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Transcriptomic and metabolomic signatures of an n-3 polyunsaturated fatty acids supplementation in a normolipidemic/normocholesterolemic Caucasian population

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Abstract

OMIC technologies, including transcriptomics and metabolomics, may provide powerful tools for identifying the effects of nutrients on molecular functions and metabolic pathways. The objective was to investigate molecular and metabolic changes following *n*-3 polyunsaturated fatty acid (PUFA) supplementation in healthy subjects via traditional biomarkers as well as transcriptome and metabolome analyses. Thirteen men and 17 women followed a 2-week run-in period based on *Canada's Food Guide* and then underwent 6-week supplementation with *n*-3 PUFA (3 g/day). Traditional biochemical markers such as plasma lipids, inflammatory markers, glycemic parameters and erythrocyte fatty acid concentrations were measured. Changes in gene expression of peripheral blood mononuclear cells were assessed by microarrays, and metabolome profiles were assessed by mass spectrometry assay kit. After supplementation, plasma triglycerides decreased and erythrocyte *n*-3 PUFA concentrations increased to a similar extent in both genders. Further, plasma high-density lipoprotein cholesterol concentrations and fasting glucose levels increased in women after *n*-3 PUFA supplementation. *N*-3 PUFA supplementation changed the expression of 610 genes in men, whereas the expression of 250 genes was altered in women. Pathway analyses indicate changes in gene expression of the nuclear receptor peroxisome proliferator-activated receptor-alpha, nuclear transcription-factor kappaB, oxidative stress and activation of the oxidative stress response mediated by nuclear factor (erythroid-derived 2)-like 2. After *n*-3 PUFA supplementation, metabolomics profiles demonstrate an increase in acylcarnitines, hexose and leucine in men only and a decrease in saturation of glycerophosphatidylcholine and lysophosphatidylcholine concentrations in all subjects. Overall, traditional and novel biomarkers suggest that *n*-3 PUFA supplementation exerts cardioprotective effects.

Keywords: Microarray; Metabolic pathways; Metabolites; Lipidomics; Nutrigenomics

1. Introduction

Omega-3 fatty acids (*n-3* FAs), including fish-oil-derived eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, are recognized to have beneficial effects on cardiovascular diseases. These cardioprotective effects of *n-3* FAs are thought to result in part from their hypotriglyceridemic and anti-inflammatory effects [1].

These beneficial effects of *n*-3 polyunsaturated fatty acid (*n-3* PUFA) are likely mediated through changes in gene expressions. A transcriptomic study in elderly subjects by Bouwens et al. (2009) [2] showed that supplementation with 1.8 g *n*-3 PUFA per day can alter gene expressions toward a more antiatherogenic and anti-inflammatory profile. In the same manner, Kabir et al. (2007) [3] concluded that adipose tissue inflammation-related genes were reduced after *n*-3 PUFA supplementation in women with type 2 diabetes without hypertriglyceridemia. Further, Gorjao et al. (2006) [4] determined that DHA-rich oil stimulates several aspects of immune function. In contrast, some other studies have demonstrated a decrease in plasma triglyceride (TG) concentrations without alteration in the expression of inflammatory genes after a high dose of *n*-3 PUFA [5] or fatty fish intake [6]. Clearly, *n*-3 PUFA supplementation has hypotriglyceridemic effects; however, the anti-inflammatory effects are still debated in humans.

In addition, alterations in the metabolome result from changes in gene expression and protein concentrations of all metabolically relevant control systems. A study by Lankinen et al. (2009) [7] found

Abbreviations: AC, acylcarnitines; CRP, C-reactive protein; DHA, docosahexaenoic; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic; FA, fatty acid; FC, fold change; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; glyPC, glycerophosphatidylcholine; HDL-C, high-density lipoprotein cholesterol; IL6, interleukin-6; LDL-C, low-density lipoprotein cholesterol; lysoPC, lysophosphatidylcholine; NrF2, nuclear factor (erythroid-derived 2)-like 2; NF-kB, nuclear factor kB; PBMCs, peripheral blood mononuclear cells; PPARA, peroxisome proliferator-activated receptor alpha; PUFA, polyunsaturated fatty acid; RIN, RNA Integrity Number; TC, total cholesterol; TG, triglyceride; TNFA, tumor necrosis factor-alpha; SM, sphingomyelin.

This trial is registered at clinicaltrials.gov as NCT01343342.

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that serum bioactive lipids associated with insulin signaling and inflammatory pathways, ceramides, lysophosphatidylcholines (lysoPCs) and diacylglycerols were decreased by fatty fish intake. Furthermore, McCombie et al. (2009) [8] found that fish oil supplementation in conjunction with body weight loss reduced total plasma TG content; in addition, weight loss without fish oil supplementation decreased the levels of plasma docosapentaenoic acid (22:5). Overall, only a limited number of clinical studies have been conducted on the impact of *n*-3 PUFA on the metabolome.

The objective of the present study was thus to investigate the effects of *n*-3 PUFA supplementation in healthy subjects on molecular and metabolic changes via traditional biomarkers as well as transcriptome and metabolome analyses. The use of multiple novel and available OMIC technologies may help us better understand the holistic impact of *n*-3 PUFA supplementation on antiatherogenic and anti-inflammatory mechanisms in healthy subjects.

2. Subjects and methods

2.1. Study population

A total of 30 subjects, including 13 men and 17 pre- and postmenopausal women, from the greater Quebec City metropolitan area were recruited. Participants had a body mass index (BMI) between 25 and 40 kg/m^2 and were not currently taking any lipid-lowering medications. Subjects were excluded from the study if they had taken *n*-3 PUFA supplements for at least 6 months prior, used oral hypolipidemic therapy or had been diagnosed with diabetes, hypertension, hypothyroidism or other known metabolic disorders such as hypertension, diabetes, severe dyslipidemia or coronary heart disease. The experimental protocol was approved by the ethics committees of Laval University Hospital Research Center and Laval University. This trial was registered at clinicaltrials.gov as NCT01343342.

2.2. Study design and diets

Thirty subjects followed a run-in period of 2 weeks. Individual dietary instructions were given by a trained dietician to achieve the recommendation from *Canada's Food Guide*. Subjects were asked to follow these dietary recommendations and maintain their body weight stable throughout the protocol. Some specifications were given regarding the *n*-3 PUFA dietary intake: not exceed two fish or seafood servings per week (max 150 g), prefer white flesh fishes instead of fatty fishes (examples were given) and avoid enriched *n*-3 PUFA dietary products such as some milks, juices, breads and eggs. Subjects were also asked to limit their alcohol consumption during the protocol; two regular drinks per week were allowed. In addition, subjects were not allowed to take *n*-3 PUFA supplements (such as flaxseed), vitamins or natural health products during the protocol.

After the 2-week run-in, each participant received a bottle containing needed *n*-3 PUFA capsules for the following 6 weeks. They were invited to take five (1 g oil each) capsules per day (Ocean Nutrition, Nova Scotia, Canada), providing a total of 3 g of *n*-3 (1.9 g EPA and 1.1 g DHA) per day. For a facilitated digestion, we recommended to take fish oil capsules while eating. Compliance was assessed from the return of bottles. Subjects were asked to report any deviation during the protocol and to write down their alcohol and fish consumption as well as the side effects. Before each phase, subjects received detailed written and oral instructions on their diet.

2.3. Biochemical parameters

2.3.1. Plasma lipids

Blood samples were collected from an antecubital vein into Vacutainer tubes containing EDTA after 12-h overnight fast and 48-h alcohol abstinence. Blood samples were taken to identify and exclude individuals with any metabolic disorders. Afterward, selected participants had blood samples taken prior and after the *n*-3 PUFA supplementation period. Plasma was separated by centrifugation (2500*g* for 10 min at 4°C), and samples were aliquoted and frozen for subsequent analyses. Plasma total cholesterol (TC) and TG concentrations were measured using enzymatic assays [9,10]. The high-density lipoprotein cholesterol (HDL-C) fraction was obtained after precipitation of very low-density lipoprotein and low-density lipoprotein (LDL) particles in the infranatant with heparin manganese chloride [11]. LDL cholesterol (LDL-C) was calculated with the Friedewald formula [12]. Fasting insulinemia was measured by radioimmunoassay with polyethylene glycol separation [13]. Fasting glucose concentrations were enzymatically measured [14].

2.3.2. Inflammatory markers

Plasma concentration of interleukin-6 (IL6) and tumor necrosis factor-alpha (TNFA) were measured with high-sensitivity enzyme-linked immunosorbent assay (ELISA) kits including Human IL-6 Quantikine HS ELISA Kit [R&D Systems, Minneapolis, MN, USA (HS600B)] and Human TNF-alpha Quantikine HS ELISA Kit [R&D Systems, Minneapolis, MN, USA (HSTA00D)]. Plasma C-reactive protein (CRP) was measured by nephelometry (Prospec equipment Behring) using a sensitive assay, as described previously [15]. CRP concentrations above 10 mg/L ($n=7$) were excluded in statistical analyses.

2.3.3. FAs analysis

FA composition of erythrocyte membranes was determined by gas chromatographic analysis. Membranes of lysed erythrocytes were isolated by centrifugation (21,000*g*, 15 min) and washed twice with 0.9% NaCl solution. Cell membranes were resuspended in 200 μl of the NaCl solution and were spiked with phosphatidylcholine C:15 (Avanti Polar Lipids, Alabaster, AL, USA), used as internal standard. Lipids were extracted using a mixture of chloroform–methanol (2:1 v/v) according to a modified Folch method [16]. FA profiles were obtained after methylation in methanol/benzene 4:1 (v/v) [17] and capillary gas chromatography using a temperature gradient on an HP5890 gas chromatograph (Hewlett Packard, Toronto, Canada) equipped with an HP-88 capillary column (100 m×0.25 mm i.d.×0.20 μm film thickness; Agilent Technologies, Palo Atto, CA, USA) coupled with a flame ionization detector. Helium was used as carrier gas (split ratio 1:80). FAs were identified according to their retention time using the following standard mixtures as a basis for comparison: the FAME 37 mix (Supelco Inc., Bellefonte, PA, USA) and the GLC-411 FA mix (NuChek Prep Inc., Elysian, MN, USA), as well as the following methylated FAs: C22:5 w6 (Larodan AB, Malmö, Sweden) and C22:5 w3 (Supelco Inc., Bellefonte, PA, USA). Erythrocyte FA profiles were expressed as the relative percentage areas of total FAs.

2.4. Transcriptomics analyses

2.4.1. Peripheral blood mononuclear cells

Blood samples were collected into an 8-ml Cell Preparation Tube (Becton Dickinson, Oakville, Ontario, Canada) pre- and postsupplementation. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation (1500*g*, 20 min, at room temperature) and washed according to the manufacturer's instructions. Total RNA was extracted with RNeasy Plus Mini Kit (QIAGEN, Mississauga, Ontario, Canada) according to manufacturer's protocol. After spectrophotometric quantification and verification of the total RNA quality via the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), samples were used for microarray analysis. Samples were excluded from additional analysis on microarray chips if they had poor RNA quality (RNA integrity number [RIN]<8).

2.4.2. Transcriptomic profiling

Two hundred nanograms of total RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion). cRNA quality was assessed by capillary electrophoresis on Agilent 2100 Bioanalyzer. Expression levels of 48,803 mRNA transcripts, to investigate 37,804 genes, were assessed by the Human-6 v3 Expression BeadChips (Illumina). Hybridization was carried out according to the manufacturer's instructions at the McGill University/Génome Québec Innovation Center (Montreal, Canada).

2.4.3. Analysis of microarray data

The microarray analysis was performed using FlexArray software [18]. The Lumi algorithm was used to normalize Illumina microarray data. Specifically, expression values were normalized by using Lumi via the robust multiarray average algorithm [19]. This step was followed by quantile normalization and $\log_2$ transformation. The samples were then grouped according to time (pre- and postsupplementation) and gender (men and women). To assess which transcripts were differentially expressed between conditions examined, we used a significance analysis of microarrays (SAM) algorithm, an adaptation of a *t* test for microarray data, on all probes. In general, the SAM application assigns a score to a gene on the basis of changes in gene expression relative to standard deviation of repeated measurements. Then, SAM uses permutations of the repeated measurements to estimate the false discovery rate [20]. A cutoff of $P\leq.05$ was used to select the regulated genes. In addition, a fold change cutoff was also computed by FlexArray software to assess the level and the direction of the gene regulation. This fold change is calculated as the absolute ratio of normalized intensities between the mean values of all individual fold-change (FC) (post-/presupplementation). Thus, two cutoff values were used to minimize the chances of false positives. Fold changes at >1.2 and $P\leq.05$ (up-regulated) or FCs at <0.8 and $P\leq.05$ (down-regulated) were taken from each treatment to determine differentially expressed transcripts, and transcript lists were generated.

2.4.4. Biological pathway analyses

Pathway analyses allowed to determine whether genes found to be differentially expressed belong to predefined networks more than expected by chance alone and help to add structure to the vast amount of data generated by microarrays. The Ingenuity Pathway Analysis (IPA) system (Ingenuity® Systems, www.ingenuity.com) was used to visualize gene expression data in the context of biological pathways. First, an input file was uploaded in the IPA system: FCs of all probe sets between pre- and post-*n*-3 PUFA supplementation and data set in Core Analysis were created. Further, the core data set was analyzed using the general settings for IPA system as "Ingenuity knowledge base (genes)" and "considered only molecules and/or relationships where

species is humans." Finally, we examined canonical pathway analysis, which allows us to link expression data to clinical endpoints for mechanistic hypothesis generation and identification of putative mechanisms.

The significance value associated with functional analysis for a data set is a measure of the likelihood that the association between a set of functional analysis molecules in the experiment and a given process or pathway is due to random chance. Smaller *P* values (<.05) indicate statistically significant, nonrandom associations. The *P* value is calculated using the right-tailed Fisher's Exact Test with a cutoff of ±1.2. IPA suggests to use *P* values as starting points for further investigation; however, pathways with larger *P* values (>.05) may be biologically relevant even if not statistically significant. In addition, there is no directionality associated with the relationship (i.e., the function cannot be interpreted as being up- or down-regulated).

2.4.5. Real-time polymerase chain reaction (PCR) validation

cDNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays; Applied Biosystems) in a final volume of 20 μl. The assays used were as follows: Hs00174128_m1 (*TNFA*), Hs00985639_m1 (*IL6*), Hs00203685_m1 (*FADS1*), Hs00188654_m1 (*FADS2*), Hs00222230_m1 (*FADS3*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene Hs99999905_ml. Assays used the same fluorescent reporter probe (FAM dye labeled), and thus, each combination of treatment and gene was analyzed in individual wells on a 96-well plate. All samples were run in duplicate on an Applied Biosystem 7500 Real-Time PCR System (Applied Biosystems) using the following thermal cycling profile: 50°C (2 min), 95°C (10 min), followed by 40 steps of 95°C for 15 s and 60°C for 60 s. The reverse transcriptase (RT)-PCR results were imported into Microsoft Excel, and the average value of duplicate C_t values was calculated. Data were adjusted for the endogenous control (*GAPDH*). Pearson correlations were performed between RT-PCR and microarray data. Statistical significance was defined as $P \leq .05$.

2.5. Metabolomic analyses

2.5.1. Metabolite profiling

The targeted metabolite profiling with the Biocrates Absolute IDQ p150 (Biocrates Life Sciences AG, Austria) mass spectrometry method was used to quantify 163 metabolites from four compound classes including 41 acylcarnitines (ACs) (AC*x*:*y*, where *x* denotes the number of carbons in the side chain and *y* the number of double bonds), 13 amino acids (proteinogenic+ornithine), hexoses (sum of hexoses-90% to 95% glucose) and 107 lipids. These lipids are subdivided into three different classes: 15 sphingomyelins (SMs) and SM derivatives, 15 lysoPCs and 77 glycerophosphatidylcholines (glyPCs). Glycerophospholipids are further differentiated with respect to the presence of ester (*a*) and ether (*e*) bonds in the glycerol moiety, where two letters (*aa*, *ae* or *ee*) denote that the first as well as the second position of the glycerol unit are bound to an FA residue, while a single letter (*a* or *e*) indicates a bond with only one FA residue; the latter molecular species are usually called lysophospholipids. Assays used 10 μl of plasma from each subject pre- and post-*n*-3 PUFA supplementation. The metabolite profiling was carried out according to the manufacturer's instructions at CHENOMX (Edmonton, Alberta, Canada). For all analyzed metabolites the concentrations are reported in μM.

2.6. Statistical analyses

Results are presented as mean±S.E.M. Variables not normally distributed were log-transformed before analyses. Data were analyzed using a paired *t* test to determine significant changes between pre- and postsupplementation periods. An unpaired *t* test was used to establish differences between men and women subgroups. Statistical analyses were performed with SAS statistical software, version 9.1 (SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as $P \leq .05$.

Sample size calculation demonstrates that a group of 30 subjects, with an 80% probability and 5% significance level of detecting an anticipated difference of 0.25 mmol/L (S.D.= 0.24) TG concentrations after 6 weeks of *n*-3 PUFA supplementation, were needed. Thus, 30 subjects were included in this study.

3. Results

3.1. Biochemical parameters

The subject baseline characteristics for men ($n=13$) and women ($n=17$) are as follows: age: 33.5±2.1 years and 34.4±2.5 years, BMI: 29.1±1.2 kg/m^2 and 29.2±0.8 kg/m^2, TC: 5.35±0.26 mmol/L and 5.51±0.30 mmol/L, LDL-C: 3.38±0.23 mmol/L and 2.93±0.27 mmol/L, HDL-C: 1.21±0.09 mmol/L and 1.65±0.13 and TG: 1.71±0.36 mmol/L and 1.32±0.17 mmol/L, respectively. No difference was observed between the gender groups for baseline characteristics except for HDL-C, which was significantly higher in women.

There was no change in weight or BMI pre- to post-*n*-3 PUFA supplementation (data not shown). Biochemical parameters pre- and post-*n*-3 PUFA supplementation are described in Table 1. Briefly, women had significantly higher presupplementation HDL-C concentrations compared to men; yet, all other parameters were similar in both subgroups pre- and postsupplementation. The *n*-3 PUFA supplementation was associated with a similar decrease in fasting TG concentrations in both genders, from 1.48 to 1.28 mmol/L (−0.20 mmol/L, −13.5%) for men and from 1.45 to 1.16 mmol/L (−0.29 mmol/L, −20%) for women. There were no other significant differences seen between the pre- and postsupplementation results of TC, LDL-C and HDL-C in either gender. However, HDL-C tended to increase in women after *n*-3 PUFA supplementation ($P=.05$). Plasma inflammatory parameters, including IL6, TNFA and CRP, did not change significantly after *n*-3 PUFA supplementation. Plasma glucose increased significantly in women without changing plasma insulin concentration after *n*-3 PUFA supplementation. There was no significant difference in fasting plasma glucose or insulin levels in men after *n*-3 PUFA supplementation. Overall, the outcome of *n*-3

Table 1
Plasma lipid concentrations, glycemic parameters, inflammatory markers and erythrocyte FA composition pre- and post-n-3 PUFA supplementation in men and women

	Men ($n=13$)			Women ($n=17$)		
	Pre-n-3 PUFA	Post-n-3 PUFA	*P* value	Pre-n-3 PUFA	Post-n-3 PUFA	*P* value
Plasma lipids						
TC (mmol/L)	5.37±0.23**	5.16±0.24**	.07	5.13±0.23	5.05±0.23	.36
LDL-C (mmol/L)	3.46±0.24	3.28±0.24	.13	2.91±0.22	2.90±0.22	.91
HDL-C (mmol/L)	1.23±0.10**†	1.29±0.11**‡	.10	1.55±0.08*†	1.62±0.09*‡	.05
TGs (mmol/L)	1.48±0.22*	1.28±0.23*	<.01	1.45±0.22*	1.16±0.13*	.01
Glycemic parameters						
Plasma glucose (mmol/L)	4.86 ±0.17	5.12± 0.11	.15	4.90 ±0.12*	5.13 ±0.13*	.02
Plasma insulin (ρmol/L)	155.69± 64.77	104.92± 20.59	.31	77.59± 6.90	81.35± 5.20	.16
Inflammation markers						
TNFA (pg/mL)	2.32±0.78	2.25±0.75	.55	1.66±0.19	1.60±0.21	.66
IL6 (pg/mL)	1.36±0.29	1.02±0.07	.31	1.60±0.31	1.30±0.20	.34
CRP (mg/L)	2.25±0.53	1.70±0.41	.30	2.66±0.77	2.51±0.77	.59
Erythrocyte FA composition						
Total *n*-3 (% of FAs)	7.45±0.39*	10.03±0.34*	<.01	7.24±0.27*	10.58±0.33*	<.01
EPA (% of FAs)	0.67±0.03*	2.12±0.13*	<.01	0.71±0.07*	2.38±0.13*	<.01
DHA (% of FAs)	4.24±0.27*	4.96±0.21*	<.01	4.23±0.20*	5.30±0.19*	<.01
Ratio *n*-3/*n*-6 FAs	0.26±0.02*	0.39±0.02*	<.01	0.25±0.01*	0.42±0.02*	<.01

Mean±S.E.M.
*Statistically significant ($P \leq .05$) or **tendency ($P \leq .10$) to be different pre- to postsupplementation. †Statistically significant ($P \leq .05$) or ‡tendency ($P \leq .10$) to be different between men and women.

PUFA supplementation on biochemical markers was similar in men and women except for fasting glucose concentrations.

In addition, as expected, the 6-week *n*-3 PUFA supplementation increased the concentration of total *n*-3, EPA and DHA content in erythrocytes, demonstrating the compliance of subjects to the supplementation regimen (Table 1). In brief, the *n*-3 PUFA supplementation was associated with a similar increase in total *n*-3 in erythrocytes in both genders, from 7.45% to 10.03 % of total free FA for men (35%) and from 7.24% to 10.58 % of total free FA for women (46%). Comparable and significant increases were also observed for EPA and DHA as well as the *n*-3/*n*-6 FAs ratio after *n*-3 PUFA supplementation in both men and women.

3.2. Changes in gene expression levels

RNA extraction from PBMCs was performed on 60 samples: 30 PBMCs before and 30 PBMCs after supplementation. After verification of the total RNA quality, none of the samples were excluded from microarray analysis because of poor RNA quality (RIN<8). Microarray analysis was performed on 60 PBMC samples to gain insight on changes in gene expression levels. However, there was one outlier who was excluded from analyses due to abnormal hybridization results. Therefore, gene expression was analyzed in a total of 29 subjects pre- and postsupplementation.

Data demonstrate that among the 47,231 transcripts present on the microarray, approximately 55% of transcripts were detected in the PBMCs of study participants. First, we compared the pre- to post-*n*-3 PUFA supplementation gene expression values to adjust for baseline difference since microarrays are more appropriate to distinguish changes in the relative gene expression. Taking the group as a whole, we determined that 170 transcripts were differentially expressed [47% (80) down-regulated and 53% (90) up-regulated] when comparing the pre to post levels of gene expression after *n*-3 PUFA supplementation. Further, we compared men to women. More precisely, 610 transcripts were differentially expressed [46% (283) down-regulated and 54% (327) up-regulated] when comparing the pre to post levels of gene expression after *n*-3 PUFA supplementation (Fig. 1). In women, only 250 transcripts were differentially expressed [49% (122) down-regulated and 51% (128) up-regulated] when comparing the pre to post levels of gene expression after *n*-3 PUFA supplementation (Fig. 1). Finally, only nine transcripts were significantly changed in both men and women, demonstrating clearly that different genes were changed in each gender group. In summary, the number and the type of transcripts were significantly different between men and women after *n*-3 PUFA supplementation. Thus, subanalyses were performed independently in men and women.

3.3. Pathway analysis results

IPA indicates that the following pathways related biologically to *n*-3 PUFA supplementation (Fig. 2) were changed to different extents in men (Fig. 2A) and women (Fig. 2B): oxidative stress response mediated by nuclear factor (erythroid-derived 2)-like 2 (NrF2), mechanism of gene regulation by peroxisome proliferators via peroxisome proliferator-activated receptor alpha (*PPARA*), hypoxia-inducible factor signaling, nuclear factor kB (NF-kB) signaling pathway and oxidative stress. Further, significantly changed genes in each pathway in men and women subgroups are described in Appendix 1.

3.4. Real-time PCR validation

In men ($n=13$), results from RT-PCR and microarray data indicate that *FADS1* ($P<.01$), *IL1B* ($P<.01$) and *TNFA* ($P<.01$) were highly correlated; however, *FADS2* [$P=$ not significant (NS)] and *IL6* ($P=$NS) were not correlated prior to the *n*-3 PUFA supplementation. Post-supplementation, *FADS1* ($P=.06$), *FADS2* ($P<.01$), *IL1B* ($P<.01$) and *TNFA* ($P<.01$) were correlated in men, although *IL6* ($P=$NS) gene expression was not correlated. In women ($n=17$), results from RT-PCR and microarray demonstrate that *FADS1* ($P=.02$), *IL1B* ($P<.01$) and *IL6* ($P=.09$) were correlated pre-*n*-3 PUFA supplementation; yet, no relation was seen in *FADS2* ($P=$NS) and *TNFA* ($P=$NS) data. Post-supplementation, the following gene expression were correlated—*FADS1* ($P<.01$), *FADS2* ($P<.01$), *IL1B* ($P<.01$) and *TNFA* ($P<.01$)—between RT-PCR and microarray results in women; only *IL6* ($P=$NS) was not

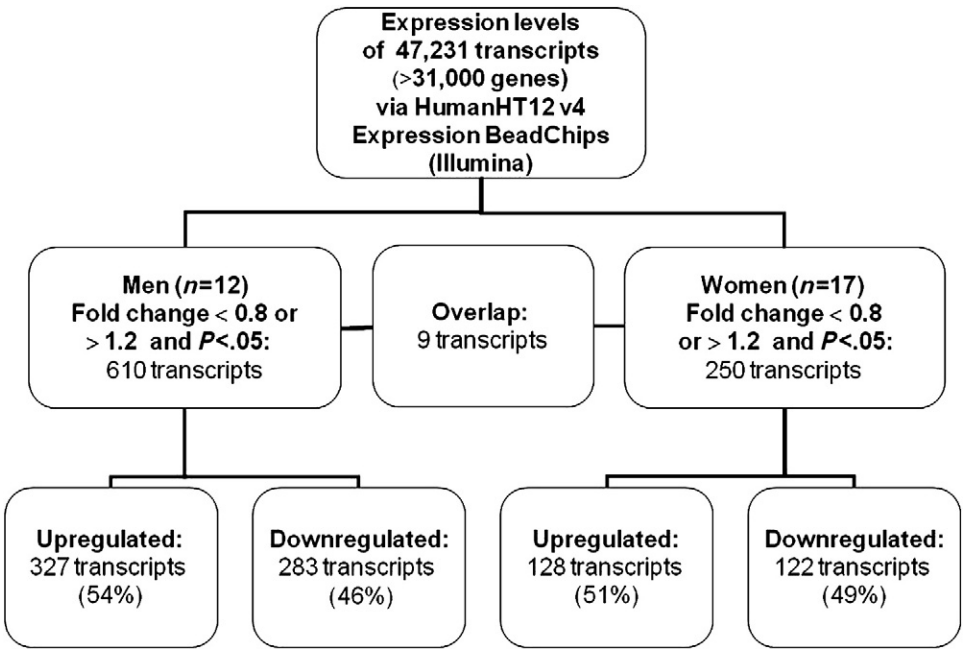
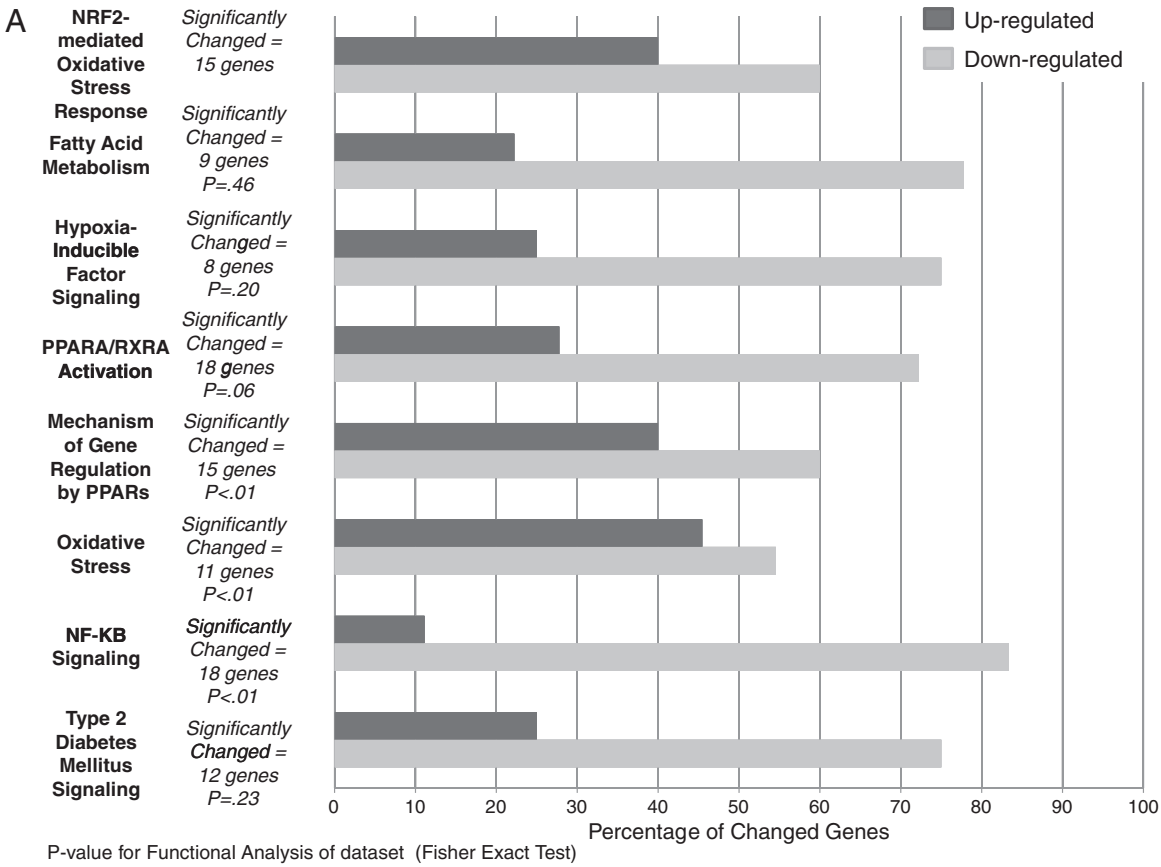


Fig. 1. Flowchart of number of genes changed in microarray analysis.



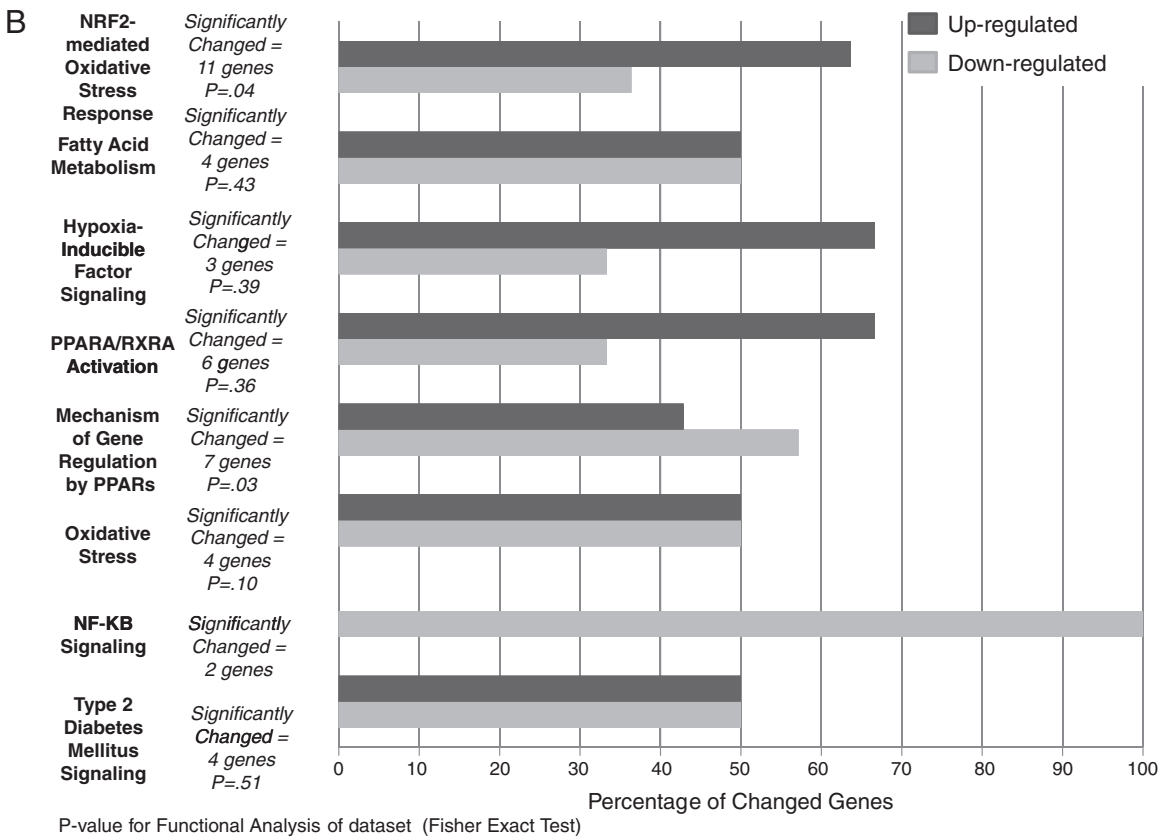


Fig. 2. Gene expression changes (≥±1.2 fold change) in canonical pathways analysis after supplementations. (A) Gene expression changes after *n*-3 PUFA supplementation in men. (B) Gene expression changes after *n*-3 PUFA supplementation in women.

interrelated. Taken together, RT-PCR and microarray data ($n=60$) were highly correlated except for *IL6* expression.

3.5. Changes in metabolites

Metabolites were quantified from four compound classes: ACs, amino acids, hexoses and phosho- and shingolipids. Appendix 2 describes the means±S.E.M. of metabolites that were quantified in men and women. First, we determined that 22 of the 41 ACs can be quantified correctly with the Absolute IDQ p150 Kit (Biocrates) in plasma. The results demonstrate that there were significant increases in 8 of the 22 ACs [AC10:2, AC18:1-OH, AC5-DC (C6-OH), AC5:1, AC5:1-DC, AC6:1, AC8:1, AC9] in men after *n*-3 PUFA supplementation. In contrast, in women, one AC (AC2) significantly decreased after *n*-3 PUFA supplementation. Further, two ACs were significantly higher in men than in women at baseline; yet, postsupplementation, all ACs were significantly higher in men than in women. Overall, these results demonstrate that *n*-3 PUFA supplementation can increase AC concentrations in men.

Secondly, amino acid concentrations demonstrated that one (glutamine) of the 14 amino acids was detected above the reference ranges recommended by the manufacturer of the kits and thus was excluded from results. Data demonstrate that men significantly increased leucine concentrations after *n*-3 PUFA supplementation; on the other hand, there were no changes in amino acid concentrations in women after *n*-3 PUFA supplementation. Presupplementation, men had higher methionine, ornithine and leucine than women. In the same way, men had higher methionine, ornithine, phenylalanine, proline, valine and leucine than women postsupplementation. Overall, results demonstrate that men have higher amino acid concentrations than women, especially leucine concentrations.

Thirdly, the sum of hexose in plasma has been examined. Data demonstrate that men had significantly increased hexose concentrations after *n*-3 PUFA supplementation; however, there were no significant differences between pre- and post-*n*-3 PUFA supplementation for women or between genders. As a whole, data suggest that hexose concentrations increased in men after *n*-3 PUFA supplementation.

Finally, the last part of the metabolomic kit investigated lipidomic profiles including SMs, lysoPCs and glyPCs pre- and post-*n*-3 PUFA supplementation. Women had one SM significantly different than men at baseline and 2 SMs significantly different than men after the *n*-3 PUFA supplementation. Examining the SM data, the results demonstrate that, in women, one SM (SM C20:2) decreased and another one (SM C22:3) increased significantly after *n*-3 PUFA supplementation. There were no significant changes in SM in men after *n*-3 PUFA supplementation. In sum, results demonstrate very little or no change in SM after *n*-3 PUFA supplementation.

Analyses show that one glyPC (PC aa C26:0) was lower than the detection range in our plasma samples. Further, results reveal that the following glyPCs were decreased significantly after *n*-3 PUFA supplementation in both genders: PC aa C34:2, PC aa C34:3, PC aa C36:4, PC aa C38:3, PC aa C38:4, PC aa C40:4, PC ae C34:3, PC ae C36:3, PC ae C36:4, PC ae C38:3, PC ae C38:4, PC ae C40:4, PC ae C42:1 and PC ae C44:5. In addition, men had decreased PC aa C32:3, PC ae C36:2 and PC ae C42:4. On the other hand, women also had decreased concentrations of PC aa C32:2, PC aa C34:1, PC aa C34:4, PC aa C36:3, PC ae C34:1, PC ae C42:3 and PC ae C42:5 after *n*-3 PUFA supplementation. Yet, *n*-3 PUFA supplementation increased the concentration of these glyPCs in both men and women: PC aa C36:5, PC aa C36:6, PC aa C38:0, PC aa C38:5, PC aa C38:6, PC aa C40:6, PC aa C42:0, PC ae C38:0 and PC ae C40:6. The ratio of glyPC aa C40:5/glyPC aa C40:6 in men and women significantly decreased after *n*-3 PUFA supplementation [from 0.46 to 0.32 ($P<.01$) and from 0.44 to 0.31 ($P<.01$), respectively, for men and women subgroups]. Men increased PC aa C42:6, and women increased PC aa C30:2, PC aa C42:1, PC ae C38:6 and PC ae C44:6. In addition, 16 PCs and 5 PCs were significantly different between the men and women at baseline and after supplementation, respectively. Overall, the results demonstrate that there was an increase in unsaturated FA after the *n*-3 PUFA supplementation period.

Examining the lysoPC data, two lysoPCs were lower than the detection range in the plasma samples analyzed (lysoPC a C14:0 and lysoPC a C26:1). Further, data demonstrate that lysoPC C20:3 decreased in both genders and lysoPC a C16:1 decreased in women after *n*-3 PUFA supplementation. In addition, at baseline, there was only one lysoPC (lysoPC a C18:2) that was significantly different between men and women. There were no differences between genders after *n*-3 PUFA supplementation. Taken as a whole, results demonstrate a modest decrease in lysoPC after *n*-3 PUFA supplementation.

4. Discussion

In the present study, we investigated the effect of *n*-3 PUFA supplementation on traditional biomarkers and investigated whether changes were also reflected in transcriptomic and metabolomic profiles in healthy adults.

Compliance in the present study is demonstrated by an increase in erythrocyte *n*-3 PUFA concentrations to a similar extent in both men and women. Further, the *n*-3 PUFA supplementation was associated with a decrease in saturation of FA side chains of the glyPC. A recent epidemiological study by Altmaier et al. (2011) [21] demonstrated that a decrease of the ratio glyPC aa C40:5/glyPC aa C40:6 indicated an increase in DHA intake. In the current study, this ratio was also significantly decreased after the *n*-3 PUFA supplementation; thus, this particular ratio could be potentially used as a biomarker of *n*-3 PUFA intake in epidemiological or clinical studies. Clearly, the results indicate that there was a high adherence of subjects to *n*-3 PUFA supplement protocol.

Transcriptomic data demonstrate that *n*-3 PUFA supplementation changed the expression of 610 genes in men, whereas the expressions of only 250 genes were altered in women; however, as demonstrated previously [22], the absolute number of genes is not as important as in which pathways these changes occur. Pathway analyses demonstrate significant changes in gene expression via the nuclear receptor *PPARA* pathways in both men and women. Literature has demonstrated that PUFA can interact directly with transcription factors such as *PPARA*, particularly the metabolism of TG-rich lipoproteins, HDL and free FA oxidation [23]. Firstly, there was a significant decrease in TG in both genders after *n*-3 PUFA supplementation. The effect of *n*-3 PUFA on TG is due to a *PPARA*-dependent stimulation of lipoprotein lipase and an inhibition of apolipoprotein C-III expressions [23]. Secondly, HDL-C concentrations increased in women and men (tendency) potentially due to *PPARA*-induced expression of apolipoprotein A-I and A-II [23]. In addition, TC concentrations did not change significantly in both men and women. Altmaier et al. (2009) [24] proposed that cholesterol concentrations and SMs are functionally related. Results show that the majority of SMs did not differ after *n*-3 PUFA supplementation, which is in accordance with the lack of change in cholesterol concentrations. Yet, it is possible that greater effects on TC and SMs could have been seen in subjects with hyperlipidemia. *PPARA* has also been shown to regulate genes involved in FA uptake, activation into acyl-CoA esters, degradation via the peroxisomal and mitochondrial beta-oxidation pathways and ketone biosynthesis [25]. Metabolomic results demonstrate that AC concentration increased significantly in men demonstrating an increase of FAs into the mitochondria for beta-oxidation. Conversely, the increase in AC concentration was not seen in women potentially due to a less significant *PPARA* activation. Gender-related differences in *PPARA* activation have previously been suggested with fibrates, which have

similar mechanisms of action as *n*-3 PUFA [26,27]. Overall, these results suggest that the activation of *PPAR*A aligns with changes in plasma lipid concentrations as well as metabolomic data.

Inflammation parameters, including IL6, TNFA and CRP, did not change; yet, the transcriptomic data demonstrate changes in inflammatory pathways after *n*-3 PUFA supplementation. The lack of significant change in plasma inflammation parameters may be due to genetic variations that influence response to *n*-3 PUFA supplementation. For example, Caron-Dorval et al. (2008) demonstrated an interaction effect between the *PPARA* L162V polymorphism and *n*-3 PUFA supplementation for plasma CRP concentrations [28]. Therefore, the effects of polymorphisms on plasma IL6, TNFA and CRP concentrations after *n*-3 PUFA supplementation should be verified. Further, gene expression profiling is thought to be more sensitive to nutritional intervention than the traditional biochemical parameters. Consequently, transcriptomic techniques may detect nutriment-induced changes even when physiological parameters are not yet changed or have near-significant effects on more recognized markers. Previous research studies have demonstrated that *n*-3 PUFA can inhibit cytokine production by direct actions on the intracellular signaling pathways that lead to activation of one or more transcription factors such as *PPAR*s [29,30] and NF-kB [1,31]. As mentioned above, both genders activated significantly the *PPAR*A pathway after *n*-3 PUFA supplementation; however, only men significantly changed the NF-kB pathway. An *in vitro* study indicated that the amino acids glutamine, leucine and proline may reduce the production of interleukin-8 probably by inhibiting NF-kB activity [32]. Thus, the higher leucine concentration in men may explain the greater changes in the NF-kB pathway. Nevertheless, women significantly changed the activation of the Nrf2 pathway; men changed this pathway to a lesser extent. Nrf2 orchestrates a group of antioxidant and other cytoprotective genes to provide a protective mechanism against detrimental stress-induced cellular and tissue damage in the cardiovascular system [33]. An increase in oxidative stress is often associated with progression of chronic diseases such as cardiovascular disease. In addition, a recent study showed that a derivative of *n*-3 PUFA may stimulate expression of the antioxidant heme oxygenase-1 — an important protecting factor of vascular tissue through activation of Nrf2 [34]. Changes in the Nrf2 pathway and related biomarkers after *n*-3 PUFA supplementation merit further investigation. In addition, *n*-3 PUFA supplementation may affect plasma inflammatory markers to a greater extent or differently in subjects with chronic inflammation compared to healthy subjects. Overall, the anti-inflammatory effects of *n*-3 PUFA may be acting via changes in gene expression in various and multiple pathways such as *PPAR*A, NF-kB and Nrf2, which should be explored in future studies.

We also found a modest decrease in lysoPC after the *n*-3 PUFA supplementation, which may be related to anti-inflammatory effects of *n*-3 PUFAs. LysoPC is the major bioactive lipid component of oxidized LDL and may be responsible for many of the inflammatory effects of oxidized LDL [35,36]. In addition, lysoPC has been associated with vascular inflammation, endothelial dysfunction and coronary atherosclerosis [37]. A study showed that lysoPC induces an increase in several inflammatory cytokines in PBMCs [38]. Therefore, the slight reduction of lysoPC in the present study may be related to a decreased production of inflammatory parameters in PBMCs of healthy adults. Overall, lipidomic profiles may provide interesting novel biomarkers for inflammatory status of subjects.

In the present study, plasma glucose increased in women and hexose concentrations increased in men without changing plasma insulin concentrations after the *n*-3 PUFA supplementation. However, the increase in glucose concentrations did not reach the “at risk” range for this population. Most clinical studies have shown that consumption of *n*-3 PUFAs has cardioprotective effects without adverse effects on glucose control and insulin activity as shown in a meta-analysis including 23 randomized controlled trials ($n=1075$ participants); however, some studies with higher doses of *n*-3 PUFA supplementation have reported detrimental effects on glycemic parameters [39]. *N*-3 PUFA supplementation may contribute to higher glucose concentrations through other mechanisms, for example, lower glucose utilization and increased glucagon-stimulated C-peptide, or increased hepatic gluconeogenesis [40]. Yet, pathway analyses demonstrate that the type 2 diabetes mellitus signaling was not modified significantly in the present study. In addition, high concentrations of lipids of the SM/ceramide pathways are connected to the attenuation insulin signaling as well as inflammatory processes [41–45]; hence, no change in SMs suggests no deleterious effects of these particular metabolites on glycemia. Higher but still normal concentrations of glucose levels should be verified in a larger sample to determine the exact impact of the *n*-3 PUFA supplementation.

In conclusion, results demonstrate that *n*-3 PUFA supplementation in healthy adults can alter favorably antiatherogenic and anti-inflammatory parameters as confirmed with a variety of well-known and novel biomarkers. This study investigated new biomarkers in clinical research that should be further verified in other samples to ascertain their validity. Furthermore, this study was conducted on healthy individuals, whereas the effect of *n*-3 PUFA supplementation on transcriptomic and metabolomic profiles should also be investigated in subjects with a deteriorated metabolic profile. In addition, future studies should examine gender differences on physiological and molecular parameters after *n*-3 PUFA supplementation. Overall, OMIC technologies may provide powerful tools for identifying new biomarkers and for establishing/confirming potential mechanisms of action of bioactive food components such as *n*-3 PUFA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.01.016.

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