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Journal of Nutritional Biochemistry 24 (2013) 54–61

Journal of Nutritional Biochemistry

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

Iwona Rudkowska[a,b], Ann-Marie Paradis[a], Elisabeth Thifault[a], Pierre Julien[b], André Tchernof[b], Patrick Couture[a], Simone Lemieux[a], Olivier Barbier[b], Marie-Claude Vohl[a,b,*]

[a]Institute of Nutraceuticals and Functional Foods (INAF), Laval University, Québec, Canada
[b]Endocrinology and Genomics, Laval University Medical Center, Quebec, Canada

Received 3 August 2011; received in revised form 2 November 2011; accepted 26 January 2012

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.

* Corresponding author. Tel.: +1 418 656 2131x4676; fax: +1 418 656 5877.
E-mail address: marie-claude.vohl@fsaa.ulaval.ca (M.-C. Vohl).

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http://dx.doi.org/10.1016/j.jnutbio.2012.01.016

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