Circulating levels of endocannabinoids and oxylipins altered by dietary lipids in older women are likely associated with previously identified gene targets

Bruce A. Watkins[1,2], Jeffrey Kim[3], Anne Kenny[2], Theresa L. Pedersen[4], Kirk L. Pappan[5], and John W. Newman[1,4]

[1]Department of Nutrition, University of California, Davis, Davis, CA (Watkins, baw@purdue.edu)

[3]Genome and biomedical sciences facility, University of California, Davis, Davis, CA (Kim jefkim@ucdavis.edu)

[2]Center on Aging, University of Connecticut Health Center, Farmington, CT, (Kenny kenny@uchc.edu)

[4]USDA, ARS, WHNRC, Davis, CA, (Newman John.Newman@ARS.USDA.GOV, Pedersen Theresa.Pedersen@ARS.USDA.GOV)

[5]Metabolon, Inc., Durham, NC (Pappan KPappan@metabolon.com)

Running title: Fish oil alters endocannabinoids and oxylipins in PMW

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Corresponding author:
Bruce A. Watkins, Ph.D.
Department of Nutrition
University of California, Davis
Davis, California 95616-5270
Purdue University Emeritus Professor
baw@purdue.edu Bruce A. Watkins, Professor

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Abbreviations:

AEA - arachidonoyl ethanolamide, i.e. anandamide

2-AG - 2-arachidonoylglycerol

DEA - docosatetraenoyl ethanolamide

DHA - docosahexaenoic acid

DHEA - docosahexaenoyl ethanolamide

DGLEA - dihomo-gamma-linolenoyl ethanolamide

EC - Endocannabinoids

ECS - endocannabinoid system

EPA - eicosapentaenoic acid

FAAH - fatty acid amide hydrolase

FAME - fatty acid methyl esters

LEA - linoleoyl ethanolamide

αLEA - alpha-linolenoyl ethanolamide

LTB_4 - Leukotriene B_4

NA-Gly - N-arachidonyl glycine

OEA - oleoyl ethanolamide

OL - Oxylipins

PEA - palmitoyl ethanolamide

PGE_2 - prostaglandin E_2

PMW - Post-menopausal women

PUFA - polyunsaturated fatty acids

SEA - stearoyl ethanolamide

5-HEPE - 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid

5,6-DiHETrE - 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid

8(9)-EpETrE - 8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid

8,9-DiHETrE - 8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid

11(12)-EpETrE - 11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid

11,12-DiHETrE -11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid

12-HEPE - 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid

12-HETE - 12-hydroxy-5E,8Z,10Z,14Z-eicosatetraenoic acid

14(15)-EpETrE - 14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid

14,15-DiHETE - 14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid

14,15-DiHETrE - 14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid

15-HEPE - 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid

16(17)-EpDPE - 16(17)-epoxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid

17-HDoHE - 17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid

17(18)-EpETE - 17(18)-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid

17,18-DiHETE - 17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid

19,20-DiHDPA - 19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid

ABSTRACT

Postmenopausal women (PMW) report marginal n-3 PUFA intakes and are at risk of chronic diseases associated with the skeletal, muscular, neuroendocrine, and cardiovascular systems. How n-3 PUFA affect the amounts of endocannabinoids (EC) and oxylipins (OL) of metabolic and physiologic importance in PMW is not clear. Based on our recent findings that dietary n-3 PUFA alter gene targets of the EC system and lower pro-inflammatory OL we proceeded to characterize these actions in blood of PMW. Our aim was to determine levels of the EC, OL, and global metabolites (GM) in white PMW (75 ± 7 y), randomized in a double-masked manner, from baseline to 6 mo after receiving a fish oil supplement of n-3 PUFA (720 mg 20:5n3 + 480 mg 22:6n3/d, n=20) or placebo (1.8g oleic acid/d, n=20). EC and OL in serum were determined by UPLC-MS/MS and GM by GC-MS and LC-MS/MS. Plasma 20:5n3 and 22:6n3 levels increased in PMW given fish oil. EC n-6 acyl-ethanolamides, arachidonate-derived diols were decreased and 20:5n3 and 22:6n3 diols, epoxides, and alcohols were increased in PMW given fish oil. GM analysis revealed that n-3 PUFA supplementation increased renal steroid hormone and proteolytic metabolite levels in PMW. Herein, we confirm that gene targets of the EC system, previously found as modifiable by n-3 PUFA result in changes in the levels of EC and OL in PMW. This study shows phenotypic responses (in levels) to n-3 PUFA supplementation in PMW and increases of n-3 acyl-ethanolamide and n-3-derived OL of clinical considerations in aging.

Keywords: postmenopausal women, endocannabinoids, oxylipins, global metabolites

1. Introduction

Postmenopausal women (PMW) are at a risk of excessive bone loss, hypertension, and metabolic syndrome. Moreover, while n-3 polyunsaturated fatty acids (PUFA) status generally increases with age [1], PMW typically have marginal intakes of n-3 PUFA. Stark et al. [2] reported that short-term supplementation with n-3 PUFA (20:5n3 EPA and 22:6n3 DHA) in PMW resulted in similarly higher EPA and DHA levels and lower linoleic acid levels that correspond to Inuit women but not the markedly lower level of arachidonic acid (AA) seen in these seal eating populations. A recent investigation revealed that aerobic exercise and n-3 PUFA supplementation with fish oil had a synergistic action on reducing inflammation and improving bone mineral density associated with osteoporosis in PMW [3].

While the specific mechanisms for the observed health improvements of n-3 PUFA are still being explored, hypotriglyceridemia [4], improved ratios of LDL/HDL [5], and reductions in coronary artery disease progression [6] and atherosclerosis incidence in PMW [7] are well documented. As n-3 PUFA are precursors for many biologically active metabolites, including endocannabinoids (EC) and oxylipins (OL), a key to understanding their health promoting properties is to identify how dietary n-3 PUFA influence the production of these metabolites.

Although the role of the endocannabinoid system (ECS) has yet to be characterized in PMW, this system affects food intake and energy metabolism in peripheral tissues, and ECS overstimulation contributes to obesity and loss of insulin sensitivity in muscle. Consequences of elevated AA-derived EC, include overstimulating of the ECS [8] leading to impaired glucose uptake in skeletal muscle [9], stimulating osteoclast proliferation [10], and generating pro-inflammatory cyclooxygenase (COX)-derived prostanoids [11]. Thus, the impact of dietary PUFA on physiology likely include mechanisms associated with alterations in lipid mediator

tone. Recently, our laboratory reported that n-3 PUFA alter expression of several genes associated with the ECS in myoblasts [12] and the mouse [13] to influence glucose uptake and fat accretion, respectively. These observation are a reason to investigate n-3 PUFA effects on EC and OL in PMW.

The OL are oxygenated fatty acids that are important regulators of physiology and health and include metabolites of all PUFA, whose balance can be altered by dietary lipid content [14]. The 20-carbon "eicosanoids" are OL derived from AA or EPA metabolized by a suite of enzymes including various cyclooxygenases, lipoygenases, and cytochrome P450 (CYPs) hydroxylase and epoxygenases. The COX- and LOX-derived OL consist of prostanoids, thromboids, and leukotrienes, and mid-chain alcohols including hydroxyeicosatetraenoic acids [15], while the CYPs yield the vasoactive and immunomodulatory omega-hydroxy and -epoxy fatty acids [16]. Several other OL are derived as secondary products of these metabolites, further adding to the potential fates of PUFA. Pertinent to PMW, prostaglandin E_2 (PGE_2) has been found to be a potent stimulator of bone resorption and regarded as the primary prostaglandin affecting bone metabolism [17]. Leukotriene B_4 (LTB_4) has also been found to increase bone resorption and affect osteoclast cell division. PGE_2 and LTB_4 are both AA metabolites which can be altered by dietary n-6/n-3 PUFA balance [18], further elaborating the influence of dietary PUFA on biological processes where OL are involved. A well-accepted effect in response to n-3 PUFA supplementation is the enrichment of membrane phospholipids with the long-chain n-3 PUFA, EPA and DHA. Given the relationship between dietary and tissue PUFA and the potential effects on circulating EC and OL levels, our research aim was to determine how dietary PUFA alter plasma levels of these metabolites in the elderly. However, little is known about the EC and OL serum levels in older adults and PMW. Therefore, the primary hypothesis for this

research is that dietary PUFA determine blood and tissue concentrations of AA, while n-3 PUFA EPA and DHA decrease AA to alter the types of EC and OL in blood. In the current study, postmenopausal subjects were given an n-3 PUFA supplement or placebo for 6 mo after which serum and plasma was collected to measure EC, OL, and PUFA, respectively. We also examined the effect of the n-3 PUFA supplement on global metabolites (GM) influenced by changes in EC and systemic macronutrient metabolism [8, 9]. The justification for this investigation with EPA and DHA is to determine the effects on the EC in PMW prior to studies on genes (cannabinoid receptors CB1 and CB2, GLUT 1, and Insulin-R) related to these compounds which was shown in myoblast cultures and mice (protein expression of CB1, CB2, GLUT 4 and insulin-R) [12, 13].

2. Materials and methods

2.1 Subjects, dietary supplements, and blood samples

Women with a mean age of 75 y (Table 1) were randomized in a double-masked manner to receive either 1.2 g EPA+DHA from fish oil (n=20; 2 - 1g capsules/d, 360 mg EPA and 240 mg DHA/ capsule; Vital Nutrients, Middletown, CT) or olive oil placebo (n=20; 2 capsules/d, 1.8g oleic acid/day, Vital Nutrients). Blood samples (serum and plasma) were collected at baseline and 6 mo into the intervention period, kept on ice during collection and stored at -80°C, with sub-aliquots designated for the analysis of total lipid fatty acids (plasma; Watkins, University of Connecticut, Farmington, CT), EC and OL (serum; USDA-ARS-WHNRC, Davis, CA), and global metabolomics (serum; Metabolon, Inc., Durham, NC). The subjects had a BMI that ranged from 26-27, were not diabetic, and non-smokers. This study was conducted according to the Declaration of Helsinki guidelines and all procedures involving human subjects

were approved by the Institutional Review Board at the University of Connecticut Health Center (ClinicalTrials.gov Identifier: NCT00634686). Written informed consent was obtained from all subjects. Blood was obtained from PMW at baseline and after 6 mo of treatment. Plasma was isolated from blood collected in heparinzed-tubes, and serum was isolated from blood collected in a sterile empty tube that was allowed to clot.

At the baseline visit a health history questionnaire was completed and height (cm) and weight (kg) for each participant were measured to calculate body mass index (BMI, kg/m2). Three-day diet records were recorded by participants at baseline to determine nutrient intake. Records were reviewed with the study dietitian and analyzed using Nutritionist Pro (ESHA version 10.1).

2.2 Fatty acid analyses

Human plasma samples (40 samples, 20 at baseline and 20 at 6 mo of placebo or n-3 PUFA supplementation) were processed to determine fatty acid composition. Briefly, 100 µl of plasma was extracted with chloroform/methanol (2:1, vol/vol). The resulting extracted lipids were treated with 0.5 N NaOH in methanol, and fatty acid methyl esters (FAME) prepared by esterification using boron trifluoride (BF_3) in methanol (10% w/w, Supelco Inc. Bellefonte, PA). The FAME were concentrated in isooctane (HPLC grade, Fisher Scientific, Pittsburg, PA) and analyzed by gas chromatography (GC) (HP 7890A series, autosampler 7693, GC ChemStation Rev.B.04.03, Agilent Technologies, Palo Alto, CA) with a DB-225 column (30 m, 0.25 mm i.d., 0.15 mm film thickness, Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector [19]. Sample peaks were identified by comparison to authentic FAME standards (Nu-

Chek-Prep Inc., Elysian, MN). Results of FAME analysis were obtained by weight percentage reports.

2.3 Endocannabinoid (EC) and oxylipin (OL) analyses

Measurements of EC and OL were performed on 40 serum samples, 20 at baseline and 20 at 6 mo of n-3 PUFA supplementation, using 250 µL of serum as previously described [20, 21]. Briefly, 60mg Oasis-HLB solid phase extraction columns (SPE) (Waters Corp., Milford, MA) were placed on a vacuum manifold, cleaned and conditioned, then spiked with deuterated EC and OL internal standards. The serum samples were thawed on ice and a 200 uL volume was transferred to the SPE, up-diluted to 3 mL with 5% MeOH/0.1% acetic acid, and gravity extracted. Columns were then washed with 3 mL the 5% MeOH solution. OL and EC were eluted from the SPE with 0.2 mL MeOH followed by 1.5 mL ethyl acetate, by gravity. Solvent was removed by vacuum and samples reconstituted in 50 µL MeOH containing the internal standard 1-cyclohexyl-3-dodecyl-urea (Sigma, Aldrich, St. Louis, MO) then, filtered at 0.1 µm. Analytes were chromatographically separated on a 2.1 × 150 mm, 1.7µm Acquity BEH C18 (Waters Corp.) column using 0.1% acetic acid and acetonitrile gradient (Table S1). The EC and OL profiles were acquired using electrospray ionization and tandem mass spectrometry by back-to-back (+)-mode/(-)-mode injections on a Sciex API 4000-QTRAP (Pleasanton, CA). A complete list of measured analytes with retention times, acquisition parameters, and detection limits are shown for the ECs and OLs in Tables S2 and S3, respectively.

2.4 Metabolite analyses

Non-targeted global metabolite profiling (GM) was conducted on 80 serum samples (all at baseline and at 6 mo, for placebo and n-3 PUFA supplemented groups, 20 subjects per group) using three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (LC/MS) optimized for basic species, LC/MS optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS) as described previously [22, 23]. Briefly, samples were extracted with methanol containing four recovery standards, aliquots dried under nitrogen and vacuum-desiccated, and reconstituted in platform-specific solvents for LC/MS or, for GC/MS, derivatized. LC/MS samples were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) consisting of an electrospray ionization source and linear ion-trap mass analyzer. Derivatized samples for GC/MS were separated on a 5% diphenyl/ 95% dimethyl polysiloxane fused silica column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) [24]. Metabolites were identified by automated matching to chemical reference library standards on the basis of retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated using software developed at Metabolon [25]. Details of this procedure and compounds identified are provided in an Excel file of supplemental materials.

2.5 Statistical analyses

Data for plasma fatty acids and those for serum EC, OL, and metabolites were expressed as the means and standard deviations. Variables for EC and OL were transformed to normal by an iterative process using imDEV v1.4 [20]. Group differences for fatty acids were evaluated by

paired t-test (SAS version 9.3; SAS Institute, Inc., Cary, NC, USA) and values for EC and OL were evaluated by 2-tailed t-test all at α 0.05. Repeated measures two-way ANOVA with post-test contrasts was used to identify GM-identified metabolites that differed significantly between experimental groups in serum, following log transformation and if necessary, imputation of missing values with the minimum observed value for each compound. A summary of the numbers of biochemicals that achieved statistical significance ($p \leq 0.05$), as well as those approaching significance ($0.05 < p < 0.1$) was reported. To account for multiple testing, the false discovery rate was computed and reported for each comparison using the q-value method [26].

Global metabolomics, fatty acid, endocannabinoid and oxylipin data were combined and analyzed using partial-least squares discriminate analysis in the R Statistical computing environment [27]. Fatty acid, endocannabinoid and oxylipin data were transformed to normality using probabilistic principal components analysis in imDEV v 1.4.2 [28]. To improve clustering performance the reduced data matrix with 473 variables (complete data set) was subjected to orthogonal signal correction using the procedures of Wehrens [29] and partial least squares discriminate analysis of Variable Importance Plot (VIP) filtered data set (n=109 variables) using the oscorespls component of the R 'pls' package [30]. Four iterative rotations provided for uniform projection of discriminate variables onto a single latent variable.

3. **Results**

3.1 Plasma fatty acids

At baseline there were no differences in plasma fatty acid levels between the placebo and n-3 PUFA groups. Supplementation with n-3 PUFA to PMW for 6 mo resulted in higher 20/22-carbon n-3 PUFA, and lower 22 carbon n-6 PUFA in plasma (Table 2). A notable change was in

higher plasma EPA, DHA, total n-3 PUFA and ratio of DHA/AA, but lower ratio of n-6/n-3 PUFA and LC n-6/n-3 PUFA in the PMW group after 6 mo of n-3 PUFA supplementation (Table 2). When compared between the placebo and n-3 PUFA supplementation groups, the increased EPA and DHA levels at 6 mo showed a reciprocal decrease in the level of 22-carbon n-6 PUFA. Other fatty acid differences include higher 17:0 and 22:5n3, as well as lower 18:1n9, 18:3n6, 22:4n6 and 22:5n6 in the n-3 PUFA supplement group compared to the placebo group at 6 mo.

The levels of EPA, DHA, 22:5n3, total PUFA, total n-3 PUFA, and ratio of DHA/AA were higher after 6 mo in PMW supplemented with n-3 PUFA compared to their baseline levels. Moreover, 20:2n6, 22:4n6 and 22:5n6 levels were lower after 6 mo of supplementation with n-3 PUFA compared to the baseline. In addition, 16:1n7, 18:1n9, and total monounsaturated fatty acids levels were lower at 6 mo compared to the baseline in the n-3 PUFA group. No changes in PUFA levels were observed in the placebo group when compared between the baseline and after 6 mo (Table 2). Despite increases in the n-3 PUFA, neither the total n-6 PUFA nor total PUFA were affected by the n-3 PUFA supplementation at 6 mo (placebo vs. control).

Based on food records, subjects did not change their dietary intake over the 6 mo of the study. A 90% compliance was reported by pill count for the subjects. The fatty acid analysis of plasma for those supplemented with n-3 PUFA was confirmed by the change in n-3 PUFA and the ratio of DHA/AA (Table 2).

3.2 Serum levels of EC and OL

After 6 mo of n-3 PUFA supplementation, levels of some n-6 ethanolamides (EA) such N-arachidonoyl glycine (NA-Gly) and docosatetraenoyl ethanolamide (DEA) but not

arachidonoyl ethanolamide (AEA) were reduced, while those for n-3 acyl-ethanolamides (e.g. DHEA) were increased in serum compared to baseline (Table 3 and Figure 1). While n-3 PUFA supplementation did not affect EAs containing palmitic, stearic, oleic, linoleic and α-linoleic acids (PEA, SEA, OEA, LEA, and αLEA) (Table 3) serum concentration of those derived from DHA (i.e. DHEA) increased (Table 3 and Figure 1).

The OL levels in PMW at baseline and after supplementation are presented in Table 4 and generally show that intake of n-3 PUFA reduces non-esterified AA-derived dihydroxyeicosatrienoic acids (DiHETrEs) but increases n-3 PUFA-derived OL in this pool. Notably, other AA-derived OLs were not significantly altered by DHA supplementation-induced changes has been observed in other studies of healthy humans [31].

The 14,15-DiHETrE, 11,12-DiHETrE, and 8,9-DiHETrE, (CYP450/soluble epoxide hydrolase metabolites) were lower in the serum of PMW supplemented with n-3 PUFA at 6 mo compared to their baseline levels (Table 4). However, after n-3 PUFA supplementation the levels of EPA and DHA diols, epoxides and alcohols (14,15-DiHETE, 17,18-DiHETE, 17(18)-EpETE, 5-HEPE, and 19,20-DiHDoPE) were higher in PMW (Table 4).

Shown in Figure 2 is the partial least squares discrimination analysis (PLS-DA) using serum EC and OL data for PMW at baseline and after 6 mo of n-3 PUFA supplementation. The arrows illustrate the subject (n = 20) change from baseline (blue) to after supplementation (green) in this multivariate space. This analysis clearly indicates that the magnitude of n-3 PUFA-induced changes were variable among subjects, and not uniformly a function of basal status. Decreases in the arachidonate-derived diols, acyl-EAs, and acyl-glycerols, and increases in DHA-derived epoxides, diols, and acyl-EAs, and EPA-derived alcohols and diols, were the primary lipid mediator discriminants of n-3 PUFA supplementation (data not shown).

301

302 3.3 Global metabolite (GM) analysis

303 GM profiling of serum from PMW at baseline and after supplementation in the placebo
304 and n-3 PUFA groups revealed a total of 394 compounds of known identity (named
305 biochemicals). The number showing significance ($p \leq 0.05$) for the major effects were as
306 follows: 26 for supplement, 21 for time and 37 for supplement X time interaction. The
307 differences are presented with respect to shared metabolism or specific metabolic pathways.

308

309 3.31 Levels of lysoglycerolipids containing DHA

310 Supplementation with n-3 PUFA (EPA and DHA) to PMW resulted in altered membrane
311 lysolipids in the serum (Table 5). The levels of 1-oleoyl-2-hydroxy-sn-glecero-3-
312 phosphoethanolamine (1-oleoyl-LysoPE), 2-oleoyl-LysoPE and 1-arachidonoyl-2-hydroxy-sn-
313 glecero-3-phosphoinositol (LysoPI) were all lower in the n-3 PUFA group compared to the
314 placebo group after 6 mo of treatment. While 2-docosahexaenoyl-LysoPE, 1-docosahexaenoyl-
315 2-hydroxy-sn-glecero-3-phosphocholine (LysoPC) and 2-docosahexaenoyl-LysoPC levels were
316 higher in the n-3 PUFA group compared to the placebo group after 6 mo. When compared
317 within the n-3 PUFA group, the levels of 1-oleoyl-LysoPE, 2-oleoyl-LysoPE and 1-
318 arachidonoyl-LysoPI were lower at 6 mo compared to baseline. In contrast the levels of 2-
319 docosahexaenoyl-LysoPE, 1-docosapentaenoyl-LysoPC and 1-docosahexaenoyl-LysoPC were
320 higher at 6 mo compared to the baseline. No changes in the levels of serum lyso-phospholipids
321 were found in PMW from baseline to 6 mo of placebo treatment (Table 5).

322

323 3.32 Sterol and steroid hormone profiles

Serum adrenal sterol levels were altered in both the placebo and n-3 PUFA supplemental groups from baseline to 6 mo of treatment (Table 6). At baseline, levels of the phytosterol beta-sitosterol and the steroid hormone cortisone were higher in the placebo group than in the n-3 PUFA group. This difference disappeared after 6 mo of n-3 PUFA supplementation. In the placebo group, pregnenolone sulfate was lower at 6 mo compared to the baseline level. In the n-3 PUFA supplemental group, 21-hydroxypregnenolone disulfate was higher from baseline to 6 mo, along with others such as 5α-pregnan-3beta,20-α-diol disulfate and androsterone sulfate. 5α-pregnan-3ß,20-α-diol disulfate was also higher in the n-3 PUFA group compared to the placebo group at 6 mo.

3.33 Amino acids and amino acid catabolites

Free modified amino acids in serum were altered in the n-3 PUFA supplemented PMW after 6 mo of treatment (Table 7). After 6 mo those supplemented with the EPA + DHA showed higher levels of free modified amino acids (Table 7). At baseline, indolepropionate was higher in the n-3 PUFA treatment group compared to the placebo group. The levels of indolepropionate did not change over time with each group. After 6 mo the levels of N-acetylglycine, N-acetylmethionine, N-acetylserine, C-glycosyltryptophan, N-methyl proline and cysteine were all higher compared to the baseline levels in the n-3 PUFA group. N-formylmethionine was lower in serum of PMW supplemented with n-3 PUFA at 6 mo compared to baseline.

3.4 Partial least squares discriminate analysis of global metabolomics, fatty acid, endocannabinoid and oxylipin data

Figure 3 presents scores plot showing group discrimination after orthogonal signal correction (baseline yellow and post-intervention in green). The data from the analysis was then used to split variables into 2 classes, those that increased over the period of intervention and those that decreased over the same period of time presented in Figure 4, and these two groups of variables were subjected to a hierarchical cluster analysis. Partial least squares (Figure 5) discriminate analysis of Variable Importance Plot (VIP) filtered data set (n=109 variables) using the oscorespls component of the R 'pls' package [30]. Data was auto scaled prior to analysis. Symbols are provided for variables with VIP scores > 1.2 – 3.4 from the initial complete data set analysis and are scaled according to these scores. A hierarchical cluster analysis using the hclust function from the base R package, 'stats', with Minkowski distances and the Ward agglomerative algorithm used to group analytes, and labels correspond to cluster labels on the PLS Loadings in the figure (Figure 4). Eight independent clusters (C1-C8) were produced, with the following characteristics of increased or decreased with n-3 PUFA treatment (Figures 5 A and B). The clusters of variables which increased with treatment (Figure 5A) were as follows: C1 n-3 PUFA, n-3 vicinal diols, and DHA-ethanolamide; C2 n-3 PUFA phospholipids; saturated fatty acid phospholipids, beta-sitosterol, campesterol; C3 n-3 alcohols, n-3 epoxides and n-6 alcohols (linoleates); C4 progesterone metabolism, amino acid metabolism, heme and heme metabolism, global metabolites. The clusters of variables which decreased with treatment (Figure 5B) were as follows: C5 n-6 PUFA, 18:1n7, n-6 vicinal diols, and n-6 ethanolamides; C6 gamma linoleic, palmitoleate, dihomo-γ-linolenic acid, phosphoinositides, monoacylglycerides, (2-AG, 1-OG, 2-OG), succinate, pyridoxate, and N-formyl methionine; C7 salicyclate and its metabolites; C8 n-6 phosphoethanoamines, di-leucine, hipurate, and quinate.

4. Discussion

Supplementation of n-3 PUFA (fish oil) to PMW led to their increase in the plasma as well as a change in the composition of the serum glycerolipids. These changes also resulted in a shift in the serum phospholipid pool, resulting in lower AA- but higher DHA-containing glycerolipids as reported in 2013 [32]. Specifically, 1-arachidonoylglycerophosphoinositol was lower while the DHA-derived 1-docosahexaenoylglycerophosphocholine, 2- docosahexaenoyl-glycerophosphocholine and 2-docosahexaenoylglycerophosphoethanolamine were higher after fish oil supplementation to PMW compared to baseline. PMW given the fish oil supplement had higher levels of 20:5n3 and 22:6n3 in plasma compared to baseline as well as the placebo group at 6 mo. Although the level of 20:4n6 did not differ between the groups after 6 mo of supplementation, the overall ratio of DHA/AA was higher and the ratio of n-6/n-3 PUFA was reduced for PMW given fish oil. A similar resistance of plasma AA to dietary n-3 PUFA supplementation has also been reported in younger cohorts [31, 33], suggesting that this finding was not associated with the age of the current cohort. Generally, the PUFA-derived metabolites were positively associated with the measured plasma PUFA. In addition, these findings confirm that PMW are responsive to changes in the type of PUFA consumed such that the glycerolipid lipid pool is plastic. The role of glycerolipids in health and disease is of great interest and rapidly taking shape due to newer analytical techniques and applications as biomarkers [20, 34].

A higher level of the DHA-derived docosahexaenoyl ethanolamide was observed in PMW supplemented with n-3 PUFA. DHEA is directly related to the intake of DHA and has been shown to enrich brain tissue at concentrations comparable to AEA [35]. DHEA as well as the metabolites produced from its oxidative metabolism have been shown to produce novel molecules possessing anti-inflammatory and organ-protective properties [36]. The consequence

of DHA tissue enrichment resulting in an increase of DHEA in PMW supports a rationale for supplementing with DHA to improve endocannabinoid tone and control age-related inflammation by altering OL. We recently reported that DHA enrichment of myoblasts increased glucose uptake and changed cannabinoid receptor gene expression in culture [12] and feeding DHA to mice improved biochemical and molecular aspects of the ECS and insulin signaling [36]. Although this is the first report demonstrating an increase in DHA containing DHEA in non-diabetic PMW after 6 mo of fish oil supplementation, it provides a rationale to explore how endocannabinoid tone may be improved to control the risk of diabetes in older subjects especially in women during post-menopause. The robust changes of EC observed in PMW that were not diabetic may suggest that such changes can occur in diabetic subjects.

A lower level of NA-Gly was observed in PMW supplemented with fish oil. NA-Gly is an AA-derived bioactive molecule found to have endocannabinoid-like properties [37] and is found primarily in the spinal cord, small intestines, and kidneys but has also been found at lower concentrations in testes, lungs, and liver [38]. This broad distribution suggests that NA-Gly could have different functions in many tissues. Recently, NA-Gly was reported to decrease fatty acid amide hydrolase (FAAH) expression, the degrading enzyme for two of the most studied EC, AEA and 2-AG [38]. Both AEA and 2-AG have been found to be elevated in adipose tissue of insulin resistant rodents [39, 40] and humans [41]. Moreover, systemic AA-derived EC levels are increased in PMW [42]. PMW have also been found to have lower cannabinoid receptor CB1 and FAAH mRNA levels in adipose tissue, suggestive of negative feedback regulation. Higher circulating levels of 2-AG have been found in insulin resistant obese PMW compared to insulin sensitive PMW [43]. Based on our findings herein, the PMW given n-3 PUFA had a higher level of DHEA, which could moderate the actions of high levels of AEA and 1/2-AG and

EC signaling in obese PMW. The effects of higher DHEA in PMW administered n-3 PUFA observed in the current study should be investigated on insulin sensitivity and obesity in older women. For example, in our laboratory a study of DHA feeding to C57BL/6 mice revealed compensatory results of ECS-related mRNA gene expression and lower levels of AA-derived endocannabinoids (1-arachidonoylglycerol, 1-AG; 2-arachidonoylglycerol, 2-AG) in quadriceps with improved muscle glucose uptake and lower epididymal fat pad mass compared to control mice [13].

AA is converted to adrenic acid (docosatetraenoic acid, 22:4n6) through chain elongation, which is found in high concentrations of cholesterol esters of the adrenal gland [44] as well as in low levels in human platelet phospholipids [31, 45-47], brain [48], and kidney medulla [44]. Adrenic acid is metabolized in these cells by cyclooxygenase, resulting in metabolites that have been shown to inhibit platelet aggregation by endothelial cells [49]. It is interesting to note that with n-3 PUFA supplementation of PMW, levels of the adrenic acid derived endocannabinoid, docosatetraenoyl ethanolamide (DEA), are lower in serum despite no difference in plasma AA level. DHA and EPA have long been identified to decrease platelet activation inhibiting aggregation [50, 51]. Perhaps the role of regulating vascular homeostasis is accomplished by the n-3 PUFA or its metabolites in place of or even preferentially over DEA. With n-3 PUFA enrichment, AA may be conserved for other functions when substitute fatty acids are available. In addition, it has been reported that when adrenic acid is liberated from platelet phospholipids it can modulate the metabolism of AA in two ways [52]. First, by the inhibition of thromboxane B2 and hydroxyl fatty acid synthesis, and second, by competing for cyclooxygenase. Adrenic acid will also increase 12-HETE synthesis and result in hydroxyl fatty acid synthesis.

In this study, PMW supplemented with n-3 PUFA after 6 mo showed lower levels of n-6 EA while those for n-3 EA were higher in serum compared to the baseline values. However, similar to its parent fatty acid AA, AEA was not affected by the n-3 PUFA dietary supplementation in PMW after 6 mo of treatment. Shorter chain EAs containing palamitic, stearic, oleic, linoleic and α-linoleic acids were not different after the supplementation with n-3 PUFA in PMW. The higher level of DHEA and lower levels of NA-Gly and DEA in serum of PMW supplemented with n-3 PUFA would support that investigating dietary PUFA on the tone of the ECS could reveal important dietary relationships in understanding obesity. The investigations should include actions on genes of the EC system.

In PMW, the intake of n-3 PUFA reduced AA-derived (DiHETrE) but increased the n-3-derived (EPA and DHA) OL in serum. In general, the levels of 14,15-DiHETE, 17,18-DiHETE 17(18)-EpETE, 5-EPE, 19,20-DiDPA were higher in PMW given the n-3 PUFA supplement. The changes in the levels of AA and n-3 PUFA derived OL in serum were strong and followed the plasma enrichment of EPA and DHA with n-3 PUFA supplementation. While large shifts in plasma prostanoids and leukotrienes were not observed, this is not surprising since these metabolic processes are poorly reflected in open circulation even in the presence of acute inflammation insults [53].

The OL have diverse actions which differ by structure and biochemical sources. On the one hand, the AA-derived mediators derived from the COX and LOX pathways tend to be associated with pro-inflammatory responses while those from EPA and DHA are either less inflammatory or anti-inflammatory mediators [14]. On the other hand, AA metabolites from the CYP epoxygenases are themselves anti-inflammatory agents, while the n-3 PUFA epoxides are even more potent anti-inflammatory agents [54-56]. Although the OL are not well characterized

461 in PMW our study showed that dietary n-3 PUFA are effective in changing the types and
462 amounts of these biological mediators and this relationship should be examined in alleviating
463 chronic inflammation associated with bone loss, muscle atrophy, and the metabolic syndrome.
464 Higher levels of free amino acids in serum of PMW supplemented with n-3 PUFA is
465 noteworthy and may suggest greater protein turnover or recycling between organs. Further, the
466 higher levels of N-acetyl amino acids might suggest greater N-acyl transferase activity associated
467 with protein synthesis in PMW given n-3 PUFA.
468 The responses of PMW from baseline to post n-3 PUFA supplementation indicates that
469 the n-3-derived EC and OL generally followed an upward trend as found in the partial least
470 squares discrimination analysis, however, not every subject responded in this fashion. As shown
471 from the PLS scores, some subjects demonstrated little to no differences in EC and OL
472 measurements. Such variable responses to supplementation are likely due to the individual's n-3
473 PUFA status and biological capacity (responders and non-responders) which have been reported
474 in such supplementation studies in the past [31].
475 When considering the discriminate analysis which incorporated all collected measures,
476 clear group discrimination was achieved after orthogonal signal correction, and discriminate
477 variables were split into 2 classes which either increased or decreased over the period of
478 intervention. A hierarchical clustering of GM, fatty acid, EC, and OL showed that the furan fatty
479 acid metabolite CMPF, DHEA, n-3 PUFA, and n-3 PUFA-diols were highly correlated, and were
480 closely related to changes in phospholipid n-3 PUFA composition and phytosterol levels. Also
481 increased by n-3 PUFA supplementation were an array of lipoxygenase and CYP450 dependent
482 mediators, and these showed associations to an array of steroids, amino acids, small organic
483 acids. Similarly, decreases in n-6 PUFA correlated strongly with reductions in diols and n-6

PUFA-EAs, with secondary linage to changes in phospholipids. These correlated responses to intervention suggest novel biochemical interactions between dietary n-3 PUFA, bioactive EC, and OL, and their potential actions on physiology and metabolism. Discovery of specific EC and OL biomarkers should continue to examine their role in understanding health and diet-related chronic disease.

In conclusion, enrichment of plasma with EPA and DHA from supplementing PMW with fish oil increased the DHA-derived EC DHEA and lowered AA family EC NA-Gly and DEA. Furthermore, the dietary supplementation also led to a substantial reduction in many AA-derived OL as well as a rise in n-3 PUFA-derived OL in serum of PMW compared to baseline. The consequences of these changes is evidence that dietary n-3 PUFA may afford potential health benefits in older women that should be investigated as a means to improve conditions associated with aging and systemic inflammation. This study provides a rationale to now determine the actions of n-3 PUFA on specific gene targets of the EC system and OL biosynthesis recently reported in cell culture and mice [12, 13]. Furthermore, in light of the GM analysis of the present study now justify targeted analysis of metabolite measurements in specific tissues to understand the effects of EC and OL changes that relate to inflammation and physiological endpoints. For example, one interesting physical outcome of n-3 PUFA supplementation in the PMW was an improvement in walking speed compared to the placebo group [57]. In this regard the percentage of frail and prefrail PMW was not different between the placebo and n-3 PUFA supplemented groups. The improvement in walking speed might be related to the changes observed in EC system genes observed in muscle [13] and OL inflammatory mediated functions[3, 4, 7, 20, 33] and both should be examined.

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Table 1. Baseline characteristics of study participant groups

	Placebo (n=20)	n-3 PUFA (n=20)	P value
Age (y)	74 ± 6	75 ± 6	0.77
Body mass index (kg/m^2)	26.3 ± 4.7	27.1 ± 6.0	0.59
PASE score	198 ± 96	269 ± 136	0.06
Dietary intake per/d			
Calories	1694 ± 700	1519 ± 780	0.45
Fat (g)	57 ± 30	58 ± 34	0.87
Protein (g)	89 ± 112	60 ± 21	0.27
Calcium (mg)	626 ± 237	740 ± 369	0.25
Vitamin D (IU)	125 ± 81	119 ± 84	0.82
n-3 PUFA (g)	0.65 ± 0.43	0.75 ± 0.53	0.54
Ethnicity %			0.32
White	97	92	
Hispanic	3	0	
Black	0	5	
Asian	0	3	
Education %			0.33
High school	15	15	
College	60	35	
Post graduate	25	40	
Marital Status %			0.90
Single	10	10	
Married	45	55	
Divorced	15	15	
Widowed	30	20	
Comorbidity %			
Coronary heart disease	10	10	1.00
Diabetes	0	0	
Hypertension	30	45	0.33
Depression	10	5	0.55
Smoker %	0	0	
Drinks alcohol %	70	70	1.00
Medications %			
Statins	56	38	0.29
Diuretic	28	23	0.62
Beta Blocker	31	25	0.69
ACE inhibitor	38	31	0.71
ARB	6	6	1.00
Calcium channel blocker	13	19	0.63
Aspirin	40	45	0.75
NSAID	10	0	0.155
Anti-acid	13	13	1.00

Values are means ± SD. Abbreviations: PASE, Physical Activity Scale for the Elderly; ACE, Angiotensin-converting enzyme; ARB, Angiotensin receptor blocker; NSAID, Nonsteroidal anti-inflammatory drug.

Table 2. Plasma fatty acid composition of postmenopausal women at baseline and after 6 mo of placebo or n-3 PUFA supplementation

Fatty acid (weight %)	Placebo		n-3 PUFA		†6 Month within groups p values
	Baseline	6 month	Baseline	6 month	
14:0	0.72±0.24	0.73±0.22	0.76±0.30	0.65±0.21	0.219
15:0	0.20±0.03	0.18±0.03	0.19±0.04	0.19±0.03	0.752
16:0	19.81±1.27	19.90±1.43	20.38±1.59	19.85±1.39	0.904
16:1t	0.51±0.11	0.51±0.10	0.49±0.12	0.45±0.12	0.121
16:1n-7	1.76±0.73	1.94±0.51	1.94±0.55[b]	1.65±0.66[a]	0.117
17:0	0.28±0.04[a]	0.26±0.04[b]	0.29±0.03	0.29±0.04	0.016
18:0	7.14±0.70	7.02±0.64	7.32±0.71	7.49±0.86	0.57
18:1n-9	19.75±1.89	19.76±2.09	20.71±1.77[a]	19.06±2.99[b]	0.040
18:1n-7	1.67±0.19	1.63±0.23	1.72±0.16	1.71±0.24	0.313
18:2n-6	31.40±4.17	31.51±3.60	29.17±2.95	30.51±3.06	0.349
18:3n-6	0.63±0.24	0.62±0.21	0.56±0.16	0.46±0.21	0.026
18:3n-3	0.71±0.21	0.69±0.18	0.64±0.17	0.62±0.18	0.244
20:2n-6	0.20±0.03	0.21±0.03	0.22±0.04[b]	0.20±0.03[a]	0.306
20:3n-6	1.50±0.44	1.64±0.29	1.67±0.38[a]	1.48±0.32[b]	0.098
20:4n-6	7.77±1.49	7.50±1.48	7.92±1.82	7.48±1.44	0.955
20:5n-3	0.83±0.38	0.93±0.62	0.87±0.67[b]	2.07±1.26[a]	0.0001
22:4n-6	0.21±0.04	0.21±0.04	0.22±0.03[a]	0.16±0.03[b]	0.001
22:5n-6	0.17±0.04	0.17±0.04	0.18±0.04[a]	0.14±0.03[b]	0.03
22:5n-3	0.54±0.10	0.56±0.11	0.52±0.10[b]	0.67±0.18[a]	0.027
22:6n-3	1.82±0.61	1.82±0.66	1.98±0.64[b]	2.95±0.94[a]	0.0001
total SA	28.17±1.42	28.15±1.55	28.99±1.91	28.48±1.60	0.51
total MONO	23.82±2.61	23.99±2.64	25.04±2.14[a]	23.05±3.44[b]	0.338
total PUFA	45.79±3.49	45.84±3.55	43.92±3.20[b]	46.67±3.54[a]	0.464
total n-3	3.90±1.06	4.00±1.28	4.01±1.22[b]	6.32±2.31[a]	0.0001
total n-6	41.89±3.62	41.84±3.53	39.91±3.33	40.35±3.25	0.174
Ratio of n-6/n-3	11.44±2.94	11.42±3.49	10.73±3.00[a]	7.54±3.84[b]	0.002
LC n-3	3.19±1.03	3.31±1.28	3.37±1.24[b]	5.69±2.29[a]	0.0001
LC n-6	9.85±1.65	9.71±1.42	10.18±1.72[a]	9.38±1.35[b]	0.456
Ratio of LCn-6/n-3	3.31±0.87	3.27±1.08	3.30±0.90[a]	1.98±1.06[b]	0.0001
Ratio of DHA/AA	0.24±0.09	0.25±0.09	0.26±0.09[b]	0.40±0.12[a]	0.0001

Values are means ± SD of n = 20 per group for both the placebo and n-3 PUFA supplementation. The data are measurements of fatty acid methyl esters (FAME) as weight percentages means ± SD of total fatty acids pool. Authentic external standards were used for the peak identification in GC chromatograms. GC detector sensitivity 10 ng/peak. Values with different superscript letters (a,b) in the columns indicate significant differences within groups from baseline to 6mo for individual fatty acids. There were no significant differences at baseline between the placebo and omega-3 PUFA groups. †Statistical comparison of month 6 data only (after placebo and n-3 PUFA treatment). The p value is for comparing between the placebo and n-3 PUFA supplementation groups at 6 mo.

Table 3. Serum endocannabinoid levels in postmenopausal women at baseline and after 6 mo of n-3 PUFA supplementation

Metabolite	Substrate	Baseline	Post supplementation	p-value	
PEA	16:0	8.04 ± 0.51	8.23 ± 0.5	0.64	
SEA	18:0	8.2 ± 0.76	7.5 ± 0.71	0.48	
OEA	18:1n9	23.1 ± 1.7	23.1 ± 1.7	0.83	
LEA	18:2n6	10.6 ± 0.7	12.0 ± 1.1	0.27	
α-LEA	18:3n3	0.1 ± 0.01	0.12 ± 0.01	0.31	
DGLEA	20:3n6	0.77 ± 0.11	0.65 ± 0.05	0.22	
NA-Gly	20:4n6	1.03 ± 0.14	0.82 ± 0.09	0.05	
AEA	20:4n6	2.54 ± 1.4	2.3 ± 0.15	0.21	
DEA	22:4n6	0.494 ± 0.03	0.43 ± 0.025	0.034	*
DHEA	22:6n3	0.491 ± 0.04	0.78 ± 0.09	0.00025	***
1-AG	22:4n6	11.4 ± 1.0	10.1 ± 1.9	0.35	
2-AG	22:4n6	12.1 ± 1	11 ± 1.4	0.2	
1-LG	18:2n6	148 ± 15	150 ± 35	0.4	
2-LG	18:2n6	135 ± 11	135 ± 15	0.6	
2-OG	18:1n9	212 ± 21	150 ± 24	0.035	*
1-OG	18:1n9	281 ± 20	240 ± 60	0.18	

Values are means ± SD of 20 samples from postmenopausal women at each time point of baseline and 6 mo of supplementation with n-3 PUFA (values expressed as nM) and significance indicated by P values and asterisks.

PEA - palmitoyl ethanolamide, SEA - stearoyl ethanolamide, OEA - oleoyl ethanolamide, LEA - linoleoyl ethanolamide, α-LEA - α-linolenoyl ethanolamide, DGLEA - dihomo-γ-linolenoyl ethanolamide, NA-Gly - N-arachidonyl glycine, AEA – anandamide, DEA - docosatetraenoyl ethanolamide, DHEA - docosahexaenoyl ethanolamide, 1-AG - 1-arachidonoylglycerol, 2-AG - 2-arachidonoylglycerol, 1-LG – 1-linoleoyl ethanolamide, 2-LG – 2-linoleoyl ethanolamide, 1-OG – 1-oleoyl ethanolamide, 2-OG – 2-oleoyl ethanolamide

Table 4. Serum oxylipin levels in postmenopausal women at baseline and after 6 mo of supplementation with n-3 PUFA

Enzyme System	Metabolite	Substrate	Baseline	Post supplementation	p-value	
LOX	15-HETE	20:4n6	0.703 ± 0.1	3.1 ± 1.1	0.58	
LOX	12-HETE	20:4n6	9.4 ± 1.6	9.6 ± 1.6	0.69	
LOX	9-HETE	20:4n6	0.287 ± 1.1	1.1 ± 0.57	0.78	
LOX	5-HETE	20:4n6	4.69 ± 32	3.2 ± 14	0.17	
LOX	5-HEPE	20:5n3	0.48 ± 0.09	1.2 ± 0.24	0.016	*
LOX	15-HEPE	20:5n3	0.21 ± 0.07	0.36 ± 0.06	0.22	
LOX	12-HEPE	20:5n3	0.75 ± 0.34	1.7 ± 0.35	0.087	
LOX	17-HDoHE	22:6n3	0.76 ± 0.1	0.88 ± 0.12	0.31	
CYP	14(15)-EpETrE	20:4n6	0.88 ± 0.1	1.0 ± 0.16	0.51	
CYP	11(12)-EpETrE	20:4n6	0.78 ± 0.11	0.92 ± 0.13	0.56	
CYP	8(9)-EpETrE	20:4n6	0.28 ± 0.04	0.27 ± 0.04	0.77	
CYP	17(18)-EpETE	20:5n3	0.224 ± 0.06	1.0 ± 0.21	0.00078	***
CYP	16(17)-EpDPE	22:6n3	0.46 ± 0.08	0.85 ± 0.15	0.12	
sEH	14,15-DiHETrE	20:4n6	0.72 ± 0.06	0.57 ± 0.04	0.0027	**
sEH	11,12-DiHETrE	20:4n6	0.53 ± 0.03	0.45 ± 0.03	0.013	*
sEH	8,9-DiHETrE	20:4n6	0.30 ± 0.02	0.26 ± 0.02	0.016	*
sEH	5,6-DiHETrE	20:4n6	0.18 ± 0.02	0.14 ± 0.02	0.2	
sEH	14,15-DiHETE	20:5n3	0.46 ± 0.05	0.75 ± 0.08	0.0019	**
sEH	17,18-DiHETE	20:5n3	4.84 ± 0.59	7.1 ± 0.76	0.0042	**
sEH	19,20-DiHDoPE	22:6n3	1.27 ± .14	1.67 ± 0.16	0.004	**

Values are means ± SD of 20 samples from postmenopausal women at each time point of baseline and 6 mo of supplementation with n-3 PUFA (values expressed as nM). LOX - lipoxygenase pathway, CYP - cytochrome P450 superfamily, sEH - soluble epoxide hydrolase (significance indicated by P values and asterisks).

LOX: 5-HEPE - 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid; 12-HEPE - 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid; 5-HETE - 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 9-HETE - 9-hydroxy-5E,7Z,11Z,14Z-eicosatetraenoic acid; 12-HETE - 12-hydroxy-5E,8Z,10Z,14Z-eicosatetraenoic acid; 15-HEPE - 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid; 15-HETE - 15-hydroxy - 15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 17-HDoHE - 17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid

CYP: 8(9)-EpETrE - 8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid; 11(12)-EpETrE - 11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid; 14(15)-EpETrE - 14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid; 16(17)-EpDPE - 16(17)-epoxy-4Z,7Z,10Z,13Z,19Z-docosapentaenoic acid; 17(18)-EpETE - 17(18)-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid

sEH: 5,6-DiHETrE - 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid; 8,9-DiHETrE - 8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid; 11,12-DiHETrE -11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid; 14,15-DiHETE - 14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid; 14,15-DiHETrE - 14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid; 17,18-DiHETE - 17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid; 19,20-DiHDoPE - 19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid

Table 5. Serum lysoglycerolipids levels in postmenopausal women at baseline and after 6 mo of placebo or supplementation with n-3 PUFA

Biochemical Name	Placebo				n-3 PUFA				Two-way ANOVA p value			Post-test contrasts p-value			
	Baseline		6 month		Baseline		6 month					n-3 PUFA vs. Placebo at baseline	n-3 PUFA vs. Placebo at 6 month	baseline vs. 6 month for Placebo	baseline vs. 6 month for n-3 PUFA
	mean	SD	mean	SD	mean	SD	mean	SD	S or P	Time	Interaction				
1-oleoylglycerophosphoethanolamine	1.14	0.42	1.05	0.29	0.99	0.26	0.85	0.33	0.033	0.052	0.32	0.24	0.020	0.49	0.04
2-oleoylglycerophosphoethanolamine	1.09	0.33	1.03	0.34	0.99	0.29	0.81	0.37	0.061	0.015	0.14	0.42	0.018	0.46	0.0066
2-docosahexaenoylglycerophospho-ethanolamine	0.97	0.33	0.96	0.48	0.94	0.23	1.21	0.32	0.15	0.11	0.017	0.97	0.013	0.55	0.0059
1-docosapentaenoylglycerophosphocholine	1.03	0.41	1.00	0.32	0.99	0.34	1.29	0.60	0.38	0.14	0.13	0.76	0.10	0.97	0.039
1-docosahexaenoylglycerophosphocholine	0.92	0.32	0.88	0.32	1.00	0.39	1.40	0.47	0.014	0.034	0.006	0.62	0.0003	0.61	0.0008
2-docosahexaenoylglycerophosphocholine	1.00	0.35	0.88	0.25	1.03	0.29	1.29	0.47	0.017	0.58	0.032	0.64	0.0013	0.25	0.06
1-arachidonoylglycerophosphoinositol	1.19	0.59	1.09	0.29	1.19	0.53	0.88	0.34	0.12	0.09	0.18	0.88	0.044	0.78	0.032

Values represent n = 20 for each group and time point of baseline and 6 mo of placebo or supplementation with n-3 PUFA. Repeated measures two-way ANOVA with post-test contrasts was used to identify biochemicals that differed significantly ($p \leq 0.05$) between experimental group, following log transformation and imputation of missing values, if any, with the minimum observed value for each compound.

Table 6. Serum sterol and steroid homone levels in postmenopausal women at baseline and after 6 mo of placebo or n-3 PUFA supplementation

Biochemical Name	Placebo				n-3 PUFA				Two-way ANOVA p value			Post-test contrasts p-value			
	Baseline		6 month		Baseline		6 month					n-3 PUFA vs. Placebo at baseline	n-3 PUFA vs. Placebo at 6 month	baseline vs. 6 month for Placebo	baseline vs. 6 month for n-3 PUFA
	mean	SD	Mean	SD	mean	SD	mean	SD	S or P	Time	Interaction				
pregnenolone sulfate	1.07	0.42	0.94	0.39	1.10	0.40	1.18	0.54	0.33	0.26	0.048	0.75	0.13	0.030	0.53
21-hydroxypregnenolone disulfate	0.86	0.39	0.87	0.31	0.89	0.36	1.03	0.33	0.34	0.049	0.23	0.72	0.17	0.57	0.027
5α-pregnan-3β,20-α-diol disulfate	0.84	0.41	0.87	0.41	0.97	0.37	1.17	0.44	0.054	0.046	0.15	0.22	0.019	0.68	0.017
β-sitosterol	1.07	0.21	0.97	0.17	0.91	0.21	0.97	0.19	0.15	0.80	0.04	0.02	0.81	0.10	0.19
cortisone	0.99	0.38	0.95	0.34	0.66	0.44	0.84	0.45	0.03	0.19	0.09	0.01	0.24	0.79	0.04
campesterol	0.80	0.38	0.96	0.41	0.81	0.48	1.00	0.55	0.91	0.03	0.87	0.86	0.99	0.14	0.09

Values represent n = 20 for each group and time point of baseline and 6 mo of placebo or supplementation with n-3 PUFA. Repeated measures two-way ANOVA with post-test contrasts was used to identify biochemicals that differed significantly ($p \leq 0.05$) between experimental group, following log transformation and imputation of missing values, if any, with the minimum observed value for each compound.

Table 7. Serum amino acid and related metabolite levels in postmenopausal women at baseline and after 6 mo of placebo or n-3 PUFA supplementation

Biochemical Name	Placebo				n-3 PUFA				Two-way ANOVA p value			Post-test contrasts p-value			
	Baseline		6 month		Baseline		6 month					n-3 PUFA vs. Placebo at baseline	n-3 PUFA vs. Placebo at 6 month	baseline vs. 6 month for Placebo	baseline vs. 6 month for n-3 PUFA
	mean	SD	mean	SD	mean	SD	mean	SD	S or P	Time	Interaction				
N-acetylglycine	1.19	0.66	1.16	0.47	1.08	0.74	1.38	1.01	0.86	0.058	0.14	0.45	0.66	0.75	0.020
N-acetylmethionine	1.00	0.40	0.96	0.32	0.87	0.22	0.94	0.16	0.53	0.27	0.074	0.22	0.97	0.61	0.043
N-acetylserine	1.14	0.37	1.06	0.31	0.98	0.29	1.08	0.22	0.52	0.51	0.034	0.11	0.65	0.28	0.049
C-glycosyltryptophan	1.02	0.20	0.99	0.20	0.98	0.25	1.06	0.27	0.96	0.28	0.019	0.53	0.46	0.35	0.017
N-methyl proline	1.31	1.02	1.04	0.80	1.60	1.82	2.41	2.60	0.30	0.34	0.042	0.99	0.065	0.43	0.036
dimethylarginine (SDMA + ADMA)	1.05	0.16	1.00	0.12	0.96	0.12	1.02	0.15	0.35	0.73	0.024	0.048	0.68	0.17	0.064
N-acetylthreonine	1.07	0.27	1.00	0.31	1.02	0.25	1.10	0.31	0.70	0.90	0.02	0.57	0.20	0.07	0.11
tryptophan	1.06	0.15	1.00	0.09	0.99	0.15	1.02	0.09	0.44	0.57	0.03	0.07	0.59	0.05	0.24
indolepropionate	1.01	0.70	0.82	0.45	1.78	1.45	1.71	1.43	0.01	0.16	0.99	0.02	0.02	0.32	0.32
cysteine	1.01	0.35	1.07	0.33	0.97	0.23	1.14	0.37	0.76	0.02	0.44	0.91	0.51	0.25	0.03
N-formylmethionine	1.07	0.35	1.07	0.27	1.04	0.18	0.95	0.19	0.43	0.10	0.03	0.95	0.15	0.69	0.01
methionine	1.04	0.14	0.97	0.09	0.96	0.13	1.00	0.11	0.41	0.45	0.02	0.04	0.46	0.03	0.26

Values represent n = 20 for each group and time point of baseline and 6 mo of placebo or supplementation with n-3 PUFA. Repeated measures two-way ANOVA with post-test contrasts was used to identify biochemicals that differed significantly ($p \leq 0.05$) between experimental group, following log transformation and imputation of missing values, if any, with the minimum observed value for each compound.

Compounds in this table are from the following sub pathways. Glycine, serine and threonine metabolism: N-acetylglycine, N-acetylserine, N-and acetylthreonine. Tryptophan metabolism: C-glycosyltryptophan, tryptophan, indolepropionate. Cysteine, methionine, SAM, taurine metabolism: N-acetylmethionine, cysteine, N-formylmethionine, methionine. Urea cycle, arginine-, proline-, metabolism: N-methyl proline, dimethylarginine (SDMA + ADMA). These compounds are different by two-way ANOVA.

Figure 1. The absolute concentration of total measured long chain ethanolamides (EA) were unchanged suggesting competition for a common deposition in postmenopausal women at baseline and 6mo of n-3 PUFA supplementation. The n = 20 for both baseline and 6 mo of supplementation [values are means ± SD (error bars)]. The raw data were checked for normality and were normalized by one of the following methods: shifted natural log, square root, or power transformation. DHEA - docosahexaenoyl ethanolamide; DEA - docosatetraenoyl ethanolamide; AEA - anandamide. The increase in DHEA is significant ($P < 0.00025$) and the ratio of DHEA/AEA is higher.

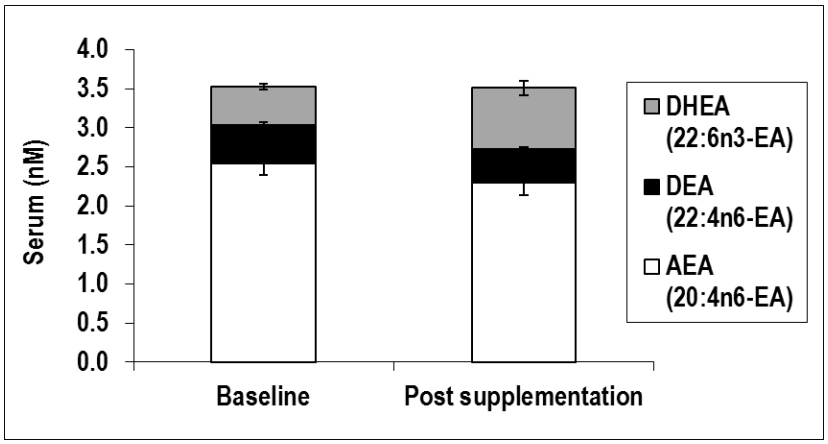


Figure 2. Partial least squares discrimination analysis (PLS-DA) for changes in serum oxylipins and endocannabinoids for postmenopausal women (PMW) at baseline (blue) and after 6mo of n-3 PUFA supplementation (green). Arrows indicate individual changes with supplementation for all (n = 20 for both time points). Baseline n-3 PUFA status shown in blue likely influences the degree of response to all variables measured after 6 mo of supplementation in PMW shown in green.

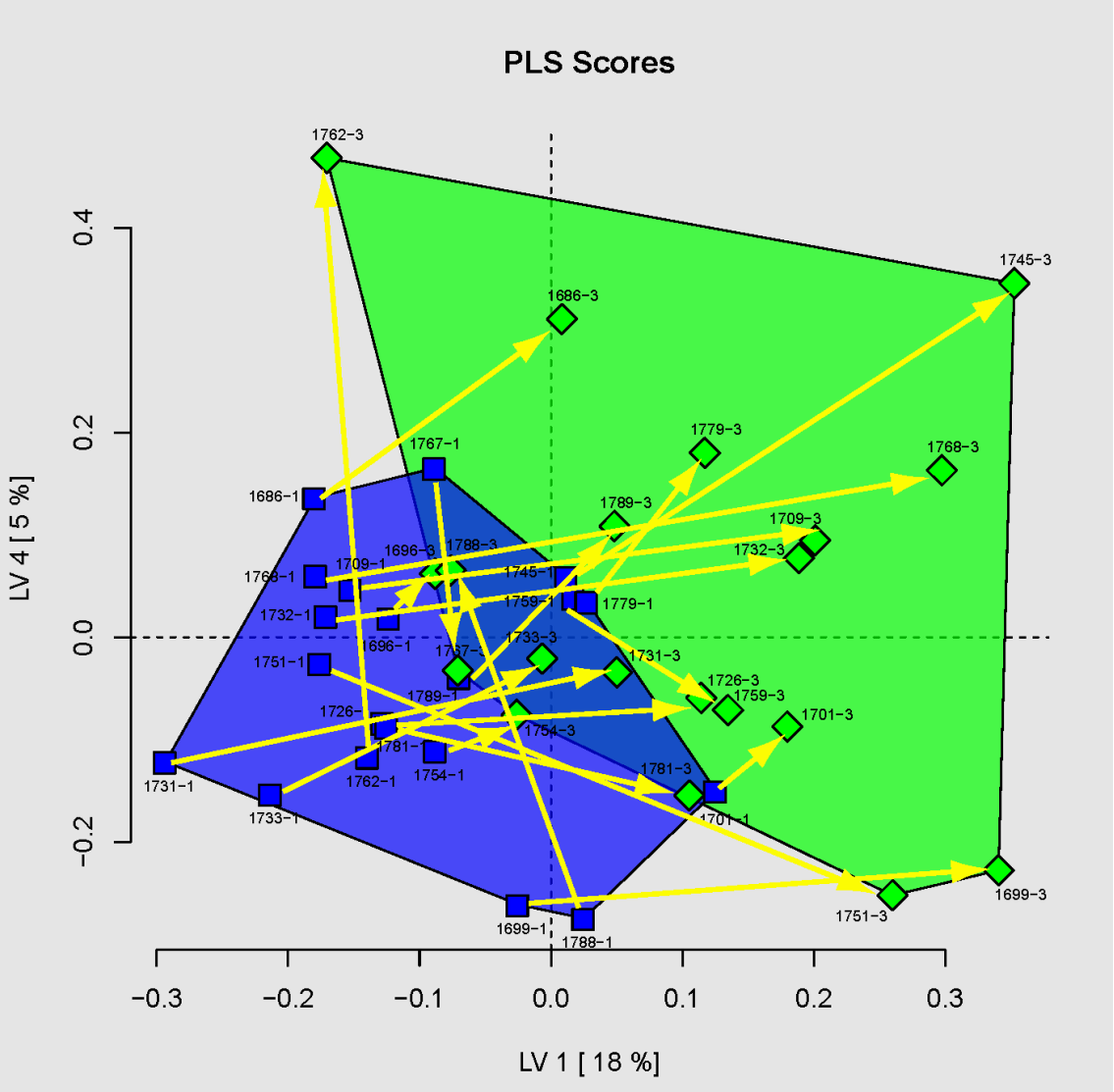


Figure 3. Partial least squares discriminate analysis scores plot showing group discrimination after orthogonal signal correction for the complete EC, OL, fatty acid and GM data set in postmenopausal women. Variables with variable importance in projection (VIP) scores were used for further analysis shown in Figure 4. (Yellow = baseline; Green = post-intervention).

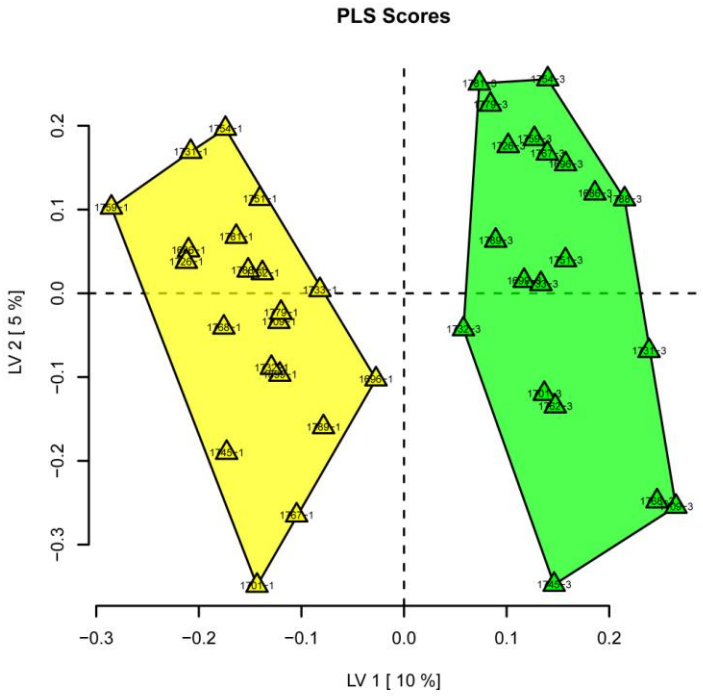


Figure 4. Partial least squares discriminate analysis of Variable Importance Plot **(**VIP) filtered data set (n=109 variables) using the oscorespls component of the R 'pls' package [28] in postmenopausal women. Data was auto scaled prior to analysis (PLS Scores and PLS Loadings). Symbols are provided for variables with VIP scores > 1.2 – 3.4 from the initial complete data set analysis and are scaled according to these scores. A hierarchical cluster analysis was performed from the clusters (C1-C8) of PLS Loadings from the n-3 PUFA intervention is shown in Figure 5.

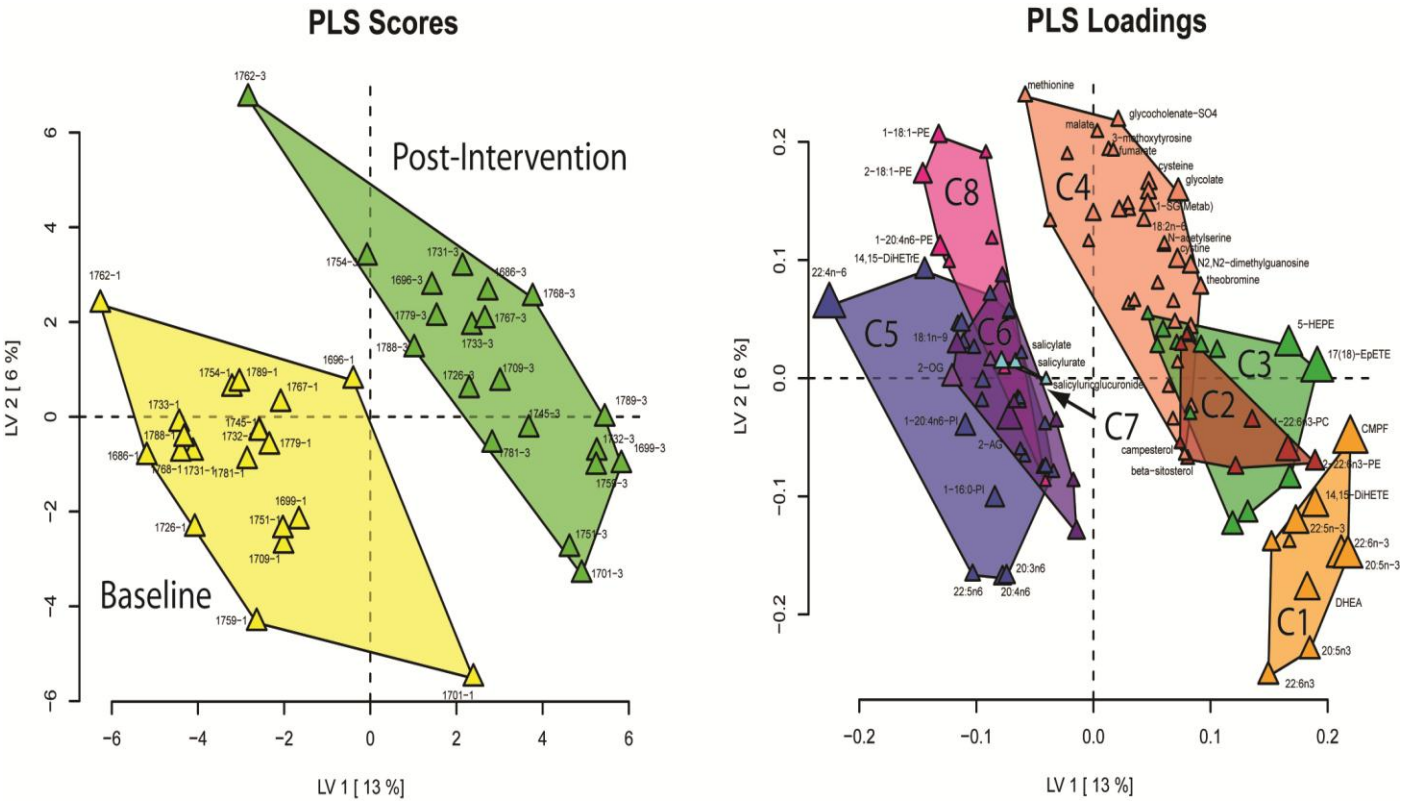
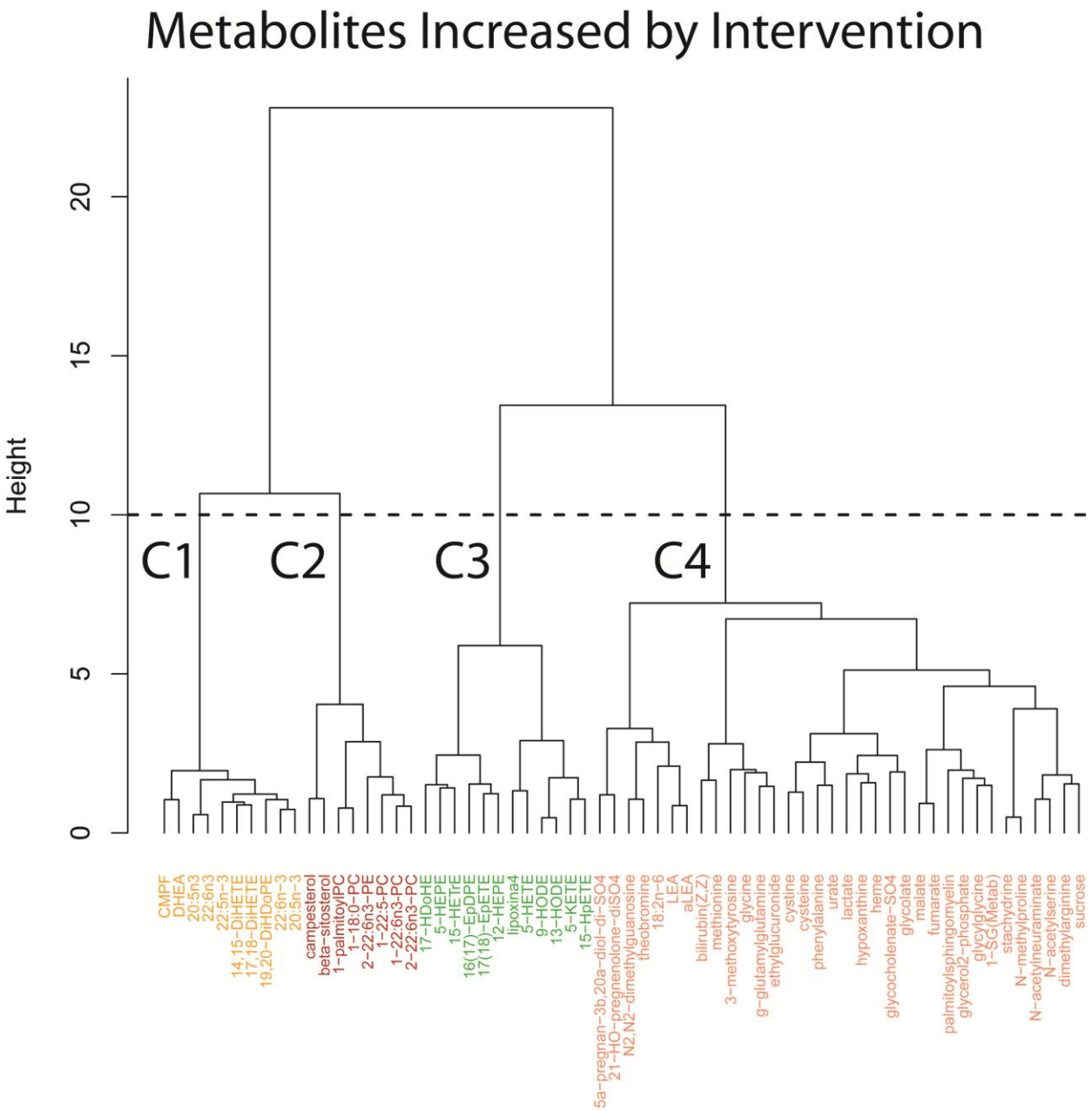


Figure 5. A hierarchical cluster analysis of intervention (Figure 5A metabolites increased by supplementation and Figure 5B metabolites decreased by supplementation) using the hclust function from the base R package, 'stats', with Minkowski distances and the Ward agglomerative algorithm used to group analytes, and labels correspond to cluster labels on the PLS Loadings in Figure 4.

5 A. Metabolites increased after 6 mo of n-3 PUFA supplementation in PMW.



5 B. Metabolites decreased after 6 mo of n-3 PUFA supplementation in PMW.