Article

Dietary Omega-3 Polyunsaturated Fatty-Acid Supplementation Upregulates Protective Cellular Pathways in Patients with Type 2 Diabetes that reduces Painful Diabetic Neuropathy

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Abstract: Background: Omega-3 polyunsaturated fatty acids (PUFAs) are increasingly reported to improve chronic neuroinflammatory diseases in peripheral and central nervous systems. Specifically, docosahexaenoic acid (DHA) protects nerve cells from noxious stimuli in vitro and in vivo. Recent reports link PUFA supplementation to improving painful diabetic neuropathy (pDN) symptoms. However, the molecular mechanism behind omega-3 PUFAs ameliorating pDN symptoms is lacking. Therefore, we sought to determine the distinct cellular pathways that omega-3 PUFAs dietary supplementation promotes in reducing painful neuropathy in type 2 diabetes mellitus (DM2) patients. Methods: Forty volunteers diagnosed with type 2 diabetes were enrolled in the "En Balance-PLUS" diabetes education study. The volunteers participated in weekly lifestyle/nutrition education and daily supplementation with 1,000 mg DHA and 200 mg eicosapentaenoic acid. The Short-Form McGill Pain Questionnaire validated clinical determination of baseline and post-intervention pain complaints. Laboratory and untargeted metabolomics analyses were conducted using blood plasma collected at baseline and after three months of participation in the dietary regimen. The metabolomics data was analyzed using random forest, hierarchical cluster, ingenuity pathway analysis, and metabolic pathway mapping. Results: We found that metabolites involved in oxidative stress and glutathione production shifted significantly to a more anti-inflammatory state post supplementation. Example of these metabolites include cystathionine (+90%), S-methylmethionine (+9%), glycine cysteine-glutathione disulfide (+157%) cysteinylglycine (+19%), glutamate (-11%), glycine (+11%) and arginine (+13.4%). In addition, the levels of phospholipids associated with improved membrane fluidity such as linoleoyl-docosahexaenoyl-glycerol (18:2/22:6) (+253 %) were significantly increased. Ingenuity pathway analysis suggested several key bio functions associated with omega-3 PUFA supplementation such as formation of reactive oxygen species ($p = 4.38 \times 10^{-4}$, z-score = -1.96), peroxidation of lipids ($p = 2.24 \times 10^{-5}$, z-score = -1.944), Ca^{2+} transport ($p = 1.55 \times 10^{-4}$, z-score = -1.969), excitation of neurons ($p = 1.07 \times 10^{-4}$, z-score = -1.091), and concentration of glutathione ($p = 3.06 \times 10^{-4}$, z-score = 1.974). Conclusion: The reduction of pro-inflammatory and oxidative stress pathways following omega-3 PUFAS supplementation is consistent with using omega-3 PUFAs as a complementary dietary strategy as part of the overall treatment of pDN.

Keywords: omega-3; polyunsaturated fatty acids; painful diabetic neuropathy; metabolism; metabolomics

1. Introduction

Painful diabetic neuropathy (pDN) is a common comorbidity of DM2 and has a significant negative impact on both quality of life and productivity of patients with DM2. Effective therapeutic interventions are sorely lacking and there is a great unmet need for novel and safe therapeutics. Currently treatment is focused on symptoms rather than targeting the disease process resulting in only a third of treated patients achieving 50% pain relief, often complicated by side effects [1-3]. In part, the development of therapies is complicated because of the inherently variable and complex nature of pDN. Although progress has been made in understanding the pathophysiology of diabetic neuropathy, attempts to use various targeted therapies has not resulted in either consistent or sustained benefits in patients with neuropathy. Progress in alleviating painful neuropathy is critically dependent on an understanding of the pathogenesis of pDN which to date is incompletely defined and we will continue to struggle in developing new pharmacotherapies that address the underlying biological dysregulation associated with pDN.

Pre-clinical models suggest that the pathophysiology of pDN involves both microvascular and metabolic changes, which ultimately lead to increased oxidative stress, inflammation, and mitochondrial dysfunction [4]. However, corroborating human data currently is insufficient in the context of pDN. This knowledge gap may be closing with the development and use of high-throughput metabolomics analysis as we can now identify critical biomarkers and elucidate major metabolic pathways involved in the pathophysiology of different disease states [5-10]. For instance, metabolomics analysis can provide a readout of interactions between an individual's genome, diet, and environment in clinical studies.

Growing evidence has implicated PUFAs in diminishing the adverse effects of chronic neuroinflammatory diseases in both the peripheral and central nervous system [11, 12]. The treatment with docosahexaenoic acid (DHA) has shown significant protection in nerve cells both in cell culture and in vivo [13-16]. Furthermore, prophylactic dietary omega-3 PUFAs protect against spinal cord injury-induced chronic pain [7]. In agreement with these findings, pre-clinical models have found that oral administration of omega-3 PUFAs can regenerate and protect peripheral nerves from injury [11, 17]. Recently, PUFA supplementation has been linked to the amelioration of painful diabetic neuropathy symptoms within a clinical cohort [10]. However, the effects on a cellular basis that are related to any benefits within humans attributed to omega-3 PUFAs and pDN is lacking.

The present study examines the underlying metabolome of participants with type 2 diabetes reporting pDN and the impact of DHA-rich supplementation on their metabolic profile. This study represents, to our knowledge, the first unbiased attempt, in humans with type-2 diabetes, to identify the biologically critical metabolic features that define pDN and how a dietary DHA-enriched supplementation alters pDN patient's metabolome during a three-month quasi experimental designed intervention. Of significance, our study identifies biochemical signatures associated with pDN and determines how a simple nutritional invention leads to significant reversal.

2. Materials and Methods

2.1. Study design and Population

En Balance-Plus is a longitudinal quasi-experimental single-arm designed study conducted in Loma Linda, California, to assess the impact of nutrition and diabetes education on type 2 diabetes among Latinos, primarily Mexican Americans. The study evaluated the effect of dietary DHA-enriched supplementation after three months on participants' metabolomic profiles. Cohort description is described in detail previously [10]. Briefly, after informed written consent was obtained, self-identified Latino Americans with type II diabetes completed a 3-month group-based intervention that consisted of taking dietary DHA-enriched capsules and attending weekly diabetes-education classes conducted in Spanish. Each participant was interviewed in person to obtain diabetes history, medication use, diet, and physical activity habits. The study was approved by the

Loma Linda University Institutional Review Board. A total of 40 Latino adults previously diagnosed with type 2 diabetes between 33 and 74 completed the 3-month study. Five participants were excluded from clinical data analysis: one due to a lab reporting error and four due to missing clinical data. The research subjects participated in weekly lifestyle and nutrition classes. They were instructed to consume a daily intake of 2,000 mg omega-3 fish-oil supplements (provided by the study) containing 1,000 mg DHA and 200 mg eicosapentaenoic acid (EPA) for the duration of the study. In addition to the health-education classes, each participant was contacted weekly by phone to confirm compliance.

2.2. Data and Study Variables

Data were collected for all participants at baseline and after three months, including fasting blood plasma samples, anthropometric measurements for weight, height, waist and hip circumferences, and dual-energy X-ray absorptiometry (Discovery A fan beam; Hologic, Marlborough, MA, USA). Plasma samples were tested at the Loma Linda University Medical Center laboratory to determine fasting blood glucose, HbA1c, and lipid profiles (high-density lipoprotein [HDL], low-density lipoprotein [LDL], total cholesterol, and triglycerides). All anthropometric measurements were taken twice for reliability, using Lohman et al.'s standardized techniques [18]. In addition, weight and height were assessed using a balance scale (Detecto, Webb City, MO, USA) and a wall-mounted stadiometer (Holtain, Crymych, England), respectively.

Blood samples were collected at baseline and three months from each participant into EDTA-treated (lavender-top) tubes between 8 and 10:30 am after a 12-hour fast. Blood was centrifuged at 2,000 g at 15°C for 15 minutes and immediately aliquoted into sterile polypropylene tubes for plasma collection. Plasma aliquots were sent to Loma Linda University Medical Center for clinical lab analysis, with the remaining aliquots stored in liquid nitrogen at –80°C for the metabolomics measurements.

Metabolic profiling was performed as previously described [19]. Briefly, untargeted semiquantitative metabolomic analysis was performed on three independent platforms: ultra-high-performance liquid chromatography (HPLC)/tandem mass spectrometry (MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and gas chromatography (GC). Metabolites were identified by comparing the ion features in the experimental samples to a reference library of chemical standards that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments, as well as associated MS. In addition, biochemical features were curated by visual inspection for quality control using the software developed at Metabolon [20].

2.3. Statistical Analysis

The *P* values reported are unadjusted for false discovery rate (*q*-values); however, in an analysis of 695 biochemicals with an assumption that most would fulfill the null hypothesis, approximately 30 would be expected to achieve a *p-value* less than 0.05 by chance due to multiple hypothesis testing. *Q* values consider false discovery rate and increase with the number of statistical tests performed. Rather than sorting by *p-value* and redefining a threshold below 0.05 to achieve more stringent statistical confidence, we have included all biochemicals that reached a *p-value* less than 0.05 in our subsequent pathway analyses since a higher *q* value does not necessarily exclude the significance of a result. We further analyzed the metabolomic data using random forest (RF), a supervised classification technique based on an ensemble of decision trees [21]. This machine-learning classification method was performed using the RF package within R to identify the most critical metabolites for separating baseline and 3-month groups.

We also performed a hierarchical clustering analysis to understand how our intervention leads to grouping the data and assessed differences amongst the groups (baseline and three months) where individuals served as their own controls. Specifically, we evaluated complete clustering using the Euclidean distance, in which each sample is a vector with all the metabolite values. Thus, the differences seen in the cluster may be unrelated to the treatment groups or study design.

An occupational threshold of 70% was applied to metabolomics data, requiring biochemicals present in at least 70% of the participants to be considered for analysis. Statistical analyses were performed using SPSS version 25 (IBM, SPSS, Inc., Armonk, NY, USA), Prism 6 (GraphPad Software, San Diego, CA, USA), R (http://cran.r-project.org), Ingenuity Pathway Analysis (IPA) platform (QIAGEN Inc., https://www.qiagenbioinformatic.com/products/ingenuity-pathway-analysis), and MetaboAnalyst 4.0 [22]. Paired-sample t-tests assessed all other data for normally distributed continuous variables and Wilcoxon signed-rank tests for abnormally distributed continuous variables to determine statistically significant differences from baseline vs. three months post-supplementation. Kolmogorov–Smirnov, and Shapiro–Wilk normality tests, together with the Grubbs's test, also known as extreme "studentized" deviate (www.graphpad.com), were used to investigate outliers and spread. Data are presented as mean ± SD. Statistical differences were considered significant at α=0.05 unless otherwise specified.

3. Results

Previous studies from our laboratory have shown that dietary omega 3 PUFAs significantly reduce pain symptoms in patients diagnosed with type 2 diabetes [9]. Experimental studies in rats from our center also demonstrated that omega 3 PUFAs treatment reduced pain after undergoing spinal cord injury [4, 7].

However, little is known about the cellular pathways involved in this effect. Interrogating the metabolome may serve to identify principal cellular pathways associated with this therapeutic efficacy in humans and provide direction towards identifying metabolites with clinical potential. This study addresses this hypothesis by characterizing the impact of omega-3 PUFA dietary supplementation on the metabolome in participants with type 2 diabetes and reporting significant pain symptoms. In addition, to ensure that changes detected in the untargeted metabolomic analysis were secondary to the dietary intervention, we monitored participants' diet, medication, and exercise habits throughout the 3-month intervention. Further, we implemented a paired analysis statistical approach measuring changes from individuals' baseline values [9], which control for extraneous sources of variation. Untargeted metabolomics allows for the detection of very subtle alterations in biochemical pathways [23].

3.1. Clinical data of "En Balance-Plus" participants pre/post-omega-3 PUFAs supplementation

Participant characteristics and clinical data were collected at baseline and after three months of DHA-enriched fish oil supplementation (Table 1). The intervention was associated with a small but significant decrease in hemoglobin A1c (HbA1c) percentage.   HbA1c percent decreased from 7.6 to 7.4 (p = 0.014). Body mass index (BMI), LDL, HDL, fasting blood glucose, and total cholesterol were unchanged during the intervention. Participants also completed food frequency questionnaires (FFQ), which allowed for estimating dietary omega-3 fatty acids consumption at baseline and three months (Table 2).   The self-reported data showed that while the participants had low plasma DHA and EPA dietary intake at baseline, there was a significant increase in consumption after three months (p=0.0001). The FFQ data were further supported with the untargeted metabolomics finding that showed a significant increase in the levels of DHA and EPA in the plasma following the three months of the study (Table 2, p ≤ 0.001).

Table 1. Characteristics of study participants and samples (n = 35)

Clinical Parameter	Baseline (BL)	3 Months (3Mo)	Difference 3Mo - BL	Significance, *P*
Age (y)	55.5 ± 11.8	-	-	-
Male %, Female %	43%, 57%	-	-	-
% Hispanic	100	-	-	-
BMI, kg/m²	29.7 ± 5.5	29.8 ± 5.7	0.1	0.706[1]
Cholesterol, mg/dL	176.3 ± 35.7	179 ± 34.3	2.7	0.294
LDL, mg/dL	117.1 ± 32.5	111.6 ± 27.9	-5.5	0.083
HDL, mg/dL	47.2 ± 13	48.2 ± 14	1	0.600
Cholesterol:HDL	3.9 ± 1.0	3.9 ± 1.1	0	0.561
Triglycerides, mg/dL	169.3 ± 83.9	163.7 ± 123	-5.6	0.347
Fasting Glucose, mg/dL	154.1 ± 71.9	142.7 ± 65.5	-11.4	0.095
HbA1c%	7.6 ± 2.3	7.4 ± 2.1	-0.2	0.014

Percentage of individuals or means ± SD of participants is shown for each variable and each time point. [1] Normally distributed.

Table 2. En Balance-Plus participants reported dietary and GC-MS relative omega-3 PUFAs levels at baseline and three months (n = 40)

	Baseline (BL)	3 Months (3Mo)	Difference 3Mo - BL	Significance, *P*
GC-MS				
DHA	.71 ± 0.4	1.85 ± 1.1	1.114	<0.001
EPA	1.03 ± 0.68	1.62 ± 1.1	0.59	0.001
Dietary Intake				
DHA, mg	57.6 ± 98.9	1035.2 ± 30.1	977.6	0.0001
EPA, mg	26.8 ± 65.5	211.7 ± 11.3	184.9	0.0001

Percentage of individuals or means ± SD of participants is shown for each variable and each time point.

3.2. Metabolomics Data Analysis

Untargeted metabolomics analysis identified 695 biochemicals of known identity. However, only a total of 106 met the occupational threshold of 70 %. In addition, the levels of 69 transformed biochemicals were significantly increased (p<0.05) at three months on matched pairs t-test while 37 were significantly decreased (p<0.05) (Table 3). Significant metabolites changes are represented by percent change $((V_2 - V_1)/|V_1|) \times 100$.

Outcomes associated with this type of intervention may be influenced by the identity of the subject and changes that occurred within each volunteer from baseline to three months. We used hierarchical cluster analysis to assess the relative magnitude of these two factors. As displayed in Figure 1, the dendrogram showed a mixture of clusters from the same time point and the same subject. Also, it appears that before and after samples from the same subject cluster together in many instances, suggesting that the intervention did not cause sweeping metabolic changes within a given individual.

Next, to understand feature importance attributed to the omega-3 intervention, we performed random forest classification using metabolite values as predictors for classifying samples at baseline or post-intervention. Classification of plasma samples collected at baseline and following fish oil intervention was 91% accurate in sorting samples when a value of 50% would be expected by random chance. Metabolite importance for group classification was expressed as a mean decrease in accuracy,

plotted in Figure 2. The top 30 metabolites contributing to group separation, as seen in Figure 2, mainly were enriched with lipid compounds followed by amino acids, nucleotides, and carbohydrate biochemicals.

After identifying principal metabolites associated with the intervention, we characterized the major metabolic pathways involved with our omega-3 supplementation. The significant pathways involved cellular oxidation, neurotoxicity, phospholipid, and acylcarnitine metabolism. We describe the metabolic maps below.

Table 3. Untargeted metabolomic compound identification and statistical comparison

|	
Total Biochemicals Identified	695
Total Biochemicals p ≤ 0.05, matched paired *t*-Test	106
Biochemicals (↑↓)	69 | 37
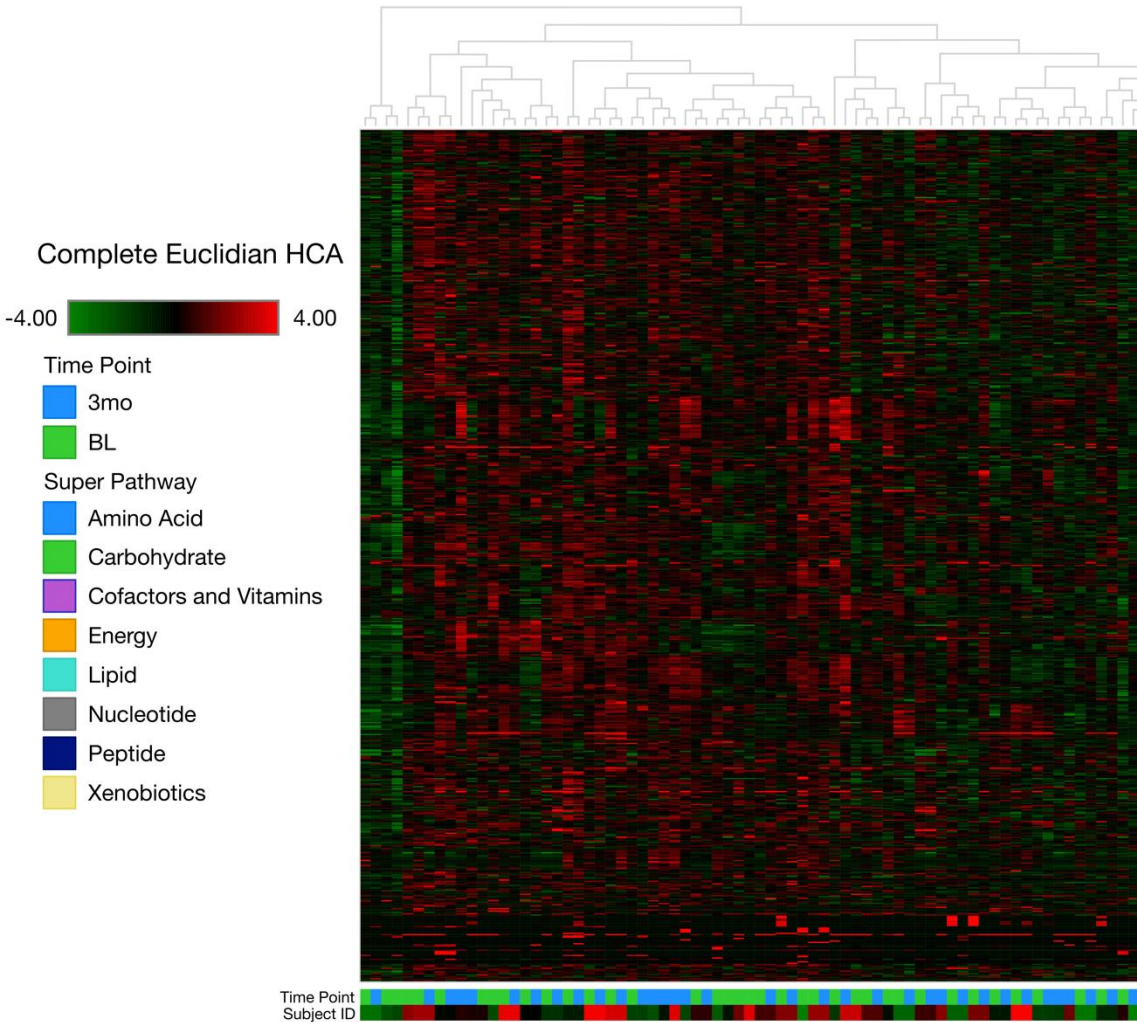
Figure 1. Dendrogram from Hierarchical Cluster Analysis. Baseline and three-month samples from the same subject clustered together in many instances. Samples from the same time point had a moderate tendency toward adjacency.
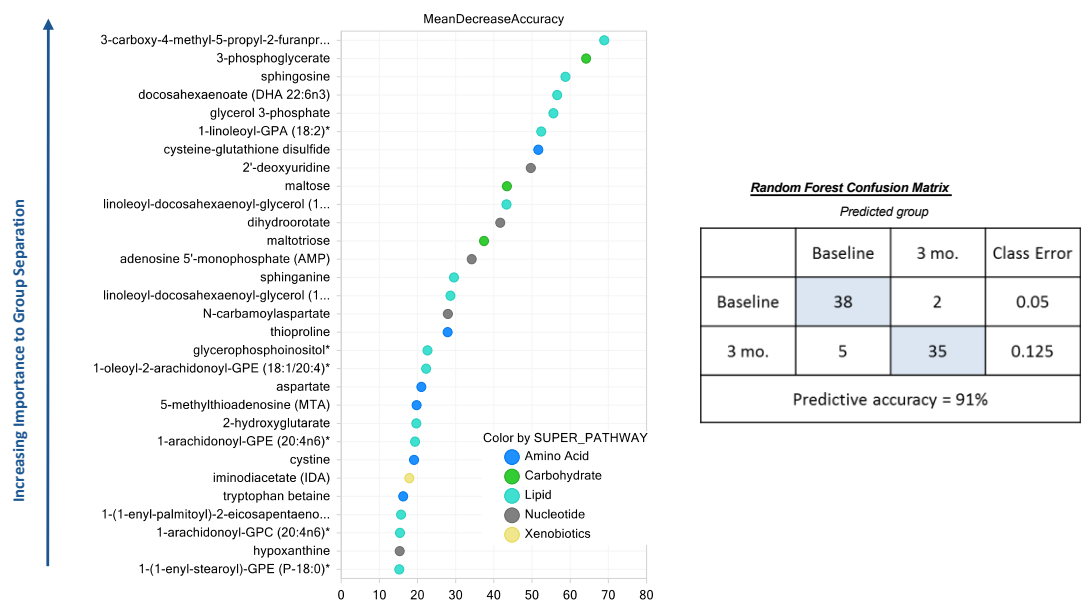
Figure 2. RF classification of plasma samples collected at baseline and 3-months after omega-3 PUFA supplementation. Classification was 91% accurate for samples when a value of 50% would be expected by random chance. Top factors contributing to group separation shown in the biochemical importance plot.

3.2.1. Omega 3 PUFAs effect on metabolites associated with the overall cellular oxidative state

Increased reactive oxygen species (ROS) and decreased antioxidant defenses have been implicated in the pathogenesis of diabetic neuropathy [24-26]. Remarkably, glutathione concentration is reduced in patients with type 2 diabetes [27, 28], exposing the sensory pain axis to biochemicals promoting neurodegeneration. Notably, our dietary intervention significantly increased several metabolites involved in the production of glutathione. Specifically, we found a dramatic increase in the relative plasma level of cystathionine (+90%), a precursor of cysteine (Table 4). Also, s-methylmethionine (+7%) and glycine (+11%) significantly increased after the dietary supplementation. Interestingly, indicators of oxidative stress, cysteine-glutathione disulfide (+156%) and cysteinylglycine (+19%), were increased at the 3-month time point. However, after dietary intervention, 2-hydroxybutyrate, usually increased in acute oxidative stress pathologies, significantly decreased by 18% [29]. Thus, it appears that the methionine-cysteine-glutathione axis functions to modulate the chronic oxidative stress given the decrease in 2-hydroxybutyrate production. Together, cysteine and methionine metabolism supported glutathione production (Figure 3). These findings align with our previous report on dietary omega-3, augmenting glutathione turnover in our spinal cord injury model [14].
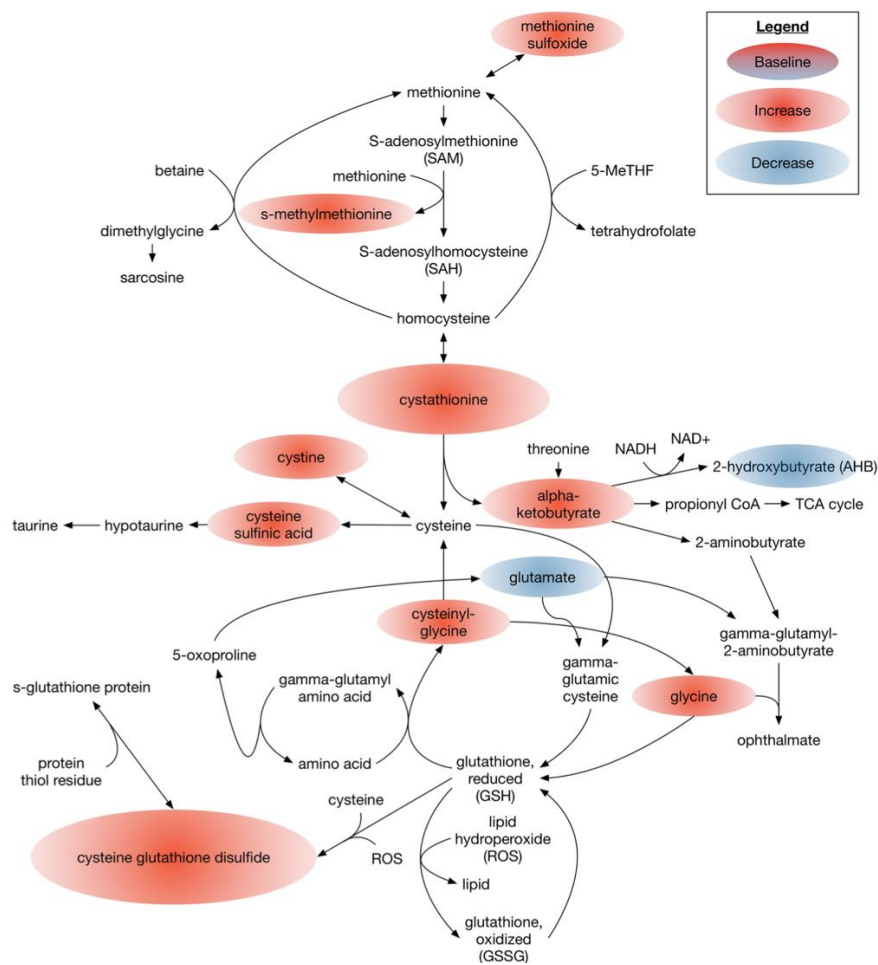
Figure 3. Changes in cysteine, methionine, and glutathione metabolism overlaid on a metabolic map. The size of each colored bubble is proportional to the fold change (increase, red; decrease, blue). The baseline bubble in the legend corresponds to a fold change of 1.

Table 4. Metabolites Related to Cellular Oxidative State

| Super Pathway	Sub Pathway	Biochemical Name	Fold Change 3Mo BL	% Change
Amino Acid	Glycine, Serine, and Threonine	glycine	1.11	+11%
	Glutamate	glutamate	0.89	-11%
		glutamine	1.07	-
	Leucine, Isoleucine, and Valine	isoleucine	1.09	-
	Methionine, Cysteine, SAM, and Taurine	methionine	1.1	-
		s-methylmethionine	1.07	+7%
		methionine sulfoxide	1.19	+19%
		cystathionine	1.9	+90%
		alpha-ketobutyrate	1.36	+36%
		2-aminobutyrate	0.94	-
		cysteine	1.01	-
		cystine	1.2	+20%
		cysteine s-sulfate	0.86	-14%
		hypotaurine	0.94	-
		taurine	0.93	-
		2-hydroxybutyrate/2-hydroxyisobutyrate	0.82	-18%
	Arginine and Proline	Arginine	1.1	+10%
	Glutathione	cysteine-glutathione disulfide	2.56	+156%
		cysteinylglycine	1.19	+19%
Carbohydrate	Fructose, Mannose, and Galactose	fructose	0.8	-
Lipid	Glycerlipid	glycerol 3-phosphate	1.78	+78%

Significant metabolite increases and decreases are highlighted in red and blue, respectively

3.2.2. Omega 3 PUFAs effects on biomarkers for neurotoxicity

It is well known that neuronal excitotoxicity is associated with increased oxidative stress and neuronal apoptosis [30]. This cellular process is at least partly mediated by sustained activation of the NMDA receptor [31]. A key neurotransmitter associated with excitotoxicity is glutamate [32]. Activation of peripheral nervous system glutamate receptors contributes to mechanical hyperalgesia in neuropathic pain animal models [32, 33]. Of significance, we found that plasma glutamate levels (-11%) decreased post dietary intervention. In contrast, the metabolomic data showed a significant increase of glycine (+11%) and arginine (+10%) in the plasma suggesting that omega-3 PUFAs trigger a rebalancing of neurotoxic amino acids (Table 4).

3.2.3. Dietary Omega-3 Supplementation regulates phospholipid profiles in plasma of patients with type 2 diabetes

We further analyzed the biochemicals involved in lipid membrane homeostasis. A hallmark of certain patients with type 2 diabetes can be an elevated plasma level of saturated (palmitic acid) free fatty acids. With this elevation the flexibility of membranes decreases, and multiple functions associated with electrical conduction and signal transduction are compromised [34]. Therefore, we were interested in the composition shift of diacylglycerols post the DHA-enriched dietary intervention. As expected, phospholipids associated with improved membrane fluidity were increased (Table 5). Specifically, a precursor for phospholipid synthesis, linoleoyl-docosahexaenoyl-glycerol (18:2/22:6) plasma level (+252%), significantly increased. Also, the dietary intervention significantly decreased 3-phosphoglycerate (3PG) and glycerate levels, -57 % and -15%, respectively (figure 4). This finding, coupled with increases in glycerol-3-phosphate (+78%), diacylglycerols, and glycerophospholipids (e.g., linolenoyl-GPC), suggests that excess glucose and free fatty acids were diverted to synthesize phospholipids (figure 4). Lastly, as predicted, the n6 fatty acid arachidonic acid plasma level decreased by 17%, and phospholipids containing arachidonate also significantly decreased post dietary intervention (see Figure 4 and Table 5).
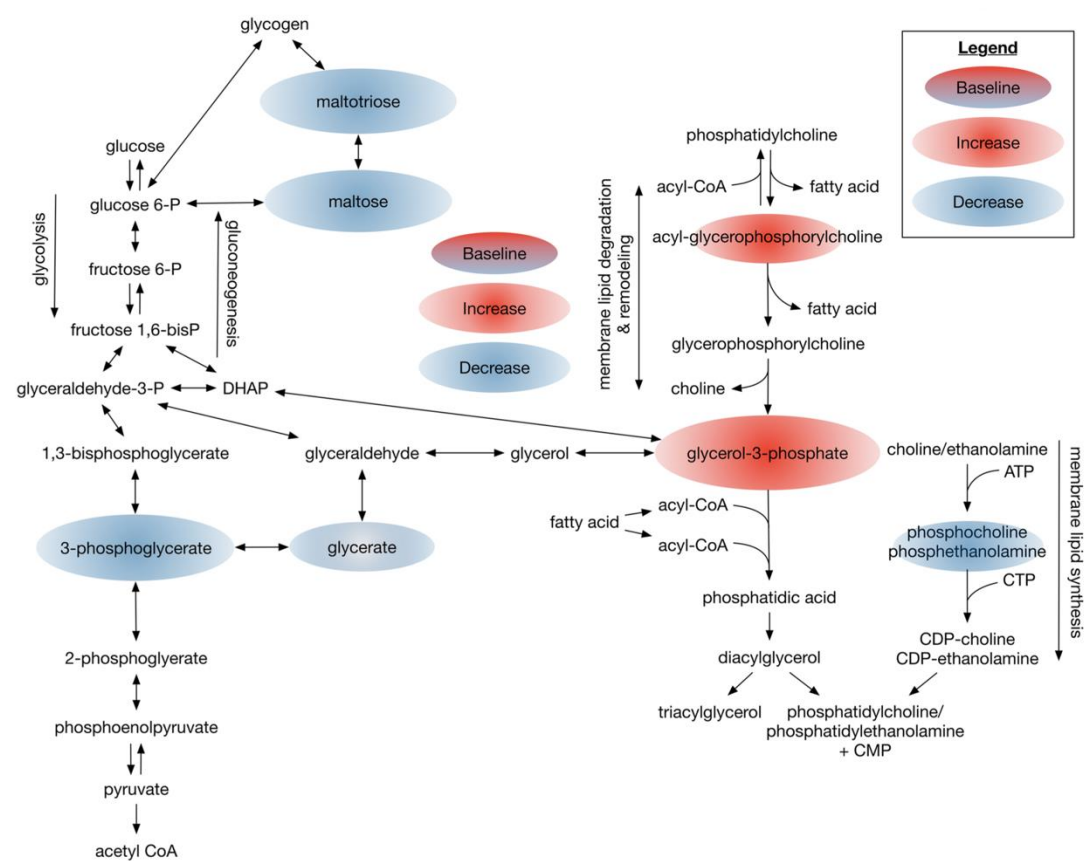
Figure 4. Glycerolipid and glucose metabolism changes overlaid on a metabolic map. The size of each colored bubble is proportional to the fold change (increase, red; decrease, blue). The baseline bubble in the legend corresponds to a fold change of 1.

Table 5. Metabolites of glucose and glycerolipid metabolism

Super Pathway	Sub Pathway	Biochemical Name	Fold Change 3Mo BL	% Change
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glucose	0.92	-
		3-phosphoglycerate	0.43	-57%
		pyruvate	1.09	-
		lactate	0.95	-
		glycerate	0.85	-15%
	Glycogen Metabolism	maltotriose	0.44	-56%
		maltose	0.52	-48%
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	eicosapentaenoate (EPA; 20:5n3)	1.57	+57%
		docosapentaenate (n3 DPA; 22:5n3)	0.97	-
		docosahexaenoate (DHA; 22:6n3)	2.62	+162%
		docosatrienoate (22:3n3)	0.67	-33%
		arachidonate (20:4n6)	0.83	-17%
		adrenate (22:4n6)	0.82	-
	Phospholipid Metabolism	phosphoethanolamine	0.81	-19%
		glycerophosphoinositol	0.5	-50%
		1,2-dipalmitoyl-GPC (16:0/16:0)	1.15	+15%
		1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	1.14	+14%
		1-stearoyl-2-oleoyl-GPC (18:0/18:1)	1.26	+26%
		1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	1.13	+13%
		1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	1.32	+32%
		1-palmitoyl-2-linoleoyl-GPI (18:2/18:3)	1.22	+22%
		1-oleoyl-2-linoleoyl-GPI (18:1/18:2)	1.42	+42%
		1-stearoyl-2-linoleoyl-GPI (18:0/18:2)	1.18	+18%
		1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	0.86	-14%
		1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	0.86	-14%
		1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	1.15	+15%
		1-palmitoyl-2-oleoyl-GPI (16:0/18:1)	1.23	+23%
		1-oleoyl-2-arachidonyl-GPE (18:1/20:4)	0.7	-30%
	Plasmalogen	1-(1-enyl-palmitoyl)-2-eicosapentaenoyl-GPE (P- 16:0/20:5)	1.88	+88%
		1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)	1.13	+13%
	Glycerolipid	glycerol 3-phosphate	1.78	+78%
		glycerophosphoglycerol	0.56	-44%
	Diacylglycerol	linoleoyl-docosahexaenoyl-glycerol (18:2/22:6) [1]	3.52	+252%
		linoleoyl-docosahexaenoyl-glycerol (18:2/22:6) [2]	4.61	+361%

Significant metabolite increases and decreases are highlighted in red and blue, respectively

3.2.4. Dietary DHA-enriched supplementation increases acylcarnitine species in participants plasma

A predictive metabolic outcome of an omega-3 rich dietary supplementation would include biochemicals involved in fatty acid oxidation. Carnitine derivatives have been demonstrated to play a pivotal role in neuroregeneration [35]. Specifically, L-carnitine and its products, acetylcarnitine (ALC) and propionyl-carnitine (PLC), are shown to have beneficial effects on neuropathic pain, degenerative axonal changes, and nerve fiber regeneration [36] in clinical studies. In our dietary intervention, we found a significant increase in several medium- and long-chain acylcarnitine species, including octanoyl carnitine (+39%), decanoylcarnitine (+37%), cis-4-decenoyl carnitine (+21%), laurylcarnitine (31%), and myristoleoylcarnitine (+27%), see figure 5. Of interest, AMP, a marker of energy stress, was significantly lower following the dietary intervention (Table 6).
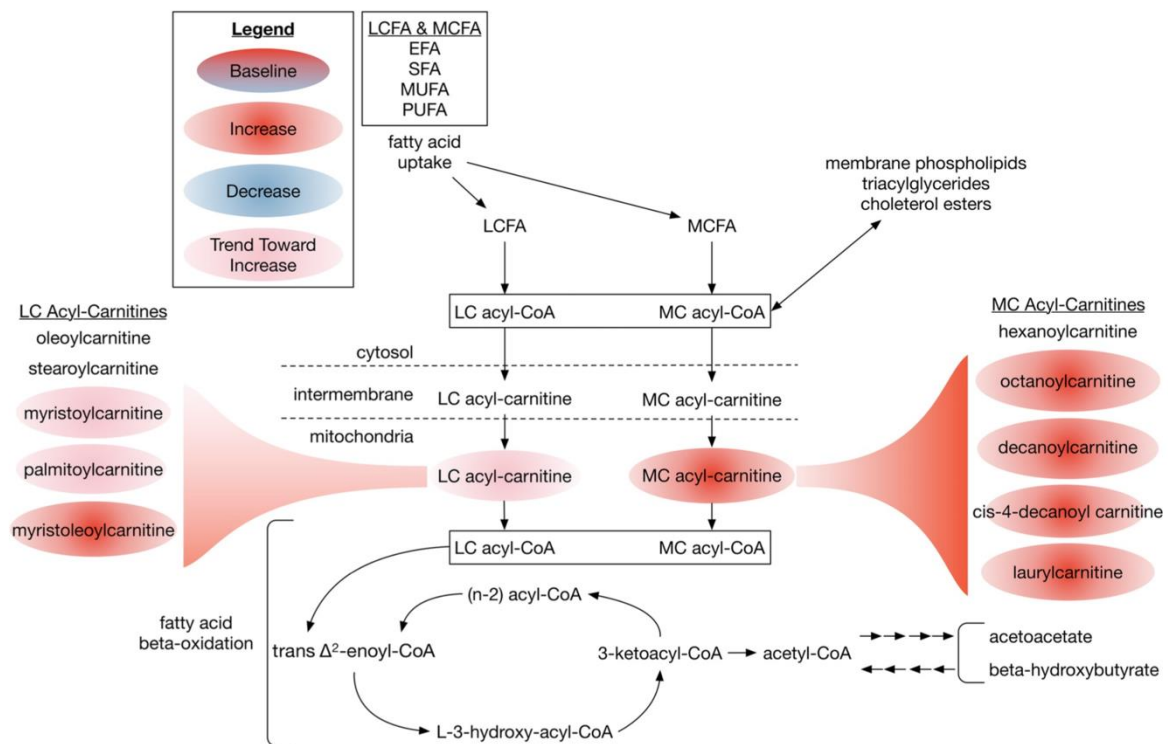
Figure 5. Changes in oxidation of fatty acids overlaid on a metabolic map. The size of each colored bubble is proportional to the fold change (increase, red; decrease, blue). The baseline bubble in the legend corresponds to a fold change of 1.

Table 6. Metabolites of fatty acid oxidation

Super Pathway	Sub Pathway	Biochemical Name	Fold Change 3Mo BL	% Change
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	octanoylcarnitine	1.39	39%
		decanoylcarnitine	1.37	37%
		cis-4-decenoyl carnitine	1.2	20%
		laurylcarnitine	1.31	31%
		myristoylcarnitine	1.17	-
		palmitoylcarnitine	1.13	-
		myristoleoylcarnitine	1.26	26%
	Ketone Bodies	acetoacetate	0.64	-
		3-hydroxybutyrate (BHBA)	0.81	-
	Fatty Acid, Monohydroxy	3-hydroxyhexanoate	1.1	-
		3-hydroxyoctanoate	1.3	-
		3-hydroxydecanoate	1.19	-
		3-hydroxylaurate	1.1	-
Nucleotide	Purine Metabolism, Adenosine Containing	adenosine 5'-monophosphate	0.58	42%

Significant metabolite increases and decreases are highlighted in red and blue, respectively

3.3. Ingenuity Pathway Analysis (IPA)

We analyzed all significantly different metabolites, which were present in at least 70 % of the participants, using the IPA platform (QIAGEN Inc., https://www.qiagenbioinformatic.com/products/ingenuity-pathway-analysis) [37] to clarify the biological significance of the experimental data. Of the 106 metabolites significantly different from baseline only 90 were recognized by the database. We obtained information regarding related bio-functions from these metabolites. The results of the IPA analysis showed significant involvement in several bio-functions, such as, free radical scavenging, cellular function and maintenance, cell signaling, and molecular transport. We categorized the top 9 enriched bio functions ($p < 5.0E-04$ and z-score > 1.5 or < -1.5) in Table 7. Of interest, formation of reactive oxygen species ($p = 4.38 \times 10^{-4}$, z-score = -1.96), peroxidation of lipids ($p = 2.24 \times 10^{-5}$, z-score = -1.944), entrance of Ca2+ ($p = 1.55 \times 10^{-4}$, z-score = -1.969), and excitation of neurons ($p = 1.07 \times 10^{-4}$, z-score = -1.091) are all suggested to be downregulated. In contrast, concentration of glutathione ($p = 3.06 \times 10^{-4}$, z-score = 1.974) bio function significantly increased.

Table 7. Top bio functions reported by IPA of significantly changed metabolites post omega-3 PUFA supplementation

Bio Function Categories	Diseases or Functions Annotation	P-Value	Activation Z-Score	Number of Biochemicals
Free Radical Scavenging	Synthesis of reactive oxygen species	7.21 E-07	-1.69	13
	Formation of reactive oxygen species	4.19E-04	-1.96	4
	Biosynthesis of hydrogen peroxide	4.95E-05	-1.95	5
Cellular Compromise, Lipid Metabolism, Small Molecule Biochemistry	Peroxidation of Lipids	2.25E-05	-1.94	6
Cell-to-Cell Signaling and Interaction, Hematological System Development and Function, Inflammatory Response	Aggregation of blood platelets	5.7E-05	-1.78	7
Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry	Uptake of D-glucose	1.19E-04	-1.72	6
Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism	Quantity of Ca2+	1.52E-04	-1.83	9
	Entrance of Ca2+	2.15E-04	-1.97	4
Drug Metabolism, Molecular Transport, Small Molecule Biochemistry	Concentration of glutathione	3.43E-04	1.97	5

Significant Bio Functions, increases and decreases are highlighted in red and blue, respectively

4. Discussion

Omega-3 PUFAs have both anti-inflammatory and antioxidant properties and supplementation has been shown to effectively ameliorates traumatic and chronic conditions such as spinal cord injury, traumatic brain injury and now painful neuropathy in DM2. [15, 38-40]. Both pre-existing pre-clinical and emerging clinical data raise the potential of using omega-3 PUFAS as a primary or complementary treatment of several human chronic and traumatic conditions. The lack of toxicity and the ready availability of omega 3- PUFAs suggest an exciting avenue for therapy conditions associate with both acute and chronic pain [11, 15, 41-43]. To date most studies with omega 3 PUFAS has been largely descriptive. Our present study defines primary underlying dietary omega-3 PUFA supplementation targets through unbiased interrogation of plasma metabolite levels. We report that omega-3 PUFA supplementation profoundly improves the neurorestorative and antioxidant metabolomic profiles of participants' plasma. This improvement was evidenced by marked and selective changes in the metabolites associated with glycerolipid, omega-3 PUFA, glucose, and cysteine/methionine/glutathione metabolism.

The pathophysiology of painful diabetic neuropathy involves a series of complex cascades that are interrelated and ultimately result in neural dysfunction [44]. Several of these processes include inflammation, cell death and dysfunction, altered peripheral blood flow, impaired spinal inhibitory function, neurotransmitter, and ionic imbalances, compromised energy metabolism, and production of free radicals [45]. Thus, our central hypothesis is that therapeutic interventions mitigating several pro-neuropathic pain processes may be required to alleviate neuropathic pain.

4.1. Overall Metabolome Indicates Targeted Metabolomic Changes

Sensitive characterization of metabolic changes associated with targeted dietary interventions is made possible with current metabolomics technology. Within the field of metabolomics, significant interest has been given to type 2 diabetes mellitus because of its extensive metabolic disturbances [46-53]. Therefore, we applied GC-MS-based metabolomics analysis to understand changes occurring during a DHA-enriched fish oil dietary intervention. We compared metabolite profiles of a Hispanic type 2 diabetes cohort with a reported low nutritional intake of omega-3 fatty acids [10] at baseline and after three months to identify changes in clinical outcomes, key metabolites, and metabolic pathways. This report is the first to use untargeted metabolic profiling to characterize changes in serum metabolites associated with omega-3 fatty acid supplementation in a population known to have low omega-3 fatty acid intake.

Given the quasi- experimental design of our intervention, we sought to determine whether differences were attributable to fish oil supplementation. The dendrogram from hierarchical cluster analysis (Figure 1) showed a moderate tendency for samples from the same time point to cluster together (baseline and three months). However, samples from the same subject taken before and after supplementation also clustered together in many instances. This clustering indicates that a subset of critical metabolic pathways contributed to group differences rather than sweeping metabolic changes.

We used Random Forest ensemble learning, a robust statistical technique for accurately classifying subjects from relatively small learning datasets to understand the most critical metabolic features for group separation between time points [54]. In Figure 5, the Random Forest classification of serum samples according to timepoint was highly accurate in classification (91%). It showed the importance of DHA, related glycerolipids, and carbohydrate metabolites in reliably differentiating between samples from before and after the intervention. Again, these results suggest dietary intervention altered targeted metabolic pathways.

Moreover, dietary DHA-enriched supplementation, as expected, did not markedly change clinical values and anthropometric measurements. Notably, fasting blood glucose, LDL, BMI, cholesterol, HDL, triglycerides, and cholesterol: HDL ratio did not significantly change. There was a small effect size on HbA1c, but the time interval and small degree of change would not be predictive of clinical benefits.   These data, taken together with no reported dietary or medication modifications by participants except for the provided DHA-enriched supplementation, suggest that changes observed in the metabolic profile can be attributed to the study's dietary intervention.

4.2. Dietary DHA-enriched supplementation leads to improved antioxidant metabolic plasma profiles of participants with type 2 diabetes

Although the measurement of plasma metabolites does not directly assess cellular metabolism, plasma metabolites are highly sensitive to changes in cellular metabolism, and models of overflow metabolism support the close relationship between intracellular and extracellular metabolic states [55, 56]. Additionally, others have demonstrated that untargeted metabolomics of plasma samples can generate significant insights regarding health status assessment and disease management [57].

While generally considered a sign of increased oxidative stress, as indicated by elevated cysteine glutathione disulfide levels in our subjects, glutathionylation may be a preferable, protective alternative to irreversible oxidation of intracellular proteins [58-61]. Interestingly, 2-hydroxybutyrate, a well-established marker of oxidative stress [62-64], was decreased in our cohort following fish oil supplementation. Moreover, while indicators of reversible oxidative stress, such as cysteine-

glutathione disulfide and cysteinylglycine, were increased post-intervention, DHA-enriched fish oil supplementation was associated with a significant reduction of 2-hydroxybutyrate (figure 5). Thus, the increased omega-3 PUFAs might have driven the increase in the methionine-cysteine-glutathione axis to address the pro-oxidative stress phenotype found at baseline [50]. Furthermore, IPA demonstrated downregulation of reactive oxygen species production and lipid peroxidation while increasing the glutathione system's activity. Together, these findings again suggest that the participants' pro-oxidative stress profile at baseline dramatically changed to a more antioxidant phenotype.

Oxidative stress is a signature of type 2 diabetes and a significant player in the pathogenesis of diabetic neuropathy. Lipotoxic stress and hyperglycemia auto-oxidation initiate extensive generation of reactive oxygen species, and lipid peroxidation overwhelming the nerve, dorsal root, and sympathetic ganglia antioxidant capacity [25, 26, 65-67].   Ultimately, increased oxidative stress leads to widespread nerve-cell dysfunction. Our previous work demonstrated that dietary DHA-enriched supplementation significantly improved volunteers' neuropathic pain scores [10]. This follow-up global metabolomic analysis shows that a dietary supplementation enriched with omega-3 PUFAs can dramatically increase the antioxidant capacity in patients with type 2 diabetes.

4.3. Dietary DHA-enriched supplementation modulates circulating excitotoxic amino acids

NMDA receptors play an important role in central sensitization in neuropathic pain [68]. Particularly, persistent over-stimulation of NMDA-type glutamate receptors leads to excitotoxic neuronal death and is essential for the long-term plastic changes in the spinal dorsal horn and the development of diabetic neuropathic pain [69-71]. Of note, preliminary evidence in animal models has shown that omega-3 fatty acids may inhibit NMDA receptor activity in the context of inflammatory pain states [72]. Interestingly, glutamate levels in the plasma increase in patients with type 2 diabetes and are elevated in chronic neuropathic pain conditions [73, 74]. Thus, our dietary intervention seems to have modulated the glutamatergic systems to a more neuroprotective state by decreasing glutamate levels. Furthermore,   given the established role of GABAergic and glycinergic dysfunction in the development of neuropathic pain [75-77], increased levels of plasma glycine and arginine support the indication that our DHA-rich supplementation improves neurorestorative pathways [78-80].   Moreover, taking what was found in the IPA, which indicated improved calcium recycling and decreased excitation of neurons (data not shown), points to the neuroprotective properties of our DHA-enriched supplementation in our type II diabetes cohort.

4.4. Dietary DHA-enriched Supplementation Changes Phospholipid Composition and Increases Acyl-carnitine Levels

In type II diabetes, increased free fatty acids lead to high cytoplasmic saturated fatty acyl-CoA, which allosterically inhibits fatty acid desaturases and reduces the synthesis of PUFA [81]. In our Mexican American population, we have previously reported dramatic low levels of dietary omega-3 and elevated levels of saturated fats [82]. In the context of neuropathic pain, this nutritional deficit can lead to neuronal dysregulation. Specifically, when cellular membranes are overly composed of saturated fatty acyl-CoA, the flexibility decreases, and multiple functions associated with electrical conduction and signal transduction may become dysregulated [34]. Our untargeted metabolomic analysis found a dramatic shift in phospholipid metabolism, suggesting increased PUFA incorporation into cellular membranes. Interestingly, palmitate levels did not change during the intervention. Therefore, it appears that merely increasing the levels of omega-3 PUFAs can restore membrane homeostasis without altering levels of saturated fatty acids.

In type II diabetes, there is typically an oversupply of lipids resulting in the accumulation of bioactive lipid metabolites; it has been proposed that Schwann cells (SC) may shift the stoichiometry of their lipid production in pDN, increasing toxic ceramide production [66]. This proposed overproduction of ceramide is mediated by diabetic SC undergoing loss of oxidative phosphorylation and ATP production due to excess acyl-CoAs cells undergoing metabolic reprogramming, leading to an accumulation of acylcarnitines [66]. SC transport of these acylcarnitines is toxic to dorsal root

ganglion neurons [83], accounting for the development of pDN [66]. Interestingly, our untargeted metabolomics analysis showed an increase of medium and long-acylcarnitines, but with associated decreases in lipid peroxidation and formation of reactive oxygen species. Furthermore, we observed a significant reduction in plasma sphingosine levels, a precursor of ceramide synthesis, suggesting a decrease in ceramide production. DHA treatment in a neuronal culture model has been previously reported to inhibit palmitic acid-induced mitochondrial membrane depolarization altogether [13]. Dietary omega-3 supplementation appears to correct the proposed metabolic reprogramming associated with pDN, leading to increased phospholipid biosynthesis and improved mitochondrial bioenergetics, supported by a decreased AMP.

Lastly, our previous studies showed that dietary omega-3 PUFAs dietary supplementation attenuated the development of thermal hyperalgesia following SCI and improved neuropathic pain scores in participants with type II diabetes [7, 10]. In agreement with data presented in this study, our previous metabolomic profiling and IPA identification of metabolite biological function of SCI tissue demonstrated a robust increase in glutathione concentration, improved calcium regulation, and decreased neuronal excitation [14]. Together, these untargeted metabolomics data sets, animal and human bio-samples, are highly suggestive that dietary DHA supplementation can directly impact neurorestorative and antioxidant pathways in chronic inflammatory disease states.

4.5. Potential uses of omega-3 intervention beyond pain

Of interest, Latinos appear to have low omega-3 PUFA intake within their diets and they suffer from other increased microvascular complications including nephropathy and retinopathy. Our supplementation study suggests that this 'nutritional deficit" may promote increasing incidence of microvascular complications and that a nutritional approach to increasing omega-3 PUFA intake could be a non-medication approach to decrease adverse outcomes.

Another significant comorbidity of type 2 diabetes is increased susceptibility and poor outcomes to respiratory viral infections [84, 85] such as influenza. Of current concern, patients with type II diabetes infected with SARS-CoV-2 and who develop COVID-19 have a worse prognosis than nondiabetics [86, 87]. Evidence suggests that an at-risk population may benefit from an anti-inflammatory diet [88]. One component of an anti-inflammatory diet includes omega-3 fatty acids. Given our current findings that our DHA-rich supplementation improved the antioxidant metabolomic profile of our participants and the known inflammatory storm caused by COVID-19 [87], adding omega-3 PUFAs to patients' diets with type 2 diabetes may serve to mitigate the effects of COVID-19.

5. Conclusions

We demonstrated that dietary DHA-enriched supplementation has a wide-ranging impact on the antioxidant metabolomic profile of our participants. Overall, this study shows that untargeted metabolomics is sensitive for defining targeted alteration in metabolism secondary to a dietary intervention. Specifically, we found that a dietary DHA-enriched supplementation improved metabolic profiles regarding omega-3 PUFA metabolism, glycerolipid metabolism, cysteine, methionine, glutathione metabolism, and glucose and fatty acid homeostasis without changing clinical parameters. Furthermore, circulating plasma metabolites showed a significant shift toward decreased reactive oxygen species biosynthesis, lipid peroxidation, improved Ca^{2+} homeostasis, and increased glutathione activity. These changes may explain previously reported reduction in neuropathic pain symptoms in our En Balance plus cohort [10]. Since omega-3 FA supplementation is safe in patients with type 2 diabetes, future studies are warranted to define further its role in the possible improvement of pDN symptoms [89].

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, A.D., A.F., M.D.; methodology, W.B, A.F., Z.C., A.D., M.D.; software, A.D.; validation, A.D., and M.D.; formal analysis, A.D.; investigation, A.D., and M.D.; resources, Z.C., and M.D.; data curation, A.D.; writing—original draft preparation, A.D., and M.D; writing—review and editing, A.D., W.B., A.F., and M.D.; visualization, A.D.;

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