.11	0.99	0.11	1.04	0.13
	maltose	HMDB00163	Fructose, mannose, galactose, starch, and sucrose metabolism	1.53	0.72	0.44	0.07	0.44	0.07	0.47	0.08
	maltotriose	HMDB01262	Fructose, mannose, galactose, starch, and sucrose metabolism	0.84	0.55	0.26	0.01	0.26	0.01	0.28	0.04
	mannitol	HMDB00765	Fructose, mannose, galactose, starch, and sucrose metabolism	0.84	0.50	0.62	0.22	0.62	0.22	0.80	0.38
	mannose	HMDB00169	Fructose, mannose, galactose, starch, and sucrose metabolism	1.65	0.45	0.57	0.14	0.57	0.14	0.61	0.16
	mannose 6-phosphate	HMDB01078	Fructose, mannose, galactose, starch, and sucrose metabolism	1.61	0.47	0.72	0.14	0.72	0.14	0.74	0.26
	N-acetylneuraminate	HMDB00230	Aminosugars metabolism	1.03	0.11	1.04	0.20	1.04	0.20	0.87	0.18
	ribitol	HMDB00508	Nucleotide sugars, pentose metabolism	1.02	0.37	1.05	0.33	1.05	0.33	0.96	0.24
	ribose	HMDB00283	Nucleotide sugars, pentose metabolism	0.99	0.17	1.16	0.31	1.16	0.31	1.45	0.60
	ribulose	HMDB00621	Nucleotide sugars, pentose metabolism	1.11	0.37	0.76	0.31	0.76	0.31	0.81	0.33
	sedoheptulose-7-phosphate	HMDB01068	Nucleotide sugars, pentose metabolism	1.37	0.55	1.61	0.36	0.61	0.36	0.94	0.41
	sorbitol	HMDB00247	Fructose, mannose, galactose, starch, and sucrose metabolism	1.13	0.40	0.86	0.25	0.86	0.25	0.93	0.27
	sucrose	HMDB00258	Fructose, mannose, galactose, starch, and sucrose metabolism	1.76	0.15	0.72	0.03	0.72	0.03	1.02	0.82
Cofactors and vitamins	3′-dephosphocoenzyme A	HMDB01373	Pantothenate and CoA metabolism	0.79	0.34	1.20	0.41	1.20	0.41	1.52	0.66
	alpha-tocopherol	HMDB01893	Tocopherol metabolism	1.23	1.15	0.91	0.08	0.91	0.08	0.93	0.24

	Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	ascorbate (Vitamin C)	HMDB00044	Ascorbate and aldarate metabolism	1.23	0.23	0.77	0.14	0.77	0.14	0.85	0.15
	CoA	HMDB01423	Pantothenate and CoA metabolism	0.76	0.32	1.58	0.77	1.58	0.77	1.74	0.87
	dehydroascorbate	HMDB01264	Ascorbate and aldarate metabolism	1.03	0.48	0.92	0.22	0.92	0.22	0.90	0.35
	dihydrobiopterin	HMDB00038	Tetrahydrobiopterin metabolism	1.11	0.28	0.71	0.15	0.71	0.15	0.62	0.00
	FAD	HMDB01248	Riboflavin metabolism	1.00	0.10	0.95	0.08	0.95	0.08	1.02	0.08
	heme	HMDB03178	Hemoglobin and porphyrin metabolism	0.71	0.12	0.68	0.05	0.68	0.05	0.80	0.27
	nicotinamide	HMDB01406	Nicotinate and nicotinamide metabolism	0.88	0.07	1.06	0.07	1.06	0.07	1.09	0.08
	NAD+	HMDB00902	Nicotinate and nicotinamide metabolism	0.88	0.13	1.07	0.21	1.07	0.21	1.07	0.17
	pantothenate (Vitamin B5)	HMDB00210	Pantothenate and CoA metabolism	1.00	0.16	1.00	0.16	1.00	0.16	1.08	0.20
	phosphopantetheine	HMDB01416	Pantothenate and CoA metabolism	0.91	0.10	1.12	0.13	1.12	0.13	1.18	0.14
	pyridoxal	HMDB01545	Pyridoxal metabolism	1.02	0.17	0.97	0.31	0.97	0.31	1.08	0.29
	riboflavin (Vitamin B2)	HMDB00244	Riboflavin metabolism	1.11	0.12	0.84	0.12	0.84	0.12	0.92	0.16
Energy	cis-aconitate	HMDB00072	Krebs cycle	1.49	0.18	0.61	0.09	0.61	0.09	0.66	0.10
	citrate	HMDB00094	Krebs cycle	1.66	0.38	0.65	0.09	0.65	0.09	0.69	0.10
	fumarate	HMDB00134	Krebs cycle	1.01	0.22	1.04	0.17	1.04	0.17	1.15	0.23
	malate	HMDB00153	Krebs cycle	1.10	0.16	0.96	0.07	0.96	0.07	1.01	0.14
	phosphate	HMDB01429	Oxidative phosphorylation	0.97	0.04	1.02	0.03	1.02	0.03	1.07	0.05
	pyrophosphate (PPi)	HMDB00250	Oxidative phosphorylation	0.85	0.50	0.75	0.41	0.75	0.41	1.03	0.66
	succinylcarnitine (C4)		Krebs cycle	1.20	0.30	0.76	0.19	0.76	0.19	0.92	0.13
Nucleotides	2′-deoxycytidine	HMDB00014	Pyrimidine metabolism, cytidine containing	1.45	0.20	0.64	0.09	0.64	0.09	0.67	0.13
	2′-deoxyinosine	HMDB00071	Purine metabolism, (hypo)xanthine/inosine containing	1.15	0.44	0.46	0.00	0.46	0.00	0.46	0.00
	5,6-dihydrouracil	HMDB00076	Purine metabolism, adenine containing	1.04	0.39	1.08	0.18	1.08	0.18	0.95	0.23
	adenine	HMDB00034	Purine metabolism, adenine containing	0.78	0.15	1.29	0.15	1.29	0.15	1.23	0.22
	adenosine	HMDB00050	Purine metabolism, adenine containing	0.33	0.16	1.56	0.43	1.56	0.43	1.75	0.39
	2′-AMP	HMDB11617	Purine metabolism, adenine containing	1.13	0.48	0.85	0.22	0.85	0.22	0.82	0.28
	3′-AMP	HMDB03540	Purine metabolism, adenine containing	1.18	0.14	0.76	0.16	0.76	0.16	0.75	0.18
	AMP	HMDB00045	Purine metabolism, urate metabolism	0.80	0.15	1.17	0.36	1.17	0.36	1.34	0.23
	allantoin	HMDB00462	Pyrimidine metabolism, cytidine containing	1.16	0.58	0.87	0.35	0.87	0.35	1.00	0.56
	cytidine	HMDB00089	Pyrimidine metabolism, cytidine containing	1.09	0.07	0.94	0.04	0.94	0.04	0.96	0.06
	3′-CMP		Purine metabolism, guanine containing	0.92	0.15	0.98	0.15	0.98	0.15	1.09	0.18
	guanosine	HMDB00133	Purine metabolism, (hypo)xanthine/inosine containing	0.54	0.09	1.33	0.10	1.33	0.10	1.25	0.18
	hypoxanthine	HMDB00157	Purine metabolism, (hypo)xanthine/inosine containing	1.02	0.07	1.00	0.09	1.00	0.09	1.02	0.10
	inosine	HMDB00195	Purine and pyrimidine metabolism	0.79	0.07	1.05	0.06	1.05	0.06	1.09	0.04

	Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	methylphosphate		Purine metabolism, adenine containing	0.94	0.14	1.07	0.11	1.07	0.11	1.09	0.13
	1-methyladenosine	HMDB03331	Pyrimidine metabolism, uracil containing	1.21	0.31	0.86	0.18	0.86	0.18	0.90	0.35
	pseudouridine	HMDB00767	Pyrimidine metabolism, uracil containing	1.36	0.31	0.76	0.19	0.76	0.19	0.81	0.08
	uracil	HMDB00300	Purine metabolism, urate metabolism	1.30	0.17	0.76	0.14	0.76	0.14	0.78	0.12
	urate	HMDB00289	Pyrimidine metabolism, uracil containing	1.64	0.37	0.54	0.24	0.54	0.24	0.48	0.12
	uridine	HMDB00296	Purine metabolism, (hypo)xanthine/inosine containing	0.77	0.10	1.17	0.12	1.17	0.12	1.10	0.16
	xanthine	HMDB00292	Purine metabolism, (hypo)xanthine/inosine containing	1.12	0.16	0.94	0.09	0.94	0.09	0.94	0.09
	xanthosine	HMDB00299	Dipeptide derivative	1.28	0.25	0.78	0.14	0.78	0.14	0.72	0.15
Peptides	anserine	HMDB00194	Dipeptide derivative	0.70	0.31	1.61	1.53	1.61	1.53	2.15	1.47
	carnosine	HMDB00033	gamma-glutamyl	0.58	0.04	0.99	0.31	0.99	0.31	1.31	0.23
	gamma-glutamylalanine		gamma-glutamyl	1.37	0.23	0.80	0.15	0.80	0.15	0.80	0.22
	gamma-glutamylglutamate		gamma-glutamyl	0.85	0.11	1.15	0.15	1.15	0.15	1.19	0.19
	gamma-glutamylglutamine	HMDB11738	gamma-glutamyl	1.02	0.21	0.92	0.10	0.92	0.10	0.94	0.14
	gamma-glutamylglycine	HMDB11667	gamma-glutamyl	0.71	0.21	1.11	0.25	1.11	0.25	1.31	0.31
	gamma-glutamylleucine	HMDB11171	gamma-glutamyl	1.20	0.15	0.75	0.21	0.75	0.21	0.80	0.10
	gamma-glutamylmethionine		gamma-glutamyl	1.18	0.16	0.92	0.18	0.92	0.18	0.81	0.20
	gamma-glutamylphenylalanine	HMDB00594	gamma-glutamyl	1.11	0.23	0.89	0.16	0.89	0.16	0.89	0.16
	gamma-glutamylthreonine*		gamma-glutamyl	1.09	0.17	0.97	0.11	0.97	0.11	0.89	0.20
	gamma-glutamyltyrosine		Dipeptide	1.08	0.11	0.82	0.33	0.82	0.33	0.91	0.29
	glycylglycine	HMDB11733	Dipeptide	1.26	0.34	0.93	0.18	0.93	0.18	0.90	0.07
	glycylleucine	HMDB00759	Dipeptide	1.17	0.21	0.90	0.09	0.90	0.09	0.97	0.15
	glycylphenylalanine		Dipeptide derivative	1.08	0.11	0.79	0.22	0.79	0.22	0.77	0.23
	homocarnosine	HMDB00745	Dipeptide	0.82	0.17	1.07	0.13	1.07	0.13	1.36	0.17
	isoleucylglycine		Dipeptide	1.15	0.27	0.95	0.12	0.95	0.12	0.93	0.13
	leucylglycine		Dipeptide	1.00	0.29	1.08	0.28	1.08	0.28	1.25	0.37
	leucylserine		Dipeptide	1.56	0.24	0.76	0.19	0.76	0.19	0.62	0.22
	phenylalanylglycine		Dipeptide	1.23	0.42	0.28	0.06	0.28	0.06	0.28	0.05
	phenylalanylphenylalanine		Dipeptide	1.30	0.31	0.31	0.08	0.31	0.08	0.46	0.23
	phenylalanylserine		Dipeptide	2.04	0.57	0.82	0.16	0.82	0.16	0.90	0.08
	prolylmethionine		Dipeptide	1.35	0.34	0.85	0.14	0.85	0.14	0.86	0.19
	threonylalanine		Dipeptide	0.98	0.27	0.98	0.30	0.98	0.30	1.11	0.37
	tryptophylglycine		Dipeptide	1.37	0.33	0.91	0.13	0.91	0.13	0.81	0.21
	tyrosylglycine		Dipeptide	1.77	0.29	0.62	0.12	0.62	0.12	0.64	0.14

	Metabolite 1 week	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	tyrosylleucine			1.55	0.29	0.63	0.09	0.63	0.09	0.68	0.17
Xenobiotics	2-pyrrolidinone	HMDB02039	Chemical	1.10	0.50	1.46	0.68	1.46	0.68	1.45	1.17
	dihydrokaempferol		Food component/Plant	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
	ergothioneine	HMDB03045	Food component/Plant	1.30	0.23	0.89	0.25	0.89	0.25	0.91	0.30
	erythritol	HMDB02994	Sugar, sugar substitute, starch	0.95	0.23	1.13	0.19	1.13	0.19	1.06	0.21
	glycerol 2-phosphate	HMDB02520	Chemical	1.12	0.35	0.90	0.24	0.90	0.24	0.87	0.32
	hippurate	HMDB00714	Benzoate metabolism	0.74	0.44	0.90	0.32	0.90	0.32	1.08	0.69
	ketamine		Drug	1.05	1.14	0.72	0.69	0.72	0.69	0.80	0.92
	pentobarbital		Drug	0.71	0.61	1.02	0.62	1.02	0.62	1.33	0.92

Table 6

Complete non-lipid metabolomic profile at eight weeks post-operation

Data represents averages of scaled metabolite amount ± standard deviation. For each small molecule, the raw area counts were re-scaled to set the median metabolite amount equal to 1. The Human Metabolome Database (HMDB) identifier has been provided. The green color in the heat map indicates averaged median values equal or lower than 1.5-fold from the median metabolite amount of 1, whereas red indicates averaged median values equal or higher than 1.5-fold from the median metabolite amount.

	Metabolite 8 weeks	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
Amino Acids	2-aminoadipate	HMDB00510	Lysine metabolism	1.06	0.44	1.20	0.46	1.20	0.46	1.08	0.50
	2-aminobutyrate	HMDB00650	Butanoate metabolism	1.64	0.80	0.89	0.68	0.89	0.68	0.79	0.48
	2-hydroxybutyrate (AHB)	HMDB00008	Cysteine, methionine, SAM, taurine metabolism	1.14	0.32	0.96	0.31	0.96	0.31	0.68	0.30
	2-methylbutyroylcarnitine	HMDB00378	Valine, leucine and isoleucine metabolism	0.92	0.41	1.04	0.30	1.04	0.30	0.60	0.22
	3-(4-hydroxyphenyl)lactate	HMDB00755	Phenylalanine & tyrosine metabolism	0.91	0.22	0.92	0.23	0.92	0.23	0.90	0.26
	3-hydroxyisobutyrate	HMDB00336	Valine, leucine and isoleucine metabolism	0.87	0.27	0.93	0.29	0.93	0.29	0.76	0.21
	3-phosphoserine	HMDB00272	Glycine, serine and threonine metabolism	0.95	0.25	1.17	0.42	1.17	0.42	0.61	0.29
	4-guanidinobutanoate	HMDB03464	Guanidino and acetamido metabolism	0.84	0.23	1.06	0.19	1.06	0.19	1.29	0.39
	5-methylthioadenosine (MTA)	HMDB01173	Polyamine metabolism	0.88	0.13	1.05	0.19	1.05	0.19	1.04	0.14
	5-oxopriline	HMDB00267	Glutathione metabolism	1.10	0.32	1.18	0.12	1.18	0.12	0.90	0.11
	agmatine	HMDB01432	Polyamine metabolism	1.16	0.40	1.47	0.58	1.47	0.58	0.87	0.27
	alanine	HMDB00161	Alanine and aspartate metabolism	1.21	0.22	1.29	0.21	1.29	0.21	0.85	0.08
	arginine	HMDB00517	Urea cycle; arginine-, proline-, metabolism	1.01	0.16	0.95	0.18	0.95	0.18	1.00	0.07
	asparagine	HMDB00168	Alanine and aspartate metabolism	1.00	0.22	0.75	0.26	0.75	0.26	1.10	0.15
	aspartate	HMDB00191	Alanine and aspartate metabolism	0.82	0.23	0.79	0.12	0.79	0.12	1.27	0.15
	beta-alanine	HMDB00056	Alanine and aspartate metabolism	1.08	0.42	0.92	0.37	0.92	0.37	1.02	0.52
	betaine	HMDB00043	Glycine, serine and threonine metabolism	1.20	0.33	1.37	0.28	1.37	0.28	0.58	0.14
	C-glycosyltryptophan*		Tryptophan metabolism	1.09	0.32	1.19	0.20	1.19	0.20	0.96	0.12
	citrulline	HMDB00904	Urea cycle; arginine-, proline-, metabolism	1.19	0.26	1.33	0.25	1.33	0.25	0.30	0.03
	creatine	HMDB00064	Creatine metabolism	0.90	0.16	0.95	0.07	0.95	0.07	1.07	0.07
	creatinine	HMDB00562	Creatine metabolism	0.95	0.21	1.03	0.14	1.03	0.14	1.01	0.22
	cystathionine	HMDB00099	Cysteine, methionine, SAM, taurine metabolism	0.91	0.16	1.01	0.25	1.01	0.25	1.10	0.21
	cysteine	HMDB00574	Cysteine, methionine, SAM, taurine metabolism	1.11	0.40	1.38	0.39	1.38	0.39	0.83	0.15
	cysteine-glutathione disulfide	HMDB00656	Glutathione metabolism	1.33	0.39	1.40	0.16	1.40	0.16	0.83	0.08
	cystine	HMDB00192	Cysteine, methionine, SAM, taurine metabolism	1.34	0.63	1.33	0.35	1.33	0.35	0.75	0.12
	gamma-aminobutyrate (GABA)	HMDB00112	Glutamate metabolism	0.87	0.32	0.73	0.22	0.73	0.22	1.44	0.46

Metabolite 8 weeks	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
			Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
glutamate	HMDB03339	Glutamate metabolism	0.80	0.14	0.86	0.09	0.86	0.09	1.19	0.10
glutamine	HMDB00641	Glutamate metabolism	0.96	0.21	1.08	0.16	1.08	0.16	1.00	0.12
glutathione, oxidized (GSSG)	HMDB03337	Glutathione metabolism	1.14	0.39	1.54	0.21	1.54	0.21	0.70	0.15
glutathione, reduced (GSH)	HMDB00125	Glutathione metabolism	0.89	0.14	1.25	0.30	1.25	0.30	0.99	0.17
glycine	HMDB00123	Glycine, serine and threonine metabolism	0.77	0.22	0.81	0.14	0.81	0.14	1.23	0.14
histidine	HMDB00177	Histidine metabolism	1.14	0.16	1.13	0.13	1.13	0.13	0.73	0.04
hydroxyisovaleroyl carnitine		Valine, leucine and isoleucine metabolism	1.12	0.41	1.37	0.24	1.37	0.24	0.85	0.22
hypotaurine	HMDB00965	Cysteine, methionine, SAM, taurine metabolism	1.50	0.67	1.58	0.61	1.58	0.61	0.84	0.35
isoleucine	HMDB00172	Valine, leucine and isoleucine metabolism	1.23	0.24	1.13	0.13	1.13	0.13	0.69	0.06
isovalerylcarnitine	HMDB00688	Valine, leucine and isoleucine metabolism	1.04	0.42	1.20	0.39	1.20	0.39	0.89	0.28
kynurenine	HMDB00684	Tryptophan metabolism	0.87	0.66	0.74	0.57	0.74	0.57	0.96	0.58
leucine	HMDB00687	Valine, leucine and isoleucine metabolism	1.21	0.24	1.14	0.13	1.14	0.13	0.71	0.08
lysine	HMDB00182	Lysine metabolism	1.22	0.30	1.34	0.15	1.34	0.15	0.91	0.09
methionine	HMDB00696	Cysteine, methionine, SAM, taurine metabolism	1.14	0.18	1.11	0.12	1.11	0.12	0.76	0.08
N-acetyl-aspartyl-glutamate (NAAG)	HMDB01067	Glutamate metabolism	0.79	0.19	0.81	0.10	0.81	0.10	1.77	0.17
N-acetylalanine	HMDB00766	Alanine and aspartate metabolism	1.05	0.32	0.92	0.14	0.92	0.14	1.10	0.16
N-acetylaspartate (NAA)	HMDB00812	Alanine and aspartate metabolism	0.77	0.22	0.82	0.09	0.82	0.09	1.55	0.07
N-acetylglutamate	HMDB01138	Glutamate metabolism	0.77	0.21	0.73	0.15	0.73	0.15	1.47	1.19
N-acetylmethionine	HMDB11745	Cysteine, methionine, SAM, taurine metabolism	1.40	0.31	1.22	0.17	1.22	0.17	0.49	0.08
N-acetyltryptophan		Tryptophan metabolism	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
N2-acetyllysine	HMDB00446	Lysine metabolism	0.93	0.25	1.38	0.38	1.38	0.38	1.03	0.46
ophthalmate	HMDB05765	Glutathione metabolism	1.21	0.61	0.95	0.58	0.95	0.58	0.93	0.41
ornithine	HMDB03374	Urea cycle; arginine-, proline-, metabolism	1.07	0.31	1.19	0.20	1.19	0.20	0.87	0.22
phenylalanine	HMDB00159	Phenylalanine & tyrosine metabolism	1.21	0.22	1.13	0.14	1.13	0.14	0.86	0.09
pipecolate	HMDB00070	Lysine metabolism	1.25	0.38	1.28	0.25	1.28	0.25	0.93	0.24
proline	HMDB00162	Urea cycle; arginine-, proline-, metabolism	1.13	0.11	1.15	0.15	1.15	0.15	0.79	0.05
putrescine	HMDB01414	Polymine metabolism	1.90	0.71	1.99	0.73	1.99	0.73	0.62	0.19
S-adenosylhomocysteine (SAH)	HMDB00939	Cysteine, methionine, SAM, taurine metabolism	0.90	0.23	0.88	0.15	0.88	0.15	1.10	0.15
S-methylcysteine	HMDB02108	Cysteine, methionine, SAM, taurine metabolism	0.86	0.42	0.96	0.33	0.96	0.33	0.69	0.16
saccharopine	HMDB00279	Lysine metabolism	0.65	0.01	0.75	0.21	0.75	0.21	0.91	0.40
sarcosine (N-Methylglycine)	HMDB00271	Glycine, serine and threonine metabolism	1.00	0.37	1.05	0.32	1.05	0.32	1.07	0.13
serine	HMDB03406	Glycine, serine and threonine metabolism	1.16	0.23	1.22	0.19	1.22	0.19	0.87	0.10
spermidine	HMDB01257	Polyamine metabolism	0.88	0.25	0.97	0.19	0.97	0.19	1.00	0.12

	Metabolite 8 weeks	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	taurine	HMDB00251	Cysteine, methionine, SAM, taurine metabolism	1.24	0.30	1.47	0.28	1.47	0.28	0.76	0.16
	threonine	HMDB00167	Glycine, serine and threonine metabolism	1.04	0.19	1.04	0.15	1.04	0.15	0.86	0.14
	trans-4-hydroxyproline	HMDB00725	Urea cycle; arginine-, proline-, metabolism	0.87	0.61	0.75	0.59	0.75	0.59	0.29	0.11
	tryptophan	HMDB00929	Tryptophan metabolism	1.10	0.19	1.10	0.13	1.10	0.13	0.97	0.07
	tyrosine	HMDB00158	Phenylalanine & tyrosine metabolism	1.17	0.21	1.18	0.14	1.18	0.14	0.64	0.06
	urea	HMDB00294	Urea cycle; arginine-, proline-, metabolism	0.76	0.49	0.98	0.54	0.98	0.54	1.24	0.47
	valine	HMDB00883	Valine, leucine and isoleucine metabolism	1.21	0.27	1.16	0.15	1.16	0.15	0.69	0.07
Carbohydrates	1,5-anhydroglucitol (1,5-AG)	HMDB02712	Glycolysis, gluconeogenesis, pyruvate metabolism	1.14	0.52	1.04	0.28	1.04	0.28	0.83	0.21
	1,6-anhydroglucose	HMDB00640	Glycolysis, gluconeogenesis, pyruvate metabolism	0.57	0.27	0.90	0.55	0.90	0.55	0.64	0.34
	arabinose	HMDB00646	Nucleotide sugars, pentose metabolism	0.52	0.29	0.69	0.53	0.69	0.53	0.66	0.46
	arabitol	HMDB01851	Nucleotide sugars, pentose metabolism	0.91	0.26	1.02	0.36	1.02	0.36	0.97	0.31
	dihydroxyacetone phosphate (DHAP)	HMDB01473	Glycolysis, gluconeogenesis, pyruvate metabolism	1.08	0.29	1.07	0.28	1.07	0.28	0.88	0.17
	erythronate*	HMDB00613	Aminosugars metabolism	1.12	0.36	1.15	0.23	1.15	0.23	0.92	0.17
	fructose	HMDB00660	Fructose, mannose, galactose, starch, and sucrose metabolism	1.28	0.47	1.38	0.31	1.38	0.31	0.69	0.15
	fructose 1-phosphate	HMDB01076	Glycolysis, gluconeogenesis, pyruvate metabolism	1.24	0.63	1.45	0.61	1.45	0.61	0.67	0.22
	fructose-6-phosphate	HMDB00124	Glycolysis, gluconeogenesis, pyruvate metabolism	1.46	0.49	1.72	0.43	1.72	0.43	0.25	0.08
	galactose	HMDB00143	Fructose, mannose, galactose, starch, and sucrose metabolism	1.31	0.37	1.39	0.27	1.39	0.27	0.29	0.09
	glucose	HMDB00122	Glycolysis, gluconeogenesis, pyruvate metabolism	1.61	0.72	2.07	0.70	2.07	0.70	0.13	0.04
	glucose-6-phosphate (G6P)	HMDB01401	Glycolysis, gluconeogenesis, pyruvate metabolism	1.44	0.47	1.69	0.41	1.69	0.41	0.23	0.07
	glycerate	HMDB00139	Glycolysis, gluconeogenesis, pyruvate metabolism	0.86	0.23	1.13	0.48	1.13	0.48	0.67	0.34
	Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate, myo-inositol 1,4 or 1		Glycolysis, gluconeogenesis, pyruvate metabolism	0.97	0.22	0.91	0.13	0.91	0.13	0.98	0.16
	Isobar: ribulose 5-phosphate, xylulose 5-phosphate		Nucleotide sugars, pantose metabolism	1.40	0.57	1.57	0.41	1.57	0.41	0.55	0.11
	lactate	HMDB00190	Glycolysis, gluconeogenesis, pyruvate metabolism	1.01	0.24	1.09	0.18	1.09	0.18	0.96	0.13
	maltose	HMDB00163	Fructose, mannose, galactose, starch, and sucrose metabolism	1.30	0.41	2.21	1.04	2.21	1.04	0.39	0.07
	maltotriose	HMDB01262	Fructose, mannose, galactose, starch, and sucrose metabolism	0.64	0.18	1.40	0.70	1.40	0.70	0.52	0.00
	mannitol	HMDB00765	Fructose, mannose, galactose, starch, and sucrose metabolism	0.98	0.46	0.88	0.49	0.88	0.49	0.75	0.26
	mannose	HMDB00169	Fructose, mannose, galactose, starch, and sucrose metabolism	1.39	0.37	1.52	0.27	1.52	0.27	0.29	0.03
	mannose-6-phosphate	HMDB01078	Fructose, mannose, galactose, starch, and sucrose metabolism	1.43	0.45	1.76	0.43	1.76	0.43	0.25	0.07
	N-acetylglucosamine 6-phosphate	HMDB02817	Aminosugars metabolism	0.99	0.43	1.35	0.42	1.35	0.42	0.83	0.36
	N-acetylneuraminate	HMDB00230	Aminosugars metabolism	1.08	0.26	1.09	0.15	1.09	0.15	0.83	0.11
	ribose	HMDB00283	Nucleotide sugars, pentose metabolism	0.90	0.32	1.09	0.35	1.09	0.35	1.01	0.13
	ribulose	HMDB00621,HMDB03371	Nucleotide sugars, pentose metabolism	0.31	0.55	1.34	0.70	1.34	0.70	0.62	0.32

	Metabolite 8 weeks	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	sedoheptulose-7-phosphate	HMDB01068	Nucleotide sugars, pentose metabolism	1.44	0.64	1.52	0.53	1.52	0.53	0.24	0.11
	sorbitol	HMDB00247	Fructose, mannose, galactose, starch, and sucrose metabolism	1.19	0.43	1.23	0.36	1.23	0.36	0.78	0.23
Cofactors and vitamins	3′-dephosphocoenzyme A	HMDB01373	Pantothenate and CoA metabolism	0.73	0.26	0.79	0.27	0.79	0.27	1.30	0.35
	acetyl CoA	HMDB01206	Pantothenate and CoA metabolism	0.82	0.19	0.89	0.16	0.89	0.16	1.30	0.28
	alpha-tocopherol	HMDB01893	Tocopherol metabolism	1.19	0.14	1.29	0.26	1.29	0.26	0.77	0.15
	ascorbate (Vitamin C)	HMDB00044	Ascorbate and aldarate metabolism	1.19	0.24	1.32	0.28	1.32	0.28	0.85	0.11
	coenzyme A	HMDB01423	Pantothenate and CoA metabolism	0.73	0.20	0.83	0.16	0.83	0.16	1.48	0.27
	dihydrobiopterin	HMDB00038	Folate metabolism	1.18	0.37	1.40	0.47	1.40	0.47	0.53	0.14
	flavin adenine dinucleotide (FAD)	HMDB01248	Riboflavin metabolism	1.09	0.20	1.13	0.16	1.13	0.16	0.83	0.11
	heme		Hemoglobin and porphyrin metabolism	1.01	0.37	1.07	0.29	1.07	0.29	0.96	0.42
	nicotinamide	HMDB01406	Nicotinate and nicotinamide metabolism	0.97	0.22	0.99	0.19	0.99	0.19	1.00	0.20
	pantothenate	HMDB00210	Pantothenate and CoA metabolism	1.24	0.24	1.36	0.28	1.36	0.28	0.82	0.17
	phosphopantetheine	HMDB01416	Pantothenate and CoA metabolism	1.00	0.19	1.20	0.33	1.20	0.33	0.94	0.16
	riboflavin (Vitamin B2)	HMDB00244	Riboflavin metabolism	1.07	0.05	1.08	0.23	1.08	0.23	0.80	0.15
Energy	acetylphosphate	HMDB01494	Oxidative phosphorylation	0.98	0.20	1.11	0.18	1.11	0.18	0.97	0.04
	citrate	HMDB00094	Krebs cycle	1.15	0.32	1.49	0.43	1.49	0.43	0.79	0.18
	fumarate	HMDB00134	Krebs cycle	0.95	0.33	1.14	0.35	1.14	0.35	1.08	0.31
	malate	HMDB00156	Krebs cycle	0.94	0.24	1.05	0.20	1.05	0.20	0.94	0.07
	phosphate	HMDB01429	Oxidative phosphorylation	0.97	0.07	0.98	0.05	0.98	0.05	1.03	0.04
	pyrophosphate (PPi)	HMDB00250	Oxidative phosphorylations	1.04	0.30	1.15	0.27	1.15	0.27	425F02;0.89	0.20
Nucleotides	adenine	HMDB00034	Purine metabolism, adenine containing	0.81	0.18	0.86	0.13	0.86	0.13	1.35	0.28
	adenosine	HMDB00050	Purine metabolism, adenine containing	0.73	0.21	0.67	0.16	0.67	0.16	2.87	0.63
	adenosine 2′-monophosphate (2′-AMP)	HMDB11617	Purine metabolism, adenine containing	1.42	0.48	1.35	0.22	1.35	0.22	0.58	0.10
	adenosine 3′-monophosphate (3′-AMP)	HMDB03540	Purine metabolism, adenine containing	1.03	0.20	1.09	0.18	1.09	0.18	0.87	0.19
	adenosine 5′-monophosphate (AMP)	HMDB00045	Purine metabolism, adenine containing	1.04	0.32	1.05	0.23	1.05	0.23	0.93	0.36
	allantoin	HMDB00462	Purine metabolism, urate metabolism	0.70	0.39	0.92	0.51	0.92	0.51	0.99	0.95
	arabinosylhypoxanthine		Purine metabolism, (hypo)xanthine/inosine containing	0.76	0.38	0.71	0.17	0.71	0.17	0.63	0.00
	cytidine	HMDB00089	Pyrimidine metabolism, cytidine containing	8F3802;1.12	0.16	1.35	0.16	1.35	0.16	0.86	0.06
	cytidine-3′-monophosphate (3′-CMP)		Pyrimidine metabolism, cytidine containing	8F3802;1.12	0.15	1.02	0.18	1.02	0.18	0.97	0.15
	guanosine	HMDB00133	Purine metabolism, guanine containing	0.65	0.28	0.70	0.18	0.70	0.18	1.76	0.30
	hypoxanthine	HMDB00157	Purine metabolism, (hypo)xanthine/inosine containing	1.13	0.32	1.14	0.19	1.14	0.19	0.94	0.10
	inosine		Purine metabolism, (hypo)xanthine/inosine containing	0.88	0.12	0.95	0.08	0.95	0.08	1.05	0.08
	methylphosphate		Purine and pyrimidine metabolism	1.00	0.33	1.26	0.20	1.26	0.20	0.95	0.09

	Metabolite 8 weeks	HMDB	Subpathway	Control: SCI		Control: Sham		O3PUFA: SCI		O3PUFA: Sham	
				Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation	Average Median Metabolite Levels	Standard Deviation
	N1-methyladenosine	HMDB03331	Purine metabolism, adenine containing	0.94	0.26	1.02	0.18	1.02	0.18	0.71	0.23
	pseudouridine	HMDB00767	Pyrimidine metabolism, uracil containing	1.06	0.17	1.02	0.15	1.02	0.15	605002;0.99	0.14
	uracil	HMDB00300	Pyrimidine metabolism, uracil containing	D11803;1.35	0.38	1.26	0.24	1.26	0.24	0.85	0.08
	urate	HMDB00289	Purine metabolism, urate metabolism	1.11	0.30	1.53	0.48	1.53	0.48	0.77	0.31
	uridine	HMDB00296	Pyrimidine metabolism, uracil containing	1.01	0.17	1.16	0.14	1.16	0.14	0.94	0.11
	xanthine	HMDB00292	Purine metabolism, (hypo)xanthine/inosine containing	0.91	0.18	1.02	0.18	1.02	0.18	0.96	0.08
	xanthosine	HMDB00299	Purine metabolism, (hypo)xanthine/inosine containing	1.25	0.42	1.54	0.36	1.54	0.36	0.81	0.14
Peptides	anserine	HMDB00194	Dipeptide derivative	1.56	1.31	0.78	0.46	0.78	0.46	0.96	0.40
	gamma-glutamylalanine		gamma-glutamyl	0.98	0.33	1.01	0.29	1.01	0.29	1.09	0.12
	gamma-glutamylglutamate		gamma-glutamyl	0.74	0.23	0.76	0.12	0.76	0.12	1.57	0.11
	gamma-glutamylglutamine	HMDB11738	gamma-glutamyl	0.84	0.13	0.97	0.15	0.97	0.15	1.14	0.16
	gamma-glutamylglycine	HMDB11667	gamma-glutamyl	0.48	0.34	0.60	0.36	0.60	0.36	1.18	0.23
	gamma-glutamylleucine	HMDB11171	gamma-glutamyl	1.24	0.33	0.11	0.32	0.11	0.32	0.99	0.12
	gamma-glutamylmethionine		gamma-glutamyl	1.07	0.28	0.96	0.25	0.96	0.25	0.92	0.14
	gamma-glutamylphenylalanine	HMDB00594	gamma-glutamyl	1.22	0.55	0.94	0.19	0.94	0.19	0.97	0.20
	gamma-glutamylthreonine*		gamma-glutamyl	0.93	0.24	764502;1.07	0.36	1.07	0.36	1.18	0.42
	glycylglycine	HMDB11733	Dipeptide	0.95	0.38	1.08	0.30	1.08	0.30	0.86	0.50
	glycylleucine	HMDB00759	Dipeptide	1.05	0.31	1.03	0.25	1.03	0.25	0.91	0.21
	homocarnosine	HMDB00745	Dipeptide derivative	0.87	0.18	1.01	0.19	1.01	0.19	1.22	0.28
	isoleucylglycine		Dipeptide	1.09	0.35	1.23	0.31	1.23	0.31	0.93	0.33
	leucylglycine		Dipeptide	1.22	0.64	1.23	0.38	1.23	0.38	0.73	0.27
	leucylserine		Dipeptide	1.32	0.65	1.50	0.35	1.50	0.35	0.85	0.20
Xenobiotics	2-ethylhexanoate (isobar with 2-propylpentanoate)		Chemical	0.97	0.23	0.93	0.13	0.93	0.13	1.04	0.16
	2-pyrrolidinone	HMDB02039	Chemical	0.85	0.42	1.07	0.34	1.07	0.34	1.28	0.47
	ergothioneine	HMDB03045	Food component/Plant	1.28	0.28	0.66	0.12	0.66	0.12	0.94	0.71
	erythritol	HMDB02994	Sugar, sugar substitute, starch	0.87	0.32	0.96	0.22	0.96	0.22	1.00	0.19
	glycerol 2-phosphate	HMDB02520	Chemical	1.24	0.43	1.49	0.37	1.49	0.37	0.73	0.23
	glycolate (hydroxyacetate)	HMDB00115	Chemical	1.14	0.30	0.97	0.38	0.97	0.38	0.76	0.14
	hippurate	HMDB00714	Benzoate metabolism	0.94	0.26	1.14	0.39	1.14	0.39	0.78	0.16
	pentobarbital		Drug	1.12	0.29	1.15	0.49	1.15	0.49	1.01	0.21